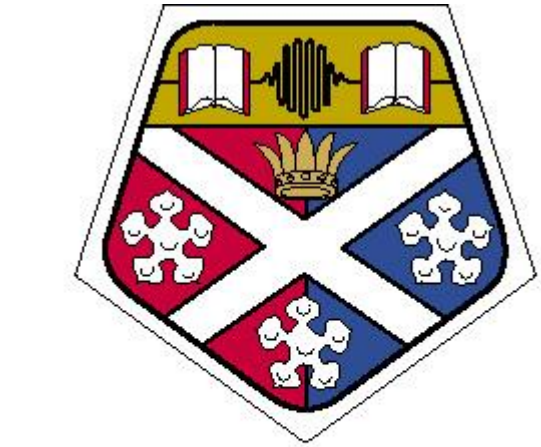

UNIVERSITY OF STRATHCLYDE

MOLECULARLY IMPRINTED POLYMERS FOR THE SELECTIVE EXTRACTION OF BIOLOGICAL MACROMOLECULES FROM AQUEOUS MEDIA

AHMED BAWAZIR

The thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Ahmed Bawazir
Date: June 2014

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Abbreviations

AIBN	2,2'azobisisobutyronitrile
AcN	Acetonitrile
AcOH	Acetic acid
ACP	*Acid phosphatase*
ALK	*Alkaline phosphatase*
BCA	Bicinchoninic assay
BET	Brunauer, Emmett and Teller
Bgo	*Beta glucosidase*
BSA	Bovine serum albumin
DEGVE	Di (ethylene glycol) vinyl ether
EGDMA	Ethylene glycol dimethacrylate
EG	Ethylene glycol
FTIR	Fourier transform infrared spectroscopy
Gly	Glycine
IPA	Isopropyl alcohol
KCl	Potassium chloride
Lys	*Lysozyme*
MAA	Methacrylic acid
MIPs	Molecular imprinted polymer
PCMC	Protein coated onto micro crystals
PEG	Polyethylene glycol
RNase A	*Ribonuclease A*
RP-HPLC	Reverse phase high pressure liquid chromatography
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulfate
Tween 20	Polysorbate 20
UV/vis	Ultraviolet/visible
4-vpy	4-vinyl pyridine

Abstract

Molecular imprinted polymer (MIPs) is established as a technology for obtaining biological mimics using artificial materials. The aim of this study is to prepare monolithic non-covalent imprinted polymers for selective extraction of proteins from aqueous media, using a novel strategy of protein coated onto micro crystals (PCMCs) as a platform for protein imprinting.

The MIPs were prepared by adding functional monomers methacrylic acid and 4-vinyl pyridine, a cross linker, ethylene glycol dimethacrylate, in a PCMCs/ acetonitrile suspension followed by the addition of the free radical initiator 2,2' azobisisobutyronitrile, in a free radical polymerisation.

The MIPs developed exhibited selectivity towards the template but non selectivity towards non specific proteins was also observed. To reduce this, we used chemical additives and non specific blocking agents, however no difference was observed. In a further attempt to combat non selective protein binding, we added a monomer di (ethylene glycol) vinyl ether (DEGVE) into the polymerisation process. Non specific protein binding was slightly reduced.

To improve polymer functionality, we developed UV initiated polymers and these were compared with temperature initiated polymers, both polymers showed similar qualities.

To gain understanding on the nature of the protein bound to the polymers, we looked at the template bound polymer activities. It was found that ~>93% of the protein bound to the imprinted polymer was active, while only ~75% of the protein bound to the control was active.

Finally, the presence of DEGVE in the polymer matrix was investigated using a FTIR and micro-analysis; however, it was not clear whether this monomer was incorporated into the polymer matrix.

Polymer physical characteristics, such as surface area and porosity, were examined using Brunauer, Emmett and Teller (BET), polymer swelling properties with a gravimetric technique and microscopic property with SEM. Most polymers demonstrated different physical characteristics.

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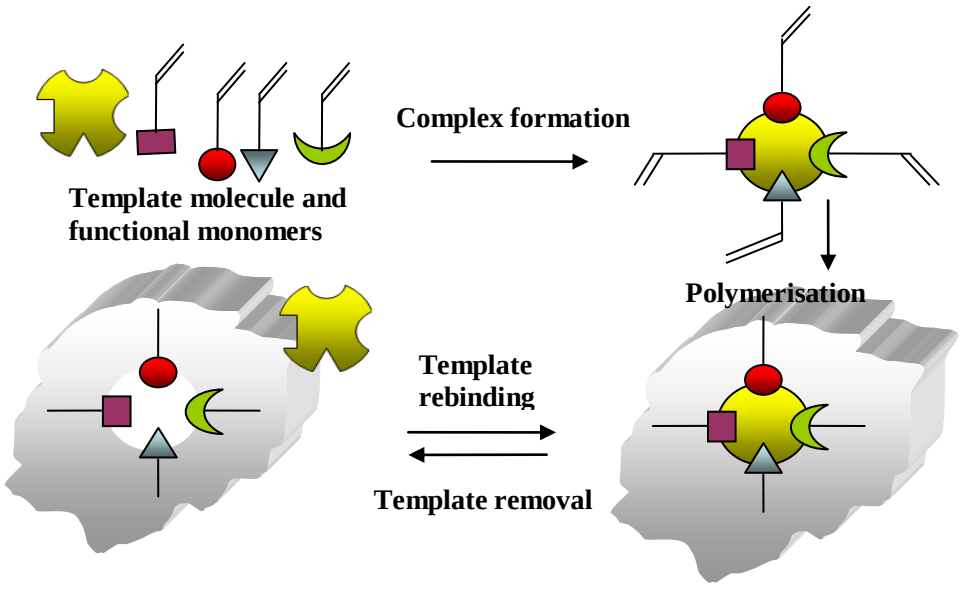
Chapter 1 : Molecular imprinted polymer

1.0 Introduction

Molecular recognition is ubiquitous and a major driving force in Nature. The mechanism of recognition is by molecules interacting with each other, such as enzyme/substrate binding, protein/receptor association, drug to biological target, antigen/ antibody recognition and complementary RNA and DNA hybridisation (Bergmann and Peppas, 2008). Many scientists are fascinated by natural phenomena and attempt to mimic natural biological systems. Molecular imprinted polymer technology offers a platform for obtaining biological mimics using artificial materials. The principle of molecular imprinting is presented in figure 1-1.The technique involves a " molecular moulding" process, where an artificial polymeric host is synthesised in the presence of a molecule of interest to generate complementary binding sites that are selective for the target molecule (Wulff *et al*, 1986; Andersson *et al*, 1984; Mosbach, 1994; Haupt, 2000). Molecular imprinting was originally envisaged to potentially follow principles akin to antibodies.

Figure 1-1: A schematic illustration of the molecular imprinting concept



The polymer is then expected to selectively and reversibly rebind the imprinted molecule similar to the antigen-antibody mechanism. Hence the term "plastic antibodies" was coined for these systems (Haupt and Mosbach, 1998).
A wide variety of templates have been employed for molecular imprinting, including ions, molecules, complexes and molecular ionic or macromolecular assemblies, including even micro-organisms" (Ge and Turner, 2008).
The molecular imprinted technique offers a platform for developing simple and inexpensive systems with a vast array of applications such as chromatography, separation, catalysts (Wulff, 1995), solid phase extraction (Andersson, 2000), biosensors (Haupt *et al*, 2000**),** medical diagnostics, drug delivery and environmental monitoring (Bossi *et al*, 2007). Given this enormous potential, efforts in this area continue to intensify and many more sectors are envisaged to emerge and progress rapidly.

1.2 Brief history of molecular imprinting

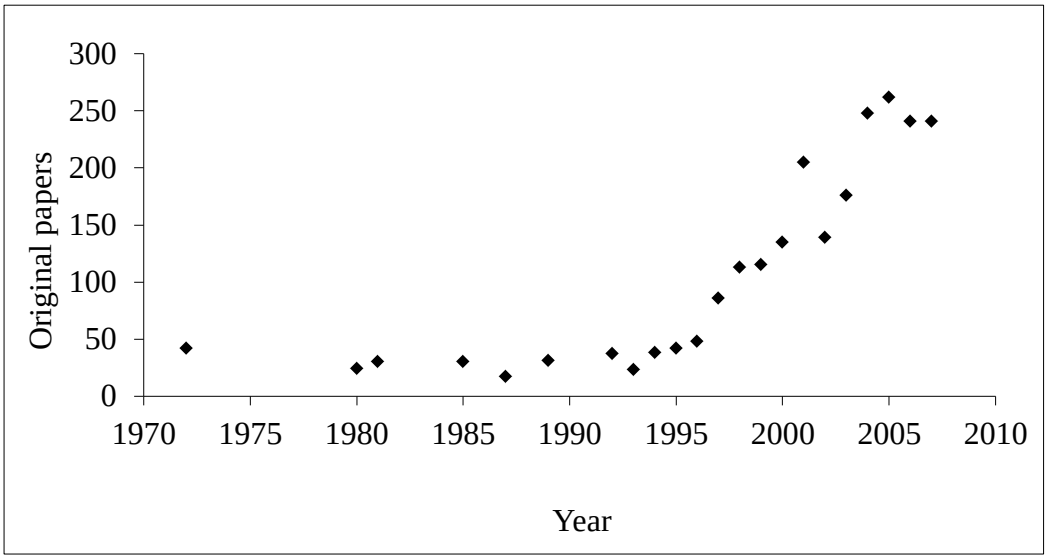
The inspiration of molecular imprinting can be traced back to two main historical sources; firstly the imaginative wonder of a Russian chemist M.V. Polyakov, from Kiev who in 1931, was interested in preparing silica materials for chromatography application. Polyakov attempted to increase the loading capacity of the silica and was studying the effect of drying on silica pore structure. Polyakov suspected that by drying the silica in the presence of small molecules, such as benzene, toluene or xylene, the pore structure might be altered in such a way as to increase the loading capacity of the silica. Polyakov used sodium silicate which was polymerised in water using $(NH_4)_2CO_3$ as the gelating agent. The polymer was left to dry for two weeks before the addition of the templates, after which it was again left to dry for another 20-30 days. Polyakov discovered unusual adsorption of the silica particles; the polymer increased the loading capacity for the imprinted template more than for a structurally analogous ligand, which suggested that some kind of memory was provided for the molecules. The second part of the story was by Linus Pauling, with the principle of "instructional theory" for

antibodies formation in 1940. This states that the primary structure of the antibody is identical and non specific but when it is in contact with an antigen, it adopts a specific form, with the template inducing structural changes to the antibody. Therefore, the antigen "instructs" the antibody to assemble around the antigen and to take the shape that is complementary to the antigen (Alexander *et al*, 2006).

In the same decade, Pauling's theory was superseded by the *clonal selection theory* which is now held to be correct. However, Pauling's student F.H. Dickey in the late 1940s was inspired by the ideas of Polyakov's theory of "induced selection" and his mentor's "instructional theory" to create silica matrices with high affinity sites. Dickey slightly modified the Polyakov method; instead of adding solvent as templates, he used methyl and other alkyl orange dyes. In addition, he added the dyes in the reaction polymer mixture unlike Polyakov who added the solvents after the silica was already dried. The addition of template alkyl orange dye in the pre-polymerisation mixture was used to induce the formation of specific sites for the dye in sol-gel imprinting. Dickey processed the imprinted polymer similarly to Polyakov, by letting it dry, followed by sieving and sedimenting before analysis with chromatography. The resultant polymers showed good recognition by preferentially adsorbing the imprinted dye in relation to other dye molecules (Alexander *et al*, 2006).

These inspirational studies led to the beginning of the molecular imprinting research fields. The research effort continued for decades, after which in 1970 marked the beginning of the development of molecular imprinting as a viable technology. Now, molecular imprinting is a rapidly maturing technology which has received substantial attention and has convinced many researchers in different aspects of the scientific field to explore the wonders of this technology; this has been reflected by the remarkable growth in the papers published from the period of 1970-2009 (figure 1-2).

Figure 1-2: Exponential growth in the MIP research papers published in the last forty years



The modern form of molecular imprinting is accredited to two scientists; Professor Günter Wulff a synthetic organic chemist and Professor Klaus Mosbach a biochemist. Their research effort in the years between 1970's to 1980's led to the discovery of the two most fundamental principles, which are discrete and commonly referred in the literature as the covalent and non covalent approaches to molecular imprinting.

1.2.1 Covalent approach

In covalent imprinting, developed by Günter Wulff and co-workers, the template-monomer association formed in a pre-polymerisation solution is maintained by reversible covalent bonds, and the recognition of the template by the imprinted polymer is dependent on the formation and cleavage of these bonds. For example, the reversible covalent bonds used for this type of imprinting are esters, Schiff bases, and ketals (Alexander *et al*, 2006). This technique generates well defined binding sites with a high degree of homogeneity because the functional groups are only associated with the template site. After polymerization, the template is extracted leaving vacant positions bearing functional groups that selectively rebind the template via covalent bonds. However, there are a number of drawbacks with this technique. Mass transfer kinetics of

the templates are poor because the kinetics of bond reforming and breaking is slow; this limits the use of the polymer in chromatography. Extracting the template is sometimes difficult because it is attached covalently to the polymer. Therefore, harsh solvents are employed to break the bonds, consequently leading to damaged binding sites. In addition, only a limited number of templates can be used. The templates must have specific functional groups, such as alcohols (diols), aldehydes, ketones, amines and carboxylic acids (Alexander *et al*, 2006), and in most cases monomers are not readily available and need to be synthesised, which requires specialist knowledge.

1.2.2 Non covalent approach

The other strategy to molecular imprinting is by a non-covalent approach conceived by Klaus Mosbach and co-workers, whereby the pre-arrangement between the template and functional monomers in a pre-polymerisation mixture is held by non covalent association and the recognition of the template is by formation of multiple weak interactions (Mosbach, 1994; Sellergren, 2001).

Mosbach and co-workers were inspired by molecular interactions in natural biological systems that lead to stable molecular complexes. Nature uses non covalent interaction such as: hydrogen bonds, ion pairing and hydrophobic interactions. Although these bonds are weak when considered individually, however if several of these weaker interactions are combined, they can lead to stable complexes, such as antibody-antigen complexes, enzyme-substrate association and receptor-hormone interactions.

The non covalent approach to molecular imprinting has been widely used (Sellergren, 2001) because it has several advantages. The synthesis of polymer imprints is simple and straightforward and a vast number of template structures possessing different functional groups can be employed such as: amino acids, peptides, steroids, carbohydrates, nucleotides, dyes, pesticides, metal ions, drugs, proteins, microorganisms and crystals (Alexander *et al*, 2006). Another advantage is that monomers are readily available for different types of molecular interaction with the template.

In contrast to covalent imprinting, the rebinding and dissociation kinetics are far better, which makes non covalent imprinting a better candidate for chromatography applications. Finally, because of the versatility of this approach, professionals from different disciplines e.g. (engineers, biologists, pharmaceutical chemists, biochemists) who are not polymer or synthetic chemists are able to appreciate this technology and have been involved in research leading to much broader applications. However, one of the drawbacks of the non covalent approach is that of binding site heterogeneity, leading to poor selectivity and performance. The inhomogeneity of the imprinted sites has previously been explained by Sellergren (2001) to be a result of the amorphous nature of the polymer where imprinted sites are suspected to reside in domains with different cross linking density, in effect leading to significant variations of sites similar to polyclonal preparations of antibodies. In addition, the incomplete interaction between the template and monomers and the self-association of monomers leading to the formation of dimers contribute to non specific site formation. Other factors, such as polymerisation medium, might interfere with template-monomers complexes and also give rise to the formation of template-template dimers or trimers, or solvent-template association again leading to formation of poor sites. Finally, the problem with template bleeding in non covalent imprinting is large. Thus typically it is almost impossible to totally extract the template from the polymer even with the most stringent washes. However, this problem can be circumvented by imprinting with a template analogue, such as imprinting of *bromoclenbuterol* for the detection of *clenbuterol*, where the bleeding of *bromoclenbuterol* did not interfere with the analysis of the targeted template (Crescenzi *et al*, 2001). Andersson and co-workers imprinted the drug sameridine analogue to resolve the problems with template leaching from the polymer (Haupt, 2001).

1.2.3 Semi covalent or sacrificial spacer approach

This approach was pioneered by Whitcombe and co-workers and combines both covalent and non-covalent techniques, therefore taking advantage of both approaches. The technique involves using covalent bonds during imprinting steps and non covalent

bonds in the recognition process. Because of the non-covalent rebinding, the technique has the advantage of fast rebinding and dissociation but has diffusion restriction. The covalent side offers the advantage of binding sites homogeneity because functional groups present in the binding site are those recovered after the cleavage of the template. Whitcombe's group used this technique for imprinting a tripeptide (Lys-Trp-Asp) using a sacrificial spacer (*o*- hydroxybenzamide) between the imprint molecules and monomers (Whitcombe *et al*, 1995).

The drawbacks with this technique are that it requires specific monomers and specific experimental conditions, and is also limited in the scope of the template that can be used. It also requires more specialist knowledge of synthetic chemistry compared with the non-covalent approach, and poor removal of the template is commonly found in covalently bound templates.

1.3 Non covalent molecular imprinting

In non covalent imprinting, there are at least five different components that are critical to the design of the imprinted matrix i.e. templates, functional monomers, cross-linkers, porogens and initiators. These components must be appropriately selected in order to shift the equilibrium towards template-functional monomer complex formation and to the creation of specific sites for the targeted template. However, the optimum combination of these components cannot be easily achieved; they are typically performed empirically until one achieves the desired imprint.

1.3.1 Template

Templates commonly used in non covalent imprinting may in principle range from wide selections of small molecules (drugs, sugars, metal ions, hormones, amino acids) to large molecular peptides and proteins and even larger molecular assemblies such as cells and viruses.

Understanding the structure of the template, the type and number of the functional groups available for the template monomer association will help to select appropriate monomers and will determine the possible functional group that will potentially form the best binding site architecture.

Overall, the number of functional groups and the distance between the groups has considerable influence on the performance of the molecular imprinted polymers (MIPs). For templates with a small number of functional groups, i.e. less than three, the mode of recognition is suggested to be the shape of the template, but for three or more functional groups the pre-organisation of the monomer is responsible for template selectivity (Simon *et al*, 2007).

The size of the template is another factor which needs careful consideration. A dense cross linked network tends to restrict the movement of large sized templates in the polymer, and poses a great challenge to extraction, leading to some template getting trapped permanently. In addition, the rebinding and dissociation kinetics of the template becomes poor and slow, thus reducing the polymer's efficiency.
Asanuma *et al*, (2001) suggested that the ideal template is in the angstrom range for production of effective MIPs.

Another factor is that the template should be soluble in monomer solution and unreactive in the polymerisation process (Sellergren, 2001). The template often needs to withstand harsh polymerisation conditions, such as organic solvents and elevated temperature, such as encountered in free radical polymerisation. This is particularly important when considering biomolecular imprinting. However, template solubility may be relinquished when template immobilisation is considered for imprinting.
Finally, the template should be available in sufficient quantities for imprinting purposes and the characterisation techniques should be available even for the template analogues. Simple and cost effective methodologies are advantageous, otherwise the analysis will be expensive, labour intensive and time consuming.

1.3.2 Functional monomer

Functional monomers are “building blocks” that form chemically reversible associations with the template (Haupt, 2001). Functional monomers are categorised according to the functional groups attached, for example acid monomers have acid groups attached, and others are: basic, neutral monomers and synthesised monomers for specific applications. In general, the type and concentration of the functional monomers typically used in non covalent imprinting are chosen from experience or from published reviews or in some cases they are determined empirically. The choice of functional monomers will determine the structure of the recognition site (Haginaka, 2007) and the concentration will influence the number of binding sites (He, 2007).

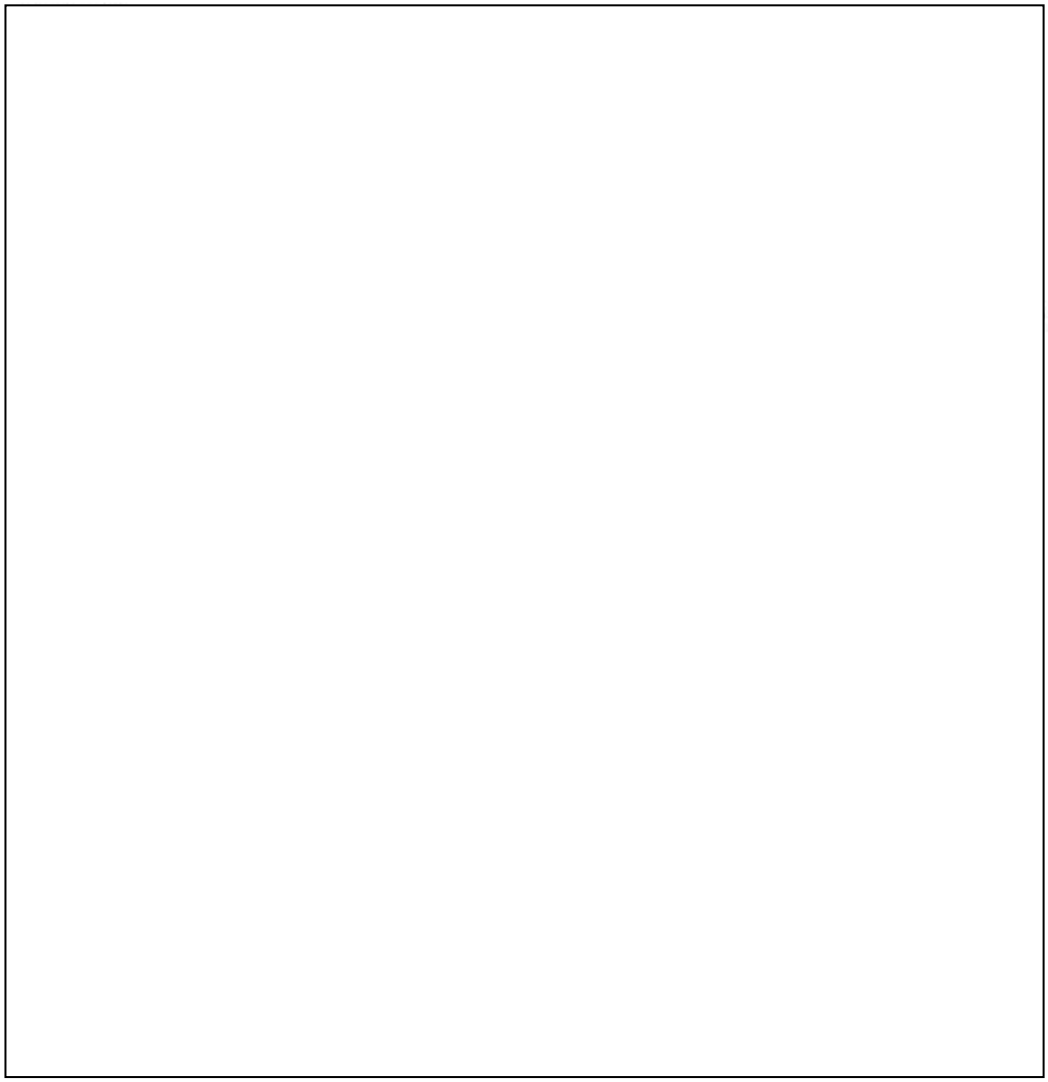
There are wide selections of functional monomers that are commercially available to give desired tailor made template-monomer interactions. The most common functional monomers in non covalent imprinting are mono-unsaturated (vinyl, acrylic, methacrylic) functional monomers (Haupt, 2001). Figure 1-3 highlights some of the functional monomers used in non covalent imprinting.

The common criteria employed towards monomer selection are by the type of functional groups exposed or available for interaction with the template. For example, if the template contains an acidic group e.g. (carboxylic group~), the basic monomer will be an appropriate choice. This will result in the formation of ionic interaction between the monomer and the template. Conversely, for a template containing a basic group, an acidic functional monomer will be ideal.

The most common and popular choice of acid and basic functional monomers are the vinyl monomers; methacrylic acid (MAA) and 4-vinyl pyridine respectively.

MAA forms both strong ionic interaction and hydrogen bonding because the carboxylic acid can act as a hydrogen bond donor or acceptor (Sellergren, 2001). Another common acid monomer is trifluoromethyl acrylic acid (TFMAA); this monomer tends to form stronger ionic interaction with a basic template compared to MAA because it has more electronegative fluorine atoms (Matsui and Takeuchi, 1997).

Figure 1-3: Functional monomers used for molecular imprinting



Acidic; aI: methacrylic acid (MAA); **aII:** *p*-vinylbenzoic acid; **aIII:** acrylic acid (AA); **aIV:** itaconic acid; **aV:** 2-(trifluoromethyl)-acrylic acid (TFMAA); **aVI:** acrylamido-(2-methyl)-propane sulfonic acid (AMPSA).
Basic; bI:4-vinylpyridine (4-VP); **bII:** 2-vinylpyridine (2-VP); **bIII:** 4-(5)-vinylimidazole; **bIV:** 1-vinylimidazole; **bV:** allylamine; **bVI:** *N*,*N*_-diethyl aminoethyl methacrylamide (DEAEM), **bVII:** *N*-(2-aminethyl)-methacrylamide; **bVIII:** *N*,*N´*_-diethyl-4-styrylamidine; **bIX:** urocanic ethyl ester.
Neutral; nI: acrylamide; **nII:** methacrylamide; **nIII:** 2-hydroxyethyl methacrylate (2-HEMA); **nIV:** trans-3-(3-pyridyl)-acrylic acid; **nV:** acrylonitrile (AN); **nVI:** methyl methacrylate (MMA); **nVII:** styrene; **nVIII:** ethylstyrene) (Cormack and Elorza, 2004).

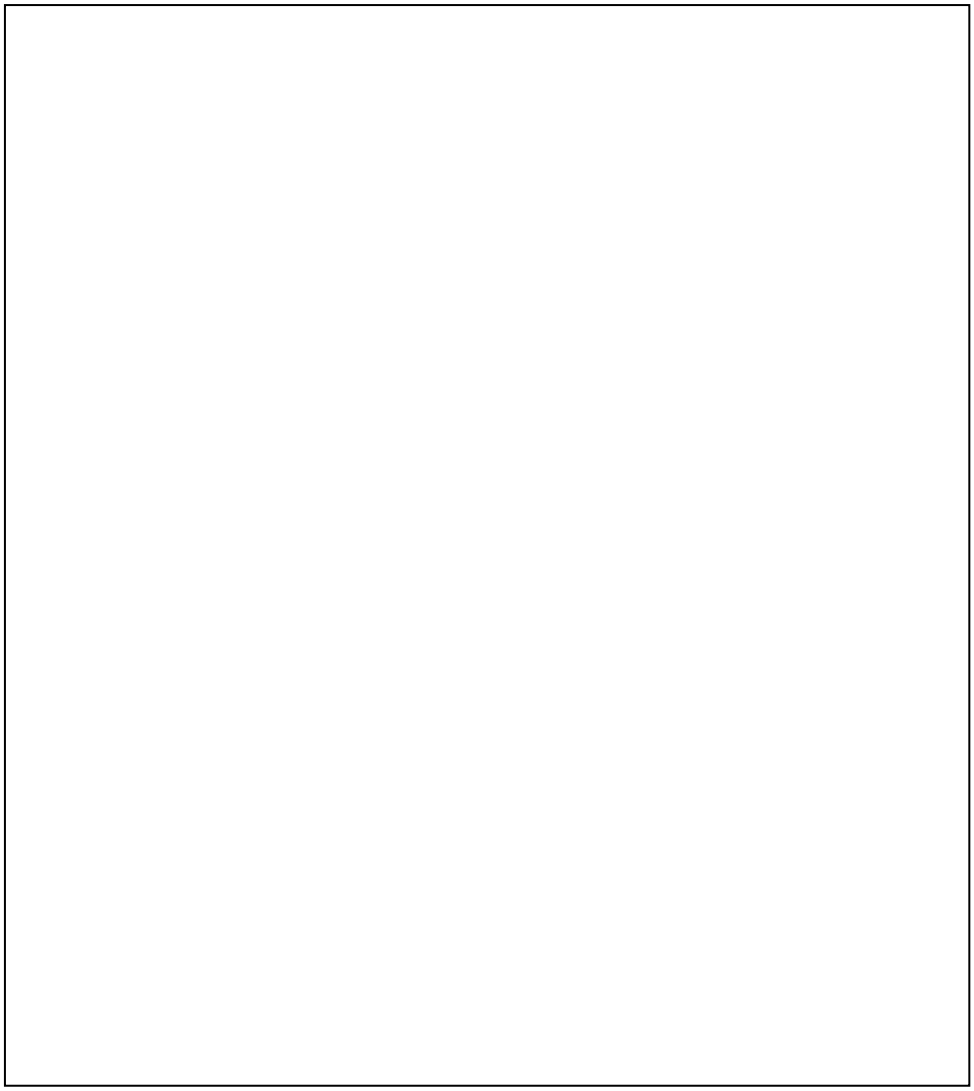
TFMAA for the basic monomer 4-vinyl pyridine also forms both ionic and hydrogen bonding with acid functional groups. When the template possesses both acid and basic groups, a cocktail of both acidic and basic monomers can be used. This has been reported to improve polymer recognition. However, a complicated system might arise especially when monomer donor-acceptor pairs have strong interactions competing with the template-monomer association and particularly if neither of the monomers has a greater preference for the template (Sellergren, 2001). Other monomers such as hydroxyethylmethacrylate (HEMA) a neutral molecule is used to induce hydrophilicity in the polymer matrix when hydrophobic interactions are causing problems in template recognition.

In order to obtain good imprinting, the functional monomers must be soluble in the polymerisation medium. For example, hydrophobic monomers such as pyridines are insoluble in water but are soluble in hydrocarbon solvents, thus appropriate selection of monomers will be essential. In addition, a suitable concentration of monomers should be used. Otherwise, if used in excess, formation of dimers or trimers will occur, contributing to the formation of non specific sites. Finally when choosing functional monomers, one should also consider the application and the environment where the imprinted polymer will be used because unreacted residual monomers would be toxic and will not be appropriate for specific applications such as blood purification.

1.3.3 Cross linker

A cross linker is a structural monomer acting as a "molecular glue" that positions and traps the template and holds the functional groups in the specific orientation around the template as formed in the pre-polymerisation complex. It forms a rigid stable matrix so that the morphology of the imprinted sites are created and preserved during and after the removal of the template. Similar to the functional monomers, there are commercially available cross linkers that differ in chain length, flexibility, freedom of rotation and solubility. Figure 1-4 lists some of the cross linkers that are commonly used in molecular imprinting.

Figure 1-4: Cross-linkers used for molecular imprinting



xI: *p*-divinylbenzene (DVB); **xII:** 1,3-diisopropenyl benzene (DIP); **xIII:** ethylene glycol dimethacrylate (EGDMA); **xIV:** tetramethylene dimethacrylate (TDMA); **xV:** *N*,*O*-bisacryloyl-l-phenylalaninol; **xVI:** 2,6-bisacryloylamidopyridine; **xVII:**1,4-phenylene diacrylamide; **xVIII:** *N*,*N*_-1,3-phenylenebis(2-methyl-2-propenamide) (PDBMP); **xIX:** 3,5-bisacrylamido benzoic acid; **xX:** 1,4-diacryloyl piperazine (DAP); **xXI:** *N*,*N*_-methylene bisacrylamide (MDAA); **xXII:** *N*,*N*_-ethylene bismethacrylamide; **xXIII:** *N*,*N*_-tetramethylene bismethacrylamide; **xXIV:** *N*,*N*_-hexamethylene bismethacrylamide; **xXV:** anhydroerythritol dimethacrylate; **xXVI:** 1,4;3,6-dianhydro-d-sorbitol-2,5-dimethacrylate; **xXVII:**isopropylenebis(1,4-phenylene) dimethacrylate; **xXVIII:** trimethylpropane trimethacrylate (TRIM); **xXIX:** pentaerythritol triacrylate (PETRA); **xXX:** pentaerythritol tetraacrylate (PETEA) (Cormack and Elorza, 2004).

The appropriate choice of the cross linker is another factor which is essential in providing stable MIPs over range of different conditions. To some extent, the choice of cross linker would depend on the size of the template because the cross linker is expected to encapsulate the template in order to create the imprinted cavities.
For example if the cross linking density is very low, this would result in the length between crosslinks to be too large, which would create large cavities, consequently leading to non specific imprints (Wiseman, 2001).

Cross linkers commonly used in non covalent imprinting are water insoluble species such as; ethylene glycol dimethacrylate (EGDMA) having two acrylate groups, trimethylpropane trimethacrylate (TRIM) with three acrylate groups and tetraethylene glycol dimethacrylate TEGDMA having four acrylate groups. The increased acrylate groups result in an increase in the cross linking density, hence formation of a more rigid polymer matrix. EDGMA and TRIM are most extensively employed as cross linkers in molecular imprinting because they provide mechanical and thermal stability to the polymer, ease of removal of the template, and produce MIPs with high affinities and selectivity (Wulff, 1995; Schweitz *et al*, 1998).
Finally, the common criteria used to determine the cross linker concentrations are derived from the amount of functional monomer used. Generally, the amount of cross linker ranges from 70-80% v/v of the total polymer composition or even as high as 90% (He *et al*, 2006). However, lower volumes have been used depending on the type of functional monomer employed (Spivak, 2005).

1.3.4 Porogen

The porogen is part of the medium where the polymerisation is carried out; typically in solvent it either drives the formation of monomers and template complexes forward or in reverse depending on the strength and the form of interactions. Various solvents have been reported in the literature, for example, water, acetonitrile (AcN), chloroform,

methanol (MeOH), a dimethyl-sulfoxide (DMSO), toluene, 1,1,1-trichloroethane (TCE), dimethylformamide(DMF) and cyclohexanol. The choice of solvents is critical in achieving a good imprint. Basically it is almost impossible to have a solvents with no interference at all. However, the ideal solvent would provide solubility to the template-monomers in both the pre-polymers and during the polymerisation process, and cause minimum interference towards the template monomer complexation (Sellergren, 2001; Wu *et al*, 2005).

Typical solvents used in imprinting have some degree of interference with template monomer association. For example protic solvents, such as water and methanol tend to disrupt hydrogen bonding formation, while highly polar aprotic solvents interfere with ionic and van der Waals interactions. Wu *et al*, (2005) used methanol as a medium for nicotinimade imprinting. The group found that the interaction energies between the porogen and the template were greater compared to the template and functional monomer (MAA). For this reason, poor nicotinamade retention and imprinting factor were observed.

For typical small molecule imprinting by free radical polymerisation, aprotic solvents, such as acetonitrile, toluene and chloroform, are commonly used. In addition, some solvents like acetonitrile induce the formation of a porous network. The pores are formed by the solvent filling out the spaces created between polymer aggregates and the acetonitrile is then released in the form of a vapour phase in a highly exothermic polymerisation reaction (Spivak and Shea, 2001; Piletsky *et al*, 2004).

There is still a concern whether the porosity has any influence on the recognition behaviour of the imprinted polymer. This concern was addressed by Spivak and Shea (2001). They used acetonitrile, a well known porous aprotic solvent, to induce pore formation in the polymer and compared it with polymer made with chloroform solvent, which exhibited an almost non-porous matrix with low specific surface area for imprinting 9-ethyladenine molecule. The resultant polymer made with chloroform

showed good recognition for the imprinted molecule 9-ethyladenine with high binding affinity when chloroform was used as mobile phase, other than when acetonitrile was used as mobile phase. A lower affinity was reported for the polymer made with acetonitrile using the chloroform as mobile phase. These results suggested that that there was some kind of memory for the imprinted solvent which influences the polymer recognition.

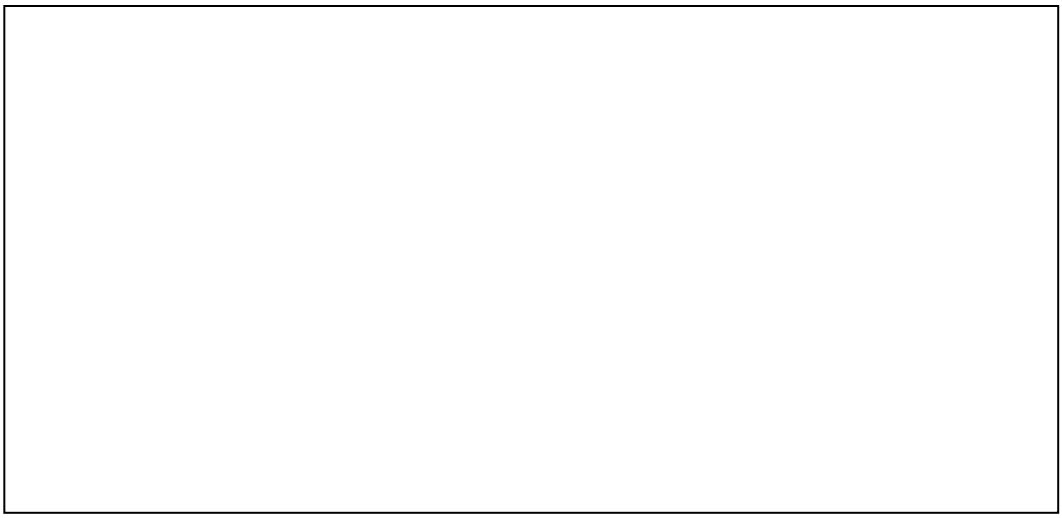
Unlike small molecules, large molecules such as proteins are insoluble in common polymerisation solvents. Exposure leads to protein aggregation and denaturation, thus rendering protein unfit for imprinting. To avoid this, an aqueous environment similar to physiological conditions or a co-solvent mixture of water and organic solvents are often used but this limits the selection of monomers to be used.
Finally, optimum concentration of mole fraction of the polymerisation solvent is essential; otherwise the excess of the solvent would lead to template-solvent association which might affect the efficiency of the polymer.

1.3.5 Initiator

Initiators have been extensively used in conventional free radical polymerisation. The initiator initiates the production of free radical species from stable precursor molecules. The free radicals produced are involved in both the propagation and termination stage of the polymerisation process. Methods of initiation may be by application of thermal energy or UV radiation.

The choice of initiators depends on factors such as the type of initiation required, the solubility of initiator in the polymerisation solvent and the temperature of initiation. There are several initiators that are water or organic soluble, are commercially available, and use either thermal or photochemical initiation to produce free radicals. The typical initiators used in molecular imprinting are shown in figure 1-5.

Figure 1-5: Initiators used for molecular imprinting



iI: azobisisobutyronitrile (AIBN); **iII:** azobisdimethylvaleronitrile (ABDV); **iIII:** dimethylacetal of benzil; **iIV:** benzoylperoxide (BPO); **iV:** 4,4′-azo(4-cyanovaleric acid) (Cormack and Elorza, 2004).

The mechanism of initiation is different for each type initiator because they have different decomposition rates when exposed to certain amounts of thermal or UV energy.

The most common and popular initiator used in molecular imprinting is azo-bis-isobutyronitrile (AIBN); this is because it has favourable decomposition kinetics, initiation efficiency and is soluble in many aprotic solvents suitable for molecular imprinting.

After selecting the initiator, another factor which is critically important is the concentration of the initiator. As for other polymer components, there are no general rules which define the amount of initiator; but it is commonly the rate-limiting step of the polymerisation process. When large concentrations of initiator are used, the polymerisation temperature might be too elevated, thus destabilising the template-monomer complex, consequently leading to formation of non specific sites (Spivak and Shea, 2001). In addition, large numbers of free radicals will be generated and produce a greater number of nuclei. This will result in the formation of smaller size globules which

will typically increase the number of smaller pores and lead to a large surface area (Piletsky *et al*, 2004). Conversely, very low concentrations of initiator, i.e. less than mol (1%) of the total double bond, result in a less rigid polymer because of poor conversion of the polymerisable double bonds, essential to producing stable MIPs (Piletsky *et al*, 2005).

The common concentration of the initiator used in a free radical polymerisation is between mol (1 - 2.5%). This range allows the maximum conversion of polymerisable vinyl double bonds and at the same time maintains the stability of the template-monomer complexation. This is because the molecular motions are slowed down and the entropic penalty associated with the self assembly process is minimised. For this reason MIPs with better performance are produced even though the polymerisation time is increased (Spivak and Shea, 2001; Mijangos, 2006).

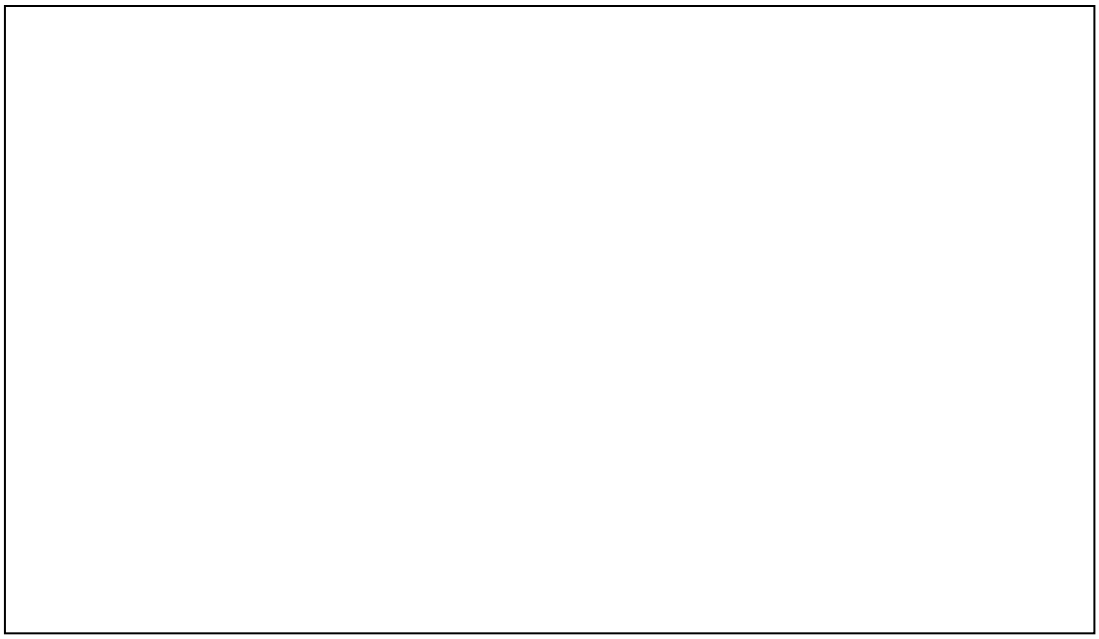
The above discussion of polymer compositions is aimed at elucidating the factors that should be considered when designing imprinted polymers. It should however not be taken as the ultimate criteria governing the selection of the polymer molecules because it might set limits on the template of which we have no knowledge. Adequate MIP performance can only be achieved after careful optimisation where the related factors are systematically varied.

1.4 Free radical non covalent imprinting

1.4.1 Pre polymerisation complex and bonding

In the pre polymerisation complexes, templates, monomers and cross linkers are mixed together to create a template polymer complex. Most non covalent bonds are weak in nature but are key to selective molecular interactions. These include hydrogen bonding, van der Waals forces, ionic interaction, π-π stacking and hydrophobic forces. The hypothetical interaction between the functional monomers (MAA and 4-vpy) and cross linker EDGMA with a hypothetical molecule is presented in figure 1-6.

Figure 1-6: Schematic illustration of a hypothetical molecule interacting with monomers



Functional monomer (MAA and 4-vpy) and cross linker EDGMA with a hypothetical molecule (This figure was adapted from Allender *et al,* 1999).

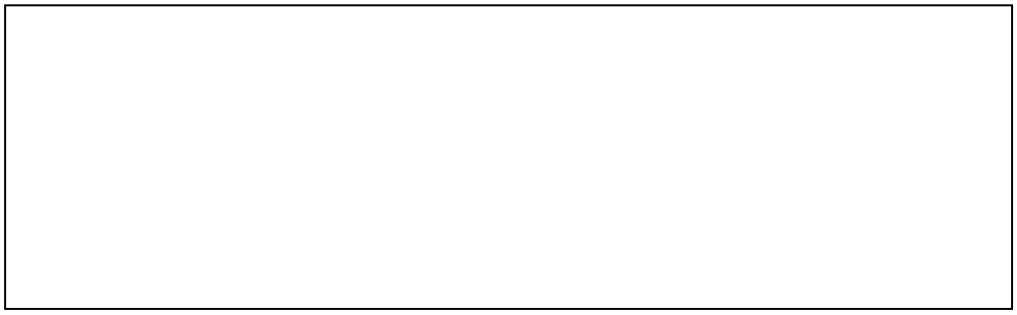
1.4.2 Free radical polymerisation process

The free radical polymerisation process involves pre-arrangement of polymerisable functional monomers around the template in a porogenic solvent forming a template-monomer porogen complex. The resulting polymer complex is then copolymerised with an excess of cross-linking reagent in the presence of free radical initiator under thermal or photochemical conditions. An imprint is formed around the template; the template is then extracted leaving the shape and orientation of functional groups intact in the polymer to form binding site pockets. Analogous to enzyme active sites, those are complementary to the template and are able to recognise the template (Piletsky *et al,* 1999). The conventional free radical polymerisation starts with initiation of monomers using either thermal or photochemical initiation, followed by propagation of chain monomers and finally termination. The process will be elucidated briefly through illustration.

1.4.2.1 Free radical initiation

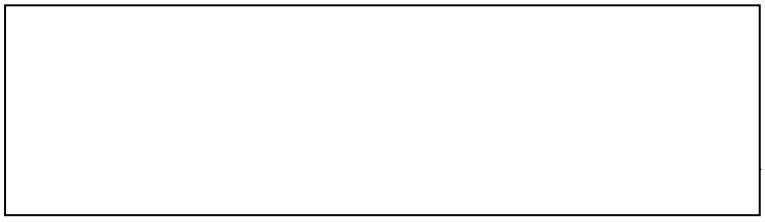
The most common and popular initiator used in molecular imprinting is azo-bis-isobutyronitrile (AIBN). The temperature required to thermally initiate decomposition of AIBN is between (60^0-64^0C) while lower temperatures are used for UV photochemical initiation (Sellergren 2001). The first step of the free radical initiation involves production of free radicals from a homolytic decomposition of the azo bond eliminating a molecule of nitrogen gas, to form two 2-cyanoprop-2-yl radicals. The homolytic cleavage of AIBN is depicted in Figure 1-7 (figure was adapted from Brown, 2005).

Figure 1-7: Homolytic cleavage of AIBN by thermal or UV energy



The second initiation step involves the generation of new radicals by the 2-cyanoprop-2-yl radicals produced by AIBN decomposition attacking functional monomer units. The new radical generated then reacts rapidly with other monomer units with a net increase in the number of free radicals in the propagation stage. Figure 1-8 (figure was adapted from Brown, 2005) depicts the 2-cyanoprop-2-yl radical attacking vinyl monomer MAA double bond to generate a functional monomer radical which is carried onto the propagation stage to yield high molecular weight polymer chains.

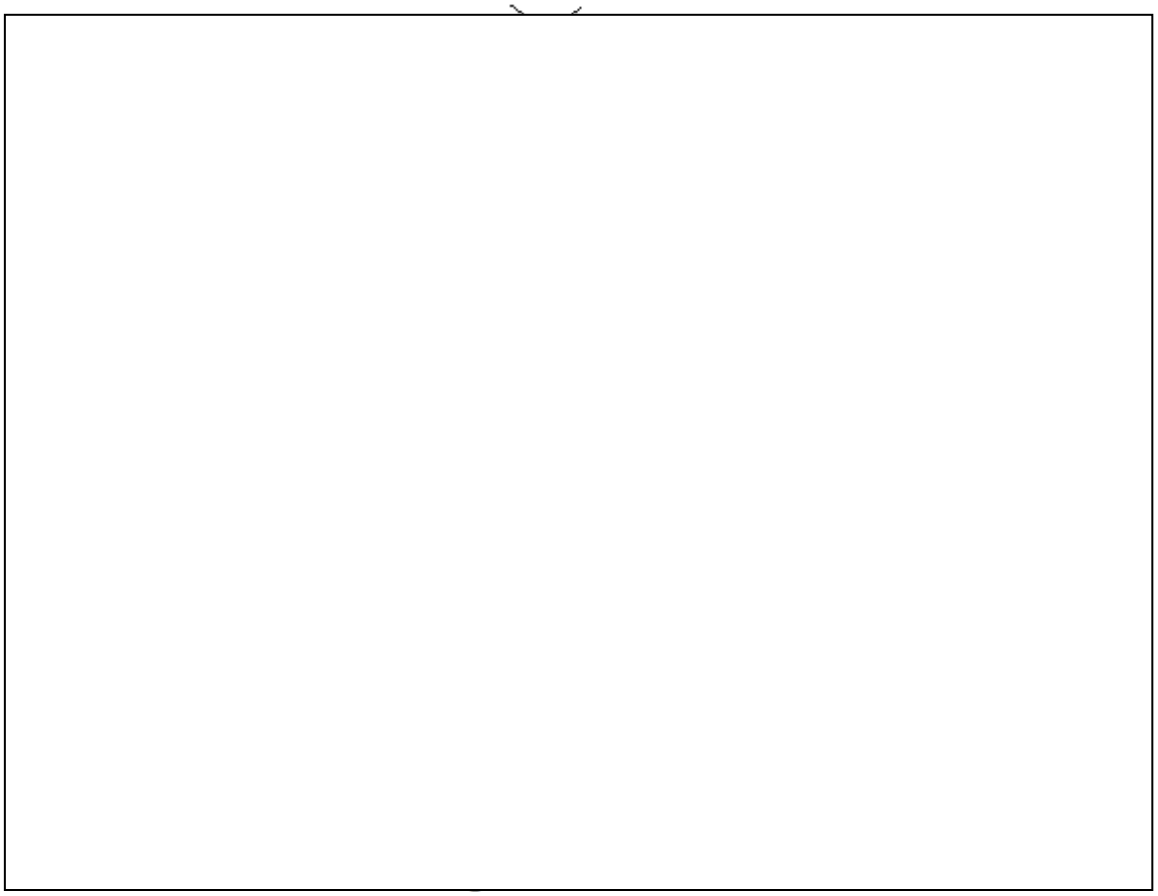
Figure 1-8: Free radical generation



1.4.2.2 Free radical propagation

The generated free radical monomer units then attack other functional monomers and the cross linking monomer to form polymer chains which trap the template molecules and form the basis of molecular imprinting. The schematic of the propagation stage is shown in figure 1-9 (figure was adapted from Brown, 2005)

Figure 1-9: Free radical monomer propagation



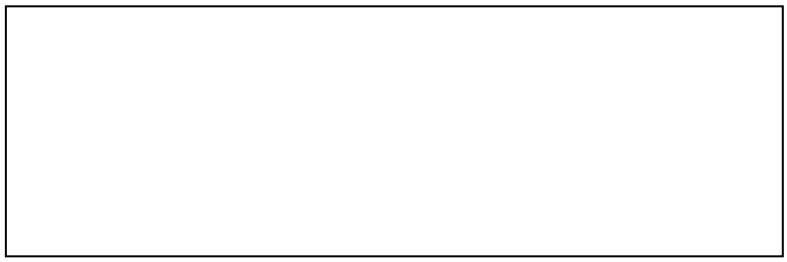
1.4.2.3 Free radical termination

The termination stage is the deactivation process of the free radical polymerisation. This process can occur when the reactive species, i.e. radicals, react by combination, disproportionation and transfer to monomer mechanisms.

1.4.2.3.1 Termination by combination

This occurs when two free radical active chain ends meet which couple together to form one long polymer chain as illustrated in figure 1-10 (figure was adapted from Brown, 2005). This could be two polymer chains with active ends or could be an active end of the polymer chain with a free radical initiator.

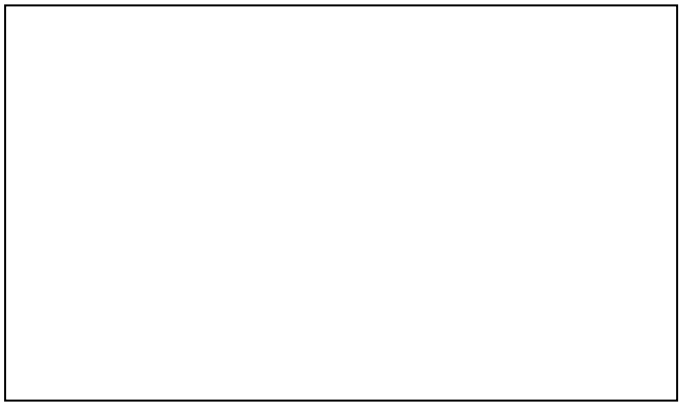
Figure 1-10: Termination by combination



1.4.2.3.2 Termination by disproportionation

Disproportionation takes place when α-hydrogen atom from one propagating polymer chain end is abstracted by another polymer chain as illustrated in figure 1-11 (figure was adapted from Brown, 2005). This allows the generation of a vinyl unsaturated group that can react with another radical to re-initiation polymer chain and one dead end polymer with a terminal saturated group that is halted from further growth.

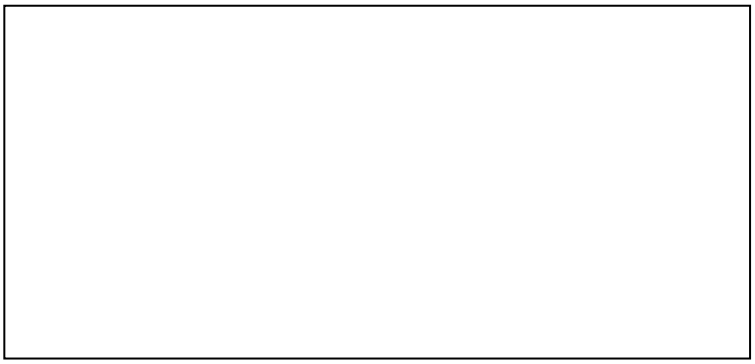
Figure 1-11: Termination by disproportionation



1.4.2.3.3 Termination through radical transfer to monomer

This method involves the termination or destruction of one polymer chain by transferring the radical from the growing chain to unreacted i.e. functional vinyl monomer therefore initiating the creation of new monomer radical, this is shown in figure 1-12 (figure was adapted from Brown, 2005).

Figure 1-12: Termination by radical transfer



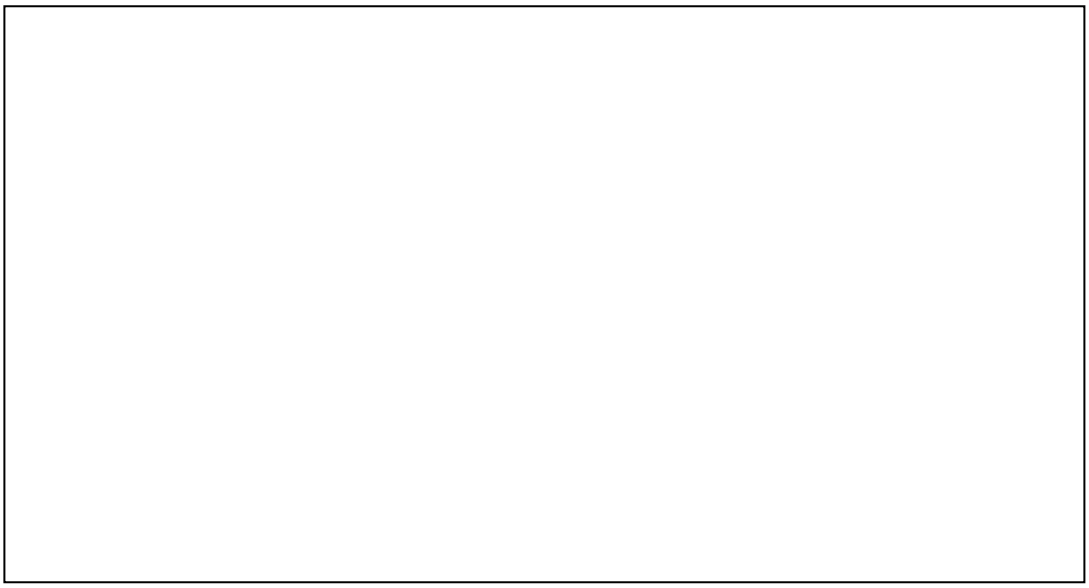
1.4.3 Post polymerisation process

After polymerisation, the monolithic polymer produced is ground into a fine manageable particle size and sieved to produce on average particle size distribution ranging from 20-50 μm. Finally, the trapped template is extracted from the polymer matrix using an appropriate extraction technique. Typically the approach used to remove templates from polymer is by solvent extraction which has been extensively documented in the literature. For example, for non covalent templates, the solvent used should be able to break the weaker bonds that formed in the pre-polymerisation complex. For templates bound covalently, extraction solvents should be employed to break the covalent bond formed. After template extraction, the site will be left vacant and will be used for subsequent rebinding of the targeted template. The specificity and selectivity of the polymer towards template and not for template analogues will prove the polymer successful and will determine the fate of polymer application.

1.5 Morphology of binding sites

A molecular imprinted polymer has a very complex structure. It is generally highly cross-linked and sometimes has pores of different sizes and shapes. Despite their vast potential, MIP applicability has so far been limited possibly due to the poor binding characteristics arising from the complex morphology and chemistry of the binding pockets (Spivak and Shea, 2001). Understanding the architecture of binding sites is critical for the prediction of the template binding. However, this has been quite a challenge due to the amorphous nature of the polymer which limits the possibility to visualise structure using standard crystallography techniques (Simon *et al*, 2007). The heterogeneous distribution of high and low affinity sites further adds to the problem (Spivak, 2005). Spivak, (2005) attempted to show a possible mechanism for binding site formation from a single molecule with two functional groups available for complexing with functional monomers. He described functional monomers rearranging around the molecule forming a monomer-template complex and contributing to a single site. Depending on how this occurs, it can be either a high or low affinity site. The schematic of hypothetical binding sites for a molecule with two functional groups interacting with a functional monomer is presented in figure 1-13.

Figure 1-13: Schematic representation of hypothetical binding sites



The dilemma envisaged ahead is how to predict the site formation mechanism for a template with many more functional monomers, such as biomolecules. The prediction of site formation is impossible for now but no one knows what the future holds and it might be resolved later with new emerging technology.

Another attempt was made to understand the behaviour of template binding. Simon *et al*, (2007) predicted that for a template with one or two functional groups interacting with monomer, the shape of the binding cavity was the prevailing feature for template recognition. In contrast, with a template containing more than two interacting functional groups, pre-organisation of functional monomers within the binding cavity was predicted to be the dominating feature for template selectivity. They suggested that MIP performance was better with templates having two functional groups compared to three, because shape selectivity was better than functional group selectivity, and these features do not work concurrently with each other. This effect is actually observed and small molecules with few functional groups are far better imprints as opposed to a multifunctional group template. They further added that for a template with three functional groups, MIPs performance should be improved with increasing distance between these groups

1.6 Conventional methods for evaluating and characterising MIPs

Molecular imprinted polymers are evaluated and characterised to determine their efficiencies and performances. There are a number of techniques that are commonly employed for binding site recognition behaviour, chemical compositions and physical morphologies of the polymers. This part of the review attempts to describe general protocols some of which have been applied in this work or are considered for future application.

1.6.1 High pressure liquid chromatography (HPLC)

HPLC has been used extensively in chemistry and related fields for separation, quantification and identification of compounds based on their polarity characteristics and interaction with column stationary phases. To describe briefly, chromatography involves distribution of compounds in two phases; the stationary phase and mobile phase. A typical stationary phase is composed of hydrophobic saturated hydrocarbons (reverse phase high pressure liquid chromatography) (RP-HPLC), which retains the compound and the retention time is measured with UV-VIS detector. The mobile phase is a solvent into which the compound is injected and distributed and transported through the column containing stationary phase, using a pump. The strength of interaction of the compound with either mobile phase, or stationary phase determine the retention time of the solute within the column. If the compound preferentially associates strongly with the mobile phase, it will be transported very quickly through the column and eluted very fast. In contrast, compounds interacting strongly with the stationary phase will be retained (retention time) and then finally eluted from the column with the mobile phase at a specific time. It can be assumed that at every point in the column a partition chemical equilibrium involving the non covalent forces acting between the solute molecules, mobile phase and the surface of the stationary phase is established. This is the principle behind the separation process of compounds in a mixture.

MIPs application as a stationary phase in HPLC has long been realised and extensively used for resolution of racemic mixtures and solid phase extractions. The imprinted and non imprinted polymers are packed into separate chromatography columns, and the sample template is injected. The retention time of the template on each polymer is measured relative to a known retention time of the solute referred to as a void marker or dead retention. The information obtained is used to determine a retention factor **(** k **')**; this is the affinity of the template for the stationary phase and is calculated as follows:

$$k' = (tr/to) - 1$$

Where, $\mathbf{t_r}$ = Time of retention time (template)

$\mathbf{t_o}$ = Time of dead retention (void marker)

The retention factor is determined for both MIPs and control non imprinted polymers (blanks), and it is used to establish specific from non specific template binding. The specific binding of the template is obtained by a normalisation method which eliminates the template binding by the non specificity known as imprinting factor **(IF)**; a modified partition coefficient. This is computed as follows:

IF = (*k' MIP) template / (k' blank) template*

Another useful parameter for determining polymer specificity is the selectivity factor α; commonly employed for enantiomeric molecules. This measures the selectivity of the template compared to structurally related analogues on the MIPs.
However, for non-enantiomeric template, the selectivity factor α of the polymer for the template and structurally unrelated molecules (template analogue) is determined by the specific selectivity factor (***S***). This is the ratio of the imprinting factor (**IF**) between the MIPs and blank polymer for the template and the analogues.

$$\boldsymbol{S} = \frac{(k' \text{ MIP) template / (k' blank) template}}{(k' \text{ MIP) template analogue/ (k' blank) template analogue}}$$

Organic solvents are typically used as mobile phase for resolving enantiomers for MIPs stationary phase; this is because of the fast diffusion kinetics and optimised MIPs performance are related to the memory of the solvent used during polymerisation. The organic phase is commonly used in a "normal phase chromatography" where the analyte binding to the stationary phase, is by polar interactions. The mobile phases are carefully tuned to discriminate the specific from non specific analyte binding, and to minimise

non specific interactions. For example, polar modifiers are added in the organic mobile phase where non specific binding is by polar interaction and vice versa; organic modifiers are used in a polar mobile phase where hydrophobicity is the cause of non specificity in the MIPs. Finally, to release the template from the polymer matrix, a mobile phase that interferes with the template polymer interactions is employed.
HPLC is a useful technique for determining the specificity, selectivity and binding capacity of the imprinted polymer. However, the technique is not straightforward because the mobile phase and flow rate have to be carefully optimised or else the affinity binding sites possessing slow rebinding kinetics might be obscured. In addition, the mobilisation of the template within polymer matrix might significantly influence the template binding.

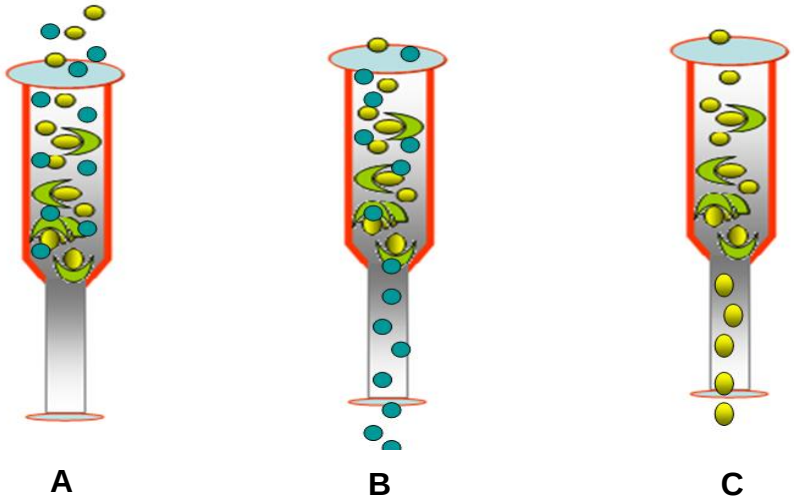
1.6.2 Solid phase extraction (SPE)

Solid phase extraction is routinely used in sample preparation and enrichment in analytical chemistry for the detection of trace and ultra trace amount of analytes. The technique is used to separate analytes of interest from a mixture by applying differential partitioning of compounds between solid and solvent phases.
SPE sorbents are employed based on the desired molecular interactions with the analyte; for example reverse phase adsorbents use hydrophobic interactions to extract analyte from aqueous, ion exchange SPE sorbents uses ionic interactions, and others combine both forms of interactions. Currently, the commercially available SPE are non specific and remove the group of compounds which have similar chemical interactions with the sorbents; the only specific sorbent is an immunosorbent which employs antibodies for specificity. Therefore, the use of (molecular imprinted polymer solid phase extraction) MIPSPE is seen as an alternative approach to produce specific sorbents with more appealing properties.
MIPs are packed into SPE cartridge and typical SPE procedures are followed, including the conditioning of the MIPs column with solvent to wet the polymer (conditioning phases), followed by the introduction of template solution mixed with other analytes

(mobile phase); the polymer is equilibrated with mobile phase to allow maximum template binding. Mobile phases used in MIPSPE are different for each template, for example pharmaceuticals, environmental and biomolecules templates; aqueous phase is commonly employed, other solvents such as organic solvents are typically used for small molecules and the techniques are rapidly becoming well established. For maximum specificity, mobile phases are carefully tuned to allow only a specific template to bind and maximally reduce non specific template interaction with sorbents.

After equilibrium is reached, the supernatant is removed from the column by filtration and the non specific bound analytes are washed off from the column using a carefully optimised solvent. The washing solvent should ideally be able to discriminate and remove only non specific analyte, leaving specific template bound. However, this in practice is difficult to achieve. Finally, the template is eluted from MIPSPE using a carefully selected solvent which disrupts the strong interaction formed between the template and sorbent, and which swells the polymer for ease of removal of the template. The SPE procedures are repeated for the non imprinted polymer and the specificity of the MIPs is determined. For template enrichment purposes, minimal elution solvent is used. The SPEs procedures are schematically presented in figure 1-14.

Figure 1-14: Solid phase extraction procedure



A. Application of sample template mixed with interferents, B. Washing off interferents, C. Elution of template.

1.6.3 Equilibrium batch rebinding

Batch evaluation of the imprinted polymers can be applied to polymers made by a non covalent as well as covalent approach, and is regarded as the better method for evaluating binding sites (Spivak, 2005). The technique can be used to measure specificity, selectivity, binding capacity of the specific template imprinted polymer, non imprinted and a competitive polymer imprinted with a template analogue similar to HPLC analysis. In addition, the particle heterogeneity is not an issue; different types of polymer configurations and size distributions can be used, with particle size ranging from 25-250μm. However, the technique is labour intensive and time consuming because the typical SPE procedures described in the following section are followed. In a selectivity assay, a known concentration of the template (C_t) solution is incubated with a known mass of the polymer and left to reach equilibrium. After equilibrium is established, the rebinding solution is separated from the polymer by filtration and the template left in the supernatant (concentration of free substrate (C_f) in solution) is measured by a standard analytical techniques such as UV-VIS or fluorescence spectroscopy. The amount of the template bound (C_b) to the polymer is determined by subtracting (C_f) from the initial template concentration (C_t) employed.

The amount of template bound from a single template solution is computed as follows:

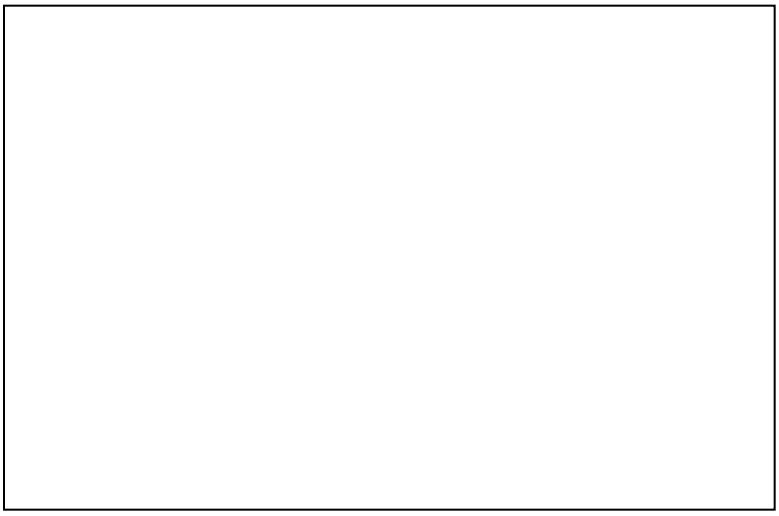
$$C_b = (C_f - C_t)/g \text{ (polymer)}$$

The equilibrium batch rebinding technique can also be used for a non competitive binding assay where a single template analogue is added in the rebinding medium and also in a competitive binding assay where both template and template analogue are added in the rebinding solution. For solid polymers, the amount of bound substrate is divided by the weight of polymer to give the amount of substrate bound per gram of polymer. Similar procedures are repeated for non imprinted and competitive polymers imprinted with template analogue. For showing specific template binding sites from non

specific sites, binding isotherms can be employed. Binding isotherms are represented by two graphs; a curvature for specific binding sites within the MIPs (solid line) and a straight line or a less defined curvature (dash line) which accounts for the existence of non-specific binding from the non imprinted polymer (figure 1-15) (The figure was modified from that of Spivak, 2005)

As for the HPLC, the binding specificity of the polymer is improved by mobile phase optimisation and by using additives such as organic and polar modifiers, depending on the nature of non specific interactions. For example, a mobile phase with a low polarity solvent; non-specificity can be minimised by the addition of a polar modifier; the changes will then be reflected by curvature in the binding isotherm.

Figure 1-15: MIPs binding isotherms for imprinted and non imprinted polymer



Another way to optimise conditions to determine the specific sites in the imprinted polymer is to increase the amount of polymer in solution; this will increase the number of specific binding sites and therefore increase template selectivity towards the imprinted polymer compared to the control polymer, and this might show change in the binding isotherms However, the amount of the polymer increased to the constant concentration of the substrate solution should be in range that will not cause template depletion to occur (Spivak, 2005).

In general, to show an imprinting effect is often cumbersome because most polymers show some degree of non specificity whether they contain binding sites or not, hence numerous tests are required. For example, several controls have to be prepared and processed similarly to the targeted MIPs and evaluated in conjunction with the imprinted polymers these include; non-imprinted polymer and competitive polymer prepared with template analogues. However, many papers have reported the imprinting effect using non imprinted polymer alone. This is not sufficient because in the absence of the template, the functional monomers have a tendency to self associate, forming dimers and trimers leading to the formation of non specific sites. Furthermore, the morphology and the physical characteristics of the blank polymer will differ in terms of porosity, surface area and polarity.

1.6.4 Physical and chemical characterisation

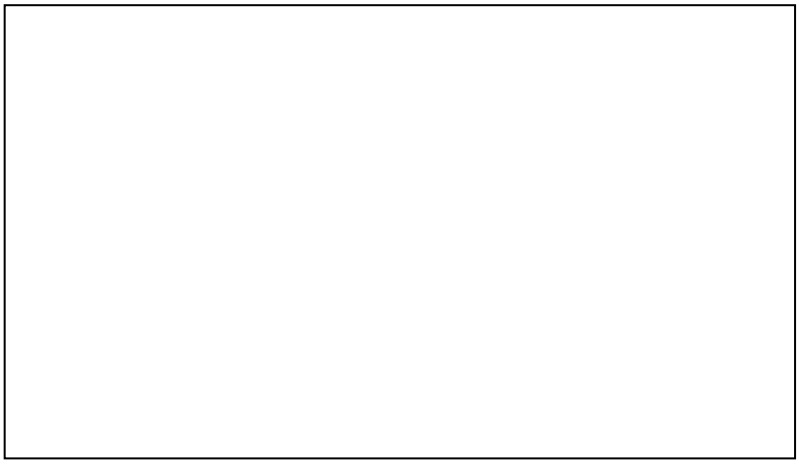
Imprinted polymers are cross linked networks that are porous. This allows mass diffusion of templates in and out of the polymer. Many imprinted polymers are solid and do not dissolve (traditional monolithic polymers). Therefore, the typical conventional polymer characteristic techniques such as gel permeation chromatography, solution NMR and UV spectroscopy, cannot be employed. In addition, the amorphous nature of the MIPs prevents the use of x-crystallography and microscopic technique to determine binding site structures (Simon *et al*, 2007)

The common techniques used for physical and chemical characterisations of MIPs include; surface area, pore size and distribution, polymer swelling and images from scanning electron and light microscopy which define the morphology of the MIPs. FTIR and solid state NMR spectroscopy, and micro-analysis are used for chemical configuration at molecular levels. Although the information provided by these techniques is limited, they are useful in governing design and application of the MIPs. For example, the swelling properties of the polymer are important in controlled release of a drug.

1.6.4.1 Surface area and porosity

The common feature of almost all MIPs is that they are porous with a very large surface area; this is an important feature if MIPs are to be applied as stationary phase in chromatography (Ousalem *et al*, 2000). The porosity and the surface area allow extensive interactions between the template and the polymer, hence improves the association and dissociation kinetics of the template within the polymer matrix. The surface area and the porosity distribution of the MIPs are controlled by the ratios and the type of cross linking monomer, porogen and the reaction temperature used. The polymer pseudo-phase diagram has been developed to give some insightful information onto the morphology of the polymer formed with variable concentration of the aforementioned compositions.

Figure 1-16: Polymer pseudo-phase diagram



The polymer pseudo-phase diagram identifies three different categories of polymer, i.e. gel-type polymers, macroporous polymers, and microgel powders (Cormack and Elorza, 2004).

In brief, a gel type polymer is formed from using a low volume of a thermodynamically compatible or good solvent regardless of the concentrations of the cross linking monomers; this is because phase separation does not occur between the solvent and the growing polymer chain. Consequently, the polymer formed has low surface area in the dry state, but when the polymer is in contact with a thermodynamically compatible solvent, it swells as a result of increasing distance between polymer chains leading to decreased mechanical stability. In contrast, using a high ratio of cross-link monomer and/or in the presence of high volumes of solvent, the growing polymer chain can separate from the solvent phase. This leads to formation of macroporous polymers with higher surface area and good mechanical strength. Finally, at much higher concentration of solvent beyond that used to form macroporous polymer, but still at high concentration of cross linkers, the more dilute conditions result in the formation of monodispersed microporous polymer or microgel powders; this is typically a precipitation polymerisation process.

Several techniques have been established for determining polymer porosity characterisation. These include mercury intrusion porosimetry, whereby the mercury is forced under pressure into a fixed amount of polymer in a dry state, a method sensitive to large pores (Cormack and Elorza, 2004), nitrogen sorption porosimetry (to be discussed) and inverse size exclusion chromatography, whereby the polymer porosity is characterised in a wet state, as opposed to the aforementioned techniques. The last-named offers additional useful information because MIPs commonly find application in solution. Other methods include small angle X-ray scattering, differential scanning calorimetry, and fluorine NMR spectroscopy (Ousalem *et al*, 2000). Surface area and porosity measurements in MIPs are routinely carried out by nitrogen adsorption porosimetry using BET (Brunauer, Emmett and Teller) analysis.
The procedure involves a fixed mass of dry polymer being subjected to nitrogen gas at a series of fixed pressures and low temperature. The sorption isotherm is then deduced from the gas sorbed on the surface of the pores as a function of applied pressure. The useful information obtained is specific surface area (m^2/g), specific pore volume (ml/g),

average pore diameter and pore size distribution. The technique is sensitive for analyzing medium-sized (meso-) and small sized (micro-) pores. The typical surface areas for imprinted polymers are in the region of 100 - 400 (m^2/g) (Spivak, 2005) but up to 700 (m^2/g) has been reported (Sellergren and Shea, 1993).

1.6.4.2 Scanning electron microscope (SEM) and light microscopy

SEM is routinely used to image pores and characterise the shape and size of polymer particles, and light microscopy is used to verify the structural integrity of polymer.

1.6.4.3 Polymer swelling and shrinking

MIPs change shape by swelling and shrinking in solvents and this influences the template binding and release kinetics. Such information is particularly useful for MIPs applications in molecular delivery. Swelling is due to flexible polymer chains moving away from each other in a thermodynamically compatible solvent. MIPs swelling behaviour can be measured using volumetric methods published by Sellergren and Shea (1993), but the problem with these techniques is that the polymer floats in chlorinated solvents. Another approach is to use a volumetric technique whereby the change in polymer volume is reflected by change in polymer size which can be captured using a microscope photograph before and after the polymer swells in solvent (Spivak, 2005). This technique is particularly useful for monodispersed homogeneous beads where an accurate size of the individual bead can be obtained. However, for heterogeneous particles, use of this technique is impractical and time consuming. Therefore, a gravimetric technique is used instead where a known mass of the polymer is weighed before and after incubation with the solvent and the solvent uptake by the polymer is measured (ml g^{-1}). This method is very crude for porous polymers because the solvent

taken up by the pores might not cause any swelling of the polymer, hence unreliable results are obtained.

1.6.4.4 Elemental micro-analysis

Elemental micro-analysis, as the name suggests, is used to measure the percentage by mass of specific elements within the polymers. Elements measured include carbon, hydrogen, nitrogen, and chlorine. The information obtained from the elemental analysis can be used to determine the percentage of a specific monomer in the polymer. For example, the nitrogen can be used to determine the composition of the heteroatom of 4-vinylpyridine in a co-monomer mixture of poly (4-vinylpyridine-co-ethylene glycol dimethacrylate). In addition, this method can be used to monitor changes of polymer compositions as a result of variables introduced in the polymer synthesis. However, the method is not very sensitive for the detection of trace amount of monomers or residual templates retained within polymers (Cormack and Elorza, 2004).

1.6.4.5 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy is useful for monitoring changes in chemical environment from the addition or removal of functional groups in the imprinted polymer (Cormack and Elorza, 2004). For example, the reactive or unreactive polymerisable double bonds species are reflected in FTIR spectra and can be analysed using standards, such as C–H out of plane bend at 900–950 cm^{-1} and the –C=C– stretch at 1639 cm^{-1}. Furthermore, FTIR results can be used to measure the degree of polymerisation by quantifying the number of unreacted double bonds using the integration of the area under the peak corresponding to a particular wavelength (Spivak, 2005). FTIR procedures involve incorporating polymer into a KBr disc (Cormack and Elorza, 2004) or using more rapid techniques based on

applying MIPs sample directly onto a diamond cell (Attenuated total reflection, ATR) for analysis.

1.7 Imprinting of biological molecules

The success of protein imprinting has been limited compared to small molecular imprinting because of the immense challenges presented by imprinting large molecules. Protein imprinting is the main interest of this thesis.
Proteins are large molecules with molecular weights ranging from 6000 Da to several million Da, and consist of many smaller units linked together via covalent bonds. Proteins have a large number of functional groups available on the surface that can potentially form template-monomer associations, leading to a large number of heterogeneous binding sites. Furthermore, proteins are often insoluble or lose activities in organic solvents and this limits the number and choices of reactive functional monomers and cross-linkers to use for imprinting purposes. In addition, proteins are very sensitive to polymerisation conditions, such as elevated temperatures, extreme pH and ionic strength. This might lead to protein denaturation, conformational changes or aggregation. Potentially, the denatured form of the protein might be captured during the imprinting, resulting in a polymer with non specific sites for the template.
Another problem is that the protein's large complex structure makes it difficult to move freely through a highly cross linked polymer matrix in order to access the binding sites hence resulting in poor binding and dissociation kinetics. Proteins are stable and active under physiological aqueous conditions and most biological recognition takes place under these conditions. However, artificial recognition of proteins in water is quite a challenge because water hydrogen bonding forces compete for binding sites.

The above factors, together with the high cost of obtaining pure proteins, make the route to effective and practical protein imprinting tortuous (Ge and Turner, 2008). However, protein imprinting research still continues and new techniques are emerging.

Protein imprinting using non covalent imprinting approaches will be reviewed in the following sections. Whenever possible we have attempted to use proteins as model molecules. However, when small molecules are used as examples, this means that we have not come across the literature regarding protein imprinting technique. Non covalent imprinting of proteins is elucidated in the form of three imprinting categories: bulk imprinting, surface imprinting and epitope imprinting.

1.7.1 Bulk imprinting

Bulk imprinting is the most simple and straightforward approach when compared to surface and epitope strategies. It involves imprinting of the whole template in a three dimensional matrix and then extraction of the template as whole molecules, from the polymer. There are a number of bulk imprinting techniques that have been developed for small and large molecules such as use of monoliths, polyacrylamide gels, hydrogels and sol gels.

1.7.1.1 Monolithic imprinting

Monolithic bulk imprinting is the most conventional technique commonly used in molecular imprinting due to its simplicity and universality. The method involves mixing all polymer components (template, monomer, solvent and initiator) together prior to polymerisation and is used almost exclusively with organic solvents. The yielded polymer is in the form of a monolithic block which is crushed, ground into a manageable size and sieved to produce particles in the desired size range. The size range is between 20 and 50 µm (Pichon, 2007) for application in chromatography and between 150-250 µm (Sellergren, 2001) for batch binding processes.

This imprinting technique has been very popular for small molecule separation using the polymer as a stationary phase in chromatography. For example, L-phenylalanine

imprinted stationary phase was prepared by free radical polymerisation for enantioseparation of chiral molecules L- from D- phenylalanine. Other molecules such as L-phenylalanine and L-phenylalanine-N-methylanilide have also been employed (Sellergren and Shea, 1995; Sellergren, 2001). Overall, this technique has been successful for enantioseparation of amino acids and their derivatives (Sellergren 2001), and for drugs and sugars (Schweitzer *et al*, 1998). Rachkov *et al*, (2000) successfully imprinted a steroid hormone β-estradiol in acetonitrile using common functional monomers MAA, EDGMA as cross linker and AIBN initiator in free radical polymerisation. The resultant polymer was reported to exhibit high selectivity for the template with sufficiently short template retention times when used in chromatography. Despite all the success, this strategy has received its share of criticism over the years because processing the monolith is time consuming, wasteful and cumbersome. The grinding and sieving process yields only moderate amounts of desirable product with non uniformity of particles. A large variation in shape and size of the particles leads to peak broadening and tailing (Schweitz *et al*, 1998), limiting its application in chromatography (Ansell and Mosbach, 1997; Ye and Mosbach, 2001; Sellergren, 2001). Substantial amounts of research work have gone into exploiting other novel monolithic synthetic strategies, such as monolithic capillary columns and, to a smaller extent, monolithic rods.

The imprinted polymers are prepared in one-step via in situ free-radical polymerisation, directly within a chromatographic column, yielding polymer monolith or film coating. This technique offers other benefits, such as reducing the bulk polymer processing steps and eradicating problems with particles heterogeneity. In addition, this approach eliminates the need to slurry and the irregular packing of ground monolith within a small diameter column which limits the length of the capillary column that can be used. Furthermore, the particles columns retaining frits, that hold the particles in place when a mobile phase is passed through, may introduce bubbles which prevent the column from working. In capillary column imprinting, these problems are avoided because the polymer is covalently attached to the inner surface of the capillary walls (Schweitz, 1998). Column monolith imprinting has been extensively researched for small molecule

imprinting. For example, MIP monoliths were prepared in a capillary column for enantioseparation of R- and S-propranolol, (S) metoprolol and (S) ropivacaine (Schweitz *et al*, 1998). Schweitz's group reported that the base line separation of propranolol and metoprolol could be carried out in less than 2 minutes. In addition the propranolol column could be used for separation of several other *β*-blockers, including prenalterol, atenolol, pidolol, and the ropivacaine column could also be used to separate other local anesthetics molecules, such as mapivacaine and bupivacaine, and Schweitz *et al*, (1998) also mentioned the separation of amino acid enantiomers of phenylalanine anilide. This technique has also been used for separation of theophylline from green tea, xanthine derivatives and caffeine (Sun *et al*, 2006). Sun's group showed that the polymer was able to separate caffeine and theophylline using imprinted column under isocratic and gradient elutions. In addition, they were able to successfully quantify the amount of caffeine in the green tea.

Other groups exploited a different imprinting approach such as monolithic polymer rod. Matsui and Takeuchi (1997) imprinted nicotine rod for separation of nicotine and reported that the imprinted rods showed higher binding capacity of the template molecule than non imprinted polymer. They also mentioned that they previously used this technique to prepare polymers imprinted with phenylalanine anilide, diaminonaphthalenes and xanthines, and the resultant polymers showed selectivity for the imprinted templates. However, the group used polar solvents for inducing micropores in the rods. Such solvents are known to interfere with the complex formation between the template and functional monomers, hence the MIPs demonstrated low retention capacity compared to a conventional monolith where a non polar porogen is commonly employed. Another drawback with this technique is that it suffers from high back pressure and low efficiency which limited their appliacation in separation technology (Huang *et al*, 2003). In addition, monoliths have been successful for many small molecules compared to MIPs rods. In effect, this rendered the MIPs rod unpopular with limited research effort being devoted to this type of imprinting.

1.7.1.2 Gel imprinting

Other bulk imprinting techniques using gels such as polyacrylamides and their derivatives for protein imprinting have shown great potential. Hjerten and co workers (Liao *et al*, 1996) imprinted bovine cytochrome C and human haemoglobin using acrylamide and N, N'-methylene bisacrylamide as functional monomers, N'N' tetramethylethylenediamine (TEMED) and ammonium persulfate as the initiator. The resultant imprinted gel showed high preferential affinities for their templates and no affinity with the blank polymer. In addition, the imprinted polymers were very specific towards their templates compared to the competitive proteins RNase A and myoglobin under competitive rebinding conditions. The group suggested that the main form of selectivity was a result of hydrogen bond formation between the templates and binding sites in aqueous solution.

In the following year, Hjerten *et al,* (1997) imprinted RNase A and human growth hormone using similar techniques. Both polymers showed excellent selectivity and specificity for their template. Furthermore, they attempted to imprint three proteins; cytochrome C, haemoglobin and lysozyme together in a single polymer. The tri-protein imprinted polymer was very selective towards all the templates and no affinity was reported for the non imprinted polymer. In addition, the group demonstrated that horse haemoglobin imprinted polyacrylamide was very specific such that it could distinguish whale haemoglobin from that of the horse haemoglobin despite their structural similarities. The group suggested that the lack of protein binding onto the non imprinted polymer might be due to the poor protein-gel interaction caused by the inert behaviour of the gels. In effect, this led to poor protein complexing because of a low number of binding sites contributing to poor loading capacities.

The work of Hjerten's group is an excellent demonstration of protein selectivity and specificity, which are the fundamental principles of molecular recognition. However, the gels have poor flow rates, which makes them unfit for high flow rate chromatography, poor binding capacity and like most soft gels their low cross linking density made the

regeneration properties unfavourable. Furthermore, they exhibited incomplete protein desorption even though the polymers possessed large pores, and even after washing with a strong surfactant (SDS). They could not identify reasons for this effect but ruled out the covalent attachment of the templates. Some of the issues, such as low flow rates, low binding capacity and reusability, were further addressed by Hjerten's group (Tong *et al*, 2001) when they immobilised the polyacrylamide gels and protein in rigid agarose gels followed by polymerisation. The group reported that the subsequent polymer showed excellent improvement in the range of problems addressed.

Another approach to polyacrylamide imprinting was by Huang *et al*, (2005) who imprinted lysozyme using both tertiary amine and carboxylic acid monomers to create amphoteric gels for use as a chromatography stationary phase. The group cleverly added solid $CaCO_3$ powder in the pre-polymerisation mixture of *N*-[3(dimethylamino) propyl] methacrylamide and methacrylic acid, and *N*,*N*-ethylenebisacrylamide (cross linker) to create large pores that would facilitate the diffusion kinetics of the proteins in the polymer matrix and to prevent permanent entrapment of the templates. The group reported that lysozyme preferentially bound to its imprinted polymer, and none bound to the non imprinted polymer. In the cross selectivity study with BSA imprinted polymer negligible binding was noted with lysozyme.

The polyacrylamide protein imprinting discussed above has shown good potential in terms of selectivity and specificity, but the gels are unattractive because of lower binding capacities and the softness of the polyacrylamide gel. This results in poor regeneration properties and after several times of using and washing, the selectivity of the gel is lost (Pang *et al*, 2005).

In an effort to further search for better gel qualities, some researchers have focused on hydrogels and sol-gels.

Hydrogels are three dimensional cross linked hydrophilic polymers that change shape and volume in response to external stimuli. For example, they can swell in liquids such as when in contact with water through absorbing significant amounts of water and are able to retain their shape without dissolving. They are very sensitive and can be made responsive to external stimuli such as light, temperature, solution pH, magnetic field, radiation, solvent composition, electric field, stress, and finally the recognition existence of molecules in solution (Byrne *et al*, 2008).

The favourable qualities of the hydrogels have been extensively exploited for template recognition; such as polymer changing shape in response to the template binding. For example, Chen *et al*, (2008) imprinted lysozyme using N-isopropylacrylamide (NIPA) as major monomer, methacrylic acid (MAA) and acrylamide (AAm) as functional co-monomers, and N,N-methylenebisacrylamide (MBAAm) as cross linker. The group reported that the imprinted polymers demonstrated high affinity for the template compared to non imprinted polymer, especially at low template rebinding concentrations. They noted that the imprinted polymer changed shape by shrinking to a specific volume in order to accommodate the template. This effect was not observed with non imprinted polymer. The group suggested that the shrinking behaviour of the polymer was due to the synergistic effect of multiple-site weak interactions (electrostatic force, hydrogen bonding and hydrophobic interaction). However, at higher template rebinding concentrations, a small reduction in polymer volume was also observed with non imprinted polymer. Under competitive binding conditions with seven different protein analogues, lysozyme MIPs showed template specificity but relatively small non specific binding of other proteins was also observed.

For small molecule imprinting, hydrogels have been used for separation of optical isomer derivative of amino acid. Liu *et al*, (1998) used sugar acrylate monomers for separation of amino acid optical isomers, such as D-isomer (CBz-D-Asp) and L-isomer (CBz-L-Asp). The group reported that the imprinted polymers demonstrated a separation factor of 1.5 for the D-isomer (CBz-D-Asp) when (CBz-D-Asp) imprinted polymer was used and 0.3 for L-isomer (CBz-L-Asp) when (CBz-L-Asp) imprinted polymer was

employed, while none was observed with non imprinted polymer. The sugar based monomers provided a high density of hydroxyl groups. However, the presence of water in the rebinding medium resulted in poor resolution between D and L isomers from both imprinted polymers because the MIPs swelled in water, altering the conformation of the imprinted sites. Another example of small molecule hydrogel imprinting was obtained for glucose. Briefly, glucose phosphate monosodium salt (glucose modified with phosphate group) was added to poly (allylamine hydrochloride) and cross linked with EGDE (ethylene glycol diglycidyl ether) to form imprinted gels (Wiseman *et al*, 2001). The addition of the amine group allowed ionic interaction as well as hydrogen bonding with the phosphate group and improved water solubility. Wiseman's group reported that the magnitude of the glucose rebinding onto the imprinted polymer was three orders of magnitude higher than non imprinted polymer and approximately two orders of magnitude greater compared to the competitive molecule fructose. This technique has been used for imprinting bioactive substances such as lectin-imprinted gels to recognise saccharide (Peppas, 2002), and also used for imprinting of large molecular assemblies, such as viruses (Bolisay *et al*, 2006) and cells (Bacskay *et al*, 2006).

The drawback with hydrogels is that the polymer is very flexible and expandable because it has plasticity in the structural chains and are therefore easily collapsible (Byrne *et al*, 2008). Hydrogels with a high concentration of hydrophilic moieties tend to swell more than gels with a high concentration of hydrophobic groups and are therefore susceptible to collapse. In addition, if the hydrogel is put in a thermodynamically non-compatible solvent, it will also collapse. For effective MIPs, the conformation of the imprinted site must be in a state similar to when the imprint was formed. If significant changes occur as a result of swelling or collapsing, they potentially comprise the selectivity of the polymer.

Sol-gels are another group of smart polymers that are prepared at ambient conditions to form colloidal suspensions of silica particles that are gelled to form a solid. The process

involves converting liquid ‘sol’ (mostly colloidal) system into a solid ‘gel’ which is then dried (Gupta and Kumar, 2008; Ge and Turner, 2008).
Sol gel imprinting involves silicon or metal alkoxide, water and a second miscible solvent (mostly alcohol) in the presence of acid or base catalysts, and use both hydrolysis and condensation reactions simultaneously once the hydrolysis reaction is initiated (Gupta and Kumar, 2008) .

Sol gel imprinting is not new. Dickey (1949) prepared sol gel matrix for imprinting alkyl orange dye and he observed that adsorption of template methyl orange was greater in imprinted silica gel compared to non-imprinted gel (Alexander, 2006). This technique has advantages of water involvement and mild conditions (i.e. pH and ionic strength) and so has been exploited for protein imprinting, such as BSA, haemoglobin, myoglobin and urease (Ge and Turner, 2009).
Lee *et al*, (2007) imprinted lysozyme and RNase A in macroporous polysiloxane silica scaffolds using sol–gel processing. The imprinted scaffolds exhibited preferential template binding of approximately three orders of magnitude higher in single template solution when compared to non imprinted polymer, and also under competitive binding with template analogues. Non imprinted scaffold bound lysozyme and RNase A similarly. However, all the imprinted polymers demonstrated some non specific binding towards non template proteins.
The problem with non specificity was not observed with small molecule imprinting using a sol gel scaffold. Tao *et al*, (2006) developed a novel interleukin-1α sensor from the sol gel derivative, xero-gel. The sensor was developed for the detection and quantification of interleukin-1α by monitoring changes of luminescence upon template binding. The luminescence molecule was cleverly tagged within the imprinted sites. The resultant polymer was selective and able to distinguish the imprinted porcine interleukin-1α from human interleukin-1α, and also was able to quantify the template bound (Ge and Turner, 2008).

As discussed above, various approaches have been researched for the construction of imprinted sites using bulk imprinting. The advantage of this approach is that the whole imprinted molecule is entrapped in the polymer matrix and can be removed and finally re-introduced into the three-dimensional cavity polymer network. The small molecular imprinted monolith and protein imprinted gels have shown many interesting qualities, such as template selectivity and specificity.

Because of the problems with bulk imprinting of biomacromolecules such as proteins, much focus was directed towards alternative imprinting strategies, such as surface imprinting and epitope imprinting.

1.7.2 Surface imprinting

Surface imprinting is an active area of protein imprinting research as it produces favourable binding and dissociation kinetics even for macromolecule templates. This is because the imprinted sites are exposed on or are in close proximity to the surface. Shi *et al*, (1999) developed a novel surface imprinting technique, where a template (protein) was immobilised onto a flat mica surface from aqueous solution. After protein adsorption onto the surface of the mica, a disaccharide layer was spin cast to cover the surface of the protein. The sugar molecules are a good source of hydroxyl groups and could potentially allow formation of hydrogen bonding between the template and the polymer binding sites. The whole sample was then exposed to a radio-frequency glow-discharge in the presence of hexafluoropropene. This led to a polymer layer being overlaid above and around the sugar covering the protein. Finally, the whole sample was mounted onto a second support via an epoxy resin layer and the mica was peeled off to expose the imprinted sites. The group reported that the shape of the imprinted site was complementary to template and the disaccharide groups oriented the molecule to fit within the cavities. The group reported that the binding sites were selective towards target templates. For lysozyme and RNase A imprinted polymers, the selectivity was between 20-26 fold greater towards their imprinted template under competitive binding conditions used with other protein analogues.

Furthermore, the group imprinted several other proteins, such as bovine serum albumin (BSA), bovine fibrinogen, bovine ribonuclease A, bovine IgG, chicken lysozyme, and bovine α –lactalbumin, using radio frequency glow discharge plasma deposition of thin fluoropolymer films around proteins coated with disaccharide molecules (Shi *et al*, 2000). The group reported again that they were successful in designing nano scale binding cavities on the surface of the polymer that has shape and chemistry complementary to the imprinted protein. They described the mechanism of their polymer recognition as due to a Vroman phenomenon between surface-adsorbed protein and solution-phase protein, whereby any low affinity protein adsorbed onto a recognition site is displaced by a protein with high affinity for the sites. However, problems with non specific proteins adsorption were greater with polymer imprinted with soft protein α - lactalbumin. The group suggested that the non specific binding observed was due to the conformational instability of the protein when immobilised onto the mica surface prior to the polymerisation. For α-lactalbumin imprinted polymer, the non native conformation of the protein might have been captured in the imprinting process and rendered the polymer unfit for recognition. This suggests that in order to get functional MIPs, the true conformation of the template must be available for imprinting. Otherwise if the template structure is not preserved or is unstable, with the template acquiring different conformations, polymers with poor recognition properties will result. The group suggested that imprinted polymers could be applied to fabricate engineered biomaterials that could control adsorbed protein at the interface with precision and accuracy.

Another imaginative imprinting technique is protein imprinted onto the surface of nanowires. Inspired by the idea of sol gel chemistry, Li *et al*, (2006) immobilised haemoglobin, bovine serum albumin, cytochrome C and horseradish peroxidase onto the surface of the nanowires in a nanoporous alumina membrane. The membrane nanopores were filled with gel forming functional monomers, cross linker and initiator, and the polymerisation was carried out by oxidation inside the nanopores. The resultant polymers demonstrated high selectivity, specificity and adsorption capacity towards the biopolymer under preferential and competitive binding conditions. The specificity of the

polymers was as a result of cooperative and multivalent hydrogen bonding, and shape selectivity of the imprinted cavities, while the high protein adsorption was a result of the large surface area provided by nanowires. The group commented that their polymer nanowires disperse well in water, which could potentially be applied to affinity chromatography and biosensors.

Another surface imprinting approach was grafting of protein imprinted thin film made of 3-aminophenylboronic acid onto microplate wells (polystyrene microtitre plates) by aromatic ring electron pairing interactions. Bossi *et al*, (2001) used this approach for imprinting horseradish peroxidase. The group demonstrated that the imprinted polymer showed high affinity for the template compared to non imprinted polymer. Furthermore, they investigated other proteins, such as lactoperoxidase, haemoglobin, cytochrome C and microperoxidase. The rebinding behaviour of each imprinted protein was reported to be different compared to the blank. However, proteins like cytochrome C could not be imprinted using this technique because the net positive charged cytochrome C was repulsed by the net positive charge of the aminophenylboronic acid hence, no template rebinding difference was observed between the imprinted polymer and the blanks. However, they successfully showed that their polymer could discriminate between Hb and its glycosylated derivatives in cross selectivity assays. The group explained that the specificity of the Hb polymer was due to the electrostatic interaction between the protein and polymer in combination with shape selectivity. They further commented that the template rebinding was dependent on the number of polymer washing cycles but it reached a certain point where further washing had no effect on the protein adsorption. The residual templates therefore had no influence on the recognition behaviour. This issue is still contradictory and no definite conclusions have been drawn yet.

In another experiment, the group compared the rebinding affinities of the imprinted film immobilised onto microplates and unimmobilised film. The template affinity for the immobilised films was 100 times more compared to unimmobilised films.

In further attempts to increase the binding capacity of imprinted thin films, Yan *et al*, (2007) grafted lysozyme mixed with 3-aminophenylboronic acid monomer solution films onto the surface of microsphere particles produced by suspension polymerisation

This technique offered the advantage of improved surface diffusion kinetics of a thin film, while the microspheres provided large surface area for high adsorption capacity. The group successfully demonstrated template selectivity and high adsorption capacity with the imprinted polymer compared to a blank, and template specificity was observed under competitive binding with other proteins in solution. However, non specific binding of other proteins was equally encountered in both imprinted and blank polymer. Under cross selectivity experiments with haemoglobin imprinted polymer, the group demonstrated that lysozyme and haemoglobin bound excellently to their respective imprinted polymers as opposed to a different polymer.

Still on the subject of polymer grafting onto the surface of beads, Fu *et al*, (2007) compared grafting of pre-polymerised BSA-acrylamide with *N'N'*methylenebis acrylamide onto a chitosan bead in acid aqueous solution with non grafted BSA imprinted gels. The acrylamide-chitosan beads produced better MIPs than non grafted BSA gel. Surface imprinting is envisaged to offer solutions to template diffusion and dissociation kinetics problems because it exposes the imprinted cavities on the surface. The surface imprinting approach has not only been used for biomacromolecules but also for larger molecular assemblies, such as yeast cell and tobacco mosaic virus (Dickert *et al*, 2002; Hayden *et al*, 2006). However, it suffers significantly from problems of non specific binding, low selectivity (Ge and Turner, 2008) and low binding capacity (Li *et al*, 2006).

In searching for better techniques, some research has been carried out on 'epitope imprinting', which combines the concepts of both surface and bulk imprinting.

1.7.3 Epitope imprinting

This approach to molecular imprinting involves imprinting an 'epitope'- a small exposed peptide fragment that can be used to represent a whole protein molecule. Epitope imprinting generally mimics the antibody-antigen system, whereby the antibody recognises a particular antigenic site (epitope) on the protein.
Rachkov and Minoura (2000) and (2001), inspired by this rationale, developed a novel epitope imprinting strategy for oxytocin molecule (a neurohypophyseal hormone). They used a tetra-peptide fragment of amino acid sequence Try-Pro-Lue-Gly-NH_2 to represent oxytocin molecule of amino acid sequence Cys–Tyr–Ile–Gln–Asn–Cys–Pro– Lue-Gly-NH_2. The resultant polymer showed good selectivity not only towards the imprinted peptide but also to the oxytocin molecule and other peptides having similar amino acid sequence (Pro-Lue-Gly-NH_2). Also, Rachkov *et al*, (2004) imprinted a larger size peptide fragment with eight amino acid sequence (octapeptide) for angiotensin II hormone. The imprinted polymer showed excellent recognition for the imprinted octapeptide but not for angiotensin II hormone.

Another group used an even larger epitope fragment comprising 16 amino acid residues and compared this with bulk imprinting of a whole lysozyme C molecule in porous silica scaffolds made by sol gel imprinting (Brown and Puleo, 2008). The preferential binding studies for whole protein and peptide-imprinted scaffolds showed similar affinity for the lysozyme C and both exhibited poor affinity for RNase A, when single protein solution was used. However, under competitive binding conditions, whole lysozyme C imprinted scaffolds bound lysozyme more strongly compared to RNase A whilst peptide-imprinted scaffolds failed to show any significant binding variation. To account for this effect, the group suggested that the lysozyme C needed to be oriented in a specific manner in order to fit into imprinted cavities. Thus, the steric effect was a limiting factor and consequently led to poor binding capacity for the peptide imprinted polymer.

Another excellent epitope imprinting strategy was presented by Shea and co-workers (Nishino *et al*, 2006). The group ingeniously used peptide fragments synthesised in house for cytochrome C, alcohol dehydrogenase and bovine serum albumin to carry out imprinting. The resultant polymer film showed excellent specificity and affinity towards imprinted template compared to non-imprinted polymer. The specificity of the polymers was attributed to hydrogen bonding and hydrophobic interactions, leading to a low number of non specific sites. In addition, the group reported that the technique was very sensitive, such that it discriminated mutant BSA peptide (same peptide with only one amino acid change) from normal BSA.

Epitope imprinting has several advantages over whole molecules in that the peptides are more stable in organic solvents and are economically cheap. However, the prior knowledge of the epitopic fragment molecules is essential. In addition, some fragments might not be readily available. Therefore, purification or modification might be necessary, which might be hard to do or expensive to manufacture. Another limitation is that the resulting polymers have low binding capacities compared to whole molecule imprinting. This is because of the problem of steric binding as opposed to the whole template binding in a multitude of orientations (Brown and Paulo, 2008).

1.7.4 Beads imprinting

In order to produce homogeneous uniform particles to improve mass transfer kinetic and for homogeneous particle distribution for chromatography application, researchers have looked into different types of particle formats and polymerisation techniques. These techniques of suspension, emulsion and precipitation polymerisation are elucidated in the following section.

1.7.4.1 Suspension polymerisation

This technique involves oil in water suspension, whereby water is a continuous phase for suspending pre-polymerisation droplets (template molecule, functional monomer, cross linker and initiator) in the (20-200 μm) range. These are maintained in suspension by mechanical stirring in the presence of additives such as stabilisers or surfactants which confer stability to the droplets. However, water interferes with hydrogen bonding formation between template and monomers and causes the partitioning of these components into the aqueous phase. For these reasons, the classical preparation of suspension beads is not readily applicable in molecular imprinting and instead perfluorocarbon (PFC) has been used (Mayes and Mosbach, 1996). A chemically inert organic solvent commonly used in bulk polymerisation is dispersed in the PFC and each droplet acts like a mini-bulk reactor (Moral and Mayes 2004). However, PFC is costly and the method requires custom made perfluorinated stabilisers for droplet formation and stabilisation. To avoid this, mineral oil has also been employed as continuous solvent for polar solvent because it eliminates the use of additives. This solvent composition was initially applied for imprinting propranolol (Kempe and Kempe, 2006). Uniform spherical particles have been employed in conjunction with protein imprinting of polyacrylamide gels to avoid the problems encountered in bulk imprinting, such as particle heterogeneity and bulk polymer processing. Pang *et al*, (2005) and (2006) used an alternative approach to protein polyacrylamide imprinting, starting with spherical gel beads of high cross linking density made by inverse-phase seed suspension polymerisation. This involved using high density cross linked beads as core material and low density polyacrylamide as imprinted shell for imprinting BSA. The group reported that the BSA gel beads demonstrated much higher adsorption capacity towards BSA, with high separation factor and good regeneration properties compared to non imprinted polymer. The recognition feature observed with imprinted beads was suggested to be as a result of multiple hydrogen bonding and shape complementary cavities induced by the template, which was absent with non imprinted beads. After thorough study to determine

the effect of cross linking concentration and the polymer reusability, the group reported that better regeneration was observed with increasing cross linking density. A similar strategy was used by Pan *et al*, (2009) for *Staphylococcus aureus* protein-A imprinting and was compared with protein imprinted bulk polyacrylamide. The group reported that the imprinted gel beads showed better specificity and high adsorption capacity compared to the non imprinted gel beads and bulk imprinted gel.

In general, a brief review of protein imprinting using polyacrylamide gels suggests gel beads have much superior qualities in terms of specificity and adsorption capacities compared to bulk gels. In addition, they avoid polymer processing and particle heterogeneity, which are not suitable for many applications. On the other hand, bulk imprinting is simple with fewer polymerisation steps than for imprinted gel beads.

1.7.4.2 Core shell particles by emulsion polymerisation

Molecular imprinted surface beads with a core-shell structure are produced through a core-shell emulsion polymerisation process. This technique involves a two-stage process. The first stage is to prepare a core or a seed particle material, the seed particle (which may be cross-linked). This would provide the mechanical stability of the imprinted core-shell particles. The second part involves the creation of the imprinted shell by emulsion polymerisation enabling imprinted sites to be positioned near or at the surface of the beads. The resulting core-shell particle provides the structure which allows the immobilisation of template or incorporation of drug releasing properties or any other useful product super paramagnetic without interfering with the imprinted shell (Tan *et al*, 2007). The continuous medium during polymerisation is water and surfactants are used to stabilise the emulsion. For example, sodium dodecyl sulfate or special polymerisable solvents, such as pyridinium 12-(4-vinylbenzyloxycarbonyl) dodecanesulfate or pyridinium 12-(cholesteryloxy carbonyloxy) dodecanesulfate, are used (Perez *et al*, 2001). The latter acted as a surfactant and a template for preparing a

cholesterol imprinted shell. Monodispersed particles are typically produced in a size range from 60-70nm (Moral and Mayes, 2004).

In general, both suspension and emulsion polymers have drawbacks in that they have lengthy polymerisation procedures and requires extensive optimisation processes (Ye *et al*, 1999). Another limitation is residual surfactants, which if not completely removed, might compromise the recognition ability of the MIPs (Wang *et al*, 2003).

1.7.4.3 Precipitation polymerisation (stabiliser free dispersion polymerisation)

The disadvantage of using surfactants and stabilisers, and the lengthy procedures required for suspension and emulsion polymerisation has led to the development of precipitation polymerisation techniques. These are similar to dispersion polymerisation but free from additives. The technique is simple and straightforward. It involves the addition of polymer components, such as template, monomers, and cross linkers, in a much larger amount of solvent than typically used in bulk polymerisation. The diluted monomer solution system allows the growth of polymer chains but because they are unable to occupy the entire volume of the vessel, a dispersion of microgel particles precipitates in the continuous phase. The microgels are formed by growing polymer chains which as they extend become more and more insoluble in the organic solvent (Ye and Mosbach, 2001). The high cross linking density produces rigid particles that do not coalesce hence there is no need to use stabilisers. This strategy has previously been used for imprinting theophylline and 17β-estradiol, with average particles size ranging between 0.2-0.3 µm (Ye *et al*, 1999). With slight modifications of the procedure, the Cormack group produced larger monodispersed imprinted particles on a 5µm scale for separation of theophylline (Wang *et al*, 2003). By 2009, the group had succeeded in doubling the particle size to 10µm for the detection of the antiepileptic drug carbamazepine and its main metabolite oxcarbazepine in urine (Beltran *et al*, 2009). One drawback with this technique is that it requires large amounts of solvent compared to monolithic technique, and therefore, large amount of the imprint molecule because of

the large dilution factor. However, this is compensated for by the high polymer yields (Haupt, 2001).

1.8 Molecular imprinting applications

More recently, the continued trend in this research area has been not only to develop new imprinting methods, but also much effort has been directed towards the potential applications.

1.8.1 Capillary electrochromatography & electrophoresis

Many applications for MIPs were initially intended for chromatography purposes because they use similar principles to conventional chromatography. For example, MIPs can be used as stationary phases for chiral separation (Haupt, 2001), enantioseparation of amino acid and their derivatives (Sellergren and Shea, 1993 and 1995), peptides and drugs in pharmaceutical industry (Schweitz *et al*, 1998; Ye *et al*, 1999). However, MIPs suffer from slow association and dissociation kinetics, resulting in a low peak and a marked sensitivity to the amount of sample introduced into the column.
MIPs application in capillary electrophoresis (CE) has received attention recently for resolving small molecule enantiomers. The MIPs integration in the CE can be through in situ generation, such as polymer coated on a capillary inner wall or by packing the polymer beads into the CE tubing system. The technique has been used for resolving recemic mixtures, such as separating S-(+)-phenylpropionic acid from R-(-)-2-phenylpropionic acid by in situ coated MIPs. However, in situ generation of the MIPs in the capillary column limits the understanding of the polymer morphological characteristics and a small change in the parameters might cause a dramatic change in the MIPs function. Therefore, reproducibility becomes essential. However, MIPs produced outside the capillary tend to be more difficult to pack in the capillary and also the narrow capillary tube diameter tends to reduce the loading capacity of the MIPs material.

1.8.2 Solid phase extraction

Solid phase extraction (SPE) is routinely used in analytical chemistry for sample preparation, sample enrichment for quantification purposes and extraction of target molecules or compounds from a complex mixture. It is also employed for cleaning up processes to remove impurities from chemical and biochemical synthetic products. However, the need for better methods in medical, food and environmental analysis is ever increasing. MIPs for MIPSPE are cheap and simple to produce, provide faster analytics, are stable over extreme conditions (high temperature, pressure and harsh organic solvents), allow small sample size and the results are reproducible.
MIPSPE can be applied directly to extract an analyte of interest from blood plasma and serum, urine, bile, liver extracts, chewing gum, environment, water and sediment and plant tissues (Haupt, 2001). For example, MIPs adsorbent has been used for solid phase extraction of atrazine from beef liver using chloroform and the pre-concentrated atrazine was eluted using 10% acetic acid in acetonitrile which was then dried and finally redissolved in acetonitrile or buffer and analysed in RP-HPLC. Other analytes detected by MIPSPE are clenbuterol and nicotine (Haupt, 2001). However, the problems with non specific binding of the template and related molecules or compounds from a sample mixture are still large. In addition, problems with leaching of the template from the polymers prevent the use of MIPs for trace and ultra trace analysis of minute target samples. This arises from the difficulty of extracting 100% of the template from the polymer even with most stringent extraction solvents. To mitigate the problem, researchers have attempted to use a close structural analogue for the desired template. For example, bromoclenbuterol was imprinted instead of the target clenbuterol; bleeding of bromoclenbuterol then did not affect the analysis of clenbuterol (Crescenzi *et al*, 2001).

Anderson and coworkers also used a drug analogue for sameridine imprinting, and the bleeding was not an issue for the subsequent sameridine SPE analysis (Haupt, 2001).

1.8.3 Artificial antibody

Antibodies by nature are pre-eminent in recognising bio-molecules; however, they are in poor supply. To combat an ever increasing demand, monoclonal antibody techniques are typically employed, although successful in application but unsuccessful in scaling up due to high cost. In general, a molecular imprinting approach offers considerable potential advantages compared to natural molecules. This is because natural molecules such as antibodies are difficult and expensive to isolate or sometimes require complex systems to purify and mostly they are unstable under non physiological conditions. In contrast, molecular imprinted polymer, are robust, simple to develop and produce inexpensive systems that are stable under harsh non physiological conditions. In addition, raising antibodies against a specific antigen is often expensive and not all proteins are immunogenic (Yan *et al*, 2007). Furthermore, proteins cannot be stored for long at ambient conditions, while MIPs can be stored in a dry powder dry form for a number of years (Schweitz *et al*, 1998).

MIPs have a common feature with antibodies in that they both bind target molecules selectively and reversibly. This could potentially be used for immunoassay type binding analysis replacing antibodies. The Mosbach group (Vlatakis *et al*, 1993) has designed a molecular imprint sorbent assay (MIA) for the detection of theophyline and tranquilizer diazepam in organic solvent as conventional immunoassays can only be used in aqueous solutions. Other compounds assayed are drugs, herbicide and corticosteroids (Haupt, 2001).

1.8.4 Biosensor

Another interesting area for application of MIPs is in chemical and optical biosensors. The area involves chemical and drug agent detection, environment monitoring, food analysis, medical diagnostics, drug screening with more potential applications continually emerging. MIPs are highly cross linking networks so they can withstand more challenging extreme environments compared to antibody sensors.

Takeuchi *et al*, (2005) imprinted cinchinidine and cinchonine signalling molecules for use in biosensors. These molecules have chromophores or flourophores which cause spectral shift for signal detection. However for molecules without these properties, the flourophores can be incorporated into the templates. Tao *et al*, (2006) developed a novel interleukin-1α sensor from xero-gel, a sol gel derivative with the aim of monitoring changes of luminescence upon template binding.

1.8.5 Tissue engineering

Everyday new biomaterials are designed which have direct or indirect biomedical application. Molecular imprinting hydrogels in tissue engineering have attracted significant interest because of the ability to control release of the active biomolecules in a localised area (Byrne and Salian, 2008). Some interesting future work of MIPS in this area includes imprinting scaffolds for delivering angiogenesis promoting vascular endothelial growth factors (VEGF), basic fibroblast growth factor (bFGF) and osteogenesis promoting bone morphogenic protein (BMP) (Drury and Mooney. 2003). Heparin-functionalised hydrogels including an imprinted scaffold to control porosity, have been used to enhance cell-adhesion and tissue in-growth (Nie *et al*, 2007). Cell imprinted scaffolds have also been suggested to increase cell interactions which provide modulatory events and aid in cellular organisation and protection. Cell-protein scaffold interactions have been exploited for specific cell binding and promoting cell differentiation and growth. For example, using hydrogels modified with RGD peptide sequences were tuned to promote specific binding of marrow stromal cells (Shin *et al*, 2002).

In addition, molecular recognition materials can potentially be applied on a biomaterial surface to specifically recognise template from the biological fluid. The adsorbed template can trigger a specific immune response instead of a normal response induced by non-specifically adsorbed antigen. This will result in a tailor made antigen response from a biomaterial or prosthestic implants coated with a specific imprinted polymer.

1.8.6 Molecule delivery

MIPs use in therapeutic delivery has sparked significant interest because of their mechanical and chemical stability as well as their ability to control release. MIPs hydrogels in particular are the centre of the molecular carriers for controlled release of therapeutic compounds, such as drugs, peptides and proteins.

Current drug delivery systems are not responsive to patients' requirements; the use of smart imprinted polymer hydrogels to switch on and off in response to the patient's needs might have considerable potential in therapeutic applications. For example, drug released or uptake in response to a patient's internal stimuli, such as changes in ionic strength, temperature or pH. The binding of a local molecule strongly triggers a release of a weakly bound therapeutic drug, e.g. release of hydrocortisone upon recognition of testosterone could be achieved in MIPS via competitive binding (Bergmann *et al*, 2008). Other examples are the use of PEG hydrogels as contact lenses for control release of the drug timolol into the eye. The drug was controlled and released only when the polymer was in contact with water in the eye (Alvarez-Lorenzo *et al*, 2004). Another eye therapeutic such as H1-antihistamines release was also developed using imprinted polymers (Venkatesh *et al*, 2007).

There is no limit to the imagination of MIPs application in drug delivery. However, a problem envisaged with MIPs applications in this field is the biocompatibility of the polymer, which must be proven safe for clinical use and meet standards by regulatory bodies.

1.8.7 Catalyst

In a continued effort to mimic enzyme functionality, molecular imprinted polymers have been prepared as a catalyst with the ability to overcome enzyme limitations in extreme

non physiological conditions, such as high ionic strength, extreme pH, high temperature and in organic solvents.

A typical template for synthetic catalytic MIPs is a transition state analogue (TSA) of the enzyme reaction. This species is expected to generate imprinted sites that stabilise the intermediate reactants on the reaction pathway, thus lowering the overall activation energy for the process.

Artificial enzymes have been extensively exploited for catalysing a range of reactions including ester and amide hydrolysis, dehydrofluorination, Diels-Alder reactions, and a transaminase reaction. For example, Mosbach and co workers have used artificial MIP catalysts for mimicking a Diels-Alder reaction involving cycloaddition of tetrachlorothiophene dioxide with maleic anhydride for the formation of carbon–carbon bonds. The resultant polymer demonstrated rate enhancements of 270-fold relative to the uncatalysed reaction (Liu and Mosbach, 1997).

Most MIPs catalyst research directed towards imprinting TSA species has shown that the resultant MIPs generates only moderate catalytic yields. This is because using TSA alone is not sufficient to produce high catalytic activities (Liu and Wulff, 2008). Recently, Wulff's group proposed a new approach to the MIPs catalyst synthetic strategy involving the addition of an amidinium group and Cu (II) or Zn (II) in the centre of the active site, in combination with TSA, for the enhancement of the catalytic yields. The group suggested that TSA binding by steric and electronic effects and positioning of functional groups were essential for construction of an effective artificial site. This artificial zinc metalloprotease carboxypeptidase A enzyme was used for the removal of the C-terminal of amino acid residues from peptide chains. The group reported successful increases of the rate of catalysed to uncatalysed reaction of 10^5-fold (Liu and Wulff, 2008).

1.8.8 Bio-transformation process

MIPs research in biotransformation processes has been limited, although there is a great potential in this area. MIPs could be used to shift unfavourable to favourable equilibrium

of enzyme-catalysed process in order to enhance the yield of product formation, isolation of a desired products, and purifying the sample from contaminants of undesired side products, MIPs could also be used in a crude extraction from a fermentation broth, or the removal of a toxic or unwanted by-product in a separation step following a biotransformation process (Alexander *et al*, 2006). For example, Ye *et al* (1998) imprinted *N*-(benzyloxycarbonyl)-β-L-aspartyl-L-phenylalanine methyl ester to separate isomer *N*-(benzyloxycarbonyl)-α-L-aspartyl-L-phenylalanine methyl ester which is a useful precursor for the artificial sweetener aspartame. The group reported that the resultant polymer demonstrated high specificity for the print molecule and can be used as downstream product polishers in the chemical synthesis of the synthetic precursor for an artificial sweetener, aspartame.

In the following year, Ye *et al* (1999) imprinted Z-$_{L}$-p-$_{L}$-Phe-OMe (Z-aspartame), a product of thermolysin condensation reaction of Z-$_{L}$-aspartic acid and $_{L}$-phenylalanine methyl ester. The group demonstrated that when the enzymatic reaction was carried out in the presence of imprinted polymer, a considerable conversion of the reactants into products was reported, while the lowest conversion was observed when the enzyme was used alone for longer reaction time (48hrs). However, the opposite effect was observed with shorter reaction time of less than 6hrs, after which the MIPs functionality prevailed. They suggested that the substrate concentration was initially decreased by the polymer and was therefore unavailable for the conversion. Others have used succinyl L-tyrosine MIPs for by-product removal of succinyl L-tyrosine from the β-lactamase inhibitor clavulanic acid in a fermentation process of therapeutics (Yu *et al*, 2002).

In general, the imagination for MIPs application in different areas discussed is limitless. However, the commercial scale up is very rare because limitations need to be overcome to be a viable technology.

1.9 Protein stabilisation

Proteins have a huge global market and tremendous efforts have been put into protein imprinting research from academia and industry. This is because it is a very attractive area of research with a vast number of applications.

Proteins such as antibodies have supreme recognition properties, whilst enzymes (another group of proteins) have both recognition and catalytic activity under normal physiological conditions. It is well known that when proteins are removed from their natural environment or isolated from solution, they can undergo reversible or irreversible, conformational changes depending on the environmental conditions. If the changes are irreversible this can lead to protein denaturation and loss of functional activity.

The work described in this thesis has focused mainly on imprinting several enzymes with a particular interest in separation and purification, but their applications are not limited.

Enzymes have been used as biocatalysts for centuries from making bread to the brewing of wines and alcohols in ancient Egypt. Most enzymes are comfortable within physiological pH and temperature conditions but have distinct characteristics which divulge their unique identity. For example taking phosphatase enzymes; acid phosphatase from potato prefers acidic pH while alkaline phosphatase from bovine prefers basic pH conditions for optimum catalytic function even though both catalyse hydrolysis of a para-nitrophenol substrate at a normal physiological temperature around 37^0C.

Enzyme structures are very complex and are determined by the amino acid sequence. Typically, they have an evolutionary selective pocket region known as the “active site”, which contains the catalytic residues that bind the substrate via non covalent interactions and carry out the catalytic process. The birth of the active site theory was initially postulated by Emil Fischer in 1894 and is famously known as “lock and key” model,

where the substrate is the "key" and the enzyme active site, the complementary region, is the "lock", which is rigid and can only fit a substrate of fixed conformation (Nicholas, 2005).

This theory was criticised because it failed to explain how the enzyme was able to accommodate the substrate in a transition state. In 1958, Daniel Koshland proposed an "induced fit" model where he proposed flexibility at the enzyme active sites on interacting with the substrate. The flexible active site continues to change until the substrate fits to the active pockets (Price, 2005)

Enzyme active sites interact with substrate by forming several non covalent associations, such as electrostatic bonds whereby charged groups on a substrate interact with oppositely charged groups in the enzyme active site, hydrogen bonds forming between electronegative groups, van der Waals forces acting on inter-atomic contacts between enzyme active site and a substrate, and finally hydrophobic association whereby hydrophobic groups interact by excluding water from their surfaces.

The ideal template is the one which is stable over non physiological polymerisation conditions. Considerable research efforts have been dedicated towards achieving protein stabilisation for use in non physiological environments such as non aqueous systems, for use at elevated temperatures and for improving shelf life. Recent advances in protein stabilisation include spray freezing into liquid and spray freeze drying. These technologies dehydrate protein into a powder suitable for therapeutic uses. The dehydration process stabilises enzymes because water facilitates or mediates physical instability and chemical degradation pathways of proteins in solution (DePaz, 2002; Liao, 2004). Although dry solid formulations provide acceptable protein shelf life, they are known to cause stresses to the protein, resulting in conformational changes such as protein unfolding and loss of activity and even stability (Engstrom, 2007).

Elversson (2006) attempted to stabilise protein by in situ coating of individual protein particles with hydroxypropyl methylcellulose. The coating was expected to reduce stress on enzyme conformation during drying. However, no improvement of protein conformation stability was noted. Other attempts were imprinting protein with substrate

analogues (Rich and Dordick, 1997), and use of additives such as cross linking agents like glutaraldehyde to stabilise protein crystals (Lalonde *et al*, 1995).
While the world was looking for better methodologies for protein stabilisation technologies, so was the innovative group from Universities of Glasgow and Strathclyde. This group developed a novel strategy to stabilise protein onto a solid support by a rapid one step protein dehydration process. The particles produced were called Protein Coated Micro Crystals (PCMCs) and will be described briefly in the following section.

1.9.1 Protein coated micro crystals (PCMCs) and molecular imprinting

PCMCs are water-soluble crystals in which proteins are co-precipitated onto an inert support matrix, such as salts, sugars and amino acids, in a steady addition of pre-blended protein and excipient mixture into organic solvents via a one-step co-precipitation process (Kreiner *et al*, 2001). PCMCs have offered a new innovative technology for producing stable protein using a robust and inexpensive technique for dehydrating proteins. Extensive research has been carried out by the Physical chemistry Group at the University of Strathclyde into the physical nature of protein attachment onto the solid support and protein conformation after immobilisation. The group suggested that PCMCs are formed by a rapid crystal-lattice mediated self assembly (CLAMS) process, in which proteins reside onto the surface of microcrystals, and the conformation of the immobilised protein is not altered during the co-precipitation process. Furthermore, the group has shown that enzyme coated on supports are stable and achieve excellent enzymatic activity in aqueous and non aqueous systems (organic solvent) (Kreiner *et al*, 2005). In addition, the stability studies have demonstrated that PCMCs are stable over long periods when stored as dry powder in non-aqueous media (Kreiner *et al*, 2005).

The design criteria for the PCMCs formulations carried out in this thesis will be discussed in the following chapters.

1.10 Our work

Protein imprinting using a conventional monolithic strategy has received very little attention in the literature. This is because it needs to overcome challenges, such as protein insolubility and conformation stability in aprotic solvents and harsh polymerisation conditions, which lead to protein denaturation and formation of aggregates. In addition, a highly cross linked polymer network will tend to trap large protein molecules, leading to poor mass transfer to and from the matrix, resulting in inadequate recognition (Turner *et al*, 2006). Furthermore, proteins are very complex structures with a large number of functional groups that form potential recognition sites over a relatively large surface area (Turner *et al*, 2006; Bossi *et al*, 2007) and may lead to significant problems with non selective binding.

Recent attempts to stabilise protein imprinting is towards template immobilisation. This approach tends to restrict template motion prior to the polymerisation and at the same time improves template-functional monomer complexes which is expected to improve the imprinting effect. In addition, only one side of the molecule is oriented and exposed to functional monomers, leading to improved homogeneity of binding cavities and reproducibility. Another advantage is that it offers potential solutions to protein insolubility and aggregation in organic solvents (Bossi *et al*, 2007).
Hitherto, only limited research efforts has been dedicated to the analysis of the role of template immobilisation on protein imprinting.

Protein imprinting was studied previously from a novel perspective using protein coated micro crystals (PCMCs) made via a rapid 1-step process developed by Moore *et al*, (2001) and co workers. The PCMCs have very interesting qualities that offer a new perspective to conventional monolithic imprinting challenges.

Firstly, proteins are unstable, or in the case of enzymes, they are known to dramatically lose their activity in organic media. PCMCs are expected to offer excellent retention of protein structural stability and for the enzyme both conformational stability and activities in organic solvents.
Secondly, the elevated levels of temperature and changes in pH in free radical polymerisation conditions are known to affect the structure and stability of the proteins. However, the PCMCs are expected to offer protein stability under these harsh conditions.
Thirdly, PCMCs are expected to create large cavities in a dense polymer network. In addition, the cavities might even be located at the surface of the polymer, thereby improving accessibility of the template to the heavily cross linked polymer network and hence improve binding and dissociation kinetics. Furthermore, the cavities produced are expected to be uniform in size, shape and binding energy, conferring binding reproducibility.
However, the downside with template immobilisation is that an extra step is introduced in the imprinting process, which adds to the cost of imprinting.

The main objectives of the work are to attempt to solve common problems found in traditional monolithic imprinting, which have been identified and described.
A summary of the main objectives is presented below:

- To identify suitable conditions for the PCMCs formulation that allow maximum immobilisation of the enzyme onto the excipients i.e. enzymes such as lysozyme and acid phosphatase which are described in their respective chapters.
- To quantify the amount of enzyme bound onto the crystals using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and UV-VIS spectrophotometer and to measure catalytic activity of the immobilised enzymes using established assay procedures specific for the enzymes in question.
- To use SEM to visualise PCMCs structures and particle analyser (MasterSizer) to determine the size of the lysozyme PCMCs particles.

- To use a novel strategy of imprinting enzyme bound to micro crystals (PCMCs) as a platform for imprinting in a non covalent traditional monolithic free radical polymerisation. This is schematically presented in figure 1-17.
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Figure 1-17: A schematics illustration of PCMCs incorporated into MIPs

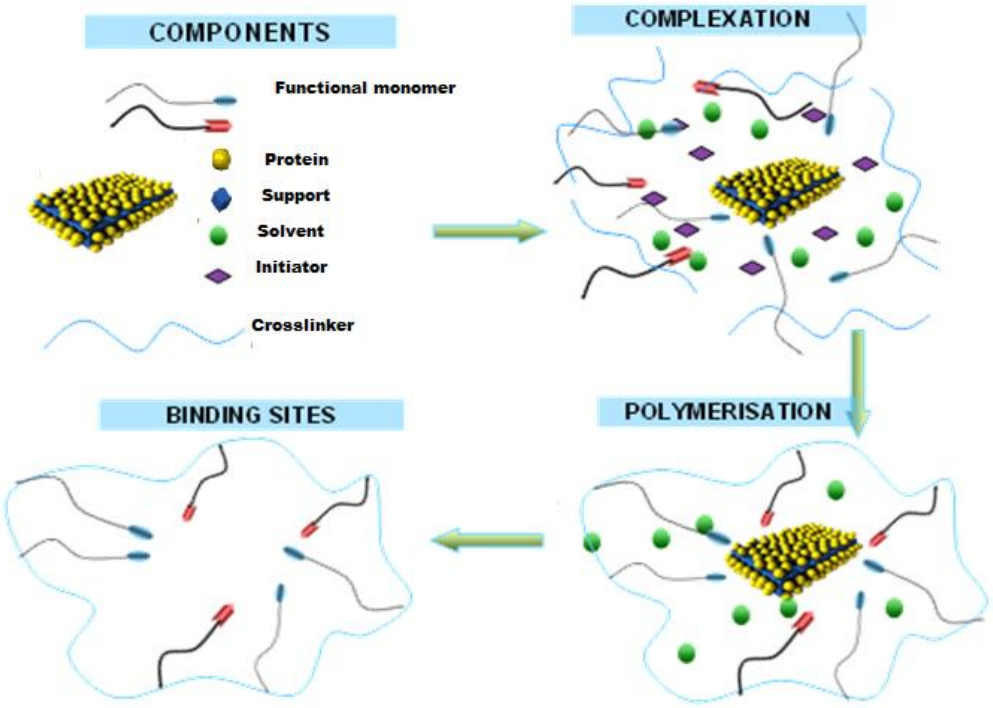


Figure 1-17 shows the general principle of non covalent imprinting, where it starts with the addition of polymer ingredients, such as template (PCMCs) and functional monomers that have functional groups which are complementary to those of the template, to form a template-functional monomer complex in a self-assembly manner. This is followed by the addition of cross linking monomer to provide the rigid dimensional structure where the template and functional monomers are locked and spatially arranged in a fixed position. The polymerisation is then initiated with free radical initiator using either thermal or UV energy, and finally the template is extracted from the polymer leaving cavities whose shape, size and chemical functional group are complementary to the template. The template (protein) is then introduced and expected to rebind selectively and reversibly to the polymer.

- To develop polymerisation protocols to improve selectivity and suppress non selective binding of the templates by optimising polymer compositions, such as:
  a) Changing the concentration of polymer components systematically with respect to others
  b) Using a cocktail of monomers
  c) Altering the polymerisation initiation process by using either thermal or UV energy so that desired imprints are achieved.

- To develop template extraction protocols for maximum removal of the templates from the highly cross linked polymer matrix.
- To develop methods for assessing molecular imprinted polymers for proteins that may distinguish selectivity from non selective template rebinding. This may include modifying rebinding conditions using either chemical reagents and/or physical conditions in:
  a) Specific template rebinding experiments whereby an imprinted template is re-introduced to the imprinted polymers and this will be used to measure the binding characteristic of the polymer
  b) Competitive binding conditions whereby the imprinted template and a template analogue (a closer molecular weight molecule) are introduced to the imprinted polymer to compete for binding sites and this will be used to measure the polymer specificity towards the imprinted template
  c) Finally, in non template selective experiments whereby a template analogue will be introduced to the polymer alone and this will be used to measure the non selectivity of the polymer

- To imprint protein analogues and compare with the targeted template in selectivity experiments. This will be used to measure the degree of non specific protein binding.

Chapter 2 : Preparation and characterisation of PCMCs

Introduction

Preliminary work towards the design of the imprinted polymer was focused on the preparation of the protein coated microcrystals (PCMCs) using different types of excipients, such as salts and amino acids, as well as different types of organic solvents. This part of the thesis highlights a brief study undertaken to develop functional PCMCs as templates for molecular imprinting and briefly discuss factors that influence the morphology and characteristics of the PCMCs. Details of the experiments can be found at the end of the chapter.

2.1 Factors affecting the morphology of PCMCs

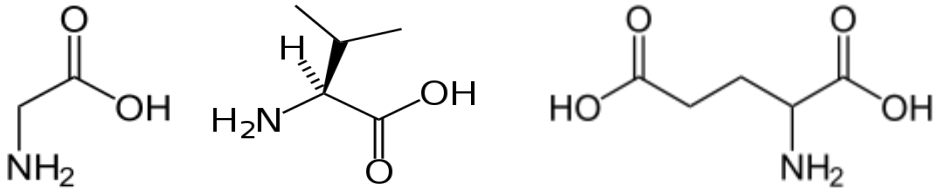
2.1.1 Choice of excipient

The excipient provides the support for immobilisation of the proteins of interest. Several categories of supports have been studied and used previously, such as salts, sugars and amino acids. For example, K_2SO_4 has been successfully used for immobilising different enzymes, such as subtilisin Carlsberg A, pepsin, albumin and trypsin. Amino acid such as DL-valine has been used for immobilising albumin and subtilisin Carlsberg A and glutamic acid has been used for albumin and pepsin (Vos, 2006).

Different crystalline supports have different charges on the surface depending on the molecular support. For example, amino acids have an isoelectric point (pI) at which the molecule carries no electrical charge. If the pH of the solution is equal to the pI, then the positive and negative charge groups in the molecule will be equal. Therefore, the molecule will be carrying no net charge. However, if the pH of the solution is below the pI of the molecule, then the overall charge will be positive and negative if above the pI. The relation between the net charge on the support and the protein has been studied previously by Moore's group at the University of Strathclyde.

This group suggested that overall charge on the support does not influence the degree of protein immobilisation even if both have the same charge (Vos, 2006).
The group proposed that protein binding onto the crystals was by non covalent forces, including hydrogen bonding, van der Waals and hydrophobic forces, but the electrostatic interaction was however inconclusive because of the absence of the repulsive charges. Furthermore, they suggested that the hydrophobic effect is contributed mainly by the presence of a side chain on the amino acid support, such as in L-valine and glutamate, whilst for glycine, a hydrophobic effect was not expected because of the lack of a side group. The molecular structures of glycine, valine and glutamic acid are illustrated in figure 2-1.

Figure 2-1: Molecular structure of amino acids; glycine, L-valine and glutamate



Glycine **L-valine** **Glutamate**

The shape of the PCMCs crystal is dependent on the type of support. Some take up a well defined cubical shape observed with salts, whilst others display leafy type morphologies as seen with some amino acid supports. Shape difference will be explained in detail with the aid of SEM images in the respective sections in this chapter.

2.1.2 Choice of precipitating solvent

Several organic solvents (e.g. acetonitrile, isopropanol and *n*-propanol) have been used as the precipitating solvent for the protein in the production of the PCMCs. The choice of the solvent may influence the shape and size of the PCMCs formed because high super saturation leading to crystallisation is the driving force for PCMC formation.

Acetonitrile is the most common solvent used in free radical molecular imprinting. However, if other solvents are employed for the PCMCs production then solvent switching is performed. This involves washing off the precipitating solvent from PCMCs with acetonitrile prior to polymerisation.

2.1.3 Protein loading

This is a ratio (or percentage) of the mass of protein relative to the amount of excipients in the isolated protein/excipient dry powder. Different protein loadings have been employed previously, ranging between 1-15%. At higher loadings, there is excellent recovery of proteins. However, at lower loading such as 1%, poorer recoveries are obtained, as reported in this investigation. In addition, the shape of the PCMCs was also influenced by the percentage of protein loading, as seen from the SEM images of the PCMCs.

2.1.4 Water content

This is the measure of the amount of water added to the organic solvent for the PCMCs production. Pure water or buffers can only be dissolved in small amounts in the precipitating organic solvent and should not be confused with water molecules that are tightly bound to the protein structure.

The amount of water used in the PCMCs formulation previously was between 3%-7%. Moore's group suggested that water content below 3% results in lower protein recovery because it limited the protein solubility in the bulk solvent and led to competitive interaction between immobilised molecules, such that they are expelled from the surface. The proposed mechanism of PCMCs formation is that the protein and excipient initially undergo a phase separation and dispersion in the solvent (Vos, 2006).

If a large amount of water is used, then its removal from the PCMCs becomes a laborious process. Residual water is undesirable, as it would compete with the protein bound to the crystals with the free radical functional monomers, interfere with the

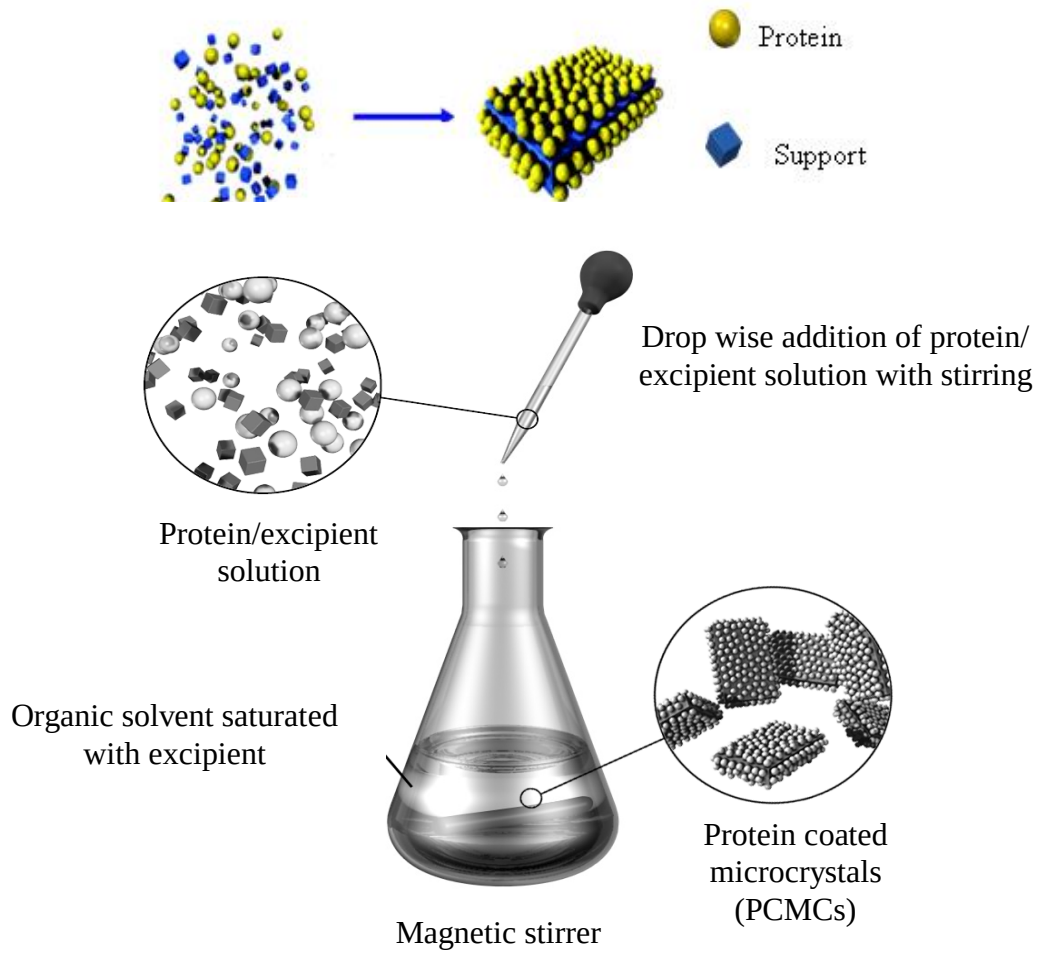
complex formation in the pre-polymerisation stage and even halt the free radical reaction in the imprinting process. Hence, moderate water content is favoured to allow maximum protein immobilisation and at the same time can easily be washed off from the PCMCs.

2.2 PCMCs preparation and morphology characterisation

The preparation of protein coated microcrystals was carried out using standard protocols similar to those reported previously by Kreiner *et al* (2001). For example, for a typical batch PCMCs production, 11.11% protein, 88.89% excipients, water 5%v/v and precipitating organic solvent 95% saturated with excipient was used (see experiment 2.1.1). Thus, 4mg of lyophilised protein powder are dissolved in 100 µl buffer (water) of desired strength and pH, and then transferred to 360 µl of excipient solution containing 36mg/ml of the same buffer. 460 µl of the protein/carrier solution are added dropwise to the 15ml vial containing 8.74ml of saturated organic solvent under agitation provided by a magnetic stirring bar for approximately 1 min. The schematic of PCMCs production by batch process is presented in figure 2-2.

Following co-precipitation, PCMCs are filtered using a membrane filter of pore size 0.22 µm and left to dry or can be centrifuged and the supernatant removed prior to the protein recovery analysis.
The initial work was carried out for developing PCMCs formulations for protein with the enzymes subtilisin Carlsberg (SC) and lysozyme (Lys). SC was chosen because the enzyme has been extensively studied and known to be excellently immobilised on a number of excipients. Therefore, it was appropriate to use this enzyme to validate that the enzyme bound to the excipient in a native conformation, and build our confidence in PCMCs formulation. SC is from the proteases family of enzymes.
It has the ability to digest itself with time and for this reason it could not be employed for the imprinting process even though the enzyme is stable in the organic solvent.

Figure 2-2: Schematic illustration of the self assembly of protein on the surface of the (PCMCs)



Lysozyme is a well known enzyme where the structure and function has long been realised. Alexander Fleming (1922), a Scottish biologist and pharmacologist, first discovered that tear fluid as well as other body secretions had an antibacterial effect that would kill the bacteria *saprophyticcocci*. Later, he isolated this lytic property and coined it lysozyme. Lysozyme is an enzyme of 14.7 kDa that is found in egg white, serum, tears, nasal mucus, saliva, blood serum and plasma, animal and human secretions polymorphonuclear leucocytes (Salton, 1957). The main function of the lysozyme is to provide immune defence against bacterial infection but it has other potential applications, such as diagnosis. For example, increased levels of lysozyme in urine and

serum can be used as early indicators of monocytic and myelomonocytic leukemia (Osserman, 1966) severe renal failure (Prockop, 1964), renal transplant rejection (Jones, 1999) and urinary tract infections. Rises in the levels of enzyme activity levels in serum can be used as diagnosis for tuberculosis, Crohn's disease, bacterial infections, arcoidosis, and ulcerative colitis. Because of all the potential applications, it is abundantly available and inexpensive and justifies the use of the enzyme as a model in the molecular imprinting.

The lysozyme structure is composed of 129 amino acid sequences, each carrying a polar or neutral charge at physiological pH. The enzyme is also known as muramidase or N-acetylmuramide glycanhydrolase because it catalyses the destruction of a peptidoglycan, (hexosaccharide) found in the cell wall of gram-positive bacteria, by hydrolysing 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine into simple sugars. The mechanism of lysozyme action is illustrated in figure 2-3 (The figure was modified from that of Bergmann, 2005).

Figure 2-3: Lysozyme activity on the peptidoglycan of bacteria cell wall

The enzyme reaction involves the hydrolysis of the β-(1-4)-glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine. This is achieved through the deprotonation of two amino acid side chains; glutamic acid 35 (Glu35) and aspartate 52 (Asp52). The Glu35 is deprotonated by donating a proton to the glycoside bond cleaving the C-O bond in the substrate, whilst Asp52 acts as a nucleophile to generate a glycosyl enzyme intermediate, which reacts with water molecule to give the product of hydrolysis.

The degree of protein immobilisation onto the excipients depends on the factors described previously. However during the co-precipitation process, it is almost impossible to immobilise all the protein. This is because some protein molecules are expected to exist freely in solution, whilst others may precipitate onto the already formed PCMCs, resulting in differential binding of the proteins onto the excipients. In order to use PCMCs as a platform for molecular imprinting, two questions need first to be addressed;

1. To determine a percentage of lysozyme immobilised onto the support so we know the amount of PCMC to use, and
2. To determine if the native conformation of the enzyme bound to the microcrystals had been preserved during PCMCs production. This is crucial if the imprinting process is to capture the true structure of the proteins.

2.2.1 Protein recovery determination

The amount of protein bound onto the microcrystals was analysed using reverse phase high performance liquid chromatography (RP-HPLC). Briefly, following the co-precipitation process, the dried PCMCs powders were re-dissolved in their respective buffer and protein solution samples were injected directly into the RP-HPLC for protein analysis. When proteins are isolated from their natural environment, they can undergo

conformational instability, leading to structural denaturation and aggregation. Therefore, finding conditions that maintain structural integrity is vital.

In the beginning of the Lys-PCMCs production, we determined the optimum pH for the PCMCs formulation ranging from pH 7-8 of phosphate buffer. At these pHs, the lysozyme having a pI of ~10.4 will carry an overall net positive charge. Phosphate buffer was used because it was commonly employed for lysozyme studies in the literature and because it offers solubility and stability to the enzyme, which is ideal for any protein isolated from its natural environment. In addition, Tris/HCl was also investigated because it demonstrated excellent immobilisation when used with subtilisin Carlsberg.

2.2.2 Buffer effect on the lysozyme PCMCs

The Lys-PCMCs were prepared as outlined in table 2-1. This was carried out using 11.11% lysozyme loading relative to KCl excipients, 5% water in the form of buffers at 0.01M strength, and saturated IPA with KCl as the precipitating solvent.

The preparation of protein coated microcrystals was carried out using standard protocols, similar to those reported previously by Kreiner *et al*, (2001). During the co-precipitation process, the lyophilised lysozyme was initially dissolved into a buffer to create near physiological conditions and transferred to the KCl solution. The lysozyme /KCl (36mg/ml) solution was added steadily in a dropwise fashion to the precipitating solvent, under agitation provided by a magnetic stirring bar, for approximately 1-2 min (see experiment 2.1.1)

Table 2-1: PCMC prepared with different buffers and pH

Sample	Protein loading %w/w	Buffer type	pH
1	10.0	Phosphate	7.23
2	10.0	Phosphate	8.23
3	10.0	Tris/HCl	7.8

Following co-precipitation, PCMCs were filtered using a membrane filter and were left to dry at room temperature. The analysis of protein recovered from PCMCs was carried out (see experiment 2.1.2) and the results are presented in table 2-2

From table 2-2, it can be suggested that almost all of the lysozyme introduced in the co-precipitation process was successfully immobilised onto the KCl support using phosphate buffers at both pHs, with the maximum immobilisation observed at pH 7.23. Satisfactory results were also noted at pH 8.23, giving PCMCs useful for molecular imprinting. However, a lower protein recovery was obtained with Tris/HCl buffer. This may be due to the instability of the enzyme in this buffer.

Table 2-2: Lysozyme recovery from Lys-PCMCs prepared at different buffer pH

Sample	Buffer type	pH	%Protein bound
1	Phosphate	7.23	97±2%
2	Phosphate	8.23	91±3%
3	Tris/HCl	7.8	88±2%

(Mean ± error, n=3)

2.2.3 Precipitating solvent effect on the lysozyme PCMCs

In an attempt to improve lysozyme binding, the various solvents that have been previously employed for PCMCs formulation, such as isopropanol, *n*-propanol and acetonitrile all saturated with KCl were investigated, using similar preparative techniques described previously, to determine the most appropriate solvent that would allow maximum immobilisation of the protein.

As suggested previously, the bulk solvent was the driving force for PCMCs formation. The particles formed with alcohols remained in suspension for a couple of minutes before sedimenting and then precipitated smoothly at the bottom of the vessel.

For PCMCs formed with acetonitrile, the particles fell out of the suspension abruptly, forming aggregated chunks of crystals. These particles sedimented very quickly and from visual observation, the particles formed were undesirable for imprinting purposes.

Albeit acetonitrile would have been a better solvent because it would eliminate the solvent switching steps in the PCMCs preparation prior to imprinting.
After optimum conditions for Lys-PCMCs formulation were obtained, the second issue regarding whether the enzyme conformation was preserved during PCMCs production was addressed. This was examined by measuring the lysozyme enzymatic activity, using an established turbidimetric assay.

2.2.4 Lysozyme PCMCs activity by turbidimetric assay

The amount of lysozyme immobilised onto the KCl crystals was initially determined by protein recovery from a known weight of lysozyme PCMCs prior to the assay so that a similar concentration of lysozyme standard could be used for comparing the degree of activity.
The assay involves measuring the degree of lysis of the bacteria *micrococcus lysodeikticus* cell wall, as described previously. This results in a decreasing population of cells in solution, reflected by a decreasing spectrophotometric absorbance. The assay was slightly modified from that of Gorin *et al*, (1971). 0.01M phosphate buffer at pH 7.23 was used instead of 0.1M at pH 6.2, the volume of substrate used was 1.5 ml instead of 3.0 ml, and the time of assay was reduced from 15min to 4min (see experiment 2.1.3). The decrease of absorbance was spectroscopically monitored at 450 nm to determine the optical density (OD) after mixing. For the base line correction of the spectrometer, 0.1ml of enzyme was added to the phosphate buffer placed in a cuvette without the bacterial cells.
From the catalytic activity graphs presented in figure 2-4, it can be observed that the activity of the lysozyme recovered from PCMCs was approximately similar to that of the free lysozyme standard at the same protein concentrations and no activity was observed from the blank KCl. This suggested that the native conformation of the lysozyme was preserved during the PCMCs production. However from closer inspection of the graphs, a slight drop on the cell number was observed initially from both Lys-PCMCs and blanks. The presence of a high concentration of KCl might have caused this effect.

Figure 2-4: Enzymatic activity graphs of Lys, Lys-PCMCs & Control (blank)

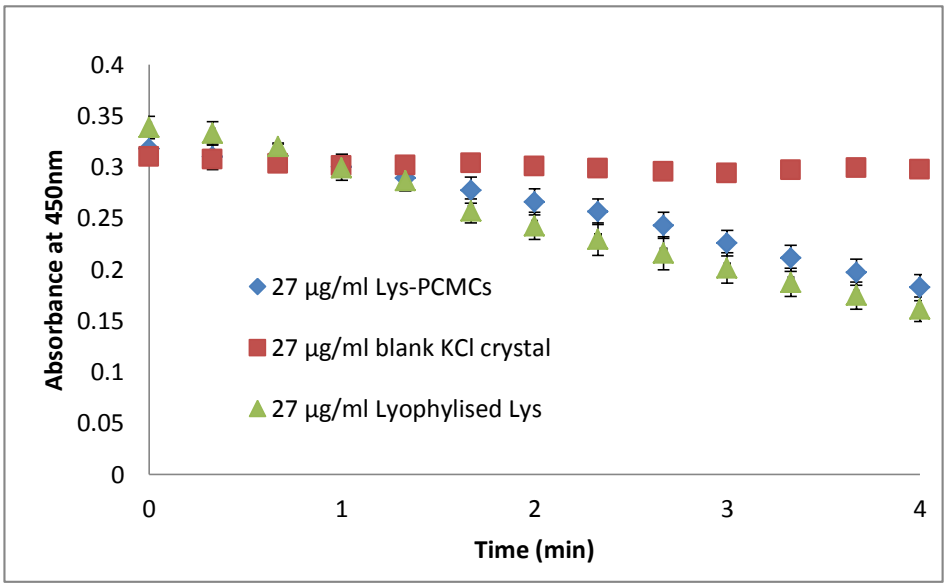


Figure 2-4 Lysozyme enzymatic activity graphs from spectrophotemetric data measured at 450nm wavelength, for lysozyme bound to PCMCs after dissolution of KCl using 0.01 M phosphate buffer pH 7.23, free standard lysozyme dissolved from the same buffer, both at 27µg/ml and blank KCl at (27µg/ml) (control).(Mean ± error, n=3)

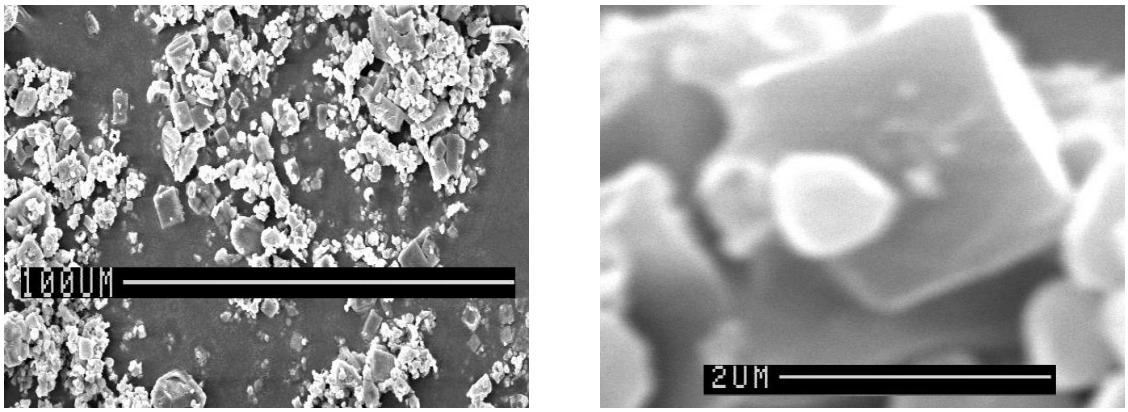
2.2.5 Lysozyme PCMCs morphology and SEM

SEM is a useful technique to visualise the structural morphology regarding the shape and size of particles. The main focus here was to determine the shape and size distribution of the particles prior to the incorporation of the PCMCs in the imprinting process, because it was envisaged that this would be reflected within the pore structure of the imprinting cavities. In addition, it would be a bonus if we could observe the position of the protein bound to the crystals, although the SEM was not an ideal tool for studying surface characteristics. For SEM, Lys-KCl-PCMCs were prepared using 10% loading, water 5%v/v and saturated IPA with KCl, as described previously. The SEM images of Lys-KCl-PCMCs are presented in figure 2-5.

The immediate feature observed with SEM images at lower magnification was that there was a significant variation of particle size. Furthermore, the particles were clustered and

far from ideal monodispersed particles. We thought that during co-precipitation, unsteady addition of the protein/excipient solution in the precipitating solvent contributed to this effect. Because of lack of particle uniformity, it was almost impossible to estimate the average particle size distributions even with increased magnification. Therefore, in order to investigate the average size distributions, the deployment of other techniques, such as particle sizing, was necessary.

Figure 2-5: SEM of Lys-PCMCs at different magnifications



Magnification X 410 Magnification X 13300

Figure 2-5 For SEM lysozyme KCl PCMCs prepared at 10% enzyme loading, 5% water (phosphate buffer at pH 7.2 0.01M) and IPA saturated with KCl as precipitating solvent.

The problem with large particle size distributions was previously addressed in our laboratory and was suggested to be overcome by the use of an automated addition system for the production of the PCMCs. In the imprinting process, large amounts of PCMCs are required. Hence, using an HPLC automated pump for addition of the enzyme/excipient into the organic solvent was expected not only to minimise the variation of particle size but also to facilitate the production of the PCMCs at a scale which otherwise would be time consuming using a batch production process.

2.2.6 Lysozyme PCMCs particle size measurement

For using PCMCs in molecular imprinting, a large variation of particle size is not ideal because this might be reflected onto the porosity distribution within the imprinted polymer. Although polymer porosity is independent from polymer recognition as discussed in chapter 1, any potential ways to minimise this would be an added advantage. It has been suggested that almost all the design factors described previously influenced the particle size and shape distribution.

For brevity, we only focused on whether to use the automated pump or batch process for PCMCs production so that the particle size variation was minimised.

PCMCs were prepared by both automated system using a HPLC pump (see experiment 2.1.4) and the batch system as described previously, using lysozyme loading10% and 5% water in IPA saturated with KCl Blank microcrystals were prepared similarly to PCMCs in the absence of protein via the same automated system and used as controls (blanks). Particle sizing measurement was achieved with a Malvern Mastersizer 2000 (Malvern Instrument Ltd) using acetonitrile as a dispersant phase. Although PCMCs were prepared in IPA, the use of acetonitrile would demonstrate the average sizes of the PCMCs and blank in the bulk porogen. Malvern Mastersizer 2000 is a wet dispersion method, where the sample powder is added in the wet phase (dispersant) and distributed by mixing with a turbulent mixer, and finally the particle size is measured by laser diffraction (see experiment 2.2).

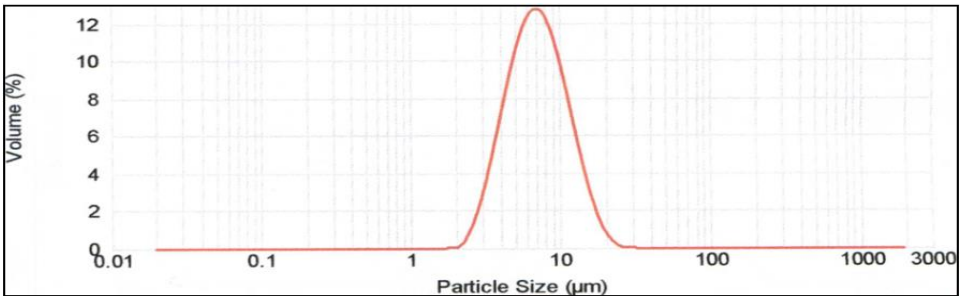
The average particle size distributions for the PCMCs by batch and pump production and blank are presented in figure 2-6 (a), (b) and (c) respectively.

The particle size results from figure 2-6 show that the average micron sized particles produced by batch and automated HPLC pump were between 2-8μm, while a larger particle size (2-13 μm) was observed with the blank. From our results, it can be suggested that for PCMCs the particle sizes on average are smaller. Although the automated system did not improve the particle size distribution, it could facilitate PCMCs production. Therefore for producing large amount of PCMCs, the automated system would be useful and will be employed. Another feature observed was the

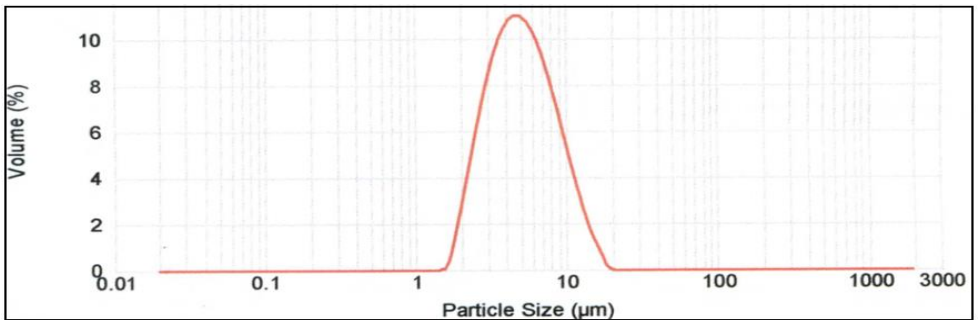
formation of large sized blank crystals which was in agreement with previous findings. It was reasoned that the protein bound to the excipient forms a protective layer on the surface, thus limiting the crystal growth and leading to formation of smaller size PCMCs. In contrast is the absence of protein large size blanks produced from the growing of the crystals.

Figure 2-6: Particle size of Lys-PCMCs and control

a) Lys-PCMCs produced by a Batch process



b) Lys-PCMCs produced by Automated HPLC pump



c) Blank KCl crystals produced by Automated HPLC Pump

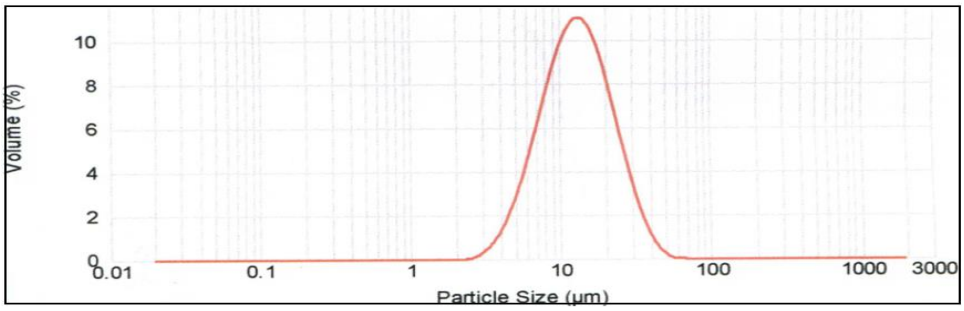


Figure 2-6 Particle size measurement with a Malvern Mastersizer 2000 (Malvern Instrument Ltd) using acetonitrile as a dispersant phase. (a) lysozyme-KCl PCMCs was prepared by batch system with the

following compositions; 10% w/w lysozyme loading and 5% water in IPA saturated with KCl, (b) lysozyme-KCl PCMCs and (c) blank KCl microcrystals were all prepared using automated HPLC pump with the same composition as batch PCMCs. 30 mg of each sample (a),(b),(c), powder were re-suspended in a 50ml of clean dry acetonitrile (dispersant) and mixing with turbulent mixer at 2000rpm. The particle laser diffraction with refractive index set at 1.490

2.2.7 Excipient effect of the lysozyme PCMCs and SEM

The effect of using high concentrations of KCl upon enzymes in organic solvent and in a molecular imprinting was not appealing and has not been thoroughly investigated in the literature. In particular, the effect of salts on zwitterionic functional monomers, which are a combination of acidic and basic monomers used in imprinting, was unknown. It was decided therefore that the use of amino acids as an alternative support was prudent because amino acids are present in the protein structure.

Amino acids as a protein carrier in the PCMCs formulation has been reported before and the use of glycine was unlikely to cause any protein structural perturbation because it does not contain a functionalised side chain (Brown, 2005). However, the problem with using glycine is that the solubility changes with buffer pH, although this could be circumvented by using buffers with pH, close to the pI of glycine which is ~5.6.

Briefly, similar investigations were carried out to determine a suitable precipitating solvent for lysozyme/glycine PCMCs. Lysozyme recovered from PCMCs with three solvents, acetonitrile, ethanol and IPA, was determined using RP-HPLC as before.

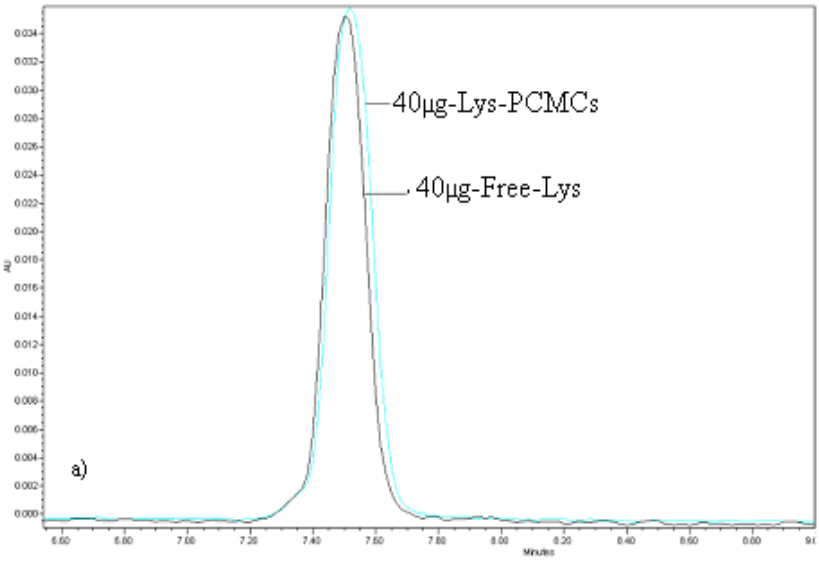
Lys-gly-PCMCs were prepared using 10% enzyme lysozyme, 5% water and organic solvents saturated with glycine, similar to the PCMCs preparations carried out as described before. IPA as a precipitating solvent produced an excellent organic media for lysozyme immobilisation onto the glycine crystals, while poor protein recovery was obtained from ethanol and acetonitrile.

Prior to the enzyme activity assay, the concentration of enzyme was determined by dissolving a known weight of lysozyme PCMCs and comparing the RP-HPLC with free unprocessed lysozyme standard as before. For brevity, we only present the RP-HPLC

chromatography measured at 280nm of lysozyme recovered from PCMCs after glycine crystals dissolution (green) and free lysozyme powder dissolved in same buffer (black) both at 40µg/ml in figure 2-7 (a). The results suggested that lysozyme was excellently immobilised onto the glycine crystals and the amount of lysozyme bound onto the microcrystals was approximately 100%. To determine the conformational stability of the lysozyme bound to glycine support, the turbidimetric assay was employed, which was fully adapted from Gorin *et al*, (1971).

The lysozyme activity recovered from PCMCs after dissolution at different amount of enzyme concentrations (10, 20, and 40 –µg/ml) was compared with similar concentrations of free standard lysozyme (see experiment 2.3). The results of lysozyme enzymatic activity graphs from spectrophotometric data measured at 450nm wavelength are presented in figure 2-7 (b). From the catalytic activity graphs, we can see that the activity of the lysozyme recovered from PCMCs was identical to the enzymatic activity of free lysozyme standard at the same protein concentrations over which no activity were observed from the blanks (glycine).

Figure 2-7: Lysozyme recovered from Lys PCMCs and the activity assay



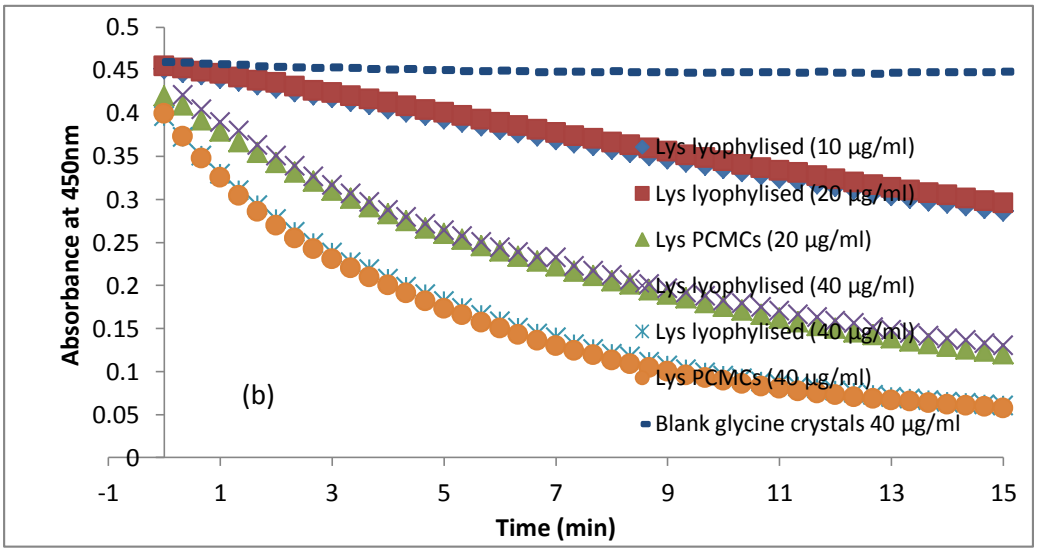
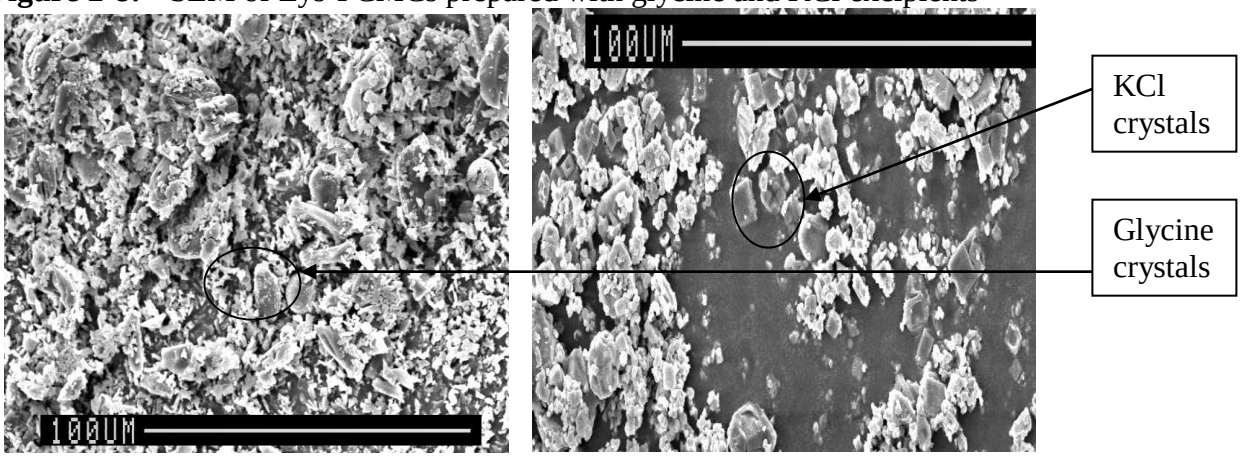


Figure 2-7 **a)** RP-HPLC spectra at 280nm of lysozyme recovered from PCMCs after glycine crystals dissolution using 0.1 M phosphate buffer pH 6.2 (green line) and free lysozyme powder dissolved in same buffer (black line), both at 40µg/ml. **b)** Lysozyme enzymatic activity graphs from spectrophotemetric data measured at 450nm wavelength, for lysozyme bound to PCMCs after dissolution of glycine using 0.1 M phosphate buffer pH 6.2 and free standard lysozyme dissolved from the same buffer, both at different concentrations (10, 20, 40 –µg/ml) and blank glycine crystals at (40µg/ml) (control). (Mean ± error, n=3)

The morphology of the new developed Lys-glycine-PCMCs was examined by SEM. The image was compared with SEM of Lys-PCMCs made with KCl crystals, both SEM are demonstrated in figure 2-8.

Figure 2-8: SEM of Lys-PCMCs prepared with glycine and KCl excipients



Lys-glycine-PCMCs Lys-KCl-PCMCs

Figure 2-8 Lys-KCl-PCMCs and Lys-gly-PCMC, both- prepared at 10% enzyme loading, 5% water (phosphate buffer at pH 7.2 0.01M) and IPA (precipitating solvent).

The particles of Lys-KCl-PCMCs were mostly cubically shaped, while those of Lys-glycine-PCMCs were a mixture of different crystal shapes, some of which are leafy and others like crystal blades, with large variation of particle size.

2.2.8 Protein loading effect onto the ACP-PCMCs and SEM

To optimise the efficiency of the molecular imprinting polymer, various techniques are employed, including changing the polymer compositions or methodology of polymer preparation; this will be described in detail in the following chapters. To improve the understanding of the conformational stability of protein bound to the imprinted cavity, we employed enzyme as template molecule, which is capable of performing catalytic activity whilst bound to the imprinted polymer cavities. The limitation of using lysozyme as the template in the imprinting was that the conformation stability of the enzyme bound onto the polymer could not be investigated through enzymatic activity. This was because the lysozyme assay involved the lysis of the bacteria cell wall and large cells cannot penetrate into polymers. Therefore, incubating the polymer in the cell solution was impractical; it was assumed that the polymer might damage the cell wall by shearing or precipitate the cells from solution leading to false results. Therefore, to increase the understanding of the functionality of enzyme bound to polymer, acid phosphatase (ACP) was employed. The enzymatic assay for this simply involves the hydrolysis of a small molecular substrate.

Acid phosphatase belongs to a metallohydrolase family of enzyme. It has metal ion centres which are involved in the hydrolysis of bonds including amides, esters of carboxylic acids and phosphoric acids (Strater, 1995; Schenk, 2005).

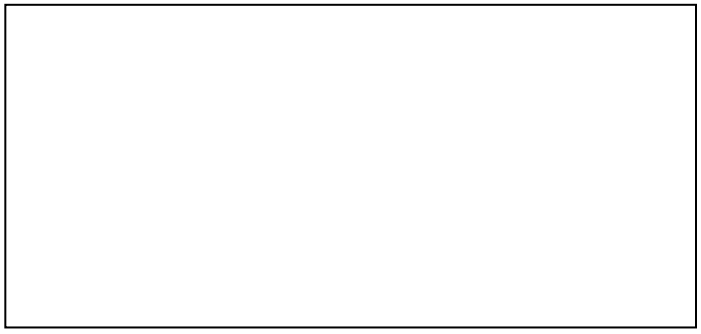
The enzyme is commonly found in a wide variety of species, such as animal lysosomes, kidney beans and sweet potatoes and fungi. It exists either as a monomer as in mammals or as a dimer in plants, sweet potatoes and kidney beans. It has a molecular weight ranging from 100 -160 kDa with an amino acid sequence of approximately 432 per

monomer in kidney beans (Strater, 1995) and a dimer of two identical polypeptide chains of 435 residues in sweet potato (Schenk, 2005).

The main functions of the acid phosphatase enzyme in mammals include iron transport, bone resorption by osteoclasts, dephosphorylation of erythrocyte phosphoproteins, and the production of hydroxyradicals and reactive oxygen species. The elevated levels of the enzyme in the prostate may be used to diagnose prostate cancer. In plants, soil microorganism's acid phosphatase is involved in the release of phosphate from organically bound phosphate nutrients, which is also used as an assay for investigating the demand of the phosphate in the soil. However, the functionality of the acid phosphatase in potatoes is still unclear (Schenk, 2005).

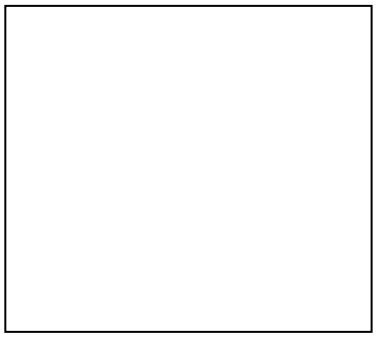
Briefly, acid phosphatase is a phosphomonoesterase; the enzymatic reaction involves the hydrolysis of a phosphate group attached to other molecules and it is optimum at lower pH ranging from 4 to 7. However, the mechanism of the enzyme reaction is not straightforward. Basically, the active sites consist of two metal ions centre coordinated with seven different amino acid residues and the second metal ion is always divalent. Acid phosphatase from sweet potato has an active site composed of Fe(III) and Mn(II) ions separated by an average of 3.26 Å which are able to form six coordinate complexes with amino acids residues, Asp134, Tyr166, and His324 coordinate to the Fe(III) ion, and Asn200 oxygen, His285 and His322 nitrogen atoms to the Mn(II) ion, and Asp163 oxygen atom acts as a bridge between the Fe(III)-O- Mn(II) which is regarded as a μ-oxo or μ-hydroxo bridge. This is schematically illustrated in figure 2-9 (a).

Figure 2-9: Acid phosphatase active site and transition state structures



(This figure was adapted from Koizumi *et al*, 2009)

The mechanism of reaction of phosphate monoester involves the rapid binding of three oxygen atoms from the phosphate ion to the Fe (III) and Mn (II) ions in the active site, the fourth oxygen atom bridging the gap between these two metal ions and finally the fifth oxygen pointing toward the substrate binding pocket (Schenk, 2005), in a tripod fashion shown in the figure 2-9 (b).



(This figure was adapted from Koizumi *et al*, 2009)

During the hydrolysis, a hydrogen bond is formed between the His 295 and other residues from the active site pocket to the metal-bound phosphorus substrate, leading to the hydrolytic cleavage of the phosphate-ester bond and the release of the alcohol group, while the phosphate is still attached to the metal ions. Two water molecules then replace the phosphate bound to Fe (III) ion by binding to the metal ion, which is likely to be deprotonated, and attack the divalent metal ion, forming an oxygen bridge, leading to the release of inorganic phosphate (Schenk, 2005).One of the attempts to improve the population of binding sites was obviously to increase the concentration of the templates. This was approached by investigating the effect of changing the acid phosphate loading onto the glycine microcrystals.Just to reiterate, one of the factors that can potentially change the structure of the PCMCs is the amount of protein loaded onto the excipients.

This part of the review outlined the investigations carried out to develop acid phosphatase-PCMCs using various concentrations of enzyme loading onto the glycine crystals. The change of PCMC morphology was investigated with SEM.

The initial investigation was carried out to determine the appropriate pH using citrate buffer of pH 4.8 and 6.2 at 10mM strength for the acid phosphate PCMC formulation. The PCMCs were prepared using 8% enzyme loading relative to the glycine excipients, 5% water and saturated IPA with glycine, using a similar procedure described previously. The acid phosphatase recovered from the dissolution of ACP-PCMCs was approximately between 90-100%, as determined by standard enzymatic assay adapted from Sigma protocols. The next step was to determine the degree of enzyme immobilisation using different 1%, 2%, and 4% enzyme loading in a citrate buffer pH 4.8 (0.01M)(see experiment 2.4.1 and 2.4.2).

The PCMCs results outlined in table 2-3 suggested that at moderate enzyme loading of 4%, the enzyme was excellently immobilised similar to 8% loading, however at lower loading the enzyme recovered from 2% was slightly lower and significantly lower with 1%. The enzyme immobilisation at lower loadings was anticipated because some enzymes are assumed to exist freely or lost in the mother liquor. In such cases, the same amount of enzyme is removed from each loading, although the effect was most apparent with lower loadings.

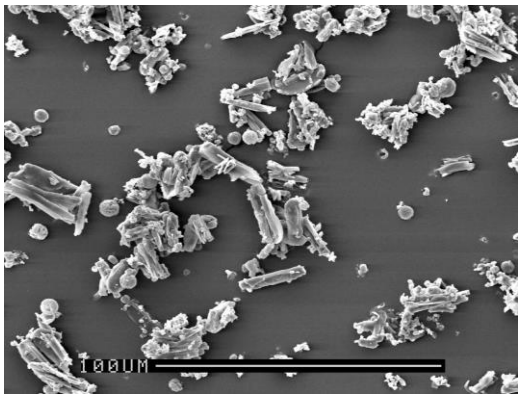
Table 2-3: ACP recovered from ACP-PCMCs prepared at different protein loading

%Protein loading	%Protein bound
1	65±5
2	80±5
4	90±5

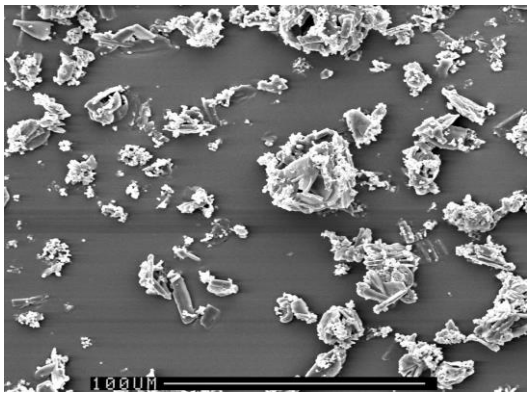
(Mean ± error, n=3)

The SEM images of ACP-PCMCs at different enzyme loadings (figure 2-10) clearly demonstrated that there were significant variations of glycine morphology with changes in the enzyme loading.

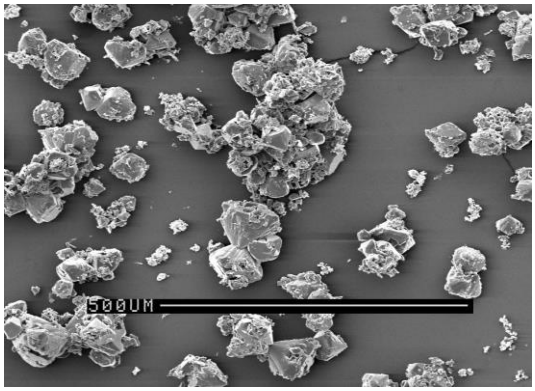
Figure 2-10: SEM of ACP PCMCs at different enzyme loading 1%, 2%, 4% and 8%



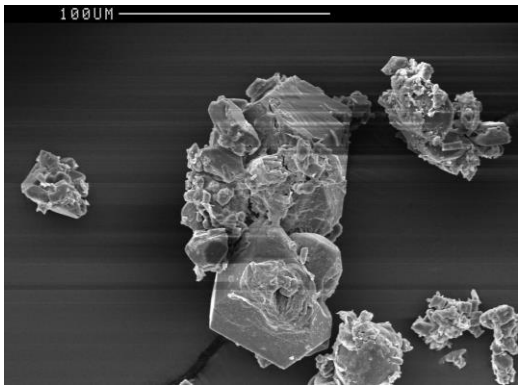
1% Magnification x 396



2% Magnification x 388



4% Magnification x 392



8% Magnification x 380

Figure 2-10 ACP PCMCs prepared at different enzyme loading 1%, 2%, 4% and 8% enzyme loading, 5% water (citrate buffer pH 4.8 (0.01M) and IPA saturated with glycine (precipitating solvent).

With closer inspection of each SEM, it can be observed that at a lower loading of 1%, the shape of the crystals is like flat long rods, some of which have sharp edges or tips similar to a blade, and these crystals begin to aggregate and attain a transitional form from 1-2D at 2% loading. The intensity of the crystal cluster and aggregates increases with enzyme loading, attaining another third dimensional form like a coned shape at 4%. Finally at 8%, these developed shaped crystals grow together in a form of large globular glycine crystals, which seem to combine all of the earlier conformations.

From the SEM images, it could be envisaged that the shape and the size of the pores formed within the imprinted polymer matrix would differ significantly from lower percentage enzyme loading to that of higher loading, where the difference would be less.

2.2.9 Porogen effect on the Lys and ACP PCMCs

In the PCMCs formulation, optimum conditions are mimicked for proteins near their physiological environments, so that the structural integrity and functionality can be maintained. However when proteins are subject to harsh environments they can undergo reversible or irreversible denaturation. Therefore, PCMCs are expected to minimise the degree of protein damage by boosting their structural stability and minimising protein aggregation outside their physiological environment. In the case where irreversible damage occurs to the structure, enzymes tend to lose both conformational stability and their ability to perform the catalytic process.

During the imprinting process, PCMCs are added into acetonitrile in the pre-polymerisation complex, after which they are subjected to free radical polymerisation. An investigation was carried out to determine the effect of porogen on the conformational stability of the enzyme bound to the microcrystals at elevated levels of polymerisation temperature.

The initial experiment was performed using Lys-gly-PCMCs prepared as described previously. Three sets of PCMCs were prepared and analysed differently; the first set of PCMCs were put in a fridge at 4^0C after washing twice with IPA, the second set was similarly washed with IPA and further washed with acetonitrile to remove all traces of IPA before placing in a fridge at 4^0C and the third set was processed similarly to the second sample before incubating at 60^0C; all samples were left for a 48hr period (see experiment 2.5).

The results of the Lys-gly-PCMCs activity are shown in figure 2-11. From the spectrophotometric graph, we can see that the PCMCs incubated with acetonitrile were extremely active compared to those incubated with IPA. This can be seen from the abrupt reduction of the spectrophotometric absorbance reflecting the damaged bacterial

cell. The Lys-PCMCs in acetonitrile left on the bench as well as those incubated at high temperature were thought to become super active. The reasonable argument to account for this effect was that the presence of acetonitrile in the *micrococcus lysodeikticus cell* substrate solution may have either killed the cell or resulted in cell precipitation from the solution; in both cases the results were inconclusive.

Figure 2-11: Porogen effect on Lys-gly-PCMCs activity at polymerisation temperature

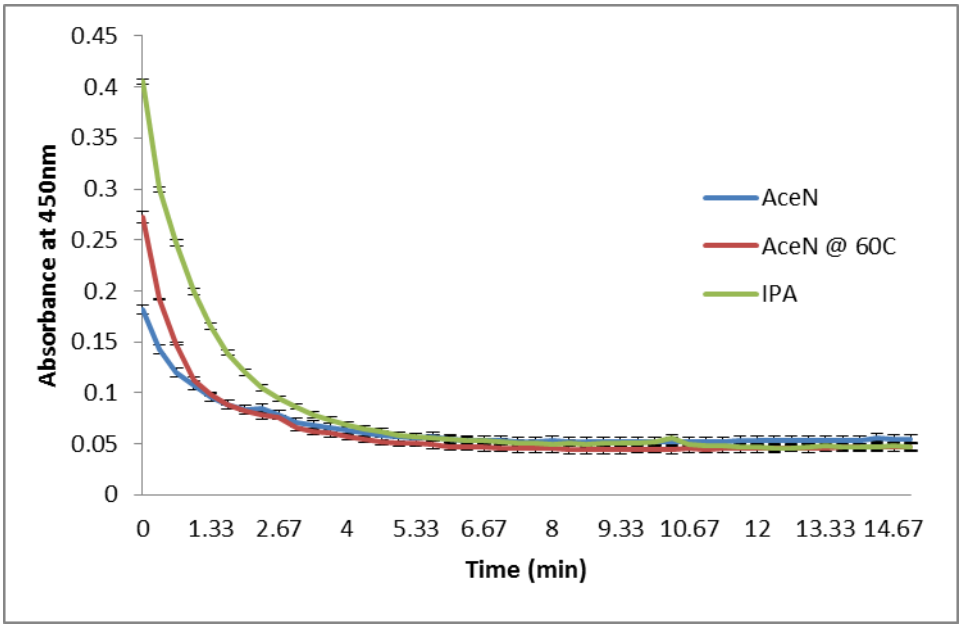


Figure 2-11 Lysozyme enzymatic activity graphs from spectrophotemetric data measured at 450nm wavelength, for lysozyme bound to PCMCs after dissolution of glycine using 0.1 M phosphate buffer pH 6.2 set at different condition. The first set of Lys-PCMCs stored in IPA at 4^0C (green line), the second set was stored in acetonitrile at 4^0C (blue line) and the third set was stored in acetonitrile at 60^0C (red line) (Mean ± error, n=3)

The use of cell substrate in this experiment to determine the effect of acetonitrile and elevated levels of temperature on the activity of lysozyme bound to the glycine microcrystals was not ideal. Therefore, the use of ACP–PCMCs was expected to give some insight into the matter and it was certain that the traces of acetonitrile residues would not interfere with the enzymatic reaction.

The experimental methodology carried out previously was to incubate PCMCs for a longer duration of time, although the free radical imprinting of the polymer is expected to gel within 1-2 hrs. Therefore, the following experiment was modified whereby the PCMCs incubation time was reduced to 2 hrs, and the enzymatic activity was measured at time 0 and 30min time intervals for 2hrs.

For this experiment, the ACP-PCMCs were prepared using two enzymes loadings of 1% and 8%. The samples were initially washed twice with IPA followed by acetonitrile prior to the incubation. In each analysis sample, ACP-PCMCs were filtered and dried, and a known amount of PCMCs was redissolved in a citrate buffer for enzyme activity assay (see experiment 2.6). The acid phosphatase activity results are presented in figure 2-12.

Figure 2-12: Porogen effect on ACP PCMCs activity at polymerisation temperature

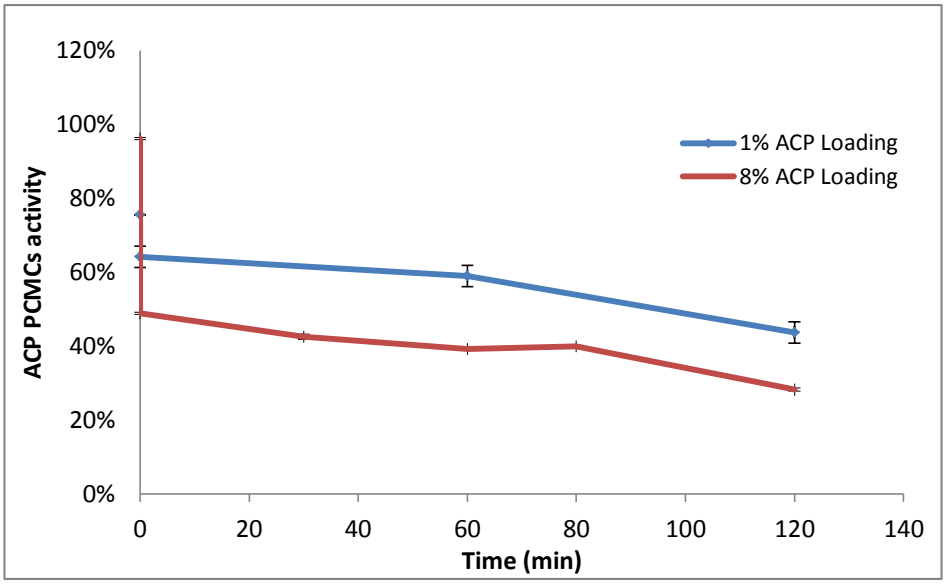


Figure 2-12 Acid phosphatase enzymatic activity graphs determined from spectrophotemetric data measured at 410nm wavelength, for ACP bound to PCMCs after dissolution of glycine using 0.01 M citrate buffer at pH 4.8. ACP PCMCs prepared at 1% and 8% enzyme loading. The ACP bound PCMCs activities were determined after 30 min time interval. (Mean ± error, n=3)

It can be observed that the acid phosphate activity at 8% loading dropped dramatically after initial contact with the acetonitrile, while a significant enzyme deactivation was noted with a lower enzyme loading of 1%. Following the ACP PCMCs incubation at 60^0C, a gradual loss of enzyme activity was observed with time from both enzyme loadings. However on closer inspection of the activity graphs, it was noted that after 1hr the enzyme deactivation was slowing coming to stationary phase for a short period of approximately 20 min, after which another significant percentage drop of enzyme activity was observed.

Overall, after two hours of the experiment, approximately a third of the enzyme activity was lost with 8% loading, while half of the activity was lost with 1% loading. Even though at higher loading much of the activity was lost, the residual active enzyme was more than that at 1% loading.
Studying the effect of porogen alone would not give the complete picture regarding the stability of protein bound to the microcrystals during pre-polymerisation and prior to the free radical process; therefore, the additional effect of functional monomers in the porogen upon PCMCs was also investigated.

2.2.10 Functional monomer effect on the Lys and ACP PCMCs

Further attempts to understand the nature of enzyme activity with some of the polymer compositions was found to be interesting and at the same time useful information could be drawn for polymer optimisation. In the free radical molecular imprinting, templates, functional monomers, cross linkers and initiator are added together in the porogen at elevated temperatures.
To study the effect of the acetonitrile alone upon the enzyme-PCMCs structural stability was insufficient. For this reason, the combined effect of functional monomers with porogen was investigated, even though the conditions are far from mimicking the real polymerisation because other compositions such as cross linker and initiator are excluded. The cross linking monomer is basically an inert monomer which is expected

to only provide backbone to the polymer chain and to encapsulate the template, not forming chemical associations with the template.

In general, the ratios are determined from the number of functional groups available on the template for complexing with functional monomers. However, to date for large molecules such as proteins, there is no standard reported in the literature, the ratios are determined emprical1y; this is a laborious and time consuming process because of the large number of polymer composition variables.

In the beginning, Lys PCMCs were imprinted using a cocktail of acidic and basic monomers, such MAA and 4vpy with a typical ratio 1:4. This will be discussed further in the following chapters. The imperative question we addressed was whether the amount of functional monomer employed was suitable for the template of interest because we were confident that non specificity of the polymer was a result of excessive amount of functional monomers.

In an attempt to minimise this effect, it was decided to add the functional monomers to the co-precipitation process so that only the monomer bound could be estimated after PCMCs dissolution. In addition, the PCMCs bound monomer developed could be added directly to the imprinting process, thus avoiding the use of excessive monomers in the polymerisation pot.

Lys PCMCs bound monomers were prepared as follows: 10% enzyme loading, 3% water and the ratio of enzyme to functional monomers was 1: 4 (MAA: 4vpy) by weight. Briefly, functional monomers were added to the enzyme/excipient solution and then were co-precipitated in the acetonitrile saturated with glycine (see experiment 2.7.1). The amount of water used was lower than before to eliminate the washing step of PCMCs with acetonitrile.

The resultant PCMCs-functional monomers had a different texture to the normal dry powder; it felt like an adsorbent soft cotton wool.

For analysis, the PCMCs-functional monomers were dried and a known weight was dissolved in a buffer for enzyme and 4vpy recovery prior to the injection into the RP-HPLC. The RP-HPLC spectra are presented in figure 2-13, showing pyridine peaks at ~ 2.3min retention time and lysozyme at ~7.5.

Figure 2-13: RP-HPLC spectra of Lys PCMCs bound monomers

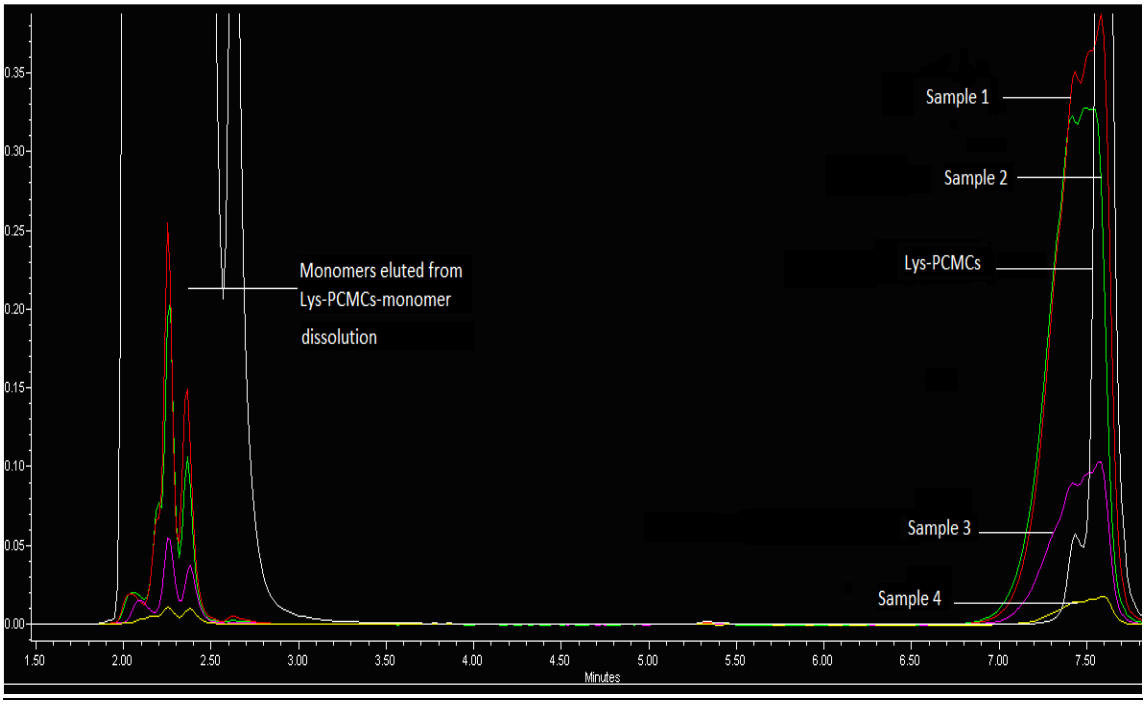


Figure 2-13 RP-HPLC spectrums at 280nm of lysozyme/functional monomers (MAA and 4vpy) recovered from 4 samples of Lys PCMCs at concentration (0.1mg/ml) after glycine crystals dissolution using 0.01 M phosphate buffer pH 7.2.

The apparent features from the lysozyme incubated with monomer spectra are that the peaks were broader compared to the normal lysozyme spectra (shown by white peak), However, the spectral changes may be due to the addition of the functional monomers, which might have complexed with the lysozyme. Another possible argument was that the lysozyme-monomer complex increased the solubility of lysozyme in acetonitrile, which therefore separated from the complex, leading to the co-precipitation of free enzyme onto the already formed monomer-bound microcrystals. These results evidently suggest that the methodology intended to precipitate lysozyme with monomers and use the monomer bound PCMCs in the polymerisation was possible. However from this, we could not realistically deduce any useful information regarding the ratio to use between the lysozyme and the functional monomer.

In a further attempt to see if we could better estimate the amount of functional monomer 4-vpy to use, we approached the problem differently; Lys-gly-PCMCs were prepared

initially using 10% loading, 3% water, saturated IPA with glycine. The Lys-gly-PCMCs formed were pre-washed with acetonitrile to remove water molecules and then incubated with 4vpy dissolved in acetonitrile for 15min to establish the complex formation between the enzyme and the functional monomer (see experiment 2.7.2).

Finally, the Lys-gly-PCMCs bound 4vpy were filtered and the enzyme and 4vpy recovered were determined by RP-HPLC. In addition, the calibration curve was drawn to determine the concentration of the functional monomer 4vpy that was bound to the PCMCs. The graph was prepared using known concentrations of 4vpy in acetonitrile and the area under the peak at 256nm was determined from RP-HPLC.

The Lys-gly-PCMCs bound monomer spectra were observed for lysozyme recovery spectra viewed at 280nm (figure 2-14) and 4vpy recovery at 256nm (figure 2-15) and their concentrations were determined from the area under their respective peaks.

Figure 2-14: Lys recovered from dissolution of Lys PCMCs bound monomers

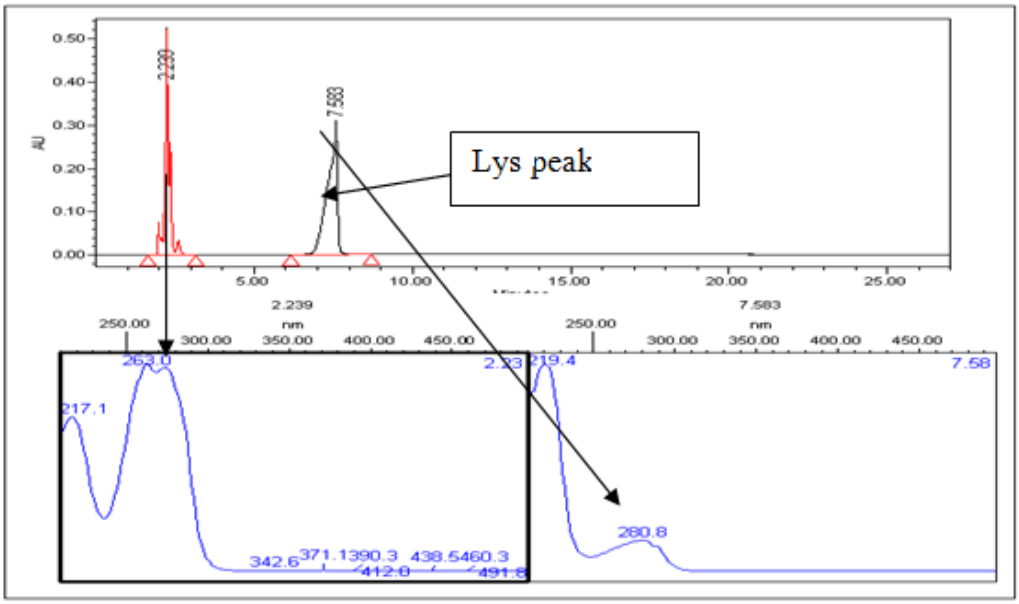


Figure 2-14 RP-HPLC spectra at 280nm of lysozyme/functional monomers (MAA and 4vpy) recovered from PCMCs after glycine crystals dissolution using 0.01 M phosphate buffer pH 7.2. RP-HPLC peak after 2.230 min corresponded to glycine absorbing at 263nm and after 7.583 minutes is lysozyme peak absorbing at 280.8nm.

The visible features from the spectra shown in figures 2-14 and 2-15 are that the shape of the lysozyme chromatography peak was broader than normal. This suggested that the addition of 4vpy might actually have resulted in a complex formation with the enzyme. This is further demonstrated by the lysozyme peak in figure 2-15. At closer inspection of the spectra, it can be observed that the lysozyme is bound to other wavelength at 260nm which is suspected to correspond to the modified 4vpy which shifted from 256 to 260nm. The changes of the 4vpy wavelength occurred into complexing of the functional monomer in the pre-polymerisation complex.

Figure 2-15: 4 vpy recovered from dissolution of Lys PCMCs bound monomers

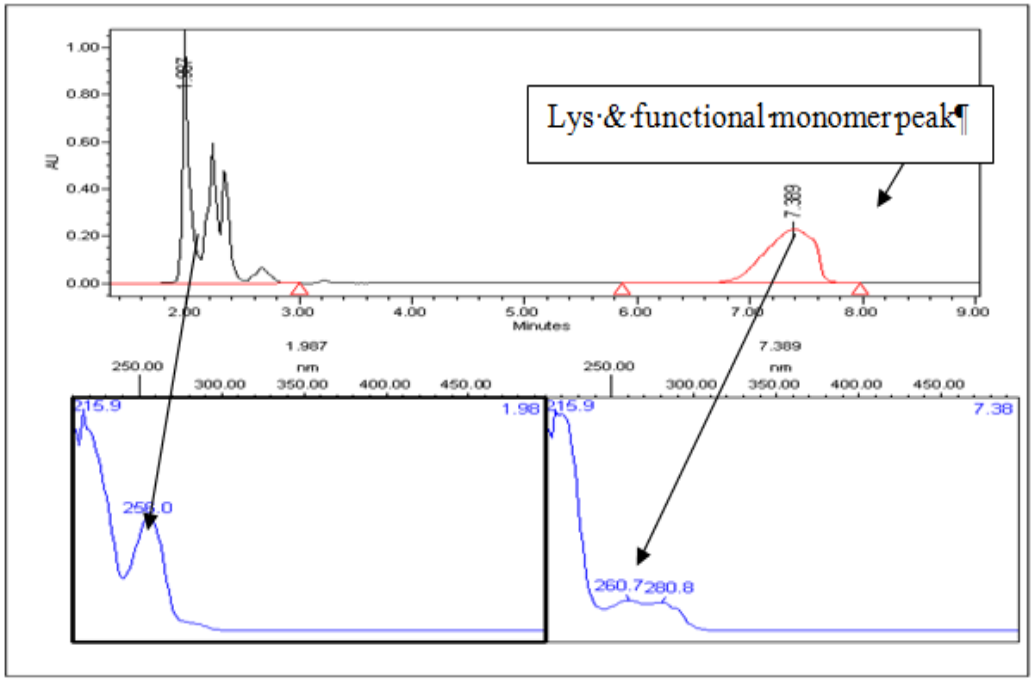


Figure 2-15 RP-HPLC spectra of lysozyme/functional monomers (MAA and 4vpy) recovered from PCMCs similar to figure 2-14. However the RP-HPLC spectra after 1.987 min at 256nm is for 4vpy and a peak after 7.38 min corresponded to lysozyme peak absorbing at 280.8nm, with traces of 4vpy at 260.7 nm.

In a further attempt to demonstrate that a complex might actually formed between the enzyme and functional monomer, a wavelength scan of the sample with Lys PCMCs and the blanks was also examined in a spectrophotometer. The resultant scan is presented in figure 2-16. From the spectra, we can observe the two humps representing two 4vpy

wavelengths at 260nm and lysozyme at 280nm, similar to what was observed previously from RP-HPLC.

Figure 2-16: Wavelength scans of Lys, Lys PCMCs, blanks & Lys PCMCs - 4vpy

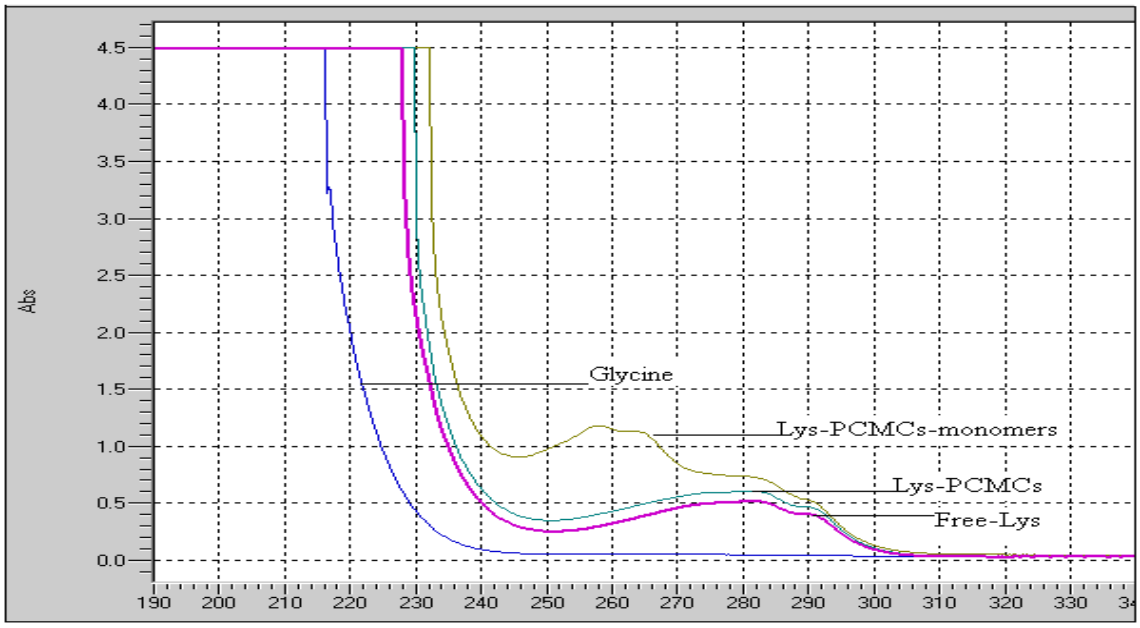


Figure 2-16 Wave length scan of lysozyme/functional monomers (MAA and 4vpy) recovered from Lys-PCMCs sample used for figure 2-14 and 2-15, glycine crystals solution, free lysozyme powder and lysozyme PCMCs, all at 100µg/ml of 0.01M phosphate buffer at pH 7.2.

Just to reiterate, the main focus was to estimate the concentration of 4vpy that was bound to a known amount of enzyme. This was determined from the area under the 4-vpy peaks at 256nm and the concentration was estimated from the 4vpy calibration curve. The amount of 4vpy corresponding to the peak shown in figure 2-15 was approximately ~6.7 µmol for each mg (~72 µmol) of lysozyme bound to microcrystals. This suggested that the ratio of 4-vinyl pyridine to lysozyme was ~ 10 times lower. Therefore, the concentration of vinyl pyridine used previously might have been in excess, and the use of a lower ratio of 4-vpy was considered appropriate for the preparation of new polymers.

A similar investigation was carried out for acid phosphatase to better estimate 4vpy and MAA; however, the experimental approach was different from that previously

described. Instead of measuring the amount of 4vpy complexed with the enzyme, we determined the amount of functional monomer bound using equilibrium binding, whereby the amount of monomers bound was calculated by subtracting the concentration of functional monomers added to the pre-polymerisation complex from the amount left after the ACP PCMCs were filtered from the solvent mixture (see experiment 2.8). The amounts of functional monomers bound to the ACP PCMCs at all loadings were lower than that observed before with the lysozyme PCMCs. The normalised results to the mg of enzyme recovered are presented in figures 2-17 and 2-18. From 4vpy histograms, the 4vpy bound was higher at 1% loading and then the lowest binding was observed with 8%, while similar 4vpy binding was observed for 2% and 4% loading, which was approximately half of that bound at 1%. A similar trend was observed with MAA, the monomer binding decreased with increasing enzyme loading.

Figure 2-17: 4vpy bound to different % of acid phosphatase loading

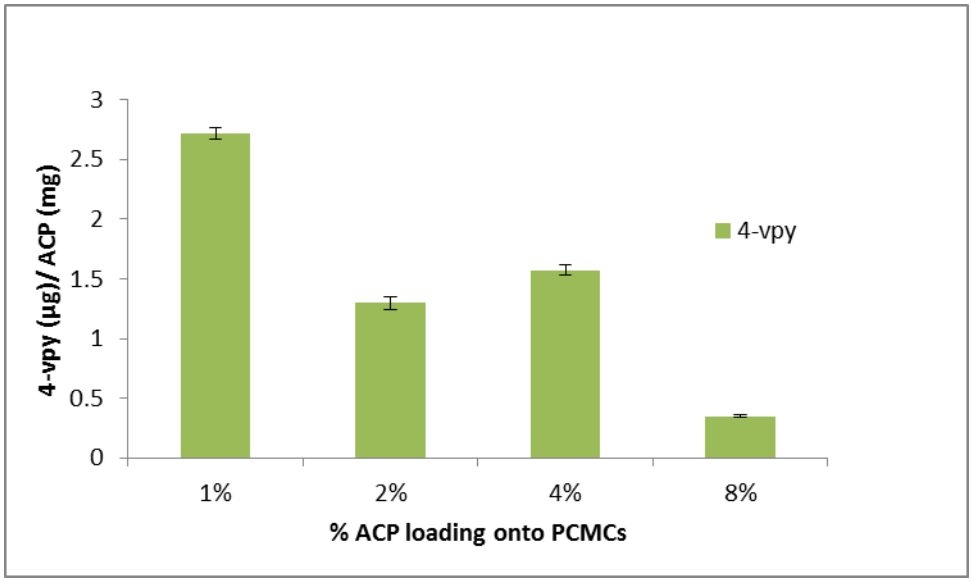


Figure 2-17 4vpy bound onto acid phosphatase PCMCs prepared at different enzyme loading 1%, 2%, 4% and 8% with 4vpy 100 µg /ml of acetonitrile at 60 ^{0}C for 1hr. (Mean ± error, n=3)

Figure 2-18: MAA bound to different % of acid phosphatase loading

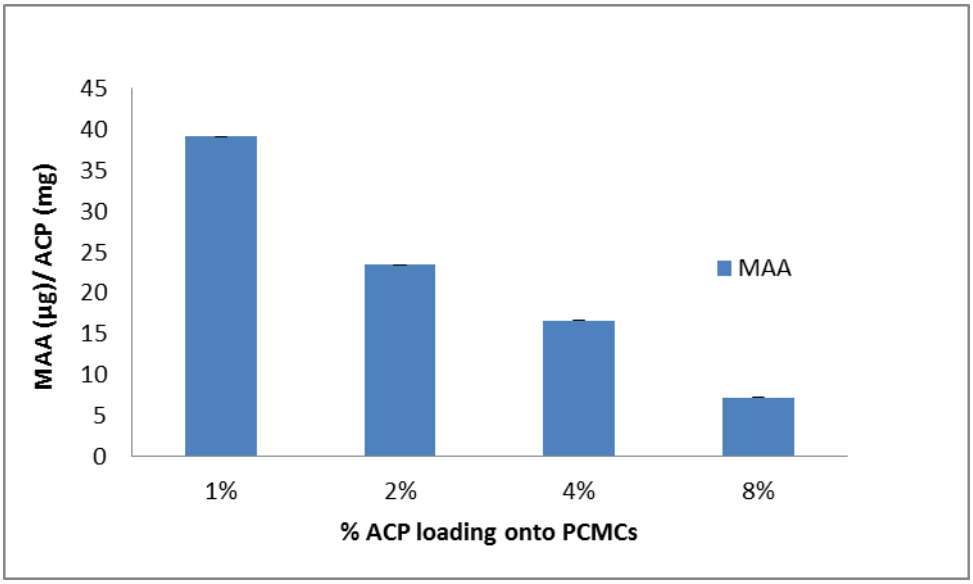
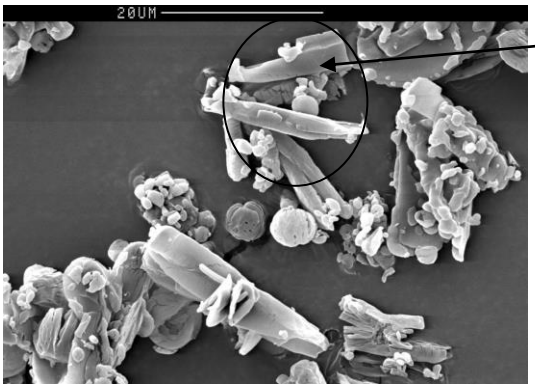


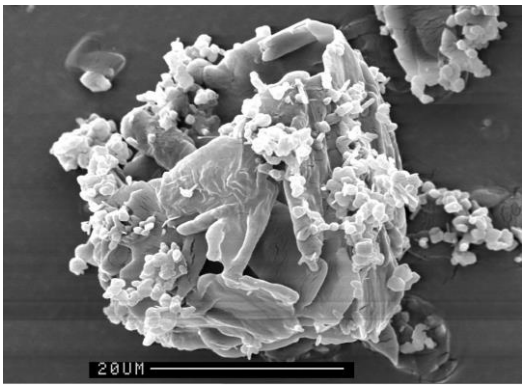
Figure 2-18 MAA bound onto acid phosphatase PCMCs prepared at different enzyme loading 1%, 2%, 4% and 8% with 4vpy 100 µg /ml of acetonitrile at 60 ^{0}C for 1hr. (Mean ± error, n=3)

It was suspected that the functional monomers were initially adsorbed onto the free surface of the microcrystals shown in figure 2-19 at higher magnification for enzyme loading at 1, 2 and 4% and then the enzyme-monomer complex is established. Therefore, at lower enzyme loading most of the surface area is exposed and available for adsorption leading to significant binding of the monomer, while lower binding occurred with high enzyme loading because of the smaller surface area exposed for this effect.

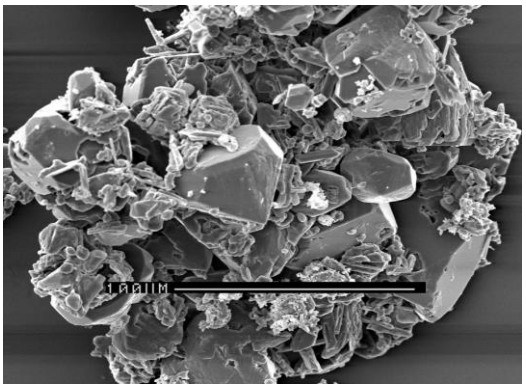
Figure 2-19: SEM of ACP PCMCs at 1, 2 and 4 % enzyme loading at higher magnification



1% Magnification x1600



2% Magnification x1600



4% Magnification x1500

Figure 2-19 Higher magnification of ACP PCMCs prepared at different enzyme loading 1%, 2% and 4% enzyme loading, 5% water (citrate buffer pH 4.8 (0.01M) and IPA saturated with glycine (precipitating solvent).

From the investigation carried out, it was impossible to get a reasonable estimation of the functional monomer to use. Therefore, we were confident that lower ratios of functional monomers were still appropriate for the formulation of the new ACP PCMCs polymer. In addition, we could not draw any conclusion whether the complex is formed between the monomers and the enzyme in the pre-polymerisation, as previously shown with lysozyme.

To address this issue, it was hypothesised that if the functional monomer associates with the enzyme, then the effect could be reflected in the enzyme activity. In addition, we could get some insight into the enzyme stability in the polymerisation conditions by simulating the combined effect of the ACP-PCMCs, functional monomer and acetonitrile at elevated temperature, even though in the absence of the cross linker from the reasons explained previously.

Still working with the lower concentration of functional monomers, a brief study was undertaken to examine the effect of the aforementioned conditions on the ACP, PCMCs activity at different enzyme loading.

The acid phosphatase PCMCs were prepared as described before. For each ACP PCMCs at different enzyme loading were incubated with both 100µg of MAA//ml of acetonitrile and 10µg of 4vpy/ml of acetonitrile in separate vials at 60^0C for 3.45 hours. After the experimental period, the proteins were recovered by dissolution of a known amount of ACP PCMCs at different loading for enzyme activity assay, as describe before (see experiment 2.9) and the results are presented in figure 2-20.

From the acid phosphate activity graph, it can be clearly observed that the percentage of active enzyme retained after incubation with 4vpy was better compared to enzyme incubated with MAA.

Overall, the percentage of active enzyme retained after 4vpy/acetonitrile incubation was obviously more with 8% and decreased with decreasing enzyme loading. However when comparing the expected amount of active enzyme initially bound, 4% loading retained the highest percentage of activity, followed by 2% and approximately similar results were obtained for both 1% and 8%, which was even more than what was retained with acetonitrile alone. A similar trend was observed with ACP PCMCs incubated with

MAA/acetonitrile. However, the retention of active enzyme for 1% and 8% was approximately similar to that observed before with acetonitrile.

Figure 2-20: ACP PCMCs activity after incubation with 4vpy and MAA

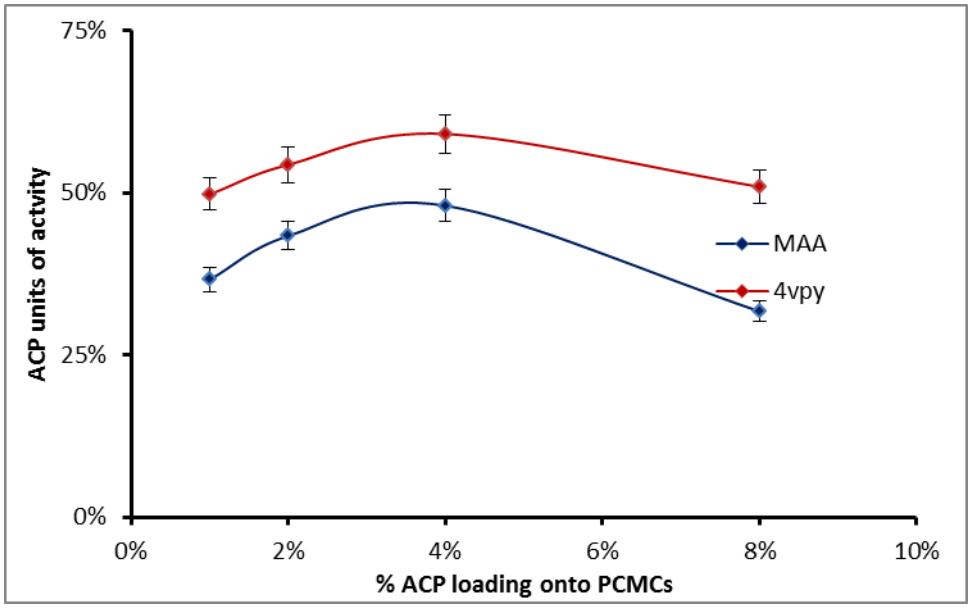


Figure 2-20 Acid phosphatase enzymatic activity graphs from spectrophotemetric data measured at 410nm wavelength bound to PCMCs after glycine crystals dissolution of glycine using 0.01 M of citrate buffer pH 4.8. 2 sets of ACP PCMCs prepared at 1, 2, 4 and 8% enzyme loading. One set was incubated with 100µg MAA/ml and the other set with 4vpy 10 µg /ml of acetonitrile at 60 ^{0}C; both for 3.45 hrs. (Mean ± error, n=3)

The immediate reaction was that 4vpy significantly improved the enzyme stability in the acetonitrile and at elevated temperature more than MAA or acetonitrile alone for 1% and 8% loading (refer to figure 2-12). This suggested that 4vpy was forming some kind of complex with functional monomers or a protective layer that shielded the enzyme from the effect of the elevated temperature and harsh organic solvent.

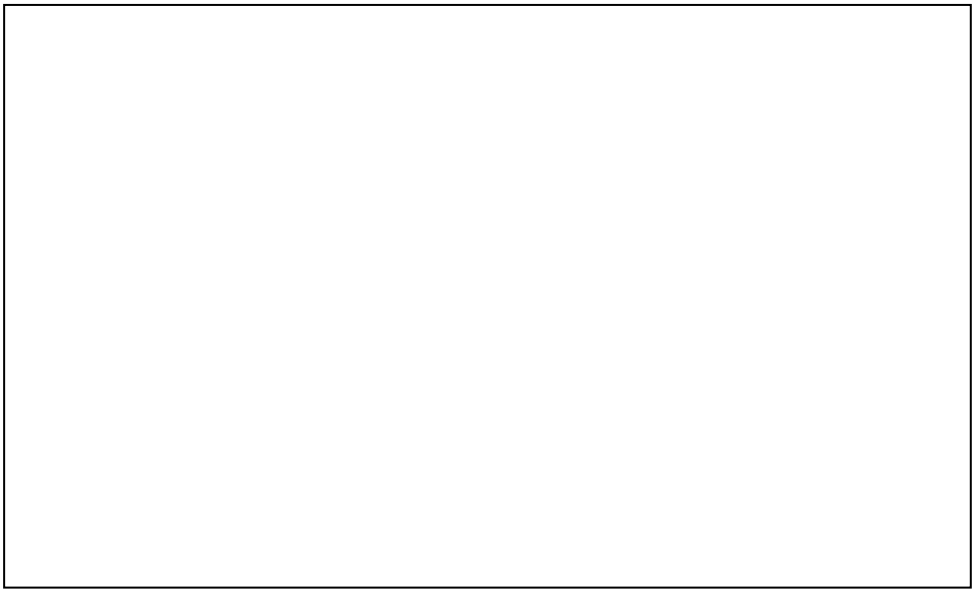
The preceding results have shed some light onto the effect of 4-vpy in boosting the stability of acid phosphatase in the porogen and at elevated polymerisation temperatures.

Most of the imprinting work carried out previously involved using a combination cocktail of acidic and basic functional monomers. In a further effort to determine better imprinting compositions, we investigated the use of functional monomer 4vpy alone. This concept was applied to alkaline phosphatase because it was very cheap compared to the acid phosphatase and it was from the same family of metallohydrolase enzymes.

2.2.11 Alkaline phosphatase PCMCs and SEM

Alkaline phosphatase is a metallohydrolase enzyme that catalyses the hydrolysis of different types of phosphate monoester bound to the groups of molecules, including nucleotides proteins and alkaloids in typical alkaline conditions between pH 8-8.5. The mechanism of enzyme activity was described by Zatalan *et al*, (2008) from a transition state perspective using a phosphate ester substrate. A schematic of the process is presented in figure 2-21.

Figure 2-21: Alkaline phosphatase active pocket in a transition state



This figure was adapted from (Zatalan *et al*, 2008)

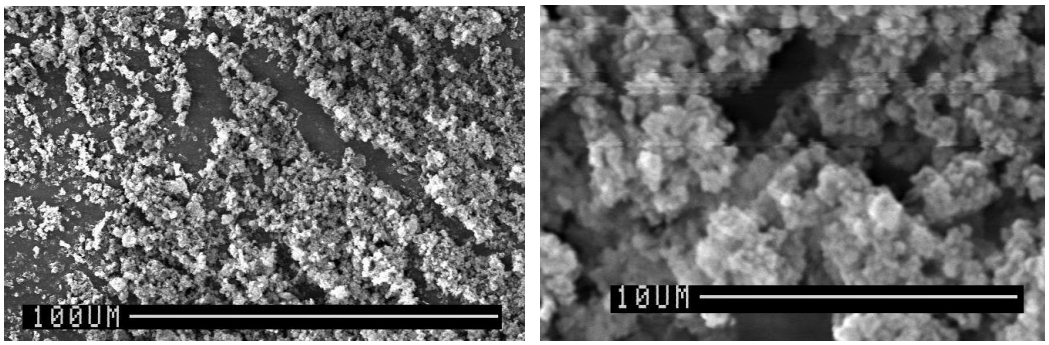
The active site pocket is composed of three metal-binding sites and amino acid residues whereby the two zinc ions (Zn^{2+}) and magnesium ion (mg^{2+}) form a catalytic metal triad. Initially, the catalysis of the phosphate monoester involves the coordination of an ester

oxygen atom from the phosphate group to the Zn1 and additional interactions between the charged non-bridging oxygen atoms of the substrate with Zn2 and two oxygen atoms with guanidinium group of residue Arg166 forming a covalent enzyme-phosphate intermediate. The ser102 hydroxyl group (nucleophile) forms hydrogen bonding with Mg^{2+}-bound by hydroxide ions, which act as a base, and the deprotonated ser102 attack the phosphorus atom by a nucleophilic reaction, forming a covalent serine-phosphate transition intermediate. In the transition state, the phosphorus centre is inverted, leading to the loss of the alcohol group, and nucleophilic hydroxide ion coordinated to Zn1 attacks the covalent enzyme-phosphate intermediate, breaking the bond into forming the non-covalent enzyme-phosphate complex, leading to a second inversion of the phosphorus centre. Finally, the water molecule bound to the Mg^{2+} acts as a general acid, donating a proton to deprotonated Ser102, leading to the release of inorganic phosphate or directly to protonate the phosphate group (Stec *et al*, 2000).

To visualise the ALK PCMCs from SEM, the PCMCs were prepared using 8% loading 5% water (diethanolamine buffer at pH 9.8) in saturated IPA with glycine, using a similar procedure to that described previously and the enzyme recovery was determined by activity assay adapted from Sigma protocols (see experiment 2.10).

The enzyme recovered was approximately between 90-100%. For SEM, the ALK-PCMCs were prepared with similar composition as above and the images are shown in figure 2-22.

Figure 2-22: SEM of ALK PCMCs at different magnifications



Magnification x 406 Magnification x 3029

Figure 2-2 Alkaline phosphatase PCMCs prepared at 8% enzyme loading, 5% water (diethanolamine buffer at pH 9.8) and IPA saturated with glycine (precipitating solvent).

The immediate feature observed from the images is that the crystal shape variation was insignificant compared to that observed with ACP-PCMCs. This might have to do with the different types of enzyme and buffer employed.

Chapter 2: Experimental Sections

Materials

Ribonuclease A from bovine pancreas Type I-A (RNase A), Lysozyme from chicken egg white, *Micrococcus lysodeikticus* , Acid phosphatase (P1146) , Alkaline phosphatase from bovine intestinal mucosa (P7640), p-Nitrophenyl phosphate (104), Diethanolamine (D-8885), Magnesium chloride hexahydrate (M-0250), Ethanol, Alumina, Na_2HPO_4, NaH_2PO_4, Isopropanol, Trifluoroacetic acid (Spectrophotometric grade), Tris-HCl and Tris-base, Sodium phosphate monobasic anhydrous and Sodium phosphate dibasic anhydrous, Sodium hydroxide anhydrous, Acetonitrile (HPLC grade) were purchased from Sigma-Aldrich Co. UK. Hydrochloric acid (3247A) Riedel-de Haën, K_2HPO_4 (MW 174.18), KH_2PO (136.09), KCl, were purchased form Fluka UK. Glycine, Citric acid (MW 210) were purchased from (Fisher scientific UK), Molecular sieves (MERCK Germany). All materials were used as received, with no further purification. Cuvette with a path length 1.0 cm (Fisher brand cuvettes, semi–micro), 15ml tall vial and 25ml vial screw on from Fisher scientific UK, Durapore Membrane filters of pore size 0.22µm and 0.45µm (Millipore Ireland). Deionised filtered water (Resistivity 18.2 MΩ) was obtained from in-house Millipore water purification system. Methacrylic acid (MAA), 4-vinly pyridine (4vpy) were purchased from Aldrich

Material Preparation

MAA and 4VPy were purified by passing through a column packed with alumina; MAA and 4VPy were then distilled under reduced pressure and acetonitrile was dried by molecular sieves.

Apparatus –Spectrophotometer (Beckman Coulter, DU 800) with a temperature controller (Beckman Coulter), Reverse Phase High Performance Liquid Chromatography (RP-HPLC) waters 2996 (photodiode array detector), waters 2487 (Dual absorbance detector), waters 2695 (separation module), Centrifuge Beckman Coulter (large Eppendorf), Centrifuge (Eppendorf AG2233, Hamburg, Germany) and Incubator (Techno, Ori-block, OB-3), Rotary evaporator. HPLC pump, Blood rotary and Incubator (Stuart Scientific), Malvern Mastersizer 2000 (Malvern Instrument Ltd), SEM microscope Cambridge Instruments Stereoscan 90, meter balance (Metter-Toledo AE200), pH meter (Metter-Toledo MP120), Magnetic stirrer and magnetic stirring bar (Fisher Scientific).

Preparation of buffers

Phosphate buffer

Stock solution of 1.0M of K_2HPO_4 was prepared by dissolving 1.74g in dH_2O and 1.0M of KH_2PO_4 were prepared by dissolving 1.36g in dH_2O.

To prepare **pH 7.2 (0.01M),** 10 mM of phosphate buffer pH 7.2 were prepared by adding 71.7 ml of 1.0M K_2HPO_4 to 28.3ml of 1.0 M KH_2PO_4. 1ml was then diluted with 99ml of dH_2O to obtain a10mM buffer strength.

To prepare **pH 8.2 (0.01M),** 10 mM of phosphate buffer pH 8.2 were prepared by adding 94.0 ml of 1.0M K_2HPO_4 to 6.0ml of 1.0 M KH_2PO_4. 1ml was then diluted with 99ml of dH_2O to obtain a10mM buffer strength.

Tris-HCl buffer pH 7.8 (0.01M)

10 mM of phosphate buffer pH 7.8 were prepared by dissolving Tris HCL (5.32g) and Tris Base (1.97g) in 500ml of dH_2O to achieve the desired pH 7.75-7.8 at room temperature in a volumetric flask.

Sodium phosphate buffer pH 6.2 (0.1M)

0.1M Sodium phosphate buffer at pH 6.2 was prepared by dissolving 1.2g of sodium phosphate monobasic anhydrous (MW; 120g) and 1.42g sodium phosphate dibasic anhydrous (MW; 142g) in 100ml of dH_2O.

Citrate buffers pH 4.8 (0.1M) and 6.2 (0.1M)

Stock solutions of 0.1 M of citric acid and 0.1M of sodium citrate were prepared by dissolving 2.10g of citric acid /l of dH_2O and 29.4g of sodium citrate /l of dH_2O.To prepare **pH 4.8 (0.1M),** 40 ml of citric acid and 60ml of tri-sodium citrate were added. 1ml of this buffer was again diluted in 9 ml of dH_2O to make 0.01M buffer strength.
To prepare **pH 6.2 (0.1M),** 8ml of citric acid were added to 92ml of tri-sodium citrate.

Diethanolamine buffer

Stock solution of 1.0 M of Diethanolamine buffer was prepared by adding 21.028g of diethanolamine in 200ml of dH_2O dissolved with $MgCl_2.6H_2O$, under magnetic stirring. The buffer is adjusted to pH 9.8 or 7.8 using 5M HCl.
To prepare 10mM of the Diethanolamine buffer, 1ml of the buffer was added to 99ml of dH_2O

Experiment 2.1.1

PCMCs preparation and characterisation

The technique employed for the preparation of enzymes, lysozyme, alkaline phosphatase and acid phosphatase PCMCs was similar to that described previously by Kreiner *et al*, (2001). For example, if a desired protein loading was 11.11% by weight then the excipient will equate to 88.89%w/w by weight (100% -11.11% proteins loaded). For example, if 4mg of enzyme were at 10% enzyme loading, then the excipient would be 36mg. Normally, protein is dissolved initially in a suitable buffer to create an environment closer to its physiological condition, to prevent it from denaturation, i.e. 4mg of protein may be added to 100µl of the buffer. However, on some occasions where amino acid such as glycine was employed as a protein support, the protein was dissolved directly into the glycine/buffer solution.

The excipient or support solution was prepared either slightly below salt saturation level or way below, depending on the amount of the crystals required. However, the amount of excipient employed should not be above salt saturation level to prevent excipient precipitating from the solution. For example, KCl salt saturates at 300mg/ml of dH_2O, while the salt solution prepared in this report was at 100mg/ml. To obtain 36mg of the excipient, 360µl of salt solution were drawn and added directly into 100µl of protein solution, and the final volume of protein/excipient solution is ~460µl (100µl + 360µl). If the desired water content of the PCMCs is 5%, for example here is (460µl), then the percentage of the precipitating bulk solvent would be 95% (100%- 5%) and this would equate to 8.47ml (460µl x 95%) /5%. The precipitating solvent is initially saturated with the excipient overnight to allow formation of microcrystals.

For a typical small scale PCMCs production, 4mg of lyophilised proteins powder were dissolved in 100µl of phosphate buffer pH 7.23 (0.01M) and then transferred to 0.36 ml of excipient solution (100mg of excipient /ml of buffer). 0.46ml of protein/carrier solution was added dropwise to the 15ml vial containing 8.74.6ml of IPA saturated with excipient under agitation provided by a magnetic stirring bar for approximately 1-2 min. Following co-precipitation, PCMCs were filtered using a membrane filter of pore size 0.22 µm and then left to dry on the lab bench at room temperature, after which protein recovery examination was carried out as described in **experiment 2.1.2.** This was repeated using other buffer; phosphate buffer pH 8.23 (0.01M) and Tris/HCl buffer pH 7.8 (0.01M).

Experiment 2.1.2

Protein recovery from PCMCs using Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The analysis of proteins recovered from the PCMCs was as follows; for example for lysozyme-KCl-PCMCs, 10mg of PCMCs were dissolved in a 0.01M phosphate buffer at e.g. pH 7.23, making the protein concentration 1mg/ml, and this was compared with free standard lysozyme powder having similar concentrations. Both samples were then

injected into RP-HPLC and the spectra generated were compared from the area under the graphs. This was repeated for PCMCs prepared using other buffers; phosphate buffer pH 8.23 (0.01M) and Tris/HCl buffer pH 7.8 (0.01M).

General settings of Reverse Phase High Performance Liquid Chromatography Settings (RP-HPLC)

All samples were analysed using RP-HPLC and were injected on a C4 column calibrated at 30°C and eluted at a flow rate of 1 ml/min in a gradient of; 0.1% trifluoroacetic acid in aqueous (mobile phase A) and 0.1% trifluoroacetic acid in acetonitrile (mobile phase B), while eluents were measured by UV/VIS at wavelengths 280 nm. **Gradient**

Mobile phase

A 90% B10% 15min

A 10% B90% 5min

A 90% B10% 5min

A 90% B10% 5min

After each sample analysis, dH_2O was passed through the column to remove any residual protein or contaminants, thus ensuring non-interference with subsequent sample analysis and to equilibrate the column back to the starting gradient.

Protein recovery is calculated as follows:

$$\text{Protein immobilised (\%)} = \frac{\text{Measured protein concentration (mg/ml)}}{\text{Expected protein concentration (mg/ml)}} \times 100$$

Experiment 2.1.3

Determination of lysozyme activity after recovery from lysozyme-KCl-PCMCS

After lysozyme PCMCs were left to dry on the bench, 20mg of lysozyme PCMCs were dissolved in 2.0ml of 0.01 M phosphate buffer at pH 7.2, making lysozyme concentration 1mg/ml. The lysozyme activity assay involved the enzyme acting upon the *Micrococcus lysodeikticus* Bacterial cell wall. The stock solution of the freeze dried *Micrococcus lysodeikticus* substrate was prepared by suspending 3mg of the substrate into 30ml of the phosphate buffer at pH 7.2 making the concentration of the substrate

0.1mg/ml. The lysozyme-KCl-PCMC solution and lyophilised powder were then diluted to concentrations 27µg/ml (1500unit of activity) and a similar unit from lysozyme standard was used. Each sample was added to a 1.5ml of standard suspension (0.1 mg/ml) of *Micrococcus lysodeikticus* placed separately into a plastic cuvette with a path length of 1cm, and assayed at 25°C for 4 min after the sample had been quickly mixed with a 1ml tip pipette. The decrease of absorbance was monitored using a spectrophotometer at 450 nm to determine the optical density (OD) after mixing. For the base line correction of the spectrometer, 0.1ml of enzyme was added to the phosphate buffer placed in a cuvette without the bacterial cells.

Experiment 2.1.4

For large scale PCMCs production for molecular imprinting purposes, a large batch of PCMCs was prepared using two Gilson-HPLC pumps; pump A for protein and pump B for saturated precipitating solvent. For example for the lysozyme PCMCs prepared for lysozyme imprinting of L1, L2 and L3, 70.56mg of enzyme were dissolved in 1.764ml of 10mM phosphate buffer pH 7.2 and transferred to 6.35 ml of excipient solution. ~8.1 ml of the protein/excipient solution were drawn using plastic tube *via* a three way tap set at a flow rate of 5ml min^{-1} and were added to 154 ml of IPA saturated with excipient set at a flow rate of 95ml min^{-1} under constant agitation provided by a magnetic stirring bar and hot plate stirrer. Following precipitation, the PCMCs were separated from the solvent by leaving to sediment and the supernatant was decanted. PCMCs were then washed with 100ml of IPA and 2-3 washes with dry acetonitrile to remove water bound to the crystals, which otherwise could potentially interfere with free radical polymerization. A similar procedure was repeated if blank crystals (control) are prepared but without the proteins.

Experiment 2.2

Lysozyme-KCl-PCMCs and blank microcrystals for particle size measurements

Sample lysozyme-KCl PCMCs and blank microcrystals were prepared using automated HPLC pump and small scale batch system with the following compositions; 10% w/w lysozyme loading and 5% water in IPA saturated with KCl.

Particle sizing measurement was achieved using a Malvern Mastersizer 2000 (Malvern Instrument Ltd) and acetonitrile as a dispersant phase. Although PCMCs were prepared in IPA, the use of acetonitrile was expected to show the actual particle sizes when the PCMCs and blank samples (control) were added to the porogen during the imprinting process. Clean dry AcN was flushed through SVS cell to purge any air present in the system because the presence of air bubble would interfere with the measurement. 30 mg polymer sample powder were initially washed with AcN to remove IPA and finally re-suspended in a 50ml of clean dry acetonitrile (dispersant) for particle sizing.

50ml of each sample suspension were measured by adding the samples directly into SVS cell and then the particles were distributed by mixing with turbulent mixer at 2000rpm. The particle sizes were finally measured by laser diffraction with refractive index set at 1.490 for KCl salt.

Experiment 2.3

Determination of lysozyme activity after recovered from Lysozyme-glycine-PCMCS

After lysozyme PCMCs were left to dry on the bench, a stock solution of lysozyme-gly PCMCs was prepared by dissolving 24 mg PCMCs in 2.4 ml of 0.1 M sodium phosphate buffer at pH 6.2 making lysozyme concentration 1mg/ml. Lysozyme standard solution was prepared by dissolving 6.5 mg of lysozyme in 6.5ml of the same buffer, making a stock solution of 1mg/ml. Briefly, the procedure and preparation of the bioassay were adapted from Gorin *et al*, (1971), using stock solution of standard lyophilised lysozyme concentration 1mg/ml and freeze dried *Micrococcus lysodeikticus* 0.1mg/ml (substrate). PCMC solutions with a calculated lysozyme concentration 1mg/ml were prepared as previously described in the protein determination **experiment 2.1.2**. 0.1ml of the PCMC solution and lyophilised powder were diluted to different concentrations (10 µg, 20 µg,

and 40 µg-ml), added to 3.0ml of standard suspension (0.1 mg/ml) of *Micrococcus lysodeikticus* in 0.1M sodium phosphate buffer at pH 6.2, placed into a plastic cuvette with a path length of 1cm, and assayed at 25°C for 15min after the sample had been quickly mixed with a 1ml tip pipette. The decrease of absorbance was spectroscopically monitored using a similar procedure to that described in the **experiment 2.1.3**.

Experiment 2.4.1

Acid phosphatase PCMCs Preparation and SEM

2 x acid phosphatase PCMCs samples were prepared in a small scale production using 4mg of acid phosphatase in the following compositions; 8% enzyme loading, 5% water and 95% IPA saturated with glycine excipient. Two citrate buffer of pH 4.8 and 6.2 at 10mM strength were investigated for the maximum enzyme binding onto the glycine excipient.

The acid phosphatase PCMCs were finally prepared at different enzyme loading; 8%, 4%, 2%, 1% and 5% water (citrate buffer at pH 4.8) in IPA saturated with glycine, using the reagents composition demonstrated in the table below. The blank microcrystals ACP B was also prepared using similar composition as ACP 4 but without the enzyme.

PCMCs %	Protein (mg)	Glycine (mg)	Glycine solution 100mg/ml	Saturated IPA (ml)
ACP 1 (1%)	1	99	0.99	18.81
ACP 2 (2%)	2	98	0.98	18.62
ACP 4 (4%)	4	96	0.96	18.24
ACP B (0%)	----	96	0.96	18.24
ACP 8 (8%)	8	92	0.92	17.48

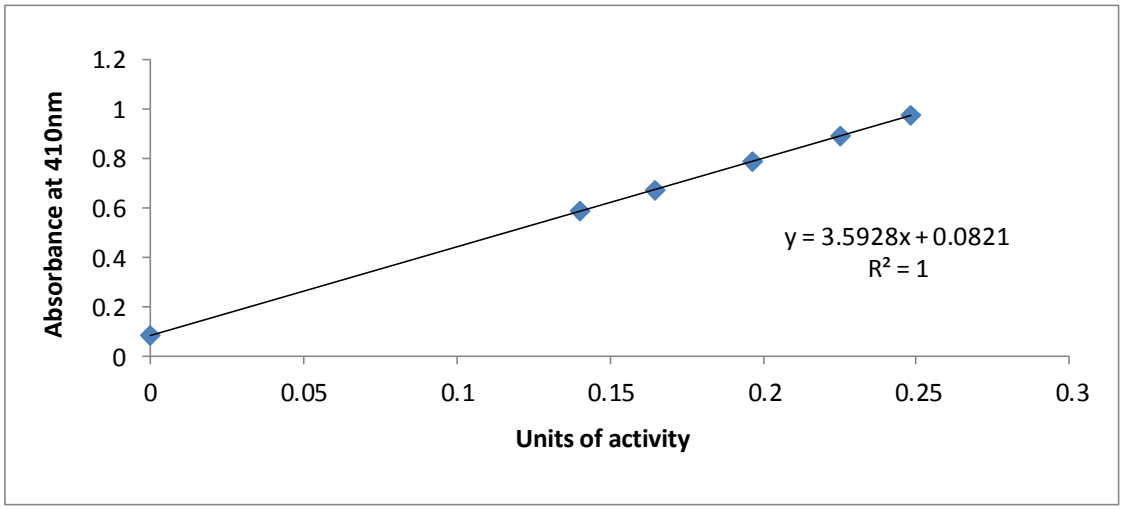
Experiment 2.4.2

Determination of acid phosphatase recovery from acid phosphatase PCMCS

For this experiment, a known weight of acid phosphatase PCMCs 1% 2%, 4% and 8% was dissolved in citrate buffer pH 4.8 (0.01M) and the enzyme recovered were determined, using an assay adapted from sigma protocols from the following link; http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/p1146enz.Par.0001.File.tmp/p1146enz.pdf) (see chapter 8 appendix 8.1).

The units of the enzyme activities were determined by comparing the enzyme recovered from the acid phosphatase after PCMCs dissolution in a cold deionised water, with that of the standard acid phosphatase units of activities having an actual units of 1mg of lyophilised enzyme powder = 4.6 units; prescribed from sigma. The calibration graph was drawn from measuring the spectrophotometric absorbance of the product generated from the enzyme hydrolysis reaction of 4-nitrophenol phosphate to 4-nitrophenol, which absorbs at 410nm.

Standard curve for acid phosphatase activity



Experiment 2.5

Lys- glycine-PCMCs and acetonitrile (porogen) effect at different temperatures.

Three lysozyme-gly-PCMCs samples were prepared in a small scale PCMCs production as described before in the **experiment 2.1.1** using 4mg of lysozyme at 10% enzyme loading, water 5%;v/v in 95%v/v IPA saturated with glycine as an excipient.

The first sample of lysozyme-gly-PCMCs was washed 3 x 4ml of IPA and was added to a vial containing 6.5ml of IPA and was put in a fridge at 4^0C. The second sample was washed with IPA similar to the first sample and further washed 3 x 4ml of acetonitrile to remove IPA and was finally added to a vial containing 6.5ml of acetonitrile before left in a fridge at 4^0C. The third sample was processed similarly to the second sample but was incubated at 60^0C. All samples were left for 48hrs prior to the enzyme activity determination using the assay protocols adapted from Gorin *et al*, (1971) (**see experiment 2.3**).

Experiment 2.6

Acid phosphatase PCMCS activity and acetonitrile (porogen) effect at elevated temperature

ACP PCMCs were prepared at 1% and 8% enzyme loading using similar reagents described before in the **experiment 2.4.1**. 100mg of each sample PCMCs ACP 1% and ACP 8% were initially washed with 2 x 5ml IPA and 2 x 7ml of acetonitrile prior to the incubation with 7 ml of the porogen (dry acetonitrile), at 60^0C for 2 hrs in a 15ml centrifugal tube. The acid phosphatase bound PCMCs activities were determined at every 30 min time interval; 1ml of each PCMCs sample suspension was drawn out from the tube and put into centrifugal eppendorfs to separate the PCMCs. The PCMCs were then dried in the in a fume hood at room temperature and ~5mg of ACP 1% and ACP 8% powder were redissolved in cold deionised water, followed by enzyme bound activities assay using sigma protocol (**see experiment 2.4.2)**

Lysozyme-functional monomers complex formation study

Experiment 2.7.1

Addition of functional monomers 4-vinyl pyridine (4vpy) and methacrylic acid (MAA) during PCMCs formulation

Lysozyme-gly-PCMCs were prepared as follows; 10% enzyme loading, 3% water in IPA saturated with glycine; the amount of water used was lower than before to eliminate the washing step of the lysozyme-gly-PCMCs with acetonitrile.

Briefly, 4mg of lysozyme were dissolved in a 40mg glycine\400µl of buffer containing 8µl of MAA (92µmol) and 8.2µl of 4vpy (76.1µmol). The ratio of enzyme to functional monomers was ~4mg: 16mg (2MAA: 24vpy). The lysozyme /excipient/functional monomer solution was then added drop-wise to the 25ml vial containing 12.93 ml of IPA saturated with glycine to create lysozyme-glycine-functional monomers-PCMCs. The developed PCMCs were measured for the enzyme recovery and 4vpy bound using similar RP-HPLC settings described before in the **experiment 2.1.2**. This was carried out by dissolving 10mg of the PCMCs in 1ml of phosphate buffer pH 7.2 (0.01m) and 50µl of the PCMCs solution were injected into the RP-HPLC. From the RP-HPLC spectra generated, lysozyme recovered was analysed at a wavelength of 280nm and 4vinyl pyridine eluted in conjunction with lysozyme was measured at 256-260nm.

Experiment 2.7.2

Addition of functional monomers 4-vinyl pyridine and methacrylic acid (MAA) after PCMCs formulation

Lysozyme-glycine-PCMCs were prepared as follows; 9.09% enzyme loading and 3% water in IPA saturated with glycine. Briefly, 4mg of lysozyme were dissolved in a 400 µl of glycine solution; the lysozyme/glycine solution was then added drop-wise into the 25ml vial containing 12.93ml of IPA saturated with glycine using similar technique described before for small scale PCMCs preparation in the **experiment 2.1.1**. PCMCs developed were left to sediment and the supernatant was decanted. The PCMCs were then washed 2 x 4ml of IPA followed by 2 x 4ml of dry acetonitrile. After wash, 11mg of the PCMCs with the amount of protein bound (1mg/ml) were suspended in 1ml of

AcN dissolved with 1mg of 4-vinyl pyridine (9.5 µmol) and MAA (11.5 µmol) in an eppendorf. The sample was rotated for 15 min to induce complex formation between lysozyme bound to the microcrystals and the functional monomers. The PCMCs were then separated from the functional monomer solution using 0.22 µm Millipore membrane and dried on the lab bench and were finally redissolved in dH_2O to recover lysozyme after carrier (glycine) dissolution. 50µl of the PCMCs solution were injected directly into RP-HPLC to quantify the amount of the lysozyme recovered at 280nm and the functional monomer 4vpy bound to the lysozyme at 256-260nm. This was also repeated for blank crystals.

For the wavelength scan experiment, similar PCMCs/functional monomer samples, blank glycine solution, and standard lyophilised lysozyme solution were each put into different cuvettes placed in a spectrophotometer for scanning at different wavelengths. The spectra generated were finally compared.

Experiment 2.8

Determination of the acid phosphatase monomers complex formation

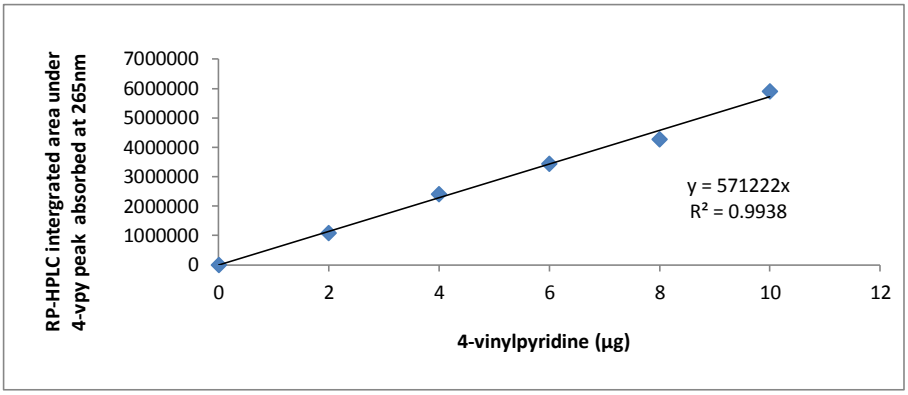
2 samples each of the acid phosphatase PCMCs were prepared at different enzyme loading at ACP1%, ACP 2%, ACP 4%, ACP 8% and ACP B (0%) a control.

One group of samples was incubated with 4ml of 100 µg of MAA /ml of acetonitrile and the other set was incubated with 100 µg/ml of 4-vinyl pyridine, both for a period of 1hr. After incubation, all samples were separated from the functional monomers solutions by centrifugation and 100µL of each supernatant were injected directly into the RP-HPLC, to determine the amount of 4vpy and MAA bound using equilibrium binding.

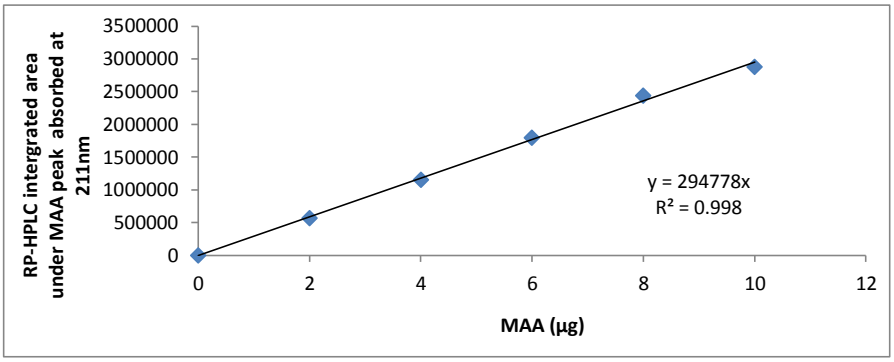
The area generated by the 4vpy was determined as follows

(Area generated from the initial concentration of the functional monomers added to the PCMCs sample before incubation) - (area generated from the final concentration of the functional monomers incubated with the PCMCs). The amount of the functional monomer bound to the PCMCs was then analysed using the calibration graphs shown below:

Standard calibration curve for 4-vinyl pyridine



Standard calibration curve for MAA



Experiment 2.9

Determination of the acid phosphatase PCMCS activity after complex formation

2 samples each of the acid phosphatase PCMCs were prepared at different enzyme loading 1%, 2%, 4% and 8%. One set was incubated with 100µg MAA/ml and the other set with 4vpy 10 µg /ml of acetonitrile at 60 ^{0}C; both for 3.45 hrs. The acid phosphatase recovered from the dissolution of known amount of the ACP PCMCs at different enzyme loading was examined for the enzyme activity using the assay adapted from Sigma **(see experiment 2.4.2).**

Experiment 2.10

Preparation of alkaline phosphatase PCMCs, PCMC, evaluation and SEM

Alkaline phosphatase PCMCs were prepared at 8% enzyme loading, 5% water (diethanolamine buffer at pH 9.8) in IPA saturated with glycine using similar procedure described previously for small scale and large scale PCMCs production. The enzyme recovered was determined by the enzyme activity assay from sigma using the following link;

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/p7640enz.Par.0001.File.tmp/p7640enz.pdf (see chapter 8 appendix 8.2).

Chapter 3 : Molecular imprinted zwitterionic polymers

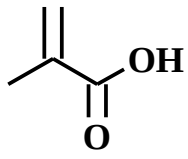
3.1 Introduction

To recap, in the free radical molecular imprinting, the polymer is prepared by adding all components, including template, functional monomers, cross linker and initiator, into the porogen and the polymerisation process is initiated using either thermal or UV initiation. Our approach to free radical monolithic imprinting uses PCMCs as a platform for protein imprinting; this involves immobilising protein onto microcrystals prior to the imprinting process. Details of the experiments can be found at the end of the chapter.

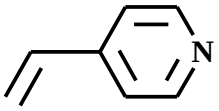
The initial work involved imprinting enzyme lysozyme PCMCs using popular monomers that include both acid and basic groups, such as methacrylic acid (MAA) and basic monomer 4-vinyl pyridine (4-vpy), along with the commonly and extensively used cross linking monomers and initiators, ethylene glycol dimethacrylate (EDGMA) and 2,2'-azobisisobutyronitrile (AIBN); all added to the porogen acetonitrile, with the polymerisation process initiated by using heating. Because of the reasons justified, it was logical to begin our study using these monomers. The molecular structures of the functional monomers, cross linker and initiator employed are presented in figure 3-1 and an extensive list of different polymer compositions and their molecular structures are presented in the introductory chapter.

The rationale of using a cocktail of functional monomers with both acid and basic groups was because lysozyme carries amino acid side chains, each having both positive or negative charges, as well as neutral charge at different pH. Therefore, charge-charge interactions and hydrogen bonding with functional monomers could be exploited. For example, MAA with a pKa value of 4.5 at physiological pH would carry a negative charge and would possibly form complementary association with lysine, histidine, and arginine, which are positively charged.

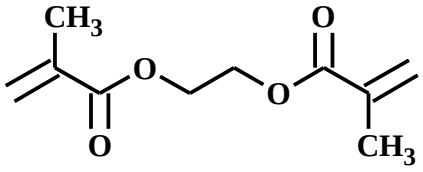
Figure 3-1: Molecular structures of molecular imprinted polymer reagents



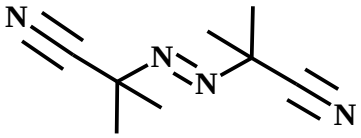
MAA



4-vpy



EDGMA



AIBN

On the other hand, positively charged 4vpy would be expected to interact with negatively charged glutamic acid and aspartic acid. Consequently, a significant number of potential template-functional monomer complexes could form, critical to formation of a selective binding site architecture.

Previously, the above monomers were employed for imprinting haemoglobin and myoglobin and were evidently shown to improve the imprinting effect. However, the charge of both proteins and functional monomers in less polar porogen was unknown, although it was assumed these interactions would still occur.

This part of the report also highlights an extensive study undertaken to develop better analytical techniques for studying protein imprinted polymers, such as using equilibrium batch rebinding process in non-competitive template rebinding, competitive and cross selective template rebinding experiments and finally tuning of mobile phases with chemical agents or additives, to discriminate selective from non selective protein binding to the polymers. Furthermore, significant attention was directed towards developing protein extraction protocols for these highly crosslinked imprinted polymers using various combinations of solvents.

3.2 Preparation of lysozyme imprinted polymer

Our approach to monolithic imprinting strategy uses PCMCs as a platform for lysozyme imprinting. Previously, we demonstrated that PCMCs technology offers an excellent technique for protein immobilisation onto the surface of microcrystals and allows the retention of the native conformation in aqueous media. This was demonstrated through protein recovery examination involving dissolution of PCMCs in aqueous phase and compared with unprocessed lysozyme of the same concentration in the same medium. These studies showed that the immobilised protein was ~100% active on the surface of the microcrystals (please refer to chapter 2).

To begin with, we investigated polymer selectivity and specificity by changing two variables, the ratio of functional monomers and the cross linker. The imprinted polymers were prepared by incorporating PCMCs compositions outlined in table 3-1, using the polymer reagents presented in table 3-2.

Table 3-1: Preparation of lysozyme PCMCs

PCMC	Protein Loading % w/w	Water content	Solvent
Lys	10.0	5%	IPA

Briefly, lysozyme PCMCs were prepared using two automated Gilson-HPLC pumps with 10% lysozyme loading, and KCl as carrier salt, 5% water and 95% IPA saturated with carrier salt. Pump A was assigned to the protein and pump B to the saturated precipitating solvent. For example, for the lysozyme PCMCs prepared for lysozyme imprinting of L1, L2 and L3; 70.56mg of enzyme were dissolved in 1.764ml of 10mM phosphate buffer pH 7.2 and transferred to 6.35 ml of excipient solution. ~8.1 ml of the protein/excipient solution were drawn using a plastic tube *via* a three way tap set at a flow rate of 5ml min^{-1} and were added to 154 ml of IPA saturated with excipient set at a

flow rate of 95ml min^{-1} under constant agitation provided by a magnetic stirring bar and hot plate stirrer. Following precipitation, the lysozyme-KCl-PCMCs were separated from the solvent by leaving to sediment and the supernatant was decanted.
PCMCs were then washed with 100ml of IPA and 2-3 washes with dry acetonitrile to remove water bound to the crystals, which otherwise could potentially interfere with free radical polymerisation. A similar procedure was repeated if blank crystals (control) were prepared but without the proteins.

Table 3-2: Reagents used for the preparation of lysozyme imprinted polymer

Polymer	Lys PCMCs (mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN g, mmol	AcN (ml)	Molar ratio of monomers: Cross linker
L1	750	0.449, 5.235	0.550, 5.235	5.0, 25.254	0.1, 0.609	7.911	2:5
L2	750	0.449, 5.236	0.550, 5.236	10.0, 50.508	0.183, 1.115	7.911	1:5
L3	750	0.899, 10.471	1.100, 10.472	10.0, 50.509	0.200, 1.219	7.911	1:10

{Volume of acetonitrile was calculated from 4/3 (sum of all monomers + cross linker)}

Briefly, the polymers were prepared using a conventional monolithic imprinting technique, whereby all polymer components, i.e. template, functional monomers, cross linker and initiator, were added together in acetonitrile (porogen), mixed gently, and the polymerisation initiated by heating. For example, for preparation of polymer L1; 750 mg of lysozyme-KCl-PCMCs containing 70.56mg (0.0048mmoles) of lysozyme were added to a 15ml vial containing 7.911 ml of dry acetonitrile (porogen). The vial was then half immersed into an ice cooled water bath, followed by the addition of functional monomers; MAA (5.236 mmoles, 0.449g), 4VPy (5.236 mmoles, 0.550g) and cross linker EDGMA (50.508 mmoles 10.0g). The mixture was purged with nitrogen for 5

min to eliminate any soluble oxygen and finally initiator AIBN (183mg) was added. The vial was then sealed with a screw top and parafilm, and incubated at 60^0C for 48 hrs under constant rotation on a blood rotor machine to ensure even distribution of PCMCs in the mixture. Similar polymerisation processes were carried out for the other polymer samples L2 and L3.

3.2.1 Processing of lysozyme imprinted polymer

All polymers obtained L1, L2 and L3 were hard, rigid and glassy block polymers, typical of monolithic polymers and similar to those reported by Sellergren and Shea, (1995); Schweitzer *et al*, (1998); Rachkov *et al*, (1999); Andersson *et al*, (1999); Sellergren (2001), with a good yield recovered from imprinting.

The polymer monoliths were ground into manageable size particles ranging between 20-50µm. The polymer powders were pre-washed with acetonitrile to remove any unreacted functional monomers MAA and 4-vpy and the size of the 4vpy peak from the RP-HPLC was taken as an indication of the complete removal of these monomers from the polymer. The acetonitrile was employed because the functional monomers are very soluble in this solvent. Just to emphasise that is quite important to completely remove unreacted residual monomers from the polymer. Otherwise if left, they might complex with free enzyme in solution during the template reintroduction step and this would interfere with the protein rebinding analysis.

At first, the concentration of the unreacted monomer left in the polymer was not known and neither was the volume of the acetonitrile required to completely eradicate the monomers. This was investigated experimentally using 100mg of polymer L3 having double concentrations of functional monomers compared to polymers L1 and L2 with different volumes of acetonitrile for 30 min and the resultant spectra are presented in figure 3-2. From the RP-HPLC traces, the most visible feature was the decreasing 4vpy peaks with increasing volume of the acetonitrile. The concentration of 4vpy removed/ml of washing solvent was approximately the same for the volumes investigated.

From this, it was clear that there was no need to consume large volumes of solvent which was not a cost effective process, one of the fundamental principles behind molecular imprinting. Using less solvent also meant that loss of polymer from using large washing vessels was minimised, because some polymer tends to stick on the wall of the container. From the results, it was found that on average 1-2ml per wash were appropriate for 100mg of polymer.

Figure 3-2: RP-HPLC of 4-vpy washed from Lys MIPs using different volumes of AcN

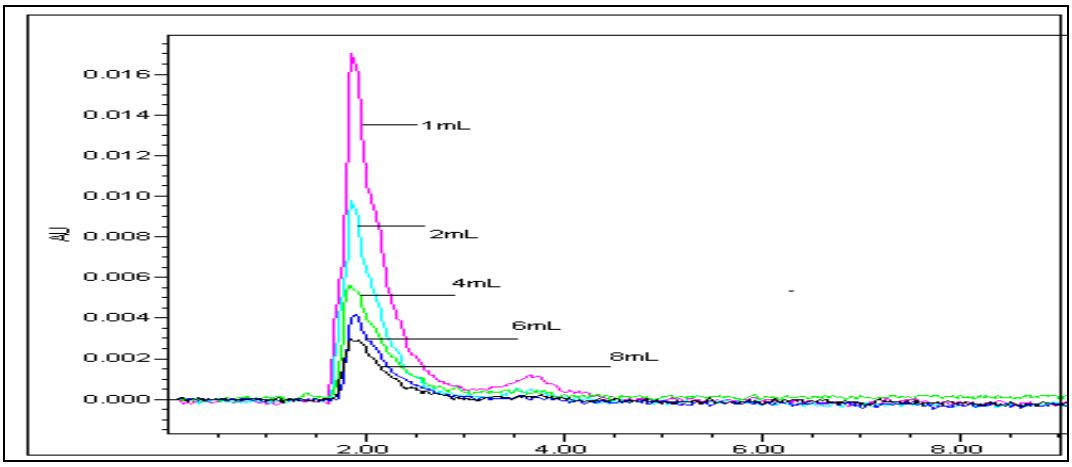


Figure 3-2 RP-HPLC spectra of 4vpy released from L3 (100mg) after polymer wash with different acetonitrile volumes (1, 2, 4, 6, and 8) ml, each for 30 min.

Another parameter looked at was the time required for maximum removal of 4vpy. This was because a longer washing time was quite a laborious and a time consuming process. The resultant spectra of 4vpy removed from the polymer at different time intervals are presented in figure 3-3. From the results, the intensity of the 4vpy peak and therefore the concentration of the 4vpy removed after different time intervals were approximately the same. From this, it was clear that on average 5min were sufficient to remove 4vpy and this greatly reduced the polymer processing time, which could instead be devoted to optimising the template extraction from the polymer, which was expected to be quite a challenge. In order to be certain that there was complete removal of 4vpy from the polymer, each wash was spectroscopically monitored and the 4-vpy RP-HPLC traces are

presented in figure 3-4. From the results, it can be clearly seen that it required a minimum of 3-4 washes in order to completely remove the 4vpy from the polymer. This was still found to be time consuming. Therefore, in a further attempt to reduce the number of washing steps, we added organic basic additives to the acetonitrile solvent.

Figure 3-3: RP-HPLC spectra of 4-vpy washed from Lys MIPs at different times

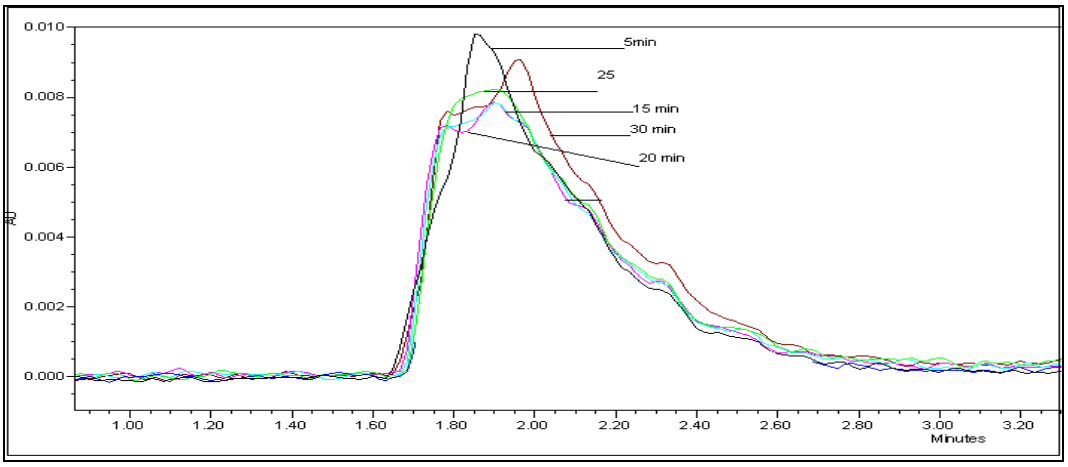


Figure 3-3 RP-HPLC spectra of 4vpy released from L3 (100mg) after polymer wash with 2ml of acetonitrile at different time intervals (5, 15, 20, 25 and 30) - minutes.

Figure 3-4: RP-HPLC spectra of 4vpy totally washed off from Lys MIPs

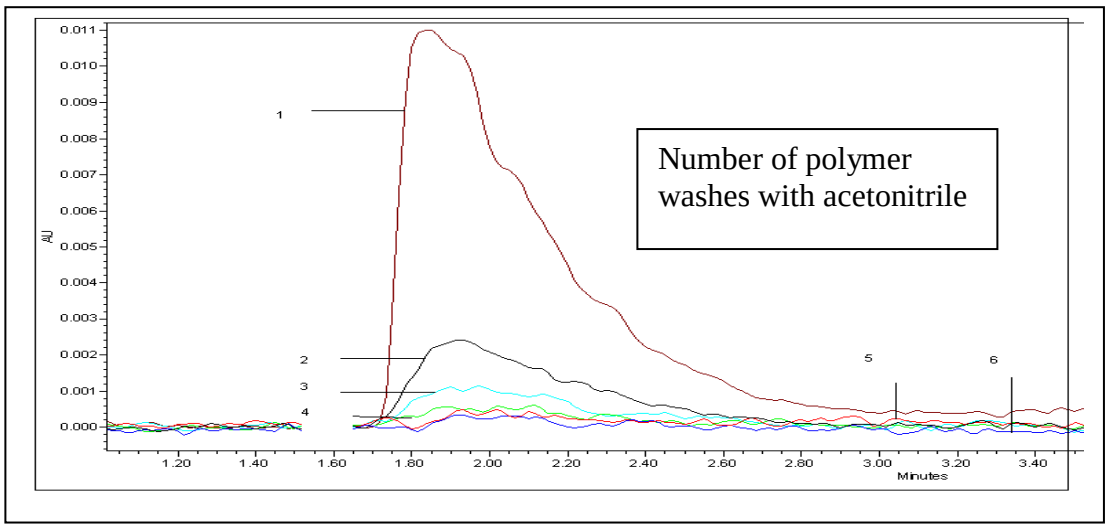
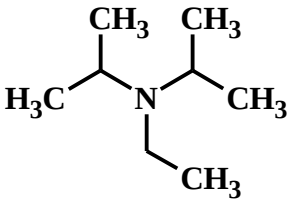


Figure 3-4, RP-HPLC spectra of 4vpy in acetonitrile wash from polymer L3. Each of 100mg of polymer was rinsed 6 times with 2ml of acetonitrile for 5 min.

N,*N*'-Diisopropylethylamine (DIPEA) is an organic base and an amine which is commonly employed in peptide chemistry. DIPEA is a poor nucleophile because the nitrogen atom is shielded by the two isopropyl groups and an ethyl group, so that only a proton is small enough to easily fit. This makes it a useful organic base and it was expected to displace the 4vpy from the polymer and hence facilitate its removal. The molecular structure of DIPEA is shown below in figure 3-5.

Figure 3-5: Molecular structure of *N*,*N*'-Diisopropylethylamine (DIPEA)



The acetonitrile washing solvent was modified by addition of DIPEA1%v/v, and the sample RP-HPLC traces of 4-vpy removed are demonstrated in figure 3-6.

From the results, it can be noticed that the shape of the spectra after seven washes was nearly the same. On closer inspection of each spectrum, a mixture of different wavelengths was observed. However even after the 5th wash, the 4-vinyl pyridine wavelength between 256-260.7 nm was still visible.

Figure 3-6: RP-HPLC of 4vpy washed from Lys MIPs with addition of 1% DIPEA

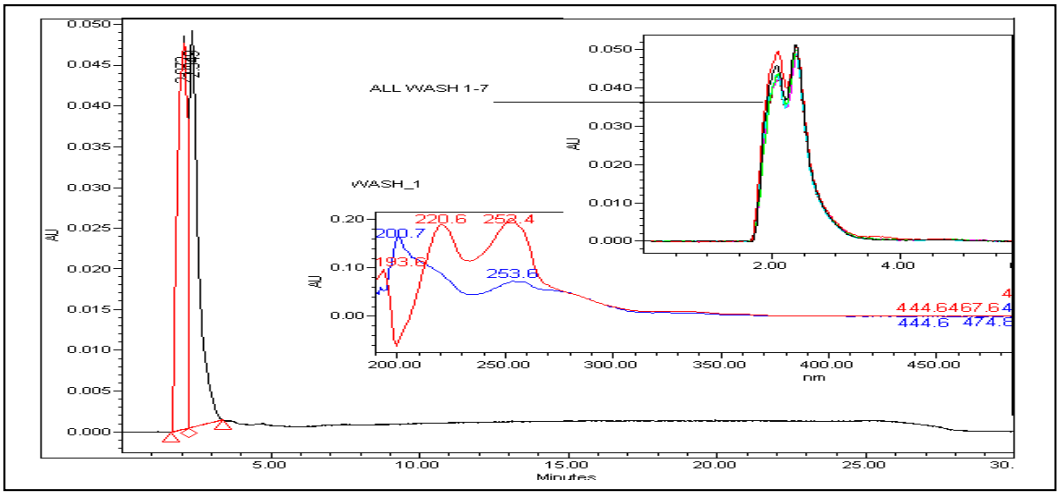
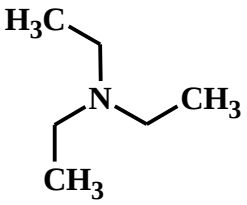


Figure 3-6 RP-HPLC spectra of 4vpy released from L3 (100mg) after polymer 5th wash with 2ml of acetonitrile modified DIPEA1%v/v, for 5 min.

The main focus here was to reduce the number of 4vpy washing steps. However, clearly the addition of DIPEA into the acetonitrile did not resolve this problem. In a further attempt to improve this, we tried another base triethylamine (Et_3N). This is also a common organic base used in organic synthesis for preparation of esters and amides but it has a strong unpleasant smell. The molecular structure is presented in figure 3-7.

Figure 3-7: Molecular structure of triethylamine (Et_3N)



The acetonitrile washing solvent was modified by addition of Et_3N 1%v/v and the RP-HPLC traces of 4-vpy removal are demonstrated in figure 3-8.

Figure 3-8: RP-HPLC of 4vpy washed from Lys MIPs with addition of 1% Et_3N

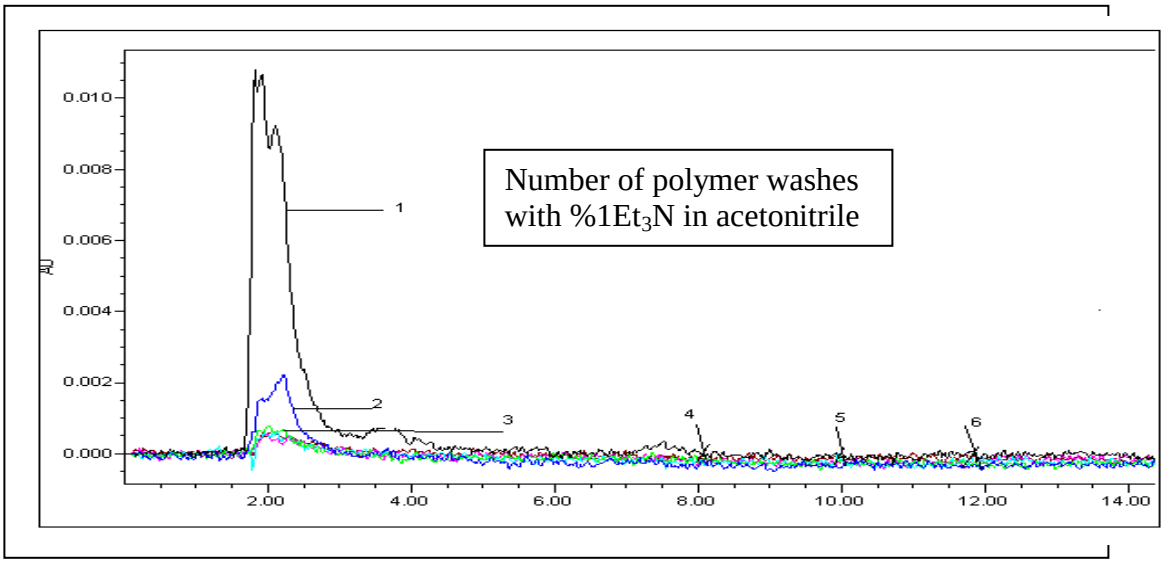


Figure 3-8 RP-HPLC spectra of 4vpy released from L3 (100mg) after polymer wash with 2ml of acetonitrile modified Et_3N 1%v/v, for 6 washes each for 5 min.

From the 4vpy spectra, the addition of Et_3N was promising because it took only 3 washes to completely eradicate 4vpy, which was one less wash compared to acetonitrile

alone. However, using Et_3N did not push the system to the limit as we wanted. In addition, another wash was required to remove the base. It was then decided to use acetonitrile alone (see experiment 3.1). The next step was to dry the polymer, followed by a dissolution of excipient bound polymer. This was carried out by washing the polymer with buffer of low ionic strength prior to the commencement of template removal.

3.2.2 Lysozyme extraction from imprinted polymer.

Often a major problem in molecular imprinting is to achieve complete extraction of the imprinted template from the polymer matrix. The highly cross linked polymer tends to trap protein macromolecules, resulting in poor mass transfer in and out of the polymer network, leading to inadequate removal. Therefore, the challenge was to find a suitable technique that would maximally extract a large percentage or ultimately all the protein from the densely cross linked polymer.

From the literature reviewed, different extraction methods have been exploited over a number of years, such as soxhlet extraction, solvent extraction with various organic solvents containing acidic or basic additives, supercritical fluids for template desorption, thermal annealing, ultra sonication and microwave assisted extraction; these were mostly useful for small molecules templates (Ellwanger *et al*; 2001)
Protein extraction using solvents is commonly employed in protein imprinting. The removal of protein is either by hydrolysing the interactions between the template and the imprinted cavities or by using surfactants such as SDS or even proteases to fragment and denature the protein therefore facilitating its removal.

To begin with, we investigated different solvents and the removal of the protein was spectroscopically monitored using RP-HPLC (see experiment 3.2). The results are tabulated in table 3-3, which gives the score for each solvent; (Poor- means no lysozyme

peak), (Good- means a weak lysozyme peak) and (Excellent- means a strong lysozyme peak).

The amount of the lysozyme extracted using many solvents was low and for some was very poor. The two solvents combining a buffer in acetonitrile, and acetic acid in methanol demonstrated a weak peak for lysozyme eluted from the polymers. However, the use of acetonitrile in the extraction phase was not desired because organic solvents tend to precipitate protein and therefore hinder its maximum removal. From this, it was clear that only acetic acid and methanol were appropriate. Further search from the literature revealed that polyethylene glycol (PEG) has been used extensively for protein extraction from crude corn extract (Gu et al, 2006) in aqueous two phase systems with addition of salts (Farruggia *et al*, 2004; Tubio, 2007).

In further attempts to optimise the lysozyme extraction solvent, PEG 200 in combination with acetic acid and methanol were employed and the new developed combination of solvents are presented in table 3-4.

The use of PEG 200 in the template extraction step removed ~5-10% of lysozyme from the imprinted polymer; however, the presence of PEG left in the polymer was not desired because it was assumed that the PEG might stick in the polymer sites and interfere with protein rebinding to the imprinted cavities. In order to wash off PEG 200, the polymer was rinsed with phosphate buffers 0.01M, followed by dH_2O until a base line was observed from the RP-HPLC. In addition, the polymer was washed with 8M urea to redissolve any protein fragments left resulted from using strong acetic acid and finally the urea was washed off with dH_20. From this, a typical sequential wash was developed.

All polymers were washed with solvent containing 30%polyethylene PEG 200 in a phosphate buffer 60% and 10% acetic acid until no lysozyme peak was observed from RP-HPLC followed by 8M urea. Each extraction solvent was monitored and only successive solvent was used when no further trace of lysozyme was observed indicating that all protein that could have been removed had been removed. Following template extraction, the polymer was dried in the oven at 60^0C for approximately 24hrs and was ready for evaluation.

Table 3-3: Summary of the Lys extracted from Lys MIPs using different solvents

Wash No.	Washing Composition	Overall Score	Wash No.	Washing Composition	Overall Score
1	30% dH_2O: 70% AcN	Poor	11	10% SDS (10 x .1% Tris/glycine/SDS in 90% dH_2O	Poor
2	40% phosphate pH 7 (0.01M) & 60% AcN	Poor	12	40% phosphate pH 7 (0.1M) & 60% AcN	Poor
3	40% phosphate pH 5 (0.01M) & 60% AcN	Poor	13	30% acetic acid & 70% methanol	Poor
4	10% formic acid & 90% can	Poor	14	10% acetic acid & 80% phosphate pH 5 (0.1M)	Poor
5	12.5% acetic acid & 87.5% methanol	Poor	15	20% acetic acid & 80% phosphate pH 7 (0.1M)	Poor
6	Phosphate buffer pH 5 (0.1M)	Poor	16	10% acetic acid & 90% phosphate pH 7 (0.1M)	Poor
7	40% phosphate buffer pH 7 (0.5M): 60% AcN	Good	17	Phosphate buffer pH 5 (0.1M)	Poor
8	Acetone	Poor	18	Phosphate buffer pH 5 (0.5M)	Poor
9	40% phosphate pH7 (0.5M) & 60% AcN	Poor	19	40% phosphate buffer pH 7 (1.0M) & 60%AcN	Poor
10	40% phosphate pH 5 (0.5M): 60% AcN	Poor	20	30% acetic acid & 70% methanol v/v	Good

Table 3-4: Summary of the Lys extracted from Lys MIPs with addition of PEG

Wash No.	Washing Composition	Overall Score	Wash No.	Washing Composition	Overall Score
21	2 washes with 20% PEG 200 & 10% acetic acid and 70% phosphate buffer pH 7 (0.1M); v/v	Good	24	12.5 % acetic acid & 85% methanol, followed by 30% PEG 200 &:10% acetic acid & 60% phosphate buffer pH 7 (0.1M); v/v	Good
22	2 washes with 30% PEG 200 &, 10% acetic acid and 60% phosphate buffer pH 7 (0.1M); v/v	Excellent	25	20% acetic acid & 80% methanol followed by 30%PEG 200 10% acetic acid: 60% phosphate buffer pH 7 (0.1M);v/v	Good
23	2 washes with 40% PEG 200 & 10% acetic acid and 50% phosphate buffer pH 7 (0.1M);v/v	Good	25	20% acetic acid in buffer followed by 30%PEG 200 and 70% phosphate buffer pH 7 (0.1M); v/v	Good

3.3 Characterisation of the lysozyme imprinted polymer

The batch rebinding process has been described previously in chapter 1. Basically, it involves evaluating the selectivity of imprinted polymers towards the imprinted template.

For example, a known concentration of the template (C_t) solution is incubated with a known mass of the polymer and left to reach equilibrium. After equilibrium is established, the rebinding solution is separated from the polymer by filtration or centrifugation and the template left in the supernatant referred as concentration of free substrate (C_f) in solution. All parameters are measured using standard analytical

techniques such RP-HPLC. The amount of the template bound (C_b) to the polymer is determined from subtracting (C_f) from the initial template concentration (C_t) employed. The concentration of template bound in a single template solution is computed as follows:

$$C_b = (C_f - C_t)/g \text{ or mg (polymer)}$$

3.3.1 Lysozyme selectivity towards lysozyme MIPs

The preferential binding ability of the polymers L1, L2 and L3 was characterised by the equilibrium selective template rebinding in aqueous medium (see experiment 3.3). The lysozyme binding data for all polymer results are presented in figure 3-9.
The immediate feature observed was that the affinities of all polymers towards imprinted protein were comparable even though they all differ slightly in their polymer compositions. However, a discrepancy was noted for lysozyme bound when compared to the expected capacity of polymer binding as determined from the equation below,

$$\text{Protein binding (\%)} = \frac{\text{Measured protein biding (mg)/ mg of polymer}}{\text{Expected protein binding (mg)/mg of polymer}}$$

The percentage binding for polymer L1 was 9.58%, L2 was 14.4% and for L3 was 19.16%. The percentage increment between L1 and L2 was equal to that between L2 and L3. This suggest that the effect of doubling the concentration of the cross linking density in L2 compared to L1 was similar to that of doubling the concentrations of functional monomer between L2 and L3. From this, it was quite difficult to identify which polymer demonstrated a true selectivity effect.
The question that we wanted to address was whether the increased selectivity of the template was due to increasing polymer selectivity or otherwise non selectivity was dominating the effect. To get an insight into the template binding characteristics of imprinted sorbents, we designed a cross selectivity study using a similar molecular weight protein RNase A, a close molecular analogue to be used in aqueous equilibrium rebinding.

Figure 3-9: Lysozyme uptake by Lys MIPs

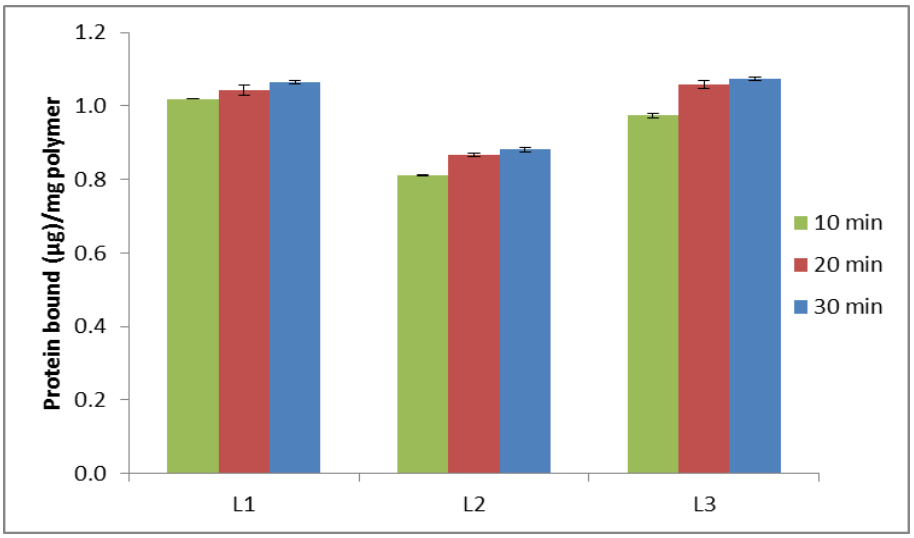


Figure 3-9 Equilibrium aqueous rebinding for Lys imprinted polymers L1, L2 and L3 with 2ml of lysozyme 0.125mg/ml of buffer pH 7.23 (0.01M) (mobile phase) using 100mg of polymer for 30 minutes. Sample were analysed after 10 min interval. (Mean ± error, n=3)

3.3.2 RNase A non specific selectivity towards lysozyme MIPs

To achieve RNase A rebinding, the procedure carried out was similar to that described previously for lysozyme (see experiment 3.4). From figure 3-10, the rebinding of RNase A to the polymer L3 was higher compared to L1, which was slightly higher compared to L2; although the overall binding of RNase A was ~3 fold lower than for the lysozyme.

It was hypothesised that the ratio of functional monomer to template and cross linker in L3 and to a smaller extent in L1 may have resulted in excess in solution. This could lead to self association forming dimers or trimmers, thus contributing largely to non selective sites. Increasing only the cross linking monomer in L2 compared to L1 may have lessened this effect.

Figure 3-10: RNase A uptake by Lys MIPs

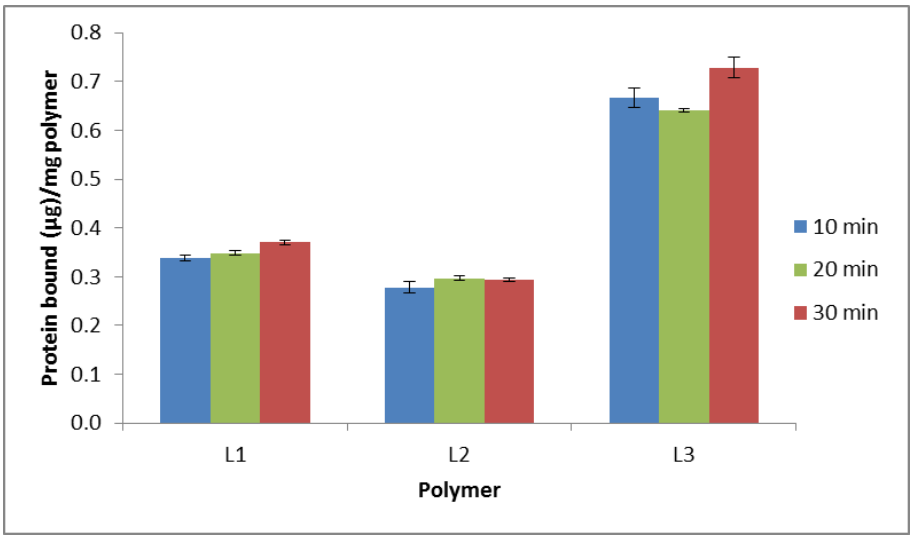


Figure 3-10 Equilibrium aqueous rebinding for Lys imprinted polymers L1, L2 and L3 with 2ml of RNase A 0.125mg/ml of buffer pH 7.23 (0.01M) (mobile phase) using 100mg of polymer for 30 minutes. Sample were analysed after 10 min interval. (Mean ± error, n=3)

This hypothesis is in agreement with that reported previously by Andersson *et al*, (1999), whereby using a very high concentration of functional monomer to template ratio was thought to lead to inclusion of a lot of non-complexed, randomly distributed monomer. This yielded increased non selective binding and therefore the selectivity of the imprinted polymer would be similar to that of the non-imprinted polymer. In contrast, increasing the concentration of cross-linking monomer should lessen the non selective binding effect by a small degree.

From this, it was logical to continue our study using only polymer L2. In order to better determine the affinity of the polymer, we designed an equilibrium rebinding experiment. There are two ways in which this could be carried out:

1. By changing the amount of polymer while keeping the protein rebinding concentration constant
2. By changing the protein rebinding concentration while keeping the amount of the polymer constant.

The first option was selected because changing the known amount of the polymer was practically simpler than analysing a range of protein concentrations which were not accurately known to begin with.

3.3.3 Equilibrium batch rebinding of lysozyme toward Lys MIPs

The equilibrium batch rebinding was carried out using different amounts of the polymer, L2 ranging from 20-120mg (see experiment 3.5). From the equilibrium binding results shown in figure 3-11, the capacity of the polymer L2 was ~0.048mg template/mg of polymer, which was below the capacity of the binding sites of 0.06mg/mg. In addition, the concentration of the lysozyme in the rebinding solution was only sufficient for saturating 1-35mg of polymer.

From this, it was realised that the low lysozyme rebinding observed earlier for all polymers L1-L3 was due to the template depletion in the rebinding solution. However, using high template loading may reduce both the template retention and selectivity because the template might shift the binding equilibrium to the weak and non selective sites (Andersson *et al,* 1999).
Therefore to avoid template depletion, it was prudent to rebind the polymer, using a template concentration only slightly higher than saturation so that it would not elevate non selective binding.

Another question that we wished to address was whether the polymer showed selective recognition towards imprinted lysozyme or whether the RNase A had poor retentive characteristics on the surface of the polymer matrix.

Figure 3-11: Equilibrium rebinding of lysozyme toward Lys MIPs

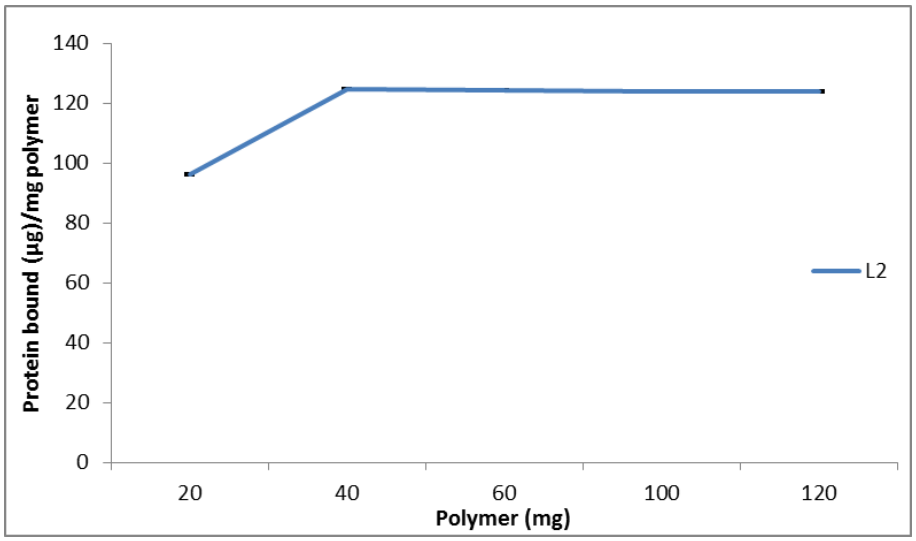


Figure 3-11 Equilibrium aqueous rebinding for Lys imprinted polymers L2 at different polymer concentration (20, 40, 60, 80,100 and 120) - mg with 2ml of lysozyme 0.125mg/ml of buffer pH 7.23 (0.01M), 5% ethanol and 0.05% Tween 20; v/v (mobile phase) for 10 minutes. (Mean ± error, n=3)

To get an insight into the template binding characteristics towards polymer L2, we also designed a competitive equilibrium binding study using specific template solutions spiked with closer molecular weight proteins, cytochrome C and RNase A.

3.3.4 Competitive rebinding of lysozyme towards Lys MIPs

To achieve this, we rebound polymer L2 with the lysozyme solution spiked with close molecular weight proteins RNase A, cytochrome C (see experiment 3.6) and the protein bound results are shown in figure 3-12.

From figure 3-12, it can be noticed that the polymer L2 adsorbed almost all of the cytochrome C compared to both lysozyme and RNase A. The indiscriminate rebinding behaviour demonstrated by this polymer suggested that non specific binding was dominating the polymer selectivity.

Figure 3-12: Proteins uptake by Lys MIPs

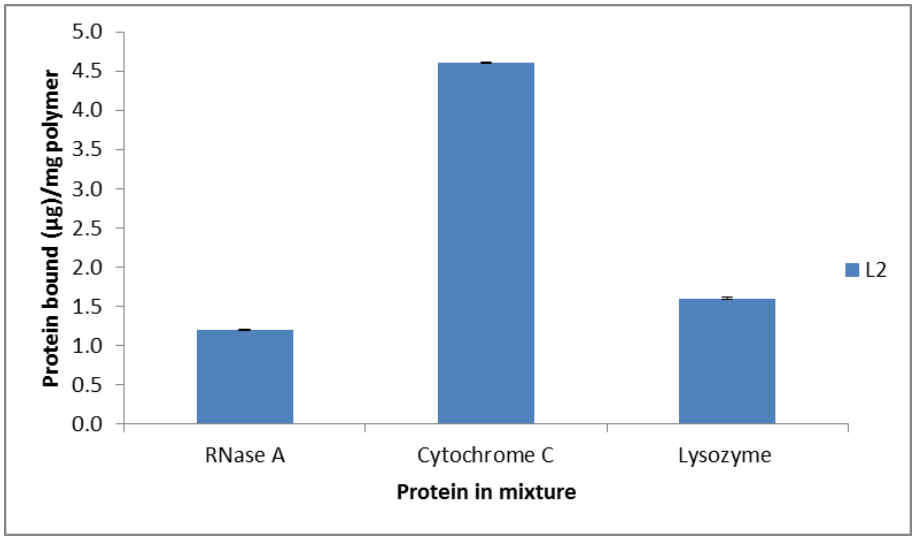


Figure 3-12 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymer L2 with lysozyme 0.1mg/ml of mobile phase (95% buffer pH 7.23 (0.01M), 5% ethanol, 0.05% Tween 20); v/v spiked with competitive molecules; RNase A (0.1mg/ml) and cytochrome C (0.1mg/ml) for 10 minutes. (Mean ± error, n=3)

In order to lessen this effect, we added BSA in the rebinding solution as a non specific blocking agent (see experiment 3.7). BSA is used extensively in ELISA as a blocking agent of non specific hydrophobic binding; therefore, it was assumed that this would lessen the effect of non selective protein binding.

From figure 3-13, it can be seen that the use of BSA did not circumvent the problem. Instead, the polymer bound both BSA and cytochrome C completely but the concentration of lysozyme bound was same as before, while RNase A binding was significantly reduced. From this, it was clear that the organic polymer may be non selective and hydrophobic interactions were the driving force preventing protein selectivity. Furthermore, it was argued that the use of cytochrome C as a competitive protein analogue was inappropriate because of the structure difference; the cytochrome C contained a metal binding group (Heme) and the BSA was very hydrophobic. Therefore, the use of RNase A was seen to be more appropriate at this time.

Figure 3-13: Protein uptake by Lys MIPs with addition of BSA

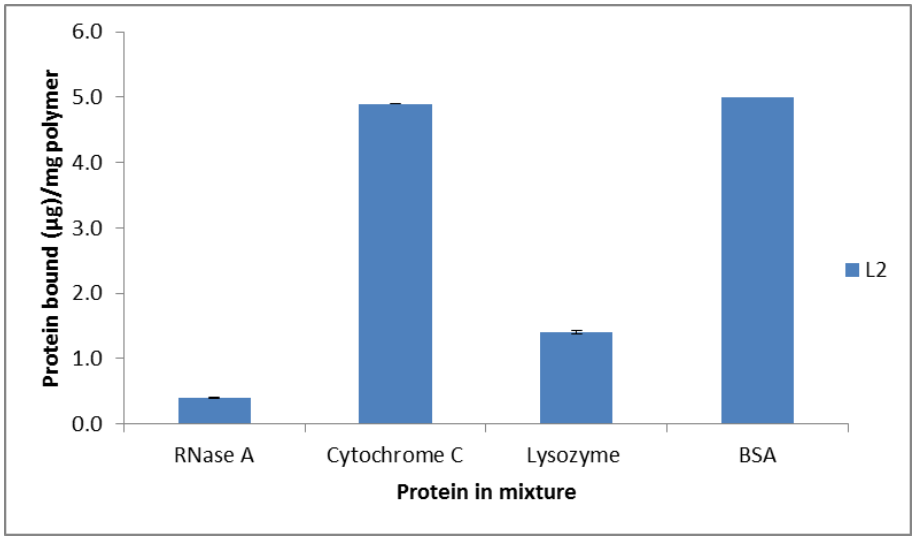


Figure 3-13 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymer L2 with lysozyme 0.1mg/ml of mobile phase (95% buffer pH 7.23 (0.01M), 5% ethanol, 0.05% Tween 20); v/v spiked with competitive molecules; BSA (1.0mg/ml), RNase A (0.1mg/ml) and cytochrome C (0.1mg/ml) for 10 minutes. (Mean ± error, n=3)

A greater understanding of the nature of the non specific interactions and how they could be reduced was clearly needed. One way to address this issue was through mobile phase tuning, because the mobile phase greatly influences the recognition abilities of imprinted polymers (Yu and Mosbach, 2000), including affinity and selectivity (Wu *et al*, 2005).

3.3.5 Lysozyme selectivity towards Lys MIPs with mobile phase tuning

Selective binding can potentially be improved using an optimum mobile phase, such as the solvent used for template polymerisation. This is because the template conformation adapted during rebinding is expected to be similar to the conformation that is captured during imprinting. The use of different rebinding media might present a different template conformation to the binding cavities and hence lead to low template selectivity (Wulff, 1995; Ansell *et al*, 1996). However, because the application of protein imprinted

polymers is mostly intended for aqueous environments, instead the mobile phase is typically tuned with addition of organic modifiers or non specific binding blockers or sometimes both. In order to attempt to suppress non selective binding, we added arginine as a blocker of non specific binding in the rebinding solution. Arginine has been used to suppress protein aggregation in protein refolding studies (Arakawa, 2003 and 2004). Arginine at neutral pH possesses an overall positive charge due to deprotonation of the guanidino group as well as being zwitterionic. Therefore, it was suspected that the positive charged arginine would bind the protein in both native state and unfolded state coating it with a zwitterionic layer and therefore suppressing protein aggregation. This could potentially reduce the non specific binding.

Initially, the investigation was carried out to determine a suitable concentration of arginine using different arginine-HCl concentrations; 0.2 M, 0.5 M, 0.7 M, all samples were equilibrated to pH 7.2 using L-arginine base prior to use (see experiment 3.8.1). From the results shown in figure 3-14, it can be seen that the addition of arginine did not interfere with the lysozyme selectivity, although slightly less lysozyme was bound with 0.5 M.

In a continued effort to tune the mobile phase, we added polysorbate 20 (commercially known as Tween 20), a surfactant commonly employed in ELISA to reduce non specific protein rebinding (see experiment 3.8.2). In order to see whether Tween 20 had any effect on the lysozyme selectivity, we looked at equilibrium template rebinding with addition of both arginine and Tween 20 in the rebinding solution.

From the results shown in figure 3-15, the lysozyme selectivity was comparable to that observed before; therefore, the addition of Tween 20 in the arginine mobile phase did not affect the lysozyme rebinding except that the equilibrium between the template and the polymer was established only after 10 min. Therefore, there was a dramatic change of the template rebinding with time. It was reasoned that the template rebinding inconsistencies may be due to the surfactant competing with the template for the binding sites or the template rebinding initially by a hydrophobic mechanism which may be very fast. Therefore it was quite a challenge to predict the amount of the template rebound with time.

Figure 3-14: Lysozyme uptake by Lys MIPs with addition of arginine-HCl

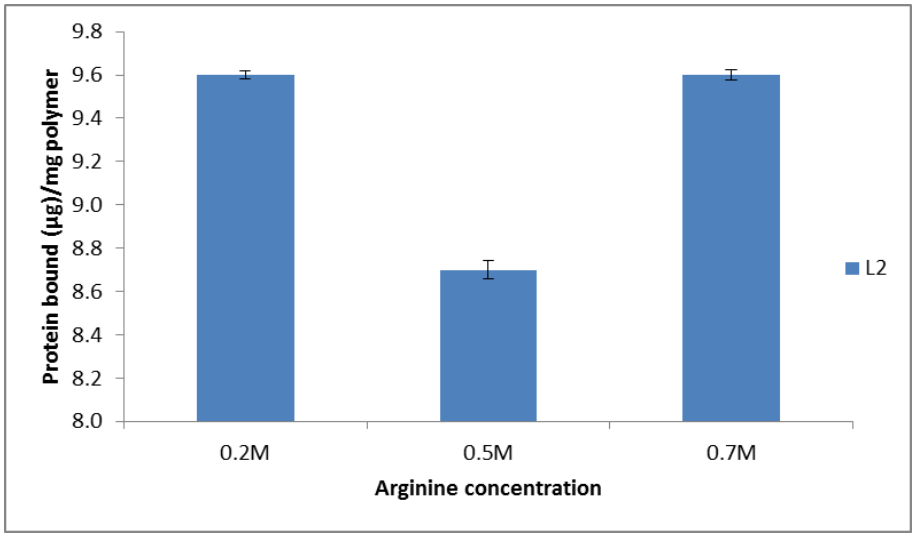


Figure 3-14 Summary of equilibrium rebinding for 100 mg Lys imprinted polymer L2 with 2ml of lysozyme 1mg/ml of mobile phase (100% buffer pH 7.23 (0.01M) +0.05% Tween 20) v/v, arginine base of different strength 0.2M, 0.5M and 0.7M equilibrated with arginine HCl (acid) to pH 7.2 for 10 minutes. (Mean ± error, n=3)

In order to eliminate this effect, we decided to add ethanol to the mobile phase as an organic modifier; the use of Tween 20 in combination with ethanol has been reported previously to combat the problems of non specific template rebinding (see experiment 3.8.3). From this, we managed to improve the template rebinding fluctuation, although a rapid increase of lysozyme binding after 7min was still apparent and finally equilibrium was again established after 10 min (figure 3-16). We concluded that the unusual binding kinetics observed might have to do with changes to the polymer structure in the mobile phase; therefore, it was decided to condition the polymer with mobile phase without protein in order to equilibrate the polymer (see experiment 3.8.4). For this experiment, a lower concentration of polymer from 100mg to 20mg/ml was used because we were running short of the sample powder. The results are presented in figure 3-17.

Figure 3-15: Lys uptake by Lys MIPs with addition of arginine-HCl & Tween 20

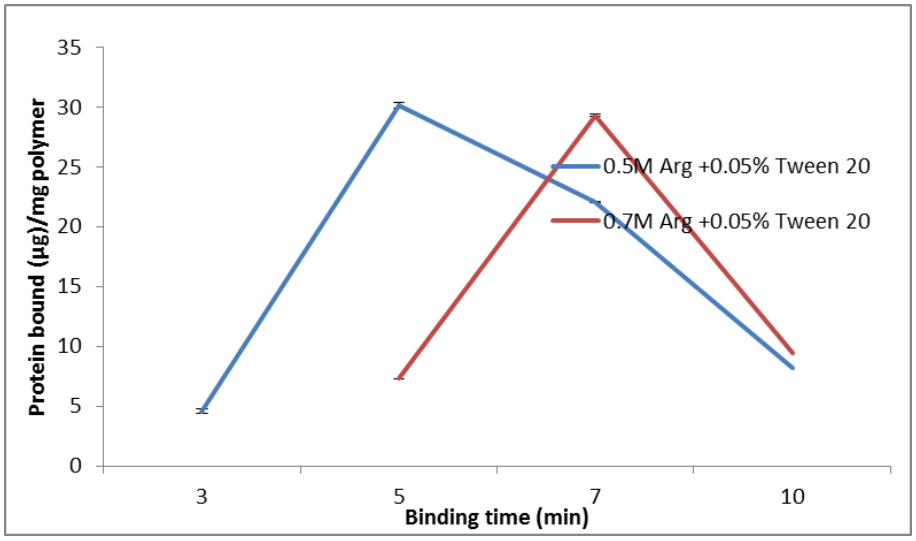


Figure 3-15 Aqueous equilibrium rebinding for 100 mg Lys polymer L2 with 2ml of lysozyme 1mg/ml of mobile phase (100% buffer pH 7.23 (0.01M) +0.05% Tween 20) v/v, arginine base of different strength 0.5M and 0.7M equilibrated with arginine HCl (acid) to pH 7.2 for 3, 5, 7 and 10 minutes. (Mean ± error, n=3)

Figure 3-16: Lys uptake by Lys MIPs with addition of arginine, Tween 20 & ethanol

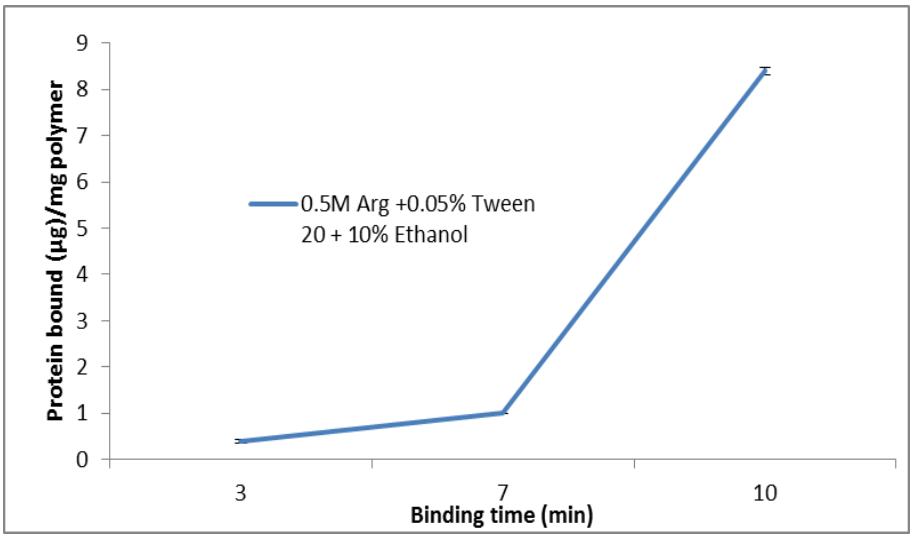


Figure 3-16 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymer L2 with 2ml of lysozyme 1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, arginine base 0.5M equilibrated with arginine HCl (acid) to pH 7.2 for 3, 7 and 10 minutes. (Mean ± error, n=3)

Conditioning and equilibrating the polymer with mobile phase prior to the rebinding seemed to lessen the dramatic increase of template rebinding with time. This is evidently shown as a gradual increase of lysozyme rebinding with time was mostly apparent up to 60 min. From the data, it was possible to predict with more certainty the amount of template bound at equilibrium even though the overall lysozyme rebinding per mg polymer was reduced. Thus, it was lower than when using a lower concentration of polymer.

Figure 3-17: Lys uptake by Lys MIPs with condition phase similar to mobile phase

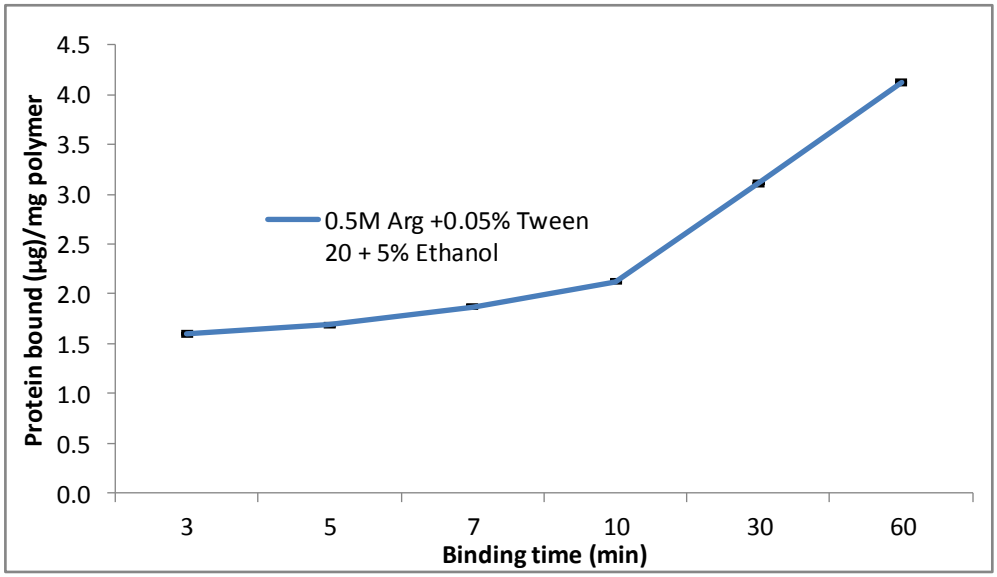


Figure 3-17 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymer L2 with 2ml of lysozyme 0.5mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, arginine base 0.5M equilibrated with arginine HCl (acid) to pH 7.2 for 3,5,7, 10, 30 and 60. (Mean ± error, n=3)

In order to see if the lysozyme rebinding characteristics were retained even in the presence of the other competitive molecule, we looked at competitive equilibrium batch rebinding using RNase A as a molecular weight analogue.

3.3.6 Competitive rebinding of Lys & RNase A towards Lys MIPs

To achieve this, we spiked lysozyme rebinding solution with RNase A both having the same concentration of 0.5mg/ml of mobile phase (see experiment 3.9.1) and the results are presented in figure 3-18. From figure 3-18, it can be seen that the presence of the competitive molecule RNase A did not compromise the selectivity of lysozyme towards the polymer L2 at each given time point. Furthermore, significantly larger, ~ 10 fold increases after 60 min rebinding were observed compared to RNase A under competitive conditions. This is more than observed before (figure 3-12 and 3-13). This suggested that the polymer was showing better selectivity towards the imprinted molecules.

Figure 3-18: Protein uptake by Lys MIPs in a competitive protein rebinding

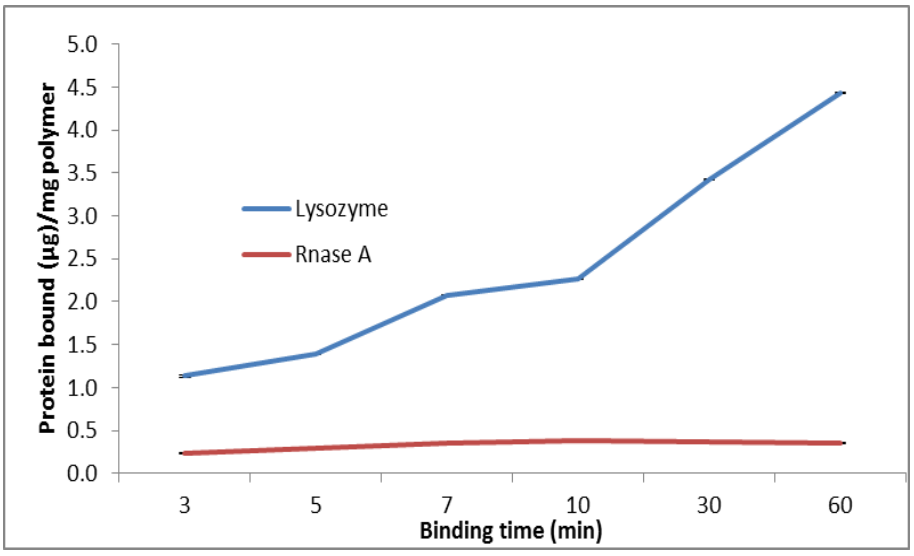


Figure 3-18 Aqueous competitive equilibrium rebinding for 20 mg Lys imprinted polymer L2 with 2ml of lysozyme 0.5mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, spiked with RNase A (0.5 mg/ml of mobile phase) for 3, 5,7,10, 30 and 60-minutes. (Mean ± error, n=3)

To get an insight into the polymer selectivity, we increased the concentration of the competitive molecule RNase A by 5 fold compared to that of the lysozyme (see experiment 3.9.2). From the results presented in figure 3-19, it can be seen that the selectivity of L2 towards lysozyme remained the same as before compared to RNase A, even though the rebinding concentration of the competitive molecule RNase A was high. This showed that the selectivity of the polymer L2 towards lysozyme was reproducible regardless of any changes in the concentration of lysozyme or RNase A in the rebinding solution, as long as the polymer concentration was the same.

Figure 3-19: Protein uptake by Lys MIPs in a competitive protein rebinding with higher concentration of RNase A

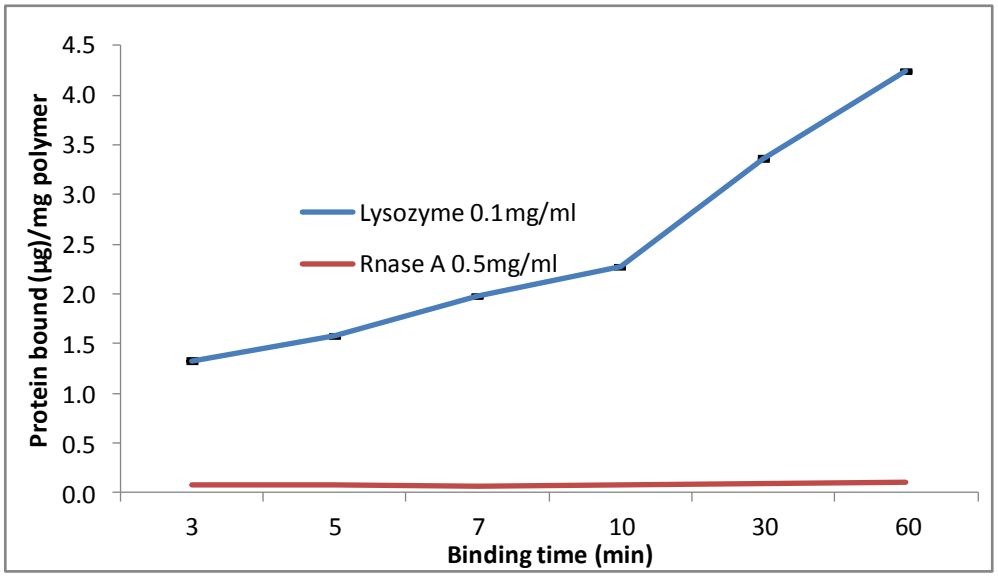


Figure 3-19 Aqueous competitive equilibrium rebinding for 20 mg Lys imprinted polymer L2 with 2ml of lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, spiked with RNase A (0.5 mg/ml of mobile phase) for 3, 5,7,10, 30 and 60-minutes. (Mean ± error, n=3)

In order to determine the lysozyme selectivity at higher lysozyme concentration in the rebinding solution, we increased the lysozyme concentration by 5 fold compared to that of the competitive molecule RNase A (see experiment 3.9.3). From the results shown in figure 3-20, it can be seen that the capacity and selectivity of the polymer towards the

imprinted lysozyme were the same regardless of the increased concentration of lysozyme in the rebinding phase, as long as the template concentration in the solution was above that causing lysozyme depletion. The binding of RNase A significantly reduces such that it could be ignored.

Figure 3-20: Protein uptake by Lys MIPs in a competitive protein rebinding with lower concentration of RNase A

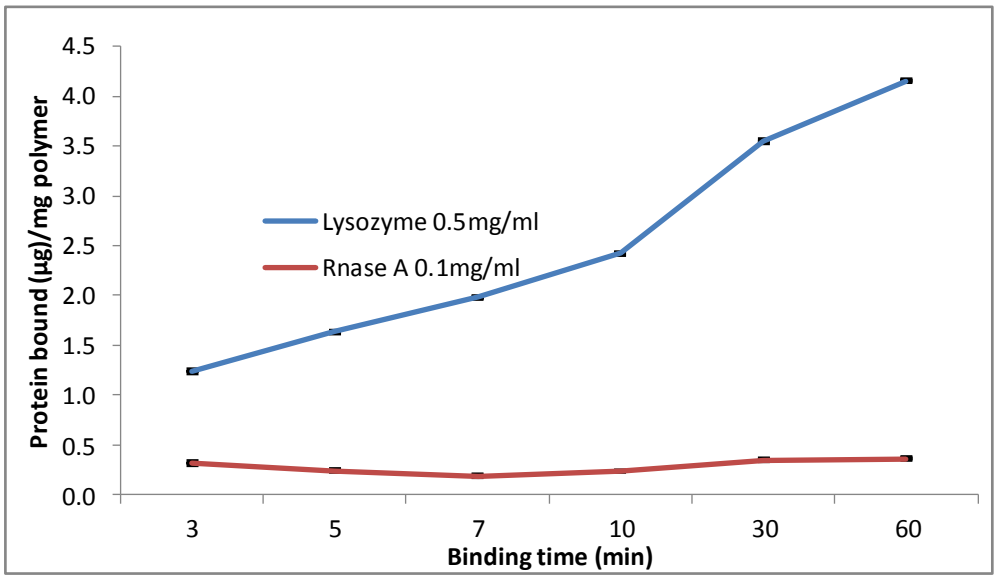


Figure 3-20 Aqueous competitive equilibrium rebinding for lysozyme polymer L2 using 2ml of lysozyme (0.1mg/ml) in mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v and RNase A (0.5 mg/ml) for 3, 5,7,10, 30 and 60-minutes. (Mean ± error, n=3)

More interestingly from increasing the polymer concentration in the rebinding solution the selectivity of the polymer L2 towards lysozyme was still large even though the concentration of RNase A in the rebinding of RNase 10 fold compared to that of lysozyme (figure 3-21). However from doubling the concentration of the lysozyme, while keeping the RNase A concentration same at 10 fold, a new equilibrium was established, whereby the lysozyme rebinding to the polymer L2 was increased by 6.6% , while that of RNase A was comparable to that observed before. The shift in binding equilibrium was towards more lysozyme selectivity while non selective binding of

RNase A was still the same. This could also be explained in that using low template concentration in the rebinding solution may have resulted in less saturation of the lysozyme binding sites, similar proportion to that observed from using 20mg polymer.

Figure 3-21: Protein uptake by Lys MIPs in a competitive binding with increased MIP

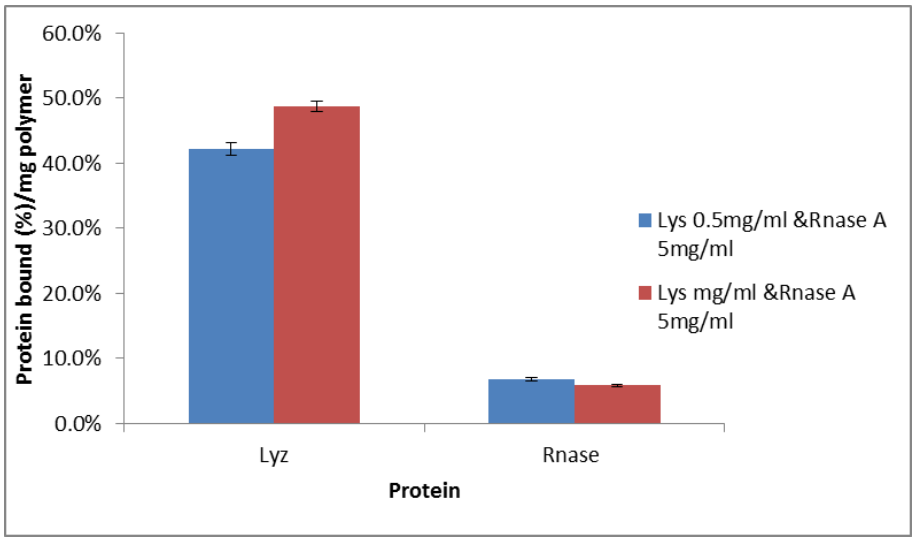


Figure 3.21 Aqueous competitive equilibrium rebinding for Lys imprinted polymer L2 using (a) 2ml of lysozyme (0.5mg/ml) spiked with RNase A (5 mg/ml), (b) lysozyme (1mg/ml) spiked with RNase A (5 mg/ml), for 10min. In both experiments the mobile phase compositions was (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v. (Mean ± error, n=3)

In order to determine the nature of the lysozyme selectivity, we designed a cross selectivity study measuring lysozyme rebound to RNase A imprinted polymer R2. This was to compare the selectivity towards lysozyme again using different concentrations of polymers and lysozyme in the rebinding solution. It was thought that the RNase A imprinted polymer would give more information than blank polymer because the absence of the protein in the blank polymer preparation might create a different polymer morphology. The use of a blank as employed in this report is a conventional technique for a comparative study and is commonly employed and approved by many molecular imprinters. However, it may not be ideal for imprinting studies.

Briefly, RNase A is an endo-ribonuclease family of enzyme that has been extensively used in a protein research and is commonly found in living organisms.

The RNase A (pI ~9.6) activity involves catalysing single stranded RNA by cleaving the phosphodiester bond between 5'-ribose of the nucleotide and the phosphate group attached to position 3'-ribose of an adjacent pyramidine nucleotide of residue C and U residues and the product of catalysis is 2',3'-cyclic phosphate, which might also be hydrolysed to the C3'-nucleoside phosphate . RNase A is also known as a hard enzyme to inactivate because it can restore its function after autoclaving (Hsu *et al*, 2006) but it is not desired in the reverse transcription-polymerase chain reaction (RT-PCR) and for *in vitro* transcription and translation because it interferes with the process; therefore, the removal of RNase A is necessary although it is quite challenging.

3.4 Preparation and characterisation of RNase A MIPs

RNase A PCMCs were prepared using similar methodology previously to that described for lysozyme PCMCs outlined in table 3-5 using KCl as a carrier material and phosphate buffer of pH 7.23.

Table 3-5: Preparation of RNase A PCMCs

PCMC	Protein Loading % w/w	Water content	Solvent
RNase A	10.0	5%	IPA

RNase polymer was prepared and processed as described for lysozyme polymer L2: table 3-6 outlines the reagent used for RNase A imprinting.

Table 3-6: Reagents used for the preparation of RNase A MIPs

Polymer	RNase A (mg)	RNase A PCMCs (mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN g, mmol	AcN (ml)
R2	70.56	750	0.449, 5.236	0.550, 5.236	10, 50.508	0.183, 1.115	7.911

The extraction of RNase A from the polymer was by sequential washes similar to that described for lysozyme polymer; 30%PEG 200,10% acetic acid, 60% phosphate buffer

pH 7 (0.1M). In this, 1-5% of RNase A was observed from extracts of the polymer as noted from RP-HPLC

The initial investigation was carried out to determine the influence of the mobile phase of different phosphate buffers pH 6,7, and 8 of buffer strength of 0.01M pH have on the RNase A rebinding onto RNase A polymer R2. The results of RNase A rebinding to the polymer are presented in figure 3-22.

Figure 3-22: RNase A uptake by RNase A MIPs using different mobile phase

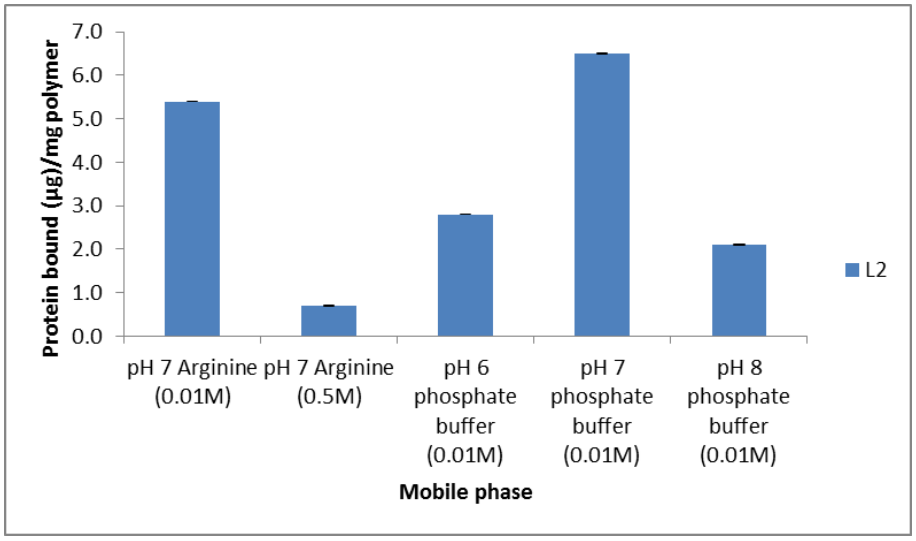


Figure 3-22 Aqueous equilibrium rebinding for 5x20mg RNase A imprinted polymer R2 with 2ml of RNase A 0.5mg/ml of different mobile phases solutions, 0.01M and 0.5M arginine-HCl equilibrated to pH 7.0, phosphate buffer pH 6, 7, and 8 at a buffer strength of 0.01M, for 10 minutes (Mean ± error, n=3)

From the histograms, it can be seen that the presence of arginine at 0.5M in the mobile phase significantly reduced the RNase A binding, while the highest binding was observed with phosphate buffer at pH 7. It was assumed that some kind of memory existed between the RNase and recognition sites, because the high RNase A selectivity was with the buffer that was used for preparation of RNase A- PCMCs.

Just to reiterate, the main focus here was to establish the nature of lysozyme selectivity towards its imprinted polymer. Therefore, the RNase A polymer was used in a cross selectivity study whereby lysozyme would be rebound to this polymer.

Because of the low selectivity of RNase observed towards the polymer when using 0.5 M arginine as mobile phase, it was suspected that the same might occur for lysozyme rebinding to this polymer.

3.4.1 Lysozyme selectivity towards Lys MIPs and RNase A MIPs

A comparative study was undertaken using different polymer concentrations of both L2 and R2 (20, 25, 50, 75, and 100- mg/2ml, see experiment 3.11.1) and the results are presented in figure 3-23.

From the results, it can be seen that the selectivity of lysozyme towards the polymer R2 was slightly higher than L2 at each concentration of lysozyme solution studied.

It was argued that although lysozyme bound slightly more to the R2 polymer, we were confident that a significant proportion bound non selectively by hydrophobic interaction, taking into consideration the influence of organic monomer in aqueous medium.

In a further attempt to discriminate the lysozyme binding, we used lower concentrations of lysozyme, such as 0.1mg/ml and 0.01mg/ml (see experiment 3.11.2). The lysozyme rebinding results are demonstrated in figures 3-24 and 3-25. It can be seen from both histograms that the lysozyme bound to R2 was slightly higher compared to L2, and this was mostly apparent with increasing concentration of the both polymers.

Figure 3-23: Lys uptake by Lys & RNase A MIPs at 0.5mg of Lys/ml

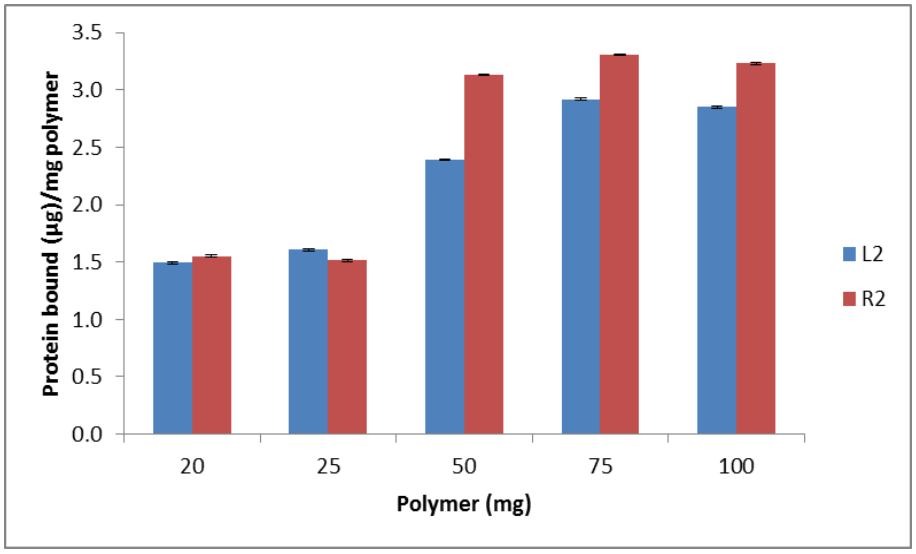


Figure 3-23 Equilibrium aqueous rebinding for Lys (L2) and RNase A (R2) imprinted polymers at different polymer concentration (20, 25, 50, 75 and100 – mg) with 2ml of lysozyme 0.5mg/ml of mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v for 10 minutes. (Mean ± error, n=3)

Figure 3-24: Lys uptake by Lys & RNase A MIPs at 0.1mg of Lys/ml

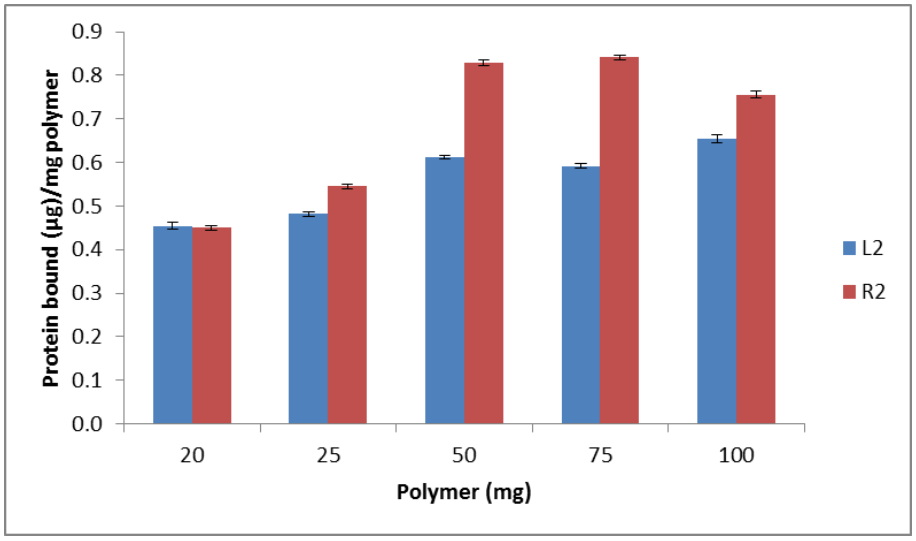


Figure 3-24 Equilibrium aqueous rebinding for Lys (L2) and RNase A (R2) imprinted polymers at different polymer concentration (20, 25, 50, 75 and100 – mg) with 2ml of lysozyme 0.1mg/ml of mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v for 10 minutes. (Mean ± error, n=3)

Figure 3-25: Lys uptake by Lys & RNase A MIPs at 0.01mg of Lys/ml

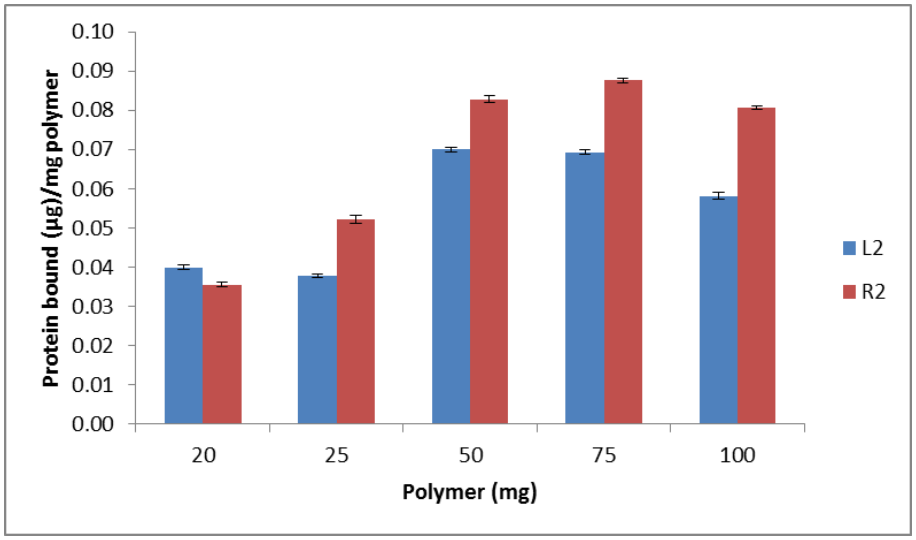


Figure 3-25 Equilibrium aqueous rebinding for Lys (L2) and RNase A (R2) imprinted polymers at different polymer concentration (20, 25, 50, 75 and100 – mg) with 2ml of lysozyme 0.01mg/ml of mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v for 10 minutes. (Mean ± error, n=3)

In order to try and eliminate non selective lysozyme rebinding, we added NaCl (0.01M) in the rebinding solution (see experiment 3.11.3). It was hypothesised that using low salts concentration might interfere with hydrophobic interaction, therefore lessening this effect, and the results are shown in figures 3-26 and 3-27.

From the results shown in both figures, it can be seen that a similar trend was repeated as before, whereby the lysozyme bound slightly more to the RNase A imprinted polymer compared to its specific polymer L2 and therefore the use of salt did not eliminate non selective binding. In further attempts to try to eliminate non selective protein binding, we added casein and BSA, shown in figure 3-27 (see experiment 3.11.4). Even with these non specific blocking agents, lysozyme rebinding to R2 polymer was almost 2 fold greater compared to L2

Figure 3-26: Lys uptake by Lys & RNase A MIPs at 0.5mg of Lys/ml with addition of NaCl (0.01M**)**

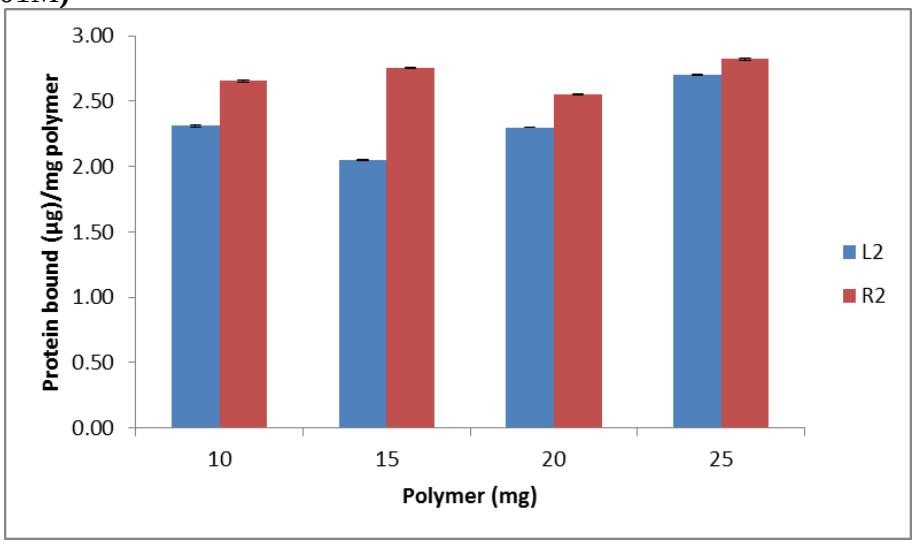


Figure 3-26 Equilibrium aqueous rebinding for Lys (L2) and RNase A (R2) imprinted polymers at different polymer concentration (20, 25, 50, 75 and100 – mg) with 2ml of lysozyme 0.5mg/ml of mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v with addition of NaCl (0.01M) for 10 minutes. (Mean ± error, n=3)

Figure 3-27: Lys uptake by Lys & RNase A MIPs at 0.1mg Lys/ml with addition of BSA & Casein

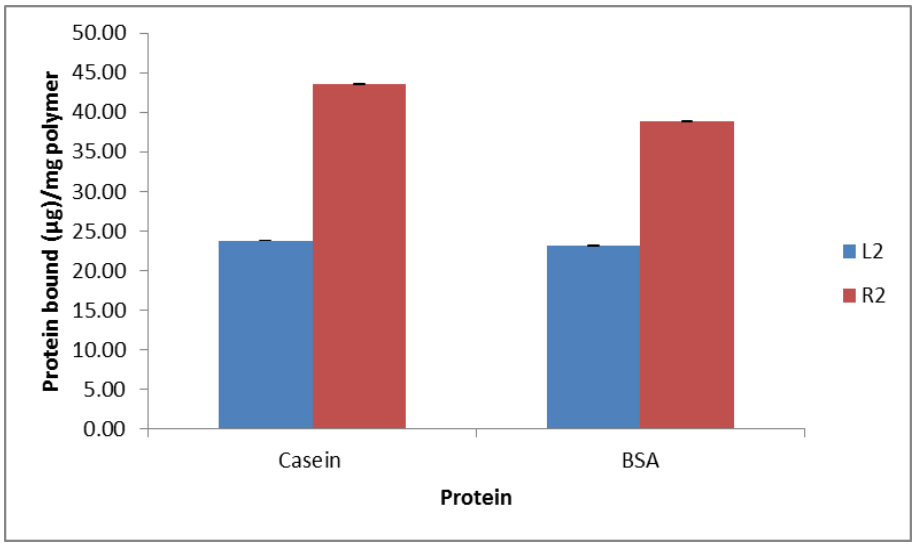


Figure 3-27 Equilibrium aqueous rebinding for Lys (L2) (2x50mg) and RNase A (R2) (2x50mg) imprinted polymers both with 2ml of lysozyme 0.5mg/ml of mobile phase (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v with addition of (a) BSA (1mg/ml) and (b) casein (1mg/ml), for 10 minutes(Mean ± error, n=3)

Overall, there was a slight increase in the lysozyme rebinding to polymer R2 compared to that of L2. A possible explanation is that may be polymer R2 had a large number of non selective sites. Alternatively, an increase in a pore diameter in R2 compared to L2 (please refer to chapter 6) may have contributed to this effect. However, if the correlation between the polymer porosity and protein binding existed was not known.

Further attempts to discriminate the template bound to the selective sites as opposed to the non selective sites involve use of a selective template extraction step to remove non-specific bound template (Rashid *et al*, 1997; Walshe *et al*, 1997) and eluting specifically bound template with solvent of increasing elutropic strength. However, protein bound to selective sites in a native conformation was expected to be difficult to elute because of the multiple interactions with the complementary sites. On the other hand, the protein bound to non selective sites is expected to change conformation or unfold which also makes it harder to remove. Extracting the template bound from both sites is quite hard and to use the elution step as a technique to discriminate specific from non specific sites presents a significant challenge.
To achieve this, we extracted lysozyme from the 100mg of both L2 and R2 10 min after rebinding (see experiment 3.12). The protein analysed from post-washes with mobile and elution phases is presented in figure 3-28.

From the template dissociation data, it can be seen that a significant amount of lysozyme was released from both polymers using mild washing conditions, although slightly more template was released from polymer R2 compared to L1. This suggested that, either a larger proportion of the lysozyme may have bound to the less strong sites in R2 compared to L1, or a slight increase in the swelling properties of the polymer R2 (refer to chapter 6) may have contributed to this effect.

More interestingly, from the elution phase, we managed to elute ~ 15% more lysozyme from L2 compared to R2. Therefore this could potentially represent the amount of lysozyme bound to the selective sites in the imprinted polymer (L2).

Figure 3-28: % Lys released from Lys & RNase A MIPs after wash with different solvents

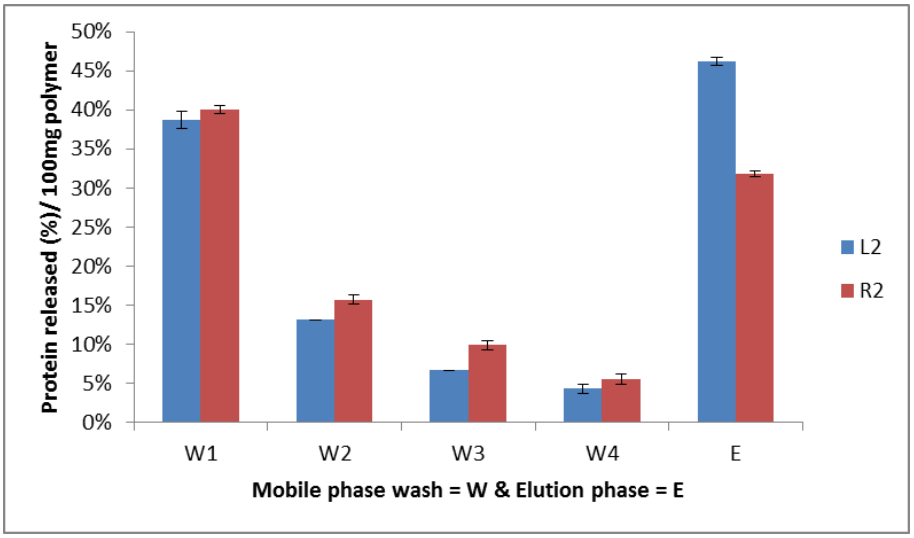


Figure 3-28 Lysozyme released from post washing 100mg polymer L2 and R2 bound lysozyme with mobile phase (W) using (95% Arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) and elution phase (E) using (10% AcOH), each wash for 10 minutes.

3.5 Conclusion

From this study, we have demonstrated that using an excess concentration of functional monomers to the template may have resulted in increased non selective binding of the polymers, possibly due to the self association of functional monomers leading to the formation of dimers or trimers. Increasing only the cross linking monomer may have lessened this effect.
From this study, we have also attempted to reduce non selective protein binding through mobile phase tuning with chemical additives and non specific blocking agent. From this, we were able to demonstrate some selectivity of the specific molecule towards its imprinted polymer compared to a competitive molecule (molecule analogue) at equal concentration of the proteins, as well as elevated levels of molecular analogue.

From this study, we have also demonstrated that the nature of the template selectivity by organic polymer in an aqueous phase may have been dominated by hydrophobic effect. In an attempt to discriminate selective from non selective protein rebinding, we employed a template selective extraction step. This has potential to differentiate the template bound to selective sites in its imprinted polymer compared to competitive polymer imprinted with molecular analogue.

Here we have also attempted to develop better analytical techniques for studying protein imprinted polymers:

1. Measure of polymer selectivity towards specific template
Specific template equilibrium rebinding - this involves measuring the selectivity and capacity of the polymer towards a specific imprinted template. This is carried out by using batch rebinding process, either changing polymer concentrations while keeping the concentrations of the protein same or vice versa.

2. Measure of polymer non selectivity towards non specific template

Non specific molecular equilibrium rebinding- this involves using a similar technique as in (**1**) to determine the selectivity and capacity of the imprinted polymer towards the non specific molecular analogue and then compare the protein binding characteristics with a specific molecule.

3. Measure of polymer specificity in competitive conditions

A competitive equilibrium binding study- this involves using specific template solution spiked with molecular analogues.

4. Validation of polymer specificity

A cross selectivity study is carried out by imprinting a polymer with closer molecular analogue and used this polymer to rebind molecules of interest. This will determine the nature of template specificity and selectivity towards imprinted polymer.

5. Measure of specific binding sites using template extraction step

This involves differentiating protein bound to the specific sites from that of non specific sites using a selective template extraction step. It is hypothesised that template bound to selective sites in native conformations is expected to be less difficult to elute because of the multiple interactions with the complementary sites. On the other hand, the protein bound to non selective sites is expected to change conformation or unfold, which also makes it harder to remove.

It was agreed then that the polymer hydrophobicity was the dominant feature in protein binding; therefore, further action was to lessen this effect by incorporating non hydrophobic elements within the polymer matrix to combat non specific effects.

Chapter 3: Experimental Section

Materials

Ribonuclease A from bovine pancreas Type I-A (RNase A), Lysozyme from chicken egg white, *Micrococcus lysodeikticus* , Cytochrome C from equine heart, Casein from bovine milk, Ethylene glycol, Ethanol, L-arginine, L-arginine monohydro-chloride, Alumina, Urea, Na_2HPO_4, NaH_2PO_4, $MgCl_2$, NaCl, Isopropanol, Trifluoroacetic acid (Spectrophotometric grade), Acetone, Acetonitrile (HPLC grade), were purchased from Sigma-Aldrich Co. UK. BSA form bovine serum, Polyethylene glycol 200 (MW 200), Polyethylene Glycol 300 (MW 300), K_2HPO_4 (MW 174.18), KH_2PO (136.09), KCl, were purchased form Fluka UK. Tween 20 and Glycine, Acetic acid, Citric acid (MW 210) were purchased from (Fisher scientific UK), Molecular sieves (MERCK Germany), N_2 (University store). All materials were used as received, with no further purification. Centrifugal concentrator Millipore ultra-free CL PTFE yellow 2.0mL 0.22µm pore size (fisher scientific), isolute double fritted (2x20um) filtrate, filtration column 3ml, (Isolute SPE accessories from Kenesis), Cuvette with a path length 1.0 cm (Fisher brand cuvettes, semi–micro, 15ml tall vial and 25ml vial screw top from Fisher scientific UK, Durapore Membrane filters of pore size 0.22µm and 0.45µm (Millipore Ireland). Deionised filtered water (Resistivity 18.2 MΩ) was obtained from in-house Millipore water purification system.

Methacrylic acid (MAA), 4-vinly pyridine (4Vpy), ethylene glycol dimethacrylate (EGDMA), were obtained from Aldrich, and 2, 2'-azobisisobutyronitrile (AIBN) from (BDH laboratory supplier England UK)

Purification of materials

EDGMA, MAA and 4VPy were purified by passing through a column packed with alumina; MAA and 4VPy were then distilled under reduced pressure. AIBN was purified by re-crystallisation from acetone and acetonitrile was dried by molecular sieves.

Apparatus –Spectrophotometer (Beckman Coulter, DU 800) with temperature controller (Beckman Coulter), HPLC waters 2996 (photodiode array detector), waters 2487 (Dual absorbance detector), waters 2695 (separation module), Centrifuge Beckman Coulter (large Eppendorf), shaking bath 58-16 (Techno), Centrifuge (Eppendorf AG2233, Hamburg, Germany) and Incubator (Techno, Ori-block, OB-3), Rotary evaporator. RP-HPLC, Blood rotary and Incubator (Stuart Scientific), Motor and Pestle and 25-50 sieves, Meter balance (Metter-Toledo AE200), pH meter (Metter-Toledo MP120), Magnetic stirrer and magnetic stirring bar (Fisher Scientific).

Preparation of buffers

Phosphate buffers

Phosphate buffers for preparing Lysozyme-KCl-PCMCs and Blank KCl crystals.

10 mM of phosphate buffer pH 7.2 were prepared from adding 71.7 ml of 1.0M K_2HPO_4 to 28.3ml of 1.0 M KH_2PO_4. 1ml was then diluted with 99ml of dH_2O to obtain a10mM buffer strength. 1.0M of K_2HPO_4 was prepared by dissolving 1.74g in dH_2O and 1.0M of KH_2PO_4 was prepared from dissolving 1.36g in dH_2O.

Arginine buffer

0.5M arginine pH 7 was prepared form equilibrating 0.5M of L-arginine monohydro-chloride with 0.5M L-arginine. For 0.5M arginine pH 5, only L-arginine monohydro-chloride was used.

Experiment 3.1

Polymer processing

After 48hrs of the polymer preparation, glass vials containing the monoliths polymers were broken to release the polymer. The monoliths were ground with a mortar and pestle into manageable fine particles, sieved at 20-50 µm mesh size and the powders were suspended in acetone to sediment similar particle size, and finally they were dried overnight in the oven at 60^0C. MIPs were then washed with acetonitrile to remove unreacted functional monomers MAA and 4-vpy. The disappearance of the 4vinyl pyridine RP-HPLC peaks from the wash solvent was an indication of the complete removal of these monomers from the polymer.

For example, 100mg of each polymer L1, L2 and L3 were weighed and were each put into separate 40 ml centrifuged tubes, followed by the addition of 20 ml of dry acetonitrile. The tubes containing polymer suspensions were put on a flask shaker machine for gentle shaking for a period of 10 min, after which the samples were again centrifuged and 50µl of the supernatants were injected directly into the RP-HPLC. To improve and facilitate 4vpy removal, the organic modifiers, such as 1% v/v of *N*,*N*'-diisopropylethylamine (DIPEA) and 1% of triethylamine (Et_3N), were added to the acetonitrile washing phase and similar washing procedures and analysis were repeated.

Experiment 3.2

Template extraction

100mg of polymer powders L1, L2 and L3 were each put into a double frit and washed with 3ml of different solvents as follows:

Wash No.	Washing Composition	Wash No.	Washing Composition
1	30% dH_2O: 70% AcN	11	10% SDS (10 x .1% Tris/glycine/SDS in 90% dH_2O
2	40% phosphate pH 7 (0.01M) & 60% AcN	12	40% phosphate pH 7 (0.1M) & 60% AcN
3	40% phosphate pH 5 (0.01M) & 60% AcN	13	30% acetic acid & 70% methanol
4	10% formic acid & 90% can	14	10% acetic acid & 80% phosphate pH 5 (0.1M)
5	12.5% acetic acid & 87.5% methanol	15	20% acetic acid & 80% phosphate pH 7 (0.1M)
6	Phosphate buffer pH 5 (0.1M)	16	10% acetic acid & 90% phosphate pH 7 (0.1M)
7	40% phosphate buffer pH 7 (0.5M): 60% AcN	17	Phosphate buffer pH 5 (0.1M)
8	Acetone	18	Phosphate buffer pH 5 (0.5M)
9	40% phosphate pH7 (0.5M) & 60% AcN	19	40% phosphate buffer pH 7 (1.0M) & 60%AcN
10	40% phosphate pH 5 (0.5M): 60% AcN	20	30% acetic acid & 70% methanol v/v

The template extraction methodology was further optimised with the addition of poly (ethylene glycol) (PEG 200)

Wash No.	Washing Composition	Wash No.	Washing Composition
21	2 wash with 20% PEG 200 & 10% acetic acid and 70% phosphate buffer pH 7 (0.1M); v/v	24	12.5 % acetic acid & 85% methanol, followed by 30% PEG 200 &:10% acetic acid & 60% phosphate buffer pH 7 (0.1M); v/v
22	2 wash with 30% PEG 200 &, 10% acetic acid and 60% phosphate buffer pH 7 (0.1M); v/v	25	20% acetic acid & 80% methanol followed by 30%PEG 200 10% acetic acid: 60% phosphate buffer pH 7 (0.1M);v/v
23	2 wash with 40% PEG 200 & 10% acetic acid and 50% phosphate buffer pH 7 (0.1M);v/v	25	20% acetic acid in buffer followed by 30%PEG 200 and 70% phosphate buffer pH 7 (0.1M); v/v

From this, the template extraction conditions were developed as follows: for example, 100mg of each polymer L1, L2 and L3 were weighed and were each put into separate 40 ml centrifuged tubes, followed by the addition of 20 ml of 30% PEG 200 in a phosphate buffer 60% and 10% acetic acid until lysozyme peak disappeared from the RP-HPLC, followed by 20ml of 8M urea. This process was repeated until no further traces of lysozyme were observed, indicating that all protein that could have been removed had been removed. All polymer sample powders were then equilibrated with 3 x 20ml phosphate buffer pH 7 (0.01M) and finally dried in the oven at 60^0C for ~ 24hrs.

Experiment 3.3

Lys selectivity towards polymers L1, L2, and L3 in equilibrium batch rebinding

For this experiment, 100mg of each polymer; L1, L2 and L3 powder were weighed and were each put into separate 3ml isolute double fritted of 2x20µm pore size. 2 ml of conditioning phase, phosphate buffer pH 7.23 (0.01M), were added to the sample and the frit was then put onto blood rotor machine for gentle mixing of about 10min, after which the conditioning phase was removed under vacuum. Polymers L1-L3 were then rebound with 2ml of mobile phase containing lysozyme 0.125 mg/ml of buffer pH 7.23 (0.01M) and mixed for 30 min. After 10 min intervals, the polymers were allowed to precipitate and 100µl of the aliquots were removed from each sample and centrifuged. 50µl of each supernatant were injected directly into RP-HPLC for template rebinding analysis.

Experiment 3.4

Non specific selectivity of RNase A towards polymers L1, L2, and L3 in equilibrium batch rebinding

100mg of each polymer; L1, L2 and L3 powder were employed in RNase A rebinding experiment. The experimental procedure and technique were similar to those described in the **experiment 3.3,** except that RNase A was added to the rebinding solution instead of lysozyme.

Experiment 3.5

Lys selectivity towards polymers L2 in equilibrium batch rebinding with different concentration of polymers

The equilibrium batch rebinding was carried out using different concentrations of polymer L2 (20, 40, 60, 80, 100, and 120) - mg. All polymers were weighed and were each put into separate 3ml isolute double fritted of 2x20µm pore size and were pre-conditioned with a solution consisting of 95% phosphate buffer pH 7.23 (0.01M), 5% ethanol and 0.05% Tween 20; v/v. The lysozyme rebinding procedure was similar to that described in the **experiment 3.3,** using a lysozyme concentration of 0.125mg lysozyme

/ml of mobile phase buffer pH 7.23 (0.01M), except that the rebinding time was reduced to 10 min.

Experiment 3.6

Lysozyme selectivity towards polymers L2 in a competitive equilibrium batch rebinding with a rebinding solution spiked with different proteins

20mg of polymer L2 were weighed and put into a double frit. The polymer was pre-conditioned with 2ml of mobile phase; 95% buffer pH 7.23 (0.01M), 5% ethanol, 0.05% Tween 20; v/v and rebound with a solution containing 0.1 mg/ml of lysozyme spiked with RNase A, cytochrome C at similar concentration for 10 min. The samples were then centrifuged and 50µl of supernatant were injected directly into RP-HPLC for protein rebinding analysis.

Experiment 3.7

Lysozyme selectivity towards polymers L1, L2, and L3 in a competitive equilibrium batch rebinding with rebinding solution spiked with different proteins and BSA

The experimental procedure was similar to that described in **experiment 3.6,** except that the BSA (0.1mg/ml) was added to rebinding solution containing protein mixture.

Experiment 3.8.1

Mobile phase tuning (1)

3 sets of 100mg of L2 were weighed and were preconditioned with 2ml of mobile phase; 95% buffer pH 7.13 (0.01M), 5% ethanol and 0.05% Tween 20; v/v and rebound with 2ml of rebinding solution containing 1mg of lysozyme /ml of mobile phase at pH 7.2 of arginine-HCl (Acid) of different strength; 0.2 M, 0.5 M and 0.7 M; all of which were equilibrated to the pH 7.2 with L-arginine (base). After 10 min of lysozyme rebinding, the samples were centrifuged and 50µl of supernatants were injected directly into RP-HPLC for protein rebinding analysis.

Experiment 3.8.2

Mobile phase tuning (2)

The experimental procedure was similar to that described in the **experiment 3.8.1,** except that only 0.5M of arginine was equilibrated to pH 7.2 and Tween 20 (0.05%) was added to the rebinding solution.

Experiment 3.8.3

Mobile phase tuning (3)

The experimental procedure was similar to that described in the **experiment 3.8.2,** except that 5% ethanol was also added to the rebinding solution.

Experiment 3.8.4

Mobile phase tuning (4)

20mg polymer L2 were weighed instead of 100mg because we were running short of the polymer. The polymer was preconditioned with 95% of 0.5M of arginine-HCl (Acid) equilibrated with L-arginine base to pH 7.2, 0.05% Tween 20 and 5% ethanol and rebound with 1ml of 0.1 mg/ml of (conditioning phase). The lysozyme rebinding procedures were similar to the described in the **experiment 3.8.2,** except that the rebinding time was 60min. The aliquots were removed for protein rebinding analysis after 10, 30 and 60-min time intervals.

Competitive equilibrium rebinding

Experiment 3.9.1

Lysozyme rebinding solution spiked with RNase A in a competitive aqueous equilibrium rebinding; using lysozyme (0.5mg/ml) & RNase A (0.5mg/ml) (1)

20mg polymer L2 were weighed and put into a double frit. The polymer was pre-conditioned with the mobile phase containing 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v and rebound with 1ml of rebinding solution containing lysozyme 0.5mg/ml of conditioning phase spiked with RNase A 0.5mg/ml (control) for

60 min, after which aliquots were removed for protein rebinding analysis after 3, 5,7,10, 30 and 60-min time intervals.

Experiment 3.9.2

Lysozyme rebinding solution spiked with RNase A in a competitive aqueous equilibrium rebinding; using lysozyme (0.1mg/ml) to RNase A (0.5mg/ml) (2)

The experimental procedure was similar to that described in the **experiment 3.9.1,** except that a lower concentration of lysozyme 0.1mg/ml was used in the rebinding solution, while that of the RNase A was left at 0.5mg/ml.

Experiment 3.9.3

Lysozyme rebinding solution spiked with RNase A in a competitive aqueous equilibrium rebinding; using lysozyme (0.5mg/ml) to RNase A (0.1mg/ml) (3)

The experimental procedure was similar to that described in the **experiment 3.9.1,** except that the concentration of lysozyme in the rebinding solution was 0.5mg/ml, while that of the RNase A was reduced to 0.1mg /ml.

Experiment 3.9.4

Lys rebinding solution spiked with RNase A in a competitive (aq) equilibrium rebinding; using different concentration of Lys & RNase A in solution (4)

2 samples of 100 mg polymer L2 were weighed and were pre-conditioned with mobile phase consisting of 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v. The first sample was rebound with RNase A (5mg/ml), while the lysozyme concentration was left at 0.5mg/ml in the mixture, and the second sample was rebound with lysozyme 1mg/ml, while that of RNase A was kept at 5mg/ml for 10 min. after which aliquots were removed for protein rebinding analysis. The experimental procedure was similar to that described in the **experiment 3.9.1**.

Experiment 3.10

RNase A selectivity towards RNase A imprinted polymer in equilibrium rebinding

5 x 20 mg samples of polymer R2 were weighed and put into double frits. The polymers were pre-reconditioned with 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v and finally rebound with 0.5 mg RNase A using different mobile phase solutions; 0.01M and 0.5M arginine-HCl equilibrated to pH 7 with L-arginine base, phosphate buffer pH 6, 7, and 8 at a buffer strength of 0.01M for 10min, after which aliquots were removed for protein rebinding analysis. The experimental procedure was similar to that described in the **experiment 3.9.1**.

Experiment 3.11.1

Lysozyme selectivity towards Lysozyme (L2) and RNase A (R2) imprinted polymers (1) using lysozyme 0.5mg/ml

A comparative study was undertaken using different polymer concentrations of both L2 and R2 (20, 25, 50, 75, and 100- mg/2ml). Each polymer was weighed and was put into double frits and then pre-conditioned with 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v. All samples were then rebound with 2ml of lysozyme 0.5mg/ml of mobile phase; 95% arginine pH 7 (0.5M), 5% ethanol, 0.05% Tween 20; v/v for 10 min, after which aliquots were removed for protein rebinding analysis. The experimental procedure was similar to that described in the **experiment 3.9.1**.

Experiment 3.11.2

Lysozyme selectivity towards Lysozyme (L2) and RNase A (R2) imprinted polymers (1) using lysozyme 0.1mg/ml (2)

A similar comparative study was undertaken to that described in the **experiment 3.11.1,** except lower concentrations of lysozyme were used in the rebinding solution, 0.1mg/ml and 0.01mg/ml.

Experiment 3.11.3

Lysozyme selectivity towards L2 and R2 imprinted polymers using lysozyme 0.5 and 0.1-mg/ml with the addition of NaCl (0.01M) (3)

A comparative study was undertaken using different polymer concentrations of both L2 and R2 (10, 15, 20 and 25- mg/2ml).This experiment was similar to that described in the **experiment 3.11.1,** except that NaCl (0.01M) was added to the rebinding solution consisting of 0.5mg of lysozyme/ml of 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v.

Experiment 3.11.4

Lysozyme selectivity towards L2 and R2 imprinted polymers using lysozyme 0.5 and 0.1-mg/ml with the addition of BSA and Casein (0.01M) (4)

A comparative study was undertaken using 50mg of both L2 and R2. This experiment was similar to that described in the **experiment 3.11.1,** except that BSA (1mg/ml) was added to the rebinding solutions consisting of: 0.1mg of lysozyme/ml of 95% arginine pH 7 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v. A similar experiment was repeated with casein (1mg/ml).

Experiment 3.12

Lysozyme release study from polymer L2 and R2 using washing and elution conditions

100mg of L2 and R2 were weighed and subjected to an experiment similar to that described in the **experiment 3.11.1**. 10 min after protein rebinding, both samples polymers were subjected to 4x 1ml wash with mobile phase (95% arginine pH 5 (0.5M), 5% ethanol, and 0.05% Tween 20; v/v) and finally with 1ml of elution phase (10% acetic acid in a 90% mobile phase), each for 10min. The polymers were centrifuged after each wash and 50µl of supernatants were injected directly into RP-HPLC for protein released study.

Chapter 4 : Molecular imprinted hydrophilic polymer

4.1 Introduction

The major problem still to be resolved in molecular imprinting is non specific protein binding. This was encountered previously where it was found impossible to discriminate protein bound to the selective sites from that bound to non selective sites. Techniques, such as mobile phase tuning with organic modifiers (ethanol, Tween 20), the use of non specific blocking agents (BSA, casein), and finally monovalent salts, were employed to overcome this problem but with variable success. Further attempts were directed towards discriminating protein already bound to the polymer high selective sites from that of low sites by using a template extraction step. From this, we observed slight differences in the amount of protein eluted from the lysozyme imprinted polymer compared to the comparative polymer imprinted with the molecular analogue RNase A. However, it was not clear if the excess protein had been eluted from the high selective sites or from the low selective sites.

The issue of non specific rebinding to the lysozyme imprinted polymer is of great concern. Previously, Moore's group attempted to incorporate hydrophilic monomers PEGDMA 1000 and PEGDMA 330 in the pre-polymerisation mixture. They reported that the hydrophilicity of the imprinted polymers was slightly improved. However, this was compromised by excessive swelling of the polymers leading to high column back pressure, which rendered them unfit for chromatography application.

To address the issue of polymer non specificity, it was hypothesised that the polymers designed previously (see chapter 3) may be too hydrophobic, as a result of using excessive basic functional monomer 4-vinyl pyridine in the polymerisation mixture. This may have contributed to the formation of non specific sites arising from monomer-monomer interactions. This could arise if there is a greater preference for the dimerisation than binding to the template (Sellergren, 2001), therefore resulting in incomplete interaction between the template and functional monomer.

In order to better estimate the concentration of template bound 4-vinyl pyridine, we designed a simple experiment (as described in chapter 2) to measure the degree of complex formation between lysozyme and this functional monomer.
From the complexing studies, it was clear that a lower ratio of functional monomer to protein should be used. New polymer compositions with ratios of (template) 1: 1 (functional monomers (0.5 4vpy + 0.5 MAA)): 40 (cross linker (EGDMA)) were considered. The use of large cross linking density was previously found to improve polymer selectivity. However, the amount of cross linker in the new formulation was >95% and was expected to result in a polymer that would be hard to break. We therefore added inert monomer di (ethylene glycol) vinyl ether (DEGVE) (figure 4-1) to facilitate breakdown and also to improve the polymer hydrophilicity. Holmberg (2008) has reported previously that DEGVE improved the hydrophilicity and non-fouling behaviour of polyethylene terephthalate (PET) polymer surfaces towards the adsorption of albumin and IgG from blood serum.

Figure 4-1: Molecular structure of Di (ethylene glycol) vinyl ether

This chapter highlights additional attempts to discriminate the protein bound to the polymer selective sites from that bound to non selective sites. Details of the experiments can be found at the end of the chapter.

4.2 Preparation of lysozyme imprinted hydrophilic polymer

Our imprinting strategy for the new developed polymer was similar to that described in chapter 3. The lysozyme was immobilised onto a glycine solid support, forming lysozyme PCMCs which acted as a platform for protein imprinting. Lysozyme PCMCs were prepared as follows: 10% lysozyme loading, 5% water, and IPA as precipitating

solvent saturated with glycine excipient (see experiment 4.1.1). The preparation of the new set of polymers with or without the addition of monomer DEGVE is outlined in table 4-1

Table 4-1: Reagents used for the preparation of lysozyme imprinted and control polymers

Polymer	PCMCs &Glycine (mg)	Lys (mg)	MAA g, mmol	4-vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
P1	1250	125	0.166, 1.94	0.333, 3.172	5.0, 25.254	------	91.33, 0.556	7.33
P2	1250	125	0.041, 0.0485	0.083, 0.792	5.0, 25.254	0.405 3.0645	91.33, 0.556	7.33
B1	Glycine 1250	-----	0.041, 0.0485	0.083, 0.792	5.0, 25.254	0.405 3.0645	91.33, 0.556	7.33

{Volume of acetonitrile was calculated from 4/3 (sum of all monomers +cross linker)}

4.2.1 Polymer processing

All polymers prepared were hard, glassy monoliths and were processed as described previously; that is, they were ground to manageable size, pre-washed with acetonitrile to remove unreacted residual monomers, followed by template extraction from the highly cross linked polymer network (see experiment 4.1.2)

Typical 4vpy traces from all polymers pre-washed with acetonitrile are presented in figure 4-2. It may be seen that the concentration of 4-vpy removed from the polymer P1 made with excess of 4vpy was approximately 5 fold greater than for P2 and B1. This suggests that the excess 4vpy employed for the preparation of the polymer P1 was not all incorporated into the monolith. Another observation was that the amount of 4vpy washed off from polymer P2 was comparable to that of B1. A possible explanation is that the presence of the lysozyme in the polymer P2 may have not consumed all of the extra monomers compared to the control polymer B1.

Figure 4-2: RP-HPLC spectra of 4vpy washed from all Lys P1, P2 & B1

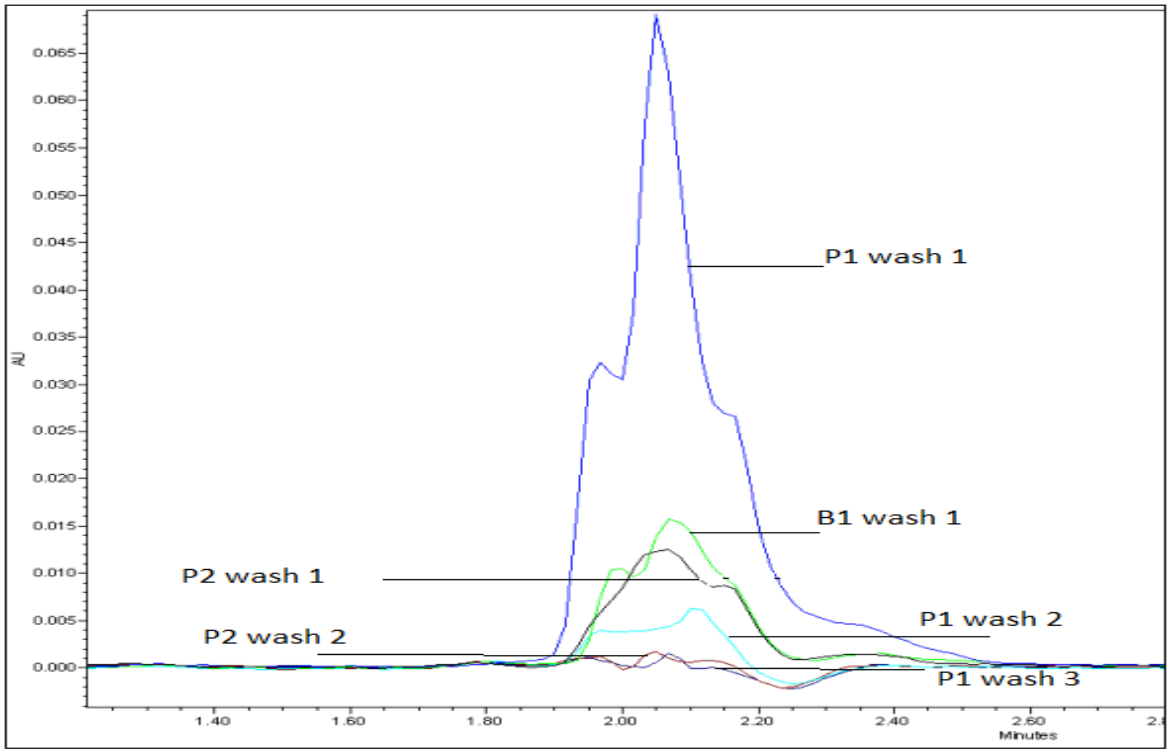


Figure 4-2 RP-HPLC spectra of 4vpy released from P1,P2 and B1 (100mg) after polymer wash 3 times with 20ml acetonitrile, each wash for 5 min

4.2.1.1 Template extraction

The next step was to extract the template from the imprinted polymers. Previously, we managed to observe very low amount of lysozyme eluted from the imprinted polymers, using the following washing conditions; PEG 200, acetic acid and buffer. For the new polymers, we developed new template extraction conditions. These are outlined in the table 4-2, together with the corresponding percentage of lysozyme extracted from polymer P2 (see experiment 4.1.3).

From the table 4-2, it can be seen that up to ~58% of the lysozyme template was extracted from P2 using the following washing conditions; polyethylene glycol 300 (PEG 300), acetic acid and either phosphate or citrate buffers. From this, it can be suggested that the addition of DEGVE or the reduction of the functional monomers

significantly improved the lysozyme released from the heavily cross linked polymer matrix.

Table 4-2: Lysozyme extracted from Lys MIPs with different solvent

Sample wash	Template Extraction Solvent	% Lys extracted
1	Citrate pH 5.08 (0.1M)	19.74
2	Citrate pH 5.08 (0.01M)	17.99
3	Phosphate pH 7.11 (0.1M)	12.71
4	Citrate pH 5.08 (0.01M) and NaCl 0.1M)	12.13
5	Citrate pH 5.08 (0.01M) and NaCl (0.01M)	18.81
6	Citrate pH 5.08 (0.1M) &10% acetic Acid	39.47
7	Citrate pH 5.08 (0.01M), PEG 200 (30%) & 10% acetic acid	55.152
8	Citrate pH 5.08 (0.01M), PEG 300 (20%) &10% acetic acid	58.84
9	dH_20, PEG 200 (30%) &10% acetic acid	52.57
10	Phosphate pH 7.11 (0.01M), PEG 300 (20%) &10 % acetic acid	58.11

Similar extraction solvent was applied to other polymers P1 and Blank B1, although the amount of lysozyme released from the P1 polymer was not analysed.

4.2.2 Lysozyme selectivity towards Lys MIPs and control

The preferential binding ability studies for P1, P2 and control polymer B1 were characterised using lysozyme equilibrium rebinding in an aqueous phase (see experiment 4.2.1). The rebinding results are demonstrated in figure 4-3.

From the results shown in figure 4-3, there was ~3 fold more binding by the imprinted polymer P1 compared to P2 and control B1, while P2 bound ~10µg (20%) more compared to B1 when 0.2mg of lysozyme in rebinding solution was used. However, the difference between P2 and B1 was reduced to ~5µg when 0.1 of lysozyme was employed.

Figure 4-3: Lysozyme uptake by Lys P1, P2 and control B1

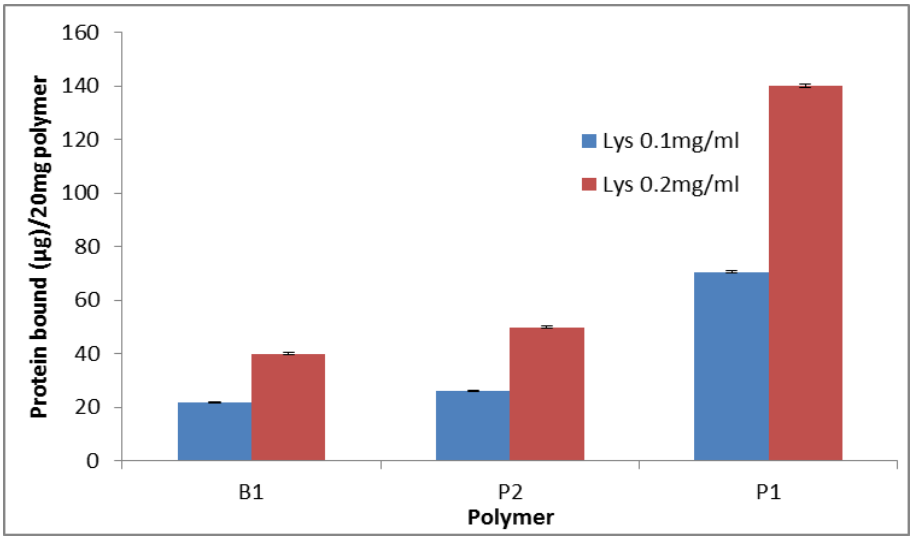


Figure 4-3 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1 with 2ml of (a) Lys 0.1mg/ml of mobile phase and (b) Lys 0.2mg/ml of mobile phase, mobile phase solution composition (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, for 10 minutes. (Mean ± error, n=3)

The increased lysozyme rebinding observed with P2 compared to the control B1 may be due to the presence of the selective sites for the lysozyme, although the concentration of these sites was low (5-10µg/20mg polymer). However, the large selectivity of the lysozyme towards P1 may be due to this polymer being too hydrophobic, with non selective binding dominating lysozyme selectivity. For the control B1, hydrophilic non selective protein binding was thought to dominate lysozyme selectivity. Furthermore, the lysozyme rebinding was found to be dependent on the concentration of the template in the rebinding solution. This is a typical characteristic commonly found with these imprinted polymers.

4.2.2.1 Lys selectivity towards Lys MIPs and control with addition of trehalose in the mobile phase

In order to try and reduce non specific hydrophilic template rebinding, we added trehalose in the mobile phase. The trehalose is known to prevent protein from denaturation and is also a suppressor of denatured protein aggregation. The trehalose holds partially folded proteins in a state from which they can be reactivated by molecular chaperones (Singer and Lindquist 1998). The use of trehalose in this investigation (experiment 4.2.2) was due to the ability of the trehalose to form hydrogen-bonding with the polar OH group of the polymers and therefore reduce non specific hydrophilic protein binding. As shown by the results given in figure 4-4, the addition of the trehalose did not suppress the non specific hydrophilic protein binding onto the control B1.

Figure 4-4: Lys uptake & released in wash & elution phase from Lys P1, P2 & B1

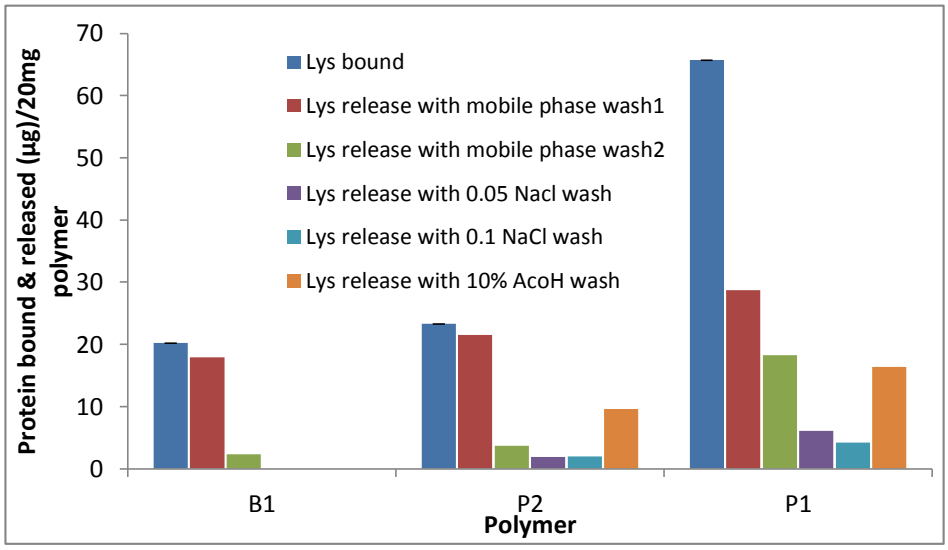


Figure 4-4 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1 with 2ml of Lys 0.1mg/ml of mobile phase composition (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, for 10 minutes. Followed by post washes to release Lys with (a) mobile phase (b) mobile phase with addition of 0.05 M NaCl and (c) mobile phase with addition of 0.01M NaCl and (d) elution phase (10% AcOH in mobile phase), each for 10 minutes. (Mean ± error, n=3)

From this, it was clear that to discriminate lysozyme rebinding between the imprinted polymer P2 and the control B1 would be a significant challenge. It was therefore decided to perform a lysozyme releasing study using template extraction steps. From the protein release study, lysozyme eluted from P1 and P2 was greater than that bound initially, and was large in P2 compared to P1. This suggests that the excess lysozyme may have leached out from the polymer even after template extraction carried out previously (section 4.2.1.1).

Template bleeding is a great challenge facing molecular imprinting in general and it is almost impossible to completely extract small molecular templates from the polymer let alone large molecular proteins.

4.2.2.2 1st Revision of template extraction methodology

At this point, it was deemed impractical to continue further with polymer analysis until the template bleeding problem had been addressed. It was therefore decided to revise and optimise the template extraction conditions in order to eliminate template bleeding. Following the failure of different concentrations of trehalose to extract substantial amount of the template (only 1-2% lysozyme washed out), it was finally realised that the surfactant Tween 20 was responsible for the template extraction phenomena observed previously. It was suspected that the surfactant may have either denatured the protein or displaced it from the imprinted cavities and therefore facilitated its removal. From this, a typical sequential wash was developed as follows: (See experiment 4.2.3).

1. 3 x 2ml of 0.5M arginine pH 5 (95%), 5% ethanol , 0.5% Tween 20 (T); v/v
2. 2 x 2ml of 8M urea in mobile phase
3. 2 x 2ml of 20% either ethylene glycol or PEG 300 or 10% acetic acid in mobile phase
4. 2 x 2ml of 8M urea in mobile phase

After each wash, the supernatant was injected directly into RP-HPLC for protein analysis. The RP-HPLC spectra of the lysozyme extracted from the polymer P1 and P2 after the first three washes with 0.5M arginine pH 5 (95%), 5% ethanol , 0.5% Tween 20 (T); v/v are shown in figure 4-5.

Before proceding to the next wash in the sequence, it was made sure that all the protein that was to be removed by washing solvent in the washing sequence had been removed. Acetic acid, ethylene glycol or PEG 300 was used to extract proteins that may have been strongly retained by the polymer. Finally, all polymers were subjected to 8M urea to dissolve protein fragments that may have developed from using strong protein denaturants, and from this, a further ~10% of lysozyme was observed in the wash.

Figure 4-5: Lys extracted from Lys P1 & P2 after adding 0.5% Tween 20 in a washing condition

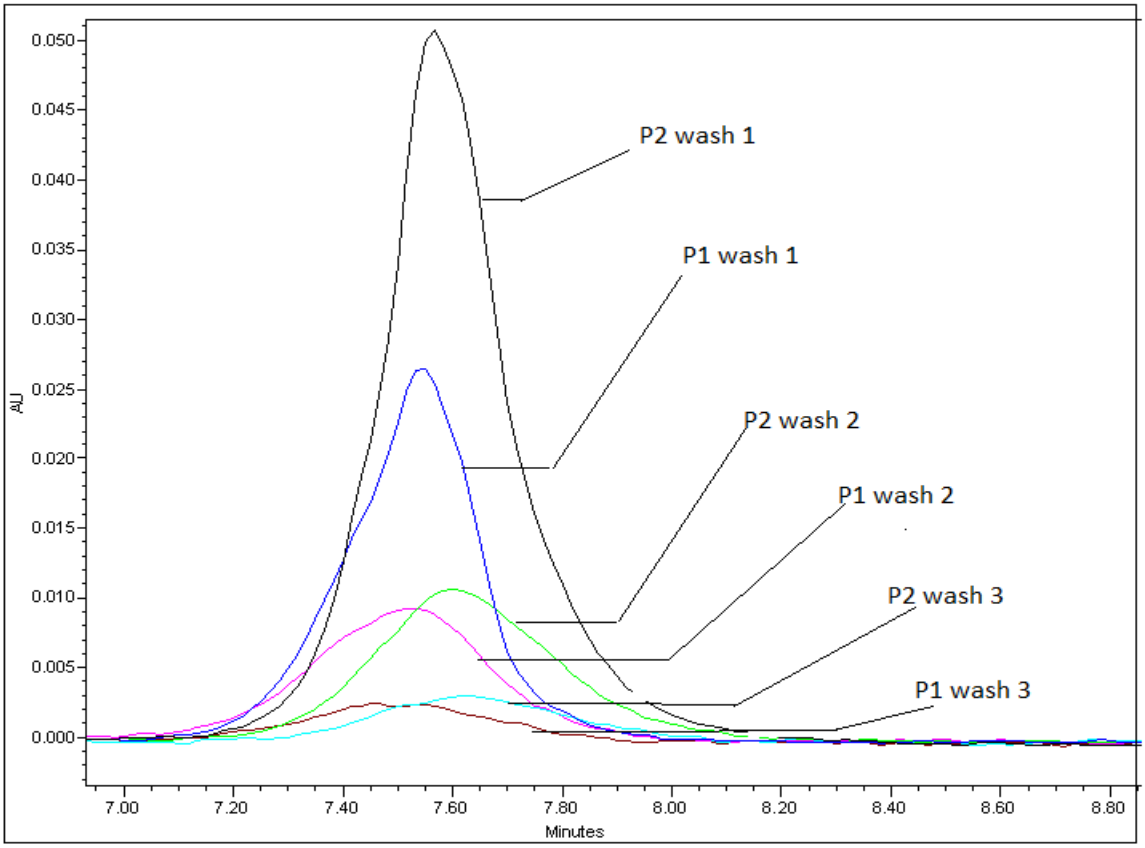


Figure 4-5 RP-HPLC spectra of lysozyme extracted from 20mg imprinted polymer P1and P2 with 3 x 2ml of 0.5M arginine pH 5 (95%), 5% ethanol , 0.5% Tween 20; v/v

From the new developed lysozyme extraction conditions, we managed to extract ~78% of the template from the P2 and ~56% from P1, which was significantly larger than that observed before (5-10%). This suggested that the modification of the polymer with the addition of DEGVE, which increased the swelling properties by ~ 0.8 (g) of solvent uptake/ polymer (g) (please refer to chapter 6), may have facilitated the release of the template from the polymer P2 compared to P1.

Furthermore, the numbers of template extraction steps were significantly reduced. However, the presence of the surfactant Tween 20 and PEG 300 was not desirable because it was suspected that these agents might compete with the template in the binding sites. All polymers were therefore thoroughly washed with isopropanol followed by low strength buffers.

4.2.2.3 Lys selectivity towards Lys MIPs & control with drying step

A typical molecular imprinting procedure after the template extraction step was to dry the polymer in the oven at elevated temperature prior to the template rebinding. It has been reported previously that the drying process might improve the polymer efficiency and chromatography performance by increasing the polymer capacity, rigidity and mass transfer kinetics, leading to reduced slow template bleeding, and at the same time complete the polymerisation process. However at very high temperatures above a glass transition temperature, this was found to reduce polymer affinity and enantioselectivity. It was reasoned that the polymer may either deform or collapse the imprinted cavities (Chen *et al*, 1999).

The question that we addressed was whether this process has any influence on the selectivity of the polymers P1 and P2. The polymers P1 and P2 were dried at 60 ^{0}C for 6 hrs and the lysozyme selectivity was compared with a similar set of polymers which was used after template extraction (see experiment 4.3). The results of the polymers affinities towards lysozyme are presented in figure 4-6.

Figure 4-6: Lys uptake by Lys P1 & P2 before and after drying

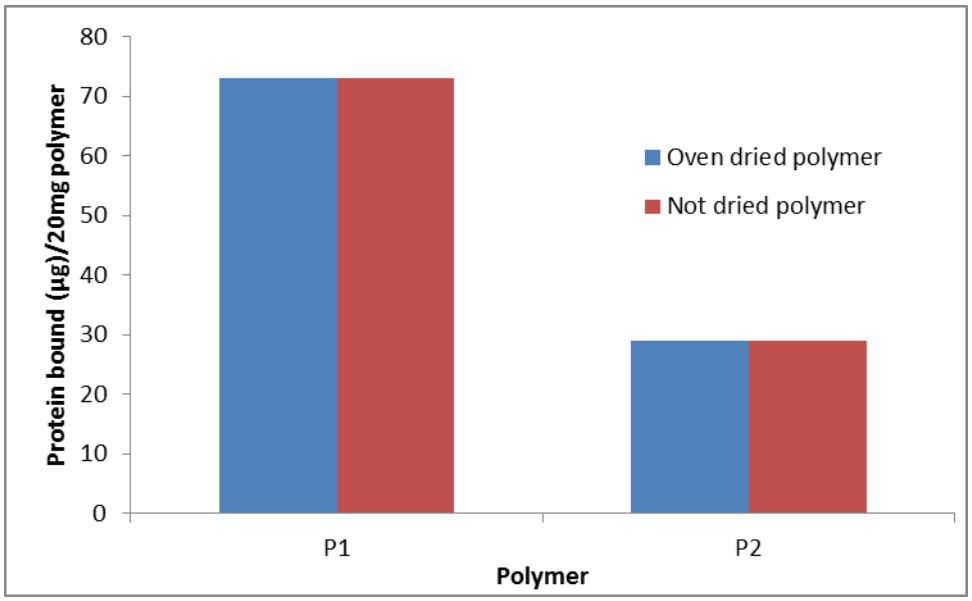


Figure 4-6 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymers, P1and P2 with 2ml of Lys 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, for 10 minutes. (Mean ± error, n=3)

The results show that the amount of lysozyme rebound was the same for each polymer before and after drying process. In addition, the selectivity of lysozyme towards the polymer was similar to that observed previously (see figure 4-3). This suggested that neither the drying process nor the additional template extracted from the polymer changed the selectivity of the lysozyme towards both imprinted polymers.

4.2.2.3.1 Template release study from Lys MIPs and control

In order to establish if there was any discrepancy in template binding to the selective sites from that of the non selective sites, we used template selective extraction conditions step. The amounts of the lysozyme released from polymers with similar compositions before and after drying were comparable (see figure 4-7).

Figure 4-7: Lysozyme in wash and elution conditions from Lys P1 & P2

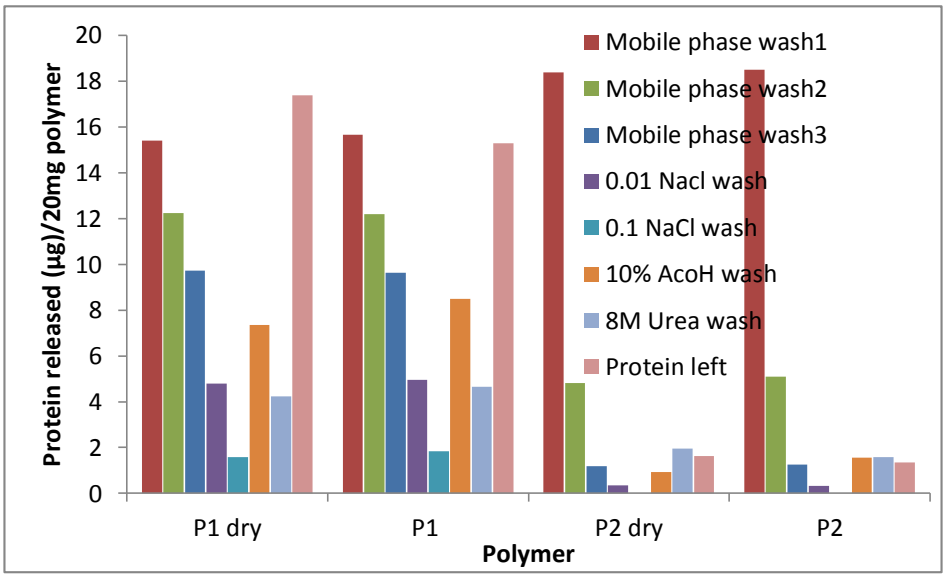


Figure 4-7 Lys released from imprinted polymers, P1,P2 oven dried and non dried using 3x2ml of mobile phase composition (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by (b) 2ml of mobile phase with addition of 0.05 M NaCl and (c) 2ml of mobile phase with addition of 0.01 M NaCl, (d) elution phase with (10% AcOH in mobile phase), (e) 2ml of 8M urea in mobile phase, all for 10 minutes and residual protein left in the polymers.

More interestingly, more lysozyme was extracted from the polymer P2 compared to P1 with mobile phase conditions. This suggested that a significant amount of the template rebound to P2 may have resided at the low selectivity sites compared to those bound to the polymer P1. The addition of 0.01M NaCl to extract the lysozyme bound hydrophobically yielded very low template, suggesting that the template bound by this interaction might have been removed in the initial mobile phase. Further tuning the mobile phase wash with 0.1 M NaCl salt to extract lysozyme bound electrostatically, yielded even less template. It is possible that by rinsing the polymer with low ion strength initially may have increased the strength of the template bound electrostatically. Conversely, using high ionic strength 0.1M would make template binding by hydrophobic interaction stronger and hence more difficult to remove. In order to overcome this, we used the elution phase containing 10% acetic acid. From this, the lysozyme released from polymers made of similar sets was comparable and more was

eluted from P1 (10-12%) compared to P2 (3-6%). This suggested that the concentration of the strong binding sites within P1 were more compared to P2.

The preceding experiment was carried out to investigate the influence of the drying process on the template association and dissociation characteristics in order to optimise each stage of the polymer process. From the results, it was clear that the drying process did not actually influence the polymer affinities towards the lysozyme. However, for easy handling and stocking of the polymer powder, it was found appropriate to dry the polymer at $37\ ^{0}$C prior to the use. In addition, the template releasing study gave us some insight into the existence of binding site hierarchies from strong to less strong selective sites.

4.2.2.4 Lys selectivity towards Lys MIPs & control with different concentration of Lys in solution

The preferential rebinding selectivity was again investigated using different concentrations of lysozyme in the rebinding solution (0.1mg, 0.05 and 0.01mg)/ml of mobile phase (see experiment 4.4). This was expected to reduce the non selective rebinding of the lysozyme to the control polymer. The lysozyme rebound results are presented in figure 4-8.

From the results, it can be seen that the lysozyme rebound significantly to P1 by more than 2 fold compared to P2, which was slightly more by ~5µg/20mg polymer compared to B1. From chapter 6, it was noted that both P2 and B1 had similar morphological properties, such as surface area and pore size. Therefore, the difference observed in the lysozyme rebinding may be solely due to the recognition site in the imprinted polymer P2, that may be absent in the non imprinted B1. Another possible explanation was that residual templates that had not been completely removed were capable of acting as a nucleation site for the template in the rebinding. This is because the actual binding sites may be complementary to the small clusters of the protein in the template (Sellergren, 2001), thus contributing to the protein recognition.

Figure 4-8: Lysozyme uptake by Lys P1 & P2 and control B1 with different concentration of Lys in the rebinding solution

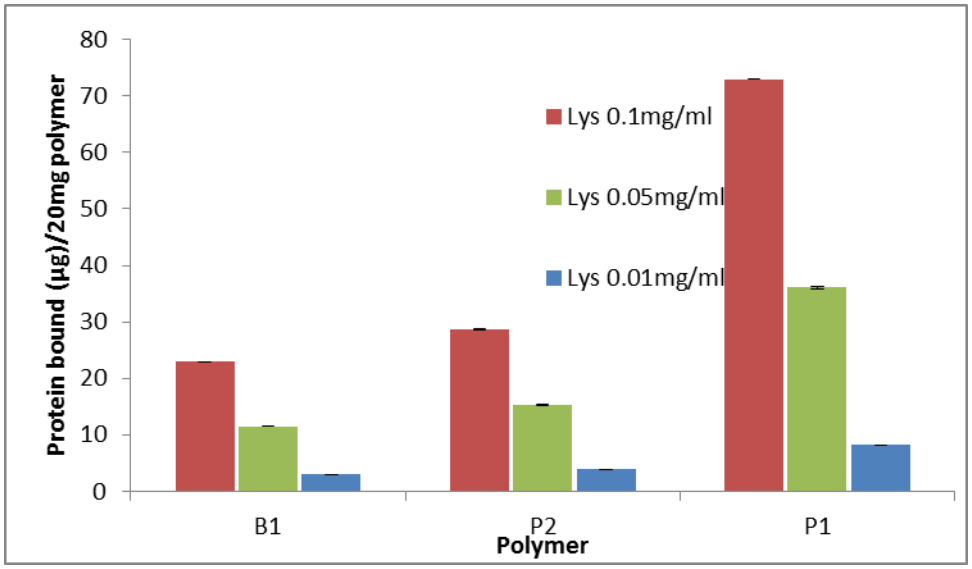


Figure 4-8 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1 with 2ml of (a) Lys 0.1mg/ml of mobile phase (b) Lys 0.05mg/ml of mobile phase (c) Lys 0.01mg/ml of mobile phase, mobile phase solution composition (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, for 10 minutes.

In order to discriminate the template rebinding to the selective sites in the imprinted polymer P2 compared to the control polymer B1, we looked at the template extraction step as before. For brevity, we only present the results of sample polymers rebound with 0.1mg lysozyme/ml for P2 and B1 (figure 4-9).

The main focus was directed towards the lysozyme eluted with the elution phase and amount of the protein retained by the polymers. From the results, it was found that the lysozyme released by P2 was 0.8µg/20mg more compared to the B1. This suggested that more templates bound to the strong sites in P2 compared to B1. In addition, more template was retained within B1, compared to P2, inferring that more lysozyme was bound to the non selective sites in B1. The lysozyme bound to these sites is difficult to remove and therefore limits the reusability of the control polymer B1 as opposed to the imprinted polymer where all the lysozyme bound initially dissociated completely.

Another possible explanation was that polymer P2 had greater swelling properties compared with control B1 (refer to chapter 6) and therefore this may have facilitated the amount of lysozyme released from the P2.

Figure 4-9: Lys in wash and elution conditions from Lys P2 & B1

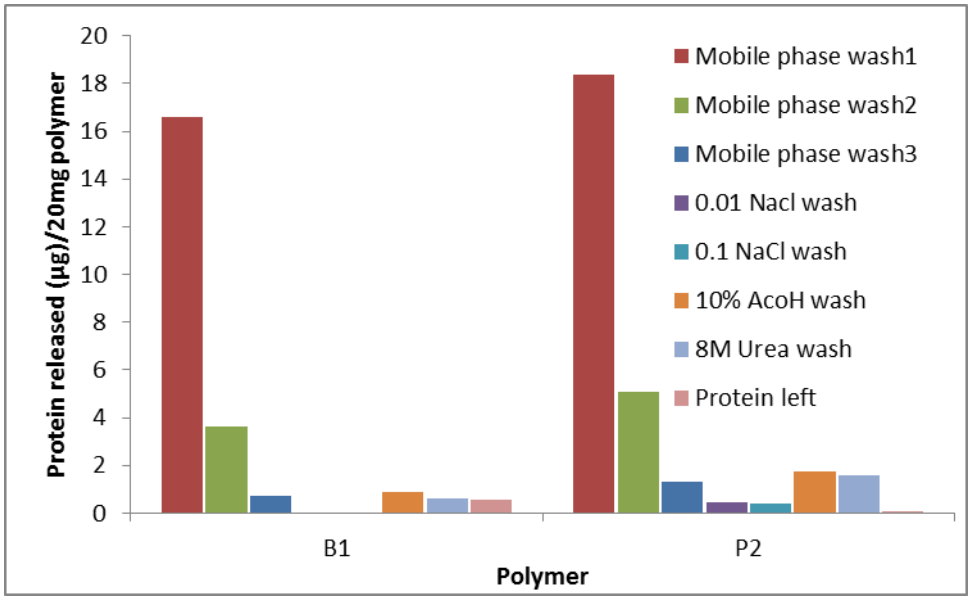


Figure 4-9 Lys released from imprinted polymers, P2 and B1 using 3x2ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by, (b) 2ml of mobile phase with addition of 0.05 M NaCl and (c) 2ml of mobile phase with addition of 0.01 M NaCl, (d) elution phase with (10% AcOH in mobile phase), (e) 2ml of 8M urea in mobile phase, all for 10 minutes and residual protein left in the polymers.

4.2.3 Competitive rebinding of lysozyme towards MIPs & control

New polymers were designed to address the issue of non specific binding. In order to see whether this effect was reduced, a competitive aqueous rebinding was performed with lysozyme solution spiked with molecules with closer molecular weight (RNase A, and cytochrome C) and slightly larger molecular weight (myoglobin) (see experiment 4.5.1). The summary of equilibrium rebinding results from figure 4-10 demonstrated that a significant reduction of non specific binding was noted with the polymers having inert monomer (DEGVE) P2 and B1. This uptake of cytochrome C was almost 4 fold on P1

compared to P2 and 7 fold with B1; this suggested that non specific binding of cytochrome C to the polymers made with DEGVE P2 and B2 was significantly reduced, while for P1 almost all of the protein was adsorbed. However, the rebinding of the specific template lysozyme towards P1 was still larger compared to both P2 and B1 which differ by only 3µg. This difference was smaller than that observed in the absence of other proteins in the mixture.No difference was noted with rebinding of RNase A, while for myoglobin ~2/3 bound to P1 and ~1/3 bound in both P2 and B1.

Figure 4-10: Protein uptake by Lys P1, P2 & B1 in a competitive rebinding

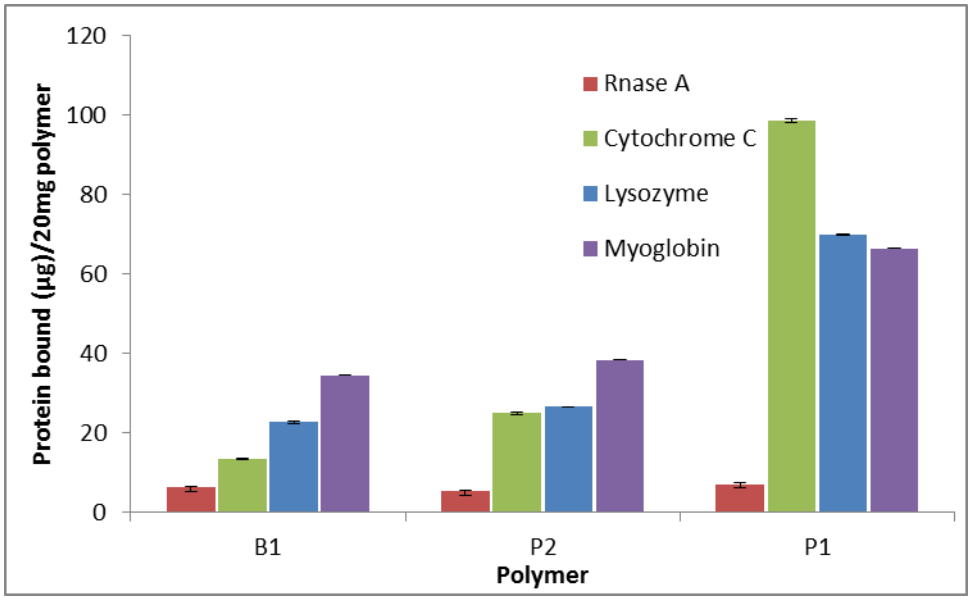


Figure 4-10 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1, with lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v spiked with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml) and myoglobin (0.1mg/ml) for 10 minutes. (Mean ± error, n=3)

It can be seen therefore that the use of a lower concentration of monomers and the addition of DEGVE might have reduced the concentration of the monomer-monomer interactions and therefore lessened the effect of the non selective protein binding.
A further attempt to try and discriminate the proteins bound to the selective from non selective sites was to carry out the protein extraction step using the different washing

conditions, as described previously. The proteins released from all polymers are presented in the table 4-3.

From the summary of the results, it can be seen that significant amounts of the proteins were released under mild washing conditions, except myoglobin, suggesting that the proteins may have primarily bound to the less strong sites. However, the exact reason for the absence of myoglobin was not known.

More interestingly, from rinsing the polymers with NaCl (0.01M) and (0.1M), only lysozyme released from P2 and B1, while lysozyme and cytochrome C were released from P1, with a 4 fold greater release of cytochrome C compared to lysozyme. This suggests that only lysozyme is bound by hydrophobic and electrostatic interaction sites in P2 and B1, while cytochrome C and lysozyme are bound by these interactions in P1, in addition, cytochrome C was more selective for these sites compared to the lysozyme. Another feature observed was that the lysozyme was eluted in conjunction with cytochrome C from polymer P1 using strong elutropic solvent conditions, although on this occasion the amount of lysozyme was ~23µg more compared to cytochrome C. Furthermore, the lysozyme eluted from P1 was 8 fold more compared to P2 and 10 fold more than B1. The lysozyme eluted from P2 was 10µg more in P2 compared to B1, but here lysozyme was eluted alone as opposed to P1.

From the results, it was clear that the concentration of the lysozyme strong sites in P1 was large compared to P2, although the non selective sites for cytochrome were significant compared to both P2 and B1. However from the elution phase, it was noted that in P2 lysozyme strong sites were independent from other proteins in the mixture, in contrast to P1. From the urea wash, lysozyme was released from all polymers, except that cytochrome C was released from only P1 but not from P2 and B1, while myoglobin was only absent in the P1 wash.

Finally, all polymers retained amounts of different proteins from the mixture, except that almost all of the lysozyme dissociated completely from P2 compared to other polymers P1 and B1. This suggests only lysozyme is bound in its native form in the P2 polymer and was therefore easier to extract, but for other non specific proteins, they may be

bound in other forms and therefore were more difficult to remove. From this, it was clear that the reusability of P2 for lysozyme rebinding was superior to P1 and B1.

Table 4-3: Protein in wash & elution phases & left in the polymers

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	Myoglobin (mg)
W1-Mobile Phase (MP)	B1	0.0056	0.0036	0.0165	
	P2	0.0054	0.0039	0.0171	
	P1	0.0058	0.0015	0.0170	
W2-Mobile Phase (MP)	B1		0.0005	0.0039	
	P2		0.0012	0.0047	
	P1		0.0054	0.0122	
W3-Mobile Phase (MP)	B1			0.0011	
	P2			0.0014	
	P1		0.0095	0.0087	
W4-NaCl 0.01M in (MP)	B1			0.0002	
	P2			0.0003	
	P1		0.0127	0.0037	
W5-NaCl 0.1M in (MP)	B1			0.0003	
	P2			0.0004	
	P1		0.0065	0.0012	
W6-Elution 10% AcOH in (MP)	B1			0.0009	
	P2			0.0019	
	P1		0.0073	0.0096	
W7-8M Urea in (MP)	B1			0.0005	0.0049
	P2			0.0013	0.0031
	P1		0.0076	0.0049	
Protein left	B1	0.0007	0.0094	0.0008	0.0295
	P2		0.0198	0.0002	0.0352
	P1	0.0012	0.0481	0.0110	0.0605

Table 4-3 Proteins released from imprinted polymers, P1, P2 and B1 using (W1-3)3x2ml of mobile phase (MP) (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by, (W4) 2ml of mobile phase with addition of 0.1 M NaCl and (W5) 2ml of mobile phase with addition of 0.01 M NaCl, (W6) elution phase with (10% AcOH in mobile phase), (W7) 2ml of 8M urea in mobile phase, all for 10 minutes and residual protein left in the polymers.

The template release study gave us some insight into the increased swelling observed in P2 compared to both B1 and P1 (please refer to chapter 6). This may have been responsible for the increased release of the specific template from the imprinted polymers as. However, other non specific proteins, such as cytochrome C and myoglobin, were strongly retained and could not be dissociated easily. This suggests that the increased swelling noted with P2 was specific to the selective sites as opposed to non selective sites in control B1.

To try and reduce non specific binding further, we added BSA in the rebinding phase (see experiment 4.5.2). BSA is known to be a very hydrophobic protein and the use of this protein in the competitive rebinding was also to see if we had improved our new polymer formulation.

From the results shown in figure 4-11, the non specific binding of cytochrome C to the polymers made with DEGVE was reduced further by almost half in P2 compared to the previous results. A slight decrease was observed with B1, but for P1 almost all of the cytochrome C was adsorbed. However, the selectivity of the lysozyme towards the imprinted polymers P2 and B1 was slightly reduced for P2 and B1 but even more for P1. This suggested that the BSA may have bound onto the hydrophobic sites on the polymer and therefore could not be displaced by the less hydrophobic lysozyme.

Another interesting feature observed was the significant reduction of myoglobin binding to almost a third in P1, while a small reduction was observed with P2 and B1 and again no difference was noted with rebinding of RNase A compared to previous data . The poor myoglobin binding could be explained by similar reasoning to that discussed in the preceding paragraph. From the results, it was clear that in addition to lowering cytochrome C binding, we were also successful in lowering the non specific binding of BSA by more than 2 fold in P2 and B1 compared to P1.

Figure 4-11: Protein uptake by Lys P1, P2 & B1 with BSA in solution

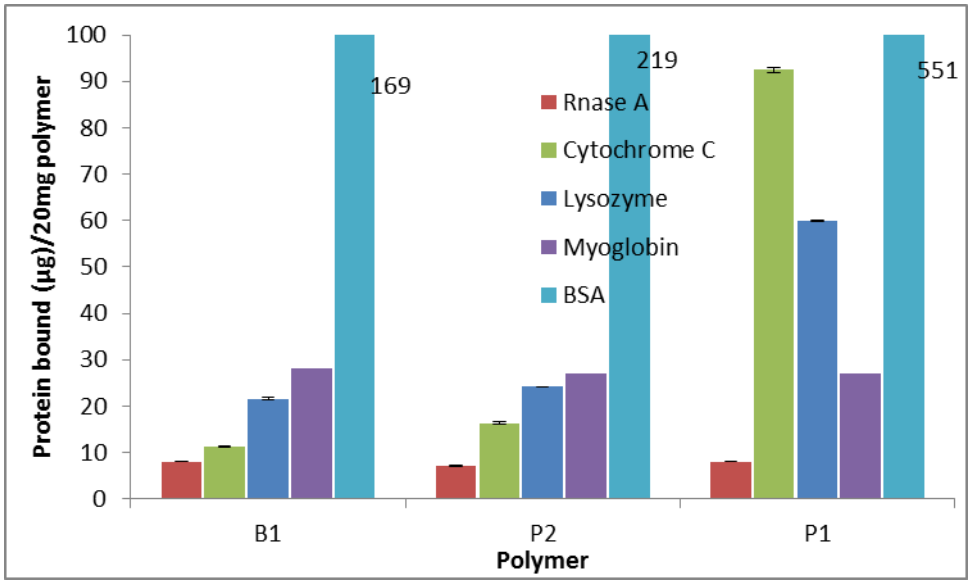


Figure 4-11 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1 with lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v spiked with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml), myoglobin (0.1mg/ml) and BSA (1mg/ml) for 10 minutes. (Mean ± error, n=3)

To determine the nature of the protein selectivity, we looked at selective template extraction studies as before. For brevity, we only show and discuss the proteins eluted with elutropic solvent (10% AcOH in buffer), 8M urea and that retained with polymers; the assumption was that these proteins were eluted from strong binding sites.

From table 4-4, it can be seen that lysozyme was again eluted with cytochrome C from P1, but on this occasion the concentrations of both proteins were comparable. However, the amount of lysozyme was higher than that of P2, which was slightly higher by ~0.8µg/20mg polymer compared to B1, but in both polymers lysozyme was eluted alone.

A similar trend was observed with the urea wash. It was clear that the addition of the BSA did slightly lessen the effect of the non selective protein rebinding. However, it elevated the amount of cytochrome C eluted with the lysozyme and the reason for this could not be explained.

Table 4-4: Protein in wash & elution phases & left with BSA in rebinding solution

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	BSA (mg)	Myoglobin (mg)
W6-Elution	B1			**0.0009**		
10%AcOH	P2			**0.0017**		
in MP	P1		**0.0105**	**0.0109**		
W7-Urea	B1			0.0004		0.0060
8M in MP	P2			0.0016		0.0050
	P1		0.0058	0.0042		0.0050
Protein	B1	0.0030	0.0068	0.0010	0.1403	0.0153
left	P2	0.0023	0.0123	0.0000	0.1899	0.0229
	P1	0.0025	0.0719	0.0046	0.5276	0.0217

Table 4-4 Proteins released from imprinted polymers, P1, P2 and B1 with elution phase mobile phase (W6) (10% AcOH in mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v and (W7) 8M urea in mobile phase, all for 10 minutes and residual protein left in the polymers.

In addition to the high concentration of BSA, other residual proteins left with all polymers were similar to that observed before, although nearly all of the lysozyme was removed from P2. This again demonstrates the reusability of P2, which was better compared to P1 and B1.

4.2.4 Competitive rebinding of Lys towards Lys MIPs & control & heat application

The indiscriminate binding demonstrated by P2 compared to the control B2 was a significant challenge, although we were still interested in determining if the imprinted polymers possessed some more specific sites compared to the non-imprinted control. We therefore decided to apply a novel technique whereby the polymer is washed after rebinding to remove lightly bound template, and then a heat shock is applied to destabilise non-specifically residual bound template. The hypothesis was that template bound to specific sites will be more stable with heat shock and would be more easily eluted from the matrix in a native conformation.

To investigate this, we looked at 10 min heat application at 60 ^{0}C to the polymer solution already bound with protein mixture without BSA (see experiment 4.5.3). The proteins bound to the polymers are shown in figure 4-12.

Figure 4-12: Protein uptake by Lys P1, P2 & control B1 before heat treatment

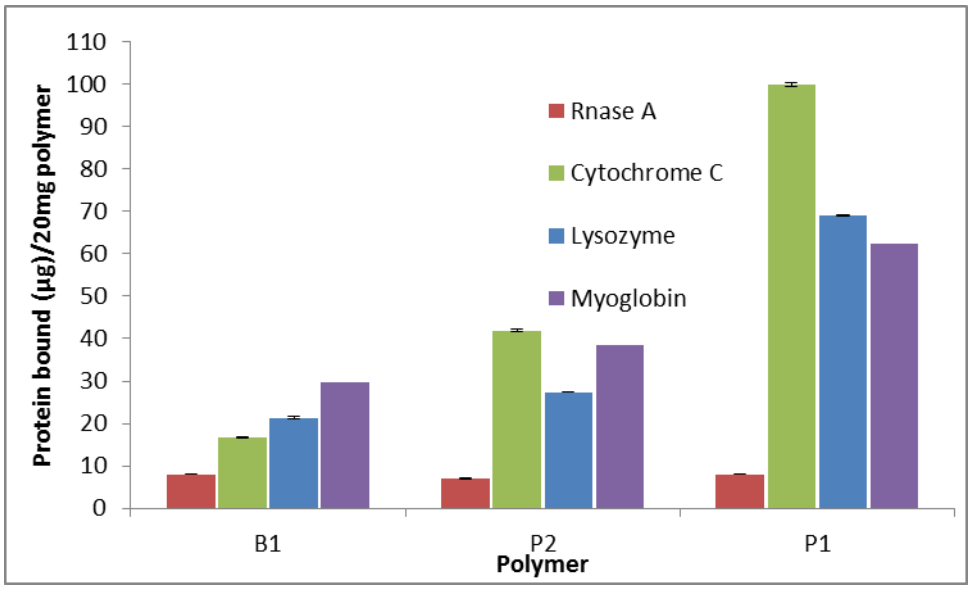


Figure 4-12 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1, with lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v spiked with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml) and myoglobin (0.1mg/ml) for 10 minutes. (Mean ± error, n=3)

The results demonstrated from figure 4-12 are comparable to those observed before (figure 4-10). The main focus here was to determine the effect of the heat shock on the already bound proteins. The proteins released after heat shock application, using the template extraction technique described previously, are presented in table 4-5.

The lysozyme amounts eluted after heat shock treatment from P2 and B1 were comparable but were almost 2 fold lower than that of the P1. In addition, the protein bound was significantly lower than observed before. However, the exact reason for indiscriminate release of lysozyme from B1 compared to P2 was not known. In addition, for the first time lysozyme was eluted alone from P1 without cytochrome C. It was

reasoned that the absence of cytochrome C in the elution phase may be due to the heat effect damaging this protein and therefore changing its conformation, such that it was difficult to extract.

It was suspected that the amount of the lysozyme eluted from P1 may represent the concentration of the lysozyme selective sites within the polymer.

Table 4-5: Protein in wash & elution phases & left in the polymers after heat treatment

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	Myoglobin (mg)
W1-Mobile Phase (MP)	B1	0.0062	0.0042	0.0132	
	P2	0.0060	0.0009	0.0152	
	P1	0.0048		0.0116	
Heat Shock					
W6-Elution 10%AcOH in MP	B1			**0.0017**	
	P2			**0.0018**	
	P1			**0.0036**	
W7-Urea 8M in MP	B1				
	P2				
	P1		0.0188	0.0043	
Protein left	B1	0.0024	0.0124	0.0063	0.0297
	P2	0.0012	0.0409	0.0107	0.0386
	P1	0.0066	0.0805	0.0491	0.0624

Table 4-5 Proteins released from imprinted polymers, P1, P2 and B1 using 2ml of (W1) mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by application of rapid heat shock in water bath at 60-67^{0}C, (W6) elution with 2ml of 10% AcOH in the mobile phase; v/v, (W7) 2ml of 8M urea in the mobile phase, all for 10 minutes and residual protein left in the polymers.

However, no traces of proteins were observed from P2 and B1 with urea washing conditions but only from P1, whereby a 4 fold greater amount of cytochrome C was released compared to lysozyme. This was larger than was observed before. It was assumed that the cytochrome C might have been damaged such that its amino acids could easily dissolve in the urea phase.

From the heat treatment technique, we managed to elute only lysozyme from P1, this has not been observed before. However using this technique, we could not discriminate the lysozyme eluted from P2 from that of the control B1.

Another interesting observation made was that more lysozyme was retained within polymer P2 compared to B1. This further suggests that the increased swelling in P2 (refer to chapter 6) may have less effect on the damaged lysozyme as a result of heat shock application.

Because of this, it was then decided to add BSA in the rebinding phase as before (see experiment 4.5.4). It was assumed that the BSA might cover non specific surfaces and therefore expose non specific protein to the prolonged heat treatment. The experiment was similar to that described before, including BSA in the rebinding solution made of lysozyme spiked with RNase A, cytochrome C and myoglobin. The protein bound results are presented in figure 4-13.

The amounts of the proteins bound to the polymers were comparable to those observed before (refer to figure 4-11)

Figure 4-13: Protein uptake by Lys P1, P2 & control B1 with BSA added to the rebinding solution

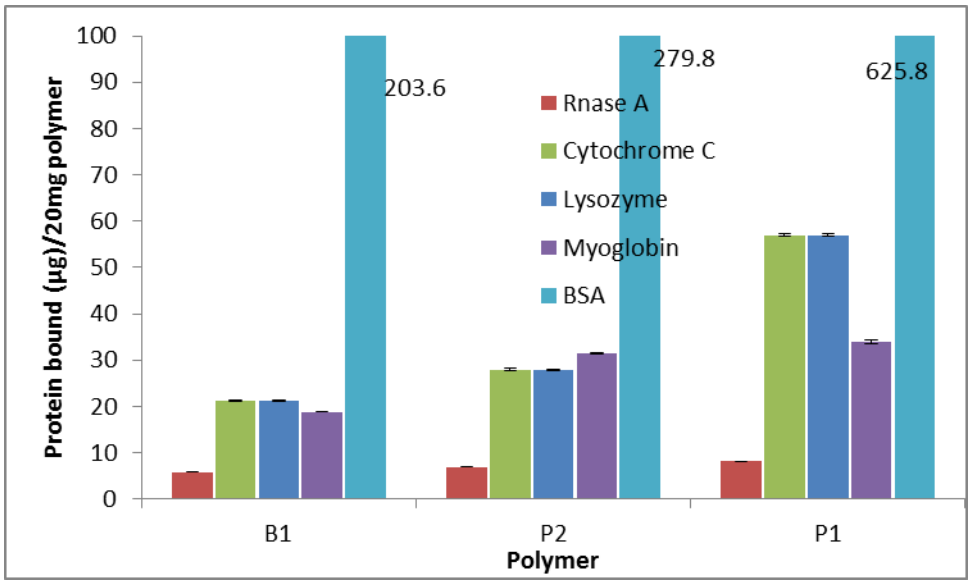


Figure 4-13 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1, with lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v spiked with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml), myoglobin (0.1mg/ml) and BSA (1mg/ml), for 10 minutes. (Mean ± error, n=3)

From the template release study shown in the table 4-6, no difference was observed with lysozyme eluted from the P2 compared to B1, and again lysozyme was eluted together with cytochrome C from P1. In addition to this, cytochrome C released in the urea phase was 3 fold lower than that of the lysozyme, and overall more proteins were released from all polymers than observed in the preceding experiment. We concluded that the presence of BSA in the rebinding solution may have lessened the effect of heat damage to non specific bound cytochrome C and also to other proteins.

Table 4-6: Protein in wash & elution & left in the polymers after heat treatment (BSA added to the rebinding solution)

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	BSA (mg)	Myoglobin (mg)
W1-Mobile Phase (MP)	B1	0.0059	0.0037	0.0120	0.0135	
	P2	0.0064	0.0010	0.0157		
	P1	0.0054	0.0012	0.0142	0.0013	
Heat Shock						
W6-Elution 10%AcOH in MP	B1			0.001665		
	P2			0.001745		
	P1		0.010772	0.011867		
W7-Urea 8M in MP	B1			0.000736		
	P2			0.001302		
	P1		0.002005	0.006569		
Protein left	B1	0.0001	0.0086	0.0066	0.1619	0.0188
	P2	0.0005	0.0280	0.0092	0.2683	0.0315
	P1	0.0027	0.0779	0.0243	0.6158	0.0340

Table 4-6 Proteins released from imprinted polymers, P1, P2 and B1 using 2ml of (W1) mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by application of rapid heat shock in water bath at 60-67^{0}C, (W6) elution with 2ml of 10% AcOH in the mobile phase; v/v, (W7) 2ml of 8M urea in the mobile phase, all for 10 minutes and residual protein left in the polymers.

However, a small discrepancy was observed from the lysozyme released in the urea phase from P2 which was 2 fold more than B1. The complete retention of myoglobin with all polymers was noted. It was reasoned that myoglobin may have bound strongly

to all polymers or may be too damaged by heat shock and therefore be very difficult to extract.

In a further attempt to discriminate the selective protein binding from non selective binding, we rebound all polymers with BSA initially and then introduced the lysozyme spiked with the other protein mixture afterwards (see experiment 4.5.5). It was hypothesised that lysozyme having a high selectivity for the imprinted sites would displace BSA bound to these sites in contrast to BSA bound to the non selective sites as described by the Vroman effect. This states that during protein adsorption from protein mixtures, those proteins with low selectivity to the polymer sites are adsorbed reversibly onto the surface, whilst proteins with strong selectivity for the sites would displace those already bound proteins.

The amount of BSA initially bound to all polymers is presented in the table 4-7.

Table 4-7: BSA uptake by Lys P1, P2 and control B1

Protein mg	**P1**	**P2**	**B1**
BSA	0.659	0.2434	0.333

From the BSA binding results shown in the table 4-7, P1 bound more BSA compared to B1 by ~2 fold and by ~2.7 fold compared to P2. The binding of BSA slightly increased in P1 and B1, but not for P2, which was similar to that observed before (see figure 4-13). The increased BSA binding to both polymers P1 and B1 suggested there were more non specific sites in these polymers compared to P2.

From figure 4-14, the lysozyme rebound to P2 was 12.3µg/20mg polymer more compared to control B1 but was less than P1 by ~17.3µg/20mg; the lysozyme bound to P2 was significantly less than that observed before (figure 4-13).

Figure 4-14: Protein uptake by Lys P1, P2 & control B1 after bound with BSA

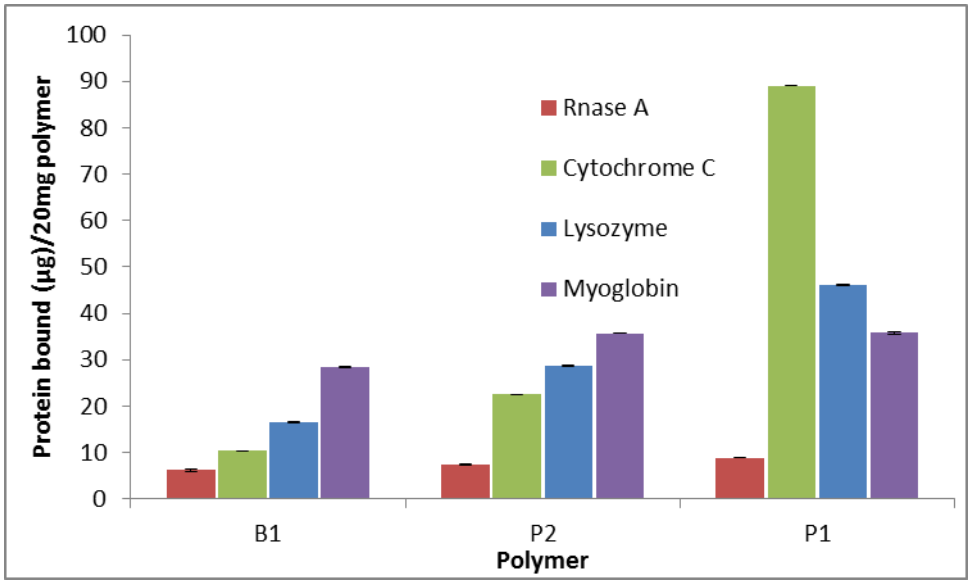


Figure 4-14 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1, with lysozyme 0.1mg/ml of mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v spiked with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml) and myoglobin (0.1mg/ml) for 10 minutes. (Mean ± error, n=3)

This indicates that there are specific sites recognising lysozyme in P2 that is absent in B1, although the concentration of these sites are small. It was also argued that the BSA bound initially may have resided onto the non specific hydrophobic surfaces and therefore hindered other proteins from binding to those sites. Lysozyme specific sites may be available in polymer P2, but are not present in control B1. However for polymer P1, being very hydrophobic, it was not clearly understood if lysozyme specific sites were present. The binding of cytochrome C was slightly increased for P2 but for B1 and P1. It was comparable to that observed before (ref figure 4-13). No significant changes were observed with both RNase A and myoglobin binding.

From the selective template extraction step (table 4-8), the lysozyme eluted from P2 was 3 fold more (0.8µg/20mg) than for B1. The difference was comparable to the amount of lysozyme rebound initially (figure 4-14).

Table 4-8: Protein in wash & elution phases & left in the polymers after heat treatment (BSA bound polymers)

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	BSA (mg)	Myoglobin (mg)
W1-Mobile Phase (MP)	B1	0.0064	0.0056	0.0117	0.0044	
	P2	0.0064	0.0030	0.0132	0.0000	
	P1	0.0064	0.0044	0.0180	0.0000	
Heat Shock						
W6-Elution 10%AcOH in MP	B1			**0.0004**		
	P2			**0.0012**		
	P1		**0.0108**	**0.0084**		
W7-Urea 8M in MP	B1					0.0014
	P2			0.0008		0.0020
	P1		0.0025	0.0028		
Protein left	B1	0.0001	0.0047	0.0042	0.2880	0.0270
	P2	0.0011	0.0195	0.0135	0.2268	0.0337
	P1	0.0025	0.0658	0.0168	0.6516	0.0358

Table 4-8 Proteins released from imprinted polymers, P1, P2 and B1 using 2ml of (W1) mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by application of rapid heat shock in water bath at 60-67$_0$C, (W6) elution with 2ml of 10% AcOH in the mobile phase; v/v, (W7) 2ml of 8M urea in the mobile phase, all for 10 minutes and residual protein left in the polymers.

However, more cytochrome C was eluted from P1 compared to lysozymes even though the binding of cytochrome C was less than before. From the urea phase, myoglobin was removed from both P2 and B1, with additional traces of lysozyme in P2. For P1, the lysozyme was again released in conjunction with cytochrome C but no trace of myoglobin was observed. Therefore, it can be seen that the BSA may have partially covered non selective sites and from this we were able to discriminate the lysozyme bound to the P2 compared to B2. However, the total lysozyme eluted from polymer P2 was significantly smaller compared to that observed before. In addition, we could not identify whether lysozyme displaced any BSA bound initially.

In order to amplify the difference of lysozyme rebinding and try to suppress further non selective binding, we rebound all the polymers with the solution containing BSA, cytochrome C, RNase A, and myoglobin without lysozyme and then introduced the

lysozyme solution afterwards (see experiment 4.5.6). The lysozyme was expected not only to displace large molecular weight BSA but also other protein bound to the strong sites. From the protein rebinding results presented in figure 4-15, the binding of all proteins to all polymers was comparable to that observed before (refer to figure 4-13)

Figure 4-15: Protein uptake by Lys P1, P2 & B1 in the absence of Lys in solution

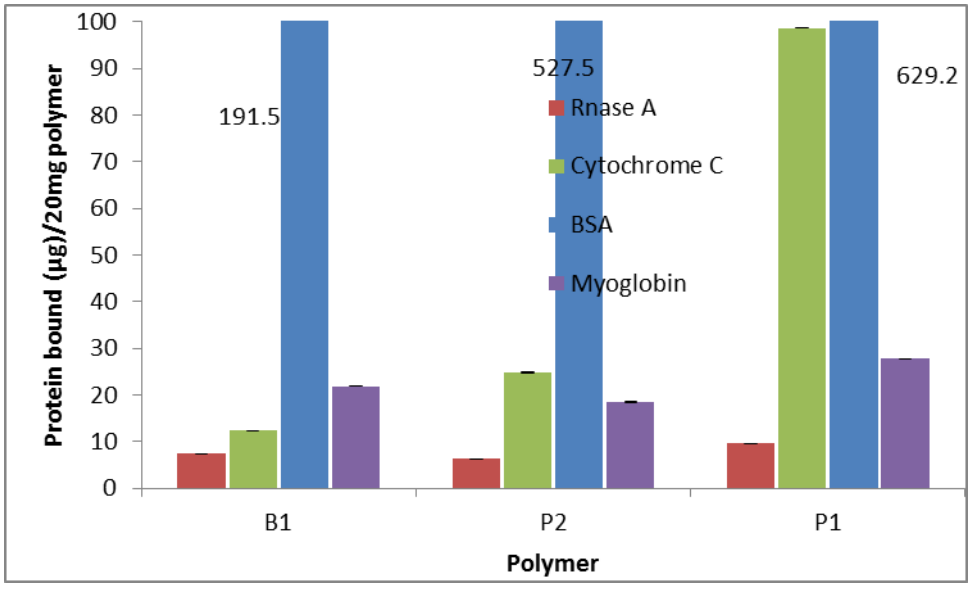


Figure 4-15 Summary of competitive equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1, with competitive molecules; RNase A (0.1mg/ml) ,cytochrome C (0.1mg/ml) and myoglobin (0.1mg/ml) in the mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) ; v/v for 10 minutes. (Mean ± error, n=3)

After the addition of lysozyme (figure 4-16), the imprinted polymer P2 bound less lysozyme than before and was comparable to B1. However for P1, the lysozyme binding was similar to the preceding experiment.

The reduced lysozyme rebinding observed from all polymers suggested that lysozyme could not displace the non specific protein bound initially.

Figure 4-16: Protein bound and released from Lys P1, P2 & control B1 after addition of Lys

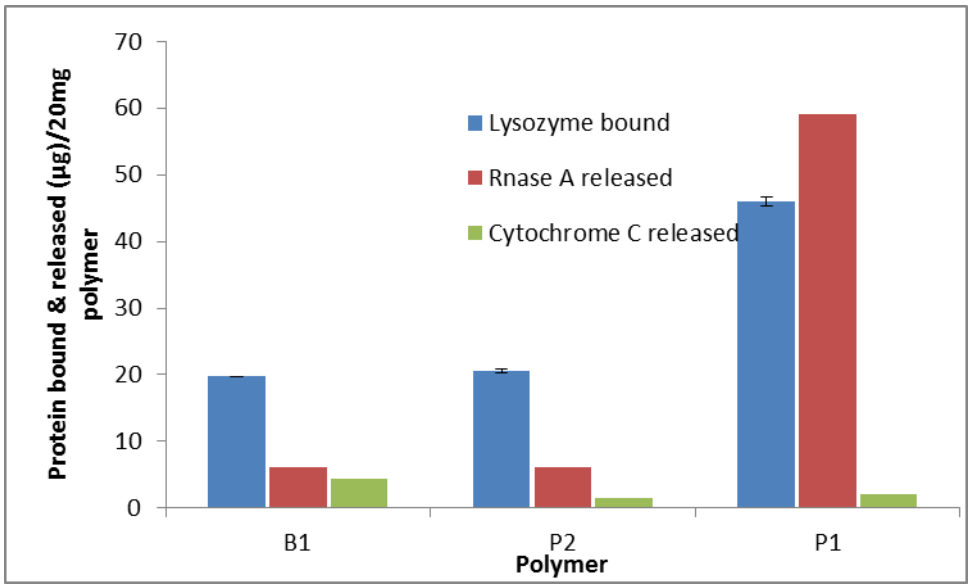


Figure 4-16 Aqueous equilibrium rebinding for 20 mg Lys imprinted polymers, P1, P2 and B1 with 2ml of Lys 0.1mg/ml of mobile phase composition (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v, for 10 minutes. RNase and Cytochrome C release with the rebinding mobile phase solution. (Mean ± error, n=3)

From table 4-9, the lysozyme amounts eluted from P2 and B1 were comparable and lower than that observed before. For P1, both lysozyme and cytochrome C were eluted, although this time the concentration of the cytochrome C was almost twice compared to that of the lysozyme. There seems to be a trend here; when BSA is rebound alone or from the protein mixture initially prior to or in the absence of lysozyme, the amount of cytochrome C eluted from P1 becomes higher than that of the lysozyme.

There seems to be a connection between the BSA binding to the polymer P1 and maybe a change in the molecular dynamics that favoured cytochrome C binding to the strong sites and therefore seen in the elution in large quantities. Another argument is that the BSA seemed to protect the cytochrome C from heat damage.

In general, it was clear that if there was any discrepancy existing between the imprinted polymer P2 and its control B1, it would be from the initial lysozyme rebinding, and this would be reflected in the amount of lysozyme eluted in the elution phase.

Table 4-9: Protein in wash & elution & left in the polymers after heat treatment (BSA added to the rebinding solution**)**

Wash (W)	Polymer	RNase A (mg)	Cytochrome C (mg)	Lysozyme (mg)	BSA (mg)	Myoglobin (mg)
W1-Mobile Phase (MP)	B1			0.0096		
	P2			0.0125	0.0024	
	P1		0.0040	0.0200		
Heat shock						
W6-Elution 10%AcOH in MP	B1			**0.0007**		
	P2			**0.0009**		
	P1		**0.0136**	**0.0076**		
Protein left	B1	0.0012	0.0079	0.0094	0.1492	0.0180
	P2	0.0001	0.0234	0.0071	0.2338	0.0219
	P1	0.0038	0.0628	0.0101	0.6200	0.0278

Table 4-9 Proteins released from imprinted polymers, P1, P2 and B1 using 2ml of (W1) mobile phase (95% arginine pH 7 (0.5M) + 5% ethanol +0.05% Tween 20) v/v; followed by application of rapid heat shock in water bath at 60-67^{0}C, (W6) elution with 2ml of 10% AcOH in the mobile phase; v/v, (W7) 2ml of 8M urea in the mobile phase, all for 10 minutes and residual protein left in the polymers.

From the preceding investigation, we were confident that the imprinted polymers P1 and P2 possessed lysozyme selective sites. For P2 polymer, these strong sites were small in number but were independent from other proteins in the rebinding phase. This was evidently demonstrated by the lysozyme, which was eluted alone from the elution phase. However P1, despite having more selective sites compared to P2, may be less specific because lysozyme was eluted together with cytochrome C.

The pre-modification of the polymer matrix with addition of DEGVE shed some light into the possibility of tailoring the imprinted material to further minimise non specific interaction in the future. The imprinted polymer made previously failed to discriminate the high selective sites from low selective sites. This was demonstrated by the small difference in lysozyme rebinding to the imprinted polymer P1 compared to the control B1, even though the non specific binding was reduced with lysozyme and other non specific molecules. However, the selectivity of the lysozyme towards the imprinted

polymers was still low, suggesting that this might be due to the smaller number of binding sites created within the polymer matrices. To further improve the polymer selectivity towards imprinted protein, it was decided to prepare UV initiated polymers in addition to thermally initiated polymers.

4.3 Preparation and characterisation of MIPs and control using UV initiation

The use of UV was expected to reduce the denaturation of proteins during polymerisation and to improve lysozyme selectivity by increasing the amount of protein complementary binding sites.

The preparation of lysozyme imprinted and non imprinted polymers compositions and procedures was similar to that described previously for P2 and control B1, except UV initiation was used instead of thermal energy (see experiment 4.6.1).

The lysozyme imprinted polymer P3 and its control B2 produced were hard glassy monolithic polymers with ~90% yield.

The polymer was processed similar to the procedures described before. However, the use of PEG 300 and Tween 20 in the template extraction was not always desirable. Therefore, it was decided to determine other solvent extraction compositions for this new set of polymers.

All new developed polymers were subjected to the developed template extraction, using the following sequential washing conditions: ethylene glycol (which is quite soluble in water) between 60-80% v/v in a 0.1 M citrate phosphate buffer pH 4.2 with dissolved $MgCl_2$ 0.1 M and finally with ethylene glycol in acetic acid, followed by 8M urea in dH_20 (see experiment 4.6.2). Just to reiterate, the rationale of using PCMCs was not only to improve the stability of the protein in organic media, but also it was envisaged to create large cavities from which protein extraction would be facilitated. Here we report that up to 80-90% of the template was successfully extracted from the imprinted polymer as quantified from the RP-HPLC, using mild extraction conditions. Therefore,

the use of PCMCs overcomes one of the major drawbacks with monolithic imprinting, i.e. the difficulty of extracting template from the polymer matrix. Up to 10% of the remaining protein might have been eluted from the matrix at concentrations that were below the limit of RP-HPLC detection. However, if the residual template is not completely removed, it might be capable of acting as a nucleation site for the template in the rebinding, because the actual binding sites are thought to be complementary to the small clusters of the protein in the template (Sellergren, 2001), thus contributing to the protein recognition.

More understanding of the nature of non specific interaction and how we can lessen this effect was needed. One way to address this issue was through mobile phase tuning as before. In this part of the report, we investigated if a relation existed between the concentration of water in the rebinding mobile phase and protein rebinding onto the imprinted polymers and control polymers.

4.3.1 Lysozyme selectivity towards MIPs & control with different mobile phase

Selective binding can be improved by an optimum mobile phase, such as solvent used for template polymerisation. This is because the template conformation adapted during rebinding is similar to the conformation that is captured during imprinting. The use of a different rebinding media might present different template conformation to the binding cavities, hence low template selectivity. However, the application of protein imprinted polymers is mostly intended for an aqueous environment. Using aqueous rebinding phase, the selectivity is improved by addition of organic solvents, and vice versa for polymer using organic solvents with low polarity. The template selectivity is improved by addition of a polar modifier as detailed in chapter 3.
In order to distinguish between specific and non specific selectivity, we carried out equilibrium lysozyme rebinding experiments using different percentage (%) of aqueous phase in the mobile phase. In addition, organic modifier ethylene glycol (EG) was introduced in order to lower the aqueous content in the phase (see experiment 4.7.1).

The experiment was carried out using 40µg lysozyme/ml of mobile phase:

1. 95% arginine pH 5 (0.5M) and 5% ethanol; v/v.
2. 90% (95% arginine pH 5 (0.5M)) and 5% ethanol and 10% ethylene glycol; v/v.
3. 60% (95% arginine pH 5 (0.5M)) and 5% ethanol and 40% ethylene glycol; v/v.

From the results shown in figure 4-17, a significant variation of protein rebinding corresponding to the change of aqueous content in the mobile phase was observed. From this figure, we can see that at (1) 95% v/v aqueous, the amount of lysozyme bound to the B2 was much greater than P3 which was slightly higher than P2 and B1.
Interestingly, more non specific rebinding occurred with B2. The lysozyme binding observed at mobile phase (2)~< 90% aqueous content with 10 % v/v ethylene glycol was still higher to B2 than to other polymers but there was a reduction of the lysozyme binding with P2 and B1. Finally, a significant reduction of the protein rebinding was observed with (3) 60% v/v aqueous 40% v/v ethylene glycol (EG), and where the lysozyme bound to all polymers was quite similar

These data show that at high aqueous content, the hydrophobic effect was the driving force for non specific protein rebinding (Andersson *et al*, 1995; Dauwe and Sellergren, 1996; Matsui *et al*, 1997). Increasing concentration of ethylene glycol might have masked the hydrophobic sites and allowed the cavities in the polymer matrix to rebind the protein. However, non specific binding was reduced by increasing the amount of ethylene glycol in aqueous phase. Therefore by tuning the mobile phase with high concentration of ethylene glycol, we managed to minimise the difference of the template binding in all polymers.

Figure 4-17: Lys uptake by Lys P2, P3 & B1, B2; mobile phase tuned with EG

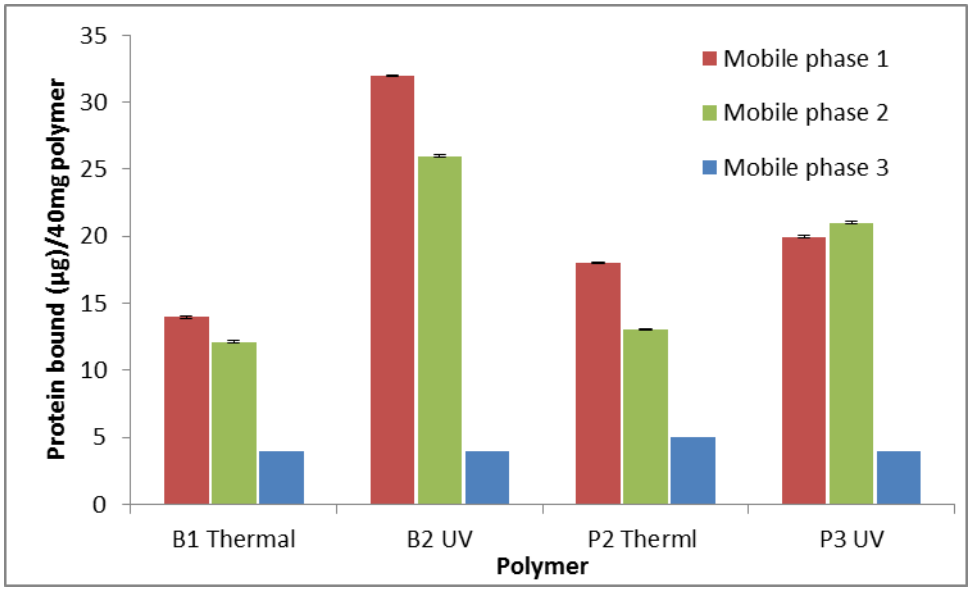


Figure 4-17 Summary of equilibrium rebinding using, 40µg lysozyme/ml of mobile phase (1) (95% Arginine pH 5 (0.5M) + 5% ethanol) v/v, 40µg lysozyme/ml of mobile phase (2) 90% (95% Arginine pH 5 (0.5M) + 5% ethanol), 10% ethylene Glycol v/v, 40µg lysozyme/ml of mobile phase (3) 60% (95% Arginine pH 5 (0.5M) + 5% ethanol) ,40% ethylene Glycol v/v, rebinding time for each experiment was 15 min. Thermal polymers were prepared using thermo chemical initiation at 60^0C and UV polymers with UV initiation. (Mean ± error, n=3)

The mobile phase tuning gave us insight into the dominating feature behind polymer selectivity. Although no discrimination between the imprinted polymer and control was observed in terms of selectivity, we were still interested to determine if imprinted polymers possessed some specific sites compared to non-imprinted matrix.

To achieve this, we looked at the application of rapid heat shock as opposed to the prolong heat application employed before. This was also expected to denature protein bound to non specific sites, but might be expected to be less damaging to specifically bound protein.

4.3.2 Lysozyme selectivity towards MIPs & controls & heat shock application

Specific sites in the imprinted and control polymers were characterised by subjecting the polymers to 1 min rapid heat shock after the template rebinding and initial wash with mobile phase, using equilibrium rebinding with mobile phase developed from the preceding experiment, for polymers P2, P3, B1 and B2 (see experiment 4.7.2).
For this experiment, lysozyme bound polymer from previous experiment using mobile phase (3); 60% v/v aqueous 40% v/v ethylene glycol (EG) was employed. This was because comparable template binding was observed with all polymers. Therefore, if any discrepancies existed, they may be as a result of the lysozyme binding to the different sites in these polymers. From figure (4-18), it can be seen that the template amounts bound from all polymers were comparable, with insignificant variation of the template released from the washes using the mobile phase.

Figure 4-18: Lys uptake and washed and eluted after heat shock application

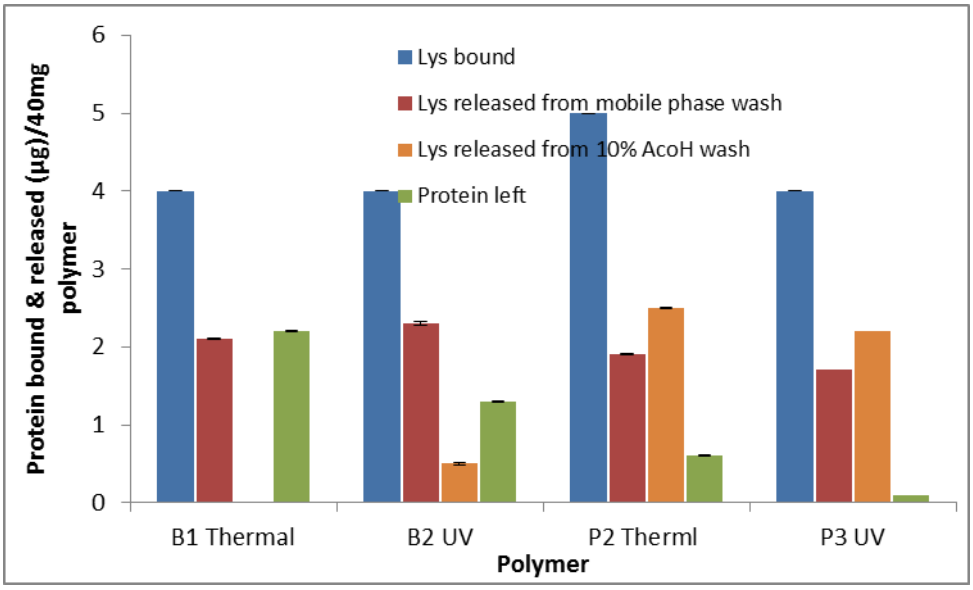


Figure 4-18 (a) Equilibrium rebinding using 40µg lysozyme/ml of mobile phase 60% (95% Arginine pH 5 (0.5M) + 5% ethanol) , 40% ethylene Glycol v/v, (b)Post-washed with mobile phase,(c) Rapid heat shock in water bath at 60-670C, (d) Elution with 20% 0.1 M citric acid in 80% ethylene glycol, (e) Residual protein left in the polymer. (For lysozyme rebound, mean ± error, n=3)

The visible feature from the results was the significant amount of the template eluted from both imprinted polymers P2 and P3 after heat shock application. In comparison, only a small concentration of template was eluted from B2, while nothing was recovered from B1. A considerably greater amount of residual template was left in control polymers B1 and B2 than with imprinted polymers P2 and P3 after the elution phase.

The lysozyme eluted from P2 and P3 and B1 and B2 showed good discrimination after heat shock. These results suggested that lysozyme imprinted polymer possesses specific imprinted sites compared to non imprinted polymer, although less than 1% quantified indicates the amount specific sites. However, low yielding of binding sites of less than 1% has been reported by others (Sellergren, 2001).

A possible argument is that a large proportion of the proteins might have been denatured by harsh imprinting conditions, such as elevated temperature in organic media and the small fraction of undamaged protein was responsible for the formation of complementary binding sites. UV initiation polymerisation was expected to cause less damage to the protein and improve the number of specific sites. However, both the UV and thermal initiated polymers showed similar qualities. This suggested that both methods have the same effect on the proteins (Chau and Tong, 2007).

4.4 Conclusion

This work has demonstrated that we were successful in reducing non specific protein binding effect with our new polymer formulations, a modification of the polymer matrix with an addition of di ethylene glycol vinyl ether (DEGVE) and/or a reduction of functional monomers in the polymerisation step changed the polymer characteristics.
A significant increase in the amount of the lysozyme (template) was extracted from a high density cross linked polymer network, relative to unmodified polymer. Here we report that up to 90% of the template was successfully extracted from the modified imprinted polymer as quantified from the RP-HPLC, using mild extraction conditions.
From this study, we also demonstrated that post treatment technique of the polymers at elevated temperatures, a typical step in a molecular imprinting process, demonstrated no difference in the polymer characteristics towards template selectivity or release.
Preferential rebinding studies demonstrated that the imprinted polymer possessed specific sites compared to non imprinted polymer, although these sites were small in number.
From competitive rebinding studies, both the modified imprinted polymer and its control demonstrated a significant reduction of polymer's non-specific binding of proteins from aqueous media. In effect, this also led to a reduction of a non specific binding of a targeted template.
From using template extraction step techniques, we demonstrated that the imprinted polymer had more strong binding sites compared to the control polymer. In addition, we were able to elute only specific template from the modified imprinted polymers, whilst the template and template analogues were eluted together from the unmodified polymer. However from post treating all polymers using a rapid heat shock, we successfully allowed the elution of only template from the unmodified polymer.
From coating all polymers with non specific blocking agent, we successfully tripled the amount of the template bound and eluted from the modified imprinted polymer compared to the modified control polymer, whilst for the unmodified polymer the

template was again higher, but the template was eluted together with non specific protein.

UV initiated polymers were developed in order to investigate and compare with the polymer prepared using initiation temperature, in terms of polymer selectivity and capacity towards the imprinted template. No difference was observed; both the UV and thermal initiated polymers showed similar qualities.

It can be concluded that this preliminary study into the use of a lysozyme imprinted polymers for the extraction of lysozyme from the protein mixture was not practical. This was because the lysozyme imprinted polymer was, however, not as selective as was expected. The most likely explanation was that the imprinted polymers may be non specific and hydrophobic effect was the major contributor of this non specific protein binding.

Further work is required, firstly to understand the nature of the template bound to the polymer as compared to the control polymer. For example, the conformation stability of the template bound to polymer binding sites. Secondly, to explore a hydrophobic interaction as a means of protein recognition in aqueous solution.

Chapter 4: Experimental section

Materials

Ribonuclease A from bovine pancreas Type I-A (RNase A), Lysozyme from chicken egg white, Myoglobin from equine heart, *Micrococcus lysodeikticus* , Cytochrome C from equine heart, Ethylene glycol, Ethanol, L-arginine, L-arginine monohydro-chloride, Alumina, Urea, Na_2HPO_4, NaH_2PO_4, $MgCl_2$, NaCl, Isopropanol, Trifluoroacetic acid (Spectrophotometric grade), Acetone, Acetonitrile (HPLC grade), Trehalose, were purchased from Sigma-Aldrich Co. UK. BSA form bovine serum, Polyethylene glycol 200 (MW 200), Polyethylene glycol 300 (MW 300), K_2HPO_4 (MW 174.18), KH_2PO (136.09), KCl, were purchased form Fluka UK. Tween 20 and Glycine,

Acetic acid, Citric acid (MW 210) were purchased from (Fisher scientific UK), Molecular sieves (MERCK Germany), Anti-freeze (local shop), N_2 (University store). All materials were used as received, with no further purification.
Centrifugal concentrator Millipore ultra-free CL PTFE yellow 2.0mL 0.22µm pore size (Fisher Scientific), isolute double fritted (2x20µm) filtrate, filtration column 3ml, (Isolute SPE accessories from Kenesis), Cuvette with a path length 1.0 cm (Fisher brand cuvettes, semi–micro, 15ml tall vial and 25ml vial screw top from Fisher scientific UK, Durapore Membrane filters of pore size 0.22µm and 0.45µm (Millipore Ireland). Deionised filtered water (Resistivity 18.2 MΩ) was obtained from in-house Millipore water purification system.
Methacrylic acid (MAA), 4-vinlypyridine (4vpy), Ethylene glycol dimethacrylate (EGDMA), Di (ethylene) glycol vinyl ether (DEGVE), were obtained from Aldrich, and 2, 2'-azobisisobutyronitrile (AIBN) from (BDH laboratory supplier England UK)

Material Preparation

EDGMA, MAA and 4VPy were purified by passing through a column packed with alumina; MAA and 4VPy were then distilled under reduced pressure. AIBN was purified by re-crystallisation from acetone, DEGVE was used without further purification and acetonitrile was dried by molecular sieves.

Apparatus –Spectrophotometer (Beckman Coulter, DU 800) with temperature controller (Beckman Coulter), HPLC waters 2996 (photodiode array detector), aters 2487 (Dual absorbance detector), waters 2695 (separation module), Centrifuge Beckman Coulter (large Eppendorf), shaking bath 58-16 (Techno), Centrifuge (Eppendorf AG2233, Hamburg, Germany) and Incubator (Techno, Ori-block, OB-3), Rotary evaporator. RP-HPLC, Blood rotary and Incubator (Stuart Scientific), UV Lamp; Black –Ray, Non-UV semiconductor Model B 180 AP, 230 V, 50 Hz, 2.A (Upland USA), Motor and pestle and 25-50 sieves, Meter balance (Metter-Toledo AE200), pH meter (Metter-Toledo MP120), Magnetic stirrer and magnetic stirring bar (Fisher Scientific),Water bath (Techno),

Preparation of buffers

Phosphate buffers

10 mM of phosphate buffer pH 7.2 were prepared by adding 71.7 ml of 1.0M K_2HPO_4 to 28.3ml of 1.0 M KH_2PO_4. 1ml was then diluted with 99ml of dH_2O to obtain a10mM buffer strength. 1.0M of K_2HPO_4 was prepared by dissolving 1.74g in dH_2O and 1.0M of KH_2PO_4 was prepared from dissolving 1.36g in dH_2O.

Arginine buffer

0.5M arginine pH 7 was prepared by equilibrating 0.5M of L-arginine monohydro-chloride with 0.5M L-arginine. For 0.5M arginine pH 5, only L-arginine monohydro-chloride was used.

Experiment 4.1.1

1st set of Lysozyme imprinted polymer P1, P2 and Blank B1

Preparation of the lysozyme PCMCs was as follows; 10% lysozyme loading, 5% water in IPA solvent saturated with glycine excipient. For example, 125mg of lysozyme were dissolved in 10.25ml of 10mM phosphate buffer pH 7.2 containing 1025mg of glycine (100mg/ml). 10.25 ml of the protein/excipient solution were drawn using plastic tube *via* a three way tap set at a flow rate of 5ml min^{-1} and were added to 195 ml of IPA saturated with excipient set at a flow rate of 95ml min^{-1} under constant agitation provided by a magnetic stirring bar and hot plate stirrer. Following precipitation, the PCMCs were allowed to sediment and the supernatant was decanted. The PCMCs formed were washed with 2 x 100ml of IPA and then centrifuged to separate the PCMCs and the supernatant IPA was decanted. Finally, the PCMCs were washed with 2 x 100ml of dry acetonitrile prior to the imprinting process to remove water presented in the bulk solvent that might potentially interfere with free radical polymerisation. A similar procedure was repeated for blank crystals (control) prepared without protein.

The reagents used for the preparations of new set of lysozyme imprinted polymers P1, P2 and control B1 are outlined in the table below,

Polymer	PCMCs /Glycine (mg)	Lys (mg)	MAA g, mmol	4-vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
P1	1250	125	0.166, 1.94	0.333, 3.172	5.0, 25.254	------	91.33, 0.556	7.33
P2	1250 Glycine	125	0.041, 0.0485	0.083, 0.792	5.0, 25.254	0.405 3.0645	91.33, 0.556	7.33
B1	1250	-----	0.041, 0.0485	0.083, 0.792	5.0, 25.254	0.405 3.0645	91.33, 0.556	7.33

{Volume of acetonitrile was calculated from 4/3(sum of all monomers +cross linker)}
The technique employed was similar to that described in chapter 3 for the preparation and production of the imprinting of monolithic polymer.

Experiment 4.1.2

Polymer processing

After 48hrs of the polymer preparation, glass vials containing the monolithic polymers were broken to release the polymers, which were then ground with a mortar and pestle into a manageable fine particles, sieved at 20-50 µm mesh size, and the powders were suspended in acetone to sediment similar particle size and some of the polymer powders were dried overnight in the oven at 60^0C. MIPs were then washed with acetonitrile to remove unreacted functional monomers MAA and 4-vpy. The disappearance of the 4vinyl pyridine RP-HPLC peaks from the wash solvent was an indication of the complete removal of these monomers from the polymer. For example, 100mg of each polymer P1, P2 and B1 were put into 40 ml centrifuged tubes, followed by the addition of 20 ml of dry acetonitrile. The tubes containing polymer suspensions were put on a flask shaker machine for gentle shaking for a period of 10 min, after which the samples were again centrifuged and 50µL of the supernatants were injected directly into the RP-HPLC.

Experiment 4.1.3

Lysozyme extraction from imprinted polymers

20 mg x 10 sample of polymer P2 were weighed and put into a different centrifuge eppendorfs tube with filter. Each sample was then subjected to a single wash with a 2ml of a single solvent outlined in table below, and the lysozyme extracted was monitored spectroscopically using RP-HPLC.

Sample	Template Extraction Solvent
1	Citrate buffer pH 5.08 (0.1M)
2	Citrate buffer pH 5.08 (0.01M)
3	Phosphate buffer pH 7.11 (0.1M)
4	Citrate buffer pH 5.08 (0.01M) and NaCl 0.1M)
5	Citrate buffer pH 5.08 (0.01M) and NaCl (0.01M)
6	Citrate buffer pH 5.08 (0.1M) &10% acetic Acid
7	Citrate buffer pH 5.08 (0.01M), PEG 200 (30%) & 10% acetic acid
8	Citrate buffer pH 5.08 (0.01M), PEG 300 (20%) &10% acetic acid
9	dH_20, PEG 200 (30%) &10% acetic acid
10	Phosphate buffer pH 7.11 (0.01M), PEG 300 (20%) &10 % acetic acid

For template extraction process, 20mg of each sample polymers, P1, P2 and control B1, were subjected to the following sequential washes:

1. 2 x 1ml of 10mM phosphate buffer at pH 7.11 to dissolve carrier glycine
2. 3 x 1ml of 30% poly ethylene glycol PEG 300, 60% 10mM citrate buffer pH 7.11 and 10% acetic acid
3. 1 x 1ml of 8M urea
4. 3 x 1ml of 10mM citrate buffer pH 7.2
5. 2 x 1ml of dH_2O

For example, the percentage of lysozyme extracted from 20 mg of the imprinted polymer P2 was calculated as follows;

$$\text{Protein extracted (\%)} = \frac{\text{Measured protein extracted (mg)}}{\text{Expected protein (mg)}} \times 100$$

The amount of lysozyme expected from 20mg polymer was 0.29 ± 0.01mg.

Experiment 4.2.1

Lysozyme selectivity towards imprinted polymers P1, P2 and control B1 in aqueous equilibrium batch rebinding

2 x 20mg of each polymer, P1, P2 and B1, were weighed and put separately into 3ml isolute double fritted of 2x20µm pore size (plastic column with double filter) and were pre-conditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20); v/v. Following this, the supernatant was removed from the frit under vacuum. One set of polymer was rebound with 1ml of 0.1 mg/ml of lysozyme and the other set with 1ml with 0.2mg /ml; both samples were suspended on a blood rotary machine for a period of 10 min. The supernatant was then removed from the frit under vacuum and 50µl were injected into the RP-HPLC for lysozyme bound analysis.

Experiment 4.2.2

Lysozyme selectivity towards imprinted polymers; P1, P2 and control B1 in aqueous equilibrium rebinding with addition of trehalose in the rebinding solution

The investigation carried out was similar to that described in the **experiment 4.2.1,** except that 0.1 M of trehalose was added to the rebinding solution containing 0.1mg of lysozyme/ml of mobile phase. After template rebinding and analysis, all polymers were subjected to the following sequential washes; 2 x 1ml mobile phases, 1 x 1ml of 0.01M of NaCl in mobile phase, 1 x 1ml of 0.1M NaCl in mobile phase and finally with 1 x 1ml of 10% acetic acid in 90% mobile phase; v/v. The supernatant from each wash were removed from the frit under vacuum and 50µl of each sample wash were injected into the RP-HPLC for lysozyme release study.

Experiment 4.2.3

1st revision of lysozyme extraction methodology from imprinted polymers P1 & P2

20mg of each polymer P1and P2 were weighed and put separately into 3ml isolute double fritted of 2x20µm pore size (plastic column with double filter) and were subjected to the following sequential washes:

1. 3 x 2ml of 0.5M arginine pH 5 (95%), 5% ethanol , 0.5% Tween 20; v/v
2. 2 x 2ml of 8M urea in mobile phase
3. 2 x 2ml of 20% either ethylene glycol or PEG 300 or 10% acetic acid in mobile phase
4. 2 x 2ml of 8M urea in mobile phase

Similar sequential wash was repeated for control polymer B1 prior to the template rebinding experiments. The percentage of lysozyme extracted was calculated as described in the **experiment 4.1.3**

Experiment 4.3

Lysozyme selectivity towards dry and undried polymers; P1 and P2 in aqueous equilibrium batch rebinding

2 x 20 mg of polymers P1 and P2 were weighed; one set of sample was dried in an oven at 60 ^{0}C for 6 hrs and was compared with similar set used after the template extraction process. The lysozyme rebinding procedure carried out was similar to that described in the **experiment 4.2.1,** using a rebinding solution containing 0.1mg of Lys/ml of mobile phase. After the template rebinding process, all polymers were subjected to the template releasing study, whereby all polymers were subjected to the following sequential washes; 3 x 1ml mobile phases, 1 x 1ml of 0.01M of NaCl in mobile phase, 1 x 1ml of 0.1M NaCl in mobile phase, 1 x 1ml of 10% acetic acid in 90% mobile phase; v/v and finally with 8M urea in mobile phase. 50µl of each supernatant were injected into the RP-HPLC for protein released analysis.

Experiment 4.4

Lysozyme selectivity aqueous equilibrium batch rebinding with different Lys concentrations in the rebinding concentration

3 x 20 mg of polymers P1, P2 and B1was dried at 37^0C. Each set of polymer (P1, P2 and B1) were rebound with different lysozyme concentration (0.1, 0.05 and 0.01-mg/ml), using a similar procedure to that describe in the **experiment 4.3.** Following this, the lysozyme releasing study was carried out using only polymer sample bound with 0.1mg of Lys/ml.

Experiment 4.5.1

Lysozyme selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with RNase A, cytochrome C and myoglobin

20mg of each polymer; P1, P2 and B1 were weighed and put separately into 2ml centrifugal tubes with filter of 2x20µm pore size and were conditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20) v/v. The samples were then centrifuged and supernatants were decanted. All polymers were then rebound with 0.1 mg/ml lysozyme solution spiked with different protein molecules; closer molecular weight RNase A and cytochrome C, and slightly large molecular weight myoglobin all at same concentration 0.1mg/ml. Following this, all polymers were again centrifuged and 50µl of each supernatant were injected into the RP-HPLC for protein bound analysis. Finally, all polymers were subjected to protein release study using similar washes described in the **experiment 4.3.**

Experiment 4.5.2

Lysozyme selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with RNase A, cytochrome C and myoglobin with addition of BSA

This investigation carried out was similar to that described in the **experiment 4.5.1,** except that BSA 1mg/ml was added to the rebinding solution.

Experiment 4.5.3

Lys selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with RNase A, cytochrome C, myoglobin and application of rapid heat shock

The investigation carried out was similar to that in the **experiment 4.5.1,** except that after the polymers were separated from the rebinding solutions, they were rinsed with mobile phase followed by centrifugation to remove the rinsing phase. Each polymer was suspended in 2ml of dH_2O in an eppendorf. The eppendorfs containing polymer suspension were then partially submerged in a water bath at temperature ranging between 60-65 ^{0}C for 10 min. After this period, the samples were again centrifuged to remove dH_2O. Finally, all polymers were subjected to protein release study using similar washes described in the **experiment 4.3** and 50µl of each supernatant were injected into the RP-HPLC for protein released analysis.

Experiment 4.5.4

Lysozyme selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with RNase A, cytochrome C, myoglobin with addition of BSA and application of rapid heat shock

The investigation carried out was similar to that described in the **experiment 4.5.3,** except that BSA 1mg/ml was added to the rebinding solution.

Experiment 4.5.5

Non blocking agent BSA binding followed by Lys selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with RNase A, cytochrome C, myoglobin and application of rapid heat shock

20mg of each polymer; P1, P2 and B1 were weighed and preconditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20) v/v; the samples were then centrifuged and supernatant decanted. All polymers were then rebound with 1 mg/ml of BSA initially for 10 min and the samples were again centrifuged and 50µl of each supernatant were injected into the RP-HPLC for BSA bound analysis. The polymer samples were then rebound with 0.1mg of Lys/ml solution

spiked with different molecules, closer molecular weight RNase A and cytochrome C, all at 0.1mg/ml. Following this, all polymers were again centrifuged and 50µl of each supernatant were injected into the RP-HPLC for protein bound analysis. Finally, all polymers were rinsed with mobile phase (without protein), followed by the rapid heat shock treatment; the experimental procedure was similar to that described in the **experiment 4.5.3.**

Experiment 4.5.6

Lys selectivity in a competitive equilibrium rebinding with Lys rebinding solution spiked with BSA, RNase A, cytochrome C and myoglobin and with application of rapid heat shock

20mg of each polymer; P1, P2 and B1 were weighed and preconditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20) v/v for 10 min after which the samples were centrifuged and supernatant decanted. All polymers were then rebound with a 1ml of rebinding solution spiked with 1 mg/ml of; BSA, RNase A, cytochrome C and myoglobin all at 0.1mg/ml without lysozyme for 10 min. All sample polymers were again centrifuged and 50µl of each supernatant were injected into the RP-HPLC for protein bound analysis. Following this, all polymer samples were rebound with 1ml of 0.1mg of Lys/ml of mobile phase for 10min, after which the samples polymer were again centrifuged and 50µl of each supernatant were injected into the RP-HPLC for lysozyme bound analysis. Finally, all polymers were subjected to rapid heat shock treatment using similar procedure described in the **experiment 4.5.3.**

Experiment 4.6.1

Preparation of lysozyme imprinted polymer using UV energy

The preparation of the lysozyme-gly-PCMCs and lysozyme imprinted polymer for P3 and its control B2 was similar to that described previously for polymers P2 and its control B1 in the **experiment 4.1.1,** except that UV energy was employed instead of thermal energy. The UV initiated polymers were prepared using UV light. The vials containing lysozyme-gly-PCMCs with polymer reagents were immersed in a water bath shaker containing 30% v/v of antifreeze agent (ethylene glycol) maintained at 0^0C and

were irradiated with UV light at a wavelength (50Hz) at a distance of 11 cm from the UV light source. The samples were constantly shaken to allow equal distribution of lysozyme PCMCs and glycine-blanks suspended in the acetonitrile (porogen). However, the water bath temperature was not tightly controlled.

Experiment 4.6.2

Polymer processing and template extraction

The lysozyme imprinted polymer P3 and its control B2 monolith were processed using similar procedures described in the **experiment 4.1.2** and the template extraction methodology was again revised as follow:

1. 2 x 1ml of 10mM citrate buffer pH 4.2 to dissolve carrier molecules glycine
2. 3 x 1ml of 80% ethylene glycol (EG), 20% 10mM citrate buffer pH 4.2 with dissolved $MgCl_2$ 0.1 M
3. 3 x 1ml of 60% of EG ,20% 10mM citrate buffer pH 4.2 and 20% AcOH
4. 1 x 1ml of 8M urea
5. 2 x 1ml of 10mM citrate buffer pH 4.2
6. 1 x 1ml of 60% 60% of EG and 20% 10mM citrate buffer pH 4.2 and 20% AcOH
7. 1 x 1ml of 8M urea
8. 2 x 1ml of 10mM citrate buffer pH 4.2
9. 2 x 1ml of dH_2O

All polymers, P2, P3, B1 and B2, were subjected to the sequential washes described above prior to the protein rebinding experiments.

Experiment 4.7.1

Lysozyme selectivity in aqueous equilibrium batch rebinding for polymers made via thermal and UV initiation; P2, B1 and P3, B2 and mobile phase tuning with of ethylene glycol (EG) in aqueous

3 sets of 20mg of polymers P2, P3 and controls B1, B2 were weighed and preconditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20) v/v; the samples were then centrifuged and supernatant decanted. All

polymers were then rebound with 2ml of 40µg lysozyme/ml of the following mobile phases:

1. 95% arginine pH 5 (0.5M) and 5% ethanol; v/v.
2. 90% (95% arginine pH 5 (0.5M)) and 5% ethanol and 10% ethylene glycol; v/v.
3. 60% (95% arginine pH 5 (0.5M)) and 5% ethanol and 40% ethylene glycol; v/v.

The lysozyme rebound analysis procedure was similar to that described in the **experiment 4.5.3.**

Experiment 4.7.2
Lysozyme selectivity in aqueous equilibrium rebinding and application of rapid heat shock

20mg of imprinted polymers P2, P3 and controls B1, B2 were weighed and preconditioned with 2ml of mobile phase (95% arginine pH 7 (0.5M), 5% ethanol and 0.05% Tween 20) v/v; the samples were then centrifuged and supernatant decanted. All polymers were then rebound with 2ml of 40µg Lys /ml of mobile phase consisted of 60% (95% arginine pH 5 (0.5M), 5% ethanol, 40% ethylene glycol); v/v. The lysozyme rebinding procedure was similar to that described in the **experiment 4.5.3.** After template rebinding analysis, all polymers were rinsed with 2ml of mobile phase for 10 min and the proteins released were analysed. After washing, all samples were re-suspended in dH_2O, followed by a rapid heat shock in a water bath at temperature ranging between 60-67 ^{0}C for 1 min, after which the samples were again centrifuged to remove dH_2O. Finally, 2ml of elution phase containing 20% (0.1M citrate) and 80% ethylene glycol were added to all samples for 15min and the template released from this was analysed using RP-HPLC.

Chapter 5 : MIPs and template stability in binding site

5.1 Introduction

To recap, it was found to be an enormous challenge to discriminate template rebinding to selective sites from that of non selective sites in both imprinted and control polymers. Various chemical attempts were explored, such as tuning of aqueous mobile phases with organic modifiers, including ethylene glycol, Tween 20, ethanol, and use of non specific block agents, arginine, BSA, casein, and application of physical stimulus using a rapid heat shock treatment. Despite this, we were unable to suppress non specific protein binding.

This part of the thesis highlights some of the work carried out to gain more understanding on the conformation stability of protein bound to the polymers. It was hypothesised that proteins from solutions would initially bind rapidly and non specifically to the polymers by a hydrophobic mechanism, and then diffuse to the binding sites of different hierarchies; the protein bound to high selective sites would be expected to be more stable compared to protein bound to low selective sites, when subject to non physiological conditions. This is because the polymer binding cavities would perhaps provide protection to these proteins and this should be reflected in the ability of the bound protein to maintain its functionality. For example, if the model template is an enzyme, then the degree of enzymatic reaction would indicate the stability of the template within the polymer matrix. The model template employed was acid phosphatase because this is capable of retuning its catalytic activity whilst bound to the imprinted polymer cavities. The limitation of using lysozyme was that the lysozyme assay involved the lysis of bacteria cell walls and such large cells cannot penetrate into polymers; therefore, incubating the polymer in the cell solution was impractical. The acid phosphatase assay on the other hand is simple and it involves the hydrolysis of a small molecular substrate 4-nitrophenol phosphate, which is quite small and could easily penetrate into the polymer matrix (refer to chapter 2). Finally, this part of the report highlights techniques that can be used to discriminate specific from non specific protein

binding, following on from those developed in the chapter 4. Details of the experiments can be found at the end of the chapter.

5.2 Preparation of acid phosphatase (ACP) imprinted polymer

A major problem still encountered in molecular imprinting is that there is no standardised methodology which defines the appropriate techniques or reagents to design molecular imprints. In order to better estimate a suitable amount of acid phosphatase to use, we investigated different amounts of the enzyme, 10, 20, 40, and 80-mg, and correlated this with the concentration of binding site cavities. Previously, it was found that the concentration of template high selective sites was ~1%; therefore, if we could avoid using less amount of the template in the imprinting process it would be cost effective, bearing in mind that some proteins are quite expensive; ~200mg acid phosphatase costs about £300. Acid phosphatase imprinted polymers were prepared using acid phosphatase PCMCs produced by a batch process at different enzyme loading onto glycine; 1%, 2%, 4%, 8%, using 5% water in IPA saturated with glycine (see experiment 5.1.1). For the preparation of the imprinted polymer, see experiment 5.1.2. The preparation of acid phosphatase imprinted polymers is outlined in table 5-1.
All polymers prepared were glassy hard monoliths and were processed as described previously, i.e. ground to manageable size, pre-washed with acetonitrile to remove unreacted residual monomers, followed by template extraction (see experiment 5.1.3). In order to see whether any enzyme activity was left after the template was subjected to harsh polymerisation conditions and the grinding process, we examined the acid phosphatase activity of the polymer prepared using 80mg enzyme (ACP 8) after the monoliths were crushed and sieved. From this experiment, it was found that the activity of enzyme still active was a ~0.03 unit, which was ~2% as determined from calculated units used ~ 1.61units/40 mg polymer. This might reflect the concentration of enzyme captured in a native conformation during the imprinting process (see experiment 5.1.4).

Table 5-1: Reagents used for preparation of acid phosphatase MIPs

Sample	PCMCs (mg)	Enzyme Loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
ACP 1	1000	1% (10)	0.0005, 0.0058	0.0005, 0.00476	3.2, 16.1624	0.319, 2.4137	57.1, 0.348	4.69
ACP 2	1000	2% (20)	0.001, 0.0116	0.001, 0.0095	3.2, 16.162	0.318 2.4062	57.1, 0.348	4.69
ACP 4	1000	4%(40)	0.002, 0.0232	0.002, 0.0190	3.2, 16.162	0.316 02.391	57.1, 0.348	4.69
ACP 8	1000	8% (80)	0.004, 0.0465	0.004, 0.03806	3.2, 16.162	0.312, 2.3608	57.1, 0.348	4.69

For the template extraction process, all polymers were subjected to the typical sequential washes described below; for example for 20mg polymer, 2ml of each solvent were employed (see experiment 5.1.5).

1. 2 x 10mM citrate buffer to dissolve carrier molecules glycine
2. 3 x 80% ethylene glycol and 20% 10mM citrate buffer
3. 3 x 60% ethylene glycol,20% 10mM citrate buffer and 20% acetic acid v/v
4. 1 x 8M urea
5. 2 x 10mM citrate buffer
6. 1 x 60% Ethylene glycol and 20% 10mM citrate buffer and 20% acetic acid
7. 1 x 8M urea
8. 2 x 10mM citrate buffer
9. 2 x dH_2O

After the template extraction step, all polymers were examined for acid phosphatase activity enzyme left within the polymers. From this assay, no activity was found with all

polymers, although protein residues were found using a Bicinchoninic assay (BCA). BCA assay is commonly used for protein quantification. Prior to the template rebinding process, all polymers were dried in an incubator at 37 ^{0}C for 48hrs.

5.3 Acid phosphatase MIPs characterisation

5.3.1 Acid phosphatase stability in mobile phase

In the preceding chapter 4, we tuned a mobile phase with organic modifier ethylene glycol to lessen the effect of non specific template rebinding. Therefore, it was logical to continue with this. To see whether ethylene glycol had any effect on the acid phosphatase activity, we designed a comparative study between the free enzyme in solution and using the same concentration of enzyme dissolved in increasing ethylene glycol concentrations ranging from 10-40%; v/v in aqueous (see experiment 5.2). From the results shown in figure 5-1,it can be seen that the free enzyme activity was comparable for enzyme dissolved in 10-30% of ethylene glycol, except that at 40%, a ~10% reduction of the enzyme activity was observed. Therefore, it was prudent to use 30% or less ethylene glycol in the mobile phase in order to maintain complete functionality of the enzymes.

5.3.2 Acid phosphatase selectivity towards ACP MIPs

An initial investigation was carried out to determine the selectivity of the template towards the newly developed polymers. The preferential binding of the acid phosphatase imprinted polymers; ACP 1 polymer was prepared with 10mg of the acid phosphatase, ACP 2 with (20mg), ACP 4 with (40mg) and ACP 8 with (80mg) of the template. The polymers were characterised by measuring specific acid phosphatase selectivity in aqueous equilibrium rebinding (see experiment 5.3.1) and the results are presented in figure 5-2.

Figure 5-1: ACP activities with different concentrations of EG in solution

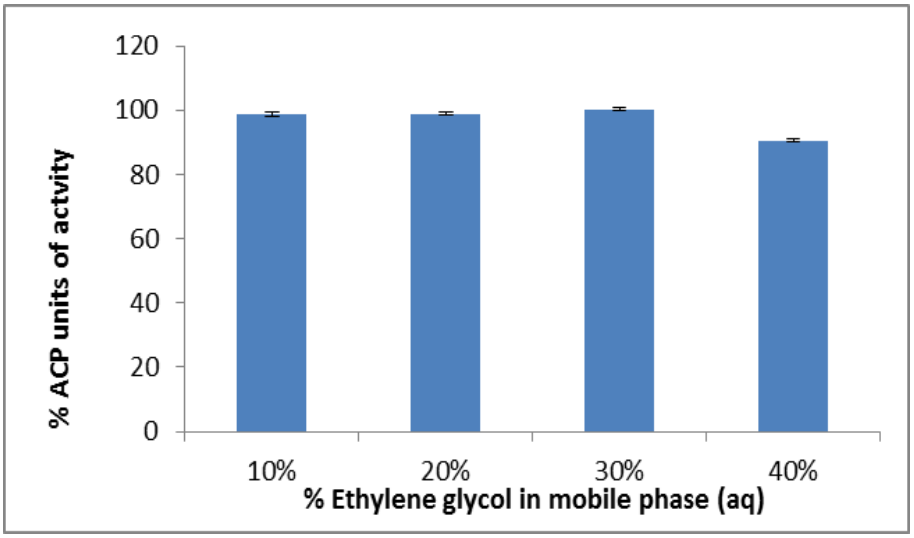


Figure 5-1 Acid phosphatase units of activity measured at 410nm. 4 sample of 0.1mg of acid phosphatase/ml of citrate buffer at pH 4.8 (0.01M) each prepared at different ethylene glycol concentration (10, 20, 30, 40-%); v/v. (Mean ± error, n=3)

Figure 5-2: Acid phosphatase uptake by all ACP (1, 2, 4 & 8) %

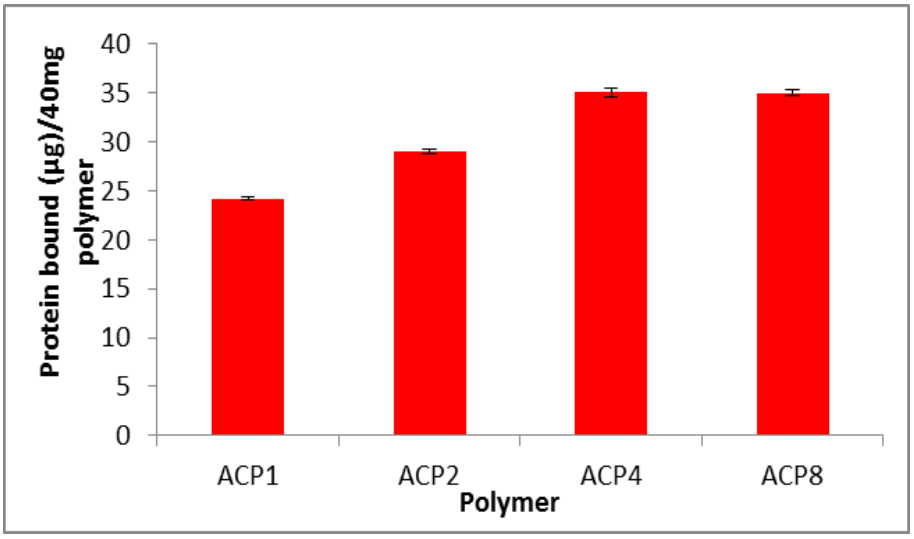


Figure 5-2 Aqueous equilibrium rebinding for 40 mg ACP imprinted polymers, ACP1, ACP2, ACP4 and ACP8 with 2ml of acid phosphatase 0.1mg/ml of mobile phase composition (70%.citrate buffer at pH 4.8 (0.01M) +30% ethylene glycol) v/v, for 15 minutes. (Mean ± error, n=3)

From the template rebinding histogram shown in figure 5-2, it can be seen that the selectivity and capacity of the imprinted polymer increased with increasing amounts of acid phosphatase employed in the polymerisation process. The template bound to both ACP 8 and ACP4 was comparable and was slightly more by 5µg than ACP2. A similar difference was observed between ACP 2 and ACP1. This suggested that on reducing the template loading by 40mg from ACP 8 to ACP 4, the selectivity of the template towards the polymer was comparable. However, on reducing the template concentration further to 20mg in ACP 2, the template selectivity was reduced by ~ 16.7 %, while reducing even further to 10mg in ACP 1, the template selectivity decreased significantly by ~31%.

In order to get an insight into the nature and stability of the template bound to these polymers, we investigated the enzymatic activity of the bound enzyme. All polymers were initially rinsed with low strength citrate buffer of pH 4.8 to remove less strongly bound enzyme, followed by measuring of enzymatic activity.

Briefly, this experiment was carried out by incubating the polymer bound enzyme with the substrate (4-nitrophenol phosphate). The activity of polymer bound enzyme was determined and compared with the expected units (see experiment 5.3.2).The results are presented in figure 5-3.

The enzyme bound activities of the imprinted polymers ACP 4 and ACP 8 were comparable at ~11%. A significant reduction was observed with ACP 2, which was ~2.9%, and significantly lower with ACP 1(1.33%), when compared to the expected units of enzyme activities. From the polymer bound enzyme activities, it can be seen that a significant amount of the enzyme bound to the imprinted polymer was inactive. It was argued that the reduced activity might be either due to template released within the mild washing solvent conditions or else the product of enzymatic reaction, 4-nitrophenol, may be too hydrophobic to be released into the reaction medium and be therefore strongly retained within the densely cross linked polymer network.

Figure 5-3: Acid phosphatase bound ACP (1, 2, 4 & 8) activity

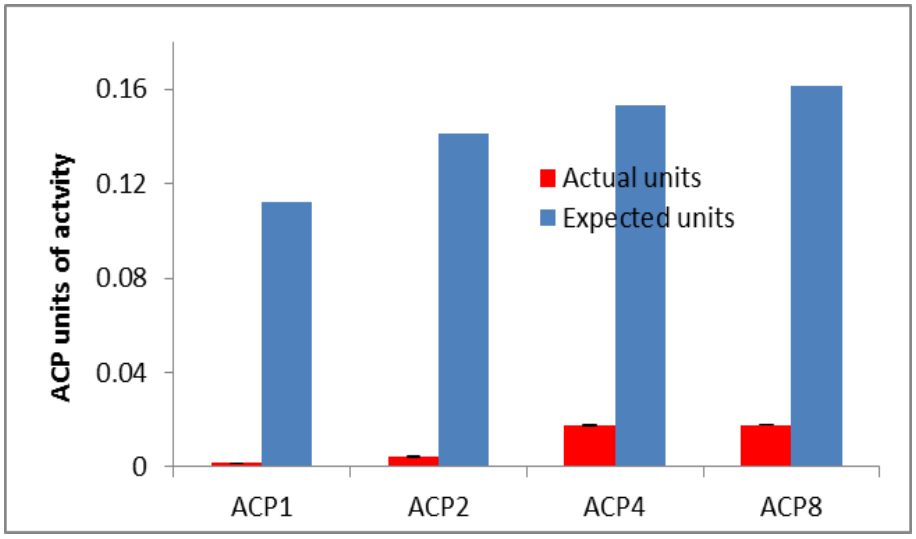


Figure 5-3 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP1, ACP2, ACP4 and ACP8 after rinse with mobile phase, measured at 410nm wavelength and free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

In order to get an insight into whether the enzyme catalysed reaction produced was retained within the polymer matrix, we added 0.1M NaOH (aq) to the polymer suspension (see experiment 5.3.3) and the results are shown in figure 5-4. It was noted that this would have a drastic effect on the enzyme activity, leading to complete deactivation.

From figure 5-4, it was clear that a considerable amount of the enzymatic product 4-nitrophenol may be too hydrophobic and actually be retained within the polymer. This is evidently seen from the significantly increased units of the enzyme activity observed with all polymers. For ACP 1, 3.36 times more nitrophenol was produced compared to the expected units of activity of the enzyme bound to the polymer while that of ACP 2 was ~4.14 units. Even larger increases were observed with both polymer ACP 4 and ACP 8 almost ~8.6 fold. This could be explained by changes in molar extinction coefficient of 4-nitrophenol with addition of Na ions.

Figure 5-4: ACP bound ACP MIPs (1, 2, 4 & 8) activities with addition of NaOH

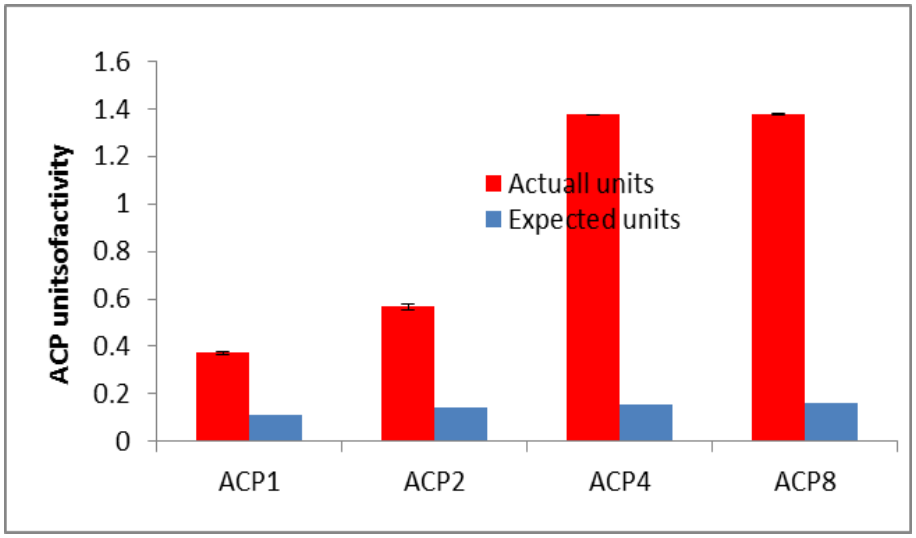


Figure 5-4 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP1, ACP2, ACP4 and CP8, data measured at 410nm wavelength. Polymer suspension and reaction medium were added to 0.1 M NaOH to halt the reaction, and free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

Another possible explanation is that the enzyme became either super active or else a control volume for the enzyme and substrate was low compared to the free enzyme and substrate in a bulk solution. From this investigation, it was clear that that using a low amount of the template in the polymer preparation reduced both the capacity and selectivity of the polymer towards the imprinted template as in ACP1and ACP2. However, there was a limit at which further increases would not elevate the number of the potential binding sites, as seen from ACP 4 compared to ACP 8.

The preliminary experiments justified continued investigations using only ACP 4 and a control polymer ACP B, prepared using a similar reagent combination, except without the template. The control polymer ACP B was processed and dried similarly to ACP 4.

5.3.3 Acid phosphatase selectivity towards ACP MIPs and control

The preferential template selectivity study was carried out for polymers ACP 4 and ACP B (see experiment 5.4). The affinities of the template towards both polymers are presented in figure 5-5.

Figure 5-5: ACP uptake by ACP 4 & ACP B

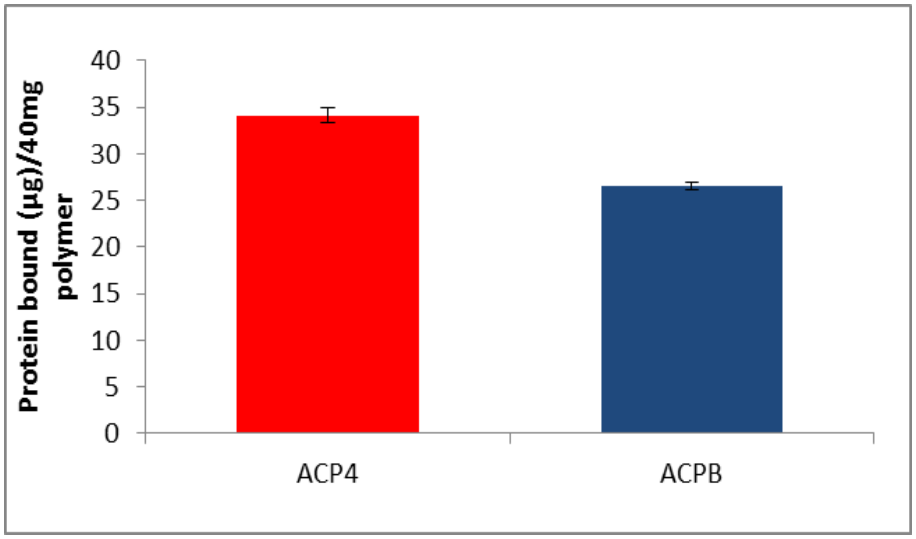


Figure 5-5 Aqueous equilibrium rebinding for 40 mg ACP imprinted polymers, ACP4 and control ACPB with 2ml of acid phosphatase 0.1mg/ml of mobile phase composition (70%.citrate buffer at pH 4.8 (0.01M) +30% ethylene glycol) v/v, for 15 minutes. (Mean ± error, n=3)

From the results, the selectivity of acid phosphatase towards imprinted polymer ACP 4 was slightly more by ~8.14 µg/40mg polymer compared to the control ACP B. The difference was comparable to that observed before with lysozyme imprinted polymer P2 and its control B1. More interestingly, these polymers have comparable surface area, pores, size and swelling capacity (please refer to chapter 6). Therefore, the difference in the template rebinding may be solely due to the template bound to the selective site.

From the results, it was also clear that the non specific rebinding was still dominating the polymer selectivity. Therefore in order to discriminate non selective bound protein,

we employed rapid heat shock treatment to the enzyme bound to both polymers at 60^0C for 1min.

The hypothesis was similar to the described before (chapter 4), whereby enzyme bound to the specific sites should be more stable in contrast to the enzyme bound to the non selective sites when subjected to the rapid heat shock treatment. This would be reflected by the degree of the polymer bound enzyme activity.

From the results shown in figure 5-6, the enzyme bound to ACP 4 polymer was more compared to ACP B by ~0.04 units, which were equivalent to 8.7µg; the difference was similar to that observed from the enzyme bound initially. This entails that 93% of the total enzyme bound to ACP 4 was active compared to ACP B, whereby only 75% was active. Therefore, a large proportion of the enzyme bound to the imprinted polymer was in a more stable conformation, as opposed to the enzyme bound to the control polymer, although the difference was relatively small.

Figure 5-6: ACP bound ACP 4 & ACP B activities before polymer wash

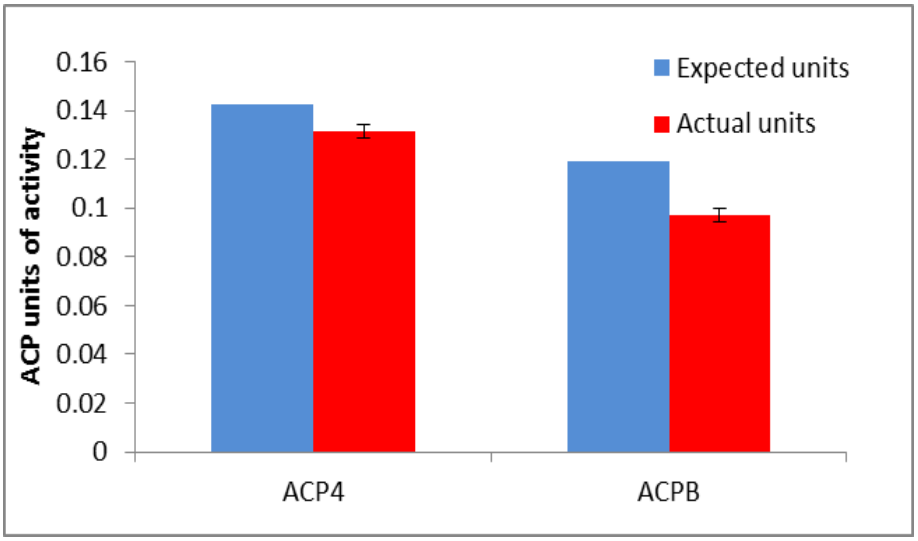


Figure 5-6 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP4 and control ACPB (rinsing step with mobile phase was eliminated) and data measured at 410nm wavelength and free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

From this, it was clear that rapid heat treatment could not completely suppress enzyme bound non-selectively in both ACP 4 and control polymer ACP B.

The indiscriminate binding observed between the ACP 4 and ACP B was very disappointing. However, we were still interested to determine if imprinted polymers possessed some specific sites compared to non-imprinted matrix.
One way to address this was to continue feeding the enzyme bound polymers with more substrates (4-nitrophenol phosphate) to overwork the enzyme bound to non specific sites and hence reduce their catalytic efficiency, while the enzyme bound to the specific was expected to continue to demonstrate high catalytic efficiency, as long as the substrate concentration was kept optimum, such that enzyme bound saturation would not occur. This was carried out after the removal of the initial products of the catalysis reaction (see experiment 5.5). The second generation enzyme bound activity is shown in figure 5-7.

Figure 5-7: ACP bound ACP 4 & ACP B activities after 2nd phase of substrate activity

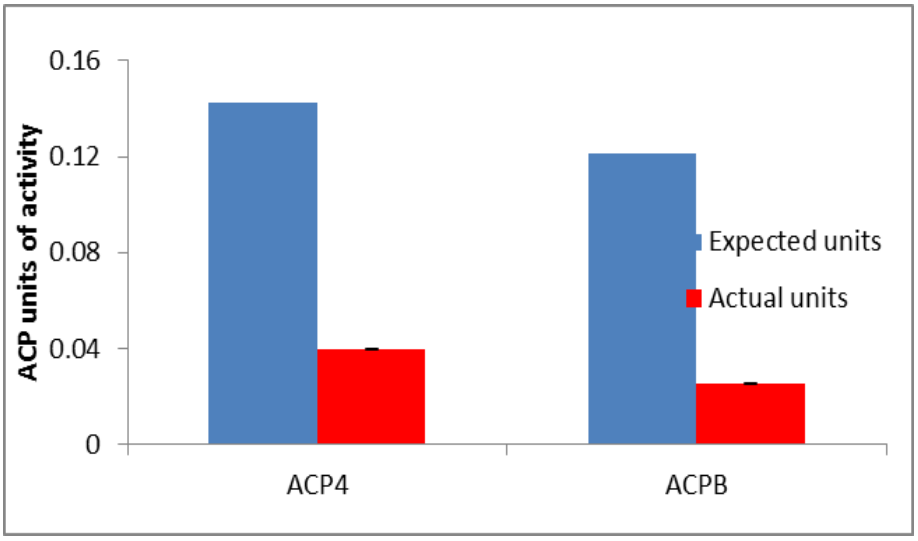


Figure 5-7 2nd phases of acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP4 and control ACPB, data measured at 410nm wavelength and free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

From the results demonstrated in figure 5-7, we can see that the difference in enzyme activity between ACP 4 and ACP B with addition of substrates was insignificant and the activity for both polymers was reduced by more than a half compared to that observed before. It was reasoned that the low activity demonstrated by both polymers may be due to the enzyme released within the reaction medium in the first phase of activity. Hence, a low concentration of enzyme was strongly retained by the polymer, leading to reduced substrate catalysed reactions in this second wave of activity.

Up until now, we were confident that there was a slight difference that existed from the enzyme bound to the specific sites compared to that bound to the non specific sites. To try and amplify this, we increased template rebinding time from 15min to 22hrs. It was assumed that using shorter rebinding time may have prevented the polymers reaching equilibrium with the template in solution, hence resulting in low template rebinding to this polymer (see experiment 5.6). The selectivity of template towards both polymers after 22hrs of rebinding is shown in figure 5-8.

Figure 5-8: ACP uptake by ACP 4 & ACP B with increasing rebinding time

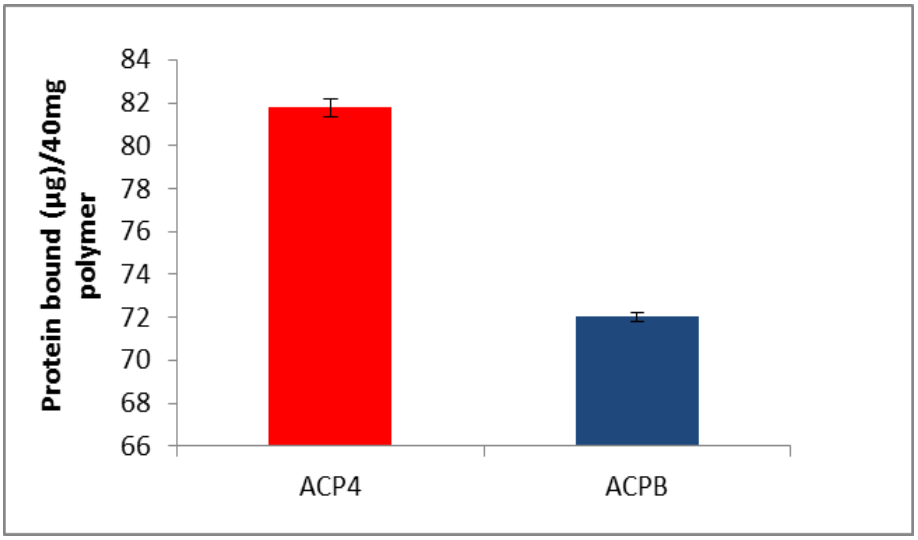


Figure 5-8 Aqueous equilibrium rebinding for 40 mg ACP imprinted polymers, ACP4 and ACPB with 2ml of acid phosphatase 0.1mg/ml of mobile phase composition (70%.citrate buffer at pH 4.8 (0.01M) +30% ethylene glycol) v/v, for 22hrs. (Mean ± error, n=3)

From the results presented in figure 5-8, it can be seen that the longer template binding time significantly increased the amount of protein uptake by both polymers. However, the difference was ~9µg/40mg polymer, and this was comparable to that observed before at extremely short rebinding time. This suggested that increasing equilibrium rebinding time did not actually amplify the difference and therefore this may be due to a kinetic effect.

Prior to measuring the polymer bound enzyme activities, the samples were rinsed with mobile phase because it was assumed that most of the template bound was non specific. From the polymer bound enzyme activities shown in figure 5-9, it was clear that most of the enzyme bound was actually removed by the mild washing conditions and the concentration of the template retained strongly by the polymer was similar to that noted with lower template rebinding time; therefore 10-15 min seemed quite sufficient. After careful inspection of the results, the selectivity of the acid phosphatase towards the imprinted polymer ACP 4 was realised to be in the region of ~35µg template /40mg polymer and was slightly lower for the ACP B.

Figure 5-9: ACP bound ACP 4 & ACP B activities with increasing rebinding time

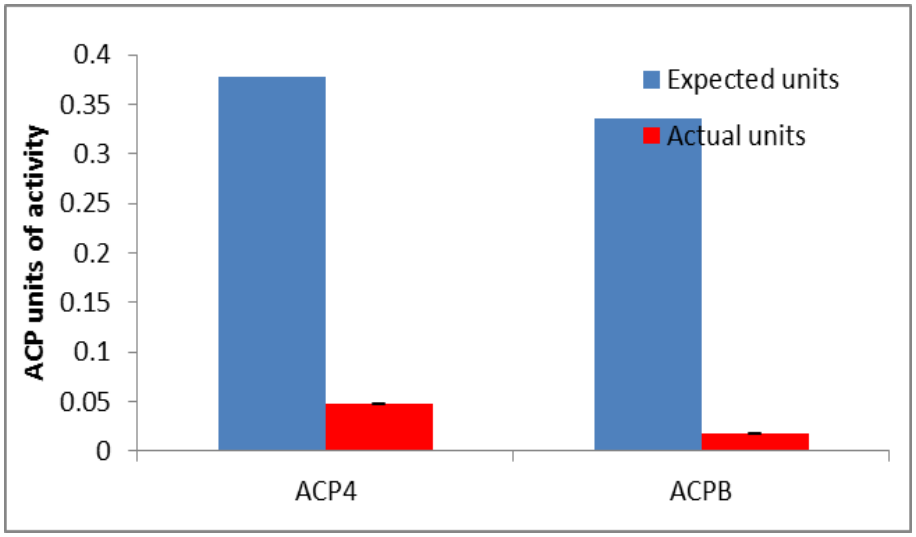


Figure 5-9 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP4 and control ACPB (ACP binding time was increased from 15 min to 22hrs) and data measured at 410nm wavelength and free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

In order to try to eliminate the non selective rebinding further, we employed a lower template concentration in the rebinding solution, 35 µg/ml instead of 100µg/ml (see experiment 5.7), and the results are shown in figure 5-10

Figure 5-10: ACP uptake by ACP 4 & ACP B with reduced concentration of ACP in solution

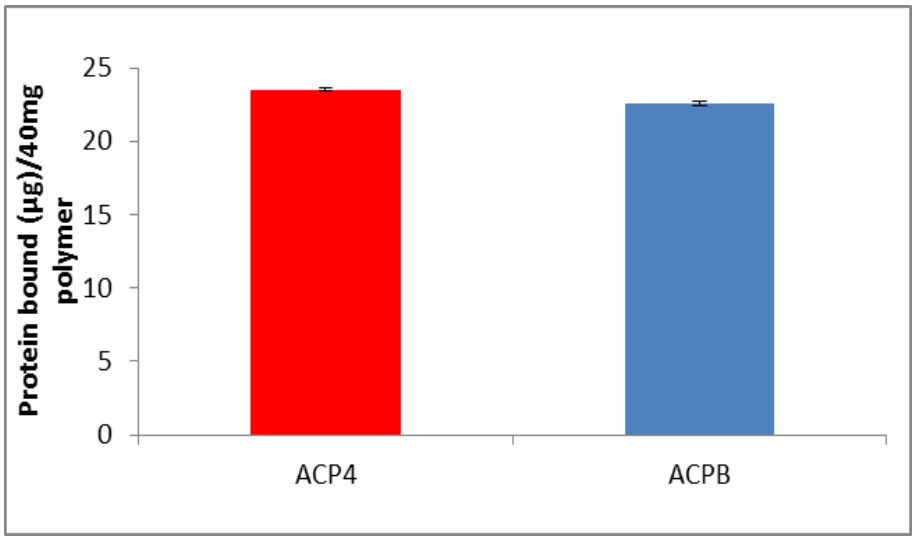


Figure 5-10 Aqueous equilibrium rebinding for 40 mg ACP imprinted polymers, ACP4 and ACPB with 2ml of acid phosphatase 23µg/ml of mobile phase composition (70%.citrate buffer at pH 4.8 (0.01M) +30% ethylene glycol) v/v, for 15 minutes. (Mean ± error, n=3)

The template amounts bound to both ACP 4 and ACP B were again comparable ~23µg/40mg, which was less than before, suggesting that the polymer affinities were dependent on the template concentrations in the rebinding solution. However, the percentage of enzyme bound activity to ACP 4 was ~97.2% while for ACP B only ~75.7% was active (figure 5-11); this was similar to that observed before. Therefore, a clear difference in stability of enzyme bound existed.

Figure 5-11: ACP bound ACP 4 & ACP B activities with reduced concentration of ACP

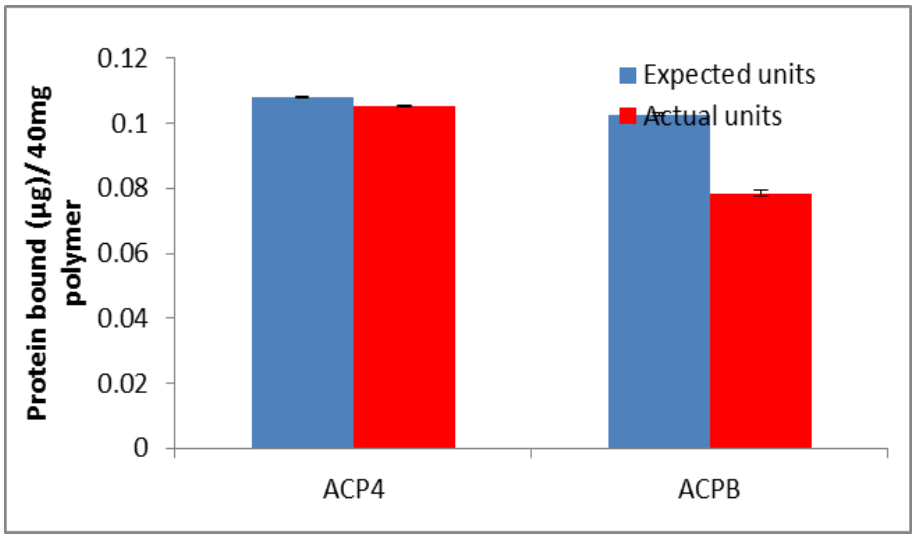


Figure 5-11 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP4 and control ACPB, (with reduced ACP concentration in the binding solution) and data measured at 410nm wavelength free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

Finally, we questioned the effect of the organic modifier ethylene glycol in reducing non specific template rebinding. It was reasoned that this organic modifier might reduce the overall selectivity of the template towards the polymer.

To address this issue, we rebound both polymers with a template rebinding solution without modifier (see experiment 5.8). The selectivity of both polymers towards the template was approximately similar (figure 5-12). However we managed to demonstrate a difference in the enzyme bound activity which again was more with ACP 4 than ACP B by 0.005 units compared to control ACP B (figure 5-13); this was equivalent to 1.1µg, which was approximately similar to the difference of the template bound observed initially (figure 5-13).

Therefore, this suggested that using ethylene glycol may have not interfered with template binding to the polymers and may have not lessened the effect of non selective protein rebinding.

Figure 5-12: ACP uptake by ACP 4 & ACP B without ethylene glycol in solution

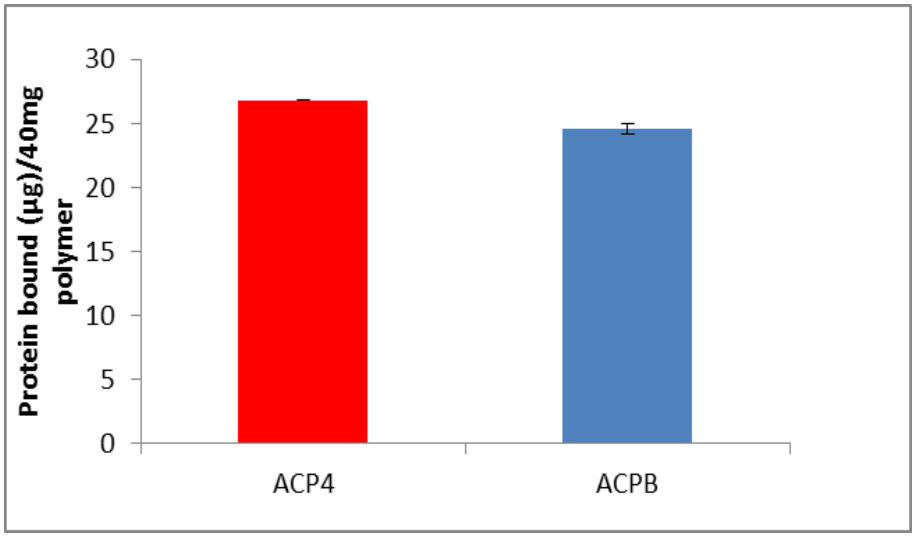


Figure 5-12 Aqueous equilibrium rebinding for 40 mg ACP imprinted polymers, ACP4 and ACPB with 2ml of acid phosphatase 23µg/ml of mobile phase (citrate buffer at pH 4.8 (0.01M)), for 15 minutes. (Mean ± error, n=3)

Figure 5-13: ACP bound ACP 4 & ACP B activities without ethylene glycol in solution

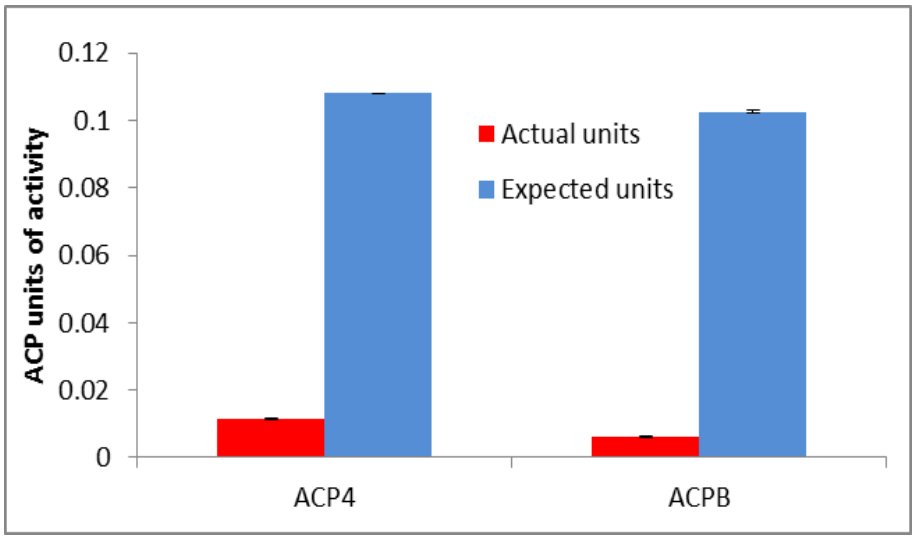


Figure 5-13 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ACP4 and control ACPB (ACP bound without ethylene glycol in the mobile phase solution) and data measured at 410nm wavelength free ACP in solution (expected units obtained from ACP standard curve). (Mean ± error, n=3)

5.4 Preparation of alkaline phosphatase (ALK) MIPs & control

The problem we still encountered with protein imprinting was prevention of non specific protein binding to both imprinted and control polymers. Previously, we managed to lessen this by the addition of Diethylene Glycol Vinyl Ether (DEGVE) in the imprinting process.
From the preceding investigation, we demonstrated that there was a slight discrepancy in the template rebinding and enzyme bound activities between the imprinted polymers compared to that of the control, although non selective protein binding was still large.

In order to try and reduce this, we reviewed the work of Piletsky *et al*, (1998); that group used hydrophobic functional monomers β-cyclodextrin and 2-acryloylamido-2-methyl-propane sulphonic acid for imprinting D-phenylalanine template in aqueous medium. The group suggested that the logic behind this was that in aqueous media, like antibodies, template recognition is a result of entropy driven hydrophobic effects in combination with the enthalpy motivated electrostatic interaction.
To use similar template recognition principles, we developed new imprinted polymers using only a single hydrophobic functional monomer 4-vinyl pyridine (4vpy) for alkaline phosphatase imprinting. Previously, we found out that using 4vpy alone improved the acid phosphatase PCMCs stability in the acetonitrile (porogen) at elevated polymerisation temperatures better than MAA or porogen alone (refer to chapter 2). However, this monomer was known to be extremely hydrophobic and may contribute largely to non specific hydrophobic rebinding.

Alkaline phosphatase was used as a model enzyme instead of acid phosphatase because they are from the same family but alkaline phosphatase much cheaper. For multiple control; blank glycine microcrystals were imprinted using only 4vpy, alkaline

phosphatase imprinted polymer using both 4vpy and MAA functional monomers and its control polymer using only glycine micro crystals.

Table 5-2 outlines the polymer compositions for the new developed polymers. The alkaline phosphatase PCMCs were prepared at 8% enzyme loading, 5% water and IPA as precipitating solvent saturated with glycine excipient (see experiment 6.9).

Table 5-2: Reagents used for the preparation of alkaline phosphatase MIPs

Sample polymers	PCMCs & Glycine (mg)	Enzyme (mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
ALK -4vpy-1	1150	100		0.4, 3.8045	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy-B1	1150	------		0.4, 3.8045	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy MAA-1	1150	100	0.2, 2.32	0.2, 0.1902	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy-MAA-B1	1150	------	0.2, 2.32	0.2, 0.1902	2.0, 10.0898	40.0, 0.244	3.2

Polymer preparation process

All polymers prepared were glassy and hard monoliths and were processed as described previously, i.e. ground to manageable size, pre-washed with acetonitrile to remove unreacted residual monomers, followed by sequential template extraction procedures, similar to that described previously for acid phosphatase imprinted polymers.

After template extraction, all imprinted polymers were examined for template enzymatic activity. After no activity was observed, the polymer powders were dried at 37^0C for 48hrs prior to the polymer evaluation.

5.4.1 ALK selectivity toward ALK MIPs and control

The preferential template selectivity study was carried out for all polymers prepared, ALK-4vp-1, ALK-4vpy-B1, ALK-MAA, ALK-MAA-B, using equilibrium alkaline phosphatase rebinding (see experiment 5.10.1) and the results are shown in figure 5-14. The imprinted polymer ALK-4vpy-1 demonstrated excellent selectivity towards the imprinted template compared to the control polymer ALK-4vpy-B1, and polymer made with a combination of both MAA and 4vpy (ALK-4py-MAA) and its control ALK-4py-MAA-B by almost 10 fold.

From the polymer physical characteristic data presented in chapter 6, we found that all these polymers had different properties; it was therefore difficult to compare and contrast the correlation between the properties, such as pore size and surface area, with the template rebinding behaviour.
The initial consideration was that because of the hydrophobic nature of the 4vpy, the template rebinding may be largely hydrophobic with ALK-4vpy-1. However, with similar composition to its control, it was expected that similar template binding should occur. From this, we were therefore confident that high selective sites might exist in the imprinted polymer using 4-vpy monomer, although these may be masked by non selective protein binding. In order to determine the nature of template bound to the polymer, we looked at the polymer bound enzyme activities.

All polymers were initially rinsed with low buffer strength to remove less strongly bound enzyme prior to the polymer enzymatic activity investigation.
The polymer enzyme activity was determined at a single point in time and a comparison was drawn between all polymers prepared (see experiment 5.10.2). The product of the catalysed reaction 4-nitrophenol was measured at 405nm. The polymer bound enzyme activity results are presented in figure 5-15. The ALK-4vpy-1 polymer demonstrated excellent activity which surpassed all other polymers significantly; this might be due to the large template rebinding observed initially compared to other polymers.

Figure 5-14: Alkaline phosphatase uptake by all polymers

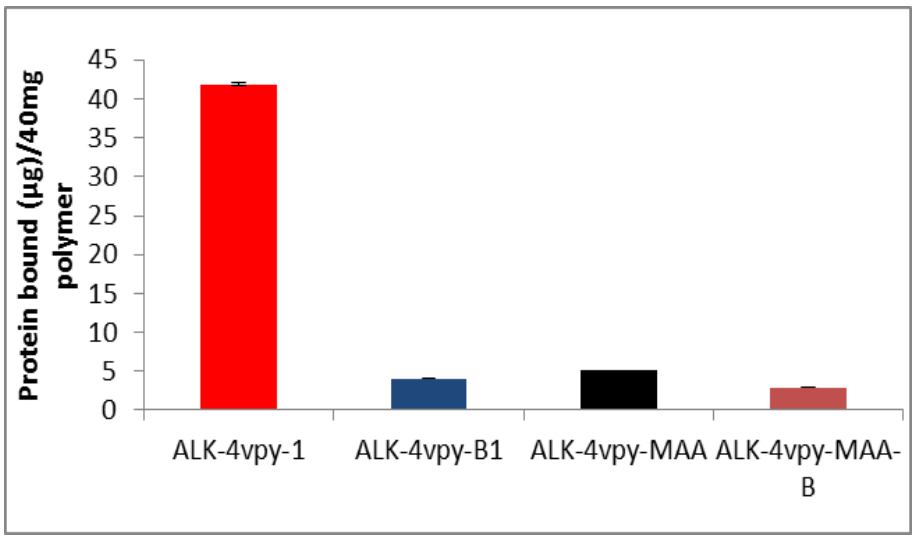


Figure 5-14 Aqueous equilibrium rebinding for 40 mg ALK imprinted polymers, ALK-4vpy-1, ALK-4vpy-B1, ALK-4vpy-MAA and ALK-4vpy-MAA-B, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8), for 10 minutes. (Mean ± error, n=3)

Figure 5-15: Alkaline phosphatase bound polymer activity for all polymers

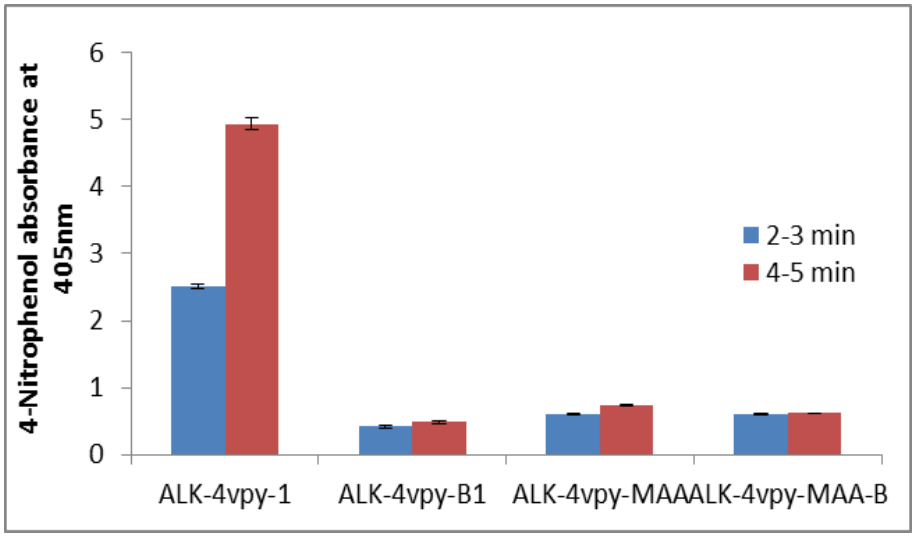
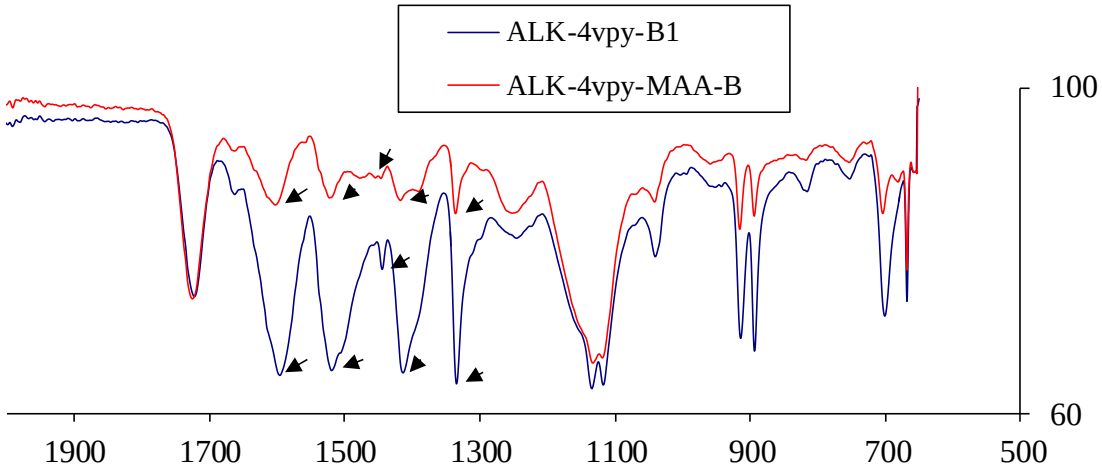


Figure 5-15 Alkaline phosphatase units of activity for 40mg ALK bound imprinted polymers, ALK-4vpy-1, ALK-4vpy-B1, ALK-4vpy-MAA and ALK-4vpy-MAA-B after polymer were rinsed with mobile phase, data measured at 405nm wavelength between 2-3 and 4-5 minutes. (Mean ± error, n=3)

In order to compare the structural compositions of the polymers prepared with the single functional monomer 4vpy to that of the combined functional monomers 4vpy and MAA, we examined the FTIR of both control polymers (ALK-4vpy-B1 and ALK-4vpy-MAA-B1) and not the complementary imprinted polymers because the residual proteins retained by these polymers may generate a large number of signals, leading to poor resolution of the FTIR spectra (see experiment 5.11).The resultant FTIR spectra of both control polymers are presented in figure 5-16.

Figure 5-16: FTIR of controls ALK-4vpy-B1 and ALK-4vpy-MAA-B



The structural differences observed between the control polymers were the much stronger signals generated by the C-H stretches from the 4vpy aromatic ring in ALK-4vpy-B1 in contrast to the weak signal produced from ALK-4vpy-MAA-B. This may be due to high concentrations of 4vpy employed in ALK-4vpy-B compared to ALK-4vpy-MAA-B.

After we had established that polymer ALK-4vpy-1 made with only 4vpy showed both high selectivity and enzyme activity, to improve polymer properties, we prepared another comparable alkaline phosphatase polymer with the addition of DEGVE (ALK-4vpy-DEGVE). In addition, a polymer was made with only the hydrophilic monomer MAA (ALK-MAA) (see experiment 5.12). It was assumed that increasing the

hydrophilicity of the polymer would reduce non specific hydrophobic protein binding and improve its dissociation kinetics. Table 5-3 outlines the polymer compositions used for the new set of polymers.

All polymers prepared were glassy and hard monoliths and were processed similar to that described previously for ALK-4vpy-1 polymer.

Table 5-3: Reagents used for 2nd preparation of ALK MIPs

Sample	PCMCs (mg)	Enzyme Loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
ALK - 4vpy-1	1725	150		0.6, 5.708		3.0 15.135	59,08 0.359	4.8
ALK – MAA	1725	150	0.6 6.984	--	--	3.0, 15.135	59.08, 0.360	4.8
ALK-4vpy – DEGVE	1725	150	--	0.3, 2.854	0.3 2.27	3.0, 15.135	59.08, 0.360	4.8

5.4.2 ALK selectivity towards ALK hydrophobic and hydrophilic MIPs

To determine the selectivity of the newly developed imprinted polymers, we used 20 mg of each polymer instead of 40 mg to reduce the concentration of template bound, hence slowing down the polymer bound enzyme reaction (see experiment 5.13). The template rebinding results, are presented in figure 5-17.

From the results, it can be seen that the template bound to the newly developed polymer ALK-4vpy-DEGVE was comparable to ALK-4vpy-1 with a slight difference of ~6µg/ 40mg. The difference was equivalent to the amount of the template bound to ALK-MAA, which was less by 4 fold compared to ALK-4vpy-1. This reduced template bound to ALK-MAA was assumed to be as a result of the pH of the rebinding solution may be unfavourable for this polymer. The addition of DEGVE monomer in ALK-4vpy-

DEGVE polymer increased the surface area by ~ 66m^2/g compared to ALK-4vpy-1 and ALK-4vpy-DEGVE. In effect, this reduced the porosity size (refer to chapter 6).

Figure 5-17: ALK uptake by ALK-4vp-1, ALK-MAA & ALK-4vpy-DEGVE at pH 9.8

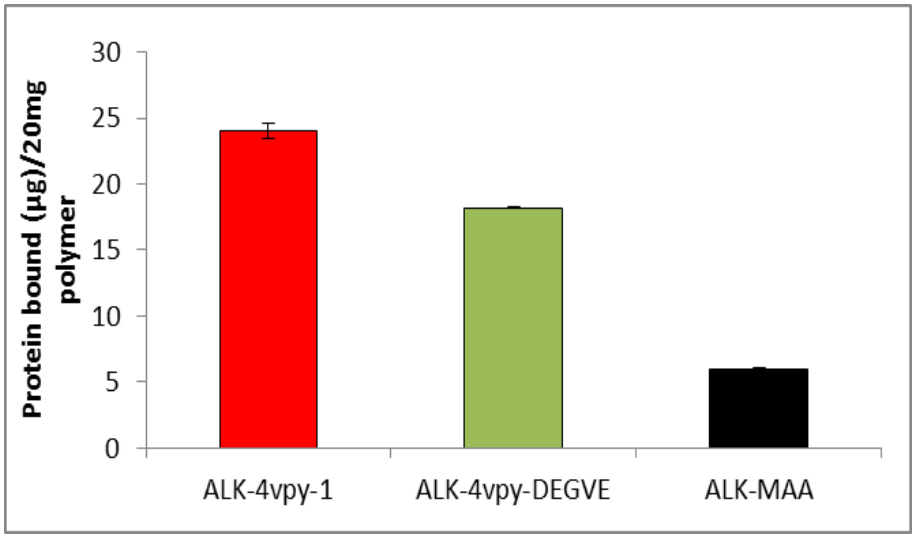


Figure 5-17 Aqueous equilibrium rebinding for 20 mg ALK imprinted polymers, ALK-4vpy-1, ALK-4vpy-DEGVE and ALK-MAA, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

In order to compare template dissociation, we rinsed all polymers with 0.1M and followed 1.0 M of diethanolamine buffer at pH 9.8. The amounts of template released from the two washing conditions are shown in figure 5-18.

From figure 5-18, it can be seen that the amount of enzyme released from both ALK-4vpy-1 and ALK-MAA in the wash with 0.1M buffer were comparable. With ALK-4vpy-DEGVE, the concentration was doubled. For the second wash with 1.0M buffer, the enzyme released from all polymers was comparable. This suggested that the use of MAA, did not alter the template dissociation characteristic compared to ALK-4vpy-1. However with poor template rebound to the ALK-MAA, this was inconclusive. However slightly more template was released with ALK-4vpy-DEGVE, suggesting that

the increased swelling observed with ALK-4vpy-DEGVE (please refer to chapter 6) may have contributed to this effect.

Figure 5-18: ALK from ALK-4vp-1, ALK-MAA & ALK-4vpy-DEGVE wash, bound at pH 9.8

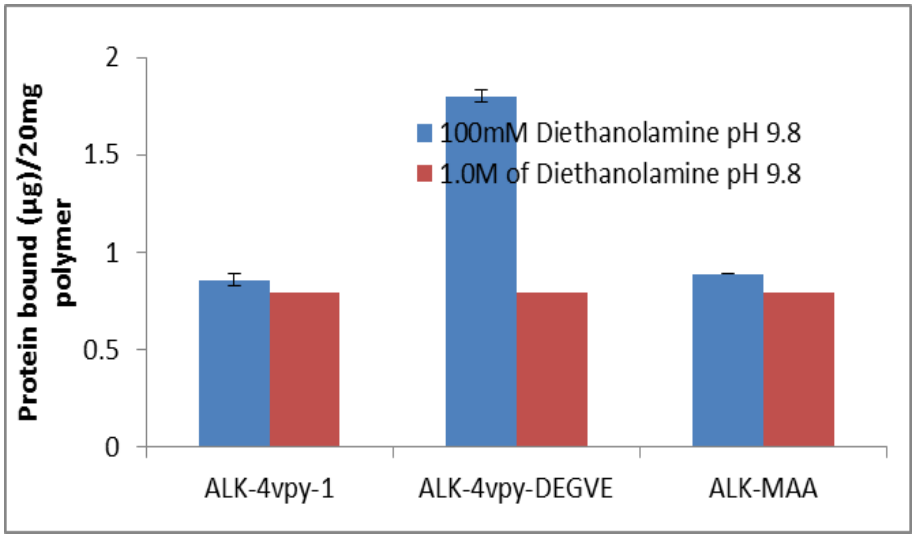


Figure 5-18 Alkaline phosphatase released from 20mg ALK bound imprinted polymers ALK-4vpy-1, ALK-4vpy-DEGVE and ALK-MAA after polymer were rinsed with 2ml of100mM and 1M diethanolamine buffer at pH 9.8, each for 10 minutes. (Mean ± error, n=3)

The polymer bound enzyme activity demonstrated by ALK-4vpy-1 (figure 5-19) was comparable to ALK-EGDVE, while the lowest activity was observed with ALK-MAA as was expected. This suggested that the activity corresponded to the amount of template bound initially and retained after polymer rinsing with mild washing conditions.

More understanding of poor template rebinding observed with ALK-MAA was required. In order to address this issue, we employed lower pH 7.8-7.9 mobile phase buffer instead of pH 9.8 (see experiment 5.14). It was recalled that during PCMCs formulation, the addition of the glycine into the diethanolamine buffer reduced the buffer from pH 9.8 to pH 7.8. Therefore because of this, it was decided to investigate the template rebinding at this pH, although the diethanolamine buffer was slightly unstable and was constantly monitored for any changes in the pH.

Figure 5-19: ALK bound at pH 9.8 ALK-4vp-1, ALK-MAA & ALK-vpy-DEGVE

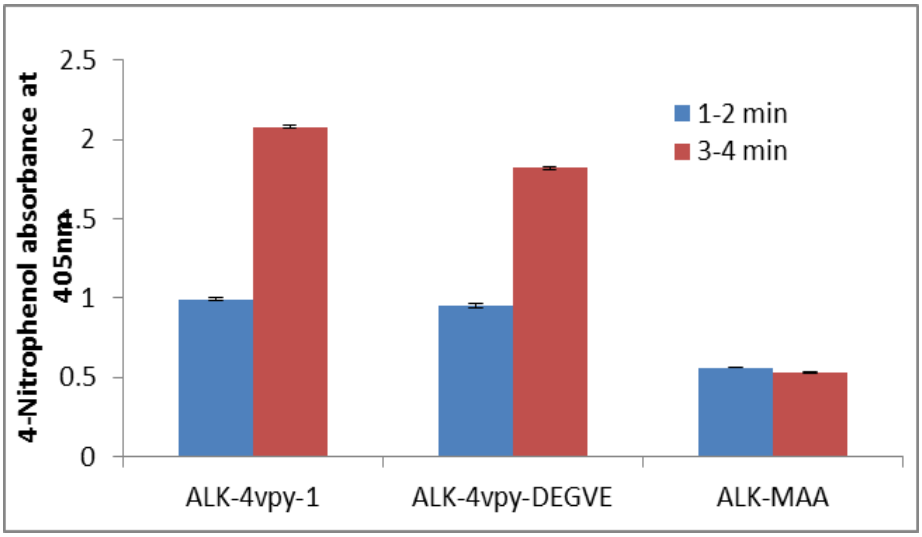


Figure 5-19 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1, ALK-4vpy-DEGVE and ALK-MAA after polymer were rinsed with diethanolamine buffer (100mM and 1M), data measured at 405nm wavelength between 1-2 and 3-4 minutes. (Mean ± error, n=3)

From figure 5-20, it can be seen that, the selectivity of the ALK-4vpy-1 and ALK-EGDVE was increased by almost half, while the largest increase was observed with ALK-MAA, whereby the template bound was ~2 fold more than the other polymers, and 12 fold more compared to that observed before at high pH. The use of lower pH mobile phase may thus have triggered the ALK-MAA memory towards the recognition of the imprinted template. Another possible argument was that the non specific hydrophilic selectivity may be the dominant feature in the template rebinding. The enzyme bound activities data shown figure 5-21after all polymers were subjected to mild washing buffer conditions was still high with ALK-4vpy-1 and ALK-4vpy-DEGVE, while lower activity was again observed with ALK-MAA, regardless of the significant increase of the templates rebound initially, although this was large compared to that observed when the template were rebound at pH 9.8. We concluded that a considerable amount of the enzyme bound to ALK-MAA may have been released in the wash phase. This may be due to the increased swelling capacity of the ALK-MAA (please refer to chapter 6) compared to both ALK-4vpy-DEGVE and ALK-4vpy-1.

Figure 5-20: ALK uptake by ALK-4vp-1, ALK-MAA & ALK-4vpy-DEGVE at pH7.9

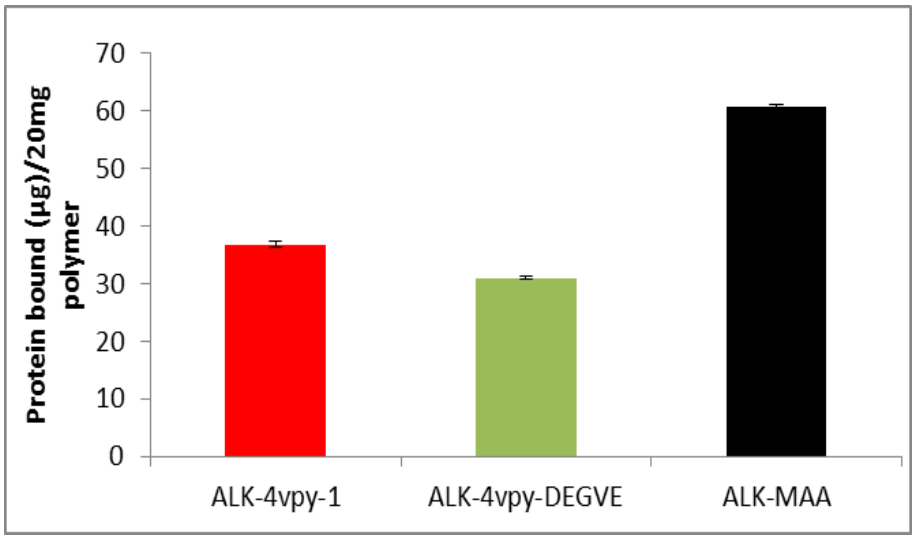


Figure 5-20 Aqueous equilibrium rebinding for 20 mg ALK imprinted polymers, ALK-4vpy-1, ALK-4vpy-DEGVE and ALK-MAA, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 7.9 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

Figure 5-21: ALK bound at pH 7.9 ALK-4vp-1, ALK-MAA & ALK-4vpy-DEGVE activities

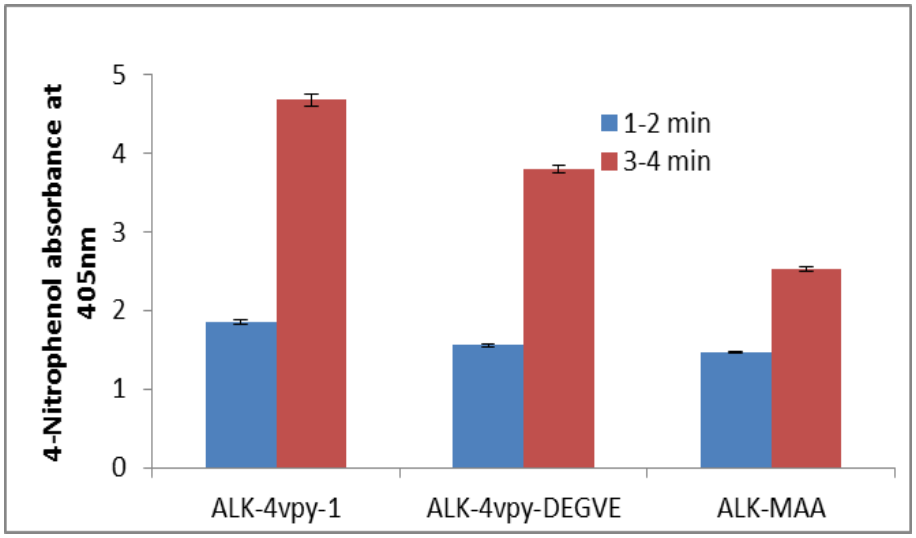


Figure 5-21 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1, ALK-4vpy-DEGVE and ALK-MAA after polymer were rinsed with diethanolamine buffer (100mM and 1M), data measured at 405nm wavelength between 1-2 and 3-4 minutes. (Mean ± error, n=3)

To further establish the nature of the alkaline phosphatase selectivity and the stability of the enzyme bound, we carried out a comparative and cross selectivity study between the ALK-4vpy-1 and a polymer imprinted with closer molecular weight Beta glucosidase (Bgo-4vpy-1).

5.4.3 Preparation of competitive beta glucosidase (Bgo) selective MIPs

Beta glucosidase PCMCs were prepared at 8%, enzyme loading, 5% water and IPA as precipitating solvent saturated with glycine as an excipient, similar to the ALK-PCMCs (see experiment 5.15). Just to reiterate that the main focus was to use only 4vpy polymer. Therefore, using only this monomer, Beta glucosidase imprinted polymer compositions are outlined in table 5-4.
The polymer was processed and template was extracted from the polymer using similar procedures described for ALK-4vpy-1

Table 5-4: Reagents used for the preparation of beta glucosidase MIPs

Sample polymer	PCMCs (mg)	Enzyme Loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
Bgo-4vpy-1	1725	150	--	0.6, 5.708	3.0, 15.135	59.08, 0.360	4.8

5.4.4 ALK selectivity towards ALK and Bgo MIPs

To establish the selectivity of the template towards specific polymers and towards the β-glucosidase polymer, 20 mg of both polymers ALK-4vpy-1 and Bgo-4vpy-1 were employed using a similar procedure to that described previously (see experiment 5.16). The amount of the template bound to both polymers is shown in figure 5-22. From the

results shown in figure 5-22, the selectivity of the alkaline phosphatase towards the specific polymer ALK-4vpy-1 was almost 2 fold higher compared to Bgo-4vpy-1, suggesting that the imprinted polymer ALK-4vpy-1 was selective compared to the non - selective polymer. However, a considerable amount of enzyme also bound to the Bgo polymer by a non selective mechanism.

More interestingly, the difference in the template rebinding observed may be solely due to the specific sites because both polymers have comparable surface area and porosity (please refer to chapter 6).From the results, it was also clear that the ALK-4vpy-1 cannot be used for SPE because of non specific binding, but if correctly packed in column it might be useful for separation. To try to release less strongly bound template, we rinsed both polymers using mild buffer conditions as before.

The amount of alkaline phosphatase released from both polymers (shown in figure 5-23) was comparable, suggesting that both polymers may have similar template dissociation characteristics, even though the swelling capacity of the ALK-4vpy-1 was larger compared to bgo-4vpy-1.

Figure 5-22: ALK uptake by ALK-4vpy-1 & Bgo-4vpy-1 at pH 9.8

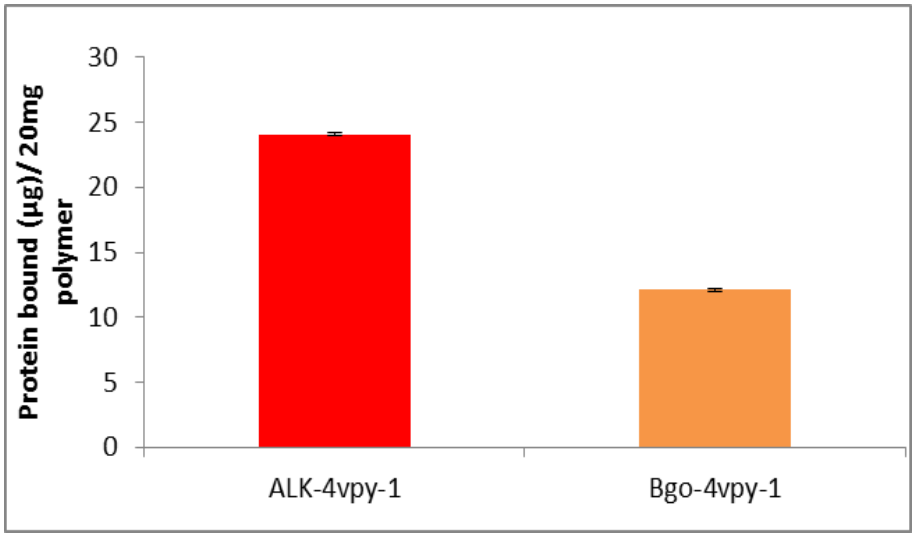


Figure 5-22 Aqueous equilibrium rebinding for 20 mg ALK-4vpy-1and Bgo-4vpy-1, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

Figure 5-23: ALK in wash from ALK-4vpy-1 & Bgo-4vpy-1

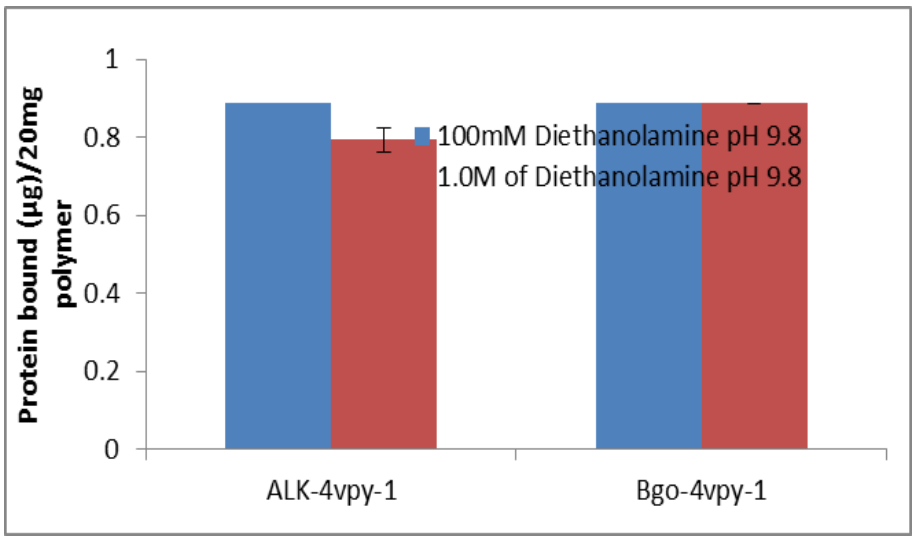


Figure 5-23 Alkaline phosphatase released from 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with 2ml of100mM and 1M diethanolamine buffer at pH 9.8, each for 10 minutes. (Mean ± error, n=3)

In addition, even though similar amount came off but much more was still left on ALK-4vpy-1. It was clear from that it was not possible to discriminate the template bound to selective sites from that of non selective sites.
In further attempts to try and differentiate this, we examined the polymer activities shown in figure 5-24.

From the enzyme bound activity, it can be seen that ALK-4vpy-1 was more active compared to Bgo-4vpy-1. This was expected because more template bound to the ALK-4vpy-1 compared to Bgo selective polymer. It was argued that although the enzyme bound to non-selective polymer, the excess binding observed with ALK-4vpy-1 might have been to the more specific sites. Another interesting feature observed was the rate of enzyme bound activity being much faster in ALK-4vpy-1 compared to Bgo-4vpy -1. This suggested that the enzyme bound to the ALK-4vpy-1 may be in a conformation that provided better reaction kinetics compared to the enzyme bound non specifically to the Bgo-4vpy-1 polymer.

Figure 5-24: ALK bound at pH 9.8 ALK-4vp-1 & Bgo-4vpy-1 activities

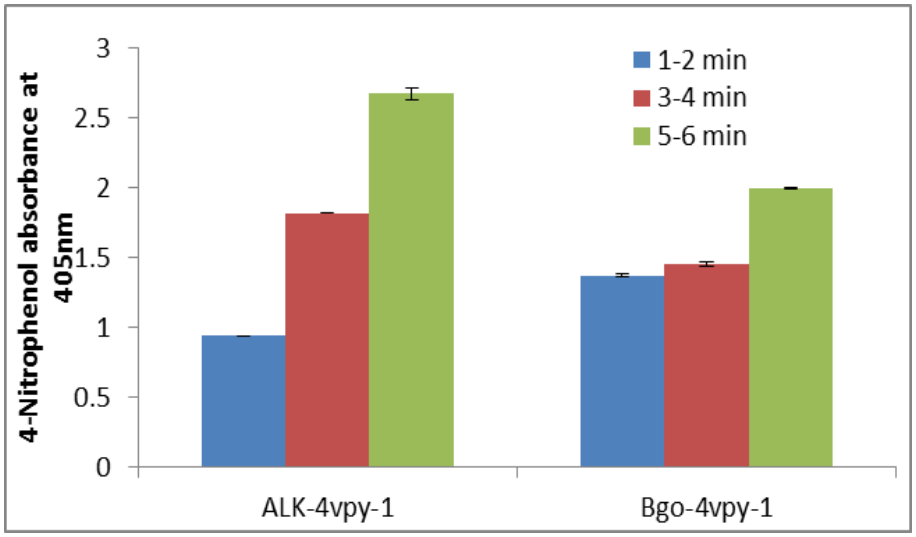


Figure 5-24 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer (100mM and 1M), data measured at 405nm wavelength between 1-2, 3-4 and 5-6 minutes. (Mean ± error, n=3)

In order to try and suppress non selective binding, we rebound the template using lower pH mobile phase buffer 7.8-7.9 (see experiment 5.17) and the results are presented in figure 5-25.

From the results, it can be seen that more alkaline phosphatase bound to ALK-4vpy-1 compared to Bgo-4vpy-1 by ~10µg, although the overall template rebinding was increased in both polymers compared to that observed before using pH 9.8 mobile phase. The polymer bound enzyme activity was also again higher with ALK-4vpy-1 compared to Bgo-4vpy-1 with faster reaction (figure 5-26). However, using lower pH buffer clearly did not suppress the binding at Bg-4vpy-1.

These results clearly demonstrate the difficulties arising from non selective template rebinding. In order to observe greater selectivity, we decided to apply rapid heat shock to try and destabilise and deactivate the enzymes bound to the non selective sites, as opposed to enzyme bound to specific sites. The assumption was that by applying heat treatment the template bound non-selectively would be expected to denature, with the treatment being less damaging to specifically bound template.

Figure 5-25: ALK uptake by ALK-4vpy-1 & Bgo-4vpy-1at pH 7.9

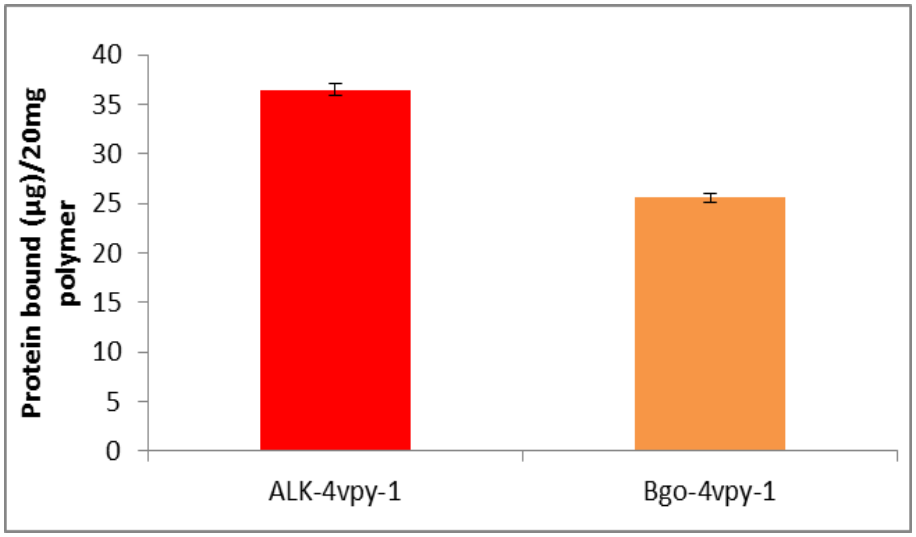


Figure 5-25 Aqueous equilibrium rebinding for 20 mg ALK-4vpy-1and Bgo-4vpy-1, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 7.9 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

Figure 5-26: ALK bound at pH 7.9 ALK-4vp-1 & Bgo-4vpy-1 activities

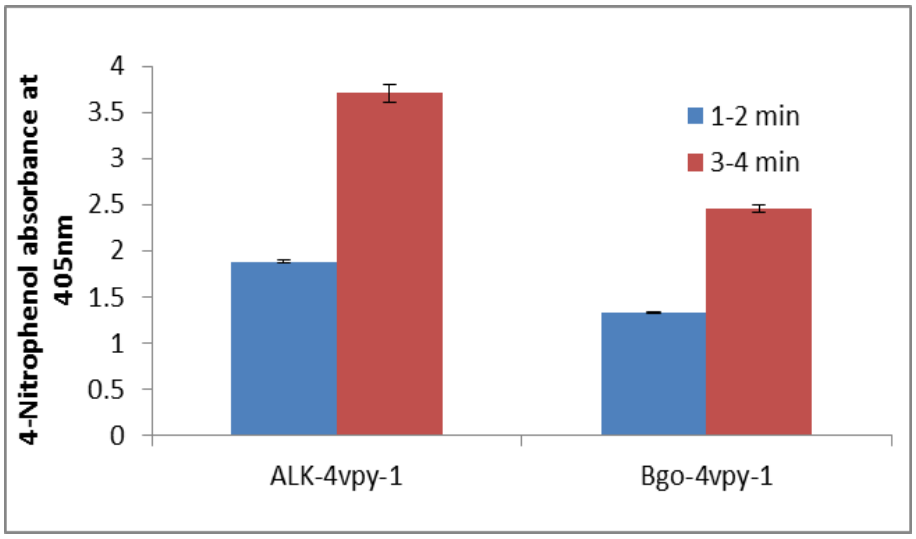


Figure 5-26 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer pH 7.9 (100mM and 1M), data measured at 405nm wavelength between 1-2, 3-4 and 5-6 minutes. (Enzyme activity carried out using diethanolamine buffer at pH 9.8) (Mean ± error, n=3)

5.4.5 ALK selectivity towards ALK & Bgo MIPs & heat application

To achieve this, we applied a rapid heat shock by incubating the polymers in buffer for 1min at 60 ^{0}C (see experiment 5.18). Following this rapid heat shock, no difference of enzyme activity was observed. This suggested that the alkaline phosphatase was either very stable at this temperature or both polymers provided conformational stability to the bound protein. It was then decided to prolong the heating application time to 40 minutes and the enzyme bound activity results are shown in figure 5-27. The enzyme bound activity to the Bgo-4vpy-1 polymer could not be suppressed even after prolonged heating.

Figure 5-27: ALK bound ALK-4vp-1 & Bgo-4vpy-1 activities after heat treatment

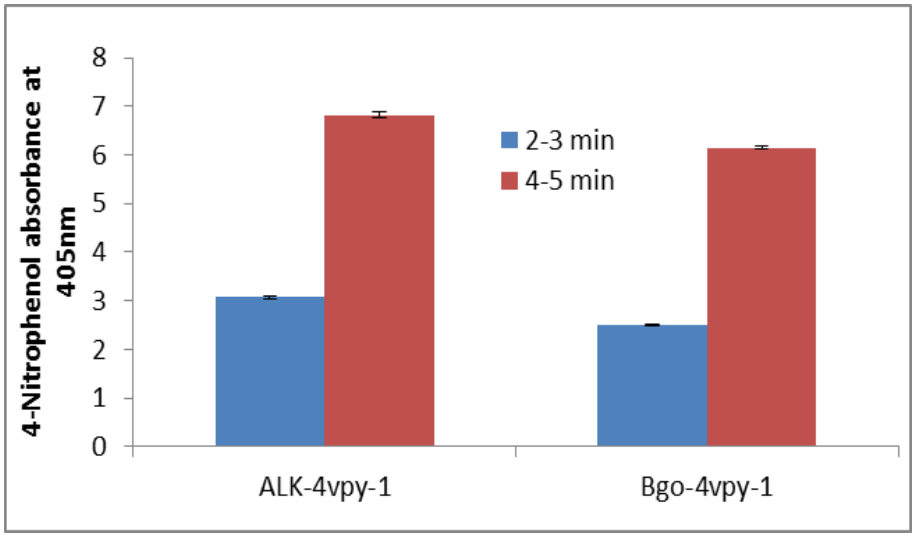


Figure 5-27 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer (100mM and 1M), heat treatment applied at 60 ^{0}C for 40 min, data measured at 405nm wavelength between 2-3 and 4-5 minutes. (Mean ± error, n=3)

In further attempts to suppress the activity of non selective template bound to the cross selective polymer, we incubated both polymers at lower temperature at ~4^{0}C in the fridge overnight to slow the enzymatic reaction of the enzyme bound non selectively

(see experiment 5.19) and the enzyme bound activity results are shown in figure 5-28. Even with this treatment, we could not see differences in the activity of alkaline phosphatase bound to ALK-4vpy-1 and Bgo-4vpy-1.

Figure 5-28: ALK bound ALK-4vp-1 & Bgo-4vpy-1 activities after MIPs stored at low temperature

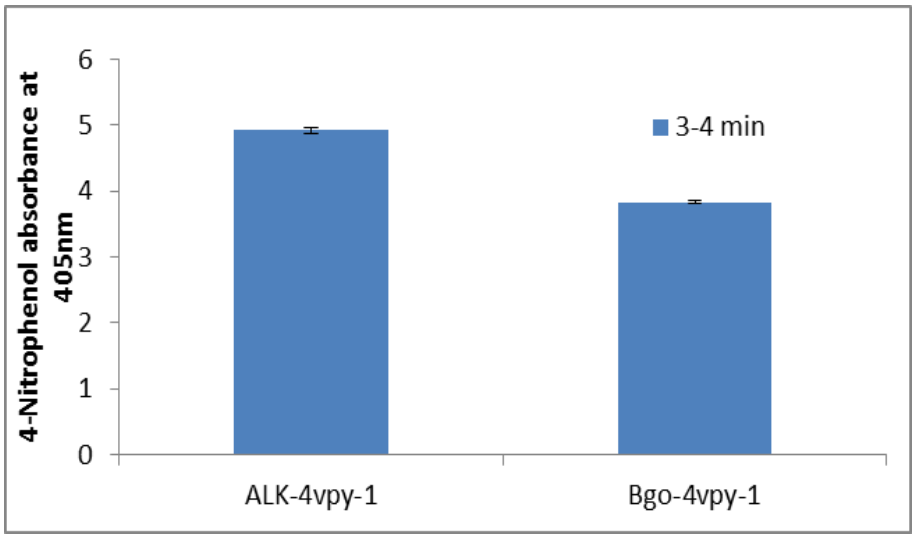


Figure 5-28 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer (100mM and 1M). Polymer bound ALK were stored at 4 ^{0}C overnight, data measured at 405nm wavelength between 3-4 minutes. (Mean ± error, n=3)

Our main focus continued to be finding a way to distinguish selective from non selective template binding using external stimuli to induce changes in the enzyme physiological conditions. From the literature, the alkaline phosphatase was reported to be very stable to slight changes in physiological conditions, such as elevated temperatures and changes in pH. This is because the enzyme has metal ions which stabilise its three dimensional structure. Therefore, in order to destabilise this enzyme would possible require stripping off these ions from the structure.

In a further effort to discriminate the template bound to specific sites from that of the non selective sites, we destabilised the alkaline phosphatase by stripping off metal ions,

using metal chelating compound ethylenediaminetetraacetic acid (EDTA). It was hypothesised that the enzymes bound to the non specific sites might be more easily unfolded into a conformation, where their ions would be acted upon by EDTA. Template bound to the recognition sites would be in a native folded conformation whereby ions would be less exposed and therefore would prevent structural destabilisation.

5.4.6 ALK selectivity towards ALK & Bgo MIPs & incubation with EDTA

The initial challenge was to find the optimum concentration of EDTA to use and the incubation time, such that it would not totally strip off every metal non-selectively from all enzymes bound. To determine the appropriate amount of EDTA, we looked at the effect of different concentrations of EDTA; (1, 5 and 10)-mM upon enzyme activity when incubated for a period of 30 min at 37^0C (see experiment 5.20.1). From this, we found out that using lower concentration of EDTA (1mM) we were able to delay the time required for total destabilisation of the enzyme for a period of more than 30 min.

From the polymer bound enzyme activity (see experiment 5.20.2) results shown in figure 5-29, it can be seen that the enzyme activity decreased from both polymers with increasing incubation time of polymers with EDTA. A possible explanation for the effect of increasing time is that the EDTA was able to penetrate more into the polymer leading to more ions being stripped off from the enzyme. However, the discrepancy in the enzyme activity between ALK-4vpy-1 and Bgo-4vpy-1 was lower than before except for after 10 min where the difference was increased.

In another attempt, we employed a novel technique using enzyme trypsin digestion. The hypothesis was similar to the described above, whereby non specifically bound enzyme may be unfolded and therefore exposed to trypsin, while template bound to the recognition sites might be in a native folded conformation and therefore less exposed to be digested by this enzyme.

Figure 5-29: ALK bound ALK-4vp-1 & Bgo-4vpy-1 activities after incubation with EDTA

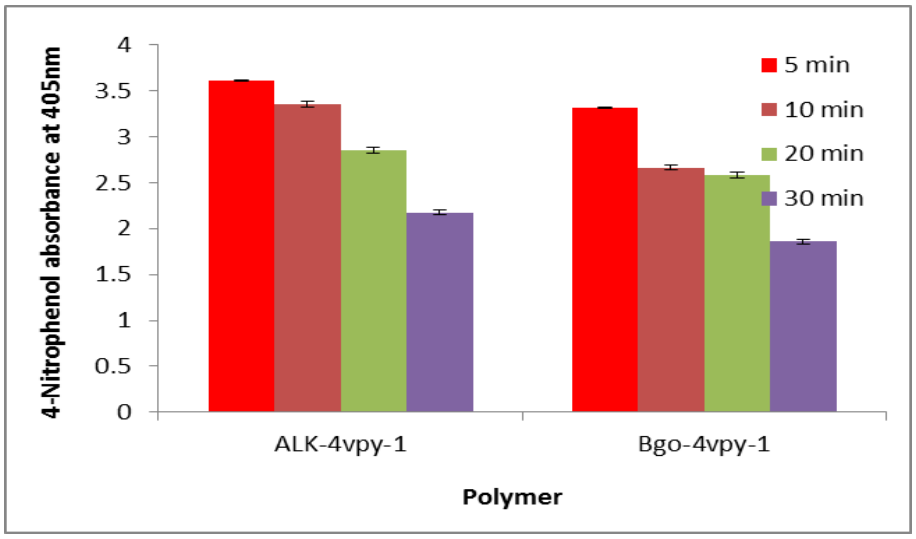


Figure 5-29 Alkaline phosphatase units of activity for 4x 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer (100mM and 1M). Polymer bound ALK were incubated with 1mM EDTA at 37^0C at different for a period of ; 5, 10, 20 and 30 minutes, data measured at 405nm wavelength between 3-4 minutes. (Mean ± error, n=3)

5.4.7 ALK selectivity towards ALK & Bgo MIPs & trypsin digestion

The polymer bound enzyme activities results after polymer was rebound with the alkaline phosphatase and then incubated with trypsin (see experiment 5.22) are shown in figure 5-30. The difference in the enzyme bound activity between the specific polymer ALK-4vpy-1 and Bgo-4vpy-1 was still low, suggesting that trypsin did not deactivate alkaline phosphatase bound to the polymers nor influenced its activity.

Figure 5-30: ALK bound ALK-4vp-1 & Bgo-4vpy-1 MIPs activities after incubation with trypsin

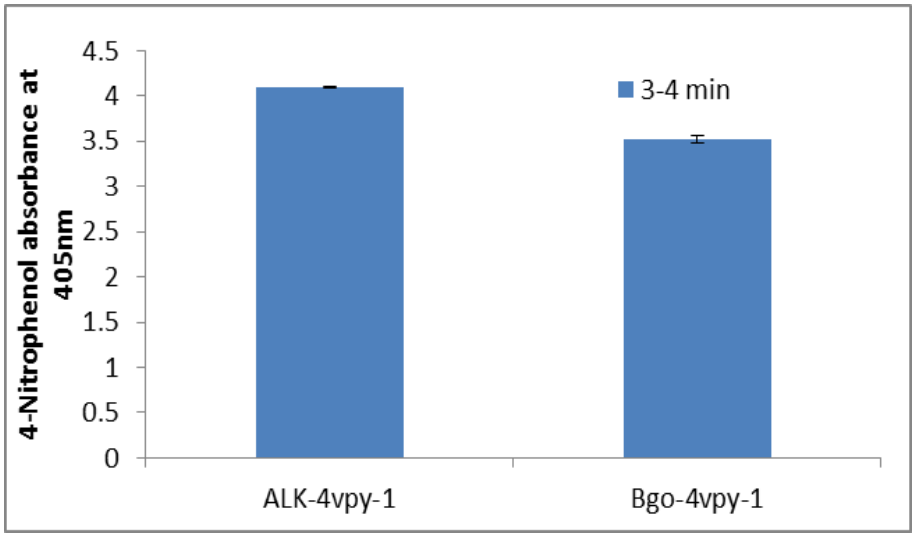


Figure 5-30 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer (100mM and 1M). Polymer bound ALK were incubated with 3ml of trypsin at 9.09µg/ml of sodium phosphate (0.06 M), at pH 8 for 19hrs at 37^0C, data measured at 405nm wavelength between 2-3 minutes. (Mean ± error, n=3)

5.4.8 ALK selectivity towards ALK & Bgo MIPs & BSA substitution

In a final attempt to discriminate the enzyme activity bound to both polymers, we employed BSA solution of different concentration to try to displace the non selective template bound to the both ALK-4vpy-1 and Bgo-4vpy-1 polymers and leave only specifically bound template. The assumption was that BSA being quite hydrophobic would have a greater selectivity for the hydrophobic non specific sites and therefore would displace templates bound to these low selective sites more than the template bound to the strong sites.

The amount of enzyme displaced by the BSA at different concentrations (4mg, 8mg, and 12mg)/ml, (see experiment 5.23) is presented in figure 5-31.

Figure 5-31: ALK in wash from ALK-4vpy-1 & Bgo-4vpy-1 using different concentration of BSA

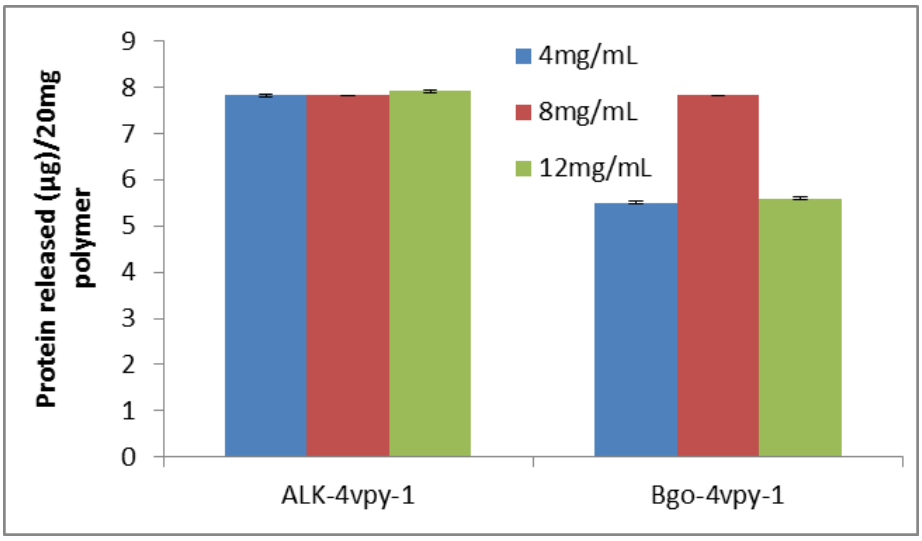


Figure 5-31 Alkaline phosphatase released from 3x 20mg ALK bound imprinted polymers,ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with 2ml of 4, 8, and 12-mg/ ml of diethanolamine buffer at pH 7.9 (10mM) for 15min. (Mean ± error, n=3)

From the results, the concentrations of the template displaced by the BSA solution were comparable for ALK-4vpy-1 and Bgo-4vpy-1 using 8mg of BSA/ml and slightly less by ~ 2µg for Bgo-4vpy-1 rinsed with 4mg and 12mg of BSA/ml.

From the polymer bound enzyme activities shown in figure 5-32, it can be seen that the enzyme activities of the polymers rinsed with 8mg of BSA/ml were comparable, while more activity was observed with ALK-4vpy-1 compared to Bgo-4vpy-1, when the polymers were rinsed with 12 mg and 4mg BSA/ml. This was similar to that observed before. Another feature noted was that even though more enzymes were released from both polymers with 8mg of BSA/ml, the overall remaining activity was greater than for polymers rinsed with 12 mg and 4mg BSA/ml. This was clearly unexpected because it would be assumed that if more templates were released from the polymer it would reduce its total activity.

Again it was clear that it was a significant challenge to discriminate template rebound to selective sites from non selective sites

To further address this issue, we designed a comparative selectivity study of alkaline phosphatase solution spiked with beta glucosidase and rebound to both ALK-4vpy-1 and Bgo-4vpy-1.

Figure 5-32: ALK bound ALK-4vp-1 & Bgo-4vpy-1 activities after wash with BSA

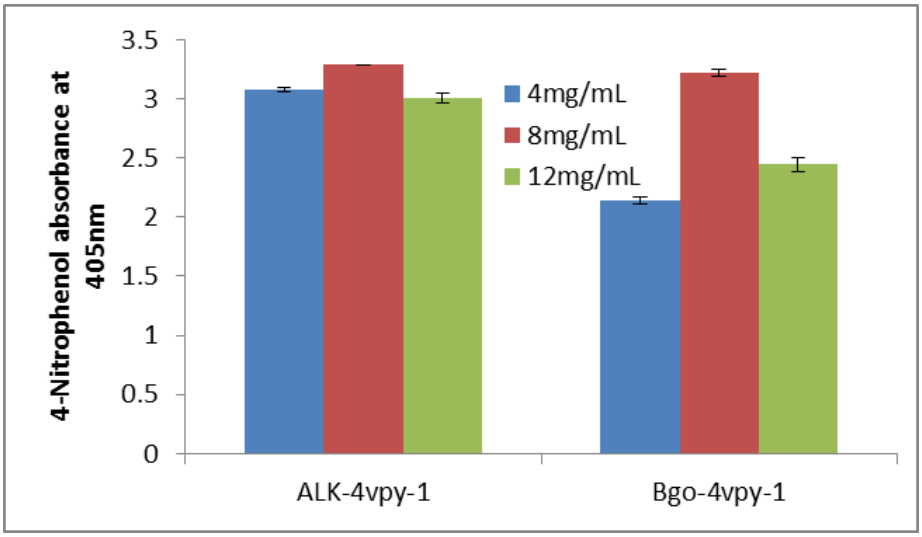


Figure 5-32 Alkaline phosphatase units of activity for 3x20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with 2ml of 4, 8, and 12-mg/ ml of diethanolamine buffer at pH 7.9 (10mM) for 15min diethanolamine data measured at 405nm wavelength between 2-3 minutes. (Mean ± error, n=3)

5.4.9 ALK selectivity toward ALK MIPs & Bgo MIPs in a competitive binding

The alkaline phosphatase selectivity was determined in a competitive environment, whereby the alkaline phosphatase solution was spiked with beta glucosidase molecules (see experiment 5.24) and the results are presented in figure 5-33. From the results, the selectivity of the ALK-4vpy-1 was ~10µg more compared to Bgo-4vpy-1 in a competitive two protein solution. This suggested that the presence of beta glucosidase in

the solution increased the selectivity of the alkaline phosphatase more towards its imprinted polymer ALK-4vpy-1 compared to previous results when alkaline phosphatase was used alone in a single protein solution, although a similar increment was also observed with Bgo-4vpy-1.

The polymer bound enzyme activities results are presented in figure 5-34. From the results, the enzyme bound activity also increased for both polymers compared to that observed previously. This could be as a result of the observed increased template binding to these polymers.

Figure 5-33: ALK uptake by ALK-4vpy-1 & Bgo-4vpy-1 in a competitive binding

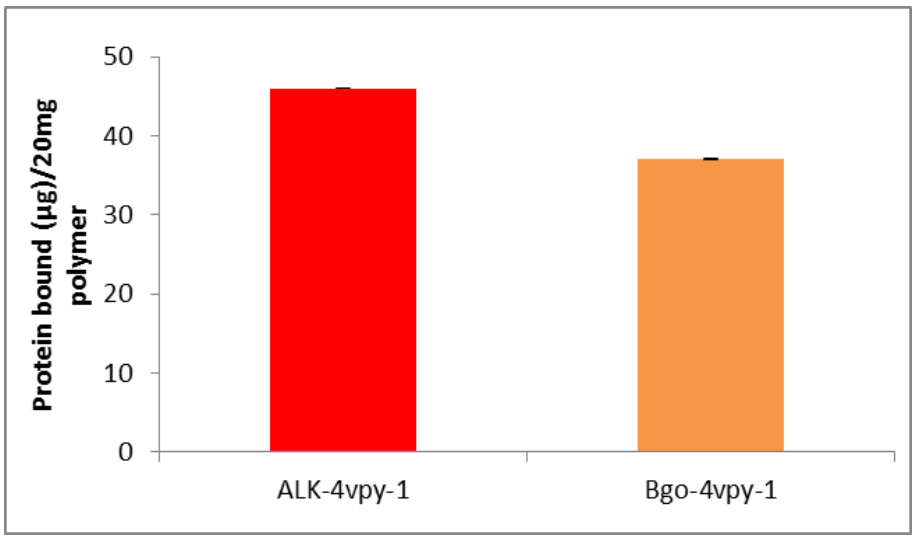


Figure 5-33 Aqueous competitive equilibrium rebinding for 20 mg ALK-4vpy-1and Bgo-4vpy-1, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

In a final attempt, we performed non specific protein binding using a closer molecular weight analogue acid phosphatase.

Figure 5-34: ALK bound ALK-4vp-1 & Bgo-4vpy-1 activities after competitive binding

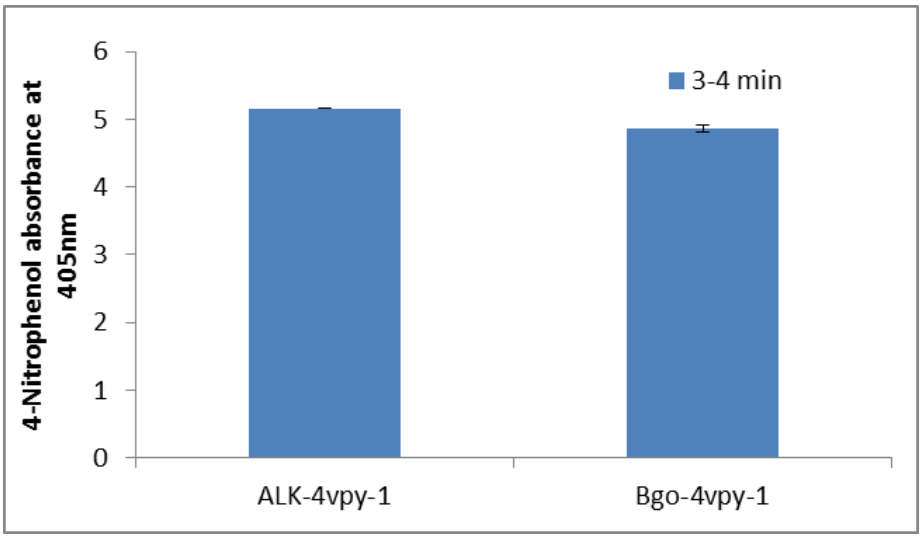


Figure 5-34 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with diethanolamine buffer pH 7.9 (100mM and 1M), data measured at 405nm wavelength between 3-4 minutes. (Mean ± error, n=3)

5.4.10 ACP non specific selectivity towards ALK & Bgo MIPs

The acid phosphatase selectivity towards both polymers is shown in figure 5-35 (see experiment 5.25). From the results, it can be seen that ~44µg bound to ALK-4vpy-1 and 48µg bound to Bgo-4vpy-1; this was large compared to that observed when specific template alkaline phosphatase was used for ALK-4vpy-1 or when acid phosphatase was used for its specific imprinted polymers (ACP 4 and ACP 8). This suggested that the polymers may be very hydrophobic or the use of high pH rebinding solution may have destabilised the acid phosphatase, such that it bound non specifically to both polymers. Disappointingly, it was also seen that the activities of the acid phosphatase bound to both polymers, shown in figure 5-36, were large. The enzyme bound activity was ~44.7% for ALK-4vpy-1 and slightly less for Bgo-4vpy-1 (42.5%) relative to the enzyme initially bound to both polymers. Hence, these polymers can be used for efficient but non-selective binding of the acid phosphatase template.

Figure 5-35: Acid phosphatase uptake by ALK-4vp-1 & Bgo-4vpy-1 in a non selective binding

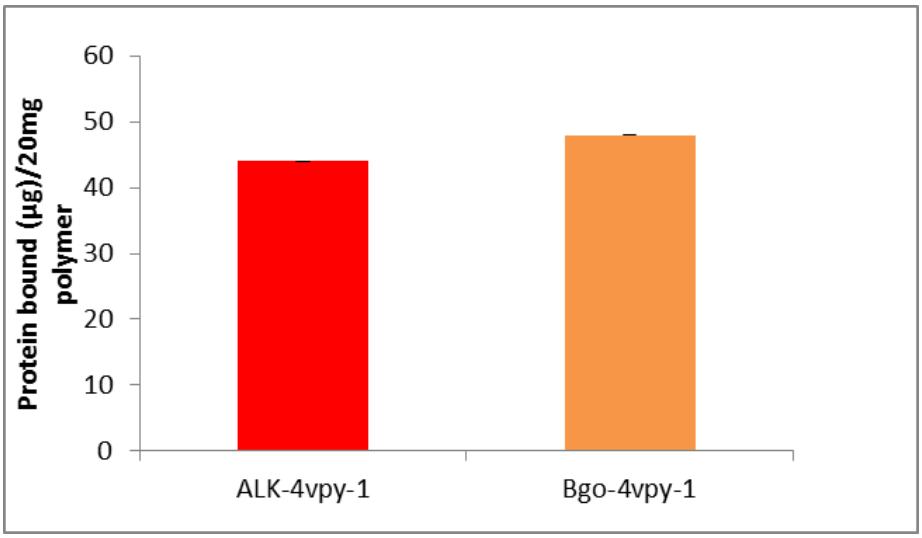


Figure 5-35 Aqueous equilibrium rebinding for 20 mg ALK imprinted polymers, ALK-4vpy-1and Bgo-4vpy-1 with 2ml of 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 7.9 + BSA (1mg/ml)), for 10 minutes. (Mean ± error, n=3)

Figure 5-36: Acid phosphatase uptake by ALK-4vp-1 & Bgo-4vpy-1 activities after non selective binding

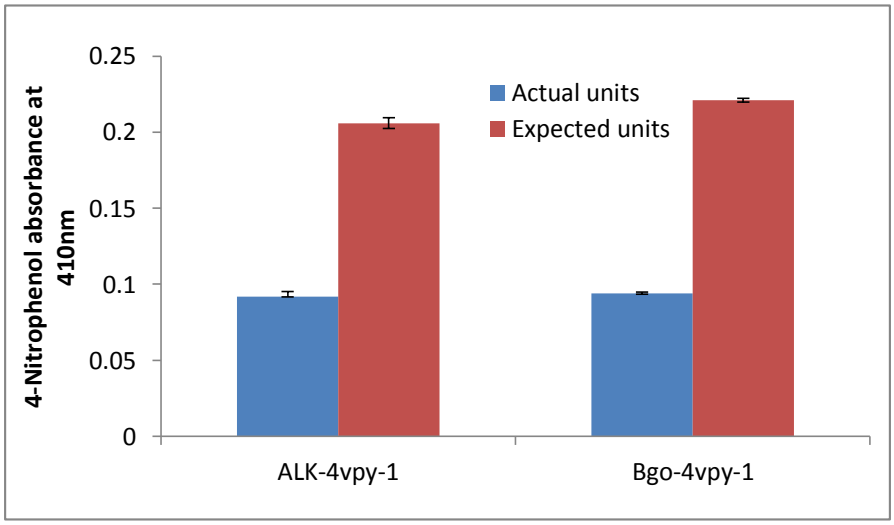


Figure 5-36 Acid phosphatase units of activity for 40mg ACP bound imprinted polymers, ALK-4vpy-1 and Bgo-4vpy-1 after polymer were rinsed with mobile phase, data measured at 410nm wavelength and free ACP using same buffer conditions (expected units obtained from ACP standard curve). (Mean ± error, n=3)

The poorly discriminating template selectivity towards specific polymers led to the design of another set of protein imprinted polymers including, alkaline phosphatase, Beta glucosidase, Bovine serum albumin (BSA)-PCMCs and control polymer made with glycine; all were prepared at low pH 8.0. The rationale behind this lower pH protein-PCMCs formulation was to see if we could imprint the alkaline phosphatase at unstable pH closer to physiological conditions. This would allow developments of this formulation with other proteins which are stable at this pH condition because few proteins are stable at extreme alkaline conditions. In addition, this investigation would give us insightful information into a possibility of imprinting a template prepared in unstable conditions. Furthermore, it would also shed some light if there are possible recognition sites specific for the alkaline phosphatase in ALK-4vpy-1.

5.5 Preparation of ALK, BSA & Bgo MIPs & control polymers using lower pH

The new developed protein imprinted polymers were designed with PCMCs prepared at low pH 8.0. The imprinted polymers were prepared using alkaline phosphatase, Beta glucosidase and BSA, having a molecular weight less than half of the former proteins. Also, the control polymer was prepared without protein. (See experiment 5.26)
All sample PCMCs were prepared at; 8% protein loading; 5% water, in IPA saturated with the excipient glycine. Table 5-5 outlines the polymer compositions used to prepare this new set of polymers.

Table 5-5: Reagents used for the preparation of ALK, BGO, BSA MIPs and control

Polymer Samples	PCMCs/ Glycine (mg)	Enzyme g (mg)	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
ALK -4vpy-2	1725	150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8

ALK-4vpy- B2 (control)	1725	----	0.6, 5.708	3.0 15.135	59,08 0.359	4.8
Bgo-4vpy-2	1725	150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8
BSA-4vpy	1725	150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8

All polymers prepared were glassy and hard monoliths and were processed similarly to that described previously for ALK-4vpy-1 polymer.

5.5.1 ALK selectivity towards ALK, Bgo & BSA MIPs and control

The preferential binding ability of the ALK-4vpy-2, ALK-4vpy-B2, Bgo-4vpy-2 and BSA-4vpy was characterised by aqueous equilibrium rebinding using alkaline phosphatase amounts only in solution (see experiment 5.27). The alkaline phosphatase bound to the imprinted polymer ALK-4vpy-2 and control polymer ALK-4vpy-B2 as well as cross selective polymer BSA-4vpy were comparable, except with Bgo-4vpy-2 whereby the enzyme bound was slightly lower by ~5µg (figure 5-37). This suggested that the non selective protein binding was the dominant feature in alkaline phosphatase selectivity and lowering the solution pH may have caused some alkaline phosphatase instability, leading to poor formation of the alkaline phosphatase binding sites.
From the alkaline phosphatase bound polymer activities shown in figure 5-38, it can be seen that the alkaline phosphatase activity bound to the cross selective polymer BSA-4vpy was higher compared to both specific polymer ALK-4vpy-2 and its control ALK-4vpy-B2 by almost 2 fold, followed by beta glucosidase polymer (Bgo-4vpy-B2) which showed slightly higher enzyme bound activity. The reasons for this are not clear.

In order to try to lessen this effect, we conditioned all polymers with BSA added to the conditioning phase to cover non specific sites (see experiment 5.28).

The alkaline phosphatase rebinding amounts shown in figure 5-39 for all polymers were comparable, except for Bgo-4vpy-2, which bound slightly less enzyme by ~ 4µg. However, the overall alkaline phosphatase binding was reduced by a factor ~1.6-1.8 compared to that observed before, suggesting that the addition of the BSA in the conditioning phase might have reduced the selectivity of the polymers towards the alkaline phosphatase.

Figure 5-37: ALK uptake by ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & control ALK-4vpy-B2

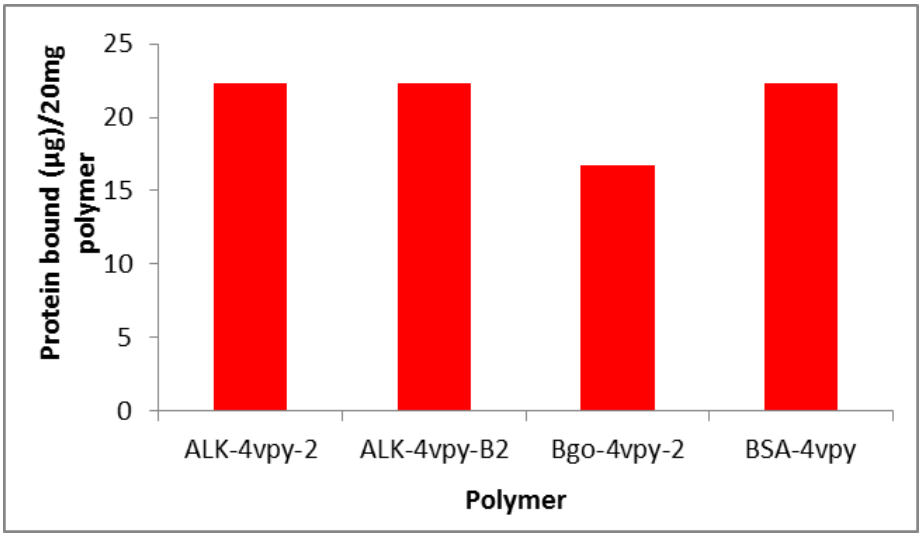


Figure 5-37 Aqueous equilibrium rebinding for 20 mg for ALK-4vpy-2, ALK-4vpy-B2 Bgo-4vpy-2 and BSA-4vpy, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8), for 10 minutes. (Mean ± error, n=3)

From the polymer bound enzyme activity shown in figure 5-40, it can be seen that the enzyme bound BSA-4vpy was again dominating the polymer activity which exceeded all other polymers; Bgo-4vpy-2, ALK-4vpy-2 and ALK-4vpy-B2, although the overall activities were lower than before. This is most likely due to the reduced alkaline phosphatase bound to the polymers initially. Another feature noted was that the activity of the Bgo-4vpy-2 exceeded both the ALK-4vpy-2 and its control ALK-4vpy-B2 even though it bound less amount of enzyme.

Figure 5-38: ALK bound ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & ALK-4vpy-B2 activities

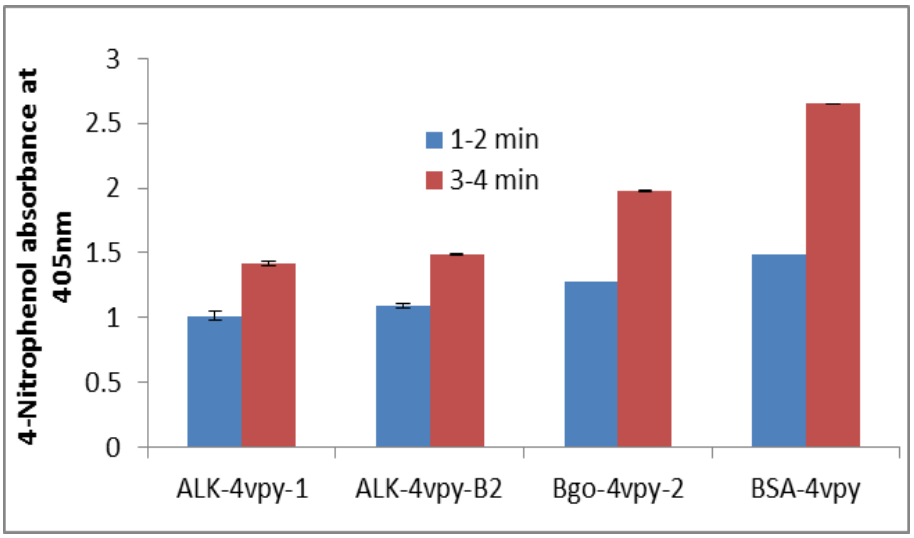


Figure 5-38 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-2, ALK-4vpy-B2, Bgo-4py-2 and BSA-4vpy after polymer were rinsed with mobile phase, data measured at 405nm wavelength between 1-2 and 3-4 minutes. (Mean ± error, n=3)

Figure 5-39: ALK uptake by ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & ALK 4vpy-B2; conditioned with BSA

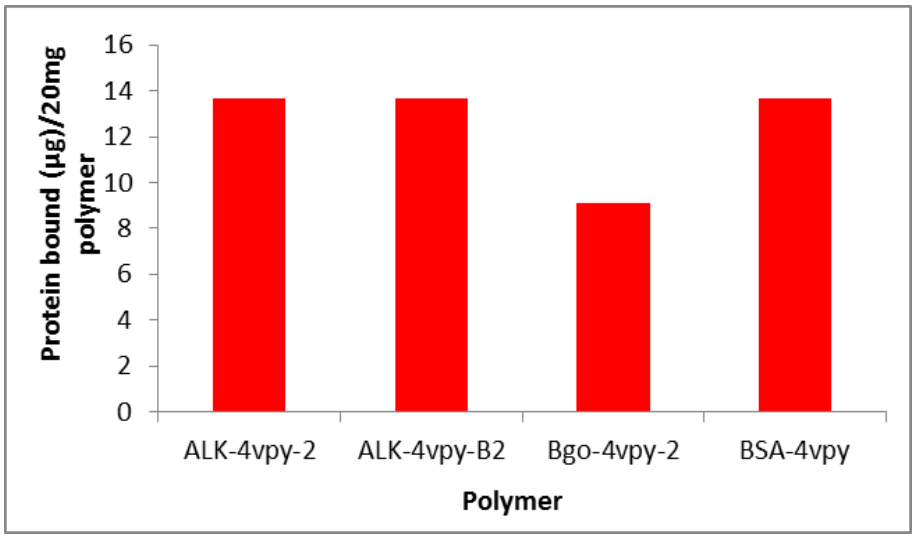


Figure 5-39 Aqueous equilibrium rebinding for 20 mg for ALK-4vpy-2, ALK-4vpy-B2 Bgo-4vpy-2 and BSA-4vpy, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8), for 10 minutes. (Mean ± error, n=3)

Figure 5-40: ALK bound ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & ALK-4vpy B2; conditioned with BSA

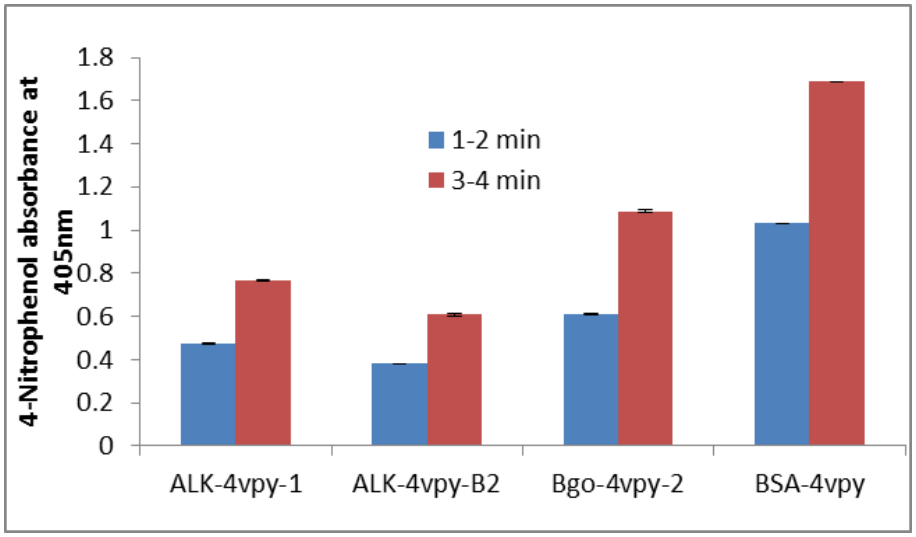


Figure 5-40 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-2, ALK-4vpy-B2, Bgo-4py-2 and BSA-4vpy after polymer were rinsed with mobile phase, data measured at 405nm wavelength between 1-2 and 3-4 minutes. (Mean ± error, n=3)

In further attempt to reduce non specific protein binding, we added BSA to both conditioning and mobile phases (see experiment 5.29). This increased alkaline phosphatase rebinding to BSA-4vpy compared to the addition of the BSA in the conditioning phase only, but reduced the enzyme binding to all other polymers (figure 5-41). We thought that the increased alkaline phosphatase binding to BSA-4vpy might be due to BSA complexed with alkaline phosphatase in the rebinding solution and therefore the complex bound to sites in the BSA-4vpy polymer, which already bound by the BSA from the conditioning phase. The BSA residues may have acted as a nucleation site for the BSA-alkaline phosphatase complex in the rebinding solution.

Another possible explanation was that the BSA may have bound hydrophobically to hydrophobic elements of the polymers and therefore inhibited the correct binding of the alkaline phosphatase towards these polymers.

The increased selectivity of alkaline phosphatase to the BSA-4vpy compared to other polymers was also reflected in the polymer bound enzyme activity, which was superior

to all other polymers (figure 5-42). The trend in the polymer bound enzyme activity results was comparable to that observed previously.

Figure 5-41: ALK uptake by ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & ALK 4vpy-B2 after conditioned and rebound with BSA in solution

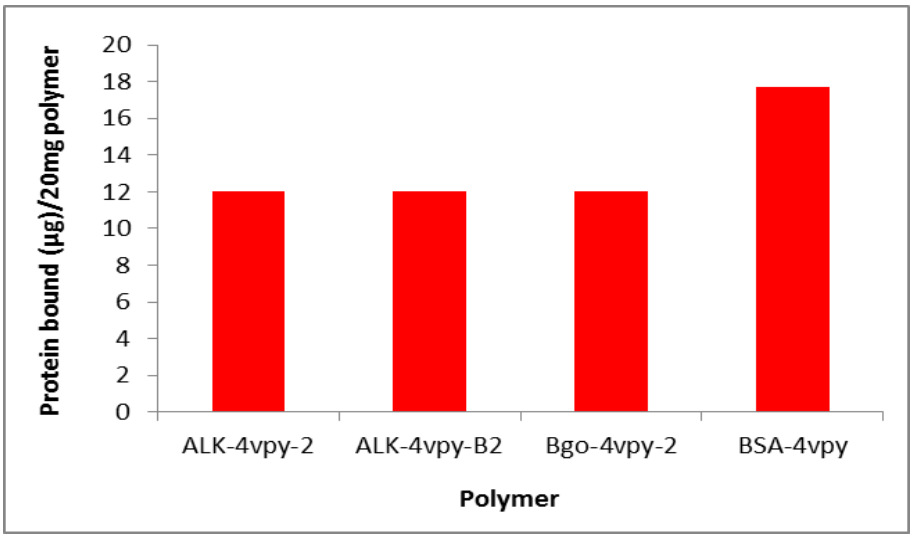


Figure 5-41 Aqueous competitive equilibrium rebinding for 20 mg for ALK-4vpy-2, ALK-4vpy-B2 Bgo-4vpy-2 and BSA-4vpy, with 2ml of alkaline phosphatase 0.1mg/ml of mobile phase composition (10mM diethanolamine buffer at pH 9.8 + BSA (1mg/ml)) with addition of BSA (0.1mg/ml) in mobile and conditioning phases, for 10 minutes. (Mean ± error, n=3)

From this investigation, it can be clearly seen that no imprinted sites were created in ALK-4vpy-2, and therefore this suggested that it was not possible to imprint alkaline phosphatase in unstable pH conditions.

Figure 5-42: ALK bound ALK-4vpy-2, Bgo-4vpy-2, BSA-4vpy & ALK-4vpy -B2 activities; conditioned and rebound with BSA

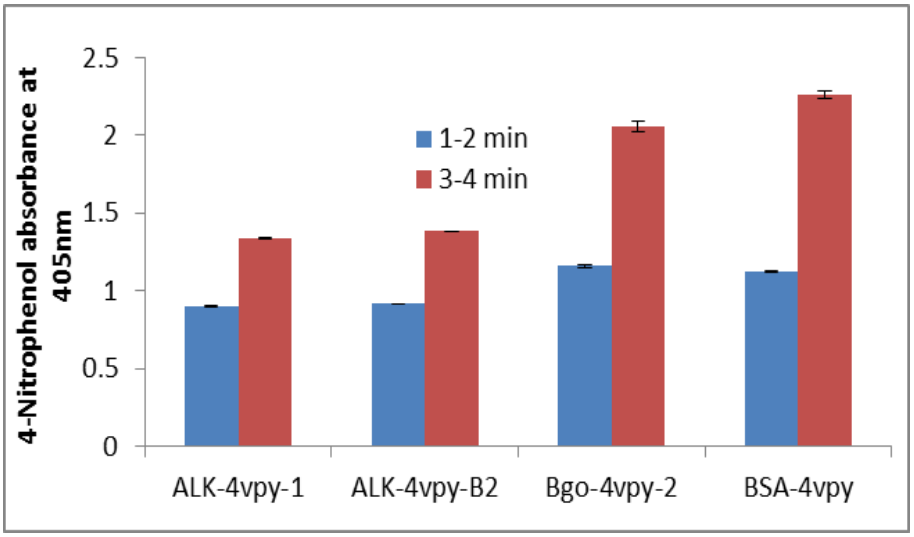


Figure 5-42 Alkaline phosphatase units of activity for 20mg ALK bound imprinted polymers, ALK-4vpy-2, ALK-4vpy-B2, Bgo-4py-2 and BSA-4vpy after polymer were rinsed with mobile phase, data measured at 405nm wavelength between 1-2 and 3-4 minutes. (Mean ± error, n=3)

5.6 Conclusion

The investigation carried out using acid phosphatase as a template successfully demonstrated that the imprinted polymer possessed specific sites compared to non imprinted polymer, although these sites were small in number. For example, ~>93% of the template bound to the imprinted polymer was in active conformation, whilst only ~75% of the template bound to the control polymer was in similar conformation. Therefore, 18% of the template may have bound to the specific sites in the imprinted polymer.

From this study, new polymer formulations were developed using hydrophobic interaction as means of alkaline phosphatase recognition. A preferential rebinding study demonstrated that the hydrophobic imprinted polymer possessed specific sites compared to non imprinted polymer and other multiple controls, i.e. hydrophilic polymer.

The pre-modification of the hydrophobic polymer with addition of diethylene glycol vinyl ether (DEGVE) showed no significant difference in template selectivity or release from the polymer.

From competitive rebinding studies, the specific template imprinted polymer demonstrated better selectivity towards the template compared to the non specific polymer imprinted with molecular analogue. Furthermore, the template bound to the specific polymer demonstrated better reaction kinetics, and therefore may be in a better conformation.

It can be concluded that using a hydrophobic mechanism as a means of template recognition may be possible. Despite certain improvements, the polymers do not possess the functionality required for use in solid phase protein extraction from aqueous solution.

Chapter 5: Experiment Section

Materials

Acid phosphatase from potato (P1146) , Alkaline phosphatase from bovine intestinal mucosa (P7640), p-Nitrophenyl phosphate (104), Diethanolamine (D-8885), Magnesium chloride hexahydrate (M-0250), Beta glucosidase from almond, Trypsin from bovine pancreas, Ethylenediaminetetraacetic acid (EDTA), Ethylene glycol, Methanol, Alumina, Urea, Na_2HPO_4, NaH_2PO_4, $MgCl_2$, NaOH, Isopropanol, Acetone, Acetonitrile (HPLC grade), were purchased from Sigma-Aldrich Co. UK. BSA from bovine serum, Polyethylene glycol 200 (MW 200), Polyethylene glycol 300 (MW 300), K_2HPO_4 (MW 174.18), KH_2PO (136.09), KCl, were purchased form Fluka UK. Glycine, Acetic acid, Citric acid (MW 210), Tri-sodium citrate were purchased from (Fisher scientific UK), Molecular sieves (MERCK Germany), N_2 (University store). All materials were used as received, with no further purification.

Centrifugal concentrator Millipore ultra-free CL PTFE yellow 2.0mL 0.22µm pore size (Fisher Scientific), Isolute double fritted (2x20µm) filtrate, Glass cuvette with a path length 1.0 cm (Fisher brand cuvettes), 15ml tall vial and 25ml vial screw top from (Fisher scientific UK), Durapore Membrane filters of pore size 0.22µm and 0.45µm (Millipore Ireland), Deionised filtered water (Resistivity 18.2 MΩ) was obtained from in-house Millipore water purification system.

Methacrylic acid (MAA), 4-vinly pyridine (4vpy), ethylene glycol dimethacrylate (EGDMA), Di (ethylene) glycol vinyl ether (DEGVE), were obtained from Aldrich, and 2, 2'-azobisisobutyronitrile (AIBN) from (BDH laboratory supplier England UK)

Material Preparation

EDGMA, MAA and 4VPy were purified by passing through a column packed with alumina; MAA and 4VPy were then distilled under reduced pressure. AIBN was purified by re-crystallisation from acetone, DEGVE was used without further purification and acetonitrile was dried by molecular sieves.

Apparatus –Spectrophotometer (Beckman Coulter, DU 800) with temperature controller (Beckman Coulter), Centrifuge Beckman Coulter (large Eppendorf), (Techno), Centrifuge (Eppendorf AG2233, Hamburg, Germany) and Incubator (Techno, Ori-block, OB-3), Rotary evaporator. Blood rotary and Incubator (Stuart Scientific), motor and pestle and 25-50 sieves, a magnetic stirring bar and hot plate stirrer (Fisher Scientific UK), water bath (Techno), FT-IR spectrometer (Perkin Elmer spectra one), Meter balance (Metter-Toledo AE200), pH meter (Metter-Toledo MP120).

Citrate buffers

Stock solutions of 0.1 M of citric acid and 0.1M of sodium citrate were prepared by dissolving 2.10g of citric acid /l of dH_2O and 29.4g of tri-sodium citrate /l of dH_2O.
To prepare **pH 4.8 (0.1M),** 40 ml of citric acid and 60ml of tri-sodium citrate were added. 1ml of this buffer was again diluted in 9 ml of dH_2O to make 0.01M buffer strength.

Diethanolamine buffer

Stock solution of 1.0 M of diethanolamine buffer was prepared by adding 21.028g of diethanolamine in 200ml of dH_2O dissolved with $MgCl_2.6H_2O$, under magnetic stirring. The buffer is adjusted to pH 9.8 or 7.8 using 5M HCl.
To prepare 10mM of the diethanolamine buffer, 1ml of the buffer was added to 99ml of dH_2O.

Experiment 5.1.1

Preparation of acid phosphatase PCMCs for imprinting

Acid phosphatase PCMCs (ACP PCMCs) were prepared at different enzyme loading; 1% (ACP 1), 2%(ACP 2), 4% (ACP 4), 8%(ACP 8) and blank crystals control without the enzyme 0% (ACP B), and 5% water in IPA saturate with glycine. The technique used for PCMCs production was similar to that described previously for the batch production process.

The reagents used for acid phosphatase PCMCs and blank control production

ACP loading (%)	Protein (mg)	Glycine (mg)	Glycine solution (100mg/ml) (ml)	Saturated IPA (ml)
ACP (1%)	10	990	9.90	188.1
ACP (2%)	20	980	9.80	186.2
ACP (4%)	40	960	9.60	182.4
ACP B	----	960	9.60	182.4
ACP (8%)	80	920	9.20	174.8

For example, the preparation of ACP at 4 % enzyme loading was as follows: 40mg of the lyophilised enzyme powder were weighed and were dissolved into 9.6 ml of citrate buffer at pH 4.8 (0.01M) containing 960mg of excipient glycine prepared at 100mg of glycine /ml of the buffer. The acid phosphatase /glycine solution was divided into two parts of 4.86 ml. Each sample solution was then added dropwise into 91.2 ml of IPA saturated with excipient (glycine) in a round bottom flask under agitation provided by a magnetic stirring bar for approximately 1-2 min. Following co-precipitation, PCMCs were allowed to sediment and the supernatant was decanted. The PCMCs formed were washed with 2 x 50ml of IPA and then centrifuged to separate the PCMCs and the supernatant IPA was decanted. Finally, the PCMCs were washed with 2 x 50ml of dry acetonitrile prior to the imprinting process.

Experiment 5.1.2

Preparation of acid phosphatase imprinted polymers

For example, the preparation of the acid phosphatase imprinted polymer ACP 4 at 4% enzyme loading was as follows: 1000 mg of acid phosphatase PCMCs were added into 8ml vial containing 4.69 ml of dry acetonitrile (porogen). The vial was then partially immersed into an ice cooled water bath, followed by the addition of functional monomers; MAA (0.0232 mmoles, 0.002), 4VPy (0.019 mmoles, 0.002g), the sample

was then put in the rotary machine for complex formation for a period of 15min and finally DEGVE (2.391mmoles, 0.316) and cross linker EDGMA (16.162 mmoles, 3.2g) were added to the mixture. The mixture was purged with nitrogen for 5 min to eliminate any soluble oxygen and finally initiator AIBN (57.1mg) was added. The vial was then sealed with a screw top and parafilm, and incubated at 60^0C for 48 hrs under constant rotation on a blood rotor machine to ensure even distribution of PCMCs in the mixture. The reagent compositions employed for the acid phosphatase imprinted polymers are presented in the table below:

Reagents used for preparation of acid phosphatase MIPs

Sample	PCMCs &Glycine (mg)	Enzyme Loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
ACP 1	1000	1% (10)	0.0005, 0.0058	0.0005, 0.00476	3.2, 16.1624	0.319, 2.4137	57.1, 0.348	4.69
ACP 2	1000	2% (20)	0.001, 0.0116	0.001, 0.0095	3.2, 16.162	0.318 2.4062	57.1, 0.348	4.69
ACP 4	1000	4%(40)	0.002, 0.0232	0.002, 0.0190	3.2, 16.162	0.316 02.391	57.1, 0.348	4.69
ACP 8	1000	8% (80)	0.004, 0.0465	0.004, 0.03806	3.2, 16.162	0.312, 2.3608	57.1, 0.348	4.69
ACP B (control)	Glycine (1000)	----	0.002, 0.0232	0.002, 0.0190	3.2, 16.162	0.316 02.391	57.1, 0.348	4.69

Experiment 5.1.3

Polymer processing

Polymer processing was similar to that described in chapter 4, whereby after 48hrs of the polymer preparation, glass vials containing the monolithic polymers were broken to release the polymers, which were then ground with a mortar and pestle into a manageable fine particles, sieved at 20-50 µm mesh size and the powders were suspended in acetone to sediment similar particle size. MIPs were then washed with acetonitrile to remove unreacted functional monomers MAA and 4-Vpy. The disappearance of the 4vinyl pyridine RP-HPLC peaks from the wash solvent was an indication of the complete removal of these monomers from the polymer. For example, 100mg of each polymer ACP 1, ACP 2, ACP 4 & ACP8 were weighed and were put into 40 ml centrifuged tube followed by the addition of 20 ml of dry acetonitrile. The tubes containing polymer suspensions were put on a flask shaker machine for gentle shaking for a period of 10 min, after which the samples were again centrifuged and 50µl of the supernatants were injected directly into the RP-HPLC.

Experiment 5.1.4

Determination of acid phosphatase activity prior to the template extraction

For this experiment, 40 mg of ACP 8 polymer powder were weighed and put into an eppendorf with filter. The sample was preconditioned with 2ml of citrate buffer at pH 4.8 for 10 min, after which the polymer was centrifuged to remove the conditioning phase. The polymer was then suspended in 0.5ml of 90mM citrate buffer pH 4.8 at 37^0C for 3 min to equilibrate the polymer, followed by the addition of 0.5ml of 15.2mM of 4-nitro phenol phosphate (substrate) and incubated for another 10min. The polymer suspensions were centrifuged and the supernatant was added directly to a vial containing 4ml of 100mM of NaOH (aq), the resulting solution mixture was spectroscopically monitored at 405nm.

Experiment 5.1.5

Acid phosphatase extraction from monoliths (template extraction process)

Acid phosphatase extraction from the polymer was carried out using a sequential washes described below. For 20mg polymer, 2ml of each solvent were employed:

1. 2 x 10mM citrate buffer to dissolve carrier molecules glycine
2. 3 x 80% ethylene glycol and 20% 10mM citrate buffer
3. 3 x 60% ethylene glycol,20% 10mM citrate buffer and 20% acetic acid v/v
4. 1 x 8M urea
5. 2 x 10mM citrate buffer
6. 1 x 60% Ethylene glycol and 20% 10mM citrate buffer and 20% acetic acid
7. 1 x 8M urea
8. 2 x 10mM citrate buffer
9. 2 x dH_2O

After template extraction, all polymers were dried in an incubator at 37^0C for ~48hrs.

Experiment 5.2

The effect of ethylene glycol upon the acid phosphatase activity

4 x of 0.1mg of acid phosphatase/ml of citrate buffer at pH 4.8 (0.01M) each containing a different percentage of ethylene glycol; (10, 20, 30, 40-%); v/v were prepared. The acid phosphatase activities were determined using a common established assay adapted from Sigma, similar to that described before (refer to chapter 2) using the following link; http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/p1146enz.Par.0001.File.tmp/p1146enz.pdf) (see chapter 8 appendix 8.1).

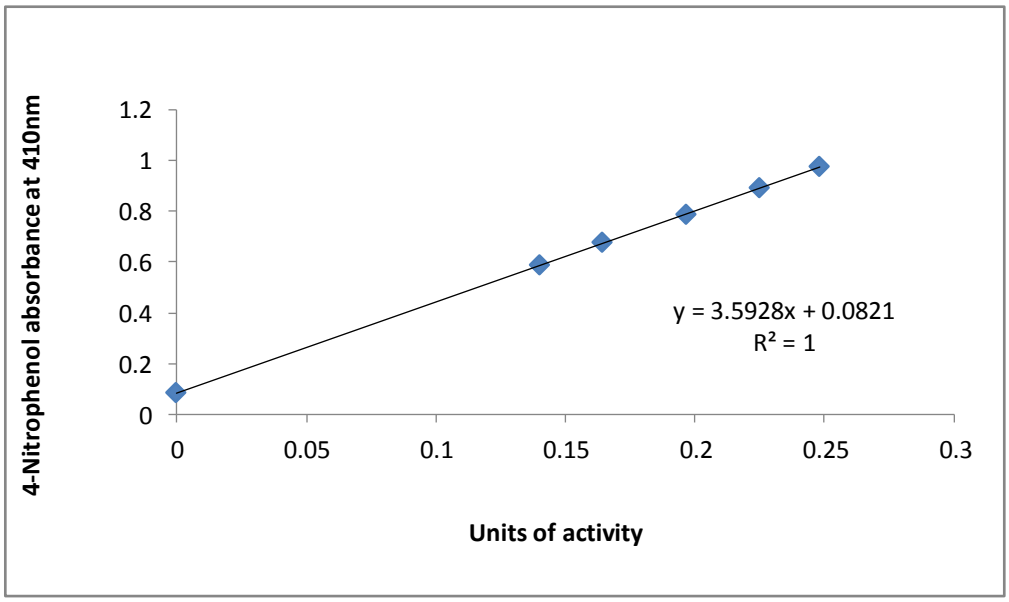
The percentage unit of the acid phosphatase activity was computed as follows:

Actual units/ expected units (1mg=4.6 units) x 100%

A standard curve was drawn using 1mg of acid phosphatase lyophilised powder with the expected units of 1mg =4.6units of activity, and the serial dilutions were carried out

ranging from 0.05-0.25 units. The enzymatic reaction involved acid phosphatase hydrolysing 4-nitropheol phosphate to 4-nitrophenol which absorb at 410 nm.

Acid phosphatase activity standard curve



Experiment 5.3.1

Acid phosphatase selectivity towards acid phosphatase imprinted polymers in aqueous equilibrium batch rebinding

40 mg of each polymer ACP 1, ACP 2, ACP 3 and ACP 4 were weighed and were put into centrifuge eppendorfs tubes with filter and were preconditioned with a phase (CP1) and rebound with solution (RBP1) shown in the table, each for 15min, after which the samples were centrifuged and the polymers were separated from the supernatant. 100µl of aliquots from each polymer were then analysed using enzyme activity assay similar to that described in the **experiment 5.2.**

The acid phosphatase bound (µg) was calculated as follows;

Initial acid phosphatase activity in solution prior to the rebinding {(actual units/ expected units) x 100µg (added to the rebinding solution)} – final acid phosphatase activity after rebinding {(Actual units/ expected units) x 100µg (added to the rebinding solution)}

	Protein	Buffer	Phase
Conditioning phase (CP1)	----	Citrate buffer pH 4.8 (10mM)	90% buffer, 10% ethylene glycol; v/v
Rebinding phase RBP1	Acid phosphatase 100 µg	Citrate buffer pH 4.8 10mM	100µg/ ml (70% buffer, 30% ethylene glycol; v/v)

Experiment 5.3.2

Determination of the polymer bound enzyme activity

The enzyme bound polymers samples ACP 1, ACP 2, ACP 4 and ACP 8 were rinsed with 10mM citrate buffer pH 4.8 for 10 min, after which the sample was centrifuged and supernatant decanted. Following this, 0.5ml of citrate buffer at pH 4.8 (90 mM) was added to the polymer suspensions. The polymer samples were then equilibrated at 37^0C for 3 min in an incubator prior to the addition of 0.5ml of 15.2mM of PNPP. The samples were again incubated for another 10 min, after which the samples were again centrifuged and the supernatant was added directly into 4ml of 0.1M of NaOH (aq). Following this, the polymers were centrifuged and 1ml of each supernatant was spectrophotometrically monitored at 410nm. The units of the acid phosphatase activity were determined and deduced from the standard curve (**see experiment 5.2**).

Experiment 5.3.3

Determination of the polymer bound enzyme activity by quenching the enzyme with NaOH (aq)

The experimental procedure was similar to that described before in the **experiment 5.3.1,** except that the enzyme bound polymer activity was slightly modified from that of **experiment 5.3.2.** Here, the polymers were not separated from the reaction medium; the

polymer suspension was then added directly into the vials containing 4ml of 0.1M NaOH (aq). Following this, the polymers were centrifuged and 1ml of each supernatant was spectrophotometrically monitored at 410nm.

Experiment 5.4

Acid phosphatase selectivity in aqueous equilibrium rebinding with the application of rapid heat shock

40mg of both ACP 4 and ACP B were weighed, preconditioned and rebound using similar procedures to those described in the **experiment 5.3.1,** except that after template rebinding, the enzyme bound polymers were suspended into 0.5ml of the buffer (90 mM) at pH 4.8 and were subjected to a rapid heat shock for 1min at 60^0 C. Following this, the enzyme bound activity procedure carried out was similar to that described in the **experiment 5.3.2**.

Experiment 5.5

Increasing the number of acid phosphatase bound polymer activities

40mg of both ACP 4 and ACP B were weighed, and were preconditioned and rebound using similar procedures to those described in the **experiment 5.3.1,** except that both polymers were again subjected to a second phase of the enzyme bound polymer activities by the addition of more substrate. The enzyme bound activity procedure carried out was similar to that described in the **experiment 5.3.2**

Experiment 5.6

Acid phosphatase selectivity in aqueous equilibrium with increasing rebinding time form 15min to 22hrs

40mg of both ACP 4 and ACP B were weighed, preconditioned and rebound using similar procedures to those described in the **experiment 5.3.1,** except that the template rebinding time was increased from 15 min to 22hrs. Both polymers were then rinsed with buffer. Following this, the enzyme bound activity procedure carried out was similar to that described in the **experiment 5.3.2**.

Experiment 5.7

Acid phosphatase selectivity in aqueous equilibrium rebinding with reduced template concentrations in the rebinding solution

40mg of both ACP 4 and ACP B were weighed and rebound using similar procedures to those described in the **experiment 5.3.1,** except that the concentration of acid phosphatase in the rebinding solution was reduced to ~35µg/ml instead of ~100µg/ml. Following this, the enzyme bound activity procedure carried out was similar to that described in the **experiment 5.3.2**, except that the polymer rinsing phase was eliminated.

Experiment 5.8

Acid phosphatase selectivity in aqueous equilibrium rebinding with reducing template concentrations in solution and elimination of ethylene glycol in the mobile phase

40mg of both ACP 4 and ACP B were weighed and preconditioned with 90% citrate (0.01M) pH 4.8 and 10% ethylene glycol for 10min using similar procedures described to those in the **experiment 5.3.1.** The polymer samples were rebound with 27µg acid phosphatase/ml of citrate buffer of pH 4.8 (0.01M) without ethylene glycol in the mobile phase. Following this, the enzyme bound activity procedure carried out was similar to that described in the **experiment 5.3.2**.

Preparation of alkaline phosphatase imprinted polymer

Experiment 5.9

Preparation of alkaline phosphatase PCMCs for imprinting

Alkaline phosphatase PCMCs were prepared at 8% enzyme loading, 5% water and IPA as precipitating solvent saturated with glycine as an excipient. The technique used for PCMCs production was similar to that described previously for the batch the production process. Alkaline phosphatase PCMCs for imprinting purposes were prepared as follows; 100mg of the lyophilised alkaline phosphatase powder were dissolved in

11.5ml of diethanolamine buffer at pH 9.8 (0.01) containing 1150mg of glycine at 100mg /ml of buffer. The sample volume was then divided into two parts of ~ 5.75 ml of protein/carrier solution. The protein/carrier solution was then added drop-wise into 110 ml of IPA saturated with excipient in a round bottom flask under agitation provided by a magnetic stirring bar for approximately 1-2 min. Following co-precipitation, PCMCs were allowed to sediment and the supernatant was decanted. The PCMCs formed were washed with 2 x 50ml of IPA and then centrifuged to separate the PCMCs and the supernatant IPA was decanted. Finally, the PCMCs were washed with 2 x 50ml of dry acetonitrile prior to the imprinting process.

Experiment 5.10

The alkaline phosphatase imprinted polymers ALK-4vp-1, ALK-4vpy-B1, ALK-MAA and ALK-MAA-B

The reagents used for the preparation of alkaline phosphatase imprinted polymers are shown in the table below:

Sample polymers	PCMCs (mg)	Enzyme (mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
ALK -4vpy-1	1150	100		0.4, 3.8045	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy-B1	Glycine 1150	------		0.4, 3.8045	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy MAA-1	1150	100	0.2, 2.32	0.2, 0.1902	2.0, 10.0898	40.0, 0.244	3.2
ALK-4vpy-MAA-B1	Glycine 1150	------	0.2, 2.32	0.2, 0.1902	2.0, 10.0898	40.0, 0.244	3.2

The techniques and procedures used for the preparation of the alkaline phosphatase polymers were similar to those described previously for acid phosphatase polymers in the **experiment 5.1.2**

Experiment 5.10.1

Alkaline phosphatase selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1, ALK-4vpy-B1, ALK-MAA, ALK-MAA-B

40 mg of each polymer ALK-4vpy-1, ALK-4vpy-MAA and their controls ALK-4vpy-B1, ALK-4vpy-MAA-B were weighed and were pre-conditioned with CP2 phase and rebound with RBP2 phase shown in the table below. Following the template rebinding, the polymer samples were centrifuged and 100µl of the aliquots were removed for enzyme activity assay, using a common established method from Sigma (see chapter 8 appendix 8.2) to determine the enzyme bound to the polymers

	Protein	Buffer	Phase solution
CP2	----	Diethanolamine pH 9.8 (10mM)	90% buffer, 10% methanol ; v/v
RBP2	alkaline phosphatase 100 µg	Diethanolamine pH 9.8 (10mM)	100µg/ml and BSA 1mg/ml; of buffer

Experiment 5.10.2

Determination of the polymer bound alkaline phosphatase activity

The enzyme bound polymers samples ALK-4vpy-1, ALK-4vpy-B1, ALK-4vpy-MAA-1, and ALK-4vpy-MAA-B1 were rinsed with 1ml of 10mM diethanolamine buffer at pH 9.8 for 10 min, after which the samples were centrifuged and supernatant decanted. The polymer samples were then added into separate 15ml centrifuge tubes containing 2.7 ml of 1.0 M diethanolamine buffer at pH 9.8. The polymer suspensions were then equilibrated at 37^0C for 3 min in an incubator prior to the addition of 300µL of 150mM of 4-nitrophenol. After 2-3 and 4-5 min time intervals, 0.5ml of each sample was pipetted into a 0.5ml eppendorfs tube with filter and centrifuged. Aliquots of 200µL were then removed from supernatant and were diluted with 800µl of same buffer. After the dilution, 1ml of each sample was spectrophotometrically monitored at 405nm. The

total absorbance was computed as follows: absorbance obtained after dilution x dilution factor (5) - absorbance obtained from the unbound polymer control (0.1)

Experiment 5.11

Chemical characterisation of ALK-4vpy-B1 and ALK-4vpy-MAA–B polymers using FTIR

For this investigation, ALK-4vpy-B1 and ALK-4vpy-MAA–B polymer powders samples were put onto the FTIR machine to determine the structural compositions of the polymers. The FTIR spectra generated were examined.

Experiment 5.12

2nd preparation of alkaline phosphatase PCMCs and imprinted polymer

Alkaline phosphatase PCMCs were prepared as follows: 150mg of the lyophilised alkaline phosphatase powder were weighed and were dissolved into 17.25 ml of diethanolamine buffer at pH 9.8 (0.01) containing 1725mg of glycine at a concentration of 100mg /ml of buffer. The sample was then divided into two parts of ~ 8.625 ml. Each sample was then added dropwise into 164 ml of IPA saturated with excipient in round bottom flask under agitation provided by a magnetic stirring bar for approximately 1-2 min. Following co-precipitation, the PCMCs were allowed to sediment and the supernatant was decanted. The PCMCs formed were washed with 2 x 50ml of IPA and then centrifuged to separate the PCMCs and the supernatant IPA was decanted. Finally, the PCMCs were washed with 2 x 50ml of dry acetonitrile prior to the imprinting process. The reagents used for the preparation of alkaline phosphatase imprinted polymers with increased template are shown in the table below:

Sample	PCMCs/ (mg)	Enzyme loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	DEGVE g mmol	AIBN mg, mmol	AcN (ml)
ALK - 4vpy-1	1725	150		0.6, 5.708		3.0 15.135	59,08 0.359	4.8

ALK – MAA	1725	150	0.6 6.984	--	--	3.0, 15.135	59.08, 0.360	4.8
ALK-4vpy – DEGVE	1725	150	--	0.3, 2.854	0.3 2.27	3.0, 15.135	59.08, 0.360	4.8

The procedure used for the preparation of the imprinted polymers was similar to that described before for acid phosphatase polymers in the **experiment 5.1.2.**

Experiment 5.13

Alkaline phosphatase selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1, ALK-MAA and ALK-4vpy-DEGVE; using mobile phase pH 9.8

20 mg of each polymer; ALK-4vp-1, ALK-MAA and ALK-4vpy-DEGVE were weighed, preconditioned and rebound with similar conditioning and rebinding phase to those employed in the **experiment 5.10.1.** Following this, all polymers were rinsed with 0.1 M and 1.0 M diethanolamine buffer for 10 min. The enzyme bound polymer activity procedure carried out was similar to that procedure described in the **experiment 5.10.2.**The enzymatic product formed was spectrophotometrically monitored at 405nm after 1-2 and 3-4 min time intervals.

Experiment 5.14

Alkaline phosphatase selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1, ALK-MAA and ALK-4vpy-**DEGVE; using mobile phase pH 7.9**

The investigation carried out was similar to that described in the **experiment 5.13,** except that the pH of the conditioning and mobile phase was reduced to pH 7.9 (0.01M) instead of pH 9.8. However, the enzyme bound polymer activities procedure carried out was similar to that described before in the **experiment 5.10.2.**

Experiment 5.15

Preparation of competitive polymer beta glucosidase (Bgo)

Beta glucosidase imprinted polymer (Bgo-4vpy-1) was prepared using Beta glucosidase-PCMCs. The preparation of the PCMCs and Bgo imprinted polymer procedure was similar to that described before for alkaline phosphatase polymers described in the **experiment 5.10**. Reagents used for the preparation of Bgo imprinted polymer are shown in the following table:

Sample	PCMCs/ (mg)	Enzyme Loading %(mg)	MAA g, mmol	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
Bgo-4vpy-1	1725	150	--	0.6, 5.708	3.0, 15.135	59.08, 0.360	4.8

Experiment 5.16

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 9.8

2 x 20 mg of ALK-4vp-1and Bgo-4vpy-1were weighed, preconditioned and rebound with alkaline phosphatase using a similar procedure to those described in the **experiment 5.10.1.** The enzyme bound polymer activities procedure carried out was similar to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm after 1-2, 3-4 min and 5-6 time intervals.

Experiment 5.17

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 7.9

2 x 20 mg of ALK-4vp-1and Bgo-4vpy-1were weighed, preconditioned and rebound using similar reagents and procedure to those described in the **experiment 5.16,** except that the pH of the conditioning and mobile phase was reduced to pH 7.9 (0.01M) instead of pH 9.8. However, the enzyme bound polymer activities procedure carried out was at pH 9.8, similar to that described before in the **experiment 5.10.2.**

Experiment 5.18

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 7.9 and application of heat shock

2 x 20 mg of ALK-4vp-1and Bgo-4vpy-1were weighed, preconditioned and rebound using a similar procedure to that described in the **experiment 5.17,** except that following this, all polymers samples were rinsed with buffer at pH 7.9 and were then suspended in diethanolamine buffer at pH 7.9. One sample was then partially submerged in a water tank set at 60 ^{0}C for 1 min rapid heat application and the other for prolonged heat application for ~40 min. Finally, the polymer bound enzyme activities procedure was carried out at pH 9.8 similar to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm after 2-3 and 4-5 min time intervals.

Experiment 5.19

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 7.9 and application of low temperature

The experimental procedure was similar to that described in the **experiment 5.18,** except that the polymers samples were rinsed with buffer at pH 7.9 and were stored in the fridge at lower temperature 4^{0}C overnight. Finally, the polymer bound enzyme activities procedure was carried out at pH 9.8, similar to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm at a single time point 3-4 min.

Experiment 5.20.1

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 7.9 and application of EDTA

3 x 40µg of ALK/ml of diethanolamine buffer at pH 7.9 with different concentration of EDTA; (1, 5 and 10)-mM/ml were each put into different eppendorfs with filter. The samples were then incubated for a period of 30 min at 37^{0}C, after which the enzyme activity procedure was carried using Sigma protocols, as described before in the **experiment 5.10.1.**

Experiment 5.20.2

4x 20mg of ALK-4vpy-1 and Bgo-4vpy-1 were weighed and the experimental procedure was similar to that described in the **experiment 5.19,** except that the polymer samples were rinsed with 1ml of diethanolamine buffer at pH 7.9 and finally 1ml of 1mM EDTA dissolved in a buffer was added to all polymers and the samples were incubated at 37^0C at different time intervals; 5, 10, 20 and 30 min. After each time point, the polymer samples were centrifuged and EDTA solution was decanted. Finally, the polymer bound enzyme activities were carried out at pH 9.8, using a similar procedure to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm at a single time point 3-4.

Experiment 5.21

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1; using mobile phase pH 7.9 and application of EDTA and prolonged heating shock application.

20mg of ALK-4vpy-1 and Bgo-4vpy-1 were weighed and the experimental procedure was similar to that described in the **experiment 5.20.1.** Following this, the samples were incubated with 1ml of 1mM EDTA for 10min. The polymer samples were then centrifuged and the supernatants were decanted. Both polymers were added into separate eppendorfs containing 1ml buffer and the samples were then partially submerged in a water tank set at 60 ^{0}C for prolonged heating treatment of ~30 min, after which the samples were again centrifuged and the supernatant decanted. Finally, the polymer bound enzyme activities determination was carried out using a similar procedure to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm at a single time point 3-4 min.

Experiment 5.22

ALK selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1 and application of Enzyme digestion technique

This investigation was similar to that described in the **experiment 5.18** except that following this, the polymer samples were rinsed with 1ml of buffer. Finally, both polymers were incubated with 3ml of trypsin at 9.09µg/ml of sodium phosphate (0.06 M), at pH 8 for 19hrs at 37^0C. In addition, two controls were prepared using 3ml of alkaline phosphatase at 9.09µg/ml sodium phosphate pH 8 (0.06 M) with and without trypsin. After the experimental period, the enzymes bound polymers activities determinations were carried out using a similar procedure to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm at a single time point of 2-3 min.

Experiment 5.23

Alkaline phosphatase selectivity in aqueous equilibrium rebinding for polymers; ALK-4vp-1 and Bgo-4vpy-1 and application of different concentration of BSA in the washing phase

3 x 20 mg were weighed and the experimental procedure carried out was similar to that described in the **experiment 5.18,** except that the polymer samples were rinsed with different buffer condition containing different concentration of BSA; 4, 8, and 12-mg/ ml of diethanolamine buffer at pH 7.9 (10mM) for 15min, after which the samples were again centrifuged and 100µl of aliquots were removed for template released analysis. . Finally, the polymer bound enzyme activities determinations were carried out using a similar procedure to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at an absorbance of 405nm at a single time point 2-3 min.

Experiment 5.24

ALK selectivity in aqueous equilibrium rebinding for; ALK-4vp-1 and Bgo-4vpy-1

20mg of ALK-4vpy-1 and Bgo-4vpy-1 were weighed and were preconditioned using a similar procedure to that described in the **experiment 5.10.1** The sample polymers were

then rebound with 1ml of rebinding phase containing; 200µg/ml of alkaline phosphatase and 200µg/ml of beta glucosidase were dissolved in a diethanolamine buffer at pH7.9 (0.01M), the final concentration of each enzyme was ~100µg/ml in the rebinding phase. Following this, the polymer samples were rinsed with 1ml of diethanolamine buffer at pH7.9 for 10min, after which the polymer samples were centrifuged and the supernatant decanted. Finally, the polymer bound enzyme activities determinations were carried out using a similar procedure to that described in the **experiment 5.10.2.** The product formed was spectroscopically monitored at 405nm after 2-3min.

Experiment 5.25

ACP selectivity in equilibrium rebinding with ALK-4vp-1& Bgo-4vpy-1

20mg of ALK-4vpy-1 and Bgo-4vpy-1 were weighed and were preconditioned as described before in the **experiment 5.10.1**. The polymer samples were rebound with rebinding phase containing 100µg of acid phosphatase/ ml of diethanolamine buffer at pH7.9. Finally, the polymer bound enzyme activities determinations were carried out using a similar procedure to that described for acid phosphatase imprinted polymer in the **experiments 5.3.1 and 5.3.2**

Experiment 5.26

Preparation of 3rd set of polymers; ALK, BSA, beta glucosidase and blank

The polymers were prepared using a similar procedure to that described in the **experiment 5.12,** except that the PCMCs were prepared at pH 8.0 instead of 9.8. The PCMCs were prepared at; 8% protein loading, 5% water, in IPA saturated with the excipient glycine. The reagents used for the preparation of ALK-4vpy-2, ALK-4vpy-B2, Bgo-4vpy-2 and BSA-4vpy-1are shown in the table below:

Polymer Samples	PCMCs (mg)	Enzyme g (mg)	4-Vpy g, mmol	EDGMA g, mmol	AIBN mg, mmol	AcN (ml)
		150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8

ALK -4vpy-2	1725					
ALK-4vpy- B2	1725		0.6, 5.708	3.0 15.135	59,08 0.359	4.8
Bgo-4vpy-2	1725	150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8
BSA-4vpy	1725	150	0.6, 5.708	3.0 15.135	59,08 0.359	4.8

Experiment 5.27

ALK selectivity in aqueous equilibrium rebinding towards ALK-4vpy-2, ALK-4vpy-B2, Bgo-4vpy-2 and BSA-4vpy-1

20mg of each polymer were weighed and the experimental procedures were similar to that described in the **experiments 5.10.1 and 5.10.2,** except that BSA was not added in the rebinding solution.

Experiment 5.28

Alkaline phosphatase selectivity in equilibrium rebinding towards ALK-4vpy-2, ALK-4vpy-B2, Bgo-4vpy-2 and BSA-4vpy-1 with BSA in the rebinding solution

20mg of each polymer were weighed and the experimental procedure was similar to that described in the **experiments 5.10.1 and 5.10.2**.

Experiment 5.29

Alkaline phosphatase selectivity towards ALK-4vpy-2, ALK-4vpy-B2, Bgo-4vpy-2 and BSA-4vpy-1 with BSA in the rebinding and conditioning phases

20mg of each polymer were weighed and the experimental procedure was similar to that described in the **experiment 5.10.1 and 5.10.2,** except that BSA was also added to the conditioning phases.

Chapter 6 : Physical evaluation and chemical characterisation of polymers

6.1 Introduction

Molecular imprinted polymers prepared in the preceding chapters (table 6-1) were evaluated to determine their structural integrity and chemical composition. This was carried out to give a better understanding their behavioural characteristics in relation to their performance. Details of the experiments can be found at the end of the chapter.

Table 6-1: Show polymers prepared in the preceding chapters

Chapter	Polymer Sample	Template	Functional monomers	Initiation method
5	ALK-4vpy-1	Alkaline phosphatase	4vpy	Thermal
,,	ALK-4vpy-B1	Control (glycine)	4vpy	Thermal
,,	ALK-4vpy-2	Alkaline phosphatase	4vpy	Thermal
,,	ALK-4vpy-B2	Control (glycine)	4vpy	Thermal
,,	ALK-MAA-4vpy	Alkaline phosphatase	MAA, 4vpy	Thermal
,,	ALK-4vpy-DEGVE	Alkaline phosphatase	4vpy, DEGVE	Thermal
,,	ALK-MAA	Alkaline phosphatase	MAA	Thermal
,,	Bgo-4vpy-1	Beta glucosidase	4vpy	Thermal
,,	Bgo-4vpy-2	Beta glucosidase	4vpy	Thermal
,,	BSA-4vpy	BSA	4vpy	Thermal
,,	ACP 4	Acid phosphatase	MAA, 4vpy, DEGVE	Thermal
,,	ACP 2	Acid phosphatase	MAA, 4vpy, DEGVE	Thermal

,,	ACP B	Acid phosphatase	MAA, 4vpy, DEGVE	Thermal
3	L2	Lysozyme	MAA, 4vpy	Thermal
3	R2	RNase A	MAA, 4vpy	Thermal
4	P1	Lysozyme	MAA, 4vpy	Thermal
,,	P2	Lysozyme	MAA, 4vpy, DEGVE	Thermal
,,	P3	Lysozyme	MAA, 4vpy, DEGVE	UV
,,	B1	Control (glycine)	MAA, 4vpy, DEGVE	Thermal
,,	B2	Control (glycine)	MAA, 4vpy, DEGVE	UV

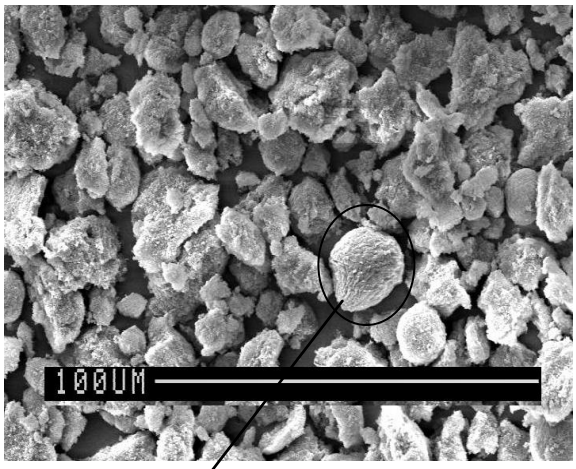
6.2 Physical evaluation

6.2.1 SEM and light microscopy of MIPs and control

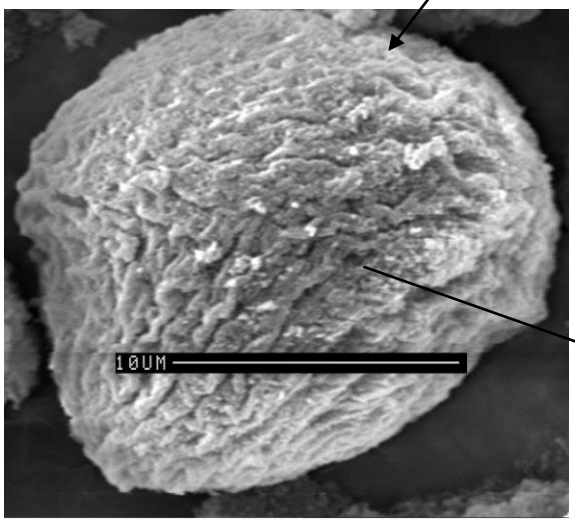
SEM and light microscopy are routinely used to visualise the shape, size and surface characteristics of polymer particles. These properties greatly influence polymer behaviour, functionality and application. For brevity, we only looked at ALK-4vpy-1 and its control, using both SEM and light microscopy.

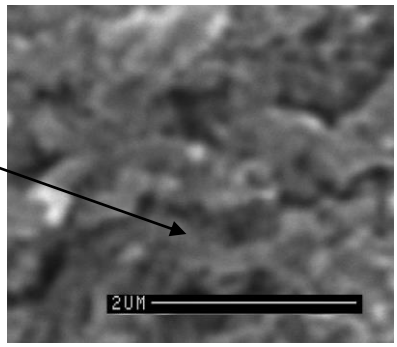
The SEMs of these monolithic polymers are shown in figure 6-1 (a-f). From figure 6-1 (a), the ALK-4vpy-1 polymer particles are far from homogeneous with large distributions of particle sizes and shapes. At higher magnification of the single particle (figure 6-1 (b)), it can be seen that the shape of the particle is not spherical and it has long groove like depressions (figure 6-1 (c)). The particles sizes of the control polymer ALK-4vpy-B1 (figure 6-1; d-e) were also heterogeneous; however, the shape and porosity were poorly defined.

Figure 6-1: SEM of ALK-4vpy-1(a-c) MIPs and control ALK-4vpy-B1 (d-f)

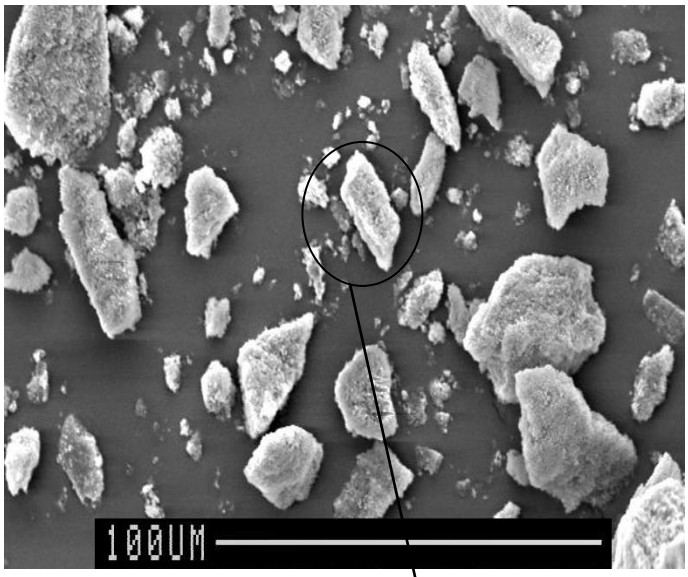


a) Magnification x 410

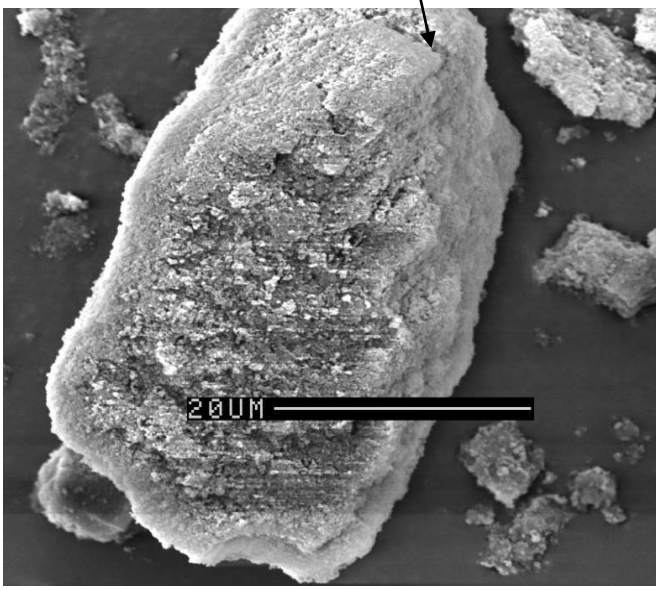




b) Magnification x 4100 **c) Magnification x 17300**



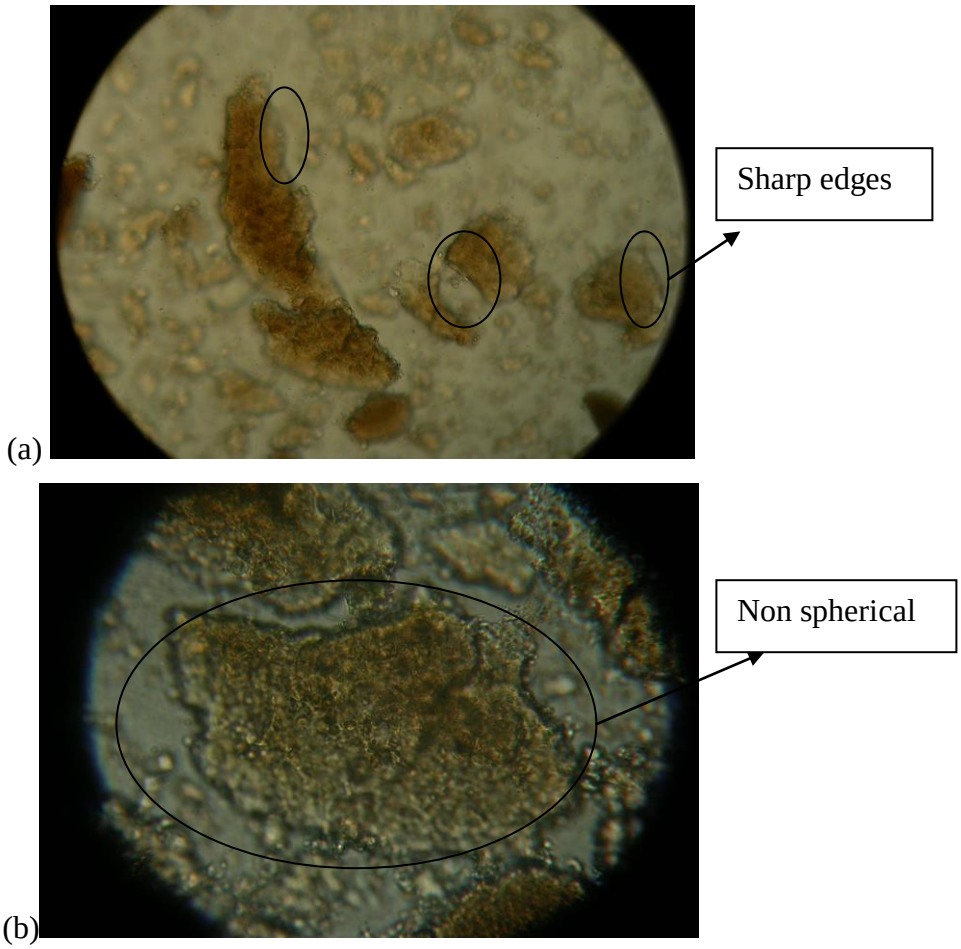
d) Magnification x 1290



e) Magnification x 1290

To further investigate the polymer structural integrity, we examined the polymer particles under light microscopy and the photographs taken are shown in figures 6-2 (a) and (b).

Figure 6-2: Light micrograph of ALK-4vpy-1 MIPs



From both images, it can be seen that the particle has sharp edges and rough surfaces, similar to that observed with SEM.

In order to get insight into the surface area and porosity characteristics, we examined a few polymer samples using Brunauer, Emmett and Teller (BET) analysis.

6.2.2 Surface area and porosity characterisation of MIPs and control

The BET technique involves a fixed mass of dry polymer subjected to nitrogen gas over a series of fixed pressures. The sorption isotherm is then deduced from the gas sorbed on the surface of the pores as a function of applied pressure. From this, the specific surface area (m^2/g), specific pore volume (ml/g), average pore diameter and pore size

distributions were determined (see experiment 6.2). For brevity, we only present sample BET graphs for the polymer ALK-4vpy-1 (figure 6-3) and its control ALK-4vpy-B1 (figure 6-4). The results of other polymers are given in table 6-2.

Figure 6-3: BET analysis of ALK-4vpy-1 MIPs

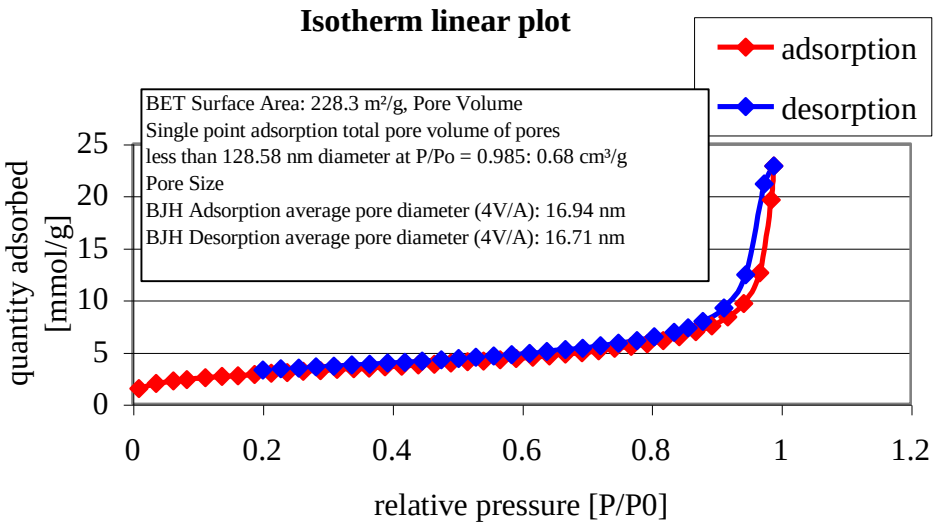


Figure 6-4: BET analysis of control ALK-4vpy-B1

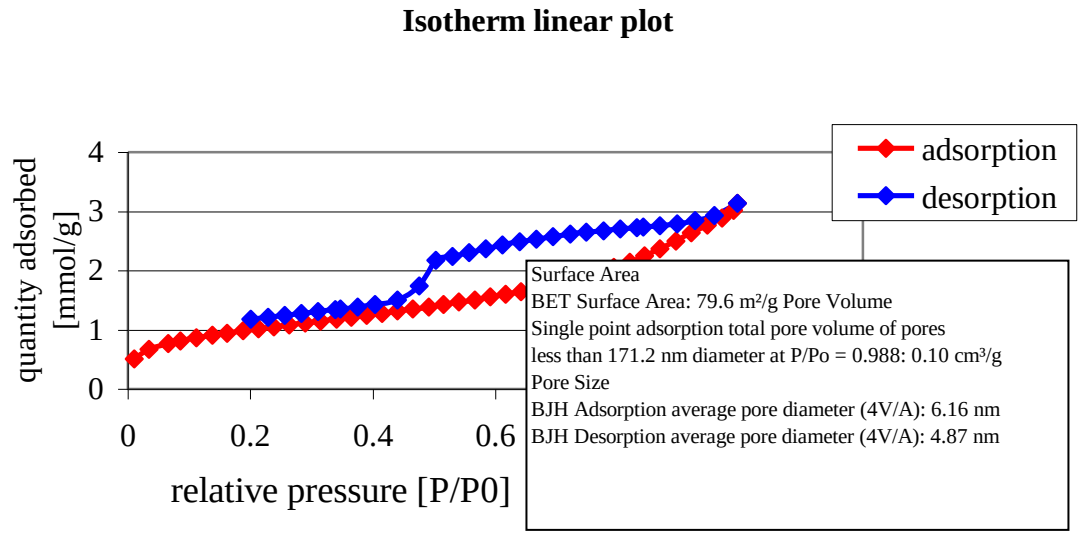


Table 6-2: BET analysis of polymers

Sample	Surface Area m^2/g	Pore Volume single point adsorption total volume of pores (cm^3/g)	Average Pore Diameter	
			Adsorption (nm)	Desorption (nm)
ALK-4vpy-1	228.3	< 128.58 nm diameter at P/Po = 0.985: 0.68	16.94	16.71
ALK-4vpy-B1	79.6	< 171.2 nm diameter at P/Po = 0.988: 0.10	6.16	4.87
ALK-MAA-4vpy	67.2	<120.55 nm diameter at P/Po = 0.984: 0.16	13.69	13.54
ALK-4vpyDEGVE	294.8	<124.77 nm diameter at P/Po = 0.984: 0.61	12.73	12.83
ALK-MAA	191.1	< 129.0 nm diameter at P/Po = 0.985: 0.41	11.95	11.91
Bgo-4vpy-1	258.3	<120.67 nm diameter at P/Po = 0.984: 0.78	16.1	15.99
BSA-4vpy	239.2	< 125.26 nm diameter at P/Po = 0.984: 0.71	17.95	17.57
ACP 4	348.66	<135.672 nm diameter at P/Po = 0.986: 0.73	12.05	12.31
ACP B	338.8	< 119.8 nm diameter at P/Po = 0.9838: 0.73	11.78	11.81
L2	227.4	<133.05 nm diameter at P/Po = 0.9855: 0.72	15.82	15.79
R2	199.8	<117.30 nm diameter at P/Po = 0.983: 0.69	17.40	16.09
P1	259.3	<123.78 nm diameter at P/Po = 0.984: 0.57	12.58	12.68
P2	323.4	<126.95 nm diameter at P/Po = 0.985: 0.67	12.64	12.95
B1	313.9	<135.87 nm diameter at P/Po = 0.986: 0.66	12.01	12.11

With reference to this table, almost all polymers had average surface areas above 100m^2/g, except for ALK-MAA-4vpy and ALK-4vpy-B1, which had areas of ~80 and ~ 67- m^2/g respectively and low pore volumes. The exact reason for this was unknown. It was assumed that from imprinting glycine above its pI in ALK-4vpy-B1 may have caused this effect. A decrease in the surface area (~2-3 fold) and pore volume (~4 fold) in ALK-MAA-4vpy was also noted.

Adding DEGVE monomer in ALK-4vpy-DEGVE polymer increased the surface area by ~ 66m^2/g compared to ALK-4vpy-1. A similar increase was also observed with lysozyme imprinted polymer P2 and its control B1 compared to P1 polymer prepared in the absence of this monomer. However, it was not clear whether the addition of DEGVE or the reduction of functional monomer may have caused this effect. This was also evidently observed with acid phosphatase imprinted polymer ACP 4 and its control ACP B. Both polymers had comparable surface area and pore volume to the lysozyme imprinted polymer P2 and its control polymer B1, suggesting that DEGVE was contributing to this effect.
More interestingly, DEGVE monomer might not only have lessened the polymer hydrophobicity but also modified polymer physical characteristics.
Overall, it was clear that monomer composition greatly influenced polymer morphological characteristics.

The other question that we addressed was whether the aforementioned parameters influenced protein binding behaviour. From comparing the ALK-4vpy-1 and ALK-4vpy-DEGVE affinities towards the imprinted alkaline phosphatase, or L2 and R2 or P2, P1 and B1, it was not clear whether these parameters influenced protein binding behaviour. In order to get insight into the physical changes of the polymers in relation to the mobile phase, we investigated the swelling properties of the polymers using a gravimetric technique.

6.2.3 Swelling properties of MIPs and control polymers

In the gravimetric technique, a known weight of polymer was measured before and after incubation with the mobile phase solution (see experiment 6.3). The solvent uptake by the polymer was determined as follows:
Solvent uptake (g) / polymer (g) =

(Initial weight of (polymer + dH_2O) - Final weight of (polymer + dH_2O) after centrifuged to remove unabsorbed dH_2O).

With reference to the results summarised in table 6-3, ALK-MAA-1 absorbed a significant amount of water/ solvent compared to all other polymers. This was expected because the polymer will be very hydrophilic as a result of using only hydrophilic monomers (in contrast to ALK-4vpy-1, a polymer made with only hydrophobic monomers).
More interestingly, the hydrophobic polymer ALK-4vpy-1 bound more solvent compared to the polymer made with the combination of both MAA and 4vpy, i.e. ALK-4vpy-MAA and its control ALK-4vpy-MAA-B. This however was not expected because it was assumed that by adding MAA in ALK-4vpy-MAA polymer hydrophilicity would improve and therefore it would retain more solvent. The addition of DEGVE in ALK-4vpy-DEGVE slightly increased solvent uptake by ~0.32g of water/g of polymer compared to ALK-4vpy-1. In addition, this was also observed with the lysozyme imprinted polymers P2 and P3 and their controls B1, B2 compared to P1 prepared without this monomer. We assumed that, the DEGVE may have contributed to this effect.

Another feature observed was lower polymer swelling capacity observed with Bgo-4vpy-1 compared to ALK-4vpy-1, even though both polymers used similar reagents. However, the exact reason for this was not known.

Table 6-3: Summary of swelling properties of MIPs and control polymers

Template	Sample Polymer	Polymer(g)	Mobile phase (g)	Mobile phase (g) /polymer g)
Alkaline phosphatase	ALK-4vpy-1	0.0247	0.1107	4.482
Alkaline phosphatase	ALK-MAA-4vpy	0.0249	0.0817	3.28
Control	ALK-MAA-4vpy-B	0.0206	0.0825	4.005
Alkaline phosphatase	ALK-4vpy-EGVE	0.0108	0.0518	4.80
Alkaline phosphatase	ALK-MAA	0.0055	0.0289	5.25
Beta glucosidase	Bgo-4vpy-1	0.0204	0.0695	3.41
Alkaline phosphatase	ALK-4vpy-2	0.0251	0.1032	4.11
Control	ALK-4vpy-B2	0.0204	0.0725	3.55
Beta glucosidase	Bgo-4vpy-2	0.0237	0.0867	3.66
BSA	Bsa-4vpy	0.0249	0.1126	4.52
Acid phosphatase	ACP2	0.0203	0.0737	3.63
Acid phosphatase l	ACP 4	0.0219	0.0924	4.22
Acid phosphatase	ACP B	0.0187	0.0784	4.19
Lysozyme	L2	0.0261	0.0504	1.93
RNase A	R2	0.0223	0.0486	2.18
Lysozyme	P1	0.0207	0.0536	2.59
Lysozyme	P2 (Thermal)	0.0197	0.0681	3.46
Control (glycine)	B1(Thermal)	0.0222	0.0639	2.88
Lysozyme	P3 (UV)	0.02	0.0656	3.28
Control ((glycine)	B2 (UV)	0.0198	0.0534	2.70

Another interesting feature observed was the swelling properties of BSA-4vpy, which was comparable to ALK-4vpy-1. Both polymers had comparable affinities towards alkaline phosphatase rebinding. This behaviour was also observed with L2 and R2, P2 and P3, and ACP 4 and ACP B. However, the selectivity of lysozyme towards P1 was greater compared to P2 and P3, even though the former polymer had low swelling capacity. From this, it was not clear whether swelling properties might have an influence on protein binding behaviour.

The other issue that we addressed was whether the surface area and porosity influence polymer swelling capacity. ALK-MAA, having low surface area, pore volume and diameter, demonstrated the highest swelling compared to polymers with large surface areas and porosities such as ALK-4vpy-1 and Bgo-4vpy-1.

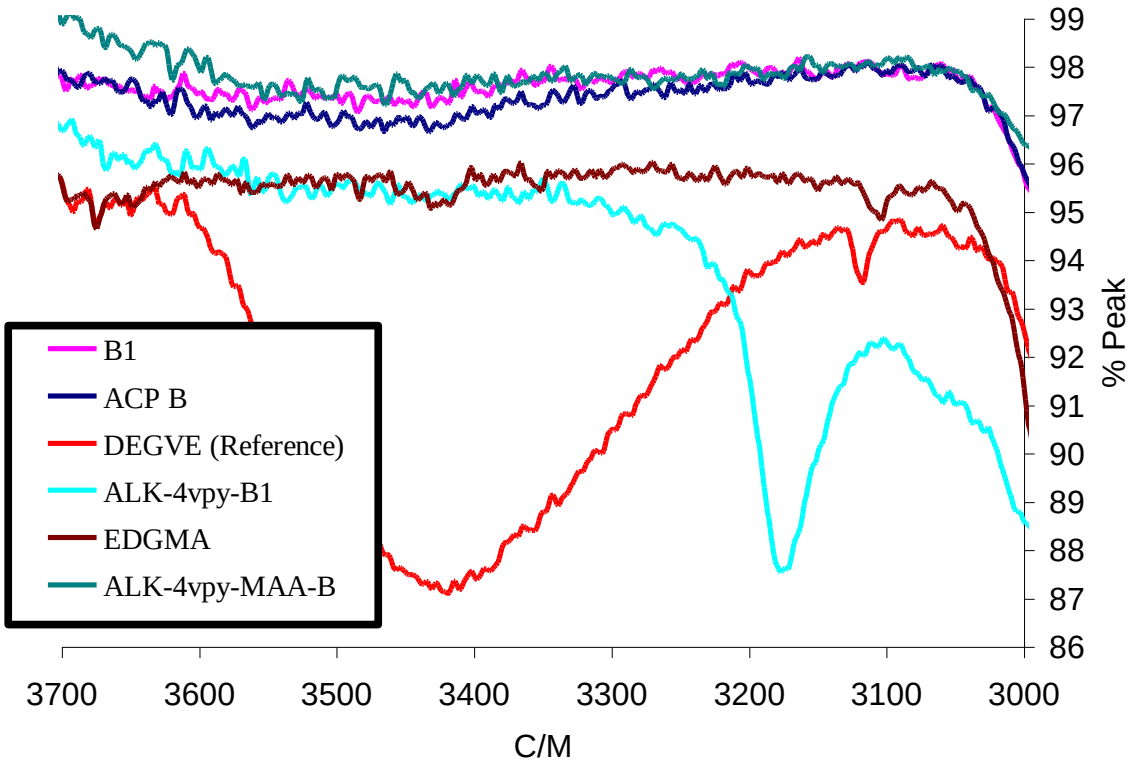
In order to determine if DEGVE was incorporated into the polymer, we examined the chemical structure of the control polymers prepared with or without the addition of the DEGVE, using Fourier Transform Infrared Spectroscopy (FTIR).

6.3 Chemical characterisation

6.3.1 Fourier transform infrared spectroscopy (FTIR) of control polymers

FTIR spectroscopy is a useful tool for examining changes in chemical structure from the addition or removal of functional groups in a polymer. Here, we investigated the presence of DEGVE in control polymer samples, ACP B and B1, and made comparison with the polymers prepared in the absence of this monomer; ALK-4vpy-B1, ALK-4vpy-MAA-B (see experiment 6.4). The resultant FTIR spectra of the polymers and reference monomers, DEGVE and DEGMA, are presented in figure 6-5

Figure 6-5: FTIR of control polymers made with and without DEGVE



From the FTIR spectra shown in figure 6-5, it can be seen that the strong O-H stretch demonstrated by reference monomer DEGVE in the region ranging from 3200-3500 cm,$^{-1}$ was not apparent in the control polymers (ALK-4vpy–B1and ALK-MAA-4vpy), but a small depression was slightly observed with ACP B polymer. This suggested that perhaps DEGVE was actually incorporated into the polymers, although the peak intensity was too small to be noticed. Another feature observed was a medium peak at ~3200 cm^{-1} representing C-H stretch from aromatic ring in ALK-4vpy-1(sky blue) compared to very weak signal generated by ALK-MAA-4vpy. This was not observed with other controls, B1 and ACP B because of the reduced concentration of 4vpy in the preparation of the polymers.

In further attempts to try and address this issue, we looked at elemental micro- analysis of the control polymers.

6.3.2 Elemental micro-analysis of control polymers

Briefly, this technique involves measuring the percentage by mass of specific elements within the polymers. Elements measured include: carbon, hydrogen, nitrogen, and can also be used to determine the composition in the specific elements in the compound. However, the determination of DEGVE would be a significant challenge because the method is not very sensitive for detecting trace amounts of monomers. For this experiment (see experiment 6.5), only control polymers were used and the elemental compositions are presented in table 6-4.

Table 6-4: Micro analysis of elements, Carbon, Hydrogen and Nitrogen in control polymers

Polymer samples	%Carbon	%Hydrogen	%Nitrogen
ALK-4vpy-B1	46.49	6.38	9.26
ALK-4vpy-B2	59.87	5.57	2.28
ALK-4vpy-MAA-B	56.76	6.8	1.6
ACP B	43.72	4.92	<0.3
B1	56.6	6.53	0.38

From table 6-4, it can be seen that the overall percentage compositions of carbon, hydrogen and nitrogen were between ~ 45-65%, while the rest of the polymer composition was oxygen. Using low concentrations of 4vpy in the preparation of ACP B and B1 polymers, led to a significant reduction of nitrogen and a slight reduction of carbon and hydrogen was also observed with ACP B, while that of polymer B1 was comparable to other polymers. This suggests that addition of DEGVE leads to increased levels of oxygen and hence increased hydrophilicity of the polymer. It can be therefore inferred that DEGVE might have been incorporated into the polymer matrix.

Another interesting observation was that the percentage of nitrogen found in the polymer made with only 4vpy as the only functional monomer (ALK-4vpy-B1) at pH 9.8, was ~4 fold greater than that the polymer of similar compositions but made at pH7 .9 (ALK-4vpy-B2). It may be concluded that the pH used to solvate proteins (template) influences polymer morphology, physical behaviour and protein binding characteristics.

Chapter 6: Experimental Section

Materials – SEM microscope Cambridge Instruments Stereoscan 90, Light microscope (Microscope Co, Olympus Vanox), Brunauer, Emmett and Teller (BET) Micromeretrices ASAP 2020, 0.5 ml eppendorfs with filters, FT-IR spectrometer (Perkin Elmer Spectrum One), CHN analyser (Perkin Elmer 2400), Meter balance (Metter-Toledo AE200), pH meter (Metter-Toledo MP120), Magnetic stirring bar and hot plate stirrer (Fisher Scientific) and all polymers prepared in this report.
Buffers - Diethylamine pH 7.8-8.0 (0.01M), Citrate pH 4.8 (0.01M), 0.5M arginine buffer pH 7 prepared as described in the previous chapters.

Experiment 6.1

Polymer characterisation with SEM and Light Microscope

For this experiment, alkaline phosphatase imprinted polymer ALK-4vpy-1 and ALK-4vpy-B1 powders were examined under SEM and light microscope.

Experiment 6.2

Surface area and pores size distributions

Brunauer, Emmett and Teller (BET) analysis was used to measure the surface area, and pores size distributions. 100mg of each polymer sample ALK-4vpy-1, ALK-4vpy-B1,

ALK-MAA-4vpy, ALK-4vpyDEGVE, ALK-MAA, Bgo-4vpy-1, BSA-4vpy, ACP 4, L2, R2, P1, P2, and B1 were weighed and put onto BET machine for analysis.

Experiment 6.3

Polymer swelling characterisation

20mg of each polymer shown in the following table were weighed and each was put in eppendorfs with filter. 0.5ml of their respective mobile phases was then added to each sample. All sample polymer suspensions were then incubated for 24 hr. After this period, all polymer sample solution was centrifuged gently at 2000 rpm for 2 minutes and supernatant decanted. Finally, all polymer samples were weighed on the balance machine, and final weight was recorded. Solvent uptake by the polymer was computed as follows

Solvent uptake (g) / polymer (g) =
(Initial weight of (polymer + dH_2O) - Final weight of (polymer + dH_2O) after centrifuged to remove unabsorbed dH_2O).

Experiment 6.4

Fourier Transform Infrared Spectroscopy (FTIR)

< 5mg of the polymer were required from each sample. Control polymers made with the addition of DEGVE; ACP B and B1 and those prepared without this monomer; ALK-4vpy-B1 and ALK-4vpy-MAA-B were used for this investigation.

Experiment 6.5

Elemental micro-analysis

For this experiment again, only control polymers were employed; ALK-4vpy-B1, ALK-4vpy-B2, ALK-4vpy-MAA-B, ACP B and B1 for the determination of the percentage concentration of carbon, hydrogen and nitrogen in the polymer samples.

Table below is a summary of polymers and mobile phases used in experiment 6.3

Template	Sample Polymer	Polymer(g)	Mobile phase 0.5ml
Alkaline phosphatase	ALK-4vpy-1	0.0247	Diethylamine pH 7.8-8.0 (0.01M)
Alkaline phosphatase	ALK-MAA-4vpy	0.0249	″
Control	ALK-MAA-4vpy-B	0.0206	″
Alkaline phosphatase	ALK-4vpy-EGVE	0.0108	″
Alkaline phosphatase	ALK-MAA	0.0055	″
Beta glucosidase	Bgo-4vpy-1	0.0204	″
Alkaline phosphatase	ALK-4vpy-2	0.0251	″
Control	ALK-4vpy-B2	0.0204	″
Beta glucosidase	Bgo-4vpy-2	0.0237	″
BSA	Bsa-4vpy	0.0249	″
Acid phosphatase	ACP2	0.0203	Citrate pH 4.8 (0.01M)
Acid phosphatase l	ACP 4	0.0219	″
Acid phosphatase	ACP B	0.0187	″
Lysozyme	L2	0.0261	0.5M arginine buffer pH 7
RNase A	R2	0.0223	″
Lysozyme	P1	0.0207	″
Lysozyme	P2 -DEGVE Thermal	0.0197	″
Control	B1-DEGVE Thermal	0.0222	″
Lysozyme	P3 -DEGVE (UV)	0.02	″
Control	B2- DEGVE (UV)	0.0198	″

Chapter 7 : Future work

An extensive study was carried out to develop and evaluate protein imprinted polymers designed using a novel protein immobilisation technique, as a platform for bio-macromolecular imprinting. From this study, a number of future studies are proposed:

1. In chapter 3, we prepared protein coated micro crystals for use as a platform for protein imprinting. Although PCMCs have been studied before and reported to enhance protein stability and activity in organic media, here we have investigated the activity of protein PCMCs in organic solvent at elevated polymerisation temperature in the presence and absence of functional monomers. However, the effects of other polymer components, such as cross linker and initiator, have not been studied and this should be given consideration.

2. In chapter 4, we investigated different ways of reducing non specific protein binding, including tuning of mobile phase with non specific blocking agents and chemical additive. In chapter 5, we modified the polymer compositions with addition of diethylene glycol vinyl ether DEGVE. All this reduced non specific protein binding. However, there are vast number of potential hydrophilic monomers that are worthy of consideration (refer to chapter 1 section 1.3.2 & 1.3.3). Finally, another investigation which is worth of consideration is to use developed polymers as size exclusion chromatography materials.

3. In chapter 5, we used hydrophobic interaction as a means of template recognition and we have demonstrated that this was perhaps possible. The functionality of alkaline phosphatase imprinted polymer in an SPE or in a size exclusion was not investigated. Therefore, it is worthy to pack polymers in columns to try and separate proteins.

4. The use of PCMCs immobilisation strategy for small protein molecular imprinting should be given great consideration. This would be useful when using defined imprinting methodologies that have shown to be promising and compare the results.

Chapter 8 : Appendix

8.1 Acid phosphatase assay from sigma protocols

ProductInformation

SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PHOSPHATASE, ACID
(EC 3.1.3.2)

PRINCIPLE:

p-Nitrophenyl Phosphate + H_2O $\xrightarrow{\text{Phosphatase, Acid}}$ p-Nitrophenol + P_i

Abbreviation:
P_i = Inorganic phosphate

CONDITIONS: T = 37°C, pH = 4.8, A_{410nm}, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination

REAGENTS:

A. 90 mM Citrate Buffer, pH 4.8 at 37°C
(Prepare 100 ml in deionized water using Citric Acid, Trisodium, Dihydrate, Sigma Prod. No. C-7254, or Citrate Buffer Solution, Sigma Stock No. 104-4. Adjust to pH 4.8 at 37°C with 1 M NaOH or 1 M HCl.)

B. 15.2 mM p-Nitrophenyl Phosphate (PNPP)
(Prepare 5 ml in deionized water using Sigma 104 Phosphatase Substrate, Sigma Stock No. 104-0.)

C. 100 mM Sodium Hydroxide Solution (NaOH)
(Prepare 50 ml in deionized water using Sodium Hydroxide, Anhydrous, Sigma Prod. No. S-5881.)

D. Acid Phosphatase Enzyme Solution
(Immediately before use, prepare a solution containing 0.15 - 0.25 unit/ml of Phosphatase, Acid in cold deionized water.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank
Reagent A (Buffer)	0.50	0.50
Reagent B (PNPP)	0.50	0.50

Enzymatic Assay of PHOSPHATASE, ACID
(EC 3.1.3.2)

PROCEDURE: (continued)

Mix by inversion and equilibrate to 37°C. Then add:

	Test	Blank
Reagent D (Enzyme Solution)	0.10	------

Immediately mix by inversion and incubate at 37°C for exactly 10 minutes. Then add:

Reagent C (NaOH)	4.00	4.00	
Reagent D (Enzyme Solution)		------	0.10

Mix by inversion and record the A_{410nm} for both the Test and Blank in a suitable spectrophotometer.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(A_{410nm}\ \text{Test} - A_{410nm}\ \text{Blank})(5.1)(df)}{(10)(18.3)(0.1)}$$

5.1 = Total volume (in milliliters) of solution
df = Dilution factor
10 = Time of assay (in minutes) as per the Unit Definition
18.3 = Millimolar extinction coefficient of p-Nitrophenol at 410 nm
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 µmole of p-nitrophenyl phosphate per minute at pH 4.8 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 1.10 ml reaction mix, the final concentrations are 41 mM citric acid, 6.9 mM p-nitrophenyl phosphate and 0.015 - 0.025 unit phosphatase, acid.

8.2 Alkaline phosphatase assay from sigma protocols

ProductInformation

SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PHOSPHATASE, ALKALINE[1]
(EC 3.1.3.1)
Glycine with Zinc Assay

PRINCIPLE:

p-Nitrophenyl Phosphate + H_2O $\xrightarrow{\text{Alkaline Phosphatase}}$ p-Nitrophenol + P_i

Abbreviation:
P_i = Inorganic Phosphate

CONDITIONS: T = 37°C, pH = 10.4, A_{405m}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Glycine Buffer with 1.0 mM Magnesium Chloride and 1.0 mM Zinc Chloride, pH 10.4 at 37°C
(Prepare 50 ml in deionized water using Glycine, Free Base, Sigma Prod. No. G-7126; Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250; and Zinc Chloride, Sigma Prod. No. Z-4875. Adjust to pH 10.4 at 37°C with 1 M NaOH. **PREPARE FRESH**.)

B. 60 mM p-Nitrophenyl Phosphate Solution (PNPP)
(Prepare 5 ml in deionized water using Sigma 104 Phosphatase Substrate, Sigma Stock No. 104-0. **PREPARE FRESH**.)

C. 1.0 mM Magnesium Chloride Solution ($MgCl_2$)
(Prepare 50 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

D. Phosphatase, Alkaline Enzyme Solution
(Immediately before use, prepare a solution containing 0.1 - 0.2 unit/ml of Alkaline Phosphatase in cold Reagent C.)

Enzymatic Assay of PHOSPHATASE ALKALINE[1]
(EC 3.1.3.1)
Glycine with Zinc Assay

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reagent A (Buffer)	2.60	2.60
Reagent B (PNPP)	0.30	0.30

Mix by inversion and equilibrate to 37°C. Monitor the A_{405nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

	Test	Blank
Reagent C ($MgCl_2$)	------	0.10
Reagent D (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the increase in A_{405nm} for approximately 5 minutes. Obtain the ΔA_{405nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{405nm}/\text{min Test} - \Delta A_{405nm}/\text{min Blank})(3)(df)}{(18.5)\,(0.1)}$$

3 = Volume (in milliliters) of assay
df = Dilution factor
18.5 = Millimolar extinction coefficient of p-nitrophenol at 405 nm
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 µmole of p-nitrophenyl phosphate per minute at pH 10.4 at 37°C.

Enzymatic Assay of PHOSPHATASE ALKALINE[1]
(EC 3.1.3.1)
Glycine with Zinc Assay

FINAL ASSAY CONCENTRATIONS:

In a 3.00 ml reaction mix, the final concentrations are 87 mM glycine, 0.90 mM magnesium chloride, 0.87 mM zinc chloride, 6.0 mM p-nitrophenyl phosphate and 0.01 - 0.02 unit alkaline phosphatase.

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