University of Strathclyde

Department of Pure and Applied Chemistry

The Single Step Purification and Immobilisation of Proteins on Molecularly Imprinted Polymer Supports

By

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A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy

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Signed: Mhairi Cayce Date: 31AUG10

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ABBREVIATIONS

2D	2-Dimensional
2-VP	2-Vinyl pyridine
3D	3-Dimensional
4-NP	4-Nitrophenol
4-VP	4-Vinyl pyridine
AA	Acrylic Acid
ACN	Acetonitrile
AIBN	N, N'-Azo-Bis-Isobutyronitrile
APL	Actual Protein Loading
BIS	*N, N*-Methylenebisacrylamide
BSA	Bovine Serum Albumin
CH_3COOH	Acetic Acid
CH_5N_3	Guanidine Hydrochloride
C_2H_6O	Ethanol
$C_6H_8O_7$	Citric Acid
CH_3OH	Methanol
CO_2	Carbon Dioxide
CLAMS	Crystal Lattice Mediated Self-assembly
dH_2O	Deionised Water
DIP	1,3-Diisopropenyl Benzene
DSC	Differential Scanning Calorimetry
DVB	Divinylbenzene
E_A	Activation Energy
EGDMA	Ethylene glycol Dimethacrylate
ES	Enzyme-Substrate
EP	Enzyme-Product
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron

HEMA	Hydroxyethyl methacrylate
HPLC	High Performance Liquid Chromatography
K_2HPO_4	Potassium Phosphate Dibasic
KH_2PO_4	Potassium Phosphate Monobasic
K_2SO_4	Potassium Sulfate
MAA	Methacrylic Acid
MAE	Microwave Assisted Extraction
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid-Phase Extraction
Na_2CO_3	Sodium Carbonate
$Na_3C_6H_5O_7$	Sodium Citrate
$(NH_2)_2CO$	Urea
NIP	Non Imprinted Polymer
NaOH	Sodium Hydroxide
PCMC	Protein Coated Micro-crystal
pKa	Acid Dissociation Constant
***p*-Nitrophenyl β-D-glucopyranoside**	PNPG
RP-HPLC	Reversed–Phase High Performance Liquid Chromatography
RPM	Rotations per Minute
SAMs	Self-Assembled Monolayers
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SPE	Solid Phase Extraction
TEM	Transmission Electron Microscopy

TEMED	*N*, *N*, *N*′, *N*′-Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TPL	Calculated Protein Loading
TRIM	Trimethylolpropane Trimethacrylate
TRIS-HCl	TRIS-Hydrochloric Acid
UV	Ultraviolet
UV/Vis	Ultraviolet-Visible

Abstract

The molecular imprinting of proteins is a highly appealing area of research since polymers that can bind selectively to proteins can be applied in a number of interesting fields, including affinity separation, chemical sensing and molecular diagnostics. Generally speaking, proteins are significantly more difficult to imprint than low molar mass templates due to their physical size, thermal instability and their tendency to denature in the solvents used typically in molecular imprinting protocols. However, this has not deterred research in this area. Previous protein imprinting reports include the imprinting of gels, the surface imprinting of silica, and the epitope approach.

In the Strathclyde approach to protein imprinting, protein in the form of an insoluble protein-coated microcrystal (PCMC) is used as the template. This PCMC strategy addresses many of the difficulties which can arise during the imprinting of proteins, including the thermal instability of proteins and their tendency to denature in many chemical environments. This novel synthetic approach has allowed for different types of protein, including enzymes, to be imprinted successfully. Molecularly imprinted solid-phase extraction (MISPE) has been used to demonstrate that MIPs prepared in this way can extract templated protein from an aqueous environment in an efficient manner, and also to probe the cross-selectivity of the imprinted sorbents for structurally-related and structurally non-related proteins.

For imprinted enzymes, where catalytic activity is an important metric, once the enzyme has been bound to the imprinted polymer, the activity of the immobilised enzyme has been assessed and its stability monitored over various time and temperature ranges. It has been found that the enzyme remains catalytically active when bound to the imprinted polymer and indeed shows enhanced stability compared to the enzyme immobilised on non-imprinted polymers.

Finally the selective extraction of enzyme from a complex biological sample (a cell lysate) has been demonstrated.

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1.0 INTRODUCTION

1.1 Molecularly Imprinted Polymers

Molecular imprinting is an area in which research interest has increased significantly within the last fifteen years the literature publication rate in recent years reflects this trend (Figure 1.1).

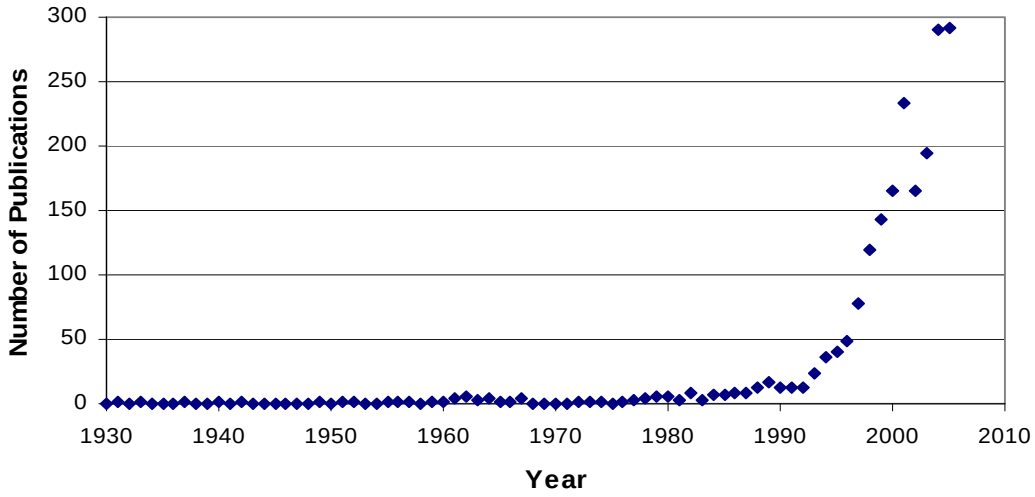


Figure 1.1: Graphical Representation of the Number of Publications within the Field of Molecular Imprinting 1931 – 2005.[1]

With various applications already demonstrated and some in use, and many more works in progress, it is easy to see why molecular imprinting has generated such an interest.

Molecularly imprinted polymers (MIPs) are polymers that are prepared in the presence of a template (Figure 1.2).[2, 3]

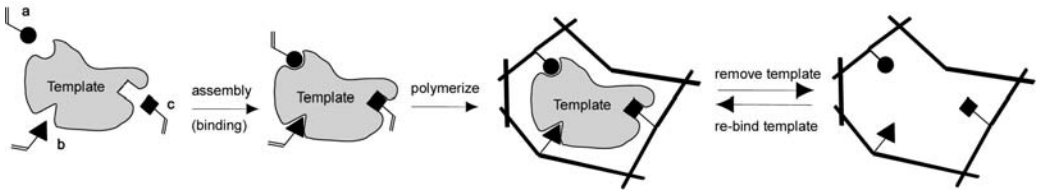


Figure 1.2: Schematic Representation of the Molecular Imprinting Process. The template is matched by functional monomers a, b & c; a template-monomer complex or covalent adduct forms; the template assembly copolymerises with a cross-linking monomer; finally, removing the template reveals the recognition site.[2, 3]

The functionality of the template which is used in the production of individual MIPs is matched with the functionality of the functional monomer(s), which in turn produces a monomer-template complex, thus the functional monomers are selected so that they have a functional group(s) present which will complement the template which has been chosen to imprint.[4]

A crosslinking agent is then introduced to the system, along with an initiator. The crosslinking agent ensures that the monomer template complex which has been formed is retained, and also ensures that the resultant MIP is hard, rigid and porous in structure. The initiator initiates the polymerisation.
The template is then removed from the MIP, revealing the binding sites which the template has forged in the MIP's structure, thus allowing rebinding of the template to be completed.

This Chapter will discuss the methods of polymerisation which are commonly used, the different approaches by which MIPs are prepared, along with further details of the components present in the production of MIPs and an in-depth review of their individual roles.

1.2 Molecularly Imprinted Polymers - Where Did It Begin?

1.2.1 Polyakov

Molecular imprinting is not a new area of research interest; the first reports of molecular imprinting date as far back as 78 years ago. The Soviet scientist, M.V. Polyakov, who was involved in research concerning the use of silica gels in

chromatography, reported unusual adsorption properties in silica particles prepared using a novel synthesis procedure in 1931.[5] Polyakov reported that, under certain circumstances, additives could be captured by silica gels, and re-adsorption of these additives onto the silica gel - within the specific conditions at which it was made - was possible.

Polyakov prepared silica gels by acidifying sodium silicate solutions using ammonium carbonate as the gelating agent. After preparation, Polyakov introduced one of the previously mentioned additives (benzene, toluene or xylene) to the silica. This was done in order to investigate the pore structure, and also with an aim to optimise the binding capacity of the silica. The silica was allowed to dry for approximately 20-30 days at room temperature, producing a hard and rigid silica matrix. Throughout the drying process the pore structure was analysed repeatedly. After this period, and with the silica now dry, the additive in question was removed by washing the silica repeatedly with hot water. Thereafter, re-adsorption studies were carried out.

It was during these studies wherein Polyakov reported that there was an increased uptake onto the silica gel of the additive in which the silica was prepared, as opposed to the other additives mentioned, i.e., the silica gel was demonstrating that it had a memory for the additive whose presence it was prepared in; truly ground-breaking work.[5]

Further research was carried out by Polyakov's group a few years later; following on from his previous findings, Polyakov reported that the increase in the uptake of the additive in which the silica was prepared could be attributed to structural changes in the silica, which arose due to the chemical nature of the additive in question.[6, 7]

Thus, Polyakov had reported the first molecular imprinting phenomena, demonstrating selectivity using what is now referred to as the "template effect", however, at this time Polyakov's findings did not create a stir in the research community, and actually went largely unnoticed.

1.2.2 Pauling

Following Polyakov's work, the next advance into the world of molecular imprinting was the work of double Nobel Prize laureate, Linus Pauling. Linus Pauling had a theory on the formation of antibodies which has a part to play in the world of molecular imprinting. After many years of research into antibodies, Pauling hypothesised that antibodies were formed when they were in the presence of an antigen template molecule (Figure 1.3)

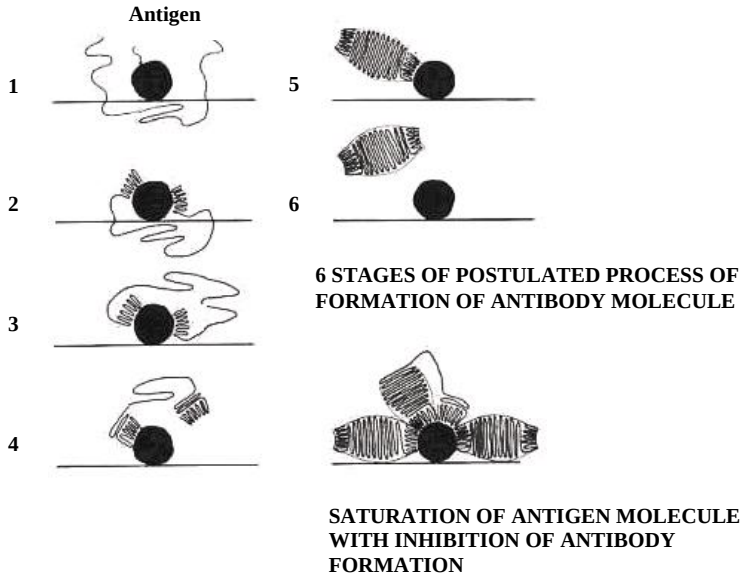


Figure 1.3: Image of the Six Stages of Antibody Formation Proposed by Pauling: Process of the formation of an antibody molecule as the result of the interaction of the globulin polypeptide chain with the antigen molecule. The lower right hand picture shows an antigen molecule surrounded by attached antibody molecules or parts of molecules and thus inhibited from further antibody formation.[8]

Pauling postulated that an antigen to which an antibody was directed acted as a template. The antigen therefore moulded the antibody protein thus ensuring that the antibody species which was specific to that individual antigen. Pauling further suggested that the initial structure of the moulded antibody would complement to the structure of the antigen, and that in the absence of an antigen the antibody would no long be specific. [8]

Papers published by Pauling and Campbell in 1942 state that this theory could be investigated by precipitating a globulin molecule i.e., a template molecule, in the presence of an antigen, followed by the slow removal of the globulins – thus, the globulins would be specific to the antigen.[9, 10]

This theory is similar to the "Lock and Key" analogy which is used to describe enzyme action (Section 1.5.1)

This hypothesis is widely referred to as the Instructional Theory.[7] Not only did it provide the first insight into bio-imprinting, but it also provided the foundations for the development of the Clonal-Selection Theory; which in turn subsequently superseded the Instructional Theory.

The Clonal-Selection Theory was published in the 1950s by F. Macfarlane Burnet and states that the antibody commits to reacting with the antigen prior to exposure. Thus, the commitment is demonstrated in the ability of the protein to fit the antigen.[11, 12]

1.2.3 Dickey

Prior to the Clonal-Selection Theory being published, Pauling continued work on his Instructional Theory, and one of his graduate students, Frank Dickey, attempted to apply this theory to a silica system.[13] Frank Dickey attempted to form specific absorbents using silica, *via* a method derived from that of Polyakov. Dickey opted to use methyl orange as his additive (template), however the key area of change was the introduction of the template; rather than introducing the template after the silica gel had been prepared as Polyakov had done, Dickey ensured that the template was present from the start.[13]

The results produced by Dickey demonstrated that the silica which was prepared in the presence of the template had a preference over any of the other alternative dyes which were present demonstrating a high level of specificity. These findings were

corroborated subsequently by further work carried out by Dickey, [14] and also Haldeman and Emmett[15] and Bernhard.[16]

Further research was carried out on molecular imprinting for the 15 years after Dickey's findings, however due to instability of the silica matrix, the irreproducibility of silica imprinted materials, and limited applications of the silica gels, research slowed down significantly.

1.3 Developments and Approaches to Molecular Imprinting

Molecular imprinting seemed to take a back seat with respect to further development in research areas until the early 1970s. Since then, development and research has increased dramatically, and a new-found enthusiastic appetite for molecular imprinting has been realised. This is mainly due to the independent research carried out by Professor Günter Wulff and Professor Klaus Mosbach. These two scientists, whose interests are in two different areas of science, have developed two different approaches to molecular imprinting, which in turn has led to the phenomenon which is molecular imprinting. This, in turn, has led to various developments within scientific research to the approaches of molecular imprinting.

1.3.1 The Covalent Approach

Professor Günter Wulff practised a different approach to Polyakov and Dickey for the preparation of molecularly imprinted polymers, an approach which is now referred to as the covalent approach (or pre-organised approach), wherein molecular imprinting was carried out in organic polymers.[17]

The covalent approach relies on a covalent attachment, wherein the template which is used for imprinting relies on using templates which have reversible covalent bonds between the monomer and the polymer (Figure 1.4).[17, 18]

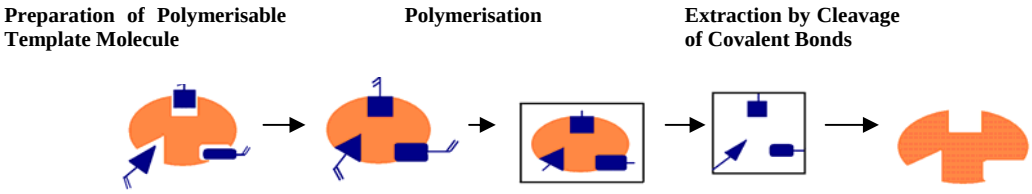


Figure 1.4: Schematic Illustration of the Covalent Approach to Molecular Imprinting.[19]

Using this approach, the monomer which has been selected is attached to the template *via* liable covalent bonds, such as boronate esters, imines, ketals and disulfides.[20] The template is then copolymerised with an excess of crosslinker in the presence of a porogenic solvent.

Once polymerisation has been completed, and the polymer has been dried, ground and sieved into a more manageable form, the template is removed from the polymer matrix. This is achieved by cleaving the labile chemical bonds that bind the template to the polymer. Once the template has been removed from the polymer, cavities are present in the matrix which matches the template both in shape, size and functional group stereochemical arrangement.

In order to rebind the template to the polymer, the bonds must be reformed, thus selectively rebinding the template back to the polymer.

Wulff's first demonstration of covalent imprinting was based upon the recognition of D-glyceric acid (a small chiral carboxylic acid bearing two additional hydroxyl groups). A divinylbenzene-based polymer was prepared in the presence of D-glyceric-(*p*-vinylanilide)-2 3-O-*p*-vinylphenylboronate, which was covalently incorporated to the polymer, before being hydrolysed (Figure 1.5).[17]

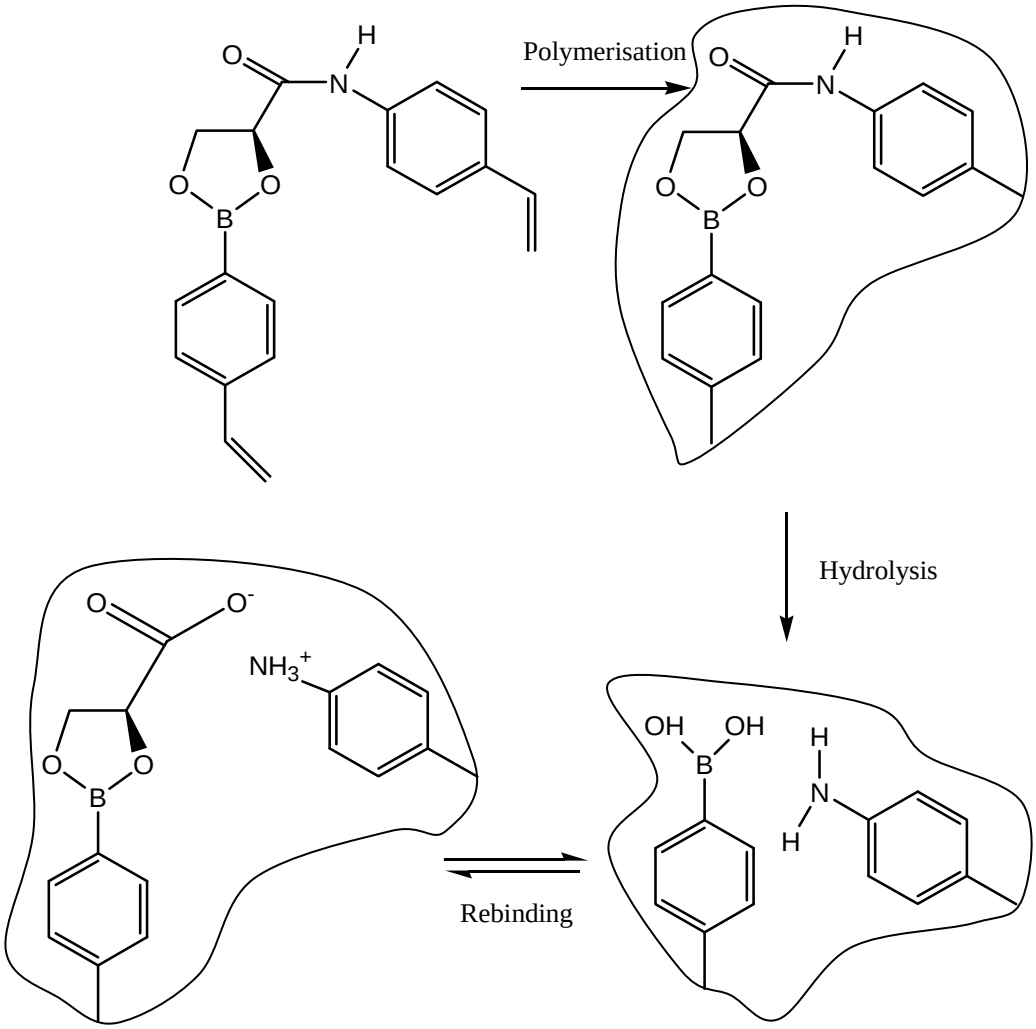


Figure 1.5: Illustration of Covalent Imprinting - The first Example by Wulff: D-glyceric-(*p*-vinylanilide)-2, 3-O-*p*-vinylphenylboronate is used as the template; Polymerisation is initiated, with a large excess of divinylbenzene as the crosslinker; hydrolysis is carried out, converting the boronate ester to boronic acid and amide to amine; Resultant polymers show chiral prejudice for template when it is introduced to a racemic mixture.[17]

The covalent approach imprinting was an exciting breakthrough in the world of molecular imprinting, and has since been involved in further research, as well as being a method which is still used by molecular imprinters today. Although the proven levels of specificity are encouraging, there are several disadvantages as well as advantages which are associated with this method. These are outlined in Table 1.1.

	ADVANTAGES	DISADVANTAGES
1	Polymerisation can be carried out in any solvent, thus providing flexibility.	The range of templates which can be imprinted is limited due to the required functional groups for this approach.
2	The functional groups are only associated with the template site.	The removal of the template from the polymer can be difficult due to the covalent bonds, thus there is low recovery of template(s) and a low number of binding sites.
3	The polymers show specific recognition for the template from which they are prepared.	Poor kinetics of re-binding due to the strength of the covalent bond; severe conditions must be employed to cleave the bond.

Table 1.1: Advantages and Disadvantages of the Covalent Approach to Molecular Imprinting[21]

1.3.2 The Non-Covalent Approach

During the 1980s, Professor Klaus Mosbach was the next scientist to step into the forefront of molecular imprinting. He developed a method of molecular imprinting, which, to date, is the most common method used for imprinting. Mosbach developed what is commonly referred to as the non-covalent approach (Figure 1.6), also known as the self-assembly approach, in organic polymers.[20]

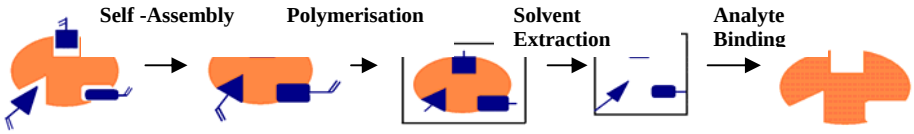


Figure 1.6: Schematic Illustration of the Non-Covalent Approach to Molecular Imprinting.[18]

The non-covalent approach works by ensuring that the non-covalent interactions between the functional monomer and the template are non-covalent both in the polymerisation and rebinding step.[22] The non-covalent interactions which are present in this approach include hydrogen bonding, hydrophobic interactions ion pair

interactions and Van Der Waals forces. A description of these interactions is provided in Table 1.2

Non-Covalent Bond	**Description of Interaction**
Hydrogen Bond	The hydrogen atom which is attached to an electronegative atom of one molecule is attracted to the electronegative atom of a different molecule
Hydrophobic Interactions	Non-polar hydrocarbon molecules form intermolecular aggregates in water
Ion Pair	A bond is form through electrostatic attraction i.e., the bond is formed between two oppositely charged ions.
Van Der Waals	The attractive or repulsive forces between molecules or parts of the same molecule. These forces are ones that are not due covalent bond formation or to the electrostatic interaction of ions or of ionic groups with one another or with neutral molecules.

Table 1.2: Non-Covalent Interactions Which May Occur Between the Functional Monomer and the Template When Using the Non-Covalent Approach to Molecular Imprinting.[23]

Fundamentally, the non-covalent approach is straightforward. The functional monomers self-assemble around the template during the polymerisation process and, as there are only non-covalent interactions present, the template can be removed easily by washing the resultant polymer with a suitable solvent repeatedly.

The functional monomer used in non-covalent imprinting is selected so that the monomers functional groups are complementary to the template that is being imprinted. The ability of the functional monomer to form stable, non-covalent interactions with the template, in a suitable solvent is also a factor in the selection process.

A variety of functional monomers have been used in the non-covalent approach to date. These can be divided into three categories: acidic, basic and neutral. Examples can be seen in Table 1.3.

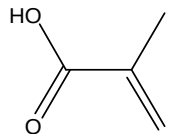
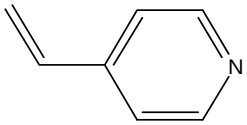
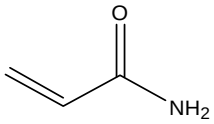
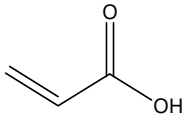
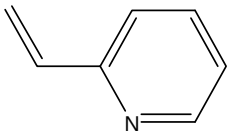
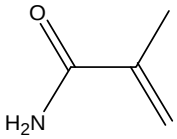
Acidic	Basic	Neutral
 Methacrylic Acid (MAA)	 4-Vinylpyridine (4-VP)	 Acrylamide
 Acrylic Acid (AA)	 2-Vinylpyridine (2-VP)	 Methacrylamide

Table 1.3 Structures of Functional Monomers Which Have Been Used Previously in Non-Covalent Imprinting

The functional monomers which are the most commonly used are methacrylic acid - which was first reported for its use by Mosbach *et al.* [24] - and vinylpyridine.[25]

Functional monomers can be used individually, or by combining them together. Depending on the template molecule, the utilisation of a combination of functional monomers for the polymerisation process can heighten the binding properties of the resultant polymer, compared to the polymer which is prepared with only an individual functional monomer present. A popular combination of functional monomers is that of methacrylic acid and 4-vinylpyridine.[26, 27, 28]

After selection of the appropriate functional monomer(s), the next stage of the polymerisation process is to combine the functional monomer(s) with the template, in the presence of a suitable porogenic solvent; the solvent must be selected to ensure that it is compatible with the non-covalent interactions which are to be formed. The role of the porogenic solvent is to mediate the strength of the non-covalent interactions (the strength of non-covalent interactions employed are influenced heavily on the polarity of the solvent utilised) and to influence the final morphology of the polymer produced.[1, 29]

In order to ensure that the non-covalent interactions are maximised, thus enhancing the recognition sites, an ideal porogenic solvent should be non-polar: the more polar the porogenic solvent, the weaker the interactions. Non-polar solvents which have been recommended include toluene.[1]

The use of a polar porogenic solvent in the non-covalent approach provides many benefits for the resultant polymer, i.e., the polymer produced will be macroporous (a rigid material, with a high specific surface area). A solvent which is used readily in order to establish this is acetonitrile. [59]

The polymer is prepared using an excess of crosslinker. The role of the crosslinker is to ensure that the resultant polymer is rigid in the recognition sites i.e., the position of the functional monomer(s) are in a fixed position around the template. This is accomplished by trapping the template/functional monomer complex which has formed and ensuring that the matrix formed is porous and mechanically robust. Common crosslinkers which have been used include ethylene glycol dimethacrylate (EGDMA) and divinylbenzene (DVB).[30]

The polymerisation process is then carried out. This is induced typically by raising the temperature or by UV irradiation. Generally, the polymerisation process takes several hours to days for completion.

Mosbach's first example of non-covalent imprinting (Figure 1.7) was for the chiral resolution of a racemic mixture of the derivatised/modified amino acid *L*-phenylalanine methyl ester; [31] the amino acid was chemically changed so that it was suitable for use as a template i.e., the L enantiomer of amino acid phenylalanine was reacted with a methyl ester to give *L*-phenylalanine methyl ester.

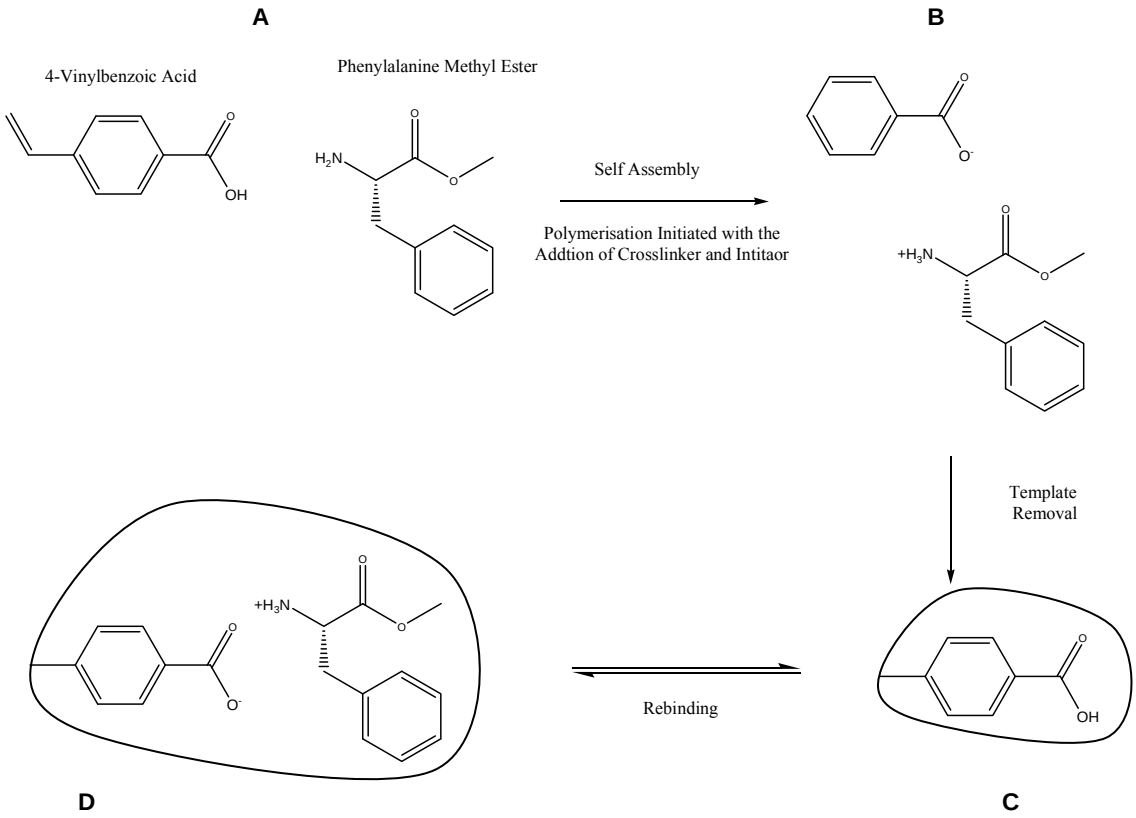


Figure 1.7: Illustration of Non-Covalent Imprinting –The First Example by Mosbach: A) 4-vinylbenzoic acid is used as the functional monomer and phenylalanine methyl ester as the template; B) The crosslinker, divinylbenzene, is added to the mixture with initiator in order to initiate polymerisation; C) Once polymerisation is complete, the template is removed from the matrix; D) The resultant polymer indicates that it has chiral prejudice for the template when it is introduced to a racemic mixture.[32,72]

Mosbach's procedure for non-covalent imprinting used 4-vinylbenzoic acid as the functional monomer and phenylalanine methyl ester as the template (A - Figure 1.7). The crosslinker, divinylbenzene, was then added to the mixture with initiator in order to initiate polymerisation (B). Once polymerisation was complete, the template was removed from the matrix (C), and thus the polymer indicates chiral prejudice for the template when it is introduced to a racemic mixture (D).[32]

As with Wulff's covalent approach to imprinting, there are also various advantages and disadvantages to the non-covalent approach. These are outlined in Table 1.4.

	ADVANTAGES	DISADVANTAGES
1	The kinetics of rebinding are several orders of magnitude higher than that of covalent imprinting.[2]	Association constant is usually relatively low, therefore in a 1:1 ratio of monomer to template, only a small quantity of the template will bind.
2	Less organic synthesis is required, i.e., synthesis of monomer-template conjugates is not required.	Large excess of functional monomer is normally used, which can result in non-specific binding sites being generated within the polymer.
3	Template removal is easier. The non-covalent interactions which bind the template are weak.	Binding sites are found throughout the polymer, and not just in the cavities. As the template cannot be fixed in place.
4		Limited re-uptake of template

Table 1.4: Advantages and Disadvantages of Non-Covalent Molecular Imprinting[21]

1.3.3 Semi-Covalent Approach

Continuing on from the research conducted by Wulff and Mosbach, semi-covalent imprinting was the next approach to be identified in the world of molecular imprinting, and was done so by Sellergren and Andersson.[33] Since the first reports of semi-covalent imprinting, however, work has been continued by Whitcombe *et al.* [34, 35] and subsequently the sacrificial spacer approach was introduced.[36]

The semi-covalent approach combines the advantages of both the covalent approach and the non-covalent approach. During semi-covalent imprinting, a covalent polymerisable template is used for the polymerisation; however the binding is non-covalent.[37]

Sellergren and Andersson first reported the semi-covalent imprinting approach when they imprinted *p*-aminophenylalanine ethyl ester. They used an ester template (that contained two polymerisable groups attached *via* ester linkages) analogue approach, which created the required imprinted site. Hydrolysis followed, and the carboxylic

acid groups which were left in the polymer binding site rebound to the amino acid through a mixture of hydrogen bonding and electrostatic interactions.[24, 33]

Other templates which have been imprinted through this approach include testosterone[38] and several phenolic species.[39]

Again, as with the covalent and non-covalent approaches, there are advantages and disadvantages with the semi-covalent approach, which are similar in nature to those outlined in Tables 1.2 and 1.3 as this approach draws from both approaches.

Some of the limitations associated with the semi-covalent approach (over-crowding in the binding site and hindered non-covalent rebinding) can be overcome with the use of a sacrificial spacer (Figure 1.8)

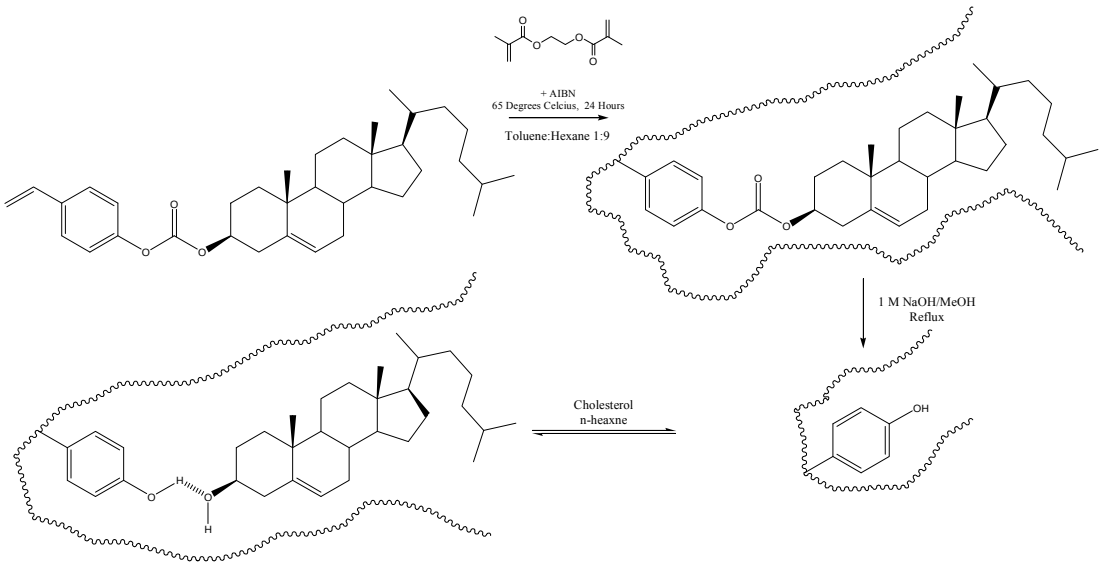


Figure 1.8: An Illustration of Molecular Imprinting of Cholesterol by the Sacrificial Spacer Method: cholesteryl (4-vinyl) phenyl carbonate is used as the template monomer; as polymerisation is initiated; B) Hydrolytic cleavage of the carbonate ester, with the loss of 'sacrificial' CO_2, leads to a recognition site which bears a phenolic residue; C) The resultant polymer shows preferential binding of cholesterol over *epi*-cholesterol, cholest-5-ene-3-one and cholesteryl acetate.[37]

In this method, Whitcombe *et al.* polymerised the template monomer, cholesterol (4-vinyl) phenyl carbonate with ethylene glycol dimethacrylate (EGDMA) in a

porogenic solvent. The template is covalently attached to the polymer. The spacer (as CO_2) and the template are removed *via* alkaline hydrolysis and a polymer-bound phenol is formed. Non-covalent rebinding of the template *via* hydrogen bonding with the phenol residue is possible with the cholesterol molecule re-occupying its original position in the site.[37]

1.4 Polymerisation and Components in Molecular Imprinting

1.4.1 Polymerisation

To obtain molecularly imprinted polymers, conventional free radical polymerisation is the most common method applied; this can be attributed to several factors, but mainly due to the fact that the functional monomers and crosslinkers required for this process are readily available, and that the polymers are relatively easy to prepare.

Conventional free radical polymerisation can be broken down into three distinct stages: initiation, chain propagation and chain termination.

Initiation:

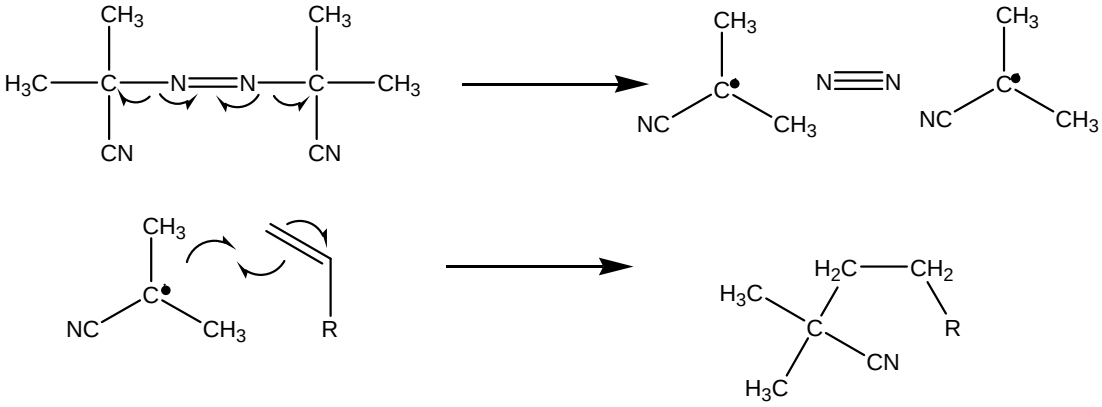


Figure 1.9: Illustration of the Decomposition and Initiation of AIBN on a Vinyl Monomer

The initiation step of the reaction generates two free radicals. It does this by breaking chemical bonds present in the initiator. The way in which the bond is broken

(homolytic scission) ensures that the two electrons which are involved in the bond end up on two separate fragments of the initiator (Figure 1.9).

Propagation:

The active centre which is present in the free radical "takes" one of the electrons from the double bond of the monomer. This then forms a new chemical bond with the initiator fragment and one of the double bonds of the monomer molecule, lengthening the chain (Figure 1.10).

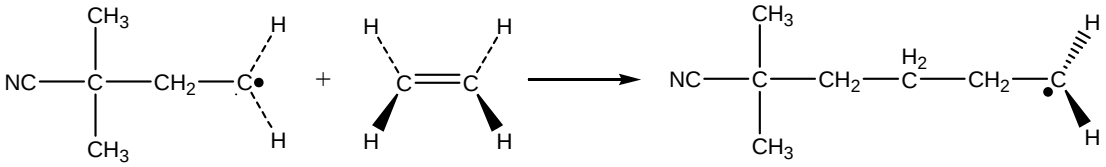


Figure 1.10: Illustration of the Propagation Step in Free Radical Polymerisation of Ethylene Using AIBN

Termination:

Termination is the third stage of free radical polymerisation. There are two main methods of termination reaction, combination and disproportionation.

During free radical polymerisation, as long as there is monomer present the polymerisation will normally continue, a term referred to as a chain reaction. During this process, however, electrons remain unpaired. As the chain reaction is generating a large flux of radicals, they will undoubtedly pair with each other without generating a new radical and form a new bond. This will terminate the chain reaction thus the reason it is aptly named coupling or combination. (Figure 1.11)

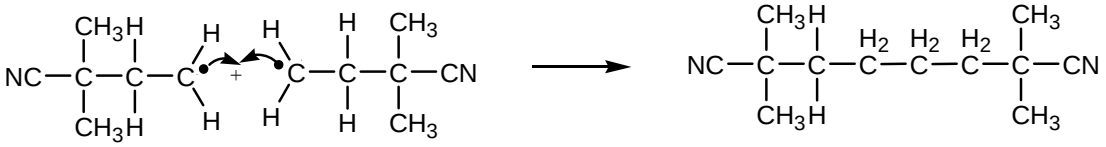


Figure 1.11: Illustration of the Termination of Two Growing Chains by Combination.[40]

The second method of termination in free radical polymerisation is termed disproportionation.

In this method, when two radicals meet the radical at the end of one chain abstracts a hydrogen atom from the ultimate monomer residue in the other active chain, i.e., it abstracts the hydrogen from an active chain. A carbon-carbon double bond is formed in place of the missing hydrogen.

This ultimately results in two terminated chains, one of which is saturated and the other with a terminal bond.

Figure 1.12 shows the termination of) radicals *via* disproportionation.

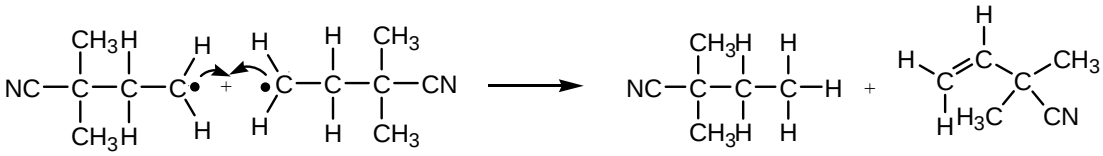


Figure 1.12: Illustration of the Termination of a Polymerisation Reaction by Disproportionation[40]

1.4.2 Functional Monomers

The role of functional monomer within the molecular imprinting process is important, and the various functional monomers which can be used in molecular imprinting were touched upon in Section 1.3.2. This section outlined three different types of functional monomer which can be employed in a non-covalent molecular imprinting system - acidic, basic and neutral (Table 1.3).

The role of the functional monomer differs with the different approaches to molecular imprinting. The predominant role, however, is that it must be able to self-assemble itself around the template throughout the polymerisation process, creating a monomer-template complex.[41]

The functionality of the monomer is therefore an extremely important factor as it must be complementary to the functionality of the template which is being imprinted. Luckily, many functional monomers are readily available to purchase, and are also relatively low in cost. To date, the two most commonly used functional monomers are methacrylic acid (MAA) and 4-vinyl pyridine (4-VP) (Figure 1.13).[30, 42]

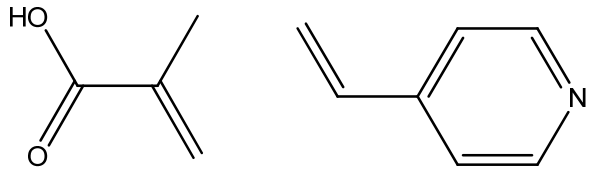


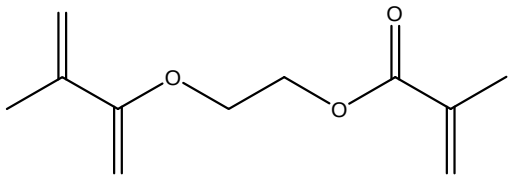
Figure 1.13: Structures of MAA & 4-VP

1.4.3 Crosslinkers

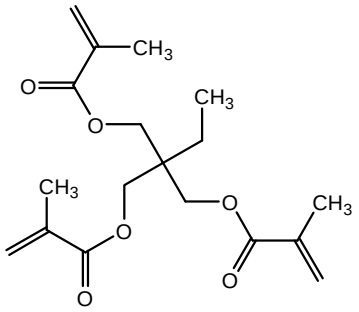
The role of a crosslinker in an imprinted polymer is to ensure that the binding site is stable, to control the morphology of the resultant polymer and to give the polymer matrix mechanical stability.[43, 44] The quantity of crosslinker used also influences the overall selectivity towards the template molecule of the imprinted polymer produced. To ensure a high degree of selectivity, a high level of crosslinking must generally be established. Approximately 70-90% of crosslinking is preferred.[45] In order for the resultant polymer have stable binding sites, and appropriate morphology, mechanical stability and demonstrate high selectivity, the cross linker selected must be able to react with the monomer(s) being utilised in the imprinting.[44,46]

The choice of crosslinker for use in molecular imprinting is restricted as only small quantities of crosslinkers have been assessed for use. This is on account of their poor miscibility monomers.[32, 45, 47, 48]The syntheses of molecular imprinted polymers carried out in solvents are generally executed using acrylate or styrene based monomers.[49] Examples of acrylate crosslinkers include ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM).[32,45,50] Styrene based monomers include p-divinylbenzene (DVB) and 1,3-diisopropenyl

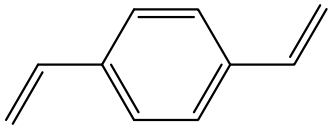
benzene (DIP).[46] The chemical structures of these crosslinkers are shown in Figure 1.14.



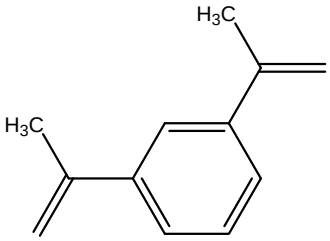
Ethylene glycol dimethacrylate (EGDMA)



Trimethylpropane trimethylacrylate (TRIM)



p-divinylbenzene (DVB)



1,3-diisopropenyl benzene (DIP)

Figure 1.14: Structures of Crosslinkers Which Have Been Used in Molecular Imprinting[46]

1.4.4 Initiators

As stated above, conventional free radical polymerisation takes place through three different stages, the first of which is free radical initiation (Section 1.4.1).

In order to initiate polymer growth an initiator is used and it is the thermal, chemical or photochemical decomposition of this initiator that allows the molecularly imprinted polymers to be prepared. The most commonly used azo-initiator is azo-*bis*-isobutyronitrile (AIBN) (Figure 1.15)

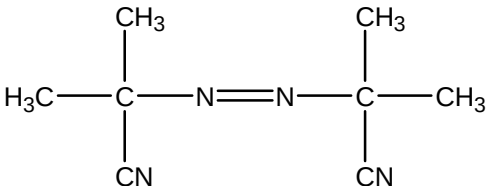


Figure 1.15– Structure of Azo-*bis*-isobutyronitrile (AIBN)

Using AIBN allows the imprinted polymers to be prepared thermally at moderate temperatures. AIBN is stable at 0 °C, but at 60 °C it decomposes rapidly to provide free radicals and it can also be used *via* UV at 0° due to its photochemical decomposition.[24, 51, 52]

The initiator used in molecular imprinting must be compatible with the template, monomer(s) and crosslinker in addition to being soluble in the porogenic solvent used in the preparation of the molecularly imprinted polymer.[46]

Typically, the polymerisation process takes anything between 24 to 48 hours to reach high monomer conversion.

1.4.5 Porogenic Solvents

The choice of porogenic solvent in the polymerisation system is of utmost importance. In non-covalent imprinting the role of the porogenic solvent is enhanced. The details of this are discussed in Section 1.3.2. The role of the solvent is to bring all of the different components of the polymerisation together (the functional monomer(s), the crosslinker, the template and the initiator) and also to create the pores within the macroporous polymer. This is the reason why the solvent is referred to as the porogenic solvent.[46]

In addition to this, the porogenic solvent is also responsible for the final morphology of the polymer matrix, as well as the pore volume; by increasing the volume of porogenic solvent, the pore volume will also increase. Figure 1.16 illustrates how the % of porogenic present can influence the final morphology of the polymer.

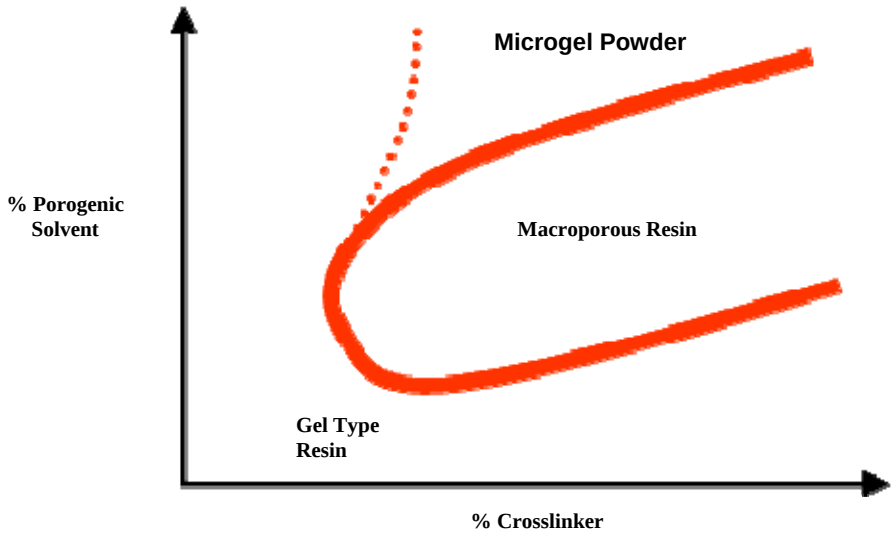


Figure 1.16: Illustration of a Polymer Morphology Pseudo-Phase Diagram[53]

As Figure 1.16 indicates, the use of high levels of crosslinker with a porogenic solvent gives a macroporous resin.

1.5 Proteins

A protein is an organic molecule comprised of amino acids that are joined together in a linear chain by peptide bonds. Proteins have high molar masses. Proteins have many biological functions; however it is the role of enzymes that is of particular interest to the present investigation.

1.5.1 Protein Characteristics and Structure

All enzymes are proteins, however not all proteins are enzymes. The structure and characteristics of a protein molecule are the same as those exhibited by an enzyme, however, enzyme molecules possess further characteristics which will be discussed in Section 1.5.2.

A protein has four level of structure: primary, secondary, tertiary and quaternary structure. Each of the structure levels is summarised in Table 1.5.[54]

Structure Level	Characteristic
Primary	Amino acid sequence in a polypeptide chain.
Secondary	The amino acid sequence of the primary structure becomes linked by hydrogen bonds. The structures formed are highly regular structures; alpha helices and beta sheets, for example.
Tertiary	Three dimensional structure of the protein molecule. This is formed when interactions occur between the alpha helices and beta sheets of the secondary structure.
Quaternary	The protein consists of more than one polypeptide chain.

Table 1.5: Protein Structure Characteristics Associated with the Primary, Secondary, Tertiary and Quaternary Structures[54]

The tertiary structure is the overall three dimensional shape of a protein. Each protein has its own unique three-dimensional structure, and this is referred to as the protein's native confirmation.

Changes in temperature, the introduction of an acidic or alkaline solution or a solvent can result in a protein becoming denatured.[55] Denaturation is the term used when a protein's native state changes shape, i.e., it is no longer in its optimal tertiary structure. The linear chains of amino acids assume a three dimensional shape when the protein is performing its biological function. Denaturation results in the interactions which hold the protein chain in shape being altered. As the biological functions of proteins are determined by their three-dimensional tertiary structure, [56] denaturation also has a detrimental effect on the function of a protein.

1.5.2 Enzymes

Enzymes are widely established as mild and selective biocatalysts in organic chemistry.[57] Enzymes catalyse reactions by lowering the activation energy (E_A) of a reaction once the enzyme has bound itself to the substrate (Figure 1.17). The figure shows the activation energy profile for a catalysed reaction with an enzyme present

in blue (blue arrow). The red profile shows the activation energy profile for a catalysed reaction with an enzyme present (red arrow). The change in free energy (Δ G) between the initial state and the final state is indicated in green (green arrow).

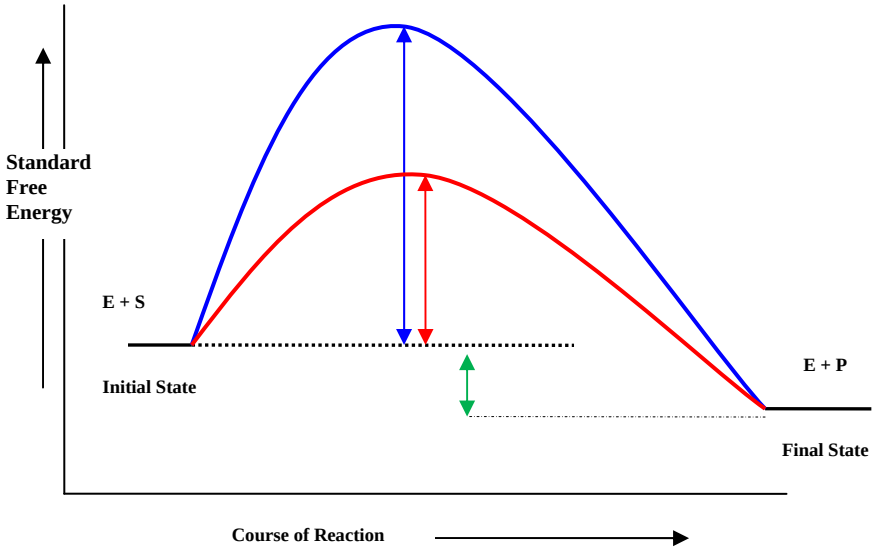


Figure 1.17: Diagram of Catalytic Reaction: A schematic diagram showing the free energy profile of the course of an enzyme-catalysed reaction involving the formation of enzyme-substrate (ES) and enzyme-product (EP) complexes[58]

Enzymes are highly specific both in the reaction catalysed and in their choice of reactants, which are called substrates.[59] An enzyme, can have one or more substrate. In the case of a single substrate, the enzyme binds to the substrate at a specific area, which is known as the active site. This is a small area of the protein and is approximately 10-20 amino acids long. The active site can be described as a groove on the surface of a protein. The shape of this groove is unique to every protein. In the case where there is more than one substrate in the enzyme, it will bind in a specific order to the active site.

The enzyme binds to the active site by means of an exact fit, i.e., the shape of the enzyme and the shape of the active site must be compatible (Figure 1.18). This hypothesis was put forward by Emile Fischer in 1895:

"…the intimate contact between the molecules…..is possible only with similar geometrical configurations. To use as a picture I would only say that the enzyme and the substrate must fit together like a lock and key."

Emile Fischer 1895

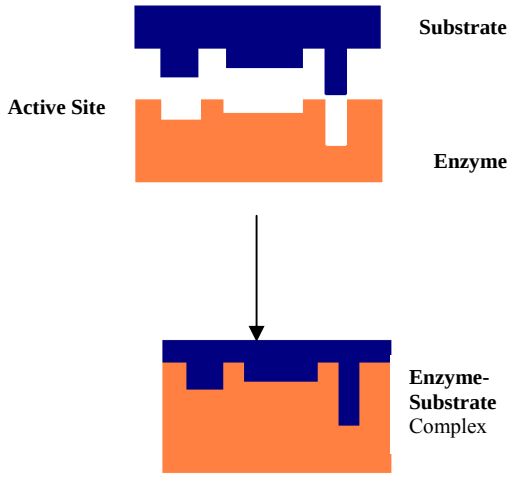


Figure 1.18: Schematic Representation of Emile Fischer's Lock and Key Mechanism[59]

Emile Fischer's hypothesis helped to explain substrate specificity; however, it did not shed any light on how the catalysis process functioned.

In 1958, Daniel Koshland introduced what is referred to as the "induced fit model". He argued that enzymes are flexible molecules, and as the active site is not a rigid structure for the substrate to conform to, the substrate instigates a change in shape in the enzyme, thus allowing the active site to fit like a "hand and glove".[60]

The enzyme (the glove) wraps around its substrate (the hand). The substrate induces a rearrangement in the atoms of the active site that enables the enzyme to cleave a chemical bond in the substrate (Figure 1.19).[59, 60]

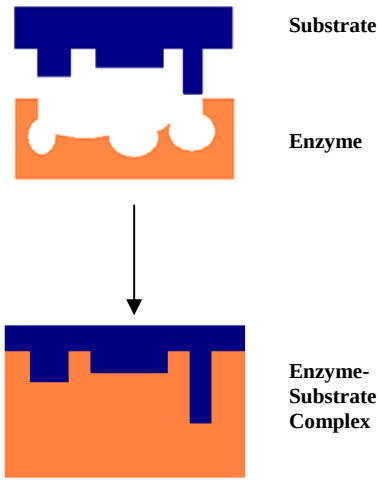


Figure 1.19: Schematic Representation of Daniel Koshland's Induced Fit Model [59, 60]

1.5.3 Protein Stabilisation

The role of biological catalyst is carried out by an enzyme when it is in its native state, and when it is in its favoured conditions. When the native state is altered, the shape of the enzyme's active site changes and the substrate molecule is not able to form the interactions with the polypeptide chain required for catalysis.

The favoured conditions of a protein are dependent on pH and temperature; however, solvents and ionic strength can also have an effect.[56]

Being able to maintain the stability and activity of a protein is of extreme importance in the area of bio-catalysis. Whilst proteins are in aqueous solutions they do exhibit enhanced stability and activity over that which they exhibit whilst in organic media.[61] In terms of molecular imprinting, this is another barrier as to why proteins cannot be used readily as templates.

Much research has been carried out on approaches to maintain the stability and activity of proteins. One method which has been reported is lyophilisation. Lyophilisation freeze-dries the protein. Once the water has been removed, it dramatically reduces the ability of the protein to undergo change, thus increasing its

stability.[62, 63, 64] This method is generally used for protein preparation for storage and use in organic media, [65] however it has been documented that this method also causes perturbation of the protein structure.[66, 67]

Other methods which have been used in an attempt to stabilise proteins include spray drying, a method in which a protein solution is nebulised, resulting in a fine spray of droplets in a heated chamber. The solvent evaporates resulting in a solid which is collected in a cyclone. Again, this is a method which exhibits many advantages, but as with lyophilisation it is an expensive and time-consuming method and requires the use of additives.[68, 69]

One novel method, which was developed by researchers working in the Universities of Strathclyde and Glasgow, is to form protein-coated micro-crystals (PCMCs) which immobilises a protein onto the surface of a crystal whilst retaining the protein in its active conformation.[70, 71]

1.5.4 Protein Coated Micro-Crystals

Research conducted by Dr Barry Moore *et al.* at the Universities of Strathclyde and Glasgow in 1998 saw the development of a method which overcame the problem of protein stability and activity in organic media; protein in the form of protein-coated micro-crystals (PCMCs) was prepared by crystal lattice mediated self-assembly (CLAMS)[70, 71] (Figure 1.20).

PCMCs are not only simple to prepare, but by following the CLAMS method it is one of the fastest and cheapest ways to overcome the problems associated with protein stability. The CLAMS method works by immobilising a protein onto the surface of a crystal, whilst the protein remains in its active conformation. A protein is dissolved in a saturated aqueous solution which contains a pre-selected excipient. Excipients that have been used include sugars and salts. This solution is then co-precipitated in a suitable solvent such as acetonitrile or isopropyl alcohol. During the

co-precipitation, the protein coats the surface of the excipient, which results in micron-sized crystals with protein molecules located at their surface - PCMCs.[72, 73, 74] The location of the protein on the surface of the microcrystal has been confirmed.

Differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and x-ray powder diffraction analysis has been employed by Dr Barry Moore *et al* to confirm that the core of the PCMCs is crystalline and that the protein is located at the surface. The coating of the protein at the surface ceases any crystal growth which may occur.[70, 74, 75]

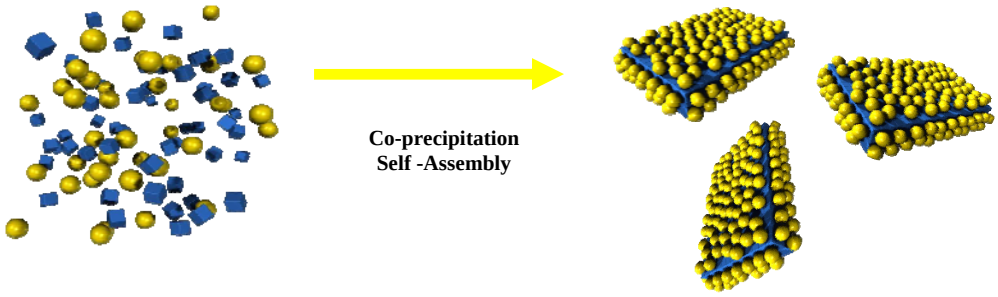


Figure 1.20: Schematic Representation of the Mechanism Associated with PCMCs Prepared by CLAMS[72, 73, 74]

Preparing proteins in this way offers a major advantage; during the CLAMS process the protein is dehydrated by the solvent in a way that denaturation is minimised, such that a large majority of the protein molecules remain in their active conformations.[70]

1.6 Proteins and Molecular Imprinting

The molecular imprinting of proteins is an area which offers great levels of promise and which can also offer uses in various applications such as separations, sensors and molecular diagnostics. Proteins are difficult to imprint due to their size, thermal instability and their tendency to denature in solvents required for imprinting. This, however, has not deterred research into this area. Early attempts to molecularly imprint proteins include the imprinting of gels,[76] surface imprinting onto silica[77] and

mica,[78] an approach which is referred to as the epitope approach[58] (wherein a short peptide sequence is imprinted), and molecular imprinting by immobilisation of the template covalently onto silica.[79]

More recently, there has been a renewed interest in imprinting proteins. In the time that this investigation was being conducted, there were several publications in the area of molecularly imprinting proteins. Studies within this area include using chitosan to increase selectivity, [80, 81] surface imprinting directly onto sensor surfaces, [82, 83] synthesising molecularly imprinted polymers as surface bound nanofilaments[84] and protein imprinting of hydrogels in an aqueous environment via coordination bonds.[85, 86]

This Section will review some of these methods for imprinting proteins, in chronological order of development.

1.6.1 Gel Imprinting

Gel imprinting is a technique which was developed by Hjertén *et al.* It is a technique which is used for the selective adsorption of a protein. Gel particles are prepared with properties which are selective towards proteins, with the first work carried out using haemoglobin, cytochrome c and transferrin.[89] The technique works by preparing a gel whilst it is in the presence of a protein template. The gel is synthesised by using pre-selected monomers and the protein template in question. Polymerisation is carried out for thirty minutes, until the gel is formed. The gel is then passed through a mesh in order to obtain granules. This is required so it is possible to pack a column for subsequent removal of the protein template. Once this has been achieved, it is possible to continue with rebinding experiments.

One key factor in this method for imprinting proteins is the use of functional monomers. Hjertén *et al*. found that the functional monomers tend to decrease the level of specificity possible. This is due to the fact that they are generally charged and thus give rise to electrostatic interactions which in turn can give the gel the property of acting as an ion-exchanger.

Since the first reports of gel imprinting, further work has been carried out using this method to imprint ribonuclease and myoglobin from horse.[76] A ribonuclease gel was prepared using acrylamide, N,N'-methylenebisacrylamide and ribonuclease whilst in the presence of ammonium persulphate (initiator) and sodium phosphate at pH 7. The resultant gel was passed through a 60 mesh net to produce granules, which were subsequently packed into a column. The column was a glass pipette with glass wool in the constriction as a support for the granules. A proteinase solution was then passed through the column in order to remove the template protein, ribonuclease.

Rebinding was carried out with a solution containing haemoglobin and ribonuclease being passed through the column, then being washed further with sodium phosphate at pH 7. The fraction was collected and analysed for haemoglobin and ribonuclease content. Analysis confirmed that there was no ribonuclease present, and thus the gel was selective towards only the template protein.

1.6.2 Surface Imprinting on Mica

Imprinting proteins onto a surface to reveal protein recognition sites was attempted by a group of scientists led by Buddy Ratner.[78] Ratner's group proposed that by using radio-frequency glow-discharge plasma decomposition, [90] polymeric thin films could be formed around template protein molecules which were covered with disaccharide molecules, which would subsequently become covalently bound to the polymer film. The cavities formed would be polysaccharide-like, thus showing great selectivity towards the template protein.

Ratner's group selected mica as the surface for this method. Mica is an aluminosilicate clay which is a naturally flat. Mica is also hydrophilic, and its surface is negatively charged. In terms of protein imprinting, this is beneficial due to the fact that denaturation is minimised.[91] The disaccharide is used so that hydrogen bonding occurs between the sugar and the template protein during dehydration. This is required in order to reduce any denaturation which may occur due to drying or plasma-induced degradation.

Using bovine serum albumin (BSA), immunoglobin and fibrinogen as template proteins, surface imprinting was carried out. The protein being used was adsorbed on the mica surface before being covered with a disaccharide layer. The disaccharide layer was then fixed in place using radio-frequency glow-discharge plasma decomposition of hexafluoropropylene. Hexafluoropropylene was used as it reacts with organic moieties on the surface yielding a smooth film. Mounting the plasma film onto a solid support ensures that the mica can be easily detached. The template protein was removed to reveal protein imprinted nano-cavities.
Analysis of the nanostructured surfaces for the templates mentioned previously, and also for blank nanostructured surfaces (no template present), showed that the level of protein which was absorbed onto the protein imprinted surface was higher than that of the blank, however there was no selectivity displayed for different proteins on the protein imprinted surface.

Although the protein template imprinted nanostructured surfaces show selectivity for protein over the blank nano-structured surfaces, they do not exhibit specificity, which is a limitation that is required to be overcome in the world of protein imprinting.

1.6.3 The Epitope Approach

This approach takes into account all of the factors which hinder the use of proteins as templates, and attempts to overcome them.

The epitope approach (Figure 1.21) is a technique which was based initially on the formation of complexes between the functional monomer and a template based on weak, non-covalent interactions such as ionic and hydrogen bonding. The complexes are fixed sterically by polymerisation and crosslinking. The template is then removed and subsequent rebinding experiments are conducted.[79]

The epitope approach relies on the idea that if a short peptide representing only a small exposed fragment of a protein structure is used as the template, the MIP

produced will recognise the peptide and also be able to recognise the whole protein molecule.

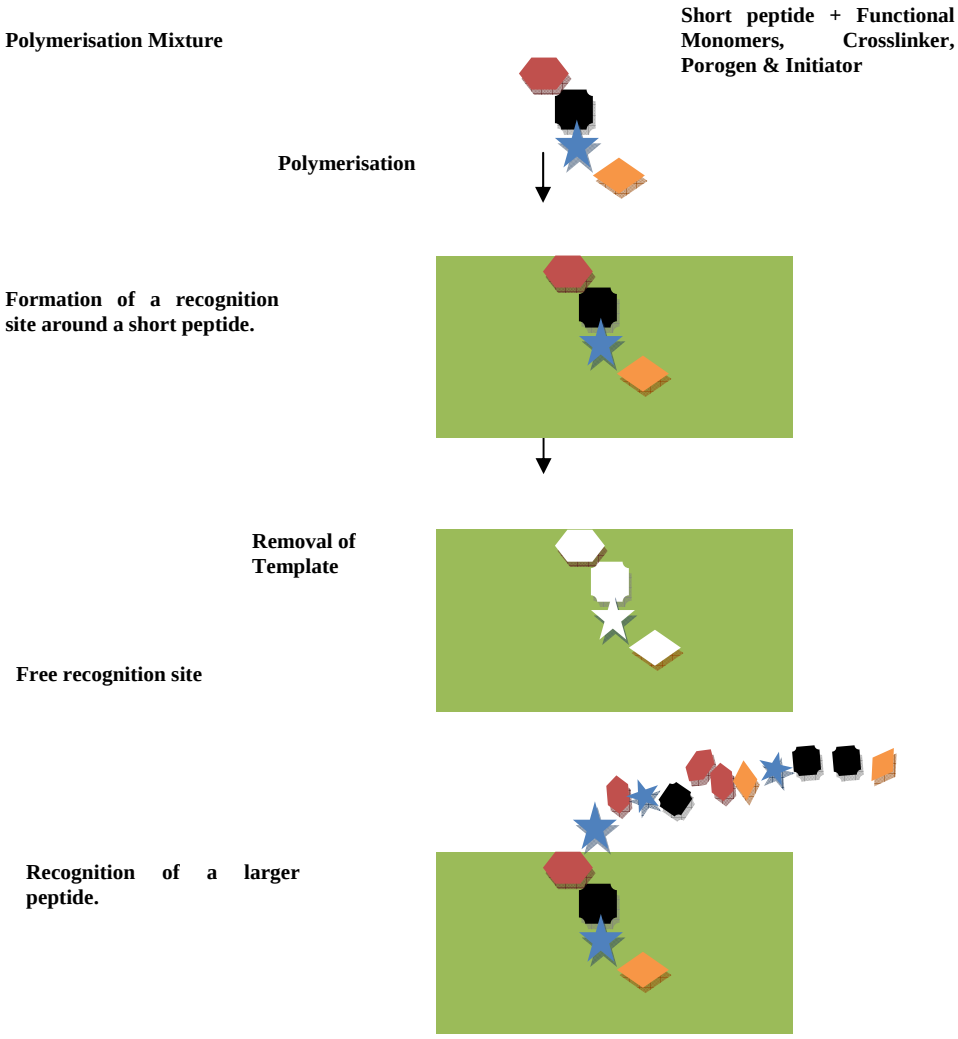


Figure 1.21: Schematic Representation of the Epitope Approach[78]

Figure 1.21 represents the epitope approach. Alexandre Rachov and Norihiko Minoura first reported this method in 2000.[91] They used methacrylic acid, ethylene glycol dimethacrylate and 2,2'-azobis(2,4-dimethylvaleronitrile) as their functional monomer, crosslinker and initiator, respectively, with acetonitrile as the porogenic solvent. The short peptide with which they worked was *Tyr-Pro-Leu-Gly*. This is the epitope for oxytocin. All components were combined and polymerisation was carried

out at 40 °C for 16 hours, with the formation of a polymer with recognition sites around the peptide template. After polymerisation was complete the template was removed and rebinding experiments were conducted. The rebinding experiments showed that the MIP showed recognition for the peptide, *Tyr-Pro-Leu-Gly,* but also for oxytocin.
Although this result can be seen as promising, it also revealed that the MIP recognises other peptides which contain this sequence, thus it is not wholly selective for the protein intended.

Shea *et al* have also carried out extensive research within this area. The Shea group utilised exposed epitopes of proteins as templates for imprinting. As the epitopes only contain a small number of a protein's amino acid sequence, it was possible to match peptide sequences from a variety of proteins: cytochrome c, alcohol dehydrogenase and BSA.

The polymerisation took place on a glass or silica surface, around the peptide chain template. Once the polymerisation was complete, the surface was removed leaving behind epitope-imprinted binding sites.[92]

Competitive rebinding analysis was conducted. The imprinted polymer was incubated in a solution containing a mixture of proteins. Specific binding was achieved *via* hydrogen bonding; the imprinted protein formed several hydrogen bonds with the imprinted sites of the polymer. The other proteins within the mixture did not form anywhere near the number of hydrogen bonds as the template molecule; thus the binding strength of these proteins was comparatively low compared to the binding strength of the template molecule.[89]

1.6.4 Surface Imprinting on Silica Surfaces using Silanes

Surface imprinting onto silica was a method which was first researched by Mosbach *et al.* Mosbach's method utilised non-covalent interactions as their recognition vehicle.[76]

Another method, which was reported by T. Shiomi *et al.* in 2005, uses a covalently immobilised template on silica, upon whose surface silane polymerisation is, performed (Figure 1.22).[93]

Using haemoglobin as the template protein molecule, solely because its characteristics are well known, it was covalently immobilised, then the silane polymerised. The haemoglobin was then removed using an acidic wash. Rebinding experiments were then carried out.

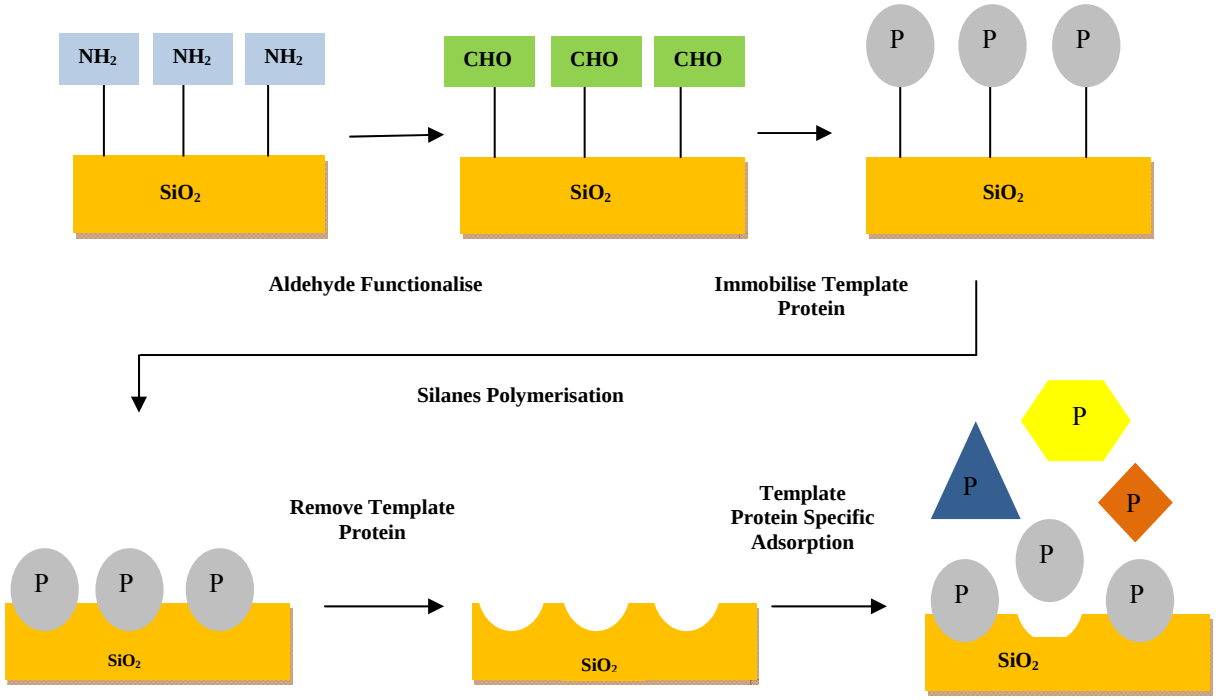


Figure 1.22: Schematic Representation of Protein Imprinting Using Immobilised Templates[93]

Prior to the immobilisation of the template (haemoglobin), imine bonds were formed between the amino groups present on the haemoglobin and the aldehyde groups which are present on the silica. The polymerisation takes place on the haemoglobin-silica surface in the presence of 3-aminopropyl trimethoxysilane and propyltrimethoxysilane. The haemoglobin was removed with 1M aqueous oxalic acid.

Rebinding experiments were then conducted. For competitive rebinding studies the proteins selected were myoglobin, transferrin and chymotrypsinogen A. Two mixtures were used for rebinding: one containing haemoglobin, transferrin and chymotrypsinogen A, and the other containing haemoglobin and myoglobin.
Analysis indicated that the haemoglobin imprinted silica showed selectivity for myoglobin over the other proteins present. This was indicated by a feint band present on the SDS-PAGE for haemoglobin.

1.6.5 Surface Molecular Imprinting

In recent years, research and development within the area of surface imprinting has become increasingly popular.[94, 95, 96] Both 3D molecular imprinting and 2D molecular imprinting have been applied to surface imprinting. 3D imprinting encompasses much of the same methodology as bulk imprinting, thus it also faces similar drawbacks such as a low re-occupation of the binding cavities and lengthy rebinding times.[82]

Surface imprinting *via* 2D imprinting offers many advantages to molecular imprinting. Advantages include a higher re-occupation percentage of the binding cavities and fast response for the target molecule to be rebound to the polymer.

Yantian Wang *et al.* utilised self-assembled monolayer's (SAMs) as their imprinting matrix material for their research in 2D imprinting. SAMs have been used in molecular imprinting previously by Zhou *et al.* and Tabushi *et al*.,[97, 98] however their research focused primarily on small organic molecules;, not proteins.

Yantian Wang *et al.* manufactured a potentiometric protein sensor which was constructed employing thiol SAMs. Using the matrix material, monolayer's of alkanethiol with hydroxyl terminal groups and the protein template, the sensing material was created on the surface of the gold-coated silicon chip - an electrochemical transducer.[82]

The template materials of choice were myoglobin from equine muscle and heamoglobin from bovine blood.

Figure 1.23 illustrates the mechanism of the 2D surface molecular imprinting.

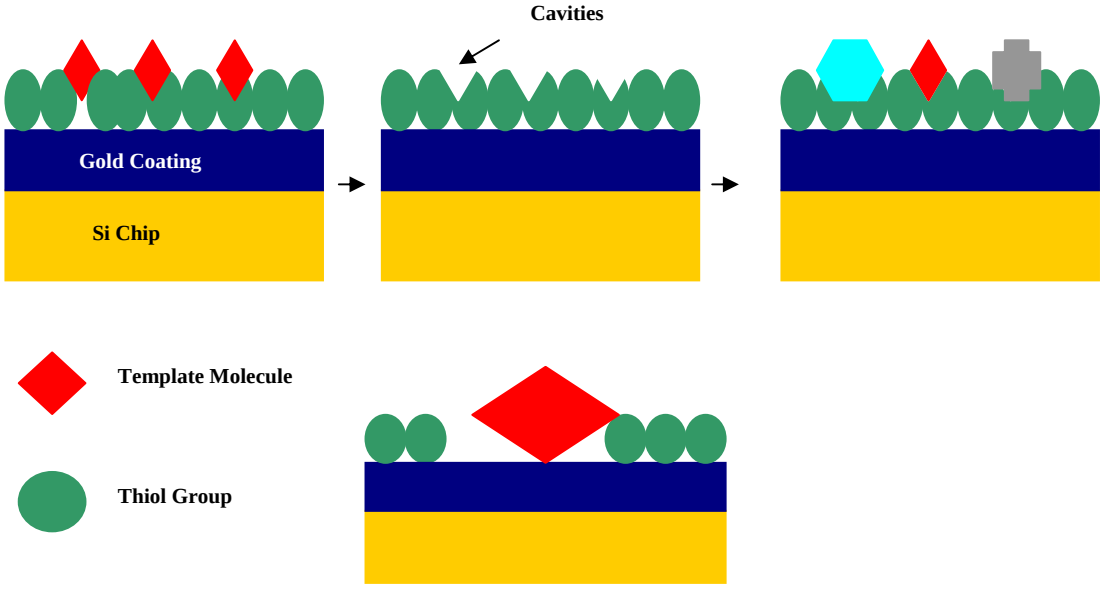


Figure 1.23: Schematic Representation of the Mechanism of the 2D Surface Molecular Imprinting[82]

The thiol SAMs and the protein template molecule are adsorbed on the gold surface (Figure 1.23). Once the polymerisation has occurred, the templates are removed by washing, to reveal template-shaped cavities. The 2D polymer was then subjected to competitive rebinding wherein selective rebinding of the protein molecule occurred *vs.* alternative protein molecules. The bottom picture suggests the binding mechanism. This is hypothesised to be *via* hydrogen bonding between the protein and –OH end groups of the thiol.

In the case of the myoglobin and haemoglobin template molecule, the group found that the sensors displayed an affinity for the template molecule, although this was dependant on the concentration of the template molecule being introduced to the sensor.

1.6.6 Surface Bound Nanofilaments

In another area of surface imprinting, is the synthesis of nano-structured molecularly imprinted polymers such as molecularly imprinted nanotubes[99, 100] and nanowires. [101, 102]

The synthesis of molecularly imprinted nanofilments has been investigated and reported by Karsten Haupt *et al* in recent years. [84, 103, 104] The group have demonstrated that by using a combination of nanomolding and molecular imprinting, nanofilaments with molecularly imprinted sites which can specifically bind proteins can be generated.

Their most recent publication, [84] discusses the preparation of myoglobin imprinted nanostructures.

The preparation of the molecular imprinted nanostructures via the use of nanofilaments is three-fold. The template is immobilised onto porous alumina substrates producing surface bound nanofilaments. Glass surfaces with attached polymer nanofilaments are then obtained. The resultant nanostructures, which are surface bound, are then synthesised in the form of molecularly imprinted polymers.[84, 104]

With respect to the protein imprinting, myoglobin was used as the template protein. Myoglobin was immobilised on to the alumina substrate *via* use of a glutaraldehyde linker to generate myoglobin nanofilaments. The nanofilaments produced were attached to glass surfaces; this was based on a co-polymer system, with the polymerisation initiated by UV light. The attachment of the nanofilaments produces the nanostructures. They were generated by nanomolding the nanofilaments to the porous alumina surface. The porous alumina surface was produced by electro-oxidation. Treating the surface with acid opened the pores.[84, 102]

For comparison purposes, the group synthesised two MIPs; each of the MIPs utilised a different functional monomer in its preparation. MIP 1 was prepared using hydroxyethyl methacrylate (HEMA), whilst the second was prepared in the presence

of MMA. The crosslinker utilised for both MIPs was EGDMA. No porogenic solvent was used as the binding sites generated were situated at the surface of the nanofilaments.

On assessment of the MIPs imprinting effects, it was found that they both demonstrated good imprinting effects, i.e., good uptake of the imprinted template myoglobin. A key observation was that the MIP prepared with HEMA as its functional monomer had a higher rebinding level than that of the MAA based MIP, and consequently also demonstrated a lower level of non-specific binding (assessments completed via the use of a control polymer). The group reported that as a hydrophilic MIP, and with an imprinting factor of 7, the HEMA based MIP was more suitable for protein imprinting than that on the MAA based, more hydrophobic MIP which had an imprinting factor of 3.

1.6.7 Preparation by Metal Coordination

Molecularly imprinted polymers prepared via metal ion coordination was first described by Kechen and Shramm in 1988.[105] It was not until 1995 however, that the Protein–metal ion coordination imprinting approach was described (Mosbach *et al*).[106] Although this was not directly "molecularly imprinting" it paved the way for further work to in developing peptide metal ion imprinting, [107, 108] and subsequently protein metal ion imprinting.[85, 86, 109]

The protein-metal ion coordination imprinting approach aims to overcome the challenges of protein imprinting which has been discussed in this Chapter. It is has been discussed thus far, that proteins favour aqueous environments vs. solvents. Unfortunately, this environment (aqueous and/or polar) does not optimise the interactions between a template protein and functional monomer(s) which is required for molecularly imprinting and can significantly reduce the effects of the crosslinker.[46,110, 111]

Metal co-ordination works by using taking advantage of the interactions which can form between the metal ions and the functional groups on the protein residues whilst in an aqueous environment; interactions which are easy to form and easy to break.[112] The protein-metal ion complex can then utilised in molecular imprinting; interactions between the functional monomer(s) and the metal-ion complex allow polymerisation to occur.

Recently, the use of bovine serum albumin (BSA) and copper ion to prepare molecularly imprinted hydrogels was reported by Wang *et al*.[85]

Wang *et al*. used the copper ion to form a metal ion complex between the BSA and itself before performing the polymerisation in an aqueous solution .They theorised that the copper ion would bridge the BSA and functional monomers together during the polymerisation to produce MIP hydrogels which demonstrated a high selectivity vs. a NIP hydrogel. The NIP hydrogel was prepared in with the copper ion absent.

Synthesis of the BSA MIP hydrogel was carried out using Acrylamide (AA), 4-VP and *N*,*N*-methylenebisacrylamide (BIS). The copper ion and the BSA were dissolved in a TRIS-hydrochloric acid (TRIS-HCl) solution, along with the functional monomers and crosslinker. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate were added to the polymerisation solution prior to it being deoxygenated. Polymerisation was carried out at room temperature for 12 hours. The resultant MIP was washed with an aqueous salt solution and deionised water (dH2O) to remove the BSA template. Upon confirmation of the template removal, the MIP was dried for 48 hours. He same protocol was executed to prepare the NIP with the absence of the copper ion and BSA.[85]

Rebinding experiments were executed on the MIP and NIP hydrogels.

The group found that when they execute the rebinding studies at the same temperature at the polymerisation was executed; it heightened the imprinting factor when the BSA was being rebound in the presence of a copper ion. They also

demonstrated that whilst carrying out the rebinding in the presence of the copper ion the MIP had enhanced selectivity *vs.* the NIP concluding that the protein-metal ion binding was effective for generating binding sites in the hydrogel.

The group concluded that they had proved their hypothesis, and that including a copper ion into the polymerisation strengthened the interaction between the functional monomers and the template protein, thus enhancing the imprinting effect.

1.7 PCMCs and MIPs

The utilisation of PCMCs in molecular imprinting offers many advantages and possibilities for overcoming many of the aforementioned difficulties in imprinting proteins. One of the key factors in the molecular imprinting of proteins is to ensure that the protein is stable and the structure is not perturbed. With PCMCs, the protein is immobilised onto the surface of the crystal thus its stability remains throughout the polymerisation process whilst demonstrating an enhanced stability to pH, solvents and temperature. In addition to this, the use of an excipient in the preparation of PCMCs generates large pores in the polymer matrix. After the polymerisation has been completed, the excipient can be dissolved readily, thus removal from the MIP is simple. This results in the large pores being revealed which facilitates the extraction of the protein and subsequent re-binding.

With regards to the production of MIPs, PCMCs are treated as the template and incorporated into a pre-polymerisation mixture (contains functional monomer, crosslinker, initiator and porogenic solvent). The polymer produced is a hard monolithic material in which the template can be removed and subsequently rebound to the MIP. The ease of template removal can be attributed to the large pores generated by the PCMCs.

1.8 Aims and Objectives

1.8.1 Aims

The aims of this investigation can be broken into two sections.

The initial or primary aim of this investigation was to generate molecularly imprinted polymers which use enzymes as templates. The resultant polymers will exhibit selectivity for the template protein over any competitors. The method used to do this involves the immobilisation of proteins onto the surfaces of crystals (PCMCs) and the subsequent imprinting of these into polymers (MIPs). After removal of the PCMCs from the MIPs, the performance and selectivity of the MIPs will be probed.

The secondary aim of this investigation is to assess the robustness of the MIP produced, i.e. the quantity of rebinding cycles the MIP can be subjected to, and, to investigate the selectivity of the MIP for its template protein when it is contained in a complex mixture.

1.8.2 Objectives

When concerned with the primary aim, the following objectives of this study are:

- Establish the optimal conditions in which PCMCs can be prepared for their employment as the template or molecularly imprinting.
- Determine which analytical methods can be utilised for the analysis of the PCMCs i.e., for determination of the presence of protein on the micro-crystal
- Establish the optimal conditions in which MIPs can be synthesised utilising PCMCs as the template molecule. The conditions should generate a hard monolithic MIP.
- Investigate and determine the conditions in which 100% of the template (protein and micro-crystals) can be removed from the polymer matrix after synthesis.

- Ascertain a rebinding method in which the performance of the MIP can be explored with respect to its rebinding capabilities and the selectivity of the MIP.
- Determine which analytical methods can be utilised for the analysis of the rebinding capabilities and the selectivity of the MIP i.e., the determination of the presence of protein.

The objectives for the secondary aim are detailed below:

- Investigate the quantity of rebinding cycles that the MIPs prepared can undergo.
- Establish the factors which may affect the MIPs ability to rebind the template molecule. Determine the optimal conditions at which rebinding can occur.
- Probe the selectivity of the MIP vs. a NIP.
- Establish the selectivity of the MIP for the template molecule whilst it is in the presence of a complex mixture.

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2.0 Analytical Techniques

Various analytical techniques were employed to assess the properties of the protein-coated micro-crystals (PCMCs), molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) development throughout this investigation.

The techniques utilised allowed a thorough assessment of the chemical, physical and morphological properties of each component (where required).

2.1 High Performance Liquid Chromatography

Reversed – Phase High Performance Liquid Chromatography (RP-HPLC) was used in several areas of the study, but was generally used to determine the presence of protein concentrations in aqueous samples.

The analysis carried out *via* RP - HPLC was achieved by injecting the sample into a Waters 2695 separations module (Waters UK, Waters Ltd, 730-740 Centennial Court, Centennial Park, Elstree, Hertfordshire, WD6 3SZ). The equipment also encompassed a 2996 Photodiode Array Detector and a 2487 Dual λ absorbance detector (λ_{210nm} – λ_{400nm}). The software utilised was Empower Pro Software.

Every analysis was performed at a flow rate of 1 mL/min and column temperature of 30 °C. The injection volume was maintained at 50 μL.

Whilst carrying out analysis *via* RP-HPLC, two columns were used; a Waters Symmetry300™ C4 column, 4.6 x 150 mm, and a Vydac 218TP™ C18 column, 250 x 4.6 mm. (Grace Vydac, 2051 Waukegan Road, Deerfield, IL 60015, USA)

Detection of the protein molecules using RP-HPLC was done performed at λ_{218}, λ_{280} or λ_{400}. The wavelength selected was specific to the protein of interest within each sample.

The proteins being analysed were subject to one of three elution protocols, two of which were gradient elutions and one of which was an isocratic elution, as Detailed in tables 2.1, 2.2 and 2.3, respectively.

Mobile Phase A is 0.1% trifluoroacetic acid (TFA) in deionised water (dH_2O). Mobile Phase B is 0.1% TFA in ACN.

Time (minutes)	**Flow Rate (mL/min)**	**% Mobile Phase A**	**% Mobile Phase B**
0	1	80	20
20	1	20	80
22	1	20	80
25	1	80	20
30	1	80	20

Table 2.1: Gradient Elution Protocol 1 for the Detection of Proteins *via* RP-HPLC – C4 Column

Time (minutes)	**Flow Rate (mL/min)**	**% Mobile Phase A**	**% Mobile Phase B**
0	1	80	20
20	1	60	40
22	1	20	80
25	1	80	20
30	1	80	20

Table 2.2: Gradient Elution Protocol 2 for the Detection of Proteins *via* RP-HPLC – C18 Column

Time (minutes)	Flow Rate (mL/min)	% Mobile Phase A	% Mobile Phase B
0	1	100	0
45	1	100	0

Table 2.3: Isocratic Elution Protocol for the Detection of Proteins *via* RP-HPLC – C4 and C18 Column

2.2 UV/Vis Spectrophotometry

As with the RP – HPLC, UV/Vis spectrophotometry was a method of protein analysis which was continually used extensively for various experiments.

All UV/Vis analysis was carried out on a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter (UK) Ltd, Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU).Each sample was 0.5 mL in volume and was placed in quartz cuvettes for analysis.

Two techniques for analysis were applied; a fixed wavelength measurement and also a wavelength scan. The fixed wavelength measurements were taken at λ_{218}, λ_{280} or λ_{410} depending on the protein of interest. The wavelength scan had a range λ_{218} - λ_{600}. All samples were measured at 25 °C.

2.3 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a technique which ascertains the morphology and topography of a substance; PCMCs, MIPs and NIPs.

SEM is an excellent tool for measuring particle size when the particle size range is relatively narrow within a sample.

Due to the nature of the MIP and NIP samples, and given that the particle size is not uniform and evenly distributed throughout the MIP and NIP samples, SEM does not provide a true reflection of the particle size throughout the MIP and NIP samples. With this in mind, additional further analysis was conducted in order to determine the size and distribution of the MIP and NIP samples.

All SEM analysis was carried out using a Hitachi S-3000N VP-SEM (Hitachi High-Technologies Corporation Europe GmbH**,** Europark Fichtenhain A12, 47807 Krefeld, Germany).

Sympatec Particle Sizing was used for this; as described below.

2.4 Particle Size Analysis

To characterise the particle size of the molecularly imprinted polymers (MIPs) produced a Sympatec Particle Sizer (Sympatec GmbH, System-Partikel-Technik, Am Pulverhaus 1, D-38678 Clausthal-Zellerfeld, Germany) was used. The characterisation was carried out whilst the MIPs were in flight.

The technique uses laser diffraction to assess the particle size. The sample is dispersed as a dry powder over a set period of time and is measured against an internal standard.[1]

The equipment used for the analysis was the Helos BF optical bench, (the laser diffraction sensor) with the particles being dispersed by a Rodos/M (a universal dry dispersing unit) which comes equipped with an Aspiros (micro-dosing device) small volume feeder. The equipment is capable of characterising particles which are 0.1 µm to 3500 µm in size. The equipment was fitted and validated to the manufacturer's standards at XstalBio Ltd; Glasgow University, UK

The MIP particles were dispersed at a 2 bar dispersion pressure. Throughout the dispersion, a series of measurements were collected. The software provided a mean of all the measurements collected, thus providing a mean particle size throughout the distribution. The equipment was calibrated prior to use.

2.5 Molecularly Imprinted Solid-Phase Extraction

Molecularly Imprinted Solid-Phase Extraction (MISPE) was used to investigate the ability of the MIPs to extract the imprinted protein from an aqueous environment, probe the cross selectivity of the MIPs in relation to structurally-related and non-structurally related proteins, and also to assesses the resultant structure (i.e. the protein has remained in its native conformation) on the protein once rebound to the MIP and the non-imprinted polymer (NIP).

In a traditional MISPE, the MIP was used as the stationary phase and packed into a column.[2, 3, 4] The column selected was dependant on the experiment carried out. Column 1 refers to a double-fritted filtration column (Figure 2.1) whereas Column 2 refers to a micro-centrifugal filter tube (Figure 2.2). A double-fritted filtration column refers to a double layer of polytetrafluoroethylene (PTFE) frit contained at the bottom of the column. The micro-centrifugal filter tube contains a single layered frit.

Figure 2.1: Double-Fritted Filtration Column for use in Molecularly Imprinted Solid-Phase Extraction

Figure 2.2: Micro-Centrifugal Filter Tube for use in Molecularly Imprinted Solid-Phase Extraction

The method by which the elution-step fractions were obtained from the columns post-MISPE was dependant on the column selected. In experiments where Column 1 was the column of choice, standard solid-phase extraction (SPE) apparatus was used i.e., an SPE vacuum manifold (Figure 2.3) When column 2 was utilised, a Microcentrifuge system was used.

Figure 2.3: SPE Vacuum Manifold with Double-Fritted Filtration Columns for use in Molecularly Imprinted Solid-Phase Extraction

Prior to loading the SPE column, the MIP was suspended in acetone then allowed to sediment for approximately 20 minutes. The MIP particles which did not sediment were removed by decantation. The MIP was allowed to dry before proceeding with the next stage.

The column of choice was packed with a known mass of the MIP. In order to allow the MIP to pre-wet prior to conducting any experiments, the MIP was suspended in 10% v/v methanol (in dH_20) for 15 minutes, allowing access to the pores within the MIP. The previously mentioned methanol solution was eluted from the column after the MIP had been pre-wet for 15 minutes. The resultant eluent was not retained as it was not required for analysis. This was carried out for all MISPE analysis.

The technique of rebinding and competitive rebinding *via* SPE is relatively simple (illustrated in Figure 2.4 and Figure 2.5) and can be summarised in four steps: conditioning, loading, washing and elution.

2.5.1 Conditioning

The column containing the sorbent (MIP or NIP) is conditioned by suspending the contents of the column in the mobile phase of choice; thus creating slurries of the sorbent and mobile phase. The mobile phase is eluted from the column after the conditioning period. As the fraction collected is not required for analysis, it is not retained.

2.5.2 Loading

The column is loaded with a solution. Examples of the loading solution include solutions containing the template protein only, a mixture of proteins, or a solution containing no protein at all; in essence a blank solution.

After the load solution has passed through the column, the fraction is collected for further analysis. This procedure was carried out for all loading solutions; all loading fractions were retained.

2.5.3 Washing

The next stage in SPE is to wash the MIP or NIP SPE cartridge with mobile phase. This step is introduced to ensure that the loading solution has passed through the column successfully and to overcome any non-specific binding which may have occurred, i.e., any free protein on the column could be easily removed.

After the wash solution has passed through the column, the fraction is collected for further analysis. This is required for all washing samples.

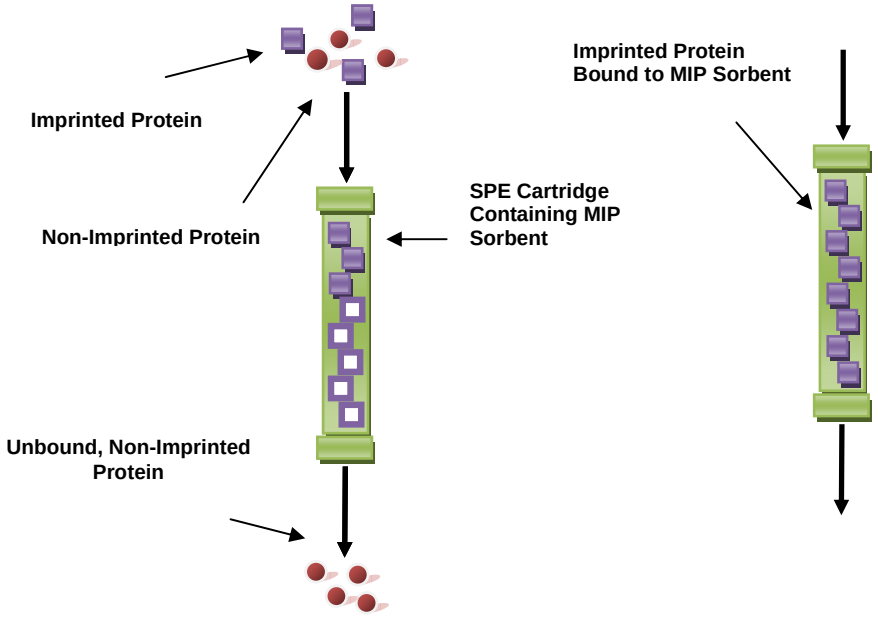

Figure 2.4: Illustration of Loading Step in Molecularly Imprinted Solid-Phase Extraction

2.5.4 Elution

A suitable eluent for the elution of the imprinted template molecule is passed through the SPE cartridge. Once the elution solution has passed through the column, the fraction is collected for further analysis. This is required for all elution samples.

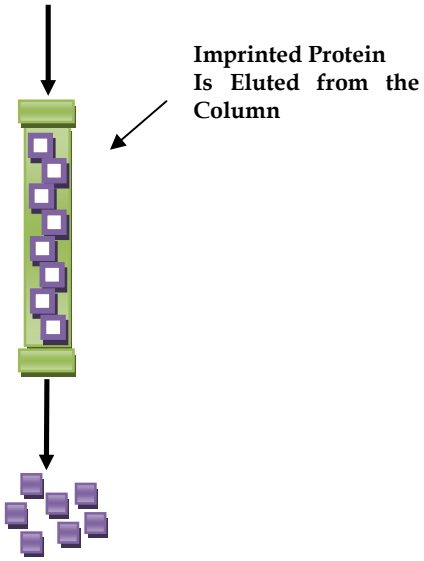

Figure 2.5: Illustration of the Solid-Phase Extraction Elution Procedure used in Molecularly Imprinted Solid-Phase Extraction

2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was employed to determine the presence of protein(s) in aqueous samples. These samples have been subjected to rebinding experiments, thus allowing the ability of the MIP to successfully rebind imprinted proteins to be probed in comparison to the binding ability of the NIP. Applying this technique also enabled the selectivity of the MIP and NIP to be probed when the imprinted protein was in the presence of a biological sample. SDS-PAGE is essential at this stage of the analysis as it allows each individual component of the biological sample (including the protein) to be separated out and identified in one single experimental procedure.

The separation of biological molecules *via* SDS-PAGE is achieved by means of a potential difference. Biological molecules possess ionisable groups, which when in solution exist as electrically charged species; cations (+) and anions (-).[5]

Applying a potential difference to biological molecules in solution (ionisable state) allows the biological molecules to be separated. The molecules move at different speeds according to their molecular weight, net charge and the shape of the molecule. The molecules will migrate towards the anode or cathode depending if they are negatively or positively charged.

A suitable matrix for the separation of the subject molecules must also be selected.

For the purposes of the experiments detailed in this thesis, pre-cast polyacrylamide gels were used. The pre-cast gels, purchased from Bio-Rad Laboratories (Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX), are cast without the SDS present, which allows either native PAGE or SDS-PAGE to be carried out. SDS-PAGE was the only method employed throughout the experimental sections utilising SDS-PAGE.

The application of polyacrylamide gels in gel electrophoresis is widely known as it was used in 1959 by two independent groups; Davis and Raymond.[6, 7] The pre-cast gel used in this investigation was a 15-well gel and had a linear gradient of 4-20%. The % value refers to the % of acrylamide present in the gel. A linear gel contains a low percentage of acrylamide at the top of the gel (where the samples are loaded) and a high percentage of acrylamide at the end of the gel i.e. 4% at the top increasing across the gel to 20% at the bottom. By using a gel with a linear gradient, a far superior resolution is obtained, and samples with a wide range of molecular weights (10-250 kDa) can be analysed.

2.6.1 Application of SDS-PAGE

In order for the technique of SDS-PAGE to be successful, the protein samples to be analysed, the proteins must be in the form of their primary structure (outlined in Chapter 1, Section 1.5). All samples are placed in the presence of SDS before analysis in order to denature them, thus allowing them to take on their primary, amino acid sequence

structure. SDS is an anionic detergent which binds to proteins in a constant mass ratio of 1.4 gm SDS/gm protein[8, 9, 10.] Once bound, the SDS denatures the proteins and coats them with a negative charge. This negative charge is uniform therefore it ensures that all proteins present for analysis have the same mass-charge ratio. By doing this, the mobility of the protein becomes a function of molecular weight; thus the migration of the protein in a polyacrylamide gel is a suitable method of determining the presence of a protein.

2.6.2 Electrophoresis

After sample preparation, the samples are loaded into the wells of the pre-cast gels. It is at this point that a constant potential difference is applied across the gel. The potential difference causes the negatively charged proteins to migrate towards the anode at different rates.

The potential difference is applied for a set time period (minutes). The potential difference was stopped when all of the proteins have migrated across the gel. The length of time the potential difference is applied varies; it is dependant on the proteins contained within the samples, and of course the size of the current applied. The larger the current, the quicker the migration will be.

2.6.2 Staining

Once the electrophoresis is complete, it is necessary to visualise the proteins on the gel. This is achieved by staining the gel.

Silver staining is used to detect the proteins which have been separated by gel electrophoresis, as described in Section 2.5.2. The utilisation of silver staining is to ensure that even minimal quantities of proteins ($\geq$ 0.3 ng) can be visulised[11].

Once the electrophoresis is complete, the gels were silver stained using the fast method, wherein a microwave oven is utilised to reduce the staining procedure to be completed in one hour.[12]

A SilverQuest™Silver Staining Kit purchased from Invitrogen (Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland, UK, PA4 9RF) contained the reagents required for silver staining.

Once stained, each protein is represented as a band on the gel. The positioning of the silver protein band is dependant on the size of the protein, thus allowing each individual sample to be identified or, in the case of the biological samples, to allow each protein within a mixture to be identified. In order to establish the molecular weight of the protein represented by a band, the first sample load should be what is referred to as a protein marker; each band left by the marker represents a protein of a particular molecular weight.

2.7 References

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3.0 Protein-Coated Micro-Crystals

3.1 Introduction

To prepare protein-coated micro-crystals (PCMCs), a protein, a suitable excipient and a suitable solvent must be selected. Since the resultant PCMCs are being used for molecular imprinting, each of the components must be compatible with the imprinting process.

Three different PCMCs were used throughout the present study; myoglobin, β-D-Glucosidase and acid phosphatase PCMCs

3.2 Components of PCMCs

When preparing PCMCs, there are components which must always be present; the excipient and the solvent. There is also an additional component which may be present; a buffer.[1]

3.2.1 Choice of Excipient

The excipient which is selected in the preparation of PCMCs must be able to be removed easily from the MIP, thus it should demonstrate high solubility in water, but should also be able to retain the structure of the PCMC whilst in the presence of an organic solvent, such as those solvents used typically for molecular imprinting. A key factor in selecting a suitable excipient it that is must be able to form crystals. For these reasons, the choice of excipient which has been used previously has included sugars, amino acids and salts.[1, 2]

Potassium sulfate (K_2SO_4) was selected as a suitable excipient for the production of PCMCs throughout this work, due to the fact that it precipitated readily from water to form micro-crystals when it was in the presence of an organic solvent such as acetonitrile or isopropyl alcohol. Another key factor in the selection of K_2SO_4 was

the cost; this salt is inexpensive to purchase, which means that potential scale-up of the PCMC-based approach would not be prohibitively expensive.

3.2.2 Choice of Organic Solvent

There are many suitable organic solvents that can be selected for the preparation of PCMCs, such as isopropyl alcohol, acetonitrile and ethanol to name a few. In an ideal system, the organic solvent used to prepare PCMCs ought to be the same as the porogenic solvent which is used in the preparation of MIPs to avoid the need to carry out a solvent switch.

By using the same solvent in the PCMC preparation stage and the MIP synthesis the solvent does not need to be changed between the PCMC preparation and the MIP preparation; the use of acetonitrile in both the production of PCMCs and MIPs would avoid a solvent switch, whereas producing PCMCs in isopropyl alcohol would require a solvent switch from isopropyl alcohol to a solvent that is compatible with non-covalent molecular imprinting.

3.2.3 Choice of Buffer

Aqueous buffers are used as a means to keep the pH of a sample constant throughout an experimental process.

An aqueous buffer solution contains a mixture of a weak acid and its conjugate base. The buffer solution can resist any change in pH when a small quantity of an acid or a base is added to it, i.e., it removes the hydroxide or hydrogen ions which may have been added to it, thus maintaining the pH.[3]

Although only slight variations of the acid and/or base can resist changes to the pH, each buffer does have what is referred to as a buffer capacity. A buffer capacity describes this ability to resist any changes in pH, regardless of the addition of an acid

or a base. The buffer capacity increases as the molarity of the buffer solution increases.[4]

An ideal buffer for a specific pH should have pKa value which is equal to that pH, ensuring that the buffer has a maximum buffer capacity for the pH.[3]

The two key things that must be considered prior to selecting a buffer are; 1) the pKa value of the sample that is being worked with - the buffer selected will have a pKa value which is close to the middle of the range required; 2) it is necessary to be aware of the working conditions - all buffers should be prepared at the working conditions (i.e., temperature and concentration). A slight variation n temperature can result in a variation of final pH.

The buffers selected were potassium phosphate and citrate buffers.

3.3 Preparing PCMCs

Prior to preparing PCMCs, there are two other factors which must be considered. The protein loading and the final water content of the resultant solution after the co-precipitation of the solubilised protein and excipient into solvent. [1]

3.3.1 Protein Loading

The amount of protein which is required for the production of PCMCs is referred to as the theoretical protein loading (TPL), and is reported as % w/w. This is based on the mass of protein present with respect to the mass of the excipient.[5, 6, 7]

3.3.2 Water Content

The water content refers to the quantity of water present in the resultant solution after the co-precipitation of the solubilised protein and excipient into solvent. [5, 6, 7]

The water content is generally reported as a % v/v and can be determined from the volume of saturated excipient - protein solution which is introduced to the organic solvent. As the PCMCs are to be used in the production of MIPs it is advisable to keep the final water content low. A final water content of 5% v/v was decided upon.[6]

To lower the % water content further, prior to the polymerisation step the acetonitrile (ACN) present was siphoned off and replaced with dry ACN. This process was repeated three times to ensure low water content for all PCMCs prepared.

3.4 PCMC Methodology

The use of PCMCs as templates in molecular imprinting requires the protein to be immobilised onto the surfaces of the micro-crystals. All PCMCs required were prepared with a 10% protein loading and 5% water content in ACN.

In order to prepare the PCMCs two different methods were employed. The method of choice depended on the mass of the PCMCs required.

The first method is the drop-wise method.[8] The PCMCs prepared using this method are prepared so on a small scale (i.e., using 20 mL of solvent), and by hand (Figure 3.1). The second method is used for the production of larger masses of PCMCs (i.e., using 1 L of solvent) and is carried out using a continuous-flow precipitator (Figure 3.2).

3.4.1 The Drop-Wise Method

An aqueous solution of saturated K_2SO_4 and protein was added drop-wise to ACN, with the aid of stirring *via* a magnetic stirrer bar. The PCMCs formed and precipitated out of solution. The resultant mixture was then filtered in order to capture the PCMCs. The filtration step was only included for the purpose of constructing a calibration curve, and was not incorporated when the PCMCs were used in the production of MIPs; these PCMCs were left suspended in solvent.

Once the PCMCs had been isolated, a calibration curve was constructed so that the quantity of protein immobilised on to the surfaces of the micro-crystals could be established.

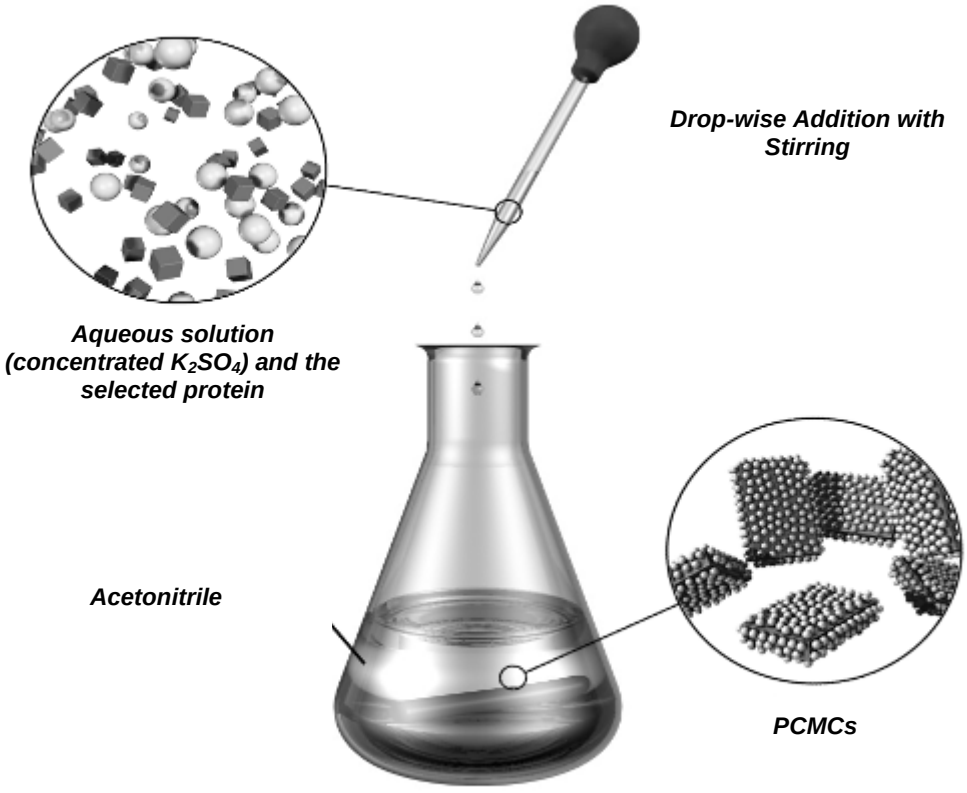

Figure 3.1: Illustration of the Drop-Wise Method for the Preparation of PCMCs

3.4.2 Continuous-Flow Precipitator Method

The Continuous-Flow Precipitator was developed by Dr Jan Vos, now of XstalBio.[5] The purpose of the Continuous-Flow Precipitator is to allow the aqueous protein/excipient solution to be added to the solvent at an increased rate, thus allowing precipitation to occur rapidly, and also allows larger quantities of PCMCs to be prepared in a smaller amount of time. The reproducibility of the method is also better than the drop-wise method.

Figure 3.2: Photographic Representation of the Continuous-Flow Precipitator Used for the Preparation of PCMCs

The equipment is constructed from two liquid chromatography pumps and a specially altered solvent mixing module. The pumps used were Gilson 303 models. The pumps are capable of delivering accurate flow rates in the range 0.5 to 150 mL/min. Two interchangeable pump heads were used: a 1 mL displacement head and a 20 mL displacement head. The pumps heads were reconditioned and calibrated prior to initial use. The 1 mL displacement head is used for the delivery of the protein/excipient solution, and the 20 mL displacement head was used for the solvent delivery.[2]

The protein solution and the precipitating solvent meet at the specially altered solvent mixing module, which in turn allows the PCMCs to precipitate out of solution. The PCMCs are then collected in a Duran bottle, and the PCMCs are given time to settle out of suspension.

All other PCMCs prepared for the polymerisations were not dried, but left in suspension in the solvent until they were ready to be incorporated in the polymerisations.

3.5 Maintaining Solubility of PCMCs

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In order to establish the actual protein loading (APL) of the PCMCs vs. the TPL (theoretical protein loading) it was necessary to re-dissolve the PCMCs into de-ionised water (dH_2O) for UV/Vis analysis.

To ensure that all PCMCs which had been prepared could be re-dissolved back into an aqueous solution i.e., dH_2O, an investigation was carried out to establish the optimum excipient concentration and the pH at which the PCMCs were soluble.

The pH that the PCMCs are prepared must be considered as this could have an effect on the eventual solubility; if the PCMCs are prepared at a pH which is close the isoelectric point of the protein, the solubility of that protein is at a minimum.

Another consideration in the PCMC preparation is the quantity of the excipient used. The level at which the excipient is no longer soluble i.e., the saturation level[9] must be determined; this will in turn allow a suitable concentration of excipient to be used which will aid in the overall solubility of the PCMCs.

Various experiments were conducted in order to establish the optimum conditions for preparing PCMCs which would easily and rapidly dissolve in dH_2O. The concentration of the excipient used was also assessed.

The pH at which the proteins were entirely soluble was established.

All of the PCMC samples prepared for this work was then prepared at this established pH.

Once the PCMCs had been re-dissolved in dH_2O, analysis was carried out *via* RP-HPLC Spectrometry. A calibration curve was constructed for each protein.

RP-HPLC was used to determine the optimum pH. The RP-HPLC method uses a column which is packed with hydrophobic material.

When a protein is in an acidic solution, the amino groups are ionised and the carboxylic acid groups are less likely to be ionised. When the protein is in an alkaline environment, the reverse is true; the carboxylic acid groups are ionised and the amino groups are not.

For any particular protein there is one pH level at which the average ionization is neutral-this is referred to as the isoelectronic pH.[10]

When a protein is at its isoelectronic pH, the hydrophobic protein interacts strongly with the hydrophobic column contents, while any other proteins, which are not at their isoelectronic point, pass through rapidly. When the protein interacts strongly the best separation is achieved.[11, 12]

The output from the HPLC is recorded as a peak. The peak area is proportional to the quantity of protein which has been injected to the HPLC, thus the peak area is a means of quantifying the concentration present. The protein which has bound to the column will record the highest peak area. The protein solution which records the highest peak area through this analysis is representative of the solution in which the protein has fully dissolved. The output from the RP-HPLC analysis is key is determining the pH at which the protein is at its most soluble.

3.6 Experimental Procedures

3.6.1 Chemicals

Sodium Citrate ($Na_3C_6H_5O_7$)
Citric Acid ($C_6H_8O_7$)
Potassium Sulfate (K_2SO_4)
Potassium Phosphate Dibasic (K_2HPO_4)

Potassium Phosphate Monobasic (KH_2PO_4)
Acetonitrile - (HPLC Grade)
Molecular Sieves - 3Å
Myoglobin from Horse Heart
β-D-Glucosidase from Almonds
Acid Phosphatase from Potato
Deionised Water (dH_2O)

Myoglobin from horse heart, β-D-Glucosidase from almonds and Acid Phosphatase from potato were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England) in the form of recrystallised powders. Potassium sulfate, potassium phosphate dibasic, potassium phosphate monobasic and acetonitrile were also purchased from Sigma-Aldrich. The acetonitrile was of HPLC grade. All solvents and chemicals were $\geq$ 90% pure. The acetonitrile was dried prior to use over activated molecular sieves (size 3Å) also purchased from Sigma Aldrich.

3.6.2 UV/Vis Spectroscopy

UV/Vis analysis of the PCMCs was carried out using the methodology described in Chapter 2 (at a fixed wavelength of 410 nm).

3.6.3 RP-HPLC

RP-HPLC analysis of the proteins was carried out using the methodology described in Chapter 2. All analysis via RP-HPLC was carried out in triplicate. The results reported are the average results.

3.6.4 Preparation of Excipient Solutions

A standard excipient solution was prepared at a concentration of 100 mg/ mL (5 g of K_2SO_4 was dissolved in 50 ml of dH_2O). The K_2SO_4 was fully soluble at 100 mg/ mL, thus this solution was not saturated.

For excipient concentrations of 80, 75, 60 and 55 mg/ml, known volumes of the stock solution were taken and diluted with dH_2O until the required concentration was reached.

3.6.5 Preparation of Protein Solutions

In order to obtain a 10% w/w loading of protein onto the resultant PCMCs, the initial protein concentration was calculated to be 6.1 mg/ml.

The protein concentration is calculated via the ratio of protein to excipient in the aqueous solution to give theoretical protein loading, based on the assumption of 100 % excipient recovery (Equation 1).

$$\left\{\frac{\text{Excipient Concentration} \times 100}{\text{Payload}}\right\} - \text{Excipient Concentration}$$

Equation 3.1: Protein Solution Concentration for PCMC Preparation

Three proteins were used to obtain three different types of PCMC (Table 3.1).

Protein	**Sample Name**	**Volume of Protein/Excipient Solution Prepared (mL)**	**Mass of Protein (g)**	**PCMC Methodology**
Myoglobin	P_1	1	0.006	Drop-wise Method
β-D-Glucosidase	P_2	30.1	0.189	Continuous Flow Precipitator
Acid Phosphatase	P_3	19	0.116	Continuous Flow Precipitator

Table 3.1: PCMC Sample Composition for Myoglobin, β-D-Glucosidase and Acid Phosphatase PCMCs used throughout this Investigation

3.6.6 Preparation of Buffers

The potassium phosphate buffer was prepared using stock solutions of dibasic potassium phosphate and monobasic potassium phosphate. The stock solutions were both prepared at a concentration on 1 M. Known volumes of the stock solution were added together and diluted with dH_2O to give the required pH and concentration of buffer. The compositions detailed in Table 3.2 are for a final concentration of 100 mM (diluted to 1 L).

pH	**Volume of Potassium Phosphate Dibasic (mL)**	**Volume of Potassium Phosphate Monobasic (mL)**
5.8	8.5	91.5
6	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7	61.5	38.5
8.1	94.0	6.0

Table 3.2: Composition of Potassium Phosphate Buffer at Varying pH Values.

The citrate buffer was prepared by adding masses of citric acid and sodium citrate to 1 L of dH_2O. This was mixed thoroughly by means of magnetic stirrer. By changing the masses of the citric acid and sodium citrate used, solutions of various pHs were prepared. The compositions are detailed in Table 3.3.

pH	Mass of Citric Acid (g)	Mass of Sodium Citrate (g)
4.42	1.09	1.42
4.68	0.94	1.62
4.82	0.87	1.73
5.01	0.76	1.87
5.2	0.66	2.01
5.41	0.55	2.17
5.61	0.45	2.31
5.83	0.34	2.46
6.01	0.26	2.58

Table 3.3: Composition of Citrate Buffer at Varying pH Values.

3.7 Results & Discussion

As reported in Section 3.3, all PCMCs were prepared under the conditions outlined in Table 3.4.

% Protein Loading (w/w %)	Solvent	% Water Content (w/w %)
10%	ACN	5%

Table 3.4: PCMC Preparation Conditions Myoglobin, β-D-Glucosidase and Acid Phosphatase PCMCs used throughout this Investigation

As the ability to re-dissolve the PCMCs back into solution after preparation was a key factor, the pH at which the PCMCs were prepared was investigated. Under the conditions outlined in Table 3.5, PCMCs were prepared at various pH values using a potassium phosphate buffer.

pH	***Solubility After 20 Mins***	***Solubility After 24 Hours***
5.03	Not Soluble	Not Soluble
5.42	Not Soluble	Not Soluble
5.97	Not Soluble	Not Soluble
6.49	Not Soluble	Not Soluble
6.97	Not Soluble	Not Soluble
8.05	Not Soluble	Not Soluble

Table 3.5: Determination of Solubility of PCMCs at Various pH Values for Myoglobin, β-D-Glucosidase and Acid Phosphatase PCMCs

As varying the pH did not result in PCMCs which could be re-dissolved back into aqueous solution it was obvious that the pH was not the single factor in the ability of the PCMCs to be re-dissolved back into an aqueous solution Following on from this result, the concentration of the excipient at which the PCMCs were prepared was assessed. For these experiments, the PCMC samples analysed were all prepared at pH 7 (Table 3.6)

Excipient Concentration (mg/ml)	**Solubility - Instantaneous**
100	Not Soluble
80	Not Soluble
75	Not Soluble
60	Not Soluble
55	Completely Soluble

Table 3.6: Determination of Myoglobin, β-D-Glucosidase and Acid Phosphatase Solubility of PCMCs at Various Excipient Concentrations

The results show that as the concentration of the excipient is increased, the ability to re-dissolve the PCMCs back in to aqueous solutions is affected.

The saturation level of potassium sulphate (the excipient) is reported to be 110 – 120 mg/mL at room temperature, [13] thus by preparing the PCMCs at saturation level of 55 mg/mL; the PCMCs should have the ability to be re-dissolved back in to an aqueous solution instantaneously.

Table 3.6 refers to concentrations less than 100 mg/mL which are not soluble. It is worth noting that this refers to instantaneous solubility and not solubility of specific time periods.

3.7.1 Myoglobin PCMCs

To determine the optimum pH at which P_1 PCMCs should be prepared, various myoglobin buffered solutions at different pH values were prepared. The buffer used was a 100 mM potassium phosphate buffer. The concentration of myoglobin remained constant throughout the experimental procedure.

The pH of the solutions used in the determination of the optimum pH and resultant average peak area reading from the RP-HPLC experiments are detailed in Table 3.7.

pH	**Average Peak Area**	**Standard Error (Peak Area)**
5.2	231288	± 200
5.42	261304	± 243
5.68	281569	± 306
5.81	305698	± 161
5.97	252569	± 96
6.18	212684	± 168

Table 3.7: Optimum pH Determination, pH Values and Peak Areas for Myoglobin PCMCs

These results were then used to construct a curve showing the average peak area variations against the different pH values (Figure 3.3).

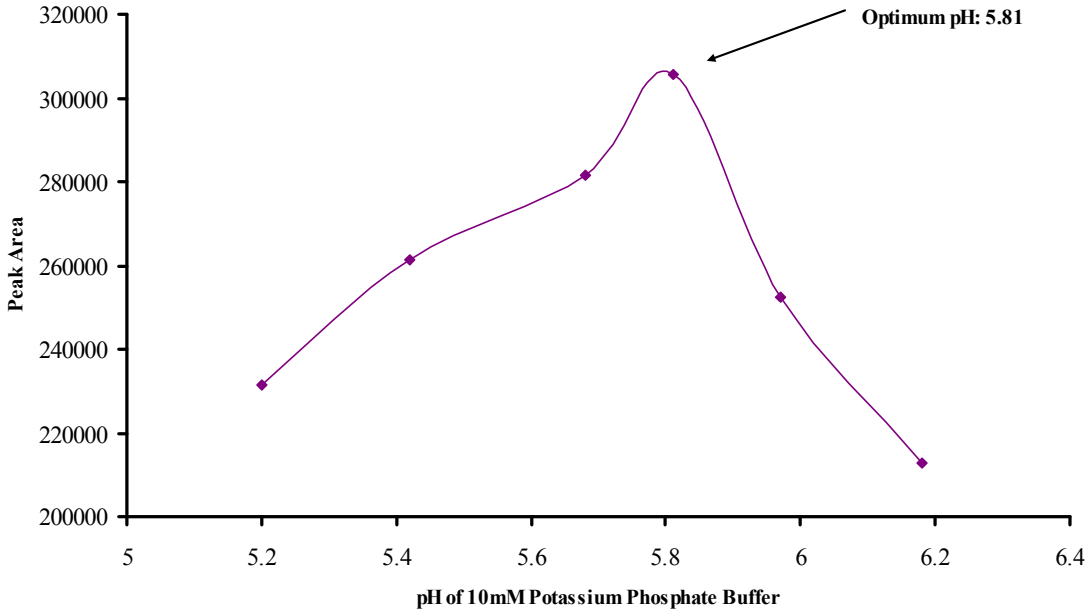

Figure 3.3: Optimum pH Calibration for Myoglobin PCMCs

The graph shows that when the protein was dissolved in an aqueous solution, pH 5.81, the highest average peak area was recorded.

As the concentration of the myoglobin was constant for all solutions, the analysis shows that varying pH of the 100 mM potassium phosphate buffer has an impact of the solubility of myoglobin and at lower pH values i.e., pH 5.2 the myoglobin is less soluble than at pH 5.81.

The mixing time for each of these solutions prior to sample extraction was the same, as was the initial myoglobin concentration. This result indicates that varying the pH of the buffer solution has a direct impact on the solubility of the protein in to the solution, as the peak area of the RP-HPLC chromatograms vary as a function of pH.

As stated previously, due to the nature of the work detailed in this investigation, the solubility of the protein in solution is a critical factor, thus the pH at which the largest quantity of protein re-dissolves into aqueous solution in the shortest time frame is the pH at which the protein will be prepared throughout this investigation.

Therefore, when working with myoglobin solutions throughout this investigation the 100 mM potassium phosphate buffer was used in the experimental procedures the pH was set at 5.81.

Once the optimal pH for myoglobin solubility in an aqueous solution was determined, two samples of the PCMCs were prepared. One of the samples was used to determine the actual protein loading (APL) of the PCMCs and the other sample was dried, at room temperature, for SEM analysis.

In order to determine the APL, PCMCs P_1 were dissolved into 100 mM potassium phosphate buffer, pH 5.81. The concentrations of each of the solutions were varied. The experiment was conducted three times. The absorbance of each solution was recorded and a calibration curve constructed using the average absorbance readings (Table 3.8 and Figure 3.4).

Myoglobin Concentration (mg/ml)	**Absorbance**	**Standard Error (Absorbance Units)**
0	0	0
0.0025	0.0122	± 0.0020
0.005	0.0251	± 0.0026
0.0075	0.0415	± 0.0017
0.01	0.0542	± 0.0026
Myoglobin on PCMCs - 0.005	**0.0253**	± 0.0022

Table 3.8: Concentration of Myoglobin vs. Absorbance Calibration Results

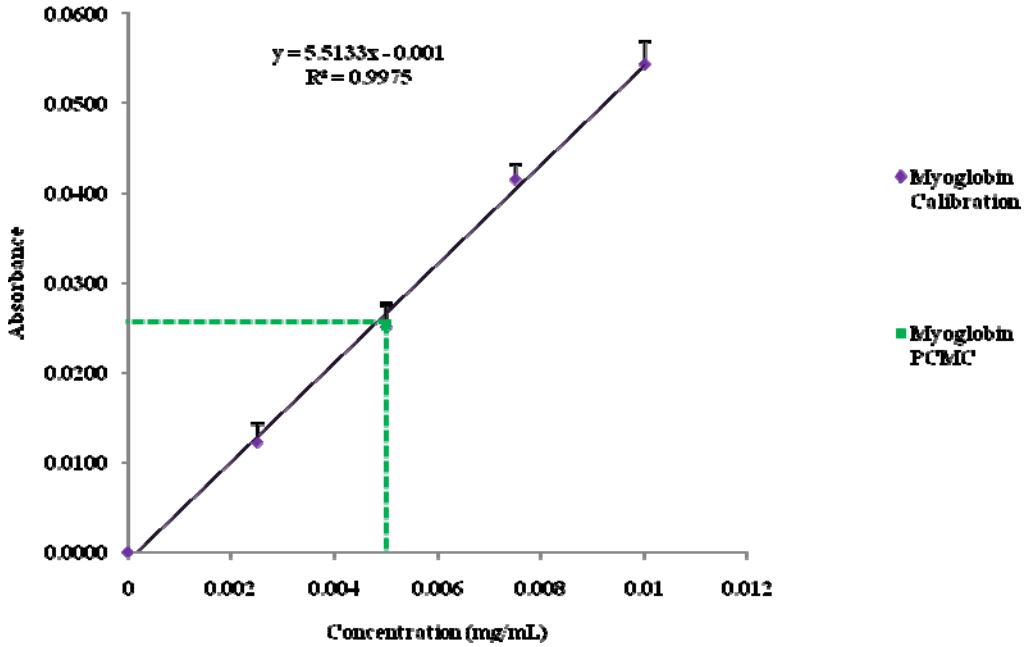

Figure 3.4: Calibration Curve for Myoglobin with Absorbance of Myoglobin PCMCs (λ_{410} nm)

The average absorbance of sample P_1 with the equivalent of 0.005 mg/ml myoglobin present was measured. It was determined that 94% of myoglobin was immobilised on the surfaces of the micro-crystals. 94% of myoglobin immobilised at the surface is an acceptable quantity. Previous work has reported levels of immobilisation on the surface of micro-crystals being as low as 60%, with the subsequent activity being 68%.[14]

The dried sample of P_1 was examined by SEM at a magnification of x 6400 (Figure 3.5).

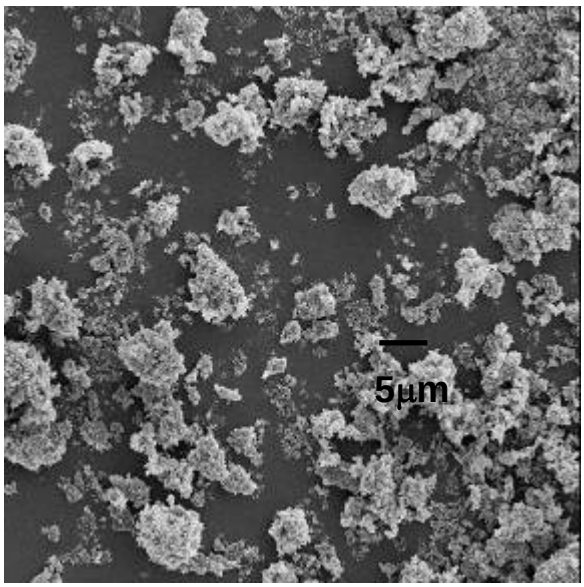

Figure 3.5: SEM Image of Myoglobin PCMCs

The SEM image indicates that there is a large distribution of particle sizes for sample P_1, in the range 5-15 μm; however it is not possible to specify the exact size of the particles. It can be seen in the SEM image that as the sample has been dried the particles have agglomerated. The only way to overcome this problem and obtain a true image would be to keep the PCMCs in suspension; which is not possible when using SEM as a means of analysis.

3.7.2 β-D-Glucosidase PCMCs

As with P_1 PCMCs, P_2 PCMCs were prepared with potassium sulfate and ACN. The pH at which the PCMCs were able to be re-dissolved back into an aqueous solution was determined (Table 3.9, Figure 3.6). The buffer used was 10 mM citrate buffer.

pH	Average Peak Area	Standard Error
4.42	1208420	± 235
4.68	1280363	± 225
4.82	1323751	± 129
5.01	1373455	± 201
5.2	1408646	± 187
5.41	1367291	± 854
5.61	1335293	± 179
5.83	1306689	± 162
6.01	1264753	± 239

Table 3.9: Optimum pH Determination, pH Values and Peak Areas for β-D-Glucosidase PCMCs

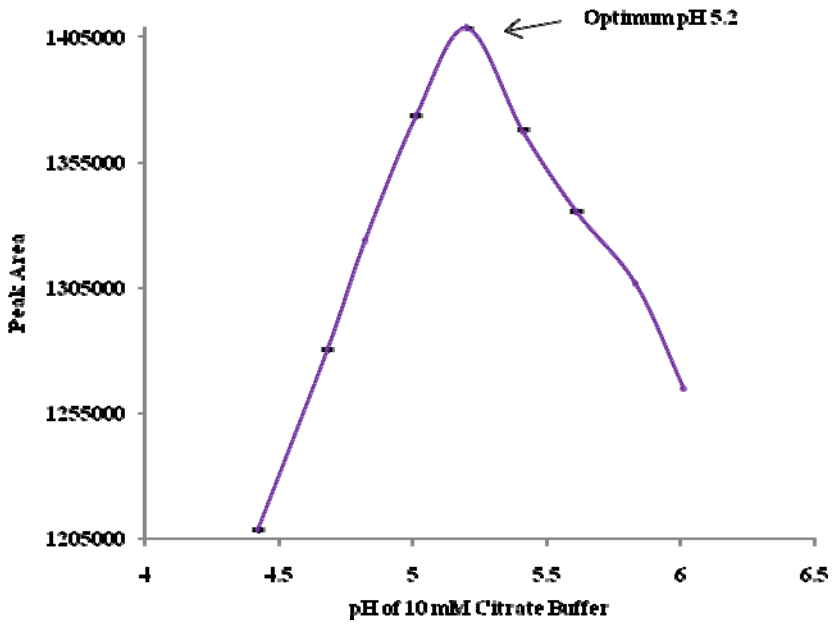

Figure 3.6: Optimum pH Calibration for β-D-Glucosidase PCMCs

Figure 3.6 show that the β-D-Glucosidase prepared at pH 5.2 has the largest average peak area, which indicates the largest quantity of β-D-Glucosidase present in the solution. For this investigation, whenever the 10 mM citrate buffer was used in the

experimental procedures for the preparation of β-D-Glucosidase aqueous solutions the pH was set at 5.2.

It was necessary to establish the APL of P_2. As the protein used is different to that of P_1, an alternative method had to be employed. The method used and the results will be detailed and discussed in full detail later in this investigation (Chapter 6).

3.7.3 Acid Phosphatase PCMCs

The pH at which the Acid Phosphatase PCMCs were able to be re-dissolved back into an aqueous solution was also determined following the same method used in Sections 3.7.1 and 3.7.1. Table 3.10 and Figure 3.7 show the results. As with the myoglobin, phosphate buffer was used and the concentration was 100 mM. The experiment was conducted in triplicate and the average results reported.

pH	Average Peak Area	Standard Error (Peak Area)
4.2	457170	± 196
4.5	464867	± 250
5.21	492232	± 262
5.5	505247	± 134
5.99	634661	± 208
6.32	510459	± 178
6.68	445411	± 158
6.8	425309	± 140
7	380079	± 151

Table 3.10: Optimum pH Determination, pH Values and Peak Areas for Acid Phosphatase PCMCs

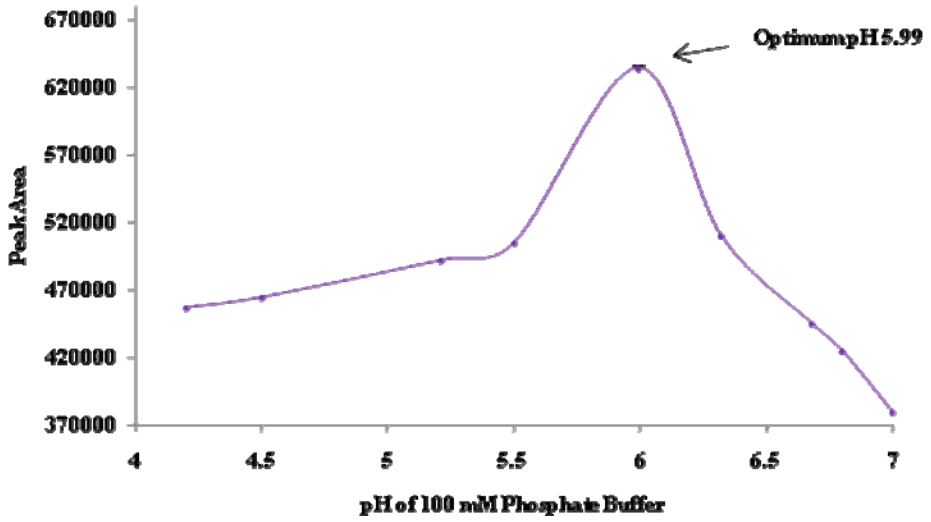

Figure 3.7: Optimum pH Calibration for Acid Phosphatase PCMCs

The graph shows that the largest average peak area is recorded for Acid Phosphatase PCMCs dissolved into a buffer solution of pH 5.99, thus verifying the largest quantity of Acid Phosphatase present. Whenever the 100 mM phosphate buffer was used in the preparation of Acid Phosphatase aqueous solutions the pH was set at 5.99.

It was necessary to establish the APL of P_3. As the protein used is different to that of P_1, an alternative method had to be employed. The method used and the results will be detailed and discussed in Chapter 6.

3.8 Conclusions

This area of the investigation has demonstrated that it is possible to immobilise myoglobin, β-D-Glucosidase and acid phosphatase onto the surfaces of potassium sulfate micro-crystals. Two methods were applied in this investigation to generate PCMCs, the drop-wise method and the continuous-flow precipitator method. The drop-wise proved to be an appropriate method to use when small quantities of PCMCs were required i.e., when ≤ 2 mL of protein solution was required for the preparation. When larger quantities of PCMCs were required i.e., ≥ 2.1 mL of

protein was required for the preparation continuous-flow precipitator method was suitable.

The pH at which the PCMCs are prepared does not influence their solubility, although the excipient concentration does ultimately control their solubility. The PCMCs were prepared at differing pH values. They were then re-suspended in aqueous solution. Each of the PCMC samples failed to re-dissolve immediately or after 24 hours. The PCMCs were the prepared once more; however, the concentration of the excipient was varied. It was found at the higher concentrations of K_2SO_4 (60 mg/mL – 100 mg/mL), the PCMCs failed to re-dissolve instantaneously. When the concentration of the K_2SO_4 was reduced to 55 mg/mL, the PCMCs readily dissolved back into aqueous solution

PCMCs prepared for use as templates in the production of MIPs should be with these guidelines in mind.

3.9 References

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4.0 Molecularly Imprinted Polymers

4.1 Introduction

PCMCs were used as the templates for protein imprinting in this investigation. The rationale behind the choice of proteins used will be explained in the following chapters, however to continue with imprinting, the functional monomer, crosslinker, porogenic solvent and initiator must also be selected.

The polymer synthesis is dependant upon the pre-arrangement stage and thus the selection of functional monomer(s) plays an important role.[1]

For this investigation it was decided that rather than using one functional monomer, two functional monomers would be used throughout. This can be attributed to the fact that the use of two monomers, which are different in structure and functionality, can enhance the imprinting efficiency of a template compared to using one single monomer alone.

Previous work[2] has indicated that two monomers which optimise the imprinting process when combined should be both acidic and basic in nature, with the crosslinker remaining neutral. This can be attributed to the fact that so doing so a zwitterionic polymer would be produced, which in turn would allow the polymer to be exploited in terms of pH, (integral to work carried out in this study) thus the two monomers selected were methacrylic acid (MAA) and 4-vinylpyridine (4-VP). The characteristics of the template molecule(s) were also considered when selecting these two monomers i.e., the functional groups and structure. As these monomers are acidic and basic respectively, a decision was made to use a crosslinker which was neutral in nature, thus ethylene glycol dimethacrylate (EGDMA) was selected.

For the polymerisations an azo-initiator was utilised; azo-*bis*-isobutyronitrile (AIBN) is a commonly available species and allows polymerisation to be thermally initiated, which was required for this system. Acetonitrile has been selected as the porogenic

solvent, as this is not only compatible for molecular imprinting, but can also be used to good effect in the preparation of PCMCs as detailed in Chapter 3.

4.2 MIP Preparation

Prior to imprinting, the functional monomers, crosslinker and initiator must all be purified. This is to ensure that any inhibitors and water are removed, along with any other impurities which may be present. Once this has been completed, the polymerisation can be carried out.[3]

4.2.1 MIP Preparation

For the preparation of MIPs, the mass of functional monomer and crosslinker required was determined based on the quantity of template which was being used in the MIP preparation. The final quantity of the template used was relative to the scale at which the polymer was being prepared.
Four times as much functional monomer was required as template, which between the two functional monomers used, was split evenly (50-50). Twenty times more crosslinker than template was required.

A known mass of PCMCs was suspended in the required volume of acetonitrile in a glass Kimax culture tube for the polymerisation process. To the glass tube, the required quantities of functional monomer and crosslinker were added. The polymerisation mixture was then purged with oxygen-free nitrogen, over an ice bath, for approximately five minutes to remove any oxygen which may have been present in the acetonitrile. The initiator was added to the tube, before it was sealed under nitrogen with a lid and Parafilm. In order for polymerisation to commence, the tube was placed in an incubator at 60 °C, on a rotor (24 rpm). The polymerisation was initiated thermally at 60 °C, and subsequently held at this temperature for 48 hours.

After polymerisation was complete, the glass tubes were broken so that access to the MIPs could be obtained. As the resultant MIPs were hard, monolithic materials, it

was necessary to grind them so that the material was of a more manageable size. This was achieved by placing the MIPs into a ball mill (Fritsch Pulverisette Model 06.102) for approximately 20 minutes.

4.2.2 Template Removal from MIP

There are various methods available for removing the template from the MIP, including Soxhlet extraction, microwave-assisted extraction and consecutively washing the MIP with fresh solvent (solvent swapping). Each of these template removal methods have been used during this investigation.[3]

Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) is a method which removes the template from the MIP by using the energy from a microwave radiation source.[4] This energy rapidly heats the extraction solvent, which is housed in a closed vessel system together with the MIP, rapidly and efficiently (Figure 4.1). Due to the fact that the vessel is sealed, extractions can be completed at higher temperatures, which also results in a significant reduction in extraction times.[5, 6]

Figure 4.1: Photographic Image of Microwave-Assisted Extraction Equipment

Solvent Switch

Solvent swapping is a relatively simple, quick and efficient method for removing the template from a MIP.

The ground MIP is placed into a round-bottomed flask, together with the minimal volume of extraction solvent required to wash the MIP (sufficient to immerse the MIP in solvent). The flask is then agitated by means of a shaker for approximately 15 minutes. The extraction solution is decanted from the MIP and analysed for template content. This procedure is repeated until the entire template has been removed successfully (typically 7-8 washes). It should be noted that the wash solutions are interchangeable, and the same wash solution may not be used for all 7-8 washes.

The purpose of the wash step is not to only to remove the template molecule from the MIP, but also removes other components present in the resultant MIP, such as the excipient, unreacted monomer(s) and initiator and soluble oligomers.[7, 8]

After the wash procedure has been completed, and the template has been successfully removed from the MIP, the MIP is dried at 70 °C for approximately 15 hours in a vacuum oven.

It is worth noting that all was procedures were carried out on the non-imprinted control polymers also (NIP).

4.3 Experimental Procedures

4.3.1 Chemicals

Methacrylic Acid (MAA)
4-Vinylpyridine (4-VP)
Ethylene glycol Dimethacrylate (EGDMA)
Acetonitrile (ACN)

Azo-*bis*-isobutyronitrile (AIBN)
Deionised Water (dH_2O)
Acetic Acid (CH_3COOH)
Guanidine Hydrochloride (CH_5N_3)
Urea $(NH_2)_2CO$
Methanol (CH_3OH)
Citric Acid ($C_6H_8O_7$)
Sodium Citrate ($Na_3C_6H_5O_7$)
Molecular Sieves - 3Å

The sodium citrate, citric acid, guanidine hydrochloride, urea, methacrylic acid, 4-vinylpyridine, ethylene glycol dimethacrylate, acetonitrile, acetic acid, methanol and azo-*bis*-isobutyronitrile were all purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). All of the solvents purchased were of HPLC grade and ≥ 95% pure. The acetonitrile was dried prior to use over activated molecular sieves (3Å).

4.3.2 UV/Vis Spectroscopy

Analysis of the PCMCs was carried out using UV/Vis spectroscopy in order to obtain a calibration curve and determine the quantity of protein immobilised on the surfaces of the micro-crystals. The procedure used is as detailed in Chapter 2.

4.3.3 Purification of Reagents

Table 4.1 outlines the purification and storage steps followed for each component.[3]

Component	Step 1	Step 2	Storage Conditions
Methacrylic Acid	Pass through a column containing neutral alumina	Distil under reduced pressure	-20°C
4-Vinylpyridine	Pass through a column containing neutral alumina	Distil under reduced pressure	-20°C
Ethylene glycol Dimethacrylate	Pass through a column containing neutral alumina	N/A	-20°C
AIBN	Recrystallise from acetone ***DO NOT HEAT***	N/A	-20°C

Table 4.1: Purification and Storage Details of Reagents Used In Molecular Imprinting

4.3.4 Polymerisations

The MIPs were prepared on a 10 g, 7 g or 5 g monomer scale, and were prepared in glass Kimax culture tubes (20 x 150 mm) fitted with screw caps. Table 4.2 details the composition and scale of each MIP, dependant on the PCMC sample (P_1, P_2 or P_3).

All imprinting experiments involved the synthesis of a non-imprinted polymer (NIP) as well as the MIP so that a point of reference and calibration could be obtained. The composition of the NIPs were exactly the same as their respective MIPs, however the template used was a micro-crystal with no protein coated on the surface; thus it was merely the excipient used in the formation of PCMCs.

Template	Mass of PCMCs (g)	Monomer Scale (g)	MAA (mL, mol)	4-VP (mL, mol)	EGDMA (mL, mol)	AIBN (g, mol)	ACN (mL)
P_1	1.54	7	0.27, 0.0032	0.35, 0.0032	6.29, 0.0322	0.116, 0.00071	9.33
P_2	1.54	7	0.27, 0.0032	0.35, 0.0032	6.29, 0.0322	0.116, 0.00071	9.33
P_3	1.10	5	0.2, 0.0023	0.25, 0.0023	4.49, 0.023	0.083, 0.0005	6.667

Table 4.2: Composition of Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs Prepared for this Investigation

All PCMCs were prepared in acetonitrile. The acetonitrile in which the PCMCs were prepared was swapped three times. This was achieved by siphoning off the ACN in which the PCMCs were prepared and replacing it with fresh ACN. This was to ensure that the water content was as close to zero as possible prior to polymerisation. The final volume of acetonitrile for each MIP is detailed in Table 4.2. Using the compositions of monomer and crosslinker outlined in Table 4.2, each component was added to the respective Kimax tube. Whilst in an ice bath, the polymerisation mixture was purged with nitrogen for five minutes. The AIBN initiator was added at ratio of 1% (mol) relative to the number of polymerisable double bonds.

This process was followed for each MIP prepared in this investigation.

The tube was sealed under nitrogen and the screw cap secured with the use of Parafilm. The tube was then transferred to an incubator (Stuart Analogue SI60 Incubator, 3 °C to 66 °C ± 0.1 °C) which had been pre-heated at 60 °C. The polymerisation was thermally initiated, and the temperature maintained at 60 °C for the duration (48 hours) of the polymerisation. Throughout the polymerisation, the tube was rotated at 24 rpm to ensure that the PCMCs were distributed evenly throughout the tube using a Stuart SB3 Tube Rotator housed within the incubator.

Once the MIP and NIP samples were prepared, they were renamed, as detailed in Table 4.3.

Template	MIP Name	NIP Name
P_1	M_1	N_1
P_2	M_2	N_2
P_3	M_3	N_3

Table 4.3: Branding of Samples – MIP and NIP Identification Coding

4.3.5 Template Removal

In order to remove the templates from the MIPs, a series of different washes were applied. Table 4.4 lists the wash solutions and their components. The application of these washes will be discussed further on in this Chapter.

Extraction Solvent	Chemical Components
dH_2O	dH_2O
20% Aqueous Acetic Acid	Acetic Acid, dH_2O
6M Guanidine Hydrochloride	Guanidine Hydrochloride, dH_2O
8M Urea	Urea, dH_2O
Acetonitrile	Acetonitrile
10% Aqueous Methanol	Methanol, dH_2O
90% Aqueous Methanol, 10% Acetic Acid	Methanol, Acetic Acid
Citrate Buffer	Citric Acid, Sodium Citrate, dH_2O

Table 4.4: Wash Solution Composition and Components used for Template Removal for Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs

4.4 Results & Discussion

After polymerisation, the MIPs that were produced were all hard and glassy in nature. In order to remove the templates from the MIPs, they were ground using a ball mill to a more manageable size prior to washing.

Using the washing techniques detailed in Section 4.2.2, template removal was attempted. The MIP samples were divided into different portions and washed. The resultant wash solutions were analysed subsequently by UV/Vis spectroscopy and HPLC to verify if there was any template from the respective MIP present.

For each of the wash techniques the template removal results were tabulated (Tables 4.5, 4.6 and 4.7, respectively).

To ensure that the template was removed from the MIP, the conditions within the MIP were changed. This was achieved by selecting wash solutions which favour desorption. For this investigation, the desorption process is performed by changing the pH and with the utilisation of organic solvents. It is worth noting the use of organic solvents as organic solvents are known to denature proteins. The requirement was to remove the template protein from the MIP, thus the final structure and activity once removed was not pertinent at this stage.

Soxhlet Extraction

Extraction Solvent	**Wash Time (Hours)**	**Volume (mL)**	**Template Removed**
dH_2O	16	200^{+}	No
20% Aqueous Acetic Acid	16	200^{+}	No

[+] The same liquid was used throughout the soxhlet extraction method

Table 4.5: Result s of Template Removal from Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs by Soxhlet Extraction

Upon completion of the soxhlet extraction, each of the wash solutions were analysed *via* UV/Vis spectroscopy and HPLC. In each of the wash solutions described in Table 4.5, there was no detection of the template protein in any of these washes.

Microwave-Assisted Extraction

Extraction Solvent	**Wash Time (Minutes)**	**Weight of MIP/NIP (g)**	**Volume (mL)**	**Template Removed**
dH_2O	15	0.5	5	No
20% Aqueous Acetic Acid	15	0.5	5	No
6M Guanidine Hydrochloride	15	0.5	5	No
8M Urea	15	0.5	5	No

Table 4.6: Results of Template Removal from Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs by Microwave-Assisted Extraction

The microwave-assisted extraction method was also unsuccessful in the extraction of the template molecule. After the extraction was complete the wash solution was removed from the MIP sample for analysis. As with the wash solution from the soxhlet extraction procedure, no template protein was detected in the wash solutions.

Solvent Switch

Extraction Solvent	Wash Time (Minutes)	Weight of MIP/NIP (g)	Volume (mL)	Template Removed
dH_2O	30	1	20	No
20% Aqueous Acetic Acid	30	1	20	No
6M Guanidine Hydrochloride	30	1	20	No
8M Urea	30	1	20	No
A series of differing washes*	30	1	20	Yes - 100%

Table 4.7: Results of Template Removal from Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs by Solvent Switch

**Washes include acetonitrile, 20% acetic acid, citrate buffer, 10%Methanol and 90 % methanol/10 % acetic acid*

It was found that carrying out individual washes with specific wash solutions was not sufficient to remove the template protein from the MIP. This was verified *via* UV/Vis spectroscopy and HPLC analysis.

Applying a combination of washes, however, did prove to be a more successful method of template removal from the MIPs.

A variation of wash solutions was applied to the MIP samples at 30 minute intervals. After each wash, the wash solution was analysed for template content. Initially this method proved to be ineffective with respect to template removal as no template was detected in the wash solutions. After perseverance, and the introduction of an initial wash of acetonitrile (ACN) prior to the application of a combination of the different wash solutions proved to be fruitful; template removal had been achieved. The success of the introduction of the ACN as the first wash in the template could be attributed to the MIPs ability to swell in the presence of solvent. If the crosslinking in a MIP is suitable, it is possible to use an organic solvent to reversibly swell the

polymer. Swelling allows access to the binding sites and template, thus facilitating the template removal.[9] The swelling required to execute this is minimal. In the case of the MIPs generated for this investigation, only minimal swelling was exhibited when they are introduced to solvent and/or an aqueous solution, ensuring that they were fit for purpose with respect to their use for solid phase extraction experiments (MISPE).[10]

Although the quantity of template was not verified, the combination of washes was applied until there was no absorbance reading detected by UV/Vis spectroscopy and there was no protein detected *via* HPLC.

It was deemed that in order to successfully remove the template from the MIPs, they first had to be washed in ACN, before a series of successive washes were carried out. This is turn, ensured that by washing the MIPs *via* the solvent switch method was sufficient to ensure that the template was removed.

With respect to the NIPs, they were subject to the same washing protocol as each of the MIP so as to ensure that they were subjected to all of the same conditions as the MIPs.

It is worth noting at this point that no specific quantitative analysis was conducted with respect to the removal of the micro-crystal (K_2SO_4), however the assumption was made that the salt was removed from the MIP and the NIP. The chromatographic output from each of the wash solutions showed a separation elution profile for neither a material which was neither part of the wash solution nor the template material. The speed of the separation (within the first minute), implied that the material was polar. It was concluded that as the only component of the MIP and NIP preparation which would also be removed during template removal, K_2SO_4, which is also polar in nature, was the material which was present in the separation elution profile.

Once it was confirmed that the template had been successfully removed from the MIP, analysis was carried out in order to ascertain the size and distribution of the MIP.

The technique uses laser diffraction to assess the particle size if they are in the range of < 0.1 µm to 3500 µm. The MIP is dispersed as a dry powder over a set period of time and is measured against an internal standard. The output for all of the MIP samples can be seen in Figures 4.2, 4.3 and 4.4.

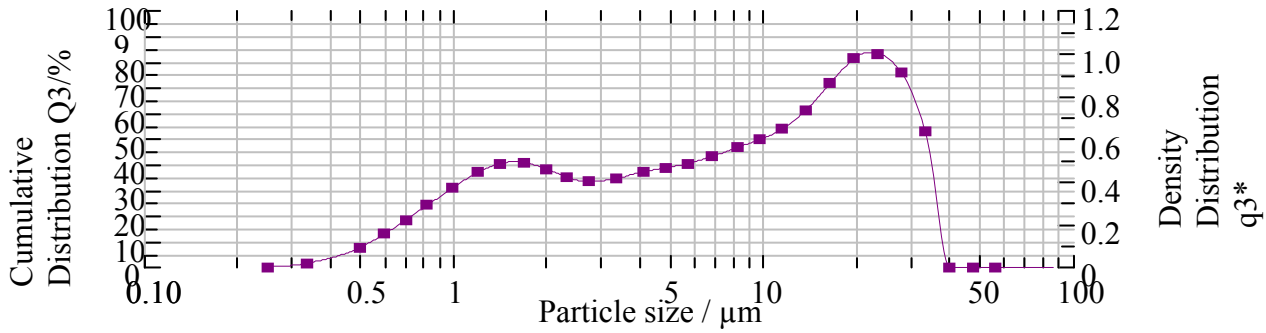

Figure 4.2: Particle Size Analysis of Myoglobin Imprinted MIP *via* Laser Diffraction

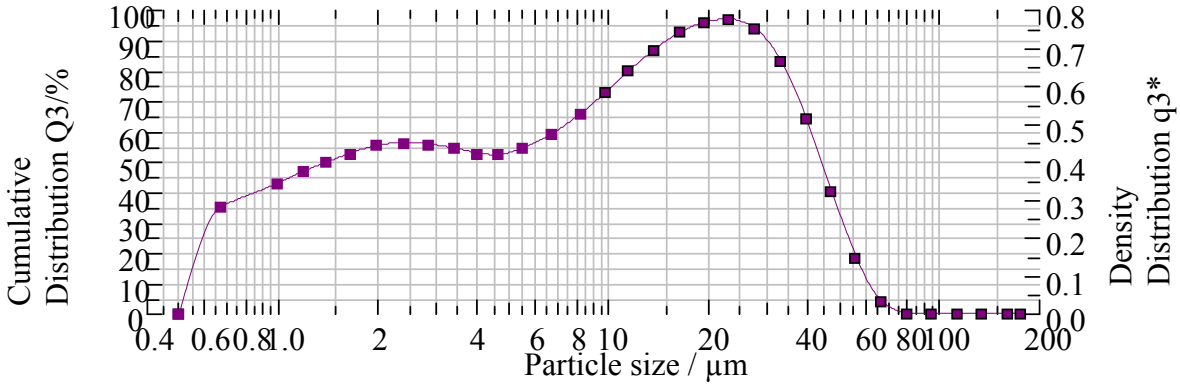

Figure 4.3: Particle Size Analysis of β-D-Glucosidase Imprinted MIP *via* Laser Diffraction

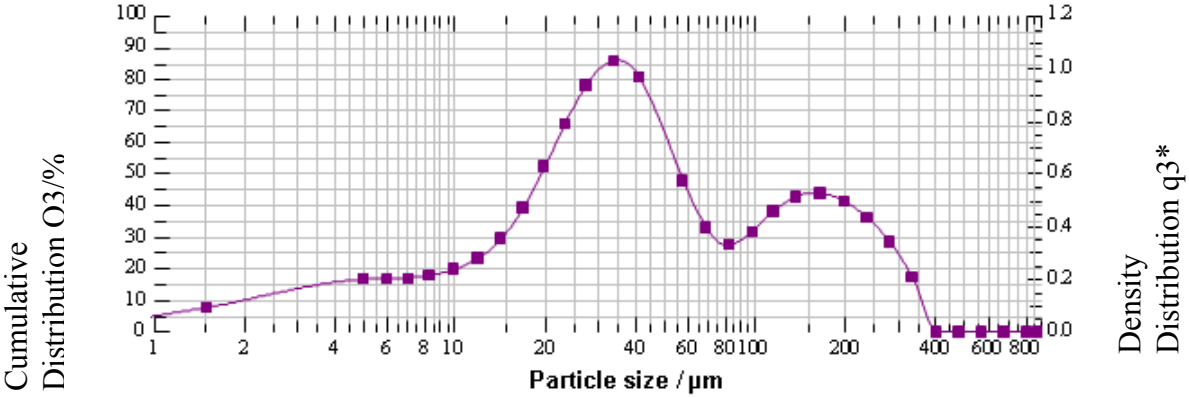

Figure 4.4: Particle Size Analysis of Acid Phosphatase MIP *via* Laser Diffraction

The size of the particles of each of the polymer samples are approximately 30-40 µm in size. This is indicated by the highest peak of each particle size analysis.

It also indicates that there are smaller particles present at ~1.5 µm, again indicated by the smaller peak visible of the particle size analysis output. This signifies that the grinding and sieving method employed to generate the MIP and NIP was not 100 % effective as there were smaller particles present. These particles must be removed prior to carrying out rebinding experiments. Sample M_3 also has larger particles, in addition to the smaller particles present. Both of the particles (small and large) must also be removed. This was accomplished by using different sizes of molecular sieves. Utilising a sieve with smaller pores allowed the small particles to be removed. Passing the MIP and NIP through a 50 µm sieve ensured that the larger particles could then be removed.

4.5 Conclusions

It has been demonstrated that molecularly imprinting of proteins utilising PCMCs as the template molecule is a viable method for imprinting proteins and the presence of the micro-crystal does not hinder the synthesis of the MIP. The MIPs which were prepared for the purpose of this investigation were all hard, glassy, monolithic materials. In addition to this the MIPs are macroporous. It has been reported previously that MIPs prepared using the method detailed in Section 4.3.4, would take on the form of a macroporous monolith.[11, 12] Each of the MIPs prepared was a

macroporous resin. They were each ground down to a manageable size so they could be used in Molecular Imprinted Solid Phase Extraction (MISPE). Macroporous monoliths, or the MIPs prepared, are suitable resins for MISPE as they have macropores which remain whether or not the material is dry or is wetted. Only minimal swelling is exhibited when they are introduced to solvent and/or an aqueous solution.[10] As the material is a monolith, it can be easily packed into a column. The pores which were generated *via* the imprinting protocol allow is to be employed in MISPE. This is work which is detailed in Chapters 5 – 10 of this investigation.

In addition to this, by using PCMCs as the template as opposed to individual protein molecules, it was hypothesized that the template would be removed without difficulty due to the fact that the PCMCs generate micrometer-sized pores within the polymer, lined with protein imprinted sites; however removing the template from the MIPs was not as straight forward as envisaged initially.

Introducing additional wash steps and the use of an organic solvent as the initial wash step, to the washing procedure did ensure that the templates were removed successfully and quantitatively. At this stage, it was also deduced that the K_2SO_4 was removed from both the MIP samples and the NIP samples. This theory will be assessed later in this investigation when rebinding experiments are conducted on all of the MIP and NIP samples which were prepared.

4.6 References

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5.0 Testing the Theory!

5.1 Introduction

Competitive rebinding is a technique which is used to determine the selectivity displayed by a MIP. The technique establishes whether or not a MIP can recognise the template molecule whilst in the presence of a competitor molecule or molecules.

For this investigation, competitive rebinding was carried out by the analytical technique of molecularly imprinted solid-phase extraction (MISPE), as detailed in Chapter 2. The competitive rebinding was carried out using only the MIP and competitor molecules.

5.2 Choice of PCMC Protein

Myoglobin was selected as the protein used to prepare PCMCs for polymer production prior to the first re-binding experiments. Myoglobin was selected for several reasons, however looking ahead to the experimental analysis required; the presence of the heme group in myoglobin enables quantitative analysis to be carried out relatively easily.

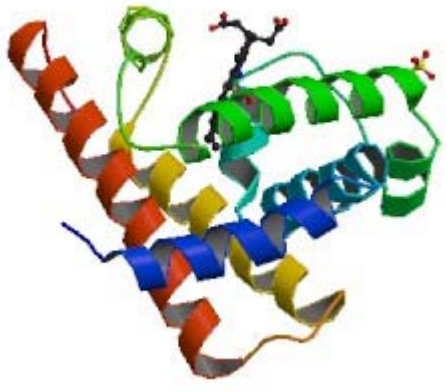

Figure 5.1: Image of the 3D Myoglobin Structure[1]

The presence of the heme group in the myoglobin molecule allows myoglobin to bind reversibly to one oxygen molecule, thus providing myoglobin with its key role; a reservoir for oxygen in the body (myoglobin is found predominantly in the muscle tissues within the body).

Myoglobin fulfills its functional role of a reservoir of oxygen when the iron, which is present, is in the ferrous oxidation state (Fe^{2+}). This is attributed to the fact that the oxygen which is carried is bound to the ferrous atom. The molecule in its ferrous state will give a red colour. When the iron in the heme group is oxidised to its ferric state (Fe^{3+}), the molecule is incapable of binding the oxygen and thus it can not fulfill its role within the body. In addition to this, the molecule will also change colour to a shade of brown.

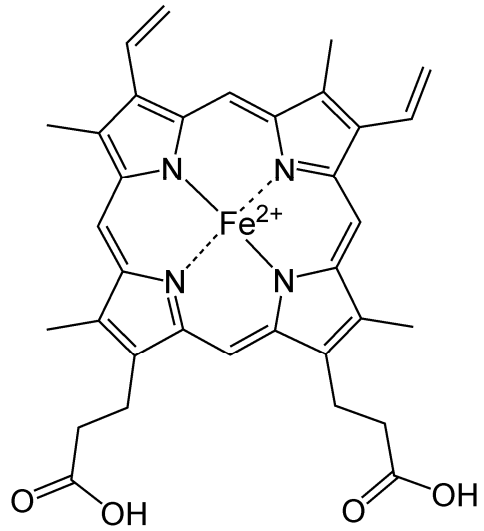

Figure 5.2: Illustrated Representation of Heme B Group[3]

It is due to the presence of the heme b group in myoglobin, and in particular its ability to absorb light in the visible region of the spectrum, with a λ_{max} of 410 nm, that analysis *via* UV/Vis Spectroscopy and High Performance Liquid Chromatography (HPLC) can be realised easily.

Another key advantage of using myoglobin in the preparation of PCMCs is that myoglobin is readily available to purchase in re-crystallised form from different animal sources, whilst remaining inexpensive.

5.3 Choice of Competitor Proteins

In order to carry out the competitive rebinding experiments, two proteins structurally related to myoglobin were selected; Haemoglobin and Cytochrome C.

5.3.1 Haemoglobin

Like myoglobin, haemoglobin has a heme b group present within its structure, thus allowing quantitative analysis to be realised readily.

Haemoglobin (Figure 5.3) is a globular protein; it consists of four polypeptide chains (globins); two alpha chains and two beta chains which are non-covalently bound to each other (α2β2). The alpha chain consists of 141 amino acids and the beta chains have 146 amino acids. Each of the chains is connected to a heme group; thus haemoglobin has four heme groups present. Each of these chains is referred to as a subunit, and has a molecular weight of 17000 Da, thus giving haemoglobin a molecular weight of 68,000 Da.

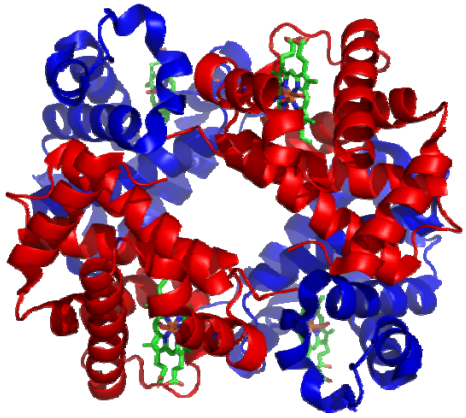

Figure 5.3: Image of the 3D Haemoglobin Structure[4]

The function of haemoglobin is to act as the oxygen carrier in the blood. It is contained within the red blood cells of mammals and other animals. Haemoglobin transfers the oxygen from the lungs to other areas of the body. When it reaches its

destination, such as the lungs, it releases the oxygen load. In addition to this, haemoglobin also plays a major part in transporting the carbon dioxide (CO_2) in the tissues back to the lungs.

5.3.2 Cytochrome C

Cytochrome C is also a heme protein, but it is structurally different from haemoglobin and myoglobin. The heme group which is present in Cytochrome C is known as heme c as opposed to the heme b group present in both myoglobin and hemaglobin. The heme c group present in cytochrome c is covalently bound and not non-covalently bound as it is in myoglobin and haemoglobin.

Cytochrome C (Figure 5.4) is a single chain heme protein containing 104 amino acids, and has a molecular weight of 12,000 Da.

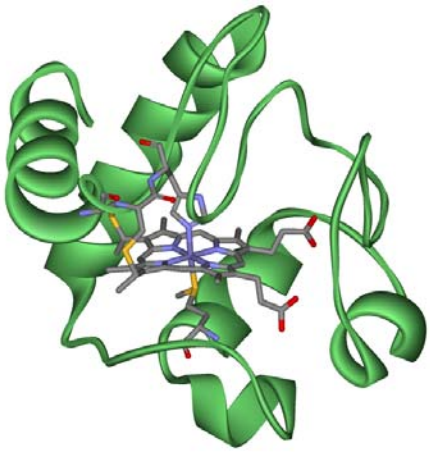

Figure 5.4: Image of the 3D Cytochrome C Structure[5]

Cytochrome C can be found in many different species such as plants, animals and organisms. The function of cytochrome C is to carry electrons. It carries the electrons through the final step of aerobic energy production and places them on oxygen. This allows the electrons to combine with hydrogen ions to form water. Cytochrome C does not bind oxygen.

5.4 Experimental Procedures

5.4.1 Chemicals

MIP M_1
Myoglobin from Horse Heart
Cytochrome C
Haemoglobin
Acetic Acid (CH_3COOH)
Methanol (CH_3OH)
Citric Acid ($C_6H_8O_7$)
Sodium Citrate ($Na_3C_6H_5O_7$)
Ethanol (C_2H_6O)
Tween 20
dH_2O

Myoglobin from horse heart, haemoglobin from horse heart and cytochrome c were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England), in the form of re-crystallised powders. Tween 20 was purchased from Fisher Scientific (Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, and Leicestershire, LE11 5RG).

The sodium citrate, citric acid, acetonitrile, methanol and acetic acid were all purchased from Sigma-Aldrich. All of the solvents purchased were of analytical grade and were ≥ 98% pure. The citric acid and sodium citrate was ≥ 99% pure. The acetonitrile was dried prior to use over activated molecular sieves (3Å).

5.4.2 High Performance Liquid Chromatography

Reversed-phase high performance liquid chromatography (HPLC) was used to determine the protein content of the wash solutions obtained during the solid-phase extraction.

5.4.3 Preparation of Mobile Phase

A standard mobile phase was prepared at a concentration of 5% ethanol and 0.05% Tween 20 (5 ml of ethanol and 0.05 ml of Tween 20 were diluted with 94.95 ml of dH_2O).

5.4.4 Preparation of Rebinding and De-binding Solutions

As the MIP which had been imprinted was being used as the solid-phase throughout the SPE experiments, three aqueous solutions were prepared (Table 5.1); one containing only myoglobin, one with both haemoglobin and myoglobin present, and the third with myoglobin and cytochrome c present. The concentrations of the rebinding solutions were selected by taking into consideration the theoretical binding capacity of the MIP.

To determine the theoretical maximum binding capacity, the knowledge of the mass of MIP prepared and the mass of myoglobin used to prepare the PCMCs as the template was essential.

The MIPs were prepared on a 7 g monomer scale, as detailed in Chapter 4. 0.15 g of myoglobin was used in the preparation of the PCMCs in order to give the 1.54 g of template required.

This means that for every 1 mg of MIP, theoretically 0.0214 mg of myoglobin had been imprinted.

Note that for this experimental section, the column selected was Column 1, the double-fritted filtration column. Each column was packed with 150 mg of MIP, thus making the theoretical maximum binding capacity 3.21 mg per column.

Rebinding Solution	SPE Column	Myoglobin (µg/ml)	Haemoglobin (µg/ml)	Cytochrome C (µg/ml)
Myoglobin	CR_1	500	-	-
Myoglobin + Haemoglobin	CR_2	250	250	-
Myoglobin + Cytochrome C	CR_3	250	-	250
Myoglobin	CR_4	500	-	-
Myoglobin	CR_5	500	-	-

Table 5.1: Loading Solution Compositions and Concentrations for Rebinding Analysis on Myoglobin MIP

In order to prepare the rebinding solutions, three standard solutions were prepared each containing 500 μg/ml of the respective protein.

Standard Solution	Mass (mg)	Volume (mL)
Myoglobin	5	10
Haemoglobin	5	10
Cytochrome C	5	10

Table 5.2: Rebinding Standard Solutions Compositions for Rebinding Analysis on Myoglobin MIP

Known volumes of the rebinding standards in Table 5.2 were combined to give the solutions listed in Table 5.1

For the elution experiments, a wash solution of 20% v/v aqueous acetic acid was used (20 mL of acetic acid was added to 80 ml of dH_2O).

5.4.5 Conditioning, Loading and Washing Experimental Procedure

Five SPE columns were packed with 150 mg of MIP M_1. All of the experiments were executed using the myoglobin MIP; no experiments were executed using the NIP.

Each of the packed columns was named CR_1, CR_2, CR_3, CR_4 and CR_5, respectively. To each of the columns, 5 ml of mobile phase (5 % ethanol and 0.05% Tween 20), was added to allow the MIP to pre-wet prior to conducting the conditioning, loading and washing stages of the experiment. The mobile phase was eluted from the column and discarded as it was not required for analysis.

The column was prepared for the experimental procedure: a cap was put on the bottom of the column, and it was sealed at the top with a cap.

The SPE apparatus was set up using a SPE vacuum manifold (Figure 2.3). To each of the columns, 5 ml of the respective loading solution was added. Using this volume of solution ensured the concentration of protein present never exceeded 2.5 mg, which is less than the maximum theoretical binding capacity of 3.85 mg.

A vacuum was applied and the loading solution was allowed to pass through the column at approximately 0.5 mL/min. The resultant eluent was collected.

To ensure all unbound protein was removed from the columns, a vacuum was applied and the wash solution (mobile phase) was passed through the column. This fraction was combined with the loading solution fraction.

The combined sample was analysed *via* HPLC for protein content (the final volume of the fractions collected was recorded in order to establish an accurate determination of protein content).

5.4.6 Elution Experimental Procedure

Continuing to use the SPE vacuum manifold, the bound analytes were eluted from the column. This was achieved by passing 5 ml of the selected elution solution through each of the five SPE columns. The elution solutions used are detailed in Table 5.3. Again, the resultant fractions were collected for analysis.

SPE Column	1st Elution Solution	2nd Elution Solution
CR_1	20% Acetic Acid (aq)	-
CR_2	20% Acetic Acid (aq)	-
CR_3	20% Acetic Acid (aq)	-
CR_4	20% Acetic Acid (aq)	1% Acetic Acid/99% Ethanol (aq)
CR_5	20% Acetic Acid (aq)	1% Acetic Acid/99% Methanol (aq)

Table 5.3: Solutions Used in the Elution of Protein Bound to the Myoglobin MIP

5.5 Results & Discussion

Each of the five columns housing the myoglobin imprinted MIP had different rebinding solutions passed through them (Table 5.1), as well as different de-binding solutions (Table 5.3). The competitive rebinding detailed in this chapter is concerned only with the myoglobin imprinted polymer; analysis was not conducted on the non-imprinted polymer (NIP) at this stage.

5.5.1 CR_1 Results

CR_1 analysis was based solely on the capacity of the MIP to rebind only the molecule which was used as the template molecule - myoglobin (loading solution R_M).

Prior to analysing the SPE fractions, a sample of myoglobin was analysed *via* HPLC in order to establish the HPLC elution profile (Figure 5.5). This was done using a C18 column and the isocratic elution procedure detailed in Chapter 2.

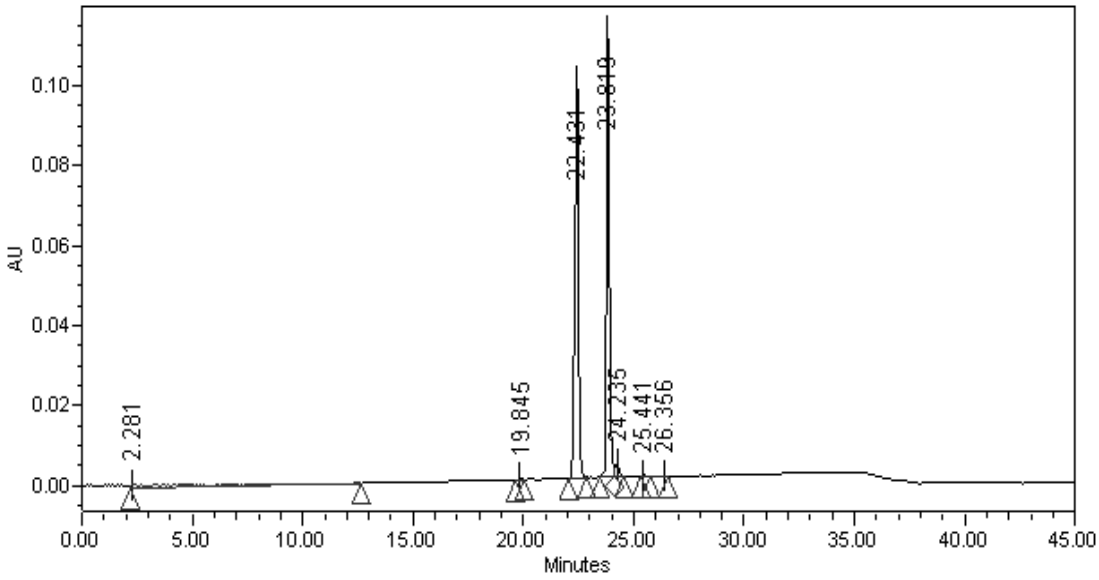

Figure 5.5: Myoglobin RP- HPLC Chromatographic Elution Profile on C18 Column

The chromatogram shows two peaks of significance. The first peak at $Rt_{22.43}$ is ascribed to the heme group present in the myoglobin molecule. The second peak is ascribed to the protein present, and this elutes at $Rt_{23.82.}$ This was determined by analysing the chromatographic profiles of the peaks. The protein is indicated at a wavelength of 280 nm, whereas the heme group is not detected at this wavelength. A further experiment was also conducted where the protein was separated from the heme group, and both samples analysed *via* HPLC. The resultant peaks were observed at the same retention times indicated above.

The two peaks displayed in the chromatographic profile both belong to myoglobin. The separation of the heme from the protein during liquid chromatography is a phenomenon which has been observed before.[6] In order to obtain one peak from myoglobin i.e., ensure that the heme does not separate from the protein, the heme group should be in the same oxidative state as the protein.[7] The conversion of the oxidative state of the heme group was not performed as part of this investigation, thus all further analysis concerning myoglobin was completed using the a double peak profile as seen in Figure 5.5.

The collected fraction containing the loading and washing sample R_M was analysed *via* HPLC after it had been passed through the HPLC column. The results are shown in the chromatogram, Figure 5.6 and Table 5.4

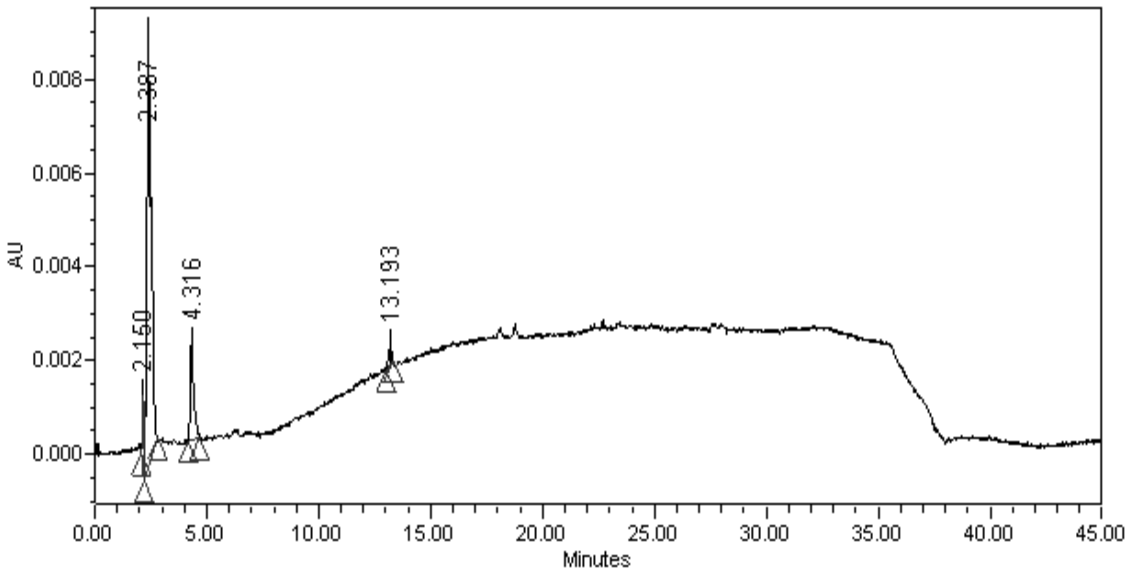

Figure 5.6: Chromatographic Profile of Loading and Wash Solution Fraction for Myoglobin Rebound on MIP

Figure 5.6 show that there are no peaks present at the retention times of the heme peak and the protein peak. This result is indicative of the fact that all of the myoglobin has bound to the MIP. As the concentration of myoglobin loaded was lower than the maximum theoretical binding capacity, the binding results are indicative of the MIPs ability to rebind the concentration of the myoglobin loaded onto the column.

The column was then loaded with a wash solution of 20% v/v aqueous acetic acid. The fraction was collected and analysed *via* HPLC.

The corresponding chromatogram for the elution of myoglobin is shown in Figure 5.7; the results are shown in Table 5.4.

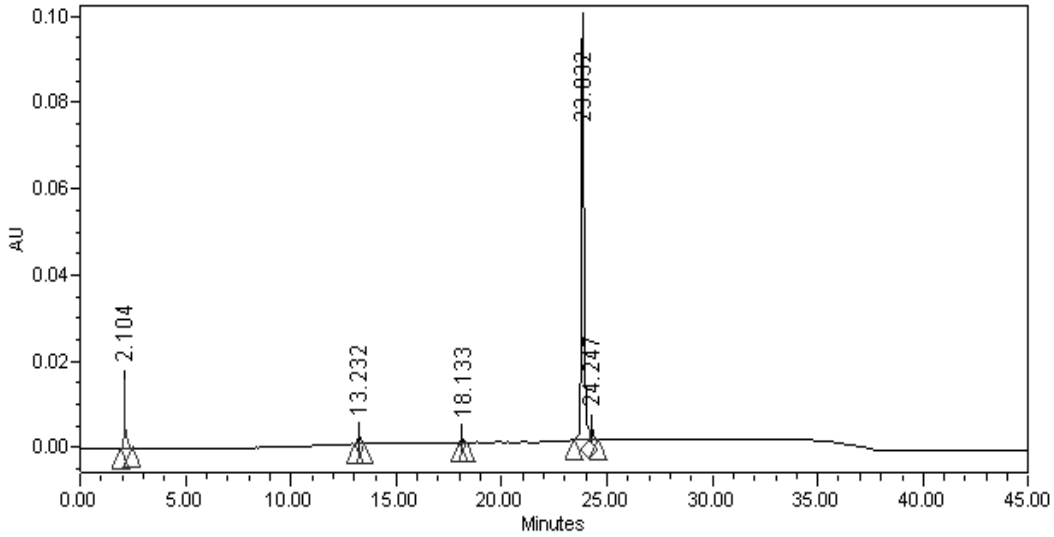

Figure 5.7: RP- HPLC Chromatographic Elution Profile of the Elution Fraction for Myoglobin Rebound on MIP

	Loading & Wash Fraction	Elution Fraction
% of Myoglobin Protein Present in Fraction	0%	89%
% of Myoglobin Heme Group Present in Fraction	0%	0%

Table 5.4: Myoglobin Rebinding Fraction ($R_{M)}$ Results for the Washing and Elution Fractions

The results presented in Figure 5.7 are quite surprising; they show a large peak present at $Rt_{23.83}$. This peak represents the protein present in the myoglobin, and corresponds to 89% of the protein loaded initially onto the column being eluted; however the heme group present in the myoglobin does not appear in the chromatogram. This indicates that the heme group is not present in the eluate and has remained within the column. The potential cause of this could be attributed to the elution procedure.

The elution solution which is applied is an acidic solution. The effects of an acid on proteins with respect to their secondary and tertiary structure are detrimental i.e.,

they denature the protein.[8, 9] Denaturation occurs when the non-covalent interactions which are responsible for the secondary and tertiary structure are disrupted.[10] Intermolecular charge repulsion is the major cause of deaturation by acids.[11] They denauture the protein by altering its structure. This in turn facilitates the removal of the protein from the MIP. Acetic acid and has been reported previously as being used as a denaturant for proteins.[12] However, in addition to being used as a denaturant for proteins, acetic acid has also been employed in the removal of heme from myoglobin.[13, 14]

Heme is bound non-covalently to myoglobin, when a dramatic drop in pH is applied, i.e., the introduction of an acid, the protein unfolds (denatures) which is turn weakens the non-covalent interactions which binds the heme to the proteins.[15] The disruption to the interactions result in the heme being disassociated from the protein.[16]

The removal of the heme group form the column is further discussed in Section 5.5.4 and 5.5.5 respectively.

5.5.2 CR_2 Results

This first competitive rebinding experiment was carried out on a loading solution containing both haemoglobin and myoglobin.

Figure 5.8 shows the chromatogram obtained upon direct injection of a haemoglobin standard solution onto the HPLC column. Once again, this was carried out on a C18 using the isocratic method detailed in Chapter 2.

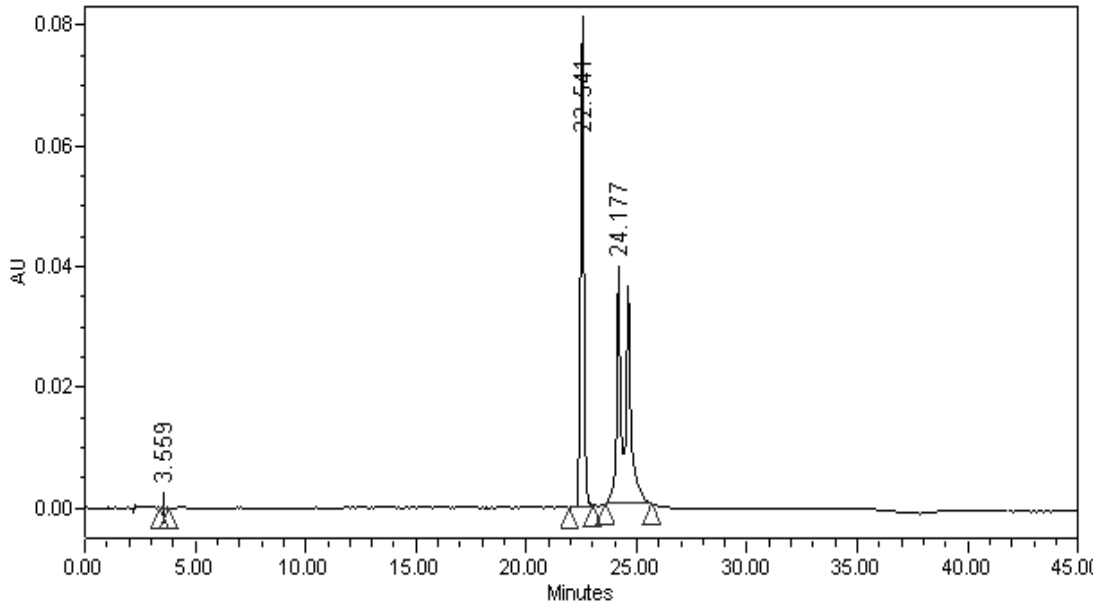

Figure 5.8: RP-HPLC Chromatographic Profile for Haemoglobin on C18 Column

As with the chromatogram for myoglobin (Figure 5.5) more than a single peak was observed; a single peak and a double peak were obtained in the chromatogram for haemoglobin.

The peaks observed in the chromatographic profile can be attributed to the separation of the globular chains present in haemoglobin. RP-HPLC is a technique which is commonly applied to separate the globular chains of haemoglobin.[17, 18, 19]

The peak eluting at $Rt_{22.54}$ represents the heme group in the haemoglobin and the double peak eluting at $Rt_{24.17}$ is the α -globin chains and the β -globin chains (the protein). This is a phenomenon which has been reported previously.[20] Both the application of a gradient elution and the use of a C18 column for the RP-HPLC separations (Chapter 2) result in this separation. The binding of the heme and the globin chains to the column occur at differing rates, thus giving different retention times.[21] The use of an isocratic gradient would avoid this phenomena but as a direct comparison of peaks was being carried out for this work; the method, volumes and concentrations used were the most appropriate for all three proteins being utilised, thus the method remain unchanged.

The Loading solution, myoglobin and haemoglobin, was passed through the SPE column followed by the wash solution. Both the fractions were collected and combined. HPLC analysis of the eluent gives rise to the chromatogram presented in Figure 5.9, and the results shown in Table 5.5.

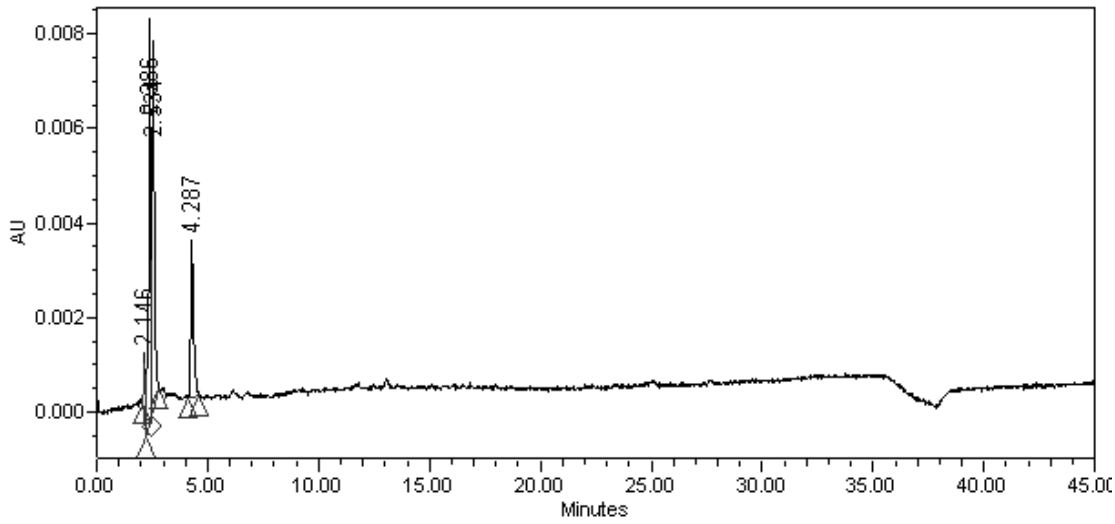

Figure 5.9: Chromatographic Profile of Loading and Wash Solution Fraction Myoglobin and Heamoglobin Loaded on to Myoglobin MIP

As with the myoglobin chromatogram, the analytes in the loading solution were bound by the MIP in the SPE column. This is indicated by the absence of any peaks of significance in the chromatogram.

The corresponding chromatogram for the elution stage is displayed in Figure 5.10, with the results shown in Table 5.5.

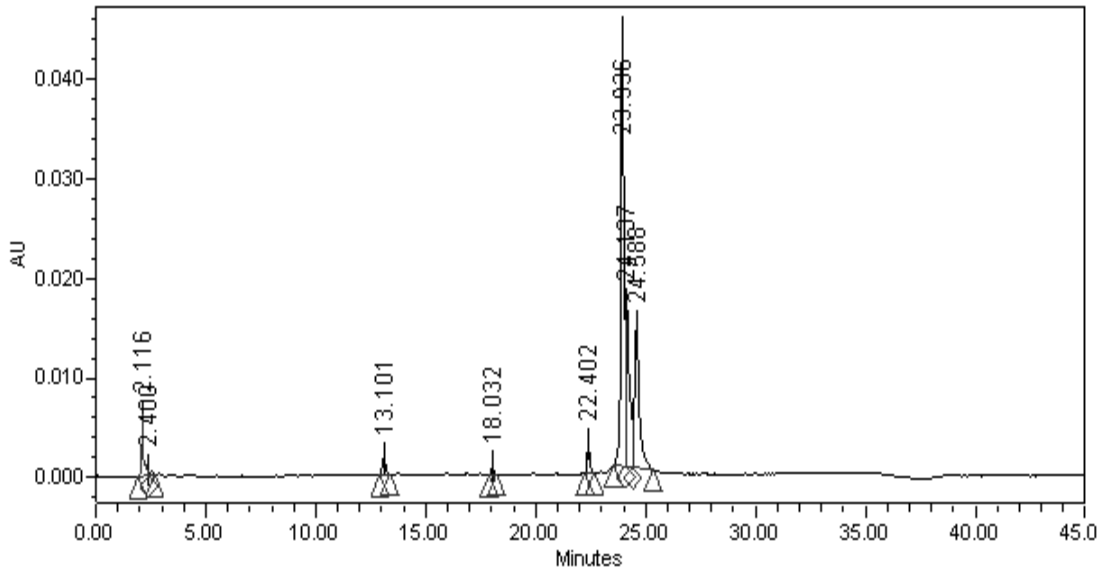

Figure 5.10: Chromatographic Profile of Eluted Fraction Myoglobin and Haemoglobin Loaded on to Myoglobin MIP

Loading Solution	Washing	Elution
% of Myoglobin Protein Present in Fraction	0%	90%
% Heme Group Present in Fraction	0%	3%
% of Haemoglobin Protein Present in Fraction	0%	79%

Table 5.5: Myoglobin and Haemoglobin Rebinding Fraction Results for the Washing and Elution Stages

This chromatogram indicates that 97% of the heme from both the myoglobin and the haemoglobin ($Rt_{22.4}$) has remained bound to the MIP. However, in the elution stage the recoveries of myoglobin and heamoglobin were 90% and 79 %, respectively.

The inability to remove the heme group from the column in the initial elution step can be explained by the presence of acetic acid in the wash solution, as discussed in Section 5.5.1. The acetic acid denatures the myoglobin and haemoglobin facilitating

its removal from the column, but also disrupts the heme-protein non-covalent binding, allowing the protein to be eluted whilst the heme remains bound.

5.5.3 CR_3 Results

A further competitive rebinding experiment was carried out using a rebinding solution containing both cytochrome c and myoglobin.

In this method a C4 column was used for the HPLC analysis as opposed to a C18 column which had been used for all previous HPLC analysis. The procedure used was gradient elution 1, as detailed in Chapter 2. Figure 5.11 displays the chromatogram produced upon direct injection of the myoglobin standard solution onto the HPLC column under the different column conditions.

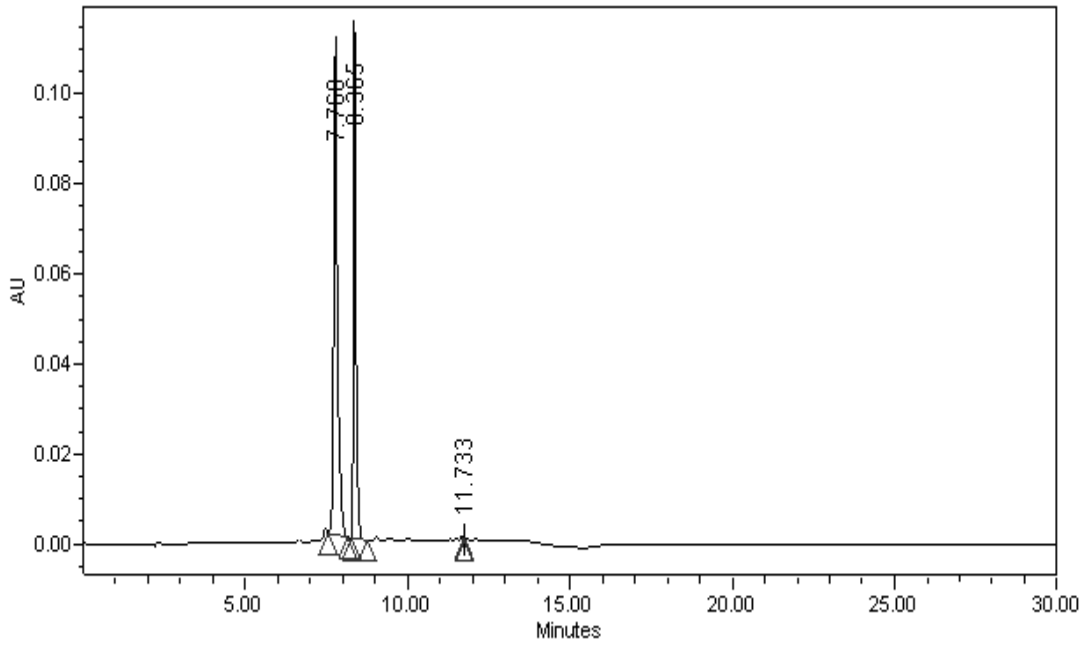

Figure 5.11: Myoglobin RP-HPLC Chromatographic Profile on a C4 Column

The peak eluting at $Rt_{7.76}$ was ascribed to the protein present and the peak eluting at $Rt_{8.37}$ was ascribed to the heme group for the myoglobin molecule. Again this was verified by the absorbance of the protein at wavelength 280 nm.

An obvious observation from the chromatographic profile of myoglobin on the C4 column *vs*. the C18 column is the retention time reversal between the protein and the heme group in addition to the shift in retention time.

The shift in retention time and the reversal of the peaks can be attributed to the column used. As a general rule, the longer the length of the chain length in the stationary phase, the longer the retention time.[22] The stationary phase within the column is non-polar, (hydrophobic) [23] thus impacts the retention time depending on the polarity of the molecule being eluted i.e., the retention time is longer for molecules which are more non-polar, while polar molecules will elute more faster.[24] The C4 column has a shorter chain length than the C18 column, [23] thus it has a lower degree of hydrophobicity than that of the C18 column.

Myoglobin has non-polar residues housed in the core of the molecule; however the residues on the surface are polar.[9] As the surface of the molecule is hydrophilic it will bond quickly with the hydrophobic, non-polar stationary phase. As the C4 column has a lower degree of hydrophobicity than that of the C18 column, the myoglobin or polar molecules will pass through, and subsequently elute more readily. As the myoglobin is eluting at a faster pace on the C4 column, the binding occurs faster which in turn results in a reversal of the elution profile for the protein and the heme group.

Figure 5.12 displays the chromatogram produced upon direct injection of the cytochrome c standard solution onto the C4 column.

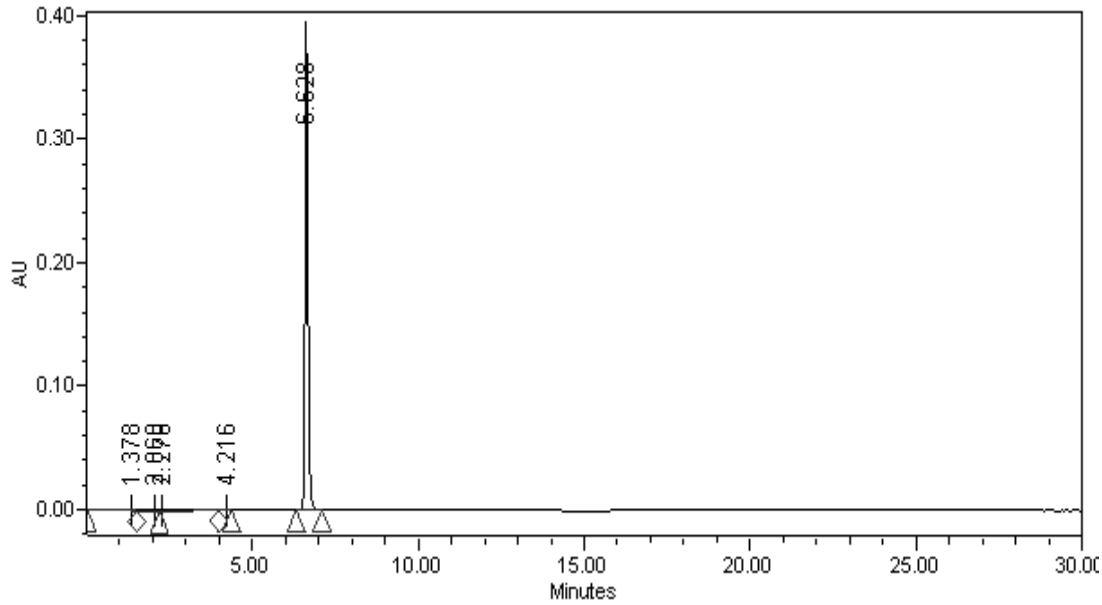

Figure 5.12: Cytochrome C RP- HPLC Chromatographic Profile on a C4 Column

The peak eluting at $Rt_{6.28}$ is ascribed the cytochrome c. It is worth noting that there is only one peak present in this chromatogram. This is a notable different between the chromatograms for myoglobin and haemoglobin wherein there was a peak for the protein present and a peak for the heme group. This can be explained by the bonding of the heme to the cytochrome c. The heme group is covalently bound to the cytochrome c, thus the molecule can elute as one entity.

The loading and washing solution were passed through the SPE column and the chromatogram of the combined eluent (Figure 5.13) obtained. It is worth noting that when the loading solution was passed through the SPE column, there were two coloured bands visible, one of which represented the heme group of the myoglobin, which was darker in colour, and the other which represented the heme group of the cytochrome c, which was a vibrant red colour, thus it was possible to determine by eye which heme group had bound (and subsequently eluted) from the SPE column by eye.

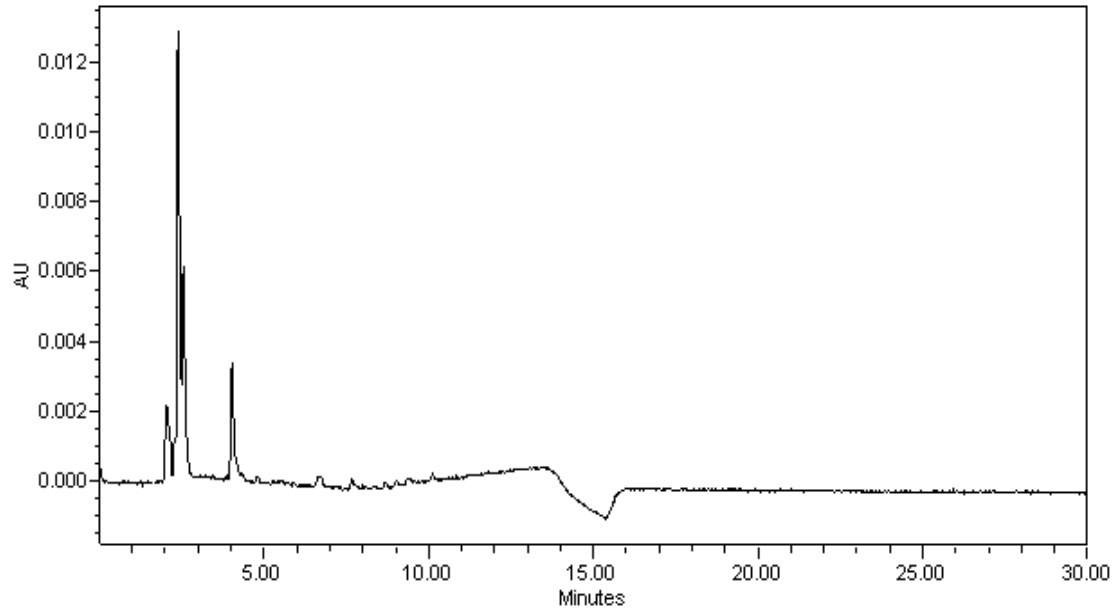

Figure 5.13: Chromatographic Profile of Loading and Washing Solution Fraction for Myoglobin and Cytochrome C Solution Bound to Myoglobin MIP

Just like previous chromatograms, all of the analytes in the loading solution were bound by the MIP in the SPE column, as indicated by the absence of any peaks of significance in the chromatogram (Figure 5.13).

After the elution solution was passed through the column the chromatogram shown in Figure 5.14 was obtained for the eluent.

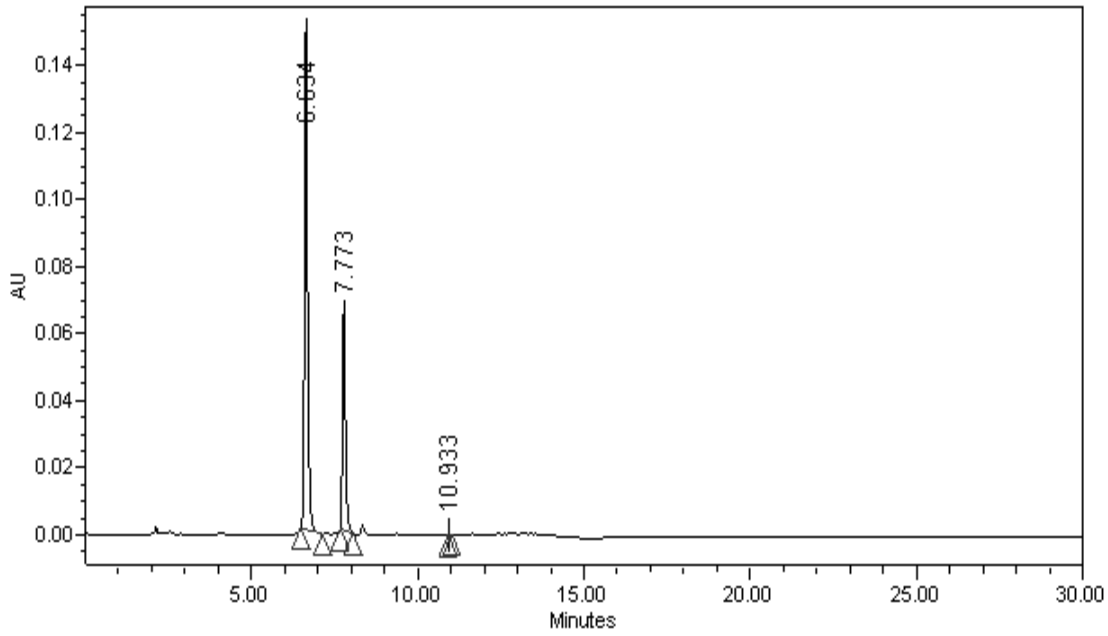

Figure 5.14: Chromatographic Profile of Eluted Fraction Myoglobin and Cytochrome C bound to Myoglobin MIP

Loading Solution	Washing	Elution
% of Myoglobin Protein Present in Fraction	0%	87%
% of Myoglobin Heme Group Present in Fraction	0%	3%
% of Cytochrome c Present in Fraction	0%	94%

Table 5.6: Myoglobin and Cytochrome C Rebinding Fraction Results for the Washing and Elution Stages

This chromatogram shows the results from the elution step; these are different to those obtained previously (Sections 5.5.1 and 5.5.2)

In the elution step, 94% of the cytochrome c was successfully recovered. 87% of the myoglobin was also recovered, with only 3% of the myoglobin heme group being recovered under the same elution conditions.

The difficulty in removing the heme group from the column when rebinding myoglobin and haemoglobin was concerned was addressed in Section 5.5.1. This can be attributed to the initial elution step. The presence of acetic acid in the wash solution, denatures the myoglobin and haemoglobin facilitating its removal from the column, but also disrupts the heme-protein non-covalent binding, allowing the protein to be eluted whilst the heme remains bound.

5.5.4 CR_4 Results

The results of the rebinding experiments for the columns indicate that although it is possible to remove the protein from the MIP after rebinding, it is a completely different matter when it comes to the heme group, as this has remained bound to the MIP in a close to quantitative manner, except in the case of cytochrome c.

This section of this investigation was therefore focussed solely on the removal of the heme group from the MIP once myoglobin had been rebound, using a solution of 1% acetic acid in 99% ethanol as the elution solvent.

The decision to introduce an organic solvent into the elution procedure was two-fold. It is commonly known that organic solvents such as alcohols act as denaturants when concerned with proteins.[25] This enabled further disruption to the structure of the remaining heme facilitating elution. By using alcohols, the polarity of the solvent is optimum to solubilise the heme, and also offers enhanced solubility *vs.* an aqueous solution.[26]

Analysis was carried out to ensure that the MIP had bound the molecule which was used as the template molecule - myoglobin.

The collected fraction containing the loading and washing sample was analysed *via* HPLC at 280 nm. The chromatographic results are shown in Figure 5.15 and Table 5.7.

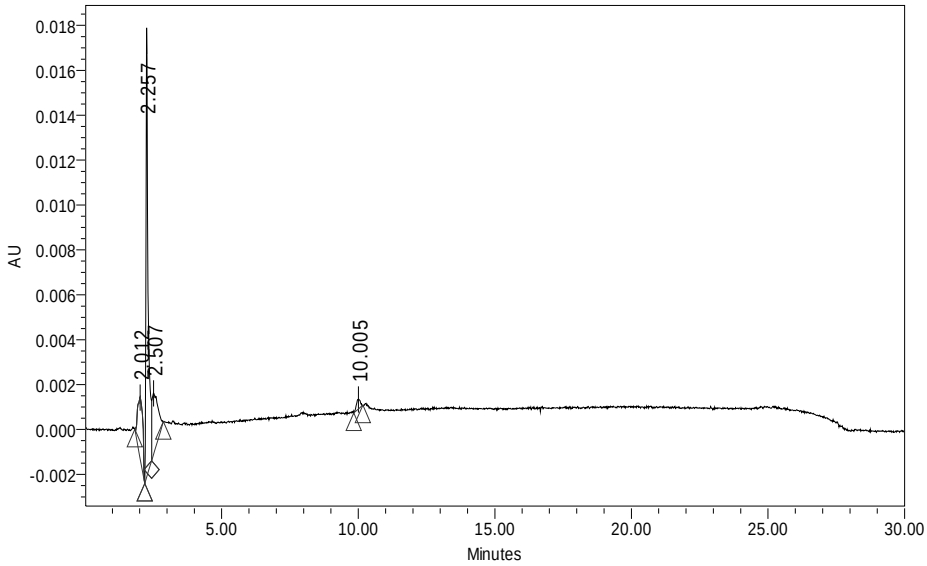

Figure 5.15: Chromatographic Profile of Loading and Washing Solution Fraction Myoglobin on Myoglobin MIP (Column CR_4)

Figure 5.15 indicates that there are no peaks present at the retention times of the myoglobin heme peak and the protein peak. This result is indicative that all of the myoglobin has bound to the MIP as indicated with the previous reported results.

The corresponding chromatogram for the elution of myoglobin is shown in Figure 5.16. The results are shown in Table 5.7.

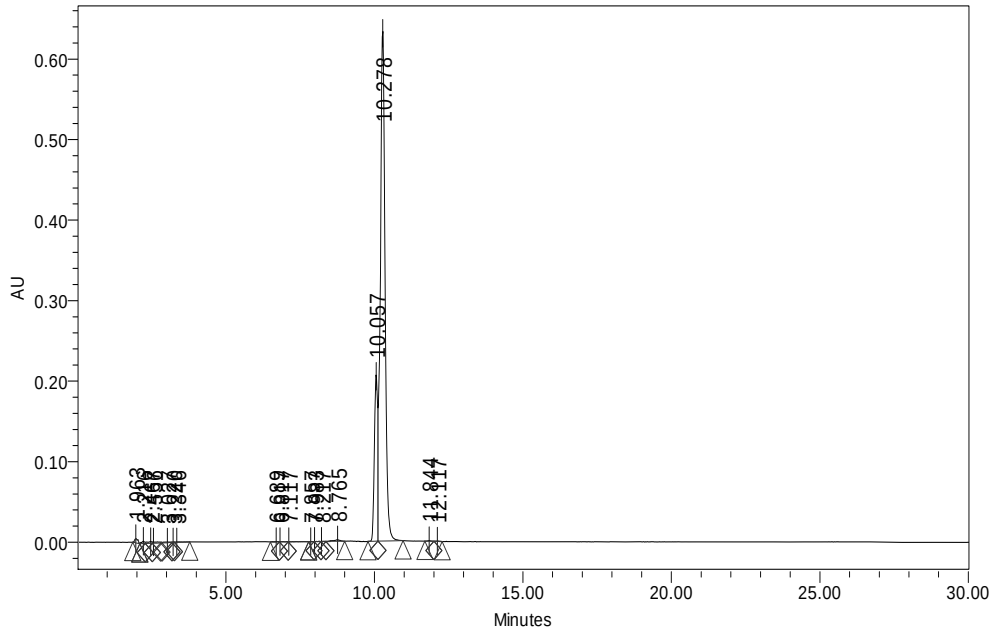

Figure 5.16: Chromatographic Profile of Myoglobin Eluted Fraction on Myoglobin MIP (Column CR_4)

	Loading & Washing Fraction	Elution Fraction
% of Myoglobin Protein Present in Fraction	0.0%	19.0%
% of Myoglobin Heme Group Present in Fraction	0.2%	63.0%

Table 5.7: Myoglobin Fraction (R_M) Results for the Washing and Elution Stages on Myoglobin MIP (Column CR_4)

The results which are reported in Figure 5.16 and Table 5.7 are dramatically different to those which have been reported in previous sections (Sections 5.5.1, 5.5.2 and 5.5.3)

Using the new acetic acid/ethanol elution wash, the removal of the heme group (63%) is more successful than the removal of the protein (26%).

5.5.5 CR_5 Results

A further experiment was also carried out to examine another wash solution.

To ensure that the myoglobin and the heme group had successfully bound to the MIP, analysis on column CR_5 was carried out using myoglobin as the loading solution. The results are shown in Figure 5.17 and Table 5.8.

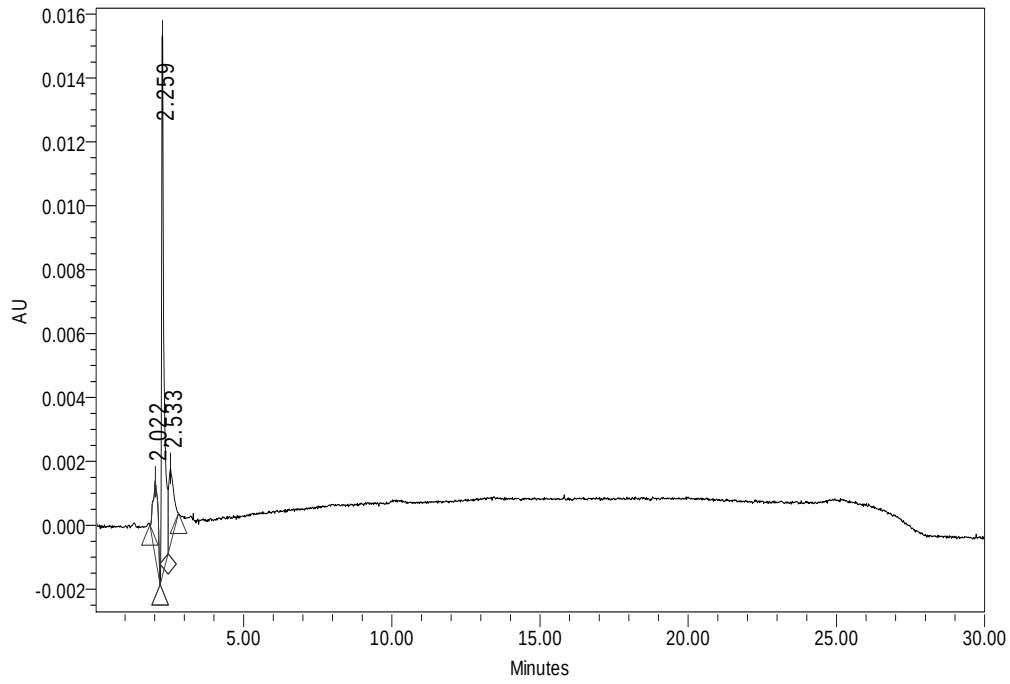

Figure 5.17: Chromatographic Profile of Loading and Washing Solution Fraction Myoglobin on Myoglobin MIP (Column CR_5)

Once again, the MIP has successfully rebound the imprinted molecule, indicated by no peaks present on the chromatogram.

To establish the removal of the heme group as well as the protein, analysis was carried out on the eluent from the wash solution containing 1% acetic and acid and 99% methanol. These results are shown in Figure 5.18 and Table 5.8.

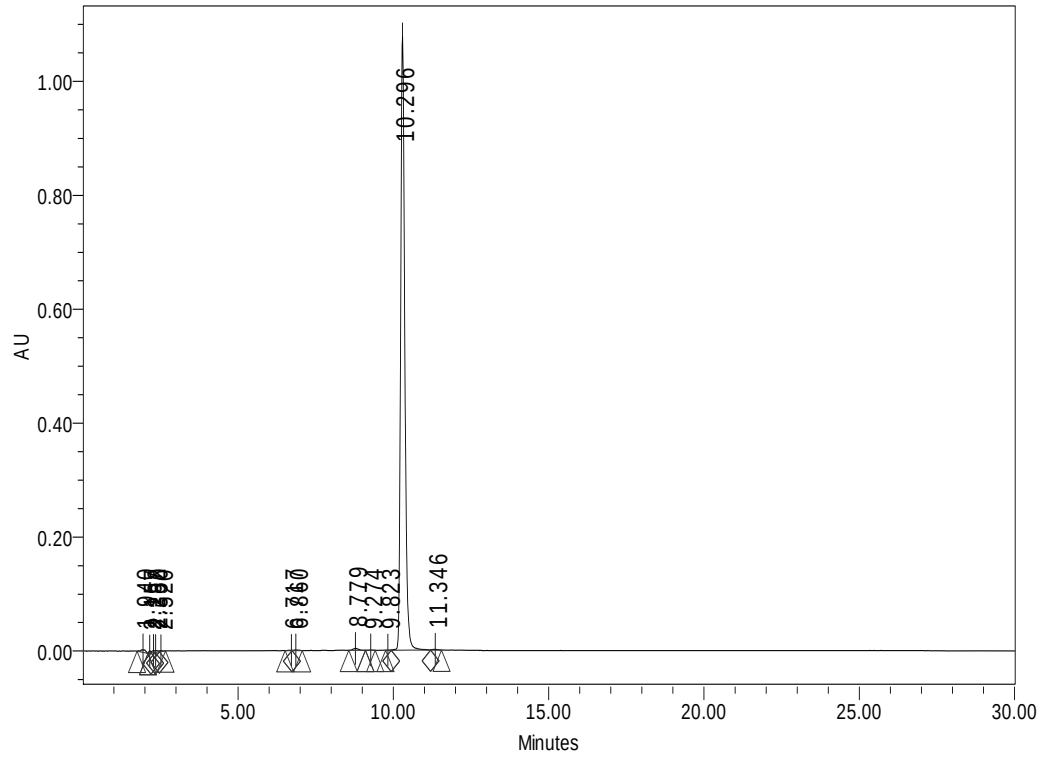

Figure 5.18: Chromatographic Profile of Myoglobin Eluted Fraction on Myoglobin MIP (Column CR_5)

	Loading & Washing Fraction	Elution Fraction
% of Myoglobin Protein Present in Fraction	0.0%	0.4%
% of Myoglobin Heme Group Present in Fraction	0.0%	89%

Table 5.8: Myoglobin Fraction (R_M) Results for the Washing and Elution Stages on Myoglobin MIP (Column CR_5)

The results from this wash solution are also interesting as they show an even more successful wash step has been identified for the removal of the heme group, with 89% being recovered from the MIP; however, it is to the detriment of the removal of the protein, with only 0.4% being recovered.

5.6 Conclusions

The competitive rebinding experiments can be carried out successfully using SPE columns. The column packing and experimental procedure is quick and effective also.

The protein content in the rebinding solution was lower than the theoretical maximum binding capacity, thus the results showing that all of the myoglobin had successfully rebound to the column were acceptable. It is worth noting however that although the protein content in the rebinding solution was lower than the theoretical maximum binding capacity, it was still close to the maximum load of myoglobin which can be applied to the column. Being close to the theoretical maximum binding capacity means that the binding sites available become limited thus non-specific binding may occur.

The heme group which is present in both myoglobin and haemoglobin remained bound to the myoglobin imprinted MIP after the initial elution procedure. This suggested that the MIP showed an affinity for the heme group which is present in both myoglobin and haemoglobin, over the protein. Further investigation into to elution solution being used countered this hypothesis. The structures of both myoglobin and haemoglobin are similar, and the heme group is non-covalently bound in the same fashion, i.e., in the case of myoglobin, the heme group is located within the protein portion of the molecule. The haemoglobin structure contains four protein chains each of which contain a heme group. Each protein chain is similar to the myoglobin molecule. Each protein chain within haemoglobin consists of a heme group.

Acetic acid is known denaturant for proteins. In addition to this acetic acid also disrupts the the non-covalent interactions which binds the heme to the proteins. The disruption to the interactions results in the heme being disassociated from the protein. As a result, the protein was readily removed from the column, but the heme remained bound.
This was indicated by the difficulty which was experienced when removing the heme group from the SPE column.

When competitive rebinding was carried out using a competing protein which has a heme group present, but which is chemically bound in a different fashion (covalently), i.e., when the MIP was loaded with cytochrome c, the MIP had demonstrated selectivity for the template molecule.

When the loading solution and washing solution were applied to the column, it was clear to see (visually) the heme group of the myoglobin present required a greater length of time to pass through the column than the heme group of the cytochrome c; the cytochrome c was eluted from the column before the myoglobin.

It was also demonstrated that whilst it is possible to remove the protein present in myoglobin in large quantities with relative ease and an appropriate wash solution, it was not so for the removal of the heme group. By altering the wash solution used, however, it was possible to remove the heme group, but to the detriment of the protein removal.

The introduction of an organic solvent into the elution procedure facilitated the elution of the heme group. The organic solvent acted as a denaturant, enabling further disruption to the structure of the remaining heme, ensuring elution with relative ease. By using alcohols as the organic solvent, the solubility of the heme was offering enhanced solubility *vs.* an aqueous solution.

5.7 References

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6.0 Introducing Enzyme-Imprinted Polymers

6.1 Introduction

This chapter describes the determination of the activity and the stability of an enzyme once it had been rebound to a MIP. The selectivity performance of the enzyme – imprinted polymer was also investigated, with comparisons being made to non-imprinted control polymers.

6.1.1 Choice of Enzyme

For this aspect of the work, the enzyme β-D-Glucosidase from almonds (systematic name β-D-glucoside glucohydrolase, EC 3.2.1.21) was selected. The name β-D-Glucosidase refers essentially to a group of hydrolases which exist widely in various sources, such as bacteria, fungi, plant and animal tissues.[1]

β-D-Glucosidase was first reported by Wöhler and Leibig in 1937 [2, 3] when it was extracted from sweet almonds (formerly known as emulsin), making it one of the oldest enzymes known and also making sweet almond emulsin a convenient source of the enzyme.[4] The origin of β-D-Glucosidase also ensures that the enzyme is readily available for purchase at a relatively low cost in the form of a lyophilised powder from various suppliers.

β-D-Glucosidase is an enzyme which catalyses the hydrolysis of glucosides. The reaction which takes place is shown in Figure 6.1.

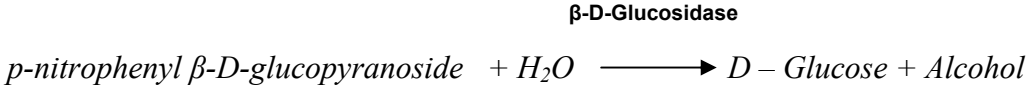

Figure 6.1: Representation of the β-D-Glucosidase Catalyzed Hydrolysis of Glucosides

The above reaction can be carried out in order to determine the specific activity of the enzyme. This aids the determination of the % of enzyme bound to the MIP during

the initial rebinding experiments and determines the stability of the enzyme bound to the MIP following subsequent rebinding experiments. This is because the product of the enzyme hydrolysis can be chosen to absorb light in the visible region of the electromagnetic spectrum, and with a λ_{max} of ~400 nm analysis I UV/Vis Spectroscopy can be realised easily.

6.1.2 β-D-Glucosidase Activity

There are two quantifiable properties of an enzyme; the total amount of protein which is present and the total activity of the enzyme.

The technique which is pertinent to measure the activity of β-D-Glucosidase is UV/vis spectrophotometry. By using UV/vis spectrophotometry both the total protein content and the activity can be established.

The total protein content is determined based on the fact that all proteins absorb light in the UV region of the electromagnetic spectrum. The activity is determined by assessing the rate of product formation or substrate utilisation during the enzyme-catalysed reaction.[5]

β-D-Glucosidase activity was determined in this investigation by assessing the quantity of 4-nitrophenoxide (Figure 6.2) released when β-D-Glucosidase is in the presence of a suitable substrate.

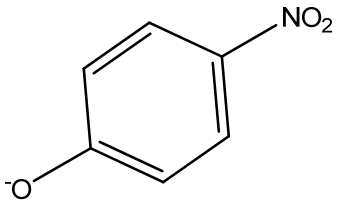

Figure 6.2: Structure of 4-Nitrophenoxide

The substrate *p*-nitrophenyl β-D-glucopyranoside was selected due to the fact that it is a chromogenic substrate, i.e., a substrate which produces an end product different in colour to the starting material, thus providing an easy and convenient way to

determine the activity of β-D-Glucosidase based on the quantity of product which is produced.

The enzymatic hydrolysis of chromogenic substrate *p*-nitrophenyl β-D-glucopyranoside (Figure 6.3) by β-D-Glucosidase produces 4-nitrophenol which in basic solution ionises to the corresponding phenoxide, as shown in Figure 6.4.

As the 4-nitrophenoxide produced is yellow in colour it allows the absorbance to be detected in the visible region of the spectrum (400 nm) using UV/vis spectrophotometry.

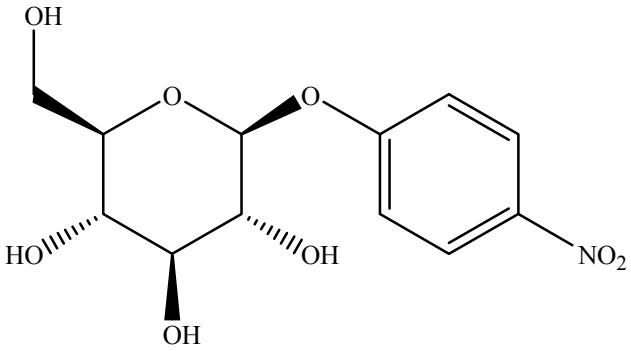

Figure 6.3: Structure of 4-Nitrophenyl β-D-Glucopyranoside

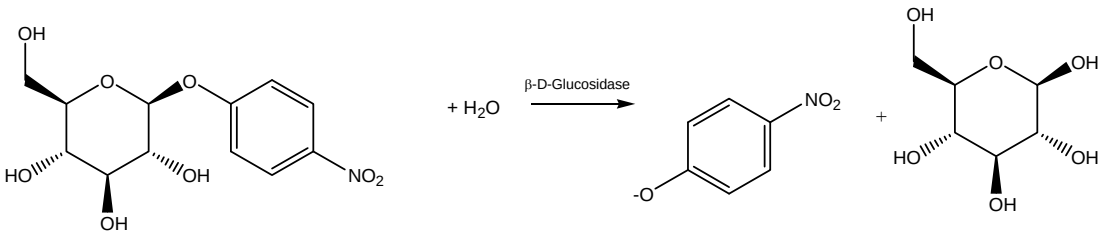

Figure 6.4: Illustration of the Chemical Reaction: Enzymatic Hydrolysis of 4-Nitrophenyl β-D-Glucopyranoside by β-D-Glucosidase.

From the information provided in Chapter 1, Section 1.5.4, pertaining to the substrate, the concentration of the substrate used was approximately 10,000 times higher; than the concentration of the enzyme.

6.2 Experimental Procedures

6.2.1 Chemicals

Sodium Citrate ($Na_3C_6H_5O_7$)
Citric Acid ($C_6H_8O_7$)
Potassium Phosphate Dibasic (K_2HPO_4)
Potassium Phosphate Monobasic (KH_2PO_4)
Sodium Carbonate (Na_2CO_3)
Methacrylic Acid (MAA)
4-Vinylpyridine (4-VP)
Ethylene Glycol Dimethacrylate (EGDMA)
Acetonitrile (ACN)
Azo-*bis*-isobutyronitrile (AIBN)
Deionised Water (dH_2O)
Molecular Sieves - 3Å
Bovine Serum Albumin (BSA)
β-D-Glucosidase from almonds
p-Nitrophenyl β-D-glucopyranoside (PNPG)
4-Nitrophenol (4-NP)
Methanol (CH_3OH)

The sodium citrate, citric acid, potassium phosphate dibasic, potassium phosphate monobasic, sodium carbonate, methacrylic acid, 4-vinylpyridine, ethylene glycol dimethacrylate, acetonitrile, azo-*bis*-isobutyronitrile were all purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). All of the solvents purchased were of HPLC grade and ≥ 95% pure. The acetonitrile was dried prior to use over activated molecular sieves (3Å).

Bovine Serum Albumin, β-D-Glucosidase from almonds, *p*-nitrophenyl β-D-glucopyranoside and 4-nitrophenol were also purchased from Sigma Aldrich. The 4-nitrophenol was of spectrophotometric grade, the Bovine Serum Albumin was ≥ 92%

pure and the *p*-nitrophenyl, β-D-glucopyranoside was ≥ 98% pure. The β-D-Glucosidase was in the form of a lyophilised powder at ≥6 units/mg.

6.2.2 4-Nitrophenol Calibration Curve

To establish the activity of β-D-Glucosidase, a calibration curve of 4-Nitrophenol (4-NP) had to be established. To obtain a calibration curve for 4-nitrophenol, solutions of various concentrations had to be prepared. This experiment was conducted in triplicate. A calibration curve was then plotted using the average absorbance readings.

4-Nitrophenol (10.8 mg) was dissolved in 20 mL of ACN; 0.1 mL of this solution was then taken and added to 9.9 mL of 10 mM citrate buffer at pH 5.2, its optimum pH. This was used as the stock solution.

Various solution concentrations of 4-nitrophenol were prepared by taking aliquots of the stock solution and diluting with buffer. This is outlined in Table 6.1.

Volume of 4-Nitrophenol Stock Solution (mL)	Volume of Buffer (mL)	Number of moles of 4-Nitrophenol (nanomoles)
0.0	1.0	**0.0**
0.1	0.9	**3.9**
0.2	0.8	**7.8**
0.3	0.7	**11.7**
0.4	0.6	**15.6**
0.5	0.5	**19.5**
0.6	0.4	**23.4**
0.7	0.3	**27.3**
0.8	0.2	**31.2**
0.9	0.1	**35.1**
1.0	0.0	**39.0**

Table 6.1: 4-Nitrophenol Calibration Compositions and Resultant Final Concentrations

Once all of the calibration standards were prepared, 0.02 mL of Na_2CO_3 solution (10.6 g of Na_2CO_3 in 500 mL of dH_2O) was added to adjust the ionisation state and subsequently the pH. The absorbance was then recorded by UV/vis spectrometry at a fixed wavelength: $\lambda = 400$ nm.

The average of the readings obtained was used to plot the 4-NP calibration curve, Figure 6.5 (Section 6.3, Page 131).

6.2.3 Enzymatic Assay of β-D-Glucosidase

The first stage of the assay for β-D-Glucosidase was to prepare all of the reagents required for the assay. This ensured that that assays were carried out immediately and that the results obtained were accurate and precise.

The activity assay was carried out at 37 ºC in an incubator. This temperature was pre-set to ensure it was ready for use once all of the preparations were complete.
To two Eppendorf tubes, E_1 and E_2, in this example, 1 mL of 10 mM citrate buffer, pH 5.2, and 0.5 mL of the substrate solution was added.

The citrate buffer was prepared in the same manner as described in Chapter 3, Section 3.4.5.

A solution of the substrate, *p*-nitrophenyl β-D-glucopyranoside (PNPG), was prepared by dissolving the substrate (603 mg) in 100 mL of dH_2O to give a 0.02 M solution (PNPG solution). It is worth noting that this should be stored at 0 ºC. This not only ensures that the substrate remains stable, but allows the same substrate solution to be used for two weeks before it must be discarded.

To E_1, 0.5 mL of the enzyme solution was then added. No enzyme was added to E_2, thus allowing it to be used in order to pre-calibrate the UV/vis spectrophotometer prior to the enzyme activity being measured.

The enzyme was prepared at a concentration of 0.0006 U/mL. This was achieved by preparing an initial solution of enzyme at a concentration of 1 mg/mL; 10 mg of β-D-Glucosidase was dissolved in 10 mL of ice-cold 0.05 M Tris-HCl buffer. The Tris-HCl buffer was prepared by adding 7.02 g of Trizma® HCl and 0.67 g of Trizma® Base in 1 L of dH_2O. The resultant pH was verified by a pH meter.

0.0079 mL of the enzyme solution was then added to 9.9921 mL (measured by weight) of a 0.2% BSA solution. The BSA solution was prepared by dissolving 0.2 g of BSA in 100 mL of 0.01 M phosphate buffer, pH 7 (Chapter 3, Section 3.4.5). The BSA present in the solution acts as a stabiliser for the enzyme.

BSA is commonly employed as a stabiliser in activity assays. [8, 9, 10] BSA binds with a low affinity to a variety of proteins and thus shields them from stress in their environment. In addition to this, BSA has no enzymatic activity by itself. Whilst

playing the role of enzyme stabiliser in this role, the BSA does not interfere with the activity assay or the enzyme which is being assayed; this is further verified in the blank activity assay. As the binding which occurs is significantly less than that of the substrate to the enzyme, the BSA will readily disassociate from the enzyme once this recognises the substrate.[11]

Eppendorfs E_1 and E_2 were then both assayed at 37 ºC, in the incubator for 15 minutes. After this time, 0.02 mL of the 0.02 M Na_2CO_3 solution (10.6 g of Na_2CO_3 in 500 mL of dH_2O) was added. The role of the Na_2CO_3 solution was to adjust the pH to pH 9 to change the ionization state of the mixture. This then ensures that the vibrant yellow colour expected is released by the reaction; this does not happen at the enzyme's optimum pH. By adding the Na_2CO_3, it also ensures that the enzymatic reaction is terminated after exactly 15 minutes.

After the 0.02 mL of the 0.02 M Na_2CO_3 solution had been added, 0.5 mL of the enzyme solution was added to the "blank" Eppendorf (E_2).

The solutions contained in Eppendorfs E_1 and E_2 were then transferred to individual cuvettes and the absorbance was read at 400 nm measured by means of UV/vis spectrometry. The cuvette which contained E_2 was used to pre-calibrate the spectrophotometer, prior to the absorbance of E_1 being measured. This procedure was executed 3 times and the average reading reported.

The average absorbance reading was obtained and recorded. The reading was then superimposed onto the 4-nitrophenol calibration curve, Section 6.2.3, and the number of moles of 4-nitrophenol released by β-D-Glucosidase after 15 minutes determined (Figure 6.5).

6.2.4 P_2: Activity Assay

In order to establish the mass of active β-D-Glucosidase present on PCMCs P_2, (Chapter 3), an activity assay was carried out. After a fixed period of time, the

absorbance was measured. This was conducted in triplicate. The average absorbance reading was superimposed onto a calibration of 4-nitrophenol concentration (Figure 6.5) to obtain the concentration of β-D-Glucosidase.

To do this, sample P_2 was diluted using buffer so that the solution theoretically contained 0.0006 *units* (0.79 mg) of β-D-Glucosidase. An additional sample containing only blank micro-crystals i.e., no protein on the surface, was also diluted using the same volume of buffer as the P_2 sample. This procedure was executed 3 times and the average reading reported.

Both of the resultant solutions were then assayed using the procedure described in Section 6.2.3. This allowed the activity to be determined.

The average activities were then compared to a calibration of 100% enzyme activity (Figure 6.7).

6.2.5 β-D-Glucosidase Enzyme on MIP and NIP: Activity Assay

Once the activity of the enzyme was established, the next stage was to determine the activity of the enzyme once it had been rebound to the MIP and NIP. This also provided a means of establishing the mass of enzyme which was capable of binding to the MIP, in addition to determining if the enzyme demonstrated selectivity in rebinding to the MIP relative to the NIP.

The first stage of this procedure was to ensure that both the MIP and the NIP were prepared for rebinding.

A suspension of MIP and NIP was prepared at a concentration of 1 mg/mL. This was achieved by adding 100 mL of 10 mM citrate buffer to 100 mg of the MIP and NIP respectively. Only 5 mg of MIP and NIP was required for the rebinding stage of the experiment respectively, thus 5 mL of each polymer suspension was required in total. To achieve this, 1 mL aliquots were removed frequently and placed in Column 2 (as

described in Chapter 2, Section 2.5). The buffer was then removed from the samples by means of centrifugation at 5,000 rpm for 5 minutes. This procedure was carried out until the filters each housed 5 mg of MIP and NIP.

Prior to bringing the polymer into contact with the enzyme, the MIP and NIP had to be wetted to allow access to the pores which had been generated during the imprinting stage. This was accomplished by adding 2 mL of 10% CH_3OH in 10 mM citrate buffer solution. Each of the columns were sealed and rotated by means of a rotor at 24 rpm for 15 minutes. The temperature was 37 ºC.
After 15 minutes, the 10% CH_3OH in 10 mM citrate buffer was removed from the MIP and NIP by means of centrifugation at 5,000 rpm for 5 minutes.

2 mL of 10 mm citrate buffer was then added to the columns. Again the columns were sealed and rotated for 15 minutes at 24 rpm at 37 ºC, before the buffer was eluted using the same centrifuge procedure described above.

The MIP and the NIP were now ready for the rebinding experiments and also for the determination of the activity of the enzyme which was bound to each polymer.

Four samples were required, two containing the MIP and two containing the NIP. The MIP and NIP samples used were M_2 and N_2 respectively (Chapter 4, Section 4.3.4).

The sample names utilised in this section of the investigation are M_{2R} and N_{2R} in addition to M_{2B} and N_{2B}.

The rebinding solutions used are shown in Table 6.2.

Sample	Rebinding Solution	Volume (mL)
M_{2R}	0.006 *u* β-D-Glucosidase	0.5 m L
M_{2B}	10 mM citrate buffer, pH 5.2	0.5 mL
N_{2R}	0.006 *u* β-D-Glucosidase	0.5 mL
N_{2B}	10 mM citrate buffer, pH 5.2	0.5 mL

Table 6.2: β-D-Glucosidase MIP and NIP (M_2 and N_2) Rebinding Solution Details Used in the Determination of Activity and Stability Once Rebound.

Each of the columns were sealed and rotated for 15 minutes at 24 rpm, 37ºC. Once this had been completed the liquids were eluted by means of centrifugation at 5,000 rpm for 5 minutes. The eluents were then transferred to separate Eppendorf tubes (2 mL) and subjected to the activity assay.

To each of the new Eppendorf tubes, 1 mL of 10 mM citrate buffer, pH 5.2, and 0.5 mL of the PNPG solution was added. The Eppendorf tubes were then sealed again and incubated at 37ºC for 15 minutes. They were rotated continually at 24 rpm.
After 15 minutes the Eppendorf tubes were removed and 0.02 mL of 0.02 M Na_2CO_3 solution was added. The absorbance at 400 nm was then measured to determine the amount of nitrophenoxide released.

During this period, the MIP and NIP housed in the columns were washed for 15 minutes with 1 mL of 10 mM citrate buffer at 37 ºC whilst being rotated at 24 rpm. This was in order to remove any remaining unbound enzyme from the MIP and NIP samples. Once the 15 minutes had passed, the buffer was eluted by means of centrifugation at 5,000 rpm for 5 minutes. Again, the eluents were collected and transferred to new Eppendorf tubes which had a capacity of 2 mL.

The eluents were then assayed using the procedure described previously and the absorbance readings recorded.

Now that the rebinding stage was complete, the final stage was to assess the activity of the enzyme remaining bound to M_{2R} and N_{2R}. M_{2B} and N_{2B} were also subjected to this assay.

The activity assays for these samples were carried out in a fashion similar to that described above, however 1 mL of 10 mM citrate buffer and 0.5 mL of the PNPG solution was added directly to the columns, before being sealed and incubated at 37 ºC for 15 minutes whilst rotating continually at 24 rpm.

After 15 minutes all of the tubes were centrifuged at 5000 rpm for 5 minutes before 0.02 mL of 0.02 M Na_2CO_3 was added. The absorbance at 400 nm was then recorded.

For each of the absorbance readings, three individual readings were taken and an average result recorded.

Using the results obtained, and by application of the 4-nitrophenol calibration curve, the percentage of enzyme rebound, the selectivity of the MIP over the NIP, and the activity of the enzyme bound to the polymers could all be deduced.

This procedure was repeated three times for each sample and the average readings reported.

6.3 Results & Discussion

In order to establish not only the activity, but also the concentration of β-D-Glucosidase for this part of the investigation, a calibration curve for 4-nitrophenol was constructed (Figure 6.5). The absorbance of 4-Nitrophenol at each concentration

was determined three times. The standard error for each point was less than ± 0.02 absorbance units.

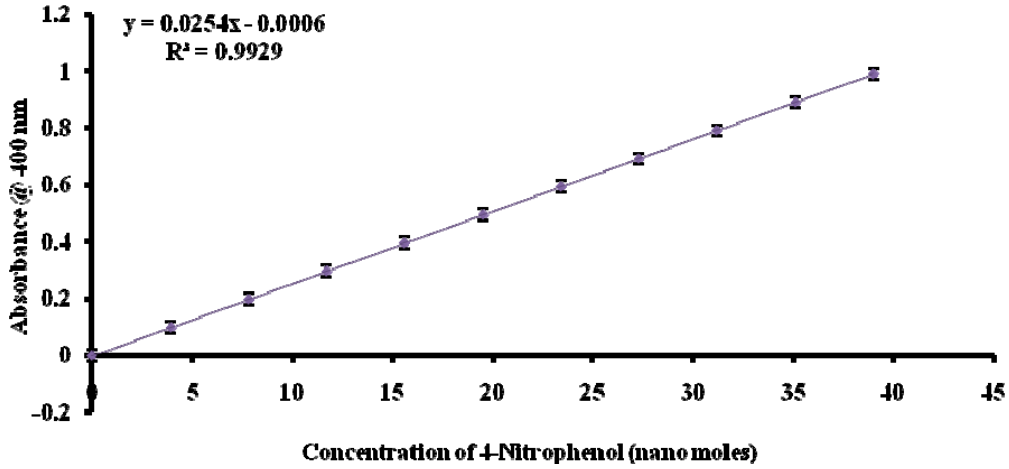

Figure 6.5: 4-Nitrophenol Calibration Curve

6.3.1 APL of P_2

Before the percentage of β-D-Glucosidase bound to the surface of the micro-crystals could be deduced, it was necessary to measure the activity of the β-D-Glucosidase purchased from Sigma-Aldrich. This was carried out so that the results of the activity assay of the micro-crystal bound enzyme could be compared to a standard of the enzyme. The results of this assay were then superimposed onto the 4-nitrophenol calibration curve (Figure 6.6). Again, the activity of the pure enzyme was determined three times.

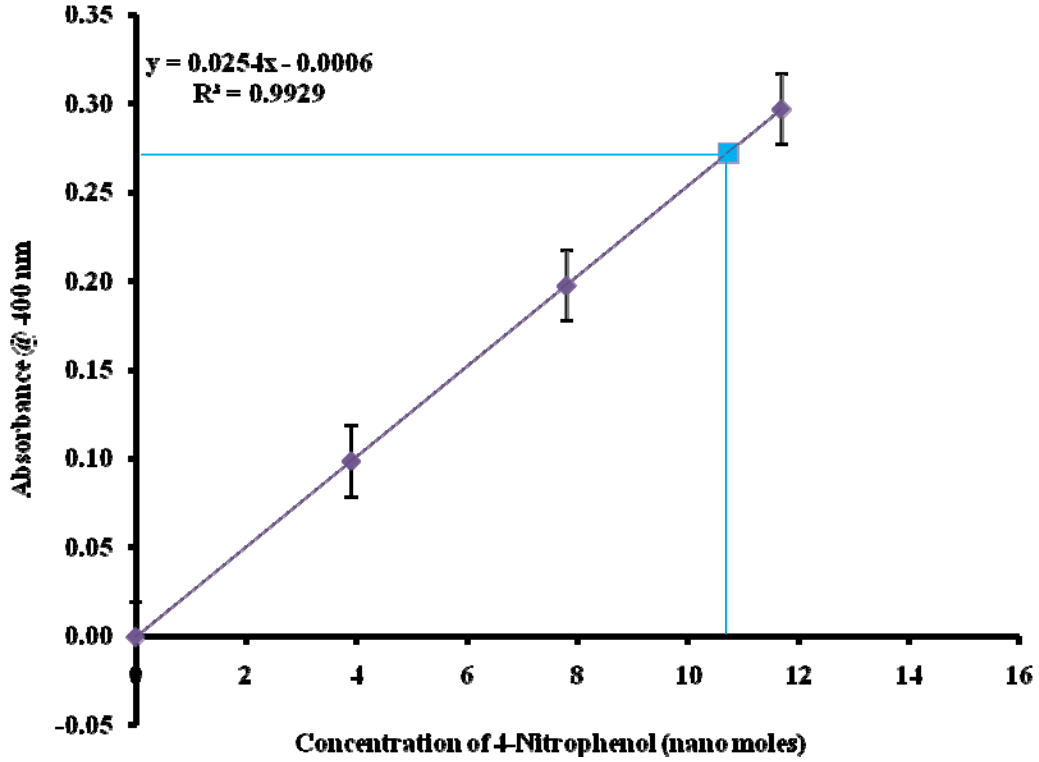

Figure 6.6: Activity of β-D-Glucosidase Superimposed on 4-Nitrophenol Calibration

The concentration of the enzyme could be determined by inputting the average absorbance reading into the calibration curve. The value obtained is shown in Table 6.3.

Enzyme	Average Absorbance	Average Number of Moles of 4-Nitrophenol (nanomoles)	Standard Error (Absorbance Units)
β-D-Glucosidase	0.272	10.732	± 0.0003

Table 6.3: β-D-Glucosidase Enzyme Absorbance Results and Activity of β-D-Glucosidase

The concentration of enzyme measured was slightly lower than the value which was predicted; 11.85 nanomoles. This prediction was made on the assumption that the enzyme was 100% active when purchased.

Theoretically, β-D-Glucosidase releases 1 μmol of 4-nitrophenol per 1 mg of enzyme, where 1 mg is equal to 7.55 *units*.

As stated, the concentration of β-D-Glucosidase used in this investigation was 0.006 *units*. 0.006 units is equal to 0.79 nmol, which is the quantity of 4-nitrophenol released per minute.

The activity assay was carried out for 15 minutes, thus the quantity of 4-nitrophenol should be 15 times this value; 11.85 nmol. As the above result determines that only 10.732 nmol of 4-nitrophenol has been released, then the enzyme used in this investigation was apparently 90.5% active.

As all subsequent experiments used the same enzyme, the results have been computed on the basis that by releasing 10.732 nmol of 4-nitrophenol, the enzyme is 100% active.

The absorbance of three β-D-Glucosidase and blank PCMC samples was measured, The average absorbance reading and subsequent enzyme loading on the PCMCs (β-D-Glucosidase and blanks) are shown in Table 6.4 and Figure 6.7.

Enzyme	**Average Absorbance**	**Average Number of Moles of 4-Nitrophenol (n moles)**	**% Enzyme Bound to Micro-Crystal**	**Standard Error (Absorbance Units)**
β-D-Glucosidase	0.2616	10.324	96.2 %	± 0.00004
Blank	0.0002	0	0%	± 0.00000

Table 6.4: Absorbance of β-D-Glucosidase PCMCs and % Enzyme Bound to Micro-Crystal with Associated Activity

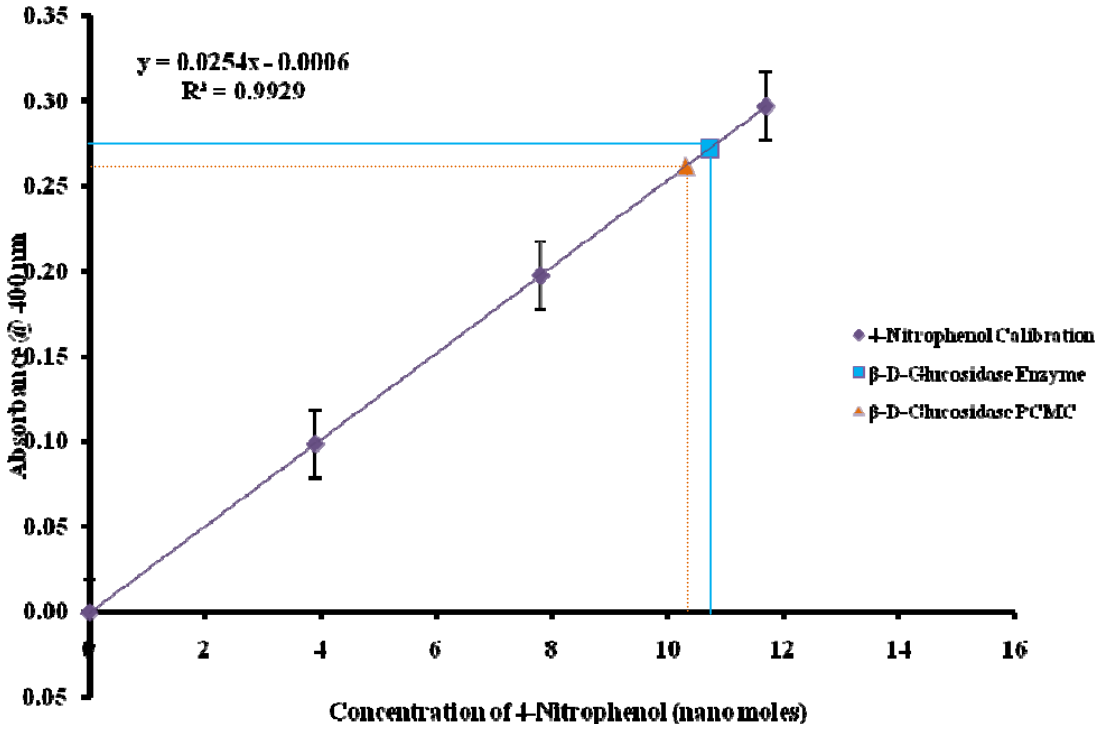

Figure 6.7: Determination of the Quantity of β-D-Glucosidase Bound to Micro-Crystals by Activity Level vs. Free Enzyme Activity

The orange triangle in the calibration curve shows the PCMC compared to the pure enzyme indicated in blue. It can be seen that the difference in activity is minimal; in fact, 96.2% of the enzyme has bound to the surfaces of the micro-crystals and remained active. This is a good result as it demonstrates that the enzyme retains its activity even after it has been immobilised. The blank PCMC has no enzyme bound to the surface, which is expected entirely as given the fact that the enzyme was not present during the production of the blank PCMCs.

The high level of uptake of active β-D-Glucosidase on the surface of the micro-crystal is a successful result; previous findings have reported quantities of enzyme immobilised on the surface of micro-crystals being as low as 60%, with the subsequent activity being 68%.[12]

6.3.2 MIP and NIP Rebinding Results

The analysis carried out for the rebinding stage was based solely on the MIPs capacity to rebind the molecule which was used as the template molecule, β-D-Glucosidase. There was not a competitive molecule present throughout this experimental section.

The uptake of β-D-Glucosidase on the NIP was assessed in order to probe the possibility of non-selective binding.

The loading of enzyme applied to each of the columns, which housed 5 mg of the MIP and NIP each, is detailed in Table 6.5.

Sample	**Rebinding Solution**	**Volume (mL)**
M_{2R}	0.006 *u* β-D-Glucosidase	0.5 m L
M_{2B}	10 mM citrate buffer, pH 5.2	0.5 mL
N_{2R}	0.006 *u* β-D-Glucosidase	0.5 mL
N_{2B}	10 mM citrate buffer, pH 5.2	0.5 mL

Table 6.5: β-D-Glucosidase MIP and NIP (M_2 and N_2) Rebinding Solution Details Used in the Determination of Activity and Stability Once Rebound.

The load solution was removed from the MIP and NIP by means of centrifugation after the initial binding studies, and the eluents collected and subsequently assayed. Figure 6.8 shows the percentage of β-D-Glucosidase bound to both of the MIP samples. This value was deduced based on the number of moles of 4-NP present in the eluents of the rebinding solutions (Figure 6.9), and shows the results for the same

experiment carried out on N_{2R} and N_{2B}. Table 6.6 shows the average results obtained for all four samples. Each experiment was executed three times.

Sample	Average Absorbance	Average Number of Moles of 4-Nitrophenol in Eluent (nanomoles)	Average % Enzyme Bound to Sample	Standard Error (% Enzyme Bound)
M_{2R}	0.0485	1.932	82%	± 2.3
M_{2B}	0.0000	0	0%	± 0.0
N_{2R}	0.0757	3.005	72%	± 1.7
N_{2B}	0.000	0	0%	± 0.0

Table 6.6: % of β-D-Glucosidase bound to MIP and NIP Samples (M_2 and N_2) Based of Activity Levels

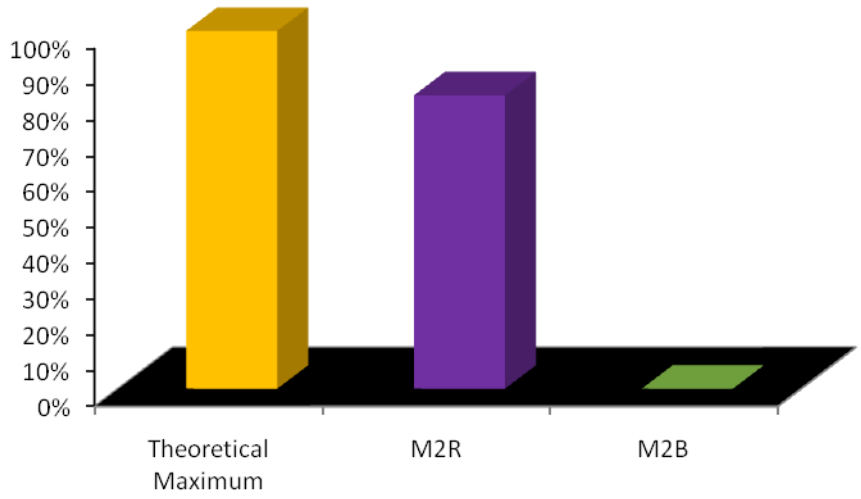

Figure 6.8: % of β-D-Glucosidase bound to M_{2R} and M_{2B}

The results show that the overall % of β-D-Glucosidase bound to M_{2R} is lower than the theoretical maximum; the final % of β-D-Glucosidase bound to the MIP is 82% ± 2.3. As expected, there is no β-D-Glucosidase bound to sample M_{2B}. This can be attributed to there being no β-D-Glucosidase present in the sample initially. It does,

however, reconfirm that there is no β-D-Glucosidase left in the MIP from the preparation stage, i.e., there is no template bleeding. No further analysis was conducted using M_{2B} as these results demonstrated that there was no β-D-Glucosidase present.

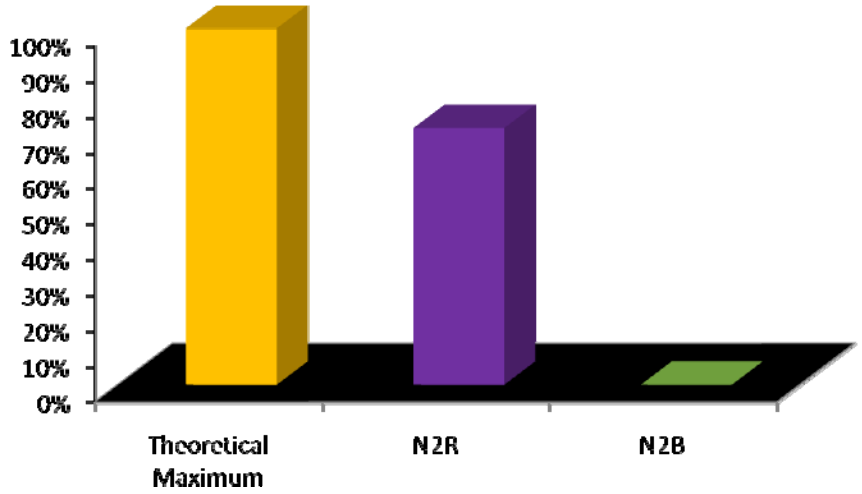

Figure 6.9: % of β-D-Glucosidase bound to N_{2R} and N_{2B}

The results shown in Figure 6.9 show that the NIP does indeed rebind the β-D-Glucosidase; in fact, 72% ± 1.7 has rebound. This result suggests that non-selective binding has occurred. As expected, there is no β-D-Glucosidase bound to sample N_{2B}. This can be attributed to there being no β-D-Glucosidase present in the sample initially. As with M_{2B}, the results show that there is no β-D-Glucosidase present at all in the NIP, thus no further analysis of N_{2B} is required.

When β-D-Glucosidase is introduced to the MIP during rebinding experiments, the β-D-Glucosidase rebinds and the enzyme retains its active conformation; further supporting that the β-D-Glucosidase has selectively bound to the MIP. With respect to the NIP, as there are no binding sites generated, the β-D-Glucosidase cannot rebind selectively, thus the binding (72%) which has occurred can be attributed to non-selective binding. [13, 14]

The next step of experimental analysis required was analysis of the eluents obtained from the wash step which was carried out. As explained previously, the wash step is

essential to ensure that any weakly bound enzyme present on the polymers is removed.

The eluents collected from samples M_{2R} and N_{2R} were analysed. These results are shown in Table 6.7.

Sample	Average Absorbance in Eluted Fraction	Average Number of Moles of 4-NP in Eluted Fraction (n Moles)	Average % Enzyme Bound to Sample	Standard Error (% Enzyme Bound)
M_{2R}	-0.00037	0	0%	± 0.0
N_{2R}	0.000333	0	0%	± 0.0

Table 6.7: % β-D-Glucosidase Present in Wash Stage Eluents of MIP and NIP (M_2 and N_2) After Rebinding Base on Activity Levels

This was a good result as it demonstrated that the results achieved in the initial rebinding stage were accurate and that enzyme which did not bind to the polymer in the application stage could be separated from the polymer at the very same stage merely by centrifugation.

The final and most crucial part of this investigation was to establish if β-D-Glucosidase, once bound to M_{2R} and N_{2R}, respectively, did indeed remain active, and if there was a difference between the activity of β-D-Glucosidase which was retained on M_{2R} and the activity of the enzyme on N_{2R}. The activity assay was carried out whilst the enzyme was still bound to M_{2R} and N_{2R}. Figure 6.10 and Table 6.8 shows the results obtained from the analysis of $M_{2R,}$ whereas Figure 6.11 and Table 6.9 show the corresponding results of N_{2R}.

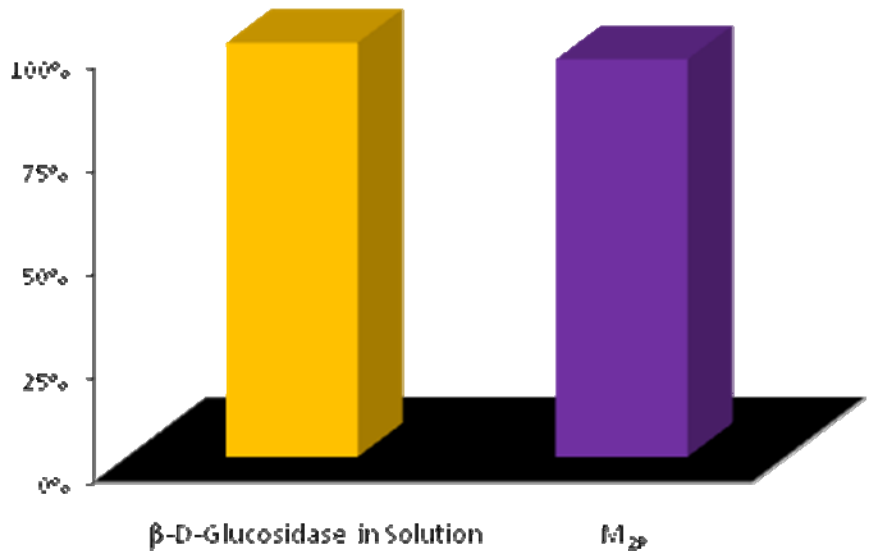

Figure 6.10: β-D-Glucosidase Activity on M_{2R}

Figure 6.10 shows that the concentration of 4-NP released by the substrate and the enzyme whilst the enzyme is immobilized on M_{2R}, (indicated in purple) is less than the theoretical prediction, indicated in the gold, based on 100% activity of the bound enzyme. Of the β-D-Glucosidase that was rebound to M_{2R}, 96% ± 1.8 of this has remained in its active conformation, demonstrating that even although it is immobilised on the MIP it retains high activity. The 4% deficit in activity may be due to problems of substrate diffusion.

Sample	Average Number of Moles of 4-Nitrophenol (n Moles)	Average Activity of β-D-Glucosidase Bound (%)	Standard Error (%β-D-Glucosidase Bound)
M_{2R}	8.12	96%	± 1.8

Table 6.8: Activity of β-D-Glucosidase Retained on MIP (M_{2R}) Based on Activity Level

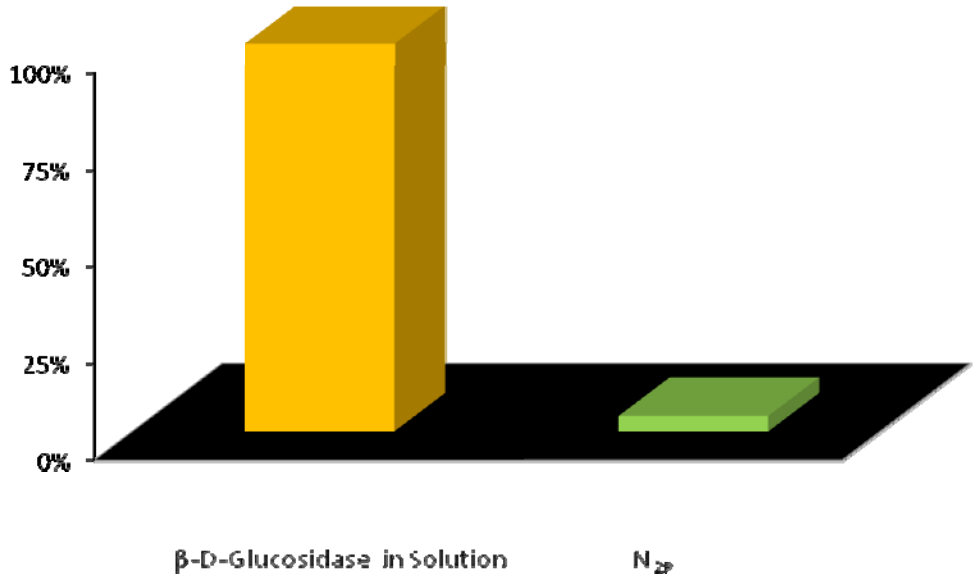

Figure 6.11: β-D-Glucosidase Activity on N_{2R}

Figure 6.11 shows that the concentration of 4-NP released by the substrate and the enzyme whilst the enzyme is immobilized on N_{2R} (indicated in green) is considerably less than the theoretical prediction, indicated in gold, based on 100% activity of the bound enzyme. Of the β-D-Glucosidase that was rebound to N_{2R}, only 4% ± 0.9 of this has remained in its active conformation, demonstrating that although a large quantity of enzyme is immobilised on the NIP, it does not remain in its active conformation.

The activity of an enzyme is determined by its tertiary structure, [15] thus it must fully retain its active conformation (tertiary structure) for the enzyme to remain active. As the integrity of the tertiary structure of the enzyme controls the overall activity of an enzyme, [16] it would suggest that the β-D-Glucosidase which rebound to NIP changed its conformation which resulted in the loss of activity.

Sample	Average Number of Moles of 4-Nitrophenol (n Moles)	Average Activity of β-D-Glucosidase Bound (%)	Standard Error (%β-D-Glucosidase Bound)
N_{2R}	0.4	4%	± 0.9

Table 6.9: Activity of β-D-Glucosidase Retained on NIP (N_{2R}) Based on Activity Level

6.4 Conclusions

6.4.1 PCMCs

The results show that the preparation of β-D-Glucosidase PCMCs in 10 mM citrate buffer (pH 5.2) *via* the Continuous Flow Precipitator provides β-D-Glucosidase PCMCs which has 96.2% of active β-D-Glucosidase immobilised on the surfaces of the micro-crystals. This was verified by the activity assay which was carried out on the PCMCs.

6.4.2 MIP and NIP

By using PCMCs as the template as opposed to individual protein molecules, it was hypothesized that, post-polymerisation, the template would be removed without difficulty due to the fact that the PCMCs generate micrometer-sized pores within the polymer, lined with protein imprinted sites.

The solvent switching method, as outlined in Chapter 4, was utilised to remove the β-D-Glucosidase which was present in the preparation of the MIPs. This method was sufficient to remove the β-D-Glucosidase and this was verified further by use of the activity assay on the MIP. The activity assay was performed on the final wash solvents of the MIP and NIP samples respectively. There was no trace of active β-D-Glucosidase, thus confirming 100% removal.

6.4.3 Rebinding Experiments & Activity Assay

The rebinding experiments were carried out successfully using Ultrafree® Microcentrifuge Filters; Column 2. By utilising the Ultrafree® Microcentrifuge Filters as columns, the introduction of the MIP and NIP resin slurries to provide a suitable bed for the rebinding and activity assay experiments was completed in an effective and timely manner. By slurring the MIP and NIP samples, loss of material due to the dry, fine particulate matter was avoided.

The size, and subsequent bed height of the packed columns was ample for the intended purpose of the column. The quantity of the material packed allowed quantitative analysis to be carried out with respect to the activity of the β-D-Glucosidase; the activity of β-D-Glucosidase can be determined whilst working at extremely low enzyme concentrations i.e., 0.006 *units* of enzyme.

The results of the initial rebinding experiments did not demonstrate clearly that the β-D-Glucosidase imprinted MIP showed an affinity for β-D-Glucosidase over the NIP. This was indicated by the activity assay results which were carried out on the eluents of the rebinding fractions; 82% of β-D-Glucosidase had successfully rebound to the MIP, however, 72% of the enzyme had also bound to the NIP.

More promising results were delivered after analysis of the enzyme which was immobilised on the MIP and NIP samples. The results demonstrated not only that once the β-D-Glucosidase was bound to the MIP it retained its activity (96%), but also that there was significant decline in the activity of the β-D-Glucosidase bound to NIP (4%). These results support the hypothesis that the binding sites which are generated in the MIP match the structure of β-D-Glucosidase, as this was the template.

The loss of activity of the β-D-Glucosidase bound to the NIP and the retention of activity of the β-D-Glucosidase which rebound to the MIP further corroborates the

theory that the binding sites which are generated in the MIP match the structure of β-D-Glucosidase.

The ability to re-use the MIP and NIP analysed in this chapter was not investigated at this stage. This will be reviewed and analysed in Chapter 7.

A key measurement which is required is to determine the long term stability of the enzyme once bound to the MIP compared to that of the enzyme whilst stored in solution.

6.5 References

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7.0 Lifetime Study

7.1 Introduction

The MIPs prepared for this investigation (M_1 and M_2) have both been found to display an affinity for its template molecule (myoglobin and β-D-Glucosidase respectively). Having demonstrated the ability to remove the template molecules after rebinding experiments, the next stage was to assess the overall robustness of the MIPs in respect of the re-usability of the MIPs.

7.2 Re-Usability

Being able to re-use a MIP for a large number of rebinding cycles is an important factor which was thoroughly investigated. The re-usability of the MIPs re-enforces the capabilities of MIPs, and the advantages which MIPs may offer in both on-line and off-line procedures.

Having touched on the potential applications that the MIPs produced may be subject to (Chapter 1), the overall economy, and hence the cycle lifetime is a key requisite if MIPs are to be used for these applications.

7.3 Experimental Procedures

7.3.1 Chemicals

MIP M_1
MIP M_2
Sodium Citrate ($Na_3C_6H_5O_7$)
Citric Acid ($C_6H_8O_7$)
Potassium Phosphate Dibasic (K_2HPO_4)
Potassium Phosphate Monobasic (KH_2PO_4)
Acetic Acid (CH_3COOH)

Deionised Water (dH_2O)
Myoglobin from Horse Heart
β-D-Glucosidase from almonds.
Methanol (CH_3OH)

β-D-Glucosidase from almonds and myoglobin from horse heart were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). Myoglobin was in the form of a re-crystallised powder and the β-D-Glucosidase was in the form of a lyophilised powder at ≥6 units/mg.

The sodium citrate, citric acid, potassium phosphate dibasic, potassium phosphate monobasic, methanol and acetic acid were all purchased from Sigma-Aldrich. All of the solvents purchased were of analytical grade and were ≥ 98% pure.

7.3.2 MISPE

The analytical technique employed to investigate the re-usability of the MIPs was MISPE, as detailed in Chapter 2, using Column 2, also detailed in Chapter 2.

7.3.3 M_1 and M_2 Column Preparation

The column used for this set of experiments was Column 2 (as described in Chapter 2, Section 2.5). In order to carry out an accurate analysis, fresh columns were packed for both M_1 and M_2. This, in turn, required the initial rebinding and elution steps to be carried out before the regeneration of the MIPs could be assessed.

With the objective of this experimental section being the regeneration of the MIP a decision was taken to use buffers in which both myoglobin and β-D-Glucosidase were at their optimum. For myoglobin, 100 mM potassium phosphate buffer, pH 5.81, was introduced (Chapter 3, Section 3.5.1) and 10 mM citrate buffer, pH 5.2 (Chapter 3, Section 3.5.2) was introduced for the β-D-Glucosidase. Preparing the myoglobin and β-D-Glucosidase MIPs in their preferred buffers allowed the function

of pH to play a pivotal role in the subsequent myoglobin and β-D-Glucosidase removal, as well as the regeneration of the MIPs.[1, 2, 3]

In order to pack the columns, two suspensions of each of the MIP samples were prepared, Table 7.1.

Sample	Buffer	Volume of Buffer (mL)	Weight of Sample (g)	Concentration (mg/mL)
M_1	100 mM Potassium Phosphate Buffer, pH 5.81	100	1	10
M_2	10 mM Citrate Buffer, pH 5.2	100	1	10

Table 7.1: MIP Sample Suspensions for Column Packing – Myoglobin (M_1) and β-D-Glucosidase (M_2) MIPs

Aliquots of the suspension were removed and added to the respective columns until each housed the required quantity of MIP, Table 7.2. The respective buffer was then removed from each column by means of centrifugation at 5000 rpm for 5 minutes. This procedure was carried out until each column housed 150 mg of the respective MIP.

Sample	Number of Aliquots	Volume of Aliquot (mL)	Quantity of Sample in Column (mg)	Binding Capacity of Sample (mg)
M_1	15	1 mL	150.00	3.14
M_2	15	1 mL	150.00	2.42

Table 7.2: Quantity of Resin (Myoglobin (M_1) and β-D-Glucosidase (M_2) MIP) Used to Pack Column and Final Binding Capacity

Prior to bringing the polymer into contact with its rebinding solution, the MIP samples had to be wetted to allow access to the pores which had been generated

during the imprinting stage. This was accomplished by adding 2 mL of 10% CH_3OH in 100 mM potassium phosphate buffer, pH 5.81, for M_1 and 2 mL of 10% CH_3OH in 10 mM citrate buffer solution, pH 5.2, for M_2. The columns were then sealed and rotated by means of a rotor at 24 rpm for 15 minutes. The temperature was kept constant at 37 ºC throughout. The liquids housed in the columns were then removed from the samples by means of centrifugation at 5,000 rpm for 5 minutes.

7.3.4 Preparation of Rebinding Solutions

The analysis carried out for the rebinding stage was based solely on each of the MIP samples capacities to rebind only the molecule which was used as the template molecule, myoglobin and β-D-Glucosidase.

The rebinding solutions prepared for analysis are detailed in Table 7.3.

Rebinding Solution	**Template Molecule**	**Buffer**	**Concentration (mg/mL)**
A	Myoglobin	100 mM potassium phosphate buffer, pH 5.81	1.56
B	β-D-Glucosidase	10 mM citrate buffer, pH 5.2	1.00

Table 7.3: Column Rebinding Solutions for Myoglobin (M_1) and β-D-Glucosidase (M_2) MIP for use in the Column Lifetime Study

Rebinding solution A was prepared by dissolving 1.56 mg of myoglobin in 15.6 mL of 100 mM potassium phosphate buffer, pH 5.81.

Rebinding solution B was prepared by dissolving 15 mg of β-D-Glucosidase in 15 mL of 10 mM citrate buffer, pH 5.2.

7.3.5 Preparation of Elution Solutions

For the elution aspect of this analysis, various elution solutions were required. These solutions and their compositions are listed in Table 7.4.

Solution Number	Elution Solution	Chemical Components
1	20% Aqueous Acetic Acid	Acetic Acid, dH_2O
2	90% Aqueous Methanol, 10% Acetic Acid	Methanol, Acetic Acid
3	10% Aqueous Methanol	Methanol, dH_2O
4	100 mM Potassium Phosphate buffer, pH 5.81	potassium phosphate dibasic, potassium phosphate monobasic, dH_2O
5	10 mM Citrate Buffer, pH 5.2	Citric Acid, Sodium Citrate, dH_2O

Table 7.4: Washes Used for Elution and Their Chemical Components during the Column Lifetime Study

In order to remove the bound protein from the respective polymers, the elution solutions were applied to their respective MIP in a series of different steps.

7.3.6 Recovery of M_1 and M_2

As the purpose of this experimental section was to investigate the re-usability of each of the MIPs prepared, each MIP was exposed to a series of rebinding and elution steps.

After the elution step for both MIPs, the MIPs had to be reconditioned to allow a further uptake of their template molecule in the subsequent further rebinding steps.

This experimental section was executed in triplicate. The results reported are the average results.

The reconditioning steps and the order of application for M_1 and M_2 are outlined in Table 7.5 and 7.6, respectively.

Reconditioning Phase	**Volume per wash (mL)**	**Number of Consecutive Washes**
10 mM Citrate Buffer, pH 5.2	2	3
10% Aqueous Methanol	2	1
10 mM Citrate Buffer, pH 5.2	2	1

Table 7.5: Reconditioning Steps for Myoglobin MIP (M_1) in Column Lifetime Study

Reconditioning Phase	**Volume per wash (mL)**	**Number Consecutive Washes**
100 mM Potassium Phosphate Buffer, pH 5.81	2 mL	3
10% Aqueous Methanol	2 mL	1
100 mM Potassium Phosphate Buffer, pH 5.81	2 mL	1

Table 7.6: Reconditioning Steps for β-D-Glucosidase MIP (M_2) in Column Lifetime Study

7.4 Results and Discussion

Each of the columns had their respective rebinding solutions applied to their columns followed by the subsequent elution washes. After reconditioning the MIPs, the process was repeated until there was a notable decrease on the each of the MIP samples ability to rebind the template molecule.

7.4.1 M_1 Regeneration

Rebinding analysis on M_1 was assessed by the MIPs capacity to rebind only the template molecule, myoglobin (loading solution A)

The chromatogram (Figure 7.1) shows that there are no peaks present at $Rt_{22.43}$ and $Rt_{23.82}$

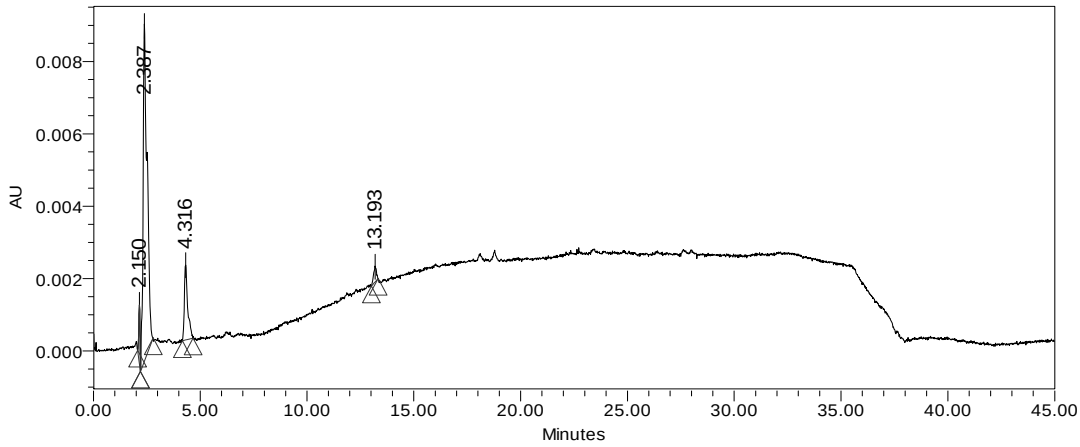

Figure 7.1: Chromatographic Profile for the Rebinding Solution Applied to Myoglobin MIP (M_1) in the Column Lifetime Study

As these are the retention times at which the heme group and the protein from the myoglobin would elute, it indicates that the template molecule has successfully rebound to M_1.

Once it was determined that the myoglobin had rebound to M_1, the MIP underwent a series of wash steps to remove the myoglobin. After each wash was applied, the eluent was analysed *via* HPLC to determine the concentration of myoglobin present. The wash stage was carried out until there was zero or negligible quantities of myoglobin present. The wash series applied to M_1 after each rebinding stage are detailed in Table 7.7.

Wash	Elution Solution
1	20% Aqueous Acetic Acid
2	90% Aqueous Methanol, 10% Acetic Acid
3	20% Aqueous Acetic Acid
4	90% Aqueous Methanol, 10% Acetic Acid
5	10% Aqueous Methanol

Table 7.7: Myoglobin MIP (M_1) Debinding Wash Protocol for Column Lifetime Study

Figure 7.2 shows that the wash series applied to M_1 in the sequence specified in Table 7.7 was successful in removing myoglobin from the MIP. The standard error for each point was less than ± 0.099 mg.

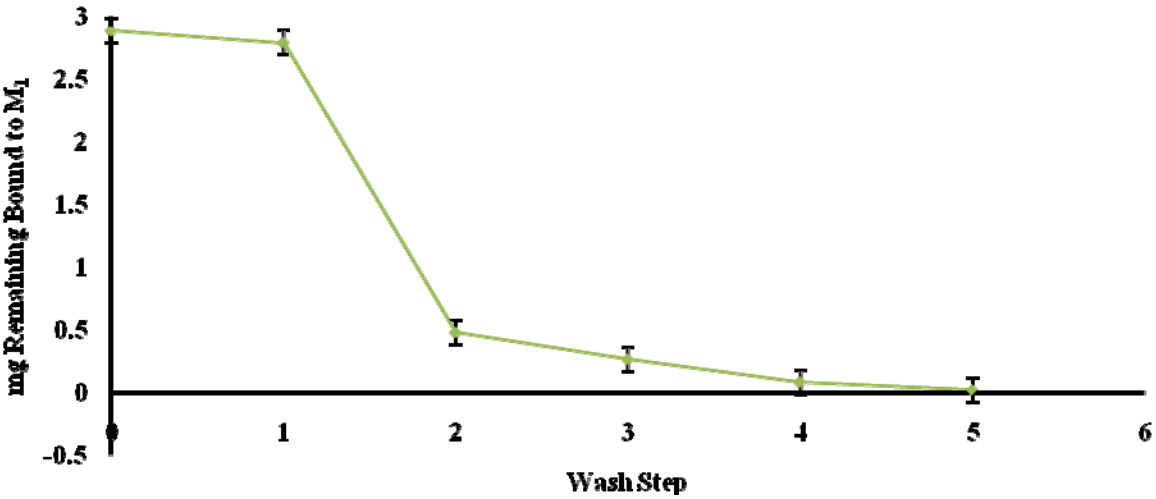

Figure 7.2: Graphical Representation of Removal of Myoglobin from Myoglobin MIP (M_1) during Wash Sequence 1 for the Column Lifetime Study

After the first rebinding and subsequent removal of myoglobin from M_1 was complete, M_1 was reconditioned as per the steps outlined in Table 7.5, thus ensuring that it was prepared for further rebinding and debinding steps.

M_1 underwent three rebinding, debinding and reconditioning steps, the results of which are shown in Table 7.8. Figure 7.3 shows the removal of myoglobin after each wash was applied following the initial rebinding step.

Regeneration Stage	Average Myoglobin Bound (mg)	Average Myoglobin Remaining (mg)	Average % Removed	Standard Error (mg of Myoglobin)
1	2.818	0.019	99.33%	± 0.011
2	2.887	0.007	99.76%	±0.019
3	2.868	0.472	83.54%	±0.026

Table 7.8: Myoglobin MIP (M_1) Regeneration Results for Column Lifetime Study

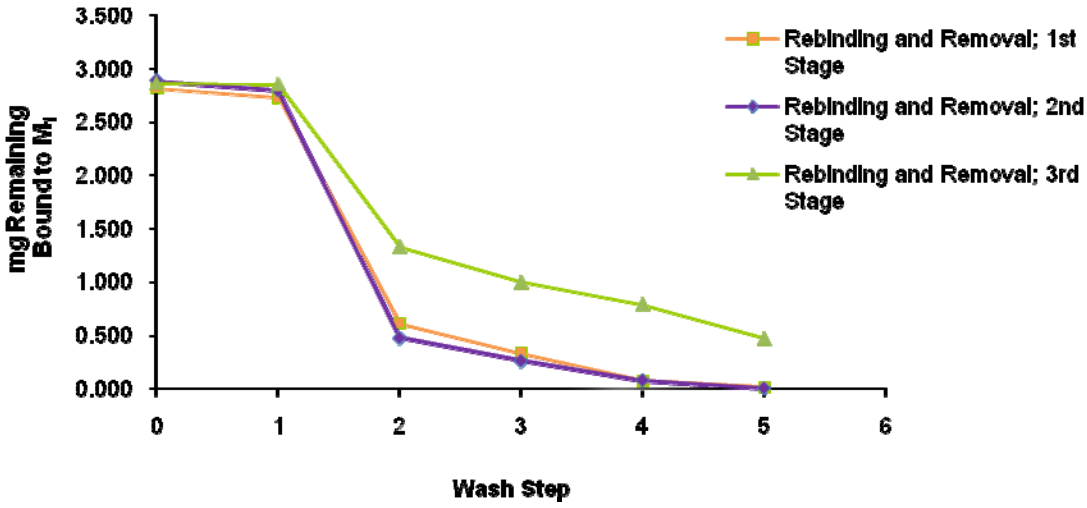

Figure 7.3: Removal of Myoglobin from Myoglobin MIP (M_1) during Each Wash Phase for Column Lifetime Study

The results shown in Figure 7.3 and Table 7.8 indicate that the wash sequence selected was adequate to remove the myoglobin from M_1 after it had been rebound twice removing 99.33% and 99.76% respectively. The MIP was capable of rebinding a third time, with an uptake of 92%, however, the wash sequence failed to remove myoglobin at the high levels that it had on the previous two washes, with only 83.54% being recovered. As the levels of myoglobin removed were significantly

lower than the two previous washes, a decision was taken to not regenerate M_1 further.

Figure 7.4 depicts the mg bound and removed from M_1. Cycle three demonstrates the MIPs failure to release similar quantities of myoglobin as it had on the first two washes.

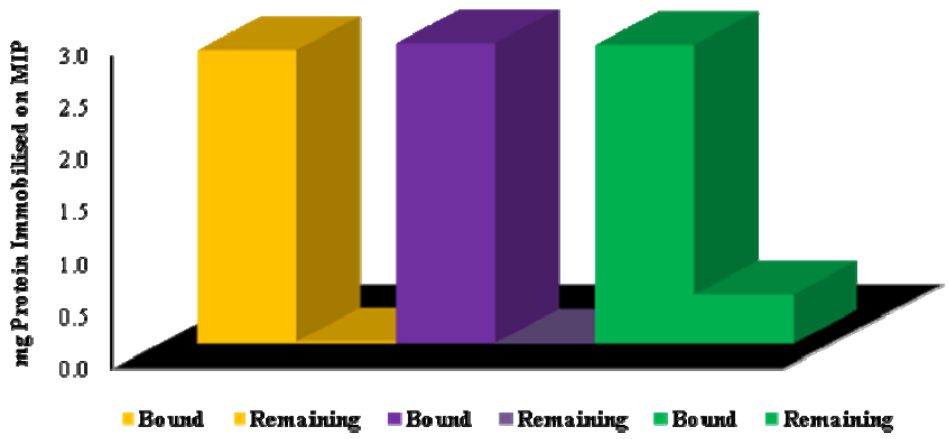

Figure 7.4: Regeneration of Myoglobin MIP (M_1) 1st –→3rd Stage for Column Lifetime Study

7.4.2 M_2 Regeneration

Rebinding analysis on M_2 was assessed by the MIP's capacity to rebind only the template molecule, β-D-Glucosidase (loading solution B). As in Chapter 6, the activity of the eluent was assessed to determine whether or not the β-D-Glucosidase had bound. By assessing if there was an activity in the eluent, the concentration of β-D-Glucosidase bound could be calculated. Figure 7.5 shows the % of enzyme bound to M_2 versus the theoretical maximum. The experiment was conducted three times, and the average result reported. This is also indicated in Table 7.9.

Sample	Average Absorbance	Average Number of Moles of 4-Nitrophenol in Eluent (nanomoles)	Average % Enzyme Bound to Sample	Standard Error (Absorbance Units)
M_2	0.2582	0.543	94.94%	± 0.0006

Table 7.9: % of β-D-Glucosidase bound to β-D-Glucosidase MIP (M_2) for Column Lifetime Study

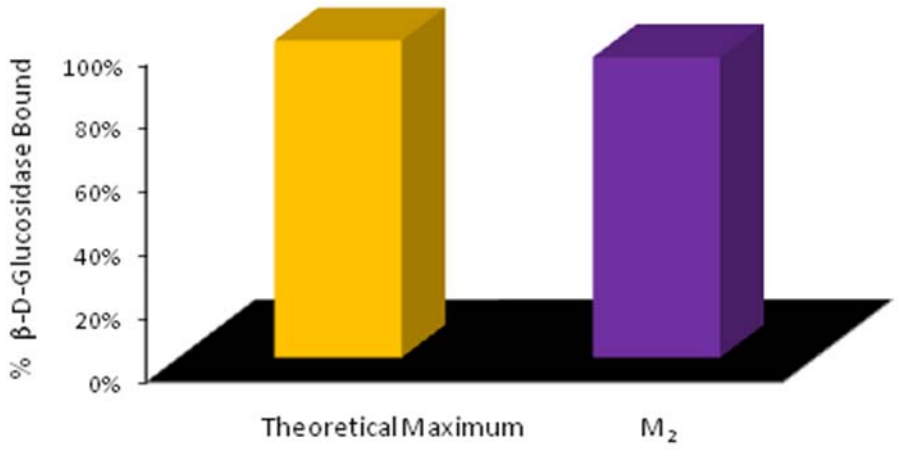

Figure 7.5: % of β-D-Glucosidase bound to β-D-Glucosidase MIP (M_2) for Column Lifetime Study

Once it was determined that the β-D-Glucosidase had rebound to M_2, the MIP underwent a series of wash steps to remove the β-D-Glucosidase. After each wash was applied, β-D-Glucosidase on the MIP was assayed in order to establish if any active β-D-Glucosidase was present. This in turn allowed the quantity, and subsequently the concentration of β-D-Glucosidase removed during each wash from M_2 to be determined. The wash stage was carried out until there was zero or minimal quantities of β-D-Glucosidase present. The wash series applied to M_2 after each rebinding stage are detailed in Table 7.10. This experiment was conducted three times and the average result reported.

Wash	Elution Solution
1	20% Aqueous Acetic Acid
2	90% Aqueous Methanol, 10% Acetic Acid
3	20% Aqueous Acetic Acid
4	90% Aqueous Methanol, 10% Acetic Acid
5	10% Aqueous Methanol

Table 7.10: β-D-Glucosidase MIP (M_2) Debinding Wash Protocol for Column Lifetime Study

Figure 7.6 shows that the wash series applied to M_2 in the sequence specified in Table 7.10 was adequate in removing β-D-Glucosidase from the MIP. The standard error for each point was less than ± 0.076 mg

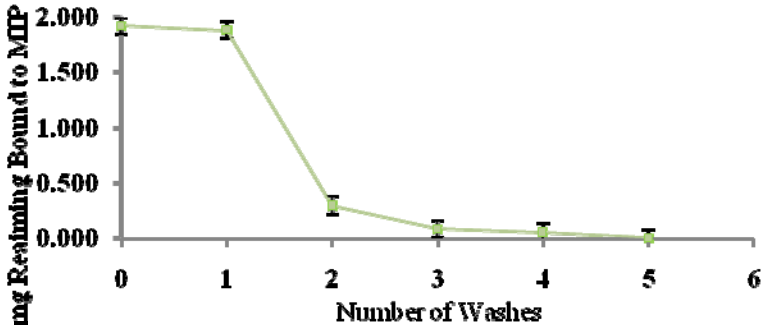

Figure 7.6: Removal of β-D-Glucosidase from β-D-Glucosidase MIP (M_2) during Wash Sequence 1 for Column Lifetime Study

After the first rebinding step and subsequent removal of β-D-Glucosidase, M_2 was reconditioned as per the steps outlined in Table 7.5 ensuring that it was prepared for further rebinding and debinding steps.

M_2 underwent three rebinding, debinding and reconditioning steps, the average results of which are presented in Table 7.11. Figure 7.7 shows the removal of β-D-Glucosidase after each wash was applied following the initial rebinding step.

Regeneration Stage	Average β-D-Glucosidase Bound (mg)	Average β-D-Glucosidase Remaining (mg)	Average % Removed	Standard Error (mg)
1	1.895	0.001	99.99	± 0.0104
2	1.922	0.010	99.50	± 0.0094
3	1.911	0.098	94.86	± 0.0098

Table 7.11: β-D-Glucosidase MIP (M_2) Regeneration Results for Column Lifetime Study

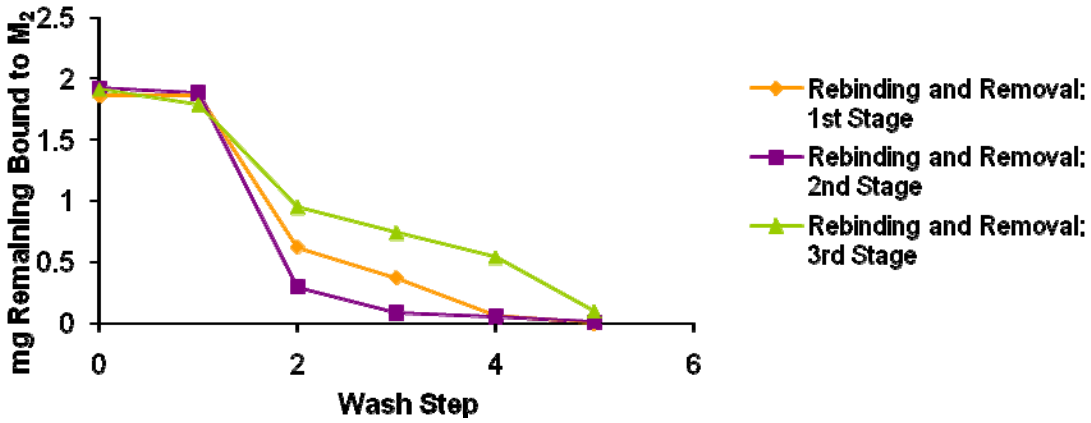

Figure 7.7: Removal of β-D-Glucosidase from β-D-Glucosidase MIP (M_2) During Each Wash Phase for Column Lifetime Study

The results shown in Figure 7.7 and Table 7.11 indicate that the wash sequence selected was adequate to remove the β-D-Glucosidase from M_2 after it had been rebound. The results also demonstrate that there is a gradual decline in the efficiency of elution of β-D-Glucosidase. After the initial rebinding stage, the first wash sequence was successful in removing 99.93% of the β-D-Glucosidase, however, the second and third wash sequence only managed to remove 99.50% and 94.86% respectively. The third wash sequence removed considerably less than the first wash sequence. As the levels of β-D-Glucosidase removed were significantly lower than the two previous washes, a decision was taken to not regenerate M_2 further.

The measured activity of the β-D-Glucosidase which remained on the MIP was indicative of the β-D-Glucosidase not being fully removed. Ten times more β-D-Glucosidase was left on the MIP after the third regeneration stage than that of the first two regeneration steps. A similar result was also seen with the myoglobin MIP. This demonstrates that the integrity of the MIPs as resin, using the current wash procedure, is questionable and comes with certain limitations. The impact of large sample loads i.e., loading close to the binding capacity and the cleaning in place for the MIP samples have a direct effect on the performance of the MIPs and the column lifetime.[4] The quantities of MIP used in the rebinding experiments are low (150 mg), however, the rebinding was carried out very close to capacity (> 73% for both) and could have an impact on the column lifetime.

Figure 7.8 depicts the mg bound and removed from M_2, indicating clearly the decrease in the wash sequence's ability to remove all of the β-D-Glucosidase.

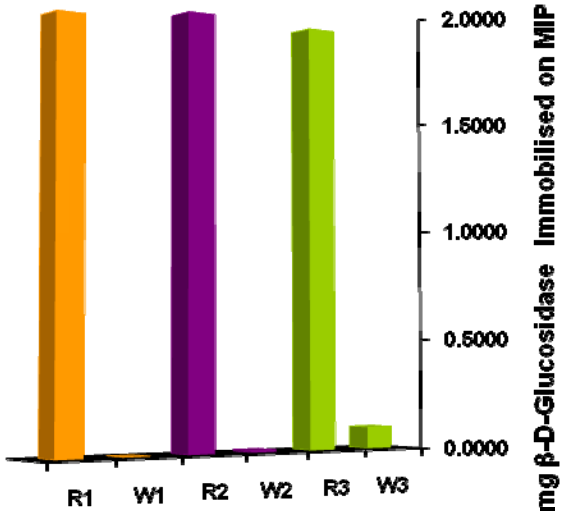

Figure 7.8: Regeneration of β-D-Glucosidase MIP (M_2); 1st ⟶3rd Stage for Column Lifetime Study

7.5 Conclusions

7.5.1 M_1 Regeneration

The results show that the wash stages used on M_1, after myoglobin had been rebound were sufficient to remove both the protein and heme from the MIP. After the 1st and second regeneration stage in excess of 99 % of the myoglobin was removed. The third regeneration cycle indicated the performance of the MIP was affected. The removal of myoglobin slightly decreased; only 83.54% of the bound myoglobin was removed, thus by using the regeneration method outlined in Table 7, the MIP was capable of being regenerated three times.

The recommendation for the utilisation of the MIP is no more than twice. Although the MIP demonstrated the ability rebind myoglobin three times, the ability to remove the protein and heme group declined somewhat. Initially, after an uptake of 90.18%, the 1st wash sequence removed 99.98% of this. The 3rd regeneration cycle saw an uptake of 91.77%, however only 83.54% of this was removed.

7.5.2 M_2 Regeneration

The results for M_2 were even better than those reported for M_1. The MIP demonstrated an excellent capability for regeneration, managing to be regenerated three times, with the β-D-Glucosidase wash sequences overall performance only decreasing from 99.93% to 94.86% after three regeneration cycles.

The recommended usage for M_2 would be a maximum of three times. This is attributed to the results which show a slight decline in β-D-Glucosidase release after the third regeneration step.

Each of the MIP samples was regenerated using the optimum buffers for both the myoglobin and β-D-Glucosidase. Cleaning of the column was out with the scope of this investigation, however may allow the lifetime of the MIPs to be extended.[5]

7.6 References

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8.0 Stability of MIP-Bound Enzymes

8.1 Introduction

To date, this investigation has demonstrated that both of the MIPs under detailed investigation (M_1 and M_2) have the capability to rebind their respective templates, and that when subjected to a suitable wash sequence the rebound molecule can subsequently be eluted from the polymer and the MIP regenerated for further use.

The next step of the investigation would be to investigate the overall stability of the rebound template when subjected to different conditions, versus the stability of the enzyme in solution. The MIP selected for this part of the investigation was M_2. The reasoning behind this decision can be attributed to the fact that the template molecule is β-D-Glucosidase, which allows the activity, and hence the stability, of the molecule to be investigated in a facile manner. The parameters selected for investigation were the temperature conditions at which the MIP was stored after the β-D-Glucosidase had been rebound to M_2 and the length of time for which it was stored under these different conditions.

8.2 Subjecting M_2 to Differing Conditions

As M_2 is an enzyme-imprinted polymer, and the activity enzymes are affected by the parameters selected for investigation, it was hypothesised that the enzyme may loose activity and become unstable when subjected to the said parameters. However, when the enzyme is immobilised on the MIP, the MIP may increase the thermal stability of the β-D-Glucosidase, allowing the enzyme to retain its enzymatic activity at elevated temperatures and for extended periods of time.

8.2.1 Temperature Effects

Each enzyme has an optimum temperature range at which its catalytic activity is highest[1], thus allowing the enzyme to participate in the chemical reactions for which it is known, i.e., in the case of β-D-Glucosidase, the reaction catalysed is the hydrolysis of glucosides,[2, 3] as explained in Chapter 6.

Temperature change can have two effects on enzymes; by increasing the temperature beyond the enzyme's optimal temperature, the enzyme will denature, hence losing its three dimensional shape, and subsequently its activity. The impact that reducing the temperature on enzymes has includes the decrease of molecular motion, which reduces the enzymatic activity. When the molecular motion is decreased, collisions between the enzyme and its substrate also decrease, resulting in a reduction of activity. An enzyme's molecular motion is decreased when the temperature is lowered.[4]

8.2.2 Enzyme Lifetime Study

As has been explained previously, the activity of an enzyme relies wholly on the structure and conformation of the enzyme. When β-D-Glucosidase binds to M_2, it is assumed that the β-D-Glucosidase has retained its structure and conformation so that it can fit into the β-D-Glucosidase shaped pockets within the MIP; thus, it can be hypothesised that the MIP will aid the β-D-Glucosidase in the retention of its activity levels. The question is however, for how long and under what conditions.

β-D-Glucosidase is stored, when not in use, at 2-8 °C. This is to ensure that it remains active whilst not in use. The recommended shelf live of β-D-Glucosidase is one year, at 2-8 °C.[5] After this period, the enzyme is deemed unsuitable for use.

When β-D-Glucosidase is not stored under these conditions, the activity will decrease. This can be seen by means of an activity assay; the activity of an enzyme will be greater if the assay is conducted immediately after removing the β-D-

Glucosidase from 2-8 °C storage as opposed to being conducted after a set period of time i.e., 60 minutes at 25 °C.

The purpose of this lifetime study was to compare the lifetime effects *in addition* to the effect of temperature on the enzyme.

8.2.3 The Storage Conditions

In order to provide an accurate study of temperature and lifetime effects, three comparisons were completed. These were β-D-Glucosidase rebound onto M_2, β-D-Glucosidase rebound onto N_2 (non-imprinted polymer, Chapter 5) and the same enzyme in solution, where the solution used throughout was the buffer in which β-D-Glucosidase was found to be optimally active.

The conditions and experimental details are outlined in Tables 8.1, 8.2, 8.3 and 8.4.

Experiment Number	**Sample**	**Time Period (Hours)**	**Temperature (°C)**
A1	Enzyme in Solution	168	4
B1	M_2	168	4
C1	N_2	168	4
A2	Enzyme in Solution	168	21
B2	M_2	168	21
C2	N_2	168	21
A3	Enzyme in Solution	168	60
B3	M_2	168	60
C3	N_2	168	60

Table 8.1: Activity and Stability Experimental Details at t = 168 Hours to Assess the Stability of MIP Bound Enzymes

Experiment Number	Sample	Time Period (Hours)	Temperature (°C)
A4	Enzyme in Solution	336	4
B4	M_2	336	4
C4	N_2	336	4
A5	Enzyme in Solution	336	21
B5	M_2	336	21
C5	N_2	336	21
A6	Enzyme in Solution	336	60
B6	M_2	336	60
C6	N_2	336	60

Table 8.2: Activity and Stability Experimental Details at t = 336 Hours Assess the Stability of MIP Bound Enzymes

Experiment Number	Sample	Time Period (Hours)	Temperature (°C)
A7	Enzyme in Solution	504	4
B7	M_2	504	4
C7	N_2	504	4
A8	Enzyme in Solution	504	21
B8	M_2	504	21
C8	N_2	504	21
A9	Enzyme in Solution	504	60
B9	M_2	504	60
C9	N_2	504	60

Table 8.3: Activity and Stability Experimental Details at t = 504 Hours Assess the Stability of MIP Bound Enzymes

Experiment Number	Sample	Time Period (Hours)	Temperature (°C)
A10	Enzyme in Solution	672	4
B10	M_2	672	4
C10	N_2	672	4
A11	Enzyme in Solution	672	21
B11	M_2	672	21
C11	N_2	672	21
A12	Enzyme in Solution	672	60
B12	M_2	672	60
C12	N_2	672	60

Table 8.4: Activity and Stability Experimental Details at t = 672 Hours Assess the Stability of MIP Bound Enzymes

8.3 Experimental Procedures

8.3.1 Chemicals

MIP M_2

NIP M_2

Sodium Citrate ($Na_3C_6H_5O_7$)

Citric Acid ($C_6H_8O_7$)

Potassium Phosphate Dibasic (K_2HPO_4)

Potassium Phosphate Monobasic (KH_2PO_4)

Sodium Carbonate (Na_2CO_3)

Deionised Water (dH_2O)

Molecular Sieves - 3Å

Bovine Serum Albumin (BSA)

β-D-Glucosidase from almonds

p-Nitrophenyl β-D-glucopyranoside (PNPG)

Methanol (CH_3OH)

The sodium citrate, citric acid, potassium phosphate dibasic, potassium phosphate monobasic, and sodium carbonate, were all purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). All of the solvents purchased were of HPLC grade and ≥ 95% pure. The acetonitrile was dried prior to use over activated molecular sieves (3Å).

Bovine Serum Albumin, β-D-Glucosidase from almonds and *p*-nitrophenyl β-D-glucopyranoside were also purchased from Sigma Aldrich. The Bovine Serum Albumin was ≥ 92% pure and the *p*-nitrophenyl; β-D-glucopyranoside was ≥ 98% pure. The β-D-Glucosidase was in the form of a lyophilised powder at ≥6 units/mg.

8.3.2 Enzymatic Assay of β-D-Glucosidase

As in Chapter 6, an enzymatic assay of β-D-Glucosidase had to be carried out prior to any other experiments. The reagents required for the assay were prepared immediately prior to the activity assay being carried out. This ensured that that assays were carried out immediately and that the results obtained were accurate and precise.

The activity assay was carried out at 37 ºC in an incubator. The incubator was heated to 37 ºC prior to use.

To two Eppendorf tubes, E_1 and E_2 in this example, 1 mL of 10 mM citrate buffer, pH 5.2 and 0.5 mL of the substrate solution was added.

The citrate buffer was prepared in the same manner as described in Chapter 3, Section 3.4.5 and the substrate solution was prepared in same manner as described in Chapter 6, Section 6.2.3

To E_1, 0.5 mL of the enzyme solution was then added. No enzyme was added to E_2, thus allowing it to be used in order to pre-calibrate the UV/vis spectrophotometer prior to the enzyme activity being measured.

Once again, the enzyme was prepared at a concentration of 0.0006 U/mL. This was achieved by preparing an initial solution of enzyme at a concentration of 1 mg/mL; 10 mg of β-D-Glucosidase was dissolved in 10 mL of ice-cold 0.05 M Tris-HCl buffer. The Tris-HCl buffer was prepared in the same manner as described in Chapter 6, Section 6.2.3.

0.0079 mL of the enzyme solution was then added to 9.9921 mL (measured by weight) of a 0.2% BSA solution (Chapter 6, Section 6.2.3).

Eppendorf' E_1 and E_2 were then both assayed at 37 ºC, in the incubator for 15 minutes. After this time, 0.02 mL of the 0.02 M Na_2CO_3 solution (10.6 g of Na_2CO_3 in 500 mL of dH_2O) was added.

After the 0.02 mL of the 0.02 M Na_2CO_3 solution had been added, 0.5 mL of the enzyme solution was added to the "blank" Eppendorf.

The solutions contained in Eppendorfs E_1 and E_2 were then transferred to individual cuvettes and the absorbance was read at 400 nm by means of a UV/vis spectrophotometer. The cuvette which contained E_2 was used to pre-calibrate the spectrophotometer, prior to the absorbance of E_1 being measured.

The absorbance reading was obtained and recorded. The reading was then superimposed onto the 4-nitrophenol calibration curve, Chapter 6, Section 6.2.2, and the number of moles of 4-nitrophenol released by β-D-Glucosidase after 15 minutes was determined (Figure 8.1).

8.3.3 Preparation of Enzyme Samples

A total of 36 0.5 mL β-D-Glucosidase samples were required for analysis. All of the samples were prepared using the same method.

A stock solution of β-D-Glucosidase was prepared in ice-cold 0.05 M Tris-HCl buffer. The final β-D-Glucosidase standard solution was prepared by taking an aliquot of the stock solution and diluting it to the required concentration with 0.2% BSA solution.

The quantities required for the stock solution are outlined in Table 8.5

Sample	**Weight of β-D-Glucosidase (mg)**	**Volume of Citrate Buffer (mL)**	**Final Concentration (mg/mL)**
Enzyme Stock Solution	10	10	1

Table 8.5: Enzyme in Solution Stock Solution Compositions for Rebinding in the Assessment of the Stability of MIP Bound Enzymes

The β-D-Glucosidase standard solution was prepared at a final concentration of 0.79 µg/mL by adding 0.0158 mL of the stock solution to 19.9842 mL to 0.2% BSA solution. Aliquots of β-D-Glucosidase standard solution (0.5 mL) were removed as required.

At this stage, 12 aliquots were removed and stored in centrifuge tubes for the enzyme in solution samples. These were labelled A1-A12, respectively.

8.3.4 Preparation of M_2 and N_2 Samples

For this section of analysis, a total of 12 MIP samples and 12 NIP samples were prepared.

M_2 and N_2 had to be packed into columns in order to be able to carry out the rebinding of the β-D-Glucosidase.
A suspension of MIP and NIP was prepared at a concentration of 1 mg/mL. This was achieved by adding 100 mL of 10 mM pH 5.2 citrate buffer to 100 mg of the MIP and NIP, respectively. Only 5 mg of MIP and NIP was required for each column and subsequently the rebinding stage of the experiment, meaning that only 5 mL of each polymer suspension was required for each column, thus a total of 60 mL of suspension of M_2 and N_2 was required.

The column used to house the M_2 and N_2 samples was Column 2 (Chapter 2, Section 2.5). To each individual column, 1 mL aliquots of M_2 and M_2 were removed frequently and placed in their respective column. The buffer was then removed from the samples by means of centrifugation at 5,000 rpm for 5 minutes. This procedure was carried out until the columns each housed 5 mg of MIP and NIP. This method was repeated until there were 12 M_2 columns and 12 N_2 columns.

Each column was labelled appropriately: B1 - B12 and C1 - C12 respectively.

8.3.5 Rebinding to M_2 and N_2

Prior to bringing the polymer into contact with the enzyme, the MIP and NIP housed in each column had to be wetted to allow access to the pores which had been generated during the imprinting stage. This was accomplished by adding 2 mL of 10% CH_3OH in 10 mM citrate buffer solution. Each of the columns were sealed and rotated by means of a rotor at 24 rpm for 15 minutes. The temperature was 37 ºC.

After 15 minutes, the liquids were separated from the solids by means of centrifugation at 5,000 rpm for 5 minutes.

2 mL of 10 mM citrate buffer was then added to the columns. Again the columns were sealed and rotated for 15 minutes at 24 rpm and 37 ºC, before the buffer was eluted using the same centrifuge procedure described above.

Each column was now prepared for rebinding.
To each of the columns 0.5 mL of the 0.79 μg/mL β-D-Glucosidase standard solution was added. The columns were then sealed and rotated for 15 minutes at 24 rpm and 37ºC. Once this had been completed the liquids were eluted by means of centrifugation at 5,000 rpm for 5 minutes.

The eluents from each column were collected and used in the determination of β-D-Glucosidase. This was accomplished by following the activity assay described in Section 8.3.2 of this chapter. The β-D-Glucosidase present in the eluents indicates the quantity of β-D-Glucosidase bound.

Following on from the rebinding stages, the MIP and NIP samples were exposed to a wash stage. The samples were washed with pH 5.2 10 mM citrate buffer.

As previously explained, the wash step is essential to ensure that any weakly bound enzyme present on the polymers is removed.

To each column, 1 mL of the pH 5.2 10 mM citrate buffer was added. The columns were then sealed and rotated for 15 minutes at 24 rpm and 37ºC. Once this had been completed, the liquids were eluted by means of centrifugation at 5,000 rpm for 5 minutes.

The eluents from each column were collected and used in the determination of β-D-Glucosidase.

8.3.6 Storage Conditions

The analysis of the temperature parameter depends on the utilisation of the correct storage conditions.

In order to carry out analysis at 4 °C, the samples were stored in the laboratory refrigerator. Each centrifuge tube and column was sealed at the top with Parafilm.

The samples which were being analysed at room temperature (21 °C) were stored on the laboratory workbench; with each sample also being sealed with Parafilm. The temperature was monitored by means of a thermometer.

The samples which were being stored at 60 °C were housed in an incubator, with the temperature pre-set to 60 °C. The temperature within the incubator was also monitored throughout the duration of the experiment. All samples housed within the incubator were sealed with Parafilm.

8.3.7 Enzyme Assay of Samples

After the set time period for each sample had elapsed, the activity of the β-D-Glucosidase for each respective sample had to be calculated.

To the respective enzyme in solution, MIP and NIP samples, 1 mL of 10 mM citrate buffer and 0.5 mL of the PNPG solution was added. The centrifuge tubes and columns were sealed and incubated at 37 ºC for 15 minutes whilst rotating continually at 24 rpm.

After 15 minutes they were all removed and centrifuged at 5000 rpm for 5 minutes before 0.02 mL of 0.02 M Na_2CO_3 was added. The absorbance at 400 nm was then recorded.

For each of the absorbance readings, three individual readings were taken and an average result recorded.

Using the results obtained and by application of the 4-nitrophenol calibration curve (Chapter 6, Section 6.2.2), the activity of the enzyme for each sample was determined.

8.4 Results and Discussion

The results of the activity levels of β-D-Glucosidase in this Chapter were established based on the data provided by the 4-nitrophenol calibration curve (Chapter 6, Section 6.3).

8.4.1 β-D-Glucosidase Activity at t = 0 Hours

In order to be able to compare directly the results of the β-D-Glucosidase activity assessed for each sample, the activity of β-D-Glucosidase was determined at t = 0 Hours.

The results are shown in Figure 8.1 and Table 8.6.

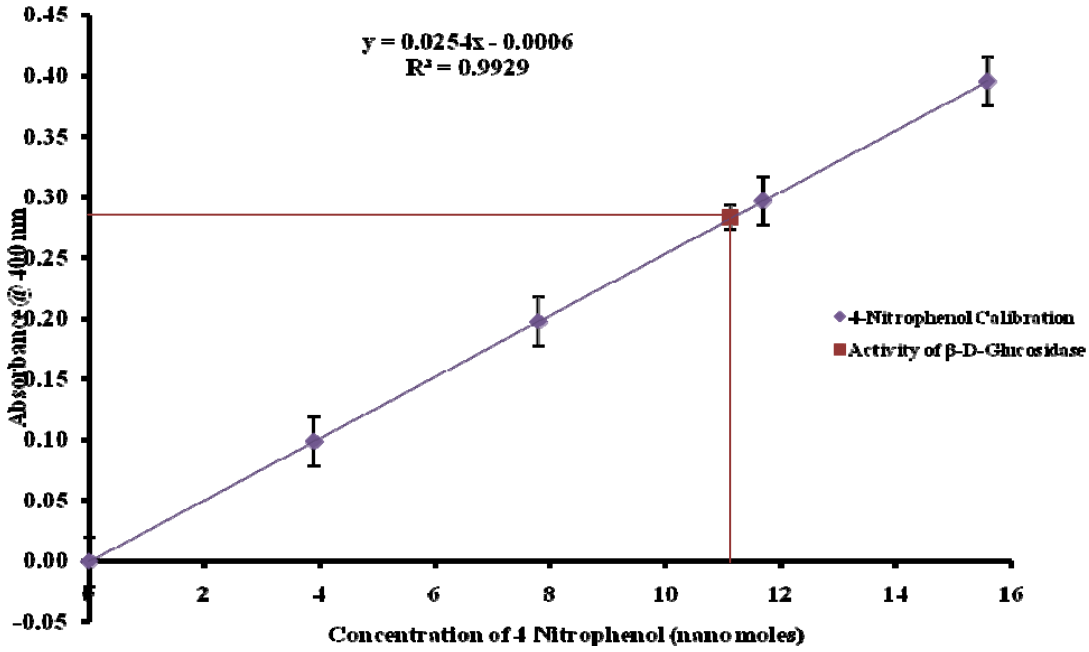

Figure 8.1: Activity of β-D-Glucosidase at t = 0 Hours

The concentration of the enzyme can be determined by placing the absorbance reading into the calibration curve. The value obtained is shown in Table 8.6.

Enzyme	Absorbance	Number of Moles of 4-Nitrophenol (nanomoles)	Standard Error (Absorbance Units)
β-D-Glucosidase	0.2833	11.13	± 0.001

Table 8.6: Enzyme Absorbance Results for the Determination of Activity of β-D-Glucosidase at t = 0 Hours

The concentration of enzyme measured is slightly lower than the value which was predicted; 11.85 nanomoles. This prediction was made on the assumption that the enzyme was 100% active when purchased.

As stated in Chapter 6, β-D-Glucosidase releases 1 μmol of 4-nitrophenol per 1 mg of enzyme, where 1 mg is equal to 7.55 *units.*

The concentration of β-D-Glucosidase used in this investigation was 0.006 *units.* 0.006 units is equivalent to 0.79 nmol. The quantity of 4-nitrophenol released in 15 minutes (the duration of the activity assay) should be 15 times this value; 11.85 nmol. As the above result determines that only 11.13 nmol of 4-nitrophenol has been released, then the enzyme used in this investigation was apparently 93.92% active.

As all further experiments use the same enzyme, the results have been recorded on the basis that by releasing 11.13 nmol, the enzyme is assumed to be 100% active. All comparisons and analyses have been completed using this result.

8.4.2 β-D-Glucosidase Bound to M_2 and N_2 Samples

The analysis carried out for the rebinding stage was based on the capacity of the MIPs and the NIPs to rebind the template molecule, β-D-Glucosidase.

The loading of enzyme applied to all 24 of the columns, which housed 5 mg of the MIP and NIP each, is detailed in Table 8.7.

Sample	Rebinding Solution	Volume (mL)
M_2	0.006 *u* β-D-Glucosidase	0.5
N_2	0.006 *u* β-D-Glucosidase	0.5

Table 8.7: Rebinding Solutions for M_2 and N_2 for the Assessment of the Stability of β-D-Glucosidase Rebound to the MIPs and NIPs

The load solution was removed from the MIP and NIP by means of centrifugation after the initial binding studies. The eluents were collected and were assayed immediately to determine the quantity of β-D-Glucosidase bound to the MIP and NIP samples.

Figure 8.2 shows the final percentage of β-D-Glucosidase bound to the 12 MIP samples. This value was deduced based on the number of moles of 4-NP present in the eluents of the rebinding solutions minus the number of moles of 4-NP present in the eluents from the wash stages. Figure 8.3 shows the results for the same experiment carried out on the twelve NIP samples. The results of the rebinding experiments are detailed in Table 8.8 and 8.9, respectively.

Sample	Number of Moles of 4-Nitrophenol in Rebinding Eluent (nanomoles)	Number of Moles of 4-Nitrophenol in Wash Eluent (nanomoles)	% Enzyme Bound to Polymer
B1	2.65	8.46	76.02
B2	2.87	8.28	74.40
B3	3.22	7.88	70.81
B4	0.99	10.16	91.29
B5	2.89	8.26	74.22
B6	3.04	8.11	72.87
B7	2.80	8.26	74.22
B8	3.37	7.78	69.91
B9	3.16	7.99	71.79
B10	0.41	10.74	96.50
B11	3.02	8.13	73.05
B12	1.59	9.56	85.90

Table 8.8: Rebinding Results for β-D-Glucosidase bound to M_2 (%)

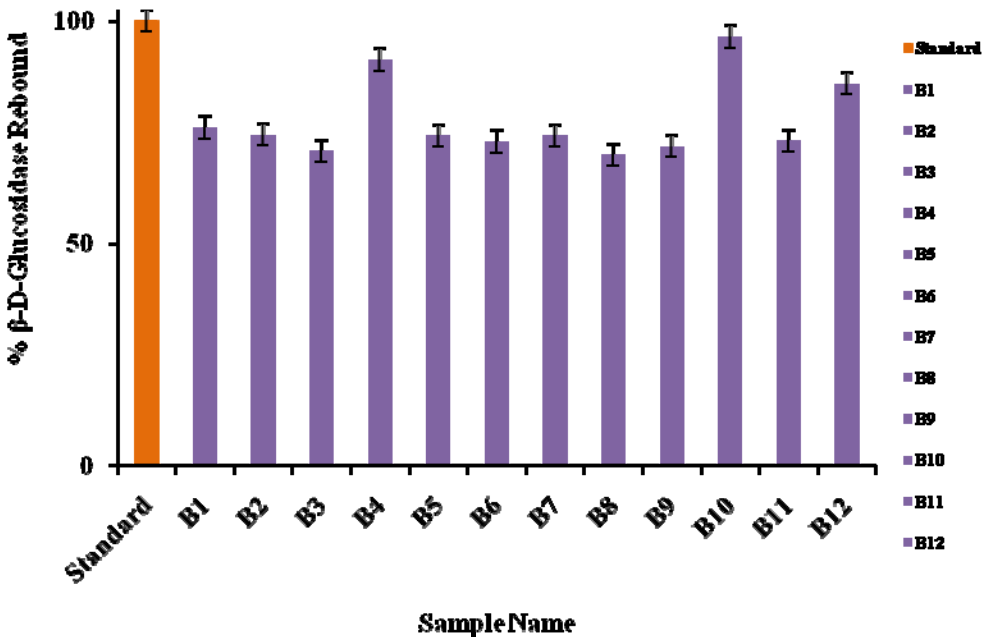

Figure 8.2: Rebinding Results for β-D-Glucosidase bound to M_2 (%)

The calculated average percentage of β-D-Glucosidase bound to the MIP samples was 77.58% ± 2.51%. Not only was this value lower than the theoretical maximum of 100%, but it was also lower than previous rebinding experiments carried out (Chapter 6). The integrity of the column packing could explain the reduced binding. If the column is packed too tightly, and the particles are not evenly distributed throughout the column, the binding potential can be significantly reduced, thus could attribute to the lower rebinding results obtained.[6] As the aim of this set of experiments was to establish the activity levels *after* the MIP samples had been subjected to different parameters, the percentage bound was not investigated further at this stage, and the experimental analysis of the temperature and time parameters were investigated.

Sample	Number of Moles of 4-Nitrophenol in Rebinding Eluent (nanomoles)	Number of Moles of 4-Nitrophenol in Wash Eluent (nanomoles)	% Enzyme Bound to Polymer
C1	1.56	9.54	85.72
C2	3.18	7.97	71.61
C3	3.10	8.01	71.97
C4	3.60	7.54	67.75
C5	4.37	6.79	61.01
C6	3.22	7.94	71.35
C7	2.22	8.94	80.33
C8	1.90	9.25	83.12
C9	3.45	7.70	69.19
C10	3.06	8.09	72.69
C11	3.11	8.04	72.24
C12	2.92	8.23	73.95

Table 8.9: Rebinding Results for β-D-Glucosidase bound to N_2 (%)

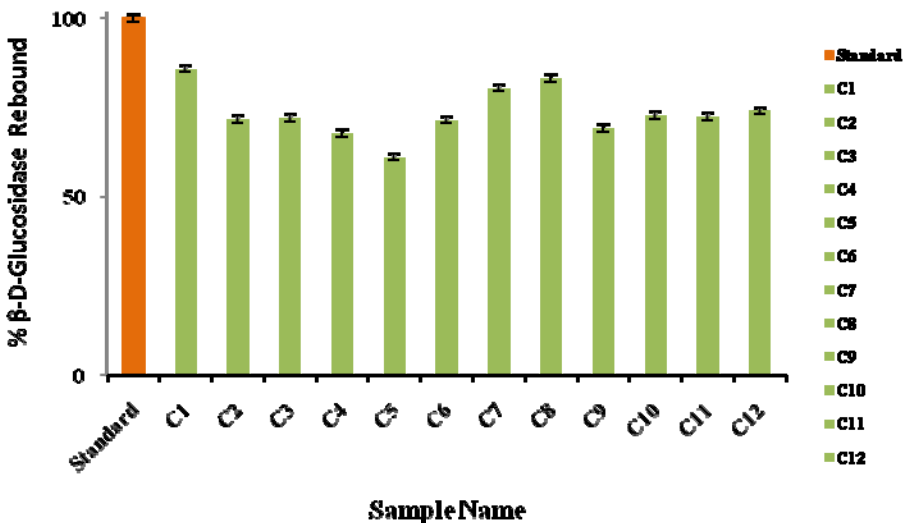

Figure 8.3: Rebinding Results for β-D-Glucosidase bound to N_2 (%)

The results displayed in Figure 8.3 show that the NIP does indeed rebind the β-D-Glucosidase. The overall average uptake of the β-D-Glucosidase in the NIP samples is 73.41% ± 1.96%. As with the MIP samples, there is a significant reduction in binding compared to the results reported in Chapter 6. The same reasoning can be applied with regards to the integrity of the column packing[6], and the distribution of the NIP particles.

As the NIP does not have β-D-Glucosidase present during the preparation of the NIP there are no β-D-Glucosidase binding sites generated for the β-D-Glucosidase to bind to, promoting non-selective binding. The NIP is prepared in the presence of a blank PCMC i.e., K_2SO_4 only. The presence of the salt (K_2SO_4) may also be a contributing factor towards the non-selective binding. Figure 8.3 demonstrates that 12 NIPs non-selectively bound β-D-Glucosidase. The average binding of β-D-Glucosidase to the NIP was 73.41% ± 1.96%. It is known that salts can impact protein-protein or protein-surface binding.[7, 8] Salts are commonly used in column chromatography to either promote binding onto the column, to reduce non-selective binding in addition to aiding the elution of the proteins from the column.[9, 10] One of the risks however is the potential for a salt to promote non-selective binding where the concentrations of the salt present is high enough.[11]

In the case of MISPE, as used throughout this investigation; the K_2SO_4 could be the contributing factor in the non-selective binding which has been demonstrated. In the absence of a protein template, the K_2SO_4 may have been imprinted. It has been reported previously that salt ions have an impact on template binding when used in MISPE.[12] The salt ions which were present followed the Hofmeister series (a classification of salts which impact the strength of the hydrophobic effect i.e., solubility of proteins. The salts in the series were also found to interact directly with proteins impacting binding) [13] and the level of non-selective binding increased. The binding was observed on both the MIP and the control polymer. As the increase in binding in the presence of the salt was seen on both polymers, the binding was reported as being non-selective in nature. The levels of inactive β-D-Glucosidase which did bind to the NIP sustain the theory that the binding is non-selective. The large quantities which bound to the NIP supports the theory that the K_2SO_4 present in the preparation of the NIP may be promoting the non-selective binding. [14]

There is also a marginal difference between the uptakes of β-D-Glucosidase between the M_2 samples and the N_2 samples; however, the area of interest for this experiment is the effects that the differing parameters have on the samples, thus no further investigation was carried out at this stage regarding the percentage rebound.

8.4.3 β-D-Glucosidase Activity at t = 168 Hours

After one week (t = 168 Hours) an activity assay was performed on samples A1-A3, B1-B3 and C1–C3. The results of this analysis are shown in Figure 8.4 and Table 8.10.

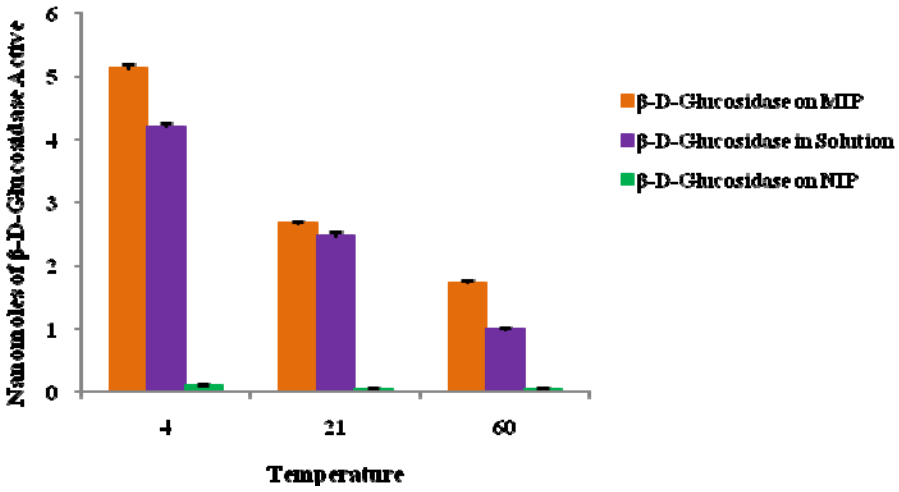

Figure 8.4: Comparison of Nanomoles of β-D-Glucosidase Active at t = 168 Hours Whilst on MIP, in Solution and on NIP

Figure 8.4 shows the average results collated for the MIP, NIP and enzyme in solution samples. Each assay was carried out in triplicate. The first area of interest is the results that were obtained for the activity levels of the MIP and NIP. Figure 8.4 shows that the concentration of 4-NP released whilst the enzyme is immobilized on M_2 (indicated in orange) is substantially higher than that of N_2, (indicated in green). This follows the trend that was detailed in Chapter 6 with respect to the selectivity of the MIP *versus* the NIP; the enzyme immobilised on the NIP does not retain its activity. This would indicate a reduced level of selectivity between the enzyme and the NIP. This would further support the conclusion that the binding which occurs is non-selective binding, as was discussed in Chapter 6.

The second key feature of the results is the enhanced activity levels of the β-D-Glucosidase that is immobilised on the MIP, in comparison with the enzyme in solution. The activity levels at room temperature are similar, however, when the β-D-Glucosidase is exposed to conditions outwith its ideal (i.e., temperature), the MIP demonstrates an apparent stabilising effect on the β-D-Glucosidase, and helps retain higher activity levels. The enzyme immobilised on the MIP released 5.13 and 1.74 nanomoles of 4-NP at 4 °C and 21 °C, whilst the enzyme in solution released 4.21 and 1.00 nanomoles under the same conditions.

Sample	Number of Moles of 4-Nitrophenol active at 4 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 21 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 60 °C (nanomoles)
Enzyme in Solution	4.21 ± 0.05	2.48 ± 0.06	1.00 ± 0.02
M_2	5.13 ± 0.06	2.68 ± 0.01	1.74 ± 0.02
N_2	0.10 ± 0.01	0.04 ± 0.01	0.04 ±0.01

Table 8.10: Number of Nano Moles Released by β-D-Glucosidase at Different Temperatures whilst n MIP, in Solution and on NIP at t = 168

Table 8.10 clearly demonstrates a trend between the numbers of nanomoles released by the samples and the temperature conditions at 168 hours. There is a visible decrease of nanomoles across the temperature range; 4 °C → 21 °C → 60 °C and each of the sample types (i.e., enzyme in solution, MIP and NIP).

8.4.4 β-D-Glucosidase Activity at t = 336 Hours

After 336 hours, the activity of the β-D-Glucosidase present for each of the three samples, at the differing temperatures was assessed in triplicate. The average results and standard error are shown in Figure 8.5 and Table 8.11.

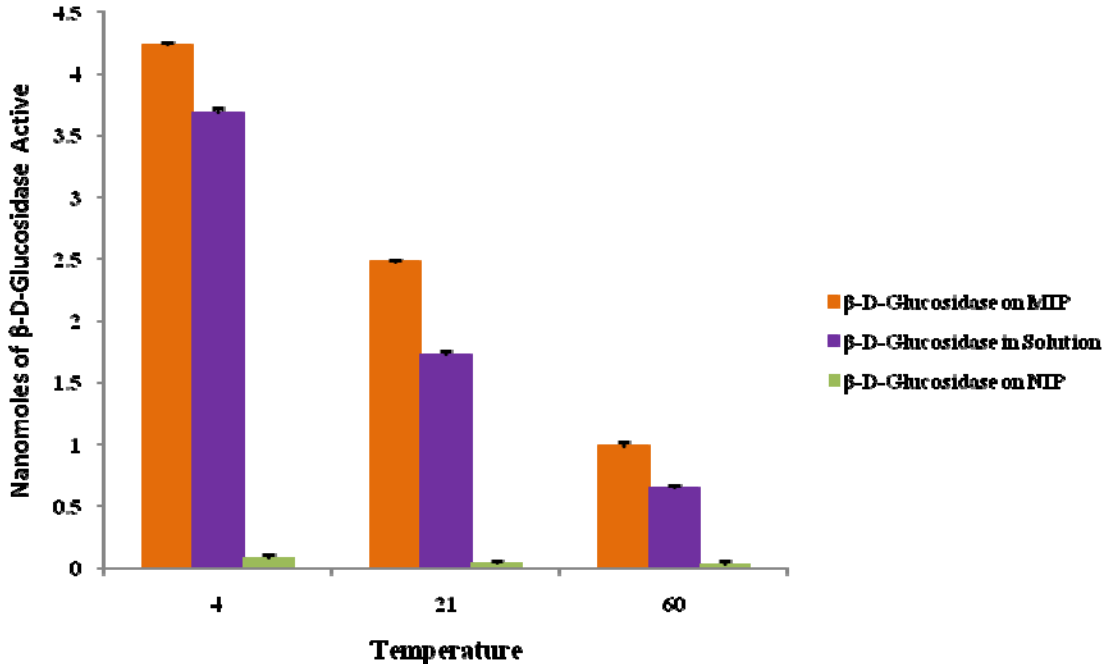

Figure 8.5: Comparison of Nanomoles of β-D-Glucosidase Active at t = 336 Hours

The enhanced activity of the β-D-Glucosidase immobilised on the MIP (indicated in orange) over the enzyme in solution samples (indicated in purple) and the NIP samples (indicated in green) is immediately apparent; thus, the samples have continued to follow the trend pertaining to the activity levels under the differing conditions, that was seen after t = 168 hours.

As the temperature increases, the number of active nanomoles decreases for all of the samples. The β-D-Glucosidase immobilised on the MIP has retained the largest percentage of its activity, followed by the β-D-Glucosidase in solution and the NIP. Once again, these results demonstrate the stabilising effect that the MIP confers to the β-D-Glucosidase, in addition to providing further evidence in support of the observation that the MIP is more selective towards the β-D-Glucosidase than the NIP.

Sample	Number of Moles of 4-Nitrophenol active at 4 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 21 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 60 °C (nanomoles)
Enzyme in Solution	3.69 ± 0.03	1.73 ± 0.02	0.65 ±0.01
M_2	4.24 ±0.01	2.48 ±0.02	0.99 ±0.03
N_2	0.08 ± 0.02	0.04 ± 0.01	0.03 ± 0.02

Table 8.11: Number of Nano Moles Released by β-D-Glucosidase at Different Temperatures whilst n MIP, in Solution and on NIP at t = 336 Hours

Table 8.11 presents the trend seen in Figure 8.5. The NIP does not seem to retain sufficient activity to allow the β-D-Glucosidase to be deemed enzymatically active. The β-D-Glucosidase present on the MIP and the enzyme in solution still retain enough activity for the β-D-Glucosidase to still be deemed enzymatically active, although the activity does decrease across the temperature range.

8.4.5 β-D-Glucosidase Activity at t = 504 Hours

Activity assays were carried out on the β-D-Glucosidase present in samples A7-A9, B7-B9 and C7-C9 at t = 504 Hours in triplicate. The average results and analysis are shown in Figure 8.6 and Table 8.12.

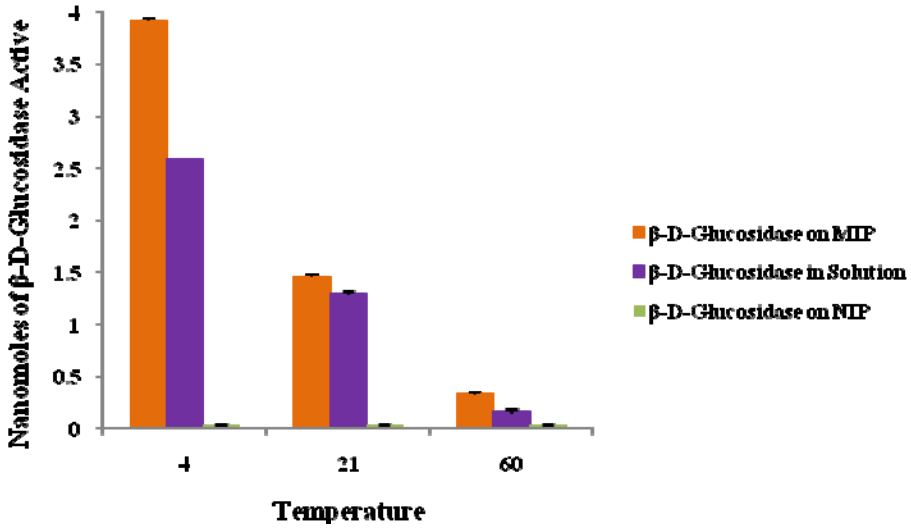

Figure 8.6: Comparison of Nanomoles of β-D-Glucosidase Active at t = 504 Hours

At 4 °C, the β-D-Glucosidase immobilised on the MIP (indicated in orange) has approximately one third (33.89%) more enzymatic activity than that of β-D-Glucosidase in solution (indicated in purple). This is a significant result as it confirms that by immobilising the β-D-Glucosidase on to the MIP, the activity levels can be sustained for a longer period of time, than if it were merely in solution.

The samples which have been stored at 21 °C do however tell a different story. There is no significant difference between the nanomoles of β-D-Glucosidase active (11.28%) with respect to the β-D-Glucosidase immobilised on the MIP and the β-D-Glucosidase in solution. A key observation at 21 °C is that the enzymatic activity is approximately half of the activity levels observed at 4 °C; the MIPs activity has reduced by 37.26% and the β-D-Glucosidase in solution by 50%.

At 60 °C, it is reasonable to say that the activity levels are at minimal levels, and thus the β-D-Glucosidase activity could not be determined.

Sample	Number of Moles of 4-Nitrophenol active at 4 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 21 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 60 °C (nanomoles)
Enzyme in Solution	2.59 ± 0.01	1.30 ±0.01	0.16 ± 0.02
M_2	3.92 ± 0.02	1.46 ±0.02	0.33 ± 0.01
N_2	0.03 ±0.01	0.03 ±0.01	0.03 ± 0.01

Table 8.12: Number of Nano Moles Released by β-D-Glucosidase at Different Temperatures whilst n MIP, in Solution and on NIP at t= 504 Hours

The table allows the points that were discussed above to be seen more clearly. Again the trend discussed at t = 168 Hours and t = 336 Hours has been followed at t = 504 hours.

The β-D-Glucosidase which has been immobilised on the NIP can not be deemed as having sufficient activity levels to be considered enzymatically active, as expected given the results at t = 336 hours.

8.4.6 β-D-Glucosidase Activity at t = 672 Hours

The β-D-Glucosidase present in samples A10-A2, B10-B12 and C10-C12 at t = 672 Hours were subject to an activity assay. This was performed three times. The average results and analysis are shown in Figure 8.7 and Table 8.13.

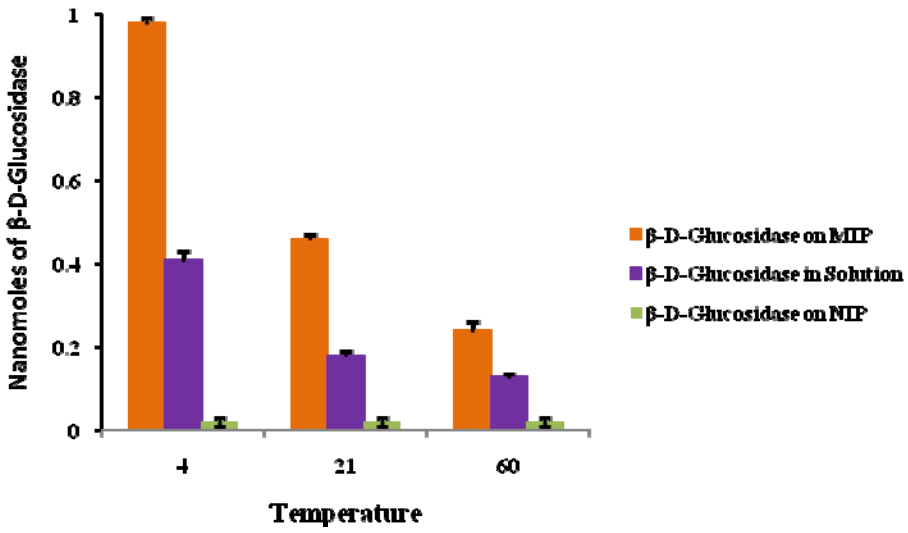

Figure 8.7: Comparison of Nanomoles of β-D-Glucosidase Active at t = 672 Hours Whilst on MIP, in Solution and on NIP

Again, the first result which is visually obvious from Figure 8.7 is the significant difference of β-D-Glucosidase activity present on the MIP (indicated in orange) across all three temperatures (4 °C, 21 °C and 60 °C). For each temperature, the MIP has retained approximately 50.00% more activity (58.39%, 60.40% and 47.03%) than the enzyme in solution samples (indicated in purple). This is in addition to retaining approximately 95.00% more activity than the β-D-Glucosidase which was immobilised on to the NIP samples (indicated in green) across the three temperatures (98.12%, 95.16% and 92.97%).

Sample	Number of Moles of 4-Nitrophenol active at 4 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 21 °C (nanomoles)	Number of Moles of 4-Nitrophenol active at 60 °C (nanomoles)
Enzyme in Solution	0.41 ± 0.02	0.18 ± 0.01	0.13 ± 0.01
M_2	0.98 ± 0.01	0.46 ± 0.01	0.24 ± 0.02
N_2	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

Table 8.13: Number of Nano Moles Released by β-D-Glucosidase at Different Temperatures whilst n MIP, in Solution and on NIP at t = 672 Hours

Another key result for t = 672 hours is the significant drop in β-D-Glucosidase activity across the MIP and β-D-Glucosidase in solution samples. At the last analysis stage (t = 336 Hours) the recorded activity ranged from 3.92 nanomoles to 0.16 nanomoles. The results for t = 504 hours show a dramatic reduction with the β-D-Glucosidase activity only spanning the range 0.98 nanomoles to 0.13 nanomoles. This key result indicates that although temperature clearly has an impact on the β-D-Glucosidase activity regardless of the sample, time also plays an important factor as it also reduces the overall β-D-Glucosidase activity.

8.4.7 Overview of Temperature Effect vs. Time on Activity Level

Figures 8.8, 8.9 and 8.10 show the trend in activity levels for the three samples (enzyme in solution, MIP and NIP) across the three temperature ranges vs. time.

Figure 8.9 shows the activity level at 4 °C across the 4 week time period.

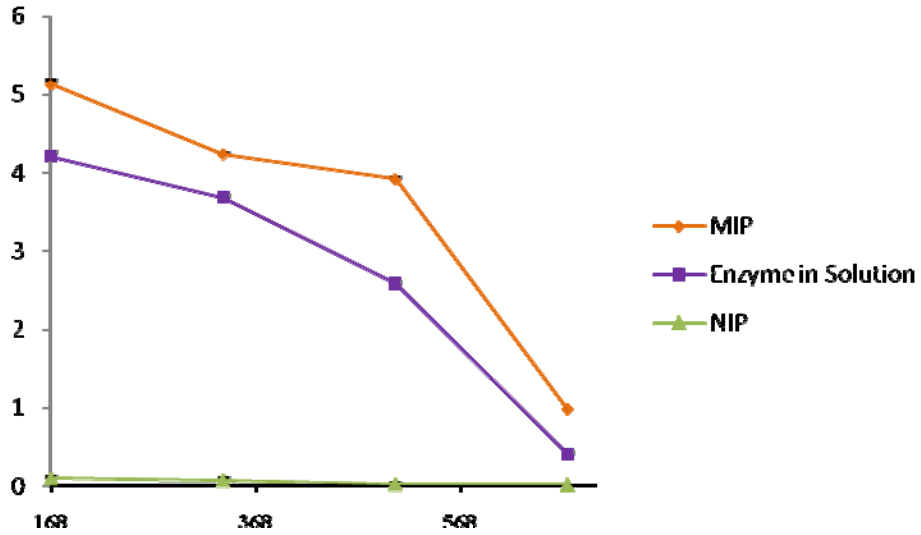

Figure 8.8: Activity Trend of β-D-Glucosidase at 4 °C Whilst Immobilised on MIP and NIP and for Enzyme in Solution

It can be seen in Figure 8.9 that the β-D-Glucosidase which is rebound to on to the MIP displays levels higher levels of activity over the β-D-Glucosidase in solution and β-D-Glucosidase on the NIP.

The activity levels of the β-D-Glucosidase rebound onto the MIP drop from 5.13 nanomoles to 0.98 nanomoles from t = 168 hours to t = 672 hours. It can be seen that the biggest decrease in activity occurs between 504 hours and 672 hours.
This is also the case with the enzyme in solution. Although the activity levels are lower than that of the β-D-Glucosidase rebound to the MIP (4.21 to 0.21 nanomoles), the trend of decreasing activity is the same, with the biggest drop in activity level occurring at t = 504 hours – t = 672 hours.

This is not the case of the β-D-Glucosidase rebound on the NIP. The initial activity at t = 168 hours is 0.10 nanomoles; this is lower than the activity levels recorded for the MIP and enzyme in solution samples after t = 672 hours. As the activity level of the β-D-Glucosidase is already close to being inactive, the decrease is minimal.

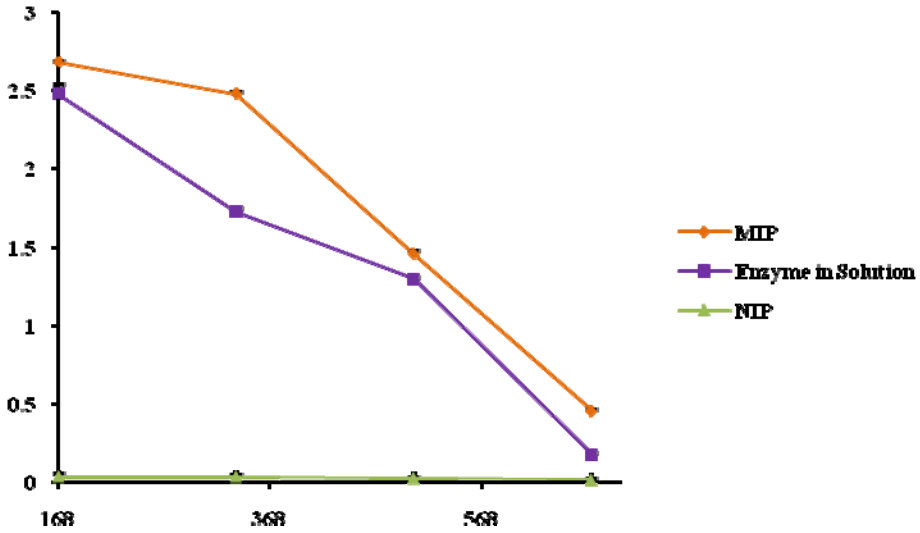

Figure 8.9: Activity Trend of β-D-Glucosidase at 21 °C Whilst Immobilised on MIP and NIP and for Enzyme in Solution

The trend displayed by the β-D-Glucosidase activity whilst rebound to the MIP and the β-D-Glucosidase in solution at 21 °C is similar to that seen at 4 °C. The activity levels decrease gradually with the larges decline in activity occurring at t = 504 hours – t = 672 hours. It is worth noting the initial activity levels however. The activity of β-D-Glucosidase rebound to the MIP is 2.68 nanomoles at t = 168 hours. This is just more than half of the activity level at t = 168 hours, 4 °C. This is also the case of the β-D-Glucosidase in solution. The activity at t = 168 hours, 21 °C is 2.48 nanomoles and 4.21 nanomoles at 4 °C.

Once again the activity levels of β-D-Glucosidase rebound onto the NIP are almost 0 from t = 168 hours (0.04 nanomoles), so the decrease across the time scale is minimal.

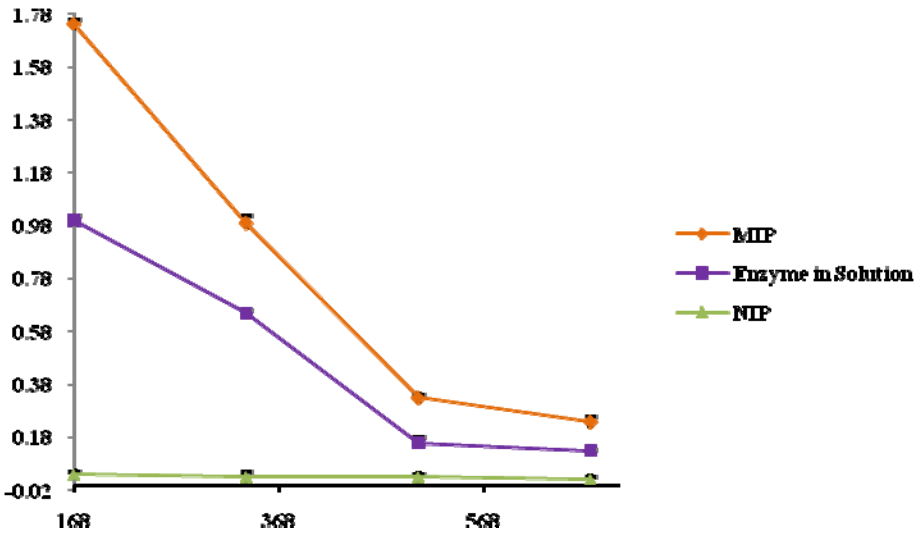

Figure 8.10: Activity Trend of β-D-Glucosidase at 60 °C Whilst Immobilised on MIP and NIP and for Enzyme in Solution

Figure 8.10 shows the activity level trend at 60 °C.

It is clear to see that the decrease in β-D-Glucosidase activity levels for all three samples follows a different trend at 60 °C that what was observed at 4 °C and 21 °C. With respect to the β-D-Glucosidase rebound on the MIP and the β-D-Glucosidase in solution, there is a rapid decline in the activity level from t = 168 hours to t = 672 hours. The initial activity levels are lower, 1.74 and 1.00 nanomoles respectively with the resultant recorded activity being 0.24 nanomoles for the β-D-Glucosidase rebound on the MIP and 0.13 for the β-D-Glucosidase in solution.

As with the 4 °C and 21 °C samples, the activity of the β-D-Glucosidase on the NIP is minimal from t = 168 hours (0.04 nanomoles), thus the decline in activity is minimal.

8.5 Conclusions

By altering the conditions that the β-D-Glucosidase is subjected to, whether it is rebound to the MIP, in solution or rebound to the NIP, the enzymatic activity is affected.

The results have shown that the MIP can play a significant role in the stabilisation of β-D-Glucosidase. It was demonstrated that over a four week time frame, the β-D-Glucosidase was more stable when rebound on the MIP compared with the β-D-Glucosidase in solution and the β-D-Glucosidase rebound to the NIP. The β-D-Glucosidase also showed an enhanced retention of activity when the storage temperature conditions were varied. This indicates that not only does temperature have an effect on activity, [15] but the MIP also has a stabilisation effect upon the β-D-Glucosidase.

The results show that once the β-D-Glucosidase has rebound or been immobilised on the MIP it reduces the denaturing effect (MIP versus β-D-Glucosidase in solution), and retains activity for approximately four weeks. This indicates that the MIP itself could play an important role in enzyme immobilisation; allowing the enzyme to be stabilised at different temperatures and for a prolonged time-frame, one of the most sought-after benefits when working with enzymes.[16, 17]

The data generated also confirms the assumption that the initial binding which occurred in the NIP can be attributed to non-selective binding, as discussed in Chapter 6.

Although only touched on, another key finding in this section of the investigation was the actual percentage of β-D-Glucosidase that initially rebound to the MIP samples. The values reported were lower than those obtained in previous Chapters of this investigation (77.58% ± 2.51% *vs*. 82% ± 0.0001). This could be attributed to the integrity of the column itself, i.e., the tightness of the column packing, the distribution of the MIP throughout the column and potentially the speed at which the rebinding occurred. These factors alone can dictate whether a column is fit for purpose within its role. [6] With more work dedicated to this area, the binding potential could be maximised.

8.6 References

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9.0 Broadening the Scope - Introducing a New Enzyme!

9.1 Introduction

Previous chapters have described a molecularly imprinted polymer's ability to utilise PCMCs as its template molecule, rebind the template molecule and, where applicable, the MIPs ability to retain the enzymatic activity once the template was rebound.

This chapter describes the transfer of the methods and procedures investigated in previous chapters. An alternative enzyme was utilised as the template, thus the activity and the stability of the enzyme once it had been rebound to the MIP was established. The manner of binding which occurred i.e., specific *vs.* non-specific with respect the template was also investigated. Direct comparisons were made between the binding to the MIP and binding to a non-imprinted control polymer (NIP).

9.1.2 Choice of Enzyme

For this area of the investigation, Acid Phosphatase from potato (systematic name phosphate-monoester phosphohydrolase [acid optimum], E.C. 3.1.3.2) was selected.

Phosphatases are enzymes which can be found in abundance.[1] They are widespread in nature, and can be obtained from various different animal and plant sources.[2] The source of the phosphatase generally determines whether the phosphatase is acidic or alkaline in form. Acid phosphatases are sourced from plant tissue, whereas alkaline phosphatases are sourced from animal tissue.[3] The isoenzyme of acid phosphatase being utilised for this aspect of the investigation has been obtained from plant tissue; potato. Due to the abundance of acid phosphatase, it is readily available for purchase from suppliers as a lyophilised powder.

Acid phosphatase is an enzyme which catalyses the hydrolysis of phosphate esters to give an alcohol and inorganic phosphate.[4] The reaction (Figure 9.1) takes place at an optimal pH of below 7.[5]

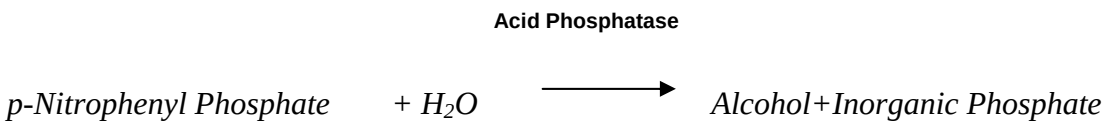

Figure 9.1: Representation of the Acid Phosphatase Catalyzed Hydrolysis of Phosphate Esters

As with the activity assay for β-D-Glucosidase, the ability to measure the specific activity of the enzyme can be carried out with relative ease. In addition to measuring the specific activity of the enzyme, the determination of % enzyme bound to the MIP during the initial rebinding experiments and the stability of the enzyme once it has been successfully rebound to the MIP can be determined. Again, as with the β-D-Glucosidase assay, this can be attributed to the fact that the product of the enzyme hydrolysis can be chosen to absorb light in the visible region of the electromagnetic spectrum, and with a λ_{max} of ~400 nm analysis *via* UV/Vis Spectroscopy can be realised easily.

9.1.3 Acid Phosphatase Activity

The instrumental technique which is pertinent to measure the activity of acid phosphatase is UV/vis spectrophotometry. By using UV/vis spectrophotometry both the total protein content and the activity of the enzyme can be determined.

The total protein content is determined based on the fact that all proteins absorb light in the UV region of the electromagnetic spectrum. The activity is determined by assessing the rate of product formation or substrate utilisation during the enzyme-catalysed reaction.[6]

The activity of the acid phosphatase is determined by assessing the quantity of p-nitrophenolate (Figure 9.2) released when the acid phosphatase is in the presence of a suitable substrate. [7]

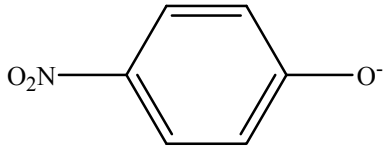

Figure 9.2: Structure of p-Nitrophenolate

The substrate, p-nitrophenyl Phosphate (PNPP) (Figure 9.3), is a non-specific, non-proteinaceous substrate which can be utilised to assay proteins, alkaline and acid phosphatases. The acid phosphatase enzyme catalyses the hydrolysis of PNPP to p-nitrophenol, which in turn is a chromogenic product (Figure 9.4).[8]

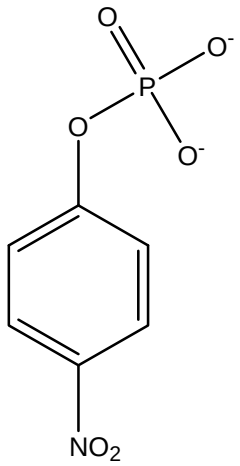

Figure 9.3: Structure of 4-Nitrophenyl Phosphate

The reaction is terminated by the addition of NaOH. The hydroxide reacts with the p-nitrophenol to produce p-nitrophenolate (the phenolic proton is removed). The p-nitrophenolate is coloured yellow thus it allows the absorbance to be detected in the visible region of the spectrum (400 nm) using UV/vis spectrophotometry and the quantity of p-nitrophenol to be determined.

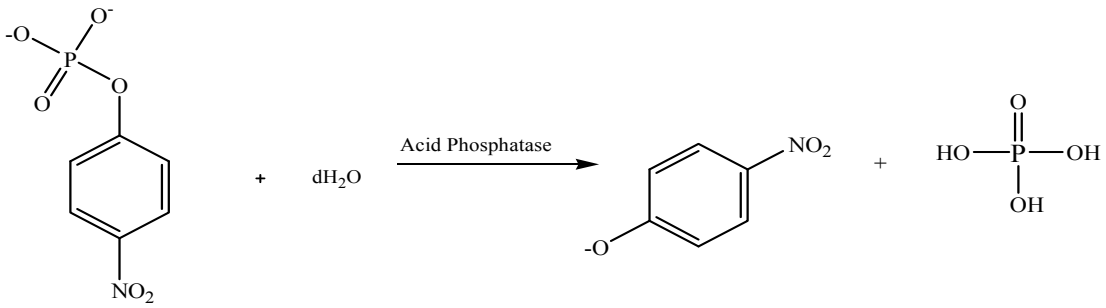

Figure 9.4: Illustrationof the Chemical Reaction: Enzymatic Hydrolysis of p-Nitrophenyl Phosphate by Acid Phosphatase[4]

From the information provided in Chapter 1, Section 1.5.4, pertaining to the substrate, the concentration of the substrate used was approximately 10,000 times more than the concentration of the enzyme used.

9.2 Experimental Procedures

9.2.1 Chemicals

MIP M_3

NIP N_3

Sodium Citrate ($Na_3C_6H_5O_7$)

Citric Acid ($C_6H_8O_7$)

Potassium Phosphate Dibasic (K_2HPO_4)

Potassium Phosphate Monobasic (KH_2PO_4)

Sodium Carbonate (Na_2CO_3)

Methanol (CH_3OH)

Deionised Water (dH_2O)

Acid Phosphatase

p-Nitrophenyl Phosphate disodium salt hexahydrate (PNPP)

Sodium Hydroxide, Anyhydrous (NaOH)

4-Nitrophenol (4-NP)

Molecular Sieves - 3Å

Acetonitrile (ACN)

The sodium citrate, citric acid, sodium carbonate, potassium phosphate dibasic, potassium phosphate monobasic and sodium carbonate were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England), as was the acetonitrile and methanol. The acetonitrile and methanol was HPLC grade and ≥ 95% pure. The acetonitrile was dried prior to use over activated molecular sieves (3Å).

Acid Phosphatase, *p*-nitrophenyl phosphate, sodium hydroxide, anhydrous and 4-nitrophenol were also purchased from Sigma Aldrich. The 4-nitrophenol was of spectrophotometric grade, and the *p*-nitrophenyl phosphate disodium salt hexahydrate was purchased in the form of a tablet. Each tablet contained 5 mg of substrate. The acid phosphatase was in the form of a lyophilised powder at 3 – 10 units/mg solid.

9.2.2 4-Nitrophenol Calibration Curve

To establish the activity of Acid Phosphatase, a calibration curve of 4-nitrophenol (4-NP) had to be established.

To obtain a calibration curve for 4-nitrophenol, solutions of various concentrations had to be prepared. Each solution then had its absorbance measured. This was conducted in triplicate and a calibration curve was plotted using the average absorbance readings obtained.

4-Nitrophenol (10.8 mg) was dissolved in 20 mL of ACN; 0.1 mL of this solution was then taken and added to 9.9 mL of 90 mM potassium phosphate buffer at pH 5.99, the optimum pH for acid phosphatase. This was used as the stock solution.

Various solution concentrations of 4-nitrophenol were prepared by taking aliquots of the stock solution and diluting with buffer. This is outlined in Table 9.1.

Volume of 4-Nitrophenol Stock Solution (mL)	**Volume of Buffer (mL)**	**Number of moles of 4-Nitrophenol (nanomoles)**
0	1	**0**
0.1	0.9	**3.9**
0.2	0.8	**7.8**
0.3	0.7	**11.7**
0.4	0.6	**15.6**
0.5	0.5	**19.5**
0.6	0.4	**23.4**
0.7	0.3	**27.3**
0.8	0.2	**31.2**
0.9	0.1	**35.1**
1	0	**39**

Table 9.1: 4-Nitrophenol Calibration Compositions and Final Concentration for Acid Phosphatase

Once all of the calibration standards were prepared, 0.02 mL of Na_2CO_3 solution (10.6 g of Na_2CO_3 in 500 mL of dH_2O) was added to adjust the ionisation state and subsequently the pH. The absorbance was then recorded by UV/vis spectrometry at a fixed wavelength: $\lambda = 400$ nm.

The readings obtained were used to plot the 4-NP calibration curve, Figure 9.5.

This procedure was similar to the calibration used for 4-NP in Chapter 6; however the potassium phosphate buffer has been changed.

9.2.3 Enzymatic Assay of Acid Phosphatase

The first stage of the assay for acid phosphatase was to prepare all of the reagents required for the assay. This ensured that that assays were carried out immediately and that the results obtained were accurate and precise.

The activity assay was carried out at 37 ºC in an incubator. This temperature was pre-set to ensure it was ready for use once all of the preparations were complete.

To two Eppendorf tubes, E_1 and E_2 in this example, 0.5 mL of 90 mM citrate buffer, pH 5.99, and 0.5 mL of the substrate solution was added.

The citrate buffer was prepared in the same manner as described in Chapter 3, Section 3.4.5.

A 15.2 mM solution of the substrate, p-nitrophenyl phosphate, was prepared by dissolving the substrate (20 mg) in 3.545 mL of dH_2O.

The buffer and the substrate were mixed together by means of inversion, before being left to equilibrate at 37 °C.

To E_1, 0.1 mL of the enzyme solution was then added. No enzyme was added to E_2, thus allowing it to be used in order to pre-calibrate the UV/vis spectrophotometer prior to the enzyme activity being measured.

The enzyme was prepared at a concentration of 0.0025 U/mL. This was achieved by preparing an initial solution of enzyme at a concentration of 0.0568 mg/mL; 1 mg of acid phosphatase was dissolved in 17.6 mL of ice-cold dH_2O. This enzyme solution was further diluted to give the final concentration of 0.0025 U/mL; 1 in 10 dilutions was carried out.

Eppendorfs E_1 and E_2 were then both assayed at 37 ºC, in the incubator for 10 minutes. After this time, 0.02 mL of 100 mM NaOH solution (4 g of NaOH in 1 L of dH_2O) was added. The role of the NaOH solution was to adjust the pH to an alkaline pH thus resulting in a change in the ionization state of the mixture. This then ensures that the vibrant yellow colour expected is released by the reaction; this does not happen at the enzyme's optimum pH. By adding the NaOH, it also ensures that the enzymatic reaction is terminated after exactly 10 minutes.

After the addition of the NaOH solution, 0.1 mL of the enzyme solution was added to the "blank" Eppendorf. Each of the Eppendorfs was mixed by inversion.

The solutions contained in Eppendorf's E_1 and E_2 were then transferred to individual cuvettes and the absorbance was read at 400 nm by means of UV/vis spectrophotometry. The cuvette which contained E_2 was used to pre-calibrate the spectrophotometer, prior to the absorbance of E_1 being measured.

This procedure was executed three times.

The absorbance reading for each execution was obtained and recorded. The average of these readings was then superimposed onto the 4-nitrophenol calibration curve, Section 9.2.2, and the average number of moles of 4-nitrophenol released by acid phosphatase after exactly 10 minutes was determined (Figure 9.5).

9.2.4 P_3: Activity Assay

In order to assess the final mass of acid phosphatase which was immobilised on to the surfaces of the micro-crystals (P_3, Chapter 3) an activity assay was performed, using the procedure detailed above on the PCMCs three times.

In order to gain an accurate determination of acid phosphatase, the PCMCs were diluted to a concentration, with buffer, at which the enzyme could be assayed; 0.0025

U/mL. An additional sample containing only micro-crystals was also diluted using the same volume of buffer as the P_3 sample.

The resultant solutions were then assayed using the procedure described in Section 9.2.3. This allowed the activity to be determined.

The average activities were then compared to a calibration of 100% enzyme activity (Figure 9.7).

9.2.5 Acid Phosphatase Enzyme on MIP and NIP: Activity Assay

Once the activity of the enzyme was established, the next stage was to determine the activity of the enzyme once it had been rebound to the MIP and NIP. This also provided an indirect means of establishing the mass of enzyme which was capable of binding to the MIP, in addition to determining if the enzyme demonstrated selectivity in rebinding to the MIP relative to the NIP. As above, the procedure was executed three times and all results reported are the average results.

The first stage of this procedure was to ensure that both the MIP and the NIP were prepared for rebinding.

A suspension of MIP and NIP was prepared at a concentration of 1 mg/mL. This was achieved by adding 100 mL of 100 mM phosphate buffer to 100 mg of the MIP and NIP respectively. Only 2 mg of MIP and NIP was required for the rebinding stage of the experiment, thus 2 mL of each polymer suspension was required in total. To achieve this, 0.5 mL aliquots were removed frequently and placed in Column 2 (as described in Chapter 2, Section 2.5). The buffer was then removed from the samples by means of centrifugation at 5000 rpm for 5 minutes. This procedure was carried out until the filters each housed 2 mg of MIP and NIP.

Prior to bringing the polymer into contact with the enzyme, the MIP and NIP had to be wetted to allow access to the pores which had been generated during the

imprinting stage. This was accomplished by adding 0.5 mL of 10% CH_3OH in 100 mM potassium phosphate buffer solution. Each of the columns were sealed and rotated by means of a rotor at 24 rpm for 15 minutes. The temperature was 37 ºC.

After 15 minutes, the liquids were separated from the solids by means of centrifugation at 5,000 rpm for 5 minutes.

0.5 mL of 100 mm potassium phosphate buffer was then added to the columns. Again the columns were sealed and rotated for 15 minutes at 24 rpm and 37 ºC, before the buffer was eluted using the same centrifuge procedure described above.

The MIP and the NIP were now ready for the rebinding experiments and also for the determination of the activity of the enzyme which was bound to each polymer.
Four samples were required, two containing the MIP and two containing the NIP. The MIP and NIP samples used were M_3 and N_3 respectively (Chapter 4, Section 4.3.4).

The sample names utilised in this section of the investigation are M_{3R} and N_{3R} in addition to M_{3B} and N_{3B}.

Sample	Rebinding Solution	Volume (mL)
M_{3R}	0.0025 *u* Acid Phosphatase	0.1
M_{3B}	100 mM potassium phosphate buffer, pH 5.99	0.1
N_{3R}	0.0025 *u* Acid Phosphatase	0.1
N_{3B}	100 mM potassium phosphate buffer, pH 5.99	0.1

Table 9.2: Rebinding Details for the Determination of the Activity of Acid Phosphatase Rebound on the Acid Phosphatase MIP and NIP

The rebinding solutions used are shown in Table 9.2.

Each of the columns were sealed and rotated for 15 minutes at 24 rpm and37ºC. Once this had been completed, the liquids were eluted by means of centrifugation at 5,000 rpm for 5 minutes.

At the same time, 0.5 mL of 90 mM citrate buffer, pH 5.99, and 0.5 mL of the PNPP solution was added to separate Eppendorf tubes. The buffer and the substrate were mixed together by means of inversion, before being left to equilibrate to 37 °C.

The eluents were then transferred to the Eppendorfs (2 mL) which housed the substrate and buffer mixtures.

The activity assay was carried out for ten minutes whilst the Eppendorfs were incubated at 37 ºC. After this time, 0.02 mL of 100 mM NaOH solution was added to stop the enzymatic reaction.

The absorbance at 400 nm was then measured to determine the amount of nitrophenolate released.

During this period, the MIP and NIP housed in the columns were washed for 10 minutes with 0.5 mL of 90 mM citrate buffer at 37 ºC whilst being rotated at 24 rpm. This was in order to remove any remaining unbound enzyme from the MIP and NIP samples. Once the 10 minutes had passed, the buffer was eluted by means of centrifugation at 5,000 rpm for 5 minutes. Again, the eluents were collected and transferred to new Eppendorf tubes which had a capacity of 2 mL.

The eluents were then assayed using the procedure described previously and the absorbance readings recorded.

Now that the rebinding stage was complete, the final stage was to assess the activity of the enzyme remaining bound to M_{3R} and N_{3R}. M_{3B} and N_{3B} were also subjected to this assay.
The activity assays for these samples were carried out in a fashion similar to that described above, however 0.5 mL of 10 mM citrate buffer and 0.5 mL of the PNPP solution was added directly to the columns, before being sealed and incubated at 37 ºC for 10 minutes whilst rotating continually 24 rpm.

After 10 minutes, all of the tubes were centrifuged at 5,000 rpm for 5 minutes before 0.02 mL of 100 mM NaOH was added. The absorbance at 400 nm was then recorded.

For each of the absorbance readings, three individual readings were taken and an average result recorded.

Using the results obtained, and by application of the 4-nitrophenol calibration curve, the average percentage of enzyme rebound, the selectivity of the MIP over the NIP, and the activity of the enzyme bound to the polymers could be deduced.

9.3 Results & Discussion

In order to establish the activity and the concentration of Acid Phosphatase for this part of the investigation, a calibration curve for 4-Nitrophenol was constructed (Figure 9.5). The standard error for each point was less than ± 0.02 absorbance units.

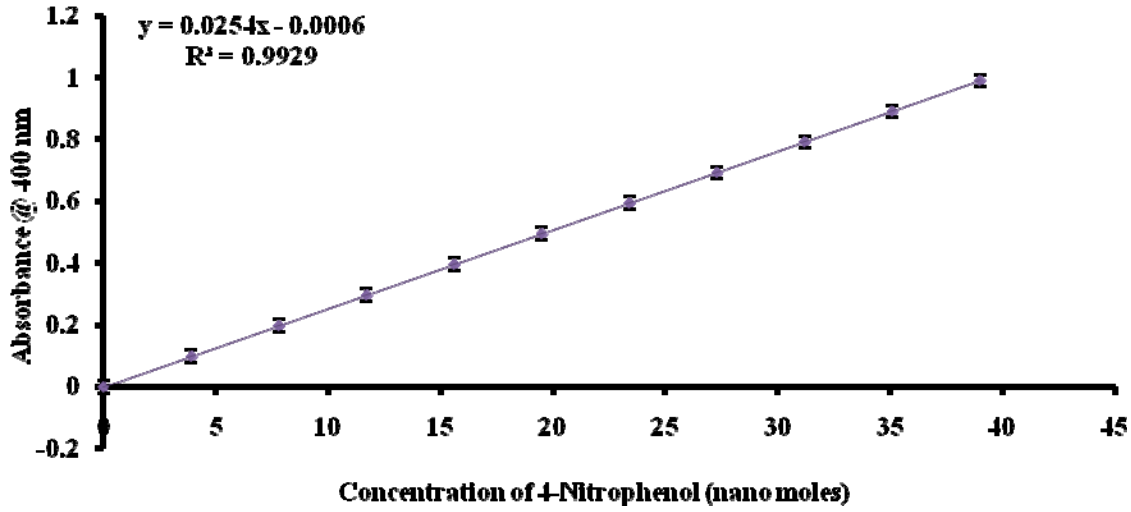

Figure 9.5: 4-Nitrophenol Calibration for Acid Phosphatase

9.3.1 APL of P_3

The activity of the Acid Phosphatase was established prior to the assessment of the percentage of Acid Phosphatase bound to the surface of the micro-crystals. This was carried out so that the results of the activity assay of the micro-crystal bound enzyme could be compared to a standard of the enzyme. The results of this assay were then superimposed onto the 4-nitrophenol calibration (Figure 9.6)

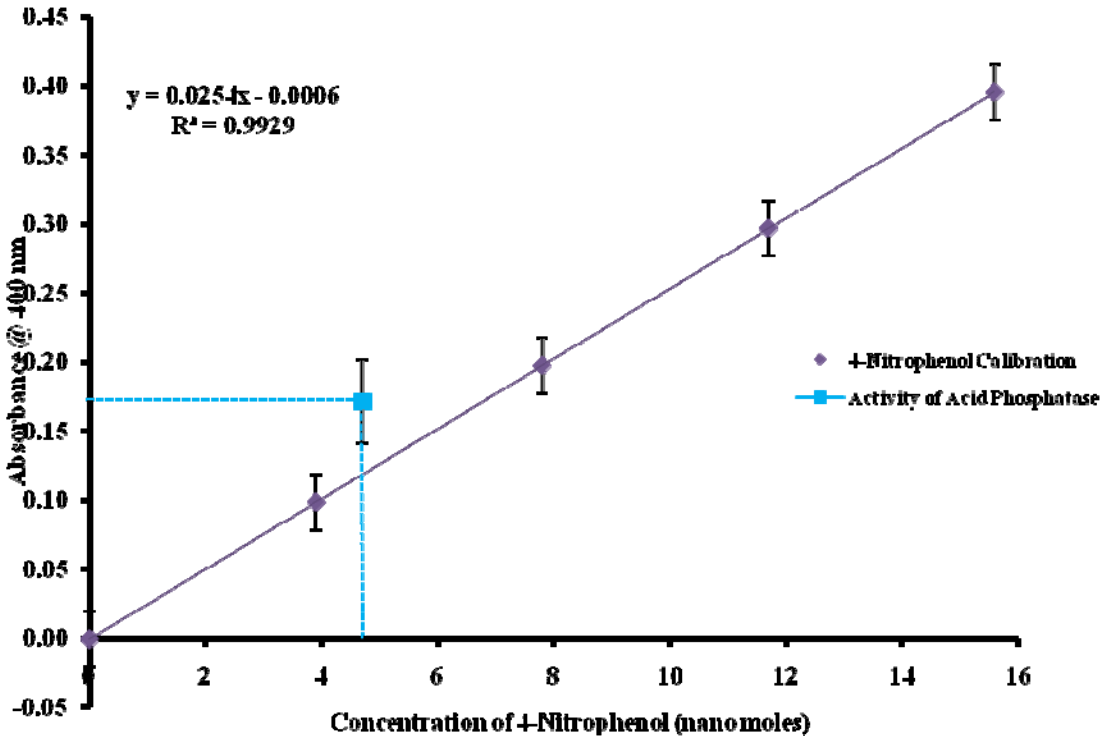

Figure 9.6: Activity of Acid Phosphatase Superimposed on the 4-Nitrophenol Calibration Curve

The concentration of the enzyme can be determined by placing the average absorbance reading into the calibration curve. The value obtained is shown in Table 9.3.

Enzyme	**Absorbance**	**Number of Moles of 4-Nitrophenol (nanomoles)**	**Standard Error (Absorbance Units)**
Acid Phosphatase	0.1713	4.7	± 0.03

Table 9.3: Acid Phosphatase Enzyme Absorbance Results and Activity of Acid Phosphatase

The measured concentration of active enzyme is lower than the theoretical prediction with respect to the concentration of enzyme assessed, i.e., the purchased enzyme is not 100% active.

Theoretically, acid phosphatase should release 1 μmol of 4-nitrophenol per 1 mg of enzyme, where 1 mg is equal to 4.4 *units.*

As stated, the concentration of acid phosphatase used in this investigation was 0.0025 *units.*

0.0025 units is equal to 0.5682 nmol, which is the quantity of 4-nitrophenol released per minute.

The activity assay was carried out for 10 minutes thus the quantity of 4-nitrophenol should be 10 times this; 5.68 nmol. As the above result determines that only 4.70 nmol of 4-nitrophenol has been released, then the enzyme used in this investigation was apparently 82.7% active.

As all further experiments use the same enzyme, the results have been recorded on the basis that by releasing 4.70 nmol, the enzyme is 100% active.

The average absorbance reading and subsequent enzyme loading on the PCMCs (acid phosphatase and blanks) are shown in Table 9.4 and Figure 9.7.

Enzyme	**Absorbance**	**Number of Moles of 4-Nitrophenol (nanomoles)**	**% Enzyme Bound to Micro-Crystal**	**Standard Error (% Enzyme Bound)**
Acid Phosphatase	0.1658	4.43	94.30	± 0.02
Blank	0.0001	0.00	0.00	± 0

Table 9.4: Absorbance of Acid Phosphatase PCMCs and % Enzyme Bound to Micro-Crystal with the Associated Activity

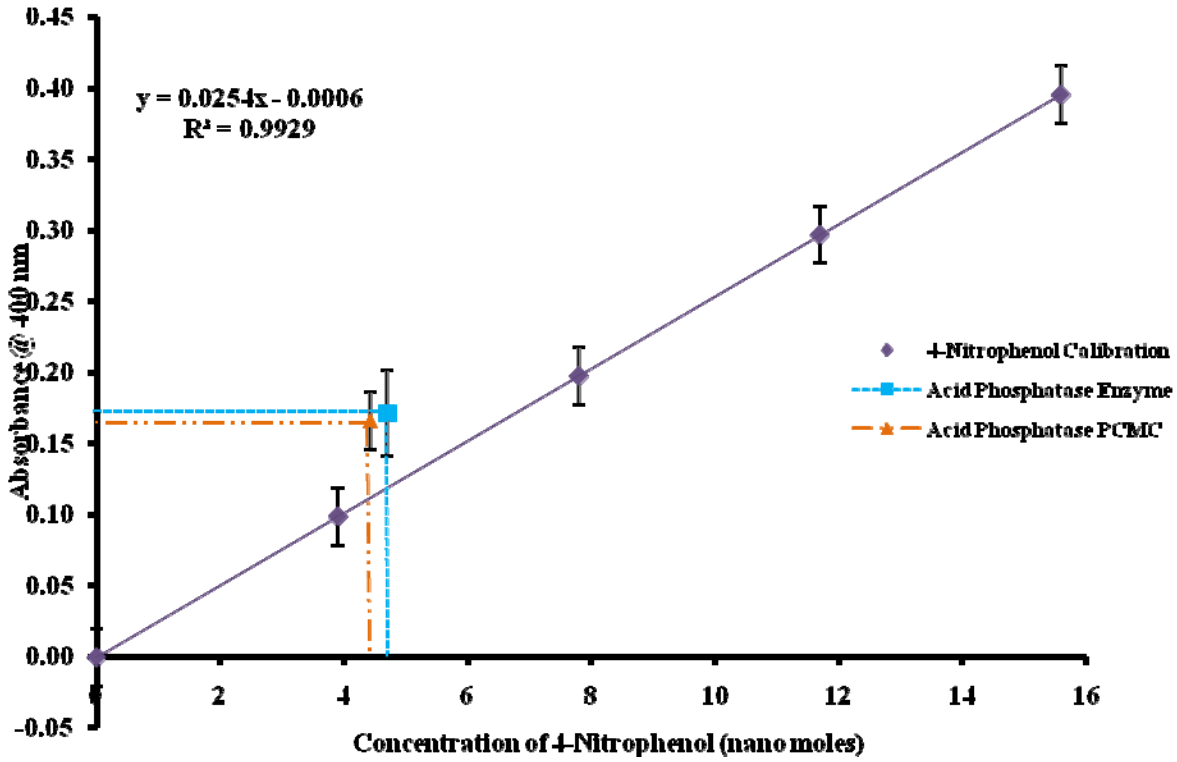

Figure 9.7: Determination of the Quantity of Acid Phosphatase Enzyme Bound to Micro-crystal by Activity Level *vs.* Acid Phosphatase Enzyme Activity

The orange triangle in the calibration curve shows the PCMC compared to the pure enzyme indicated in blue. It can be seen that the difference in activity is minimal; in fact, 94.3% of the enzyme has bound to the surface of the micro-crystals and remained active. This is a good result as it demonstrates that the enzyme retains its activity even after it has been immobilised. The blank PCMC has no enzyme bound to the surface, which is an expected result.

9.3.2 MIP and NIP Rebinding Results

The analysis carried out for the rebinding stage was based solely on the MIPs capacity to rebind only the molecule which was used as the template molecule, Acid Phosphatase.

The uptake of Acid Phosphatase on the NIP was assessed in order to probe the possibility of non-selective binding.

The loading of enzyme applied to each of the columns, which housed 2 mg of the MIP and NIP each, is detailed in Table 9.5.

Sample	**Rebinding Solution**	**Volume (mL)**
M_{3R}	0.0025 *u* Acid Phosphatase	0.1
M_{3B}	100 mM potassium phosphate buffer, pH 5.99	0.1
N_{3R}	0.0025 *u* Acid Phosphatase	0.1
N_{3B}	100 mM potassium phosphate buffer, pH 5.99	0.1

Table 9.5: Rebinding Solutions for Acid Phosphatase MIP and NIP (M_3 and N_3)

The load solution was removed from the MIP and NIP by means of centrifugation after the initial binding studies, and the eluents collected and subsequently assayed. Figure 9.8 shows the percentage of Acid Phosphatase bound to both of the MIP samples. This value was deduced based on the number of moles of 4-NP present in the eluents of the rebinding solutions. Figure 9.9 and shows the results for the same experiment carried out on N_{3R} and N_{3B}. Table 9.7 shows the results obtained for all four samples.

Sample	Absorbance	Number of Moles of 4-Nitrophenol in Eluent (nano moles)	% Enzyme Bound to Sample	Standard Error (% Enzyme Bound)
M_{3R}	0.1017	1.3380	72	± 1.5
M_{3B}	0.0000	0.0000	0	± 0.0
N_{3R}	0.1203	2.2380	52	± 2.0
N_{3B}	0.000	0.0000	0	±0.0

Table 9.6: % of Acid Phosphatase bound to MIP and NIP Samples (M_3 and N_3) Based of Activity Levels

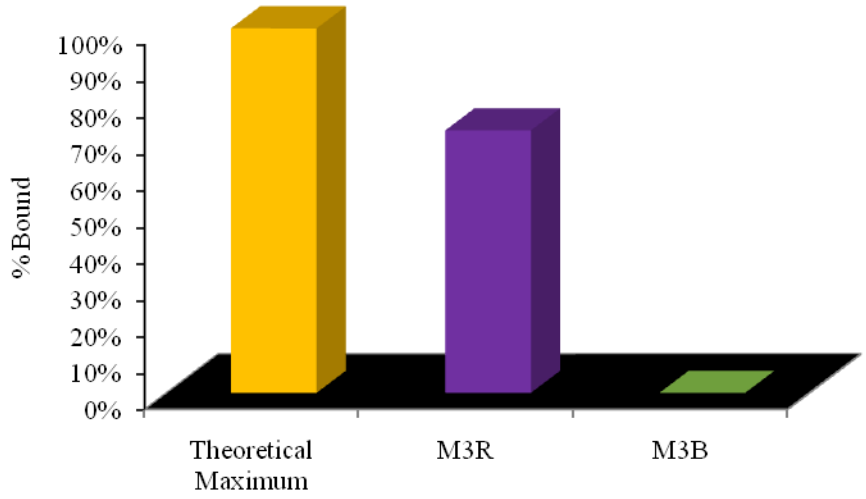

Figure 9.8: % of Acid Phosphatase bound to M_{3R} and M_{3B}

Figure 9.8 and the Table 9.6 show that the levels of Acid Phosphatase bound to the MIP is actually lower than the predicted 100%; only 72% ± 1.5 % of the Acid Phosphatase present in the rebinding sample remained bound to the polymer (indicated in purple in Figure 9.8). As expected, there is no Acid Phosphatase bound to sample M_{3B}. This can be attributed to there being no Acid Phosphatase present in the sample initially. It does, however, reconfirm the fact that there is no Acid Phosphatase left in the MIP from the preparation stage, .i.e., there is no sign of template bleeding.

As these results indicate that there is no Acid Phosphatase left in the M_{3B} sample, no further analysis is required using M_{3B}

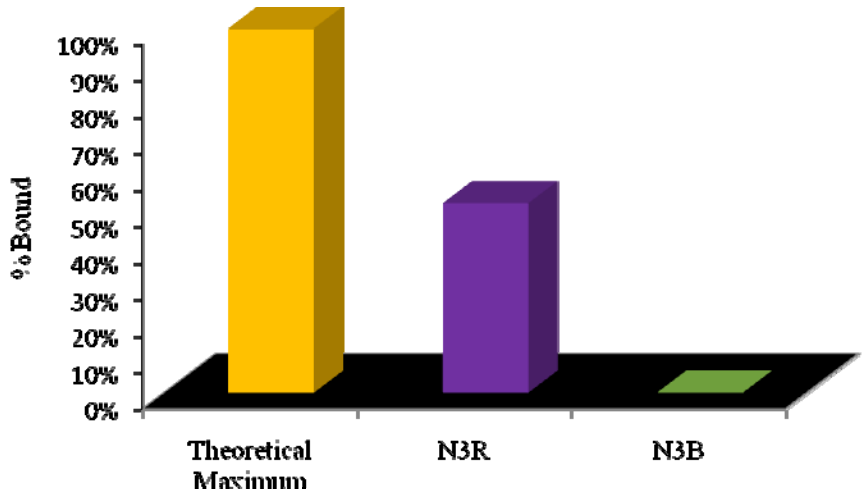

Figure 9.9: % of Acid Phosphatase bound to N_{3R} and N_{3B}

The results shown in Figure 9.9 show that the NIP does indeed rebind the Acid Phosphatase, however it binds at a lower level than that of the MIP; 52% ± 2 % has rebound. The rationale for this occurring can be attributed to the binding which has occurred in the NIP; non-selective binding due to the absence of template shaped binding sites, [9, 10] or the presence of salt (K_2SO_4).[11]

As expected, there is no Acid Phosphatase bound to sample N_{3B}. This can be attributed to there being no Acid Phosphatase present in the sample initially. As with M_{3B}, the results show that there is no Acid Phosphatase present at all in the NIP, thus no further analysis of N_{3B} is required.

The next step of the experimental work was analysis of the eluents obtained from the wash step which was carried out. As explained previously, the wash step is essential to ensure that any weakly bound enzyme present on the polymers is removed.

The eluents collected from samples M_{3R} and N_{3R} were analysed. These results are shown in Table 9.7.

Sample	Absorbance in Eluted Fraction	Number of Moles of 4-NP in Eluted Fraction (nanomoles)	% Enzyme Bound to Sample	Standard Error (% Enzyme Bound)
M_{3R}	0.0042	0	0	± 0.0
N_{3R}	-0.0002	0	0	± 0.0

Table 9.7: % Acid Phosphatase Present in Wash Stage Eluents of MIP (M_3 and N_3) After Rebinding Base on Activity Levels

This was a good result as it reconfirmed the results achieved in the initial rebinding stage. It also re-confirmed that enzyme which did not bind to the polymer in the application stage could be separated from the polymer at the very same stage merely by centrifugation.

As with the analysis of the β-D-Glucosidase imprinted polymer, the final stage of this part of the investigation was to establish if the Acid Phosphatase could retain its enzymatic activity once bound to M_{3R} and N_{3R}.

If the Acid Phosphatase did indeed remain active, the difference between the activity levels of M_{3R} and N_{3R} would also have to be clarified.

Figure 9.10 and Table 9.8 shows the results obtained from the analysis of M_{3R} and Figure 9.11; Table 9.9 shows the corresponding results for N_{3R}.

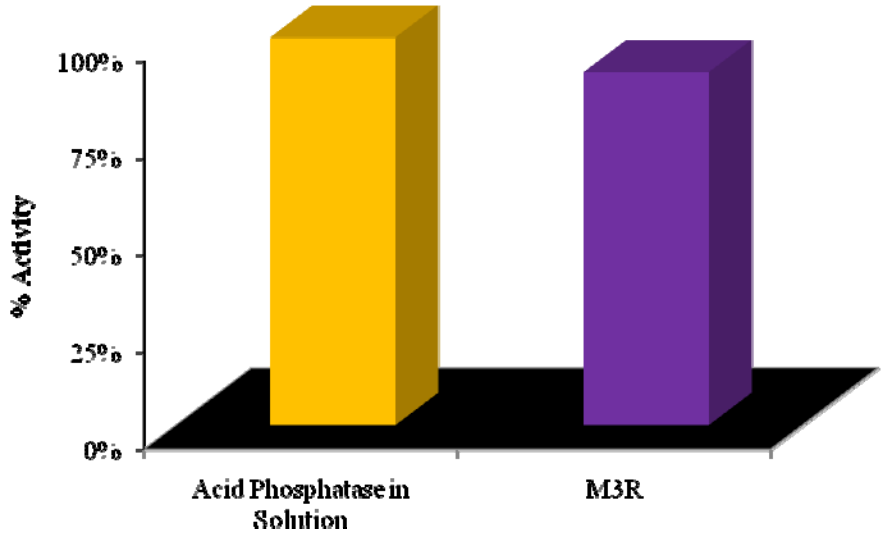

Figure 9.10: Acid Phosphatase Activity on M_{3R}

Figure 9.10 shows that the concentration of 4-NP released by the substrate and the enzyme whilst the enzyme is immobilized on M_{3R} (indicated in purple) is less than the theoretical prediction, indicated in orange, based on 100% activity of the bound enzyme. Of the Acid Phosphatase that was rebound to M_{3R}, 91% ± 1.8 of this has remained in its active conformation, demonstrating that even although it is immobilised on the MIP it retains its activity. The 9% deficit in activity, however, could be due to problems of substrate diffusion.

Sample	**Number of Moles of 4-Nitrophenol (nanomoles)**	**Activity of Acid Phosphatase Bound (%)**	**Standard Error (Activity of Acid Phosphatase Bound (%))**
M_{3R}	4.27	91.00	± 1.8

Table 9.8: Activity of Acid Phosphatase Retained on MIP (M_{3R}) Based on Activity Levels

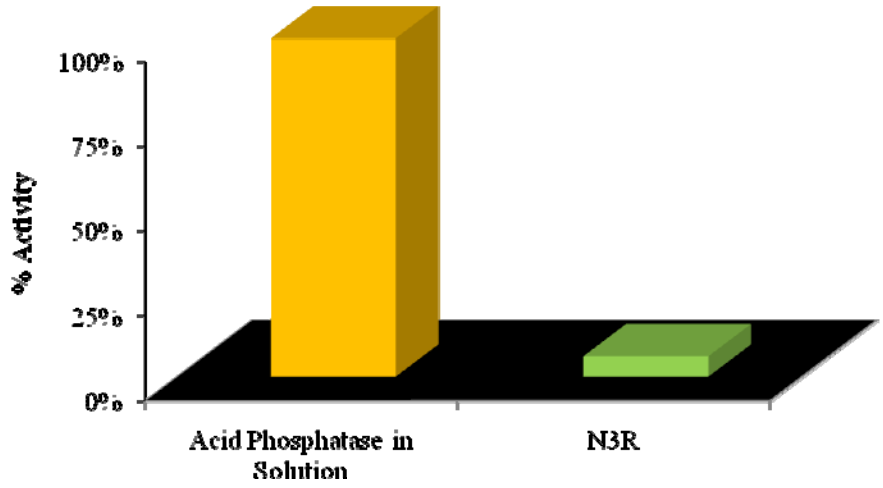

Figure 9.11: Acid Phosphatase Activity on N_{3R}

Figure 9.11 shows that the concentration of 4-NP released by the substrate and the enzyme whilst the enzyme is immobilized on N_{3R}, (indicated in green) is considerably less than the theoretical prediction, indicated in orange, based on 100% activity of the bound enzyme. Of the Acid Phosphatase that was rebound to N_{3R}, 6% ± 0.9 of this has remained in its active conformation, demonstrating that although a large quantity of enzyme is immobilised on the NIP, it does not remain in its active conformation.

It is probable that this drop in activity level of the Acid Phosphatase bound to the NIP is related to the absence of template shaped binding sites in the NIP and likelihood that there have been K_2SO_4 binding sites generated instead. As previously discussed in Chapters 6 and 8, for an enzyme to remain active it must remain in its active conformation; changing its shape to access the K_2SO_4 binding sites would result in a change of its active conformation leading to the loss of activity.[12]

Sample	Number of Moles of 4-Nitrophenol (nanomoles)	Activity of Acid Phosphatase Bound (%)	Standard Error (Activity of Acid Phosphatase Bound (%))
N_{3R}	0.3	6.0	± 0.9

Table 9.9: Activity of Acid Phosphatase Retained on NIP (N_{3R}) Based on Activity Level

9.4 Conclusions

9.4.1 PCMCs

Experiments to establish the optimum buffer for the preparation of Acid Phosphatase PCMCs were successful in identifying 100 mM phosphate buffer, pH 5.99 as the optimum buffer. When the Acid Phosphate was dissolved in the 100 mM phosphate buffer, pH 5.99, it was 82.7% active. The Acid Phosphatase was then immobilised on to the surface of a micro-crystal. The assessment of the activity retained by the enzyme showed that it was 94.3% active.

These results also further confirmed the conclusions reached in Chapter 4 with respect to the methodology in which the PCMCs are prepared. The Acid Phosphatase PCMCs were prepared *via* The Continuous-Flow Precipitator. Given that 94.3% of the Acid Phosphatase on the surface of the micro-crystal is active, it can be assumed that this method is transferrable across enzymes, in addition to being quick and effective.

9.4.2 MIP and NIP

By using PCMCs as the template as opposed to individual protein molecules, it was hypothesized that, post-polymerisation, the template would be removed without difficulty due to the fact that the PCMCs generate micrometer-sized pores within the polymer, lined with protein imprinted sites.

Use of the solvent switching method outlined in Chapter 4 ensured that the Acid Phosphatase was removed successfully. This was verified further by use of the activity assay on the MIP and NIP, respectively, revealing that there was no Acid Phosphatase present in the final wash solvents.

9.4.3 Rebinding Experiments & Activity Assay

The rebinding experiments were carried out successfully using centrifugal filters. Although the volume capacity was smaller than that of Column 2 (Ultrafree® Microcentrifuge Filters), which were used in the assessment of the rebinding and activity assay of β-D-Glucosidase, they still housed a 0.2 μm membrane filter required for this stage of the analyses. The packing of the filters and experimental procedure was quick and effective. By slurring the MIP and NIP samples, loss of material due to the dry, fine particulate matter was avoided.

The activity assay was performed using these membrane centrifugal filters was also successful. After packing, the resultant column(s) was appropriate for the needs of the experiment. The overall size, and column bed height was sufficient to determine activity of Acid Phosphatase whilst working at extremely low enzyme concentrations, 0.0025 *units* of enzyme.

Again, the results resemble those which were obtained for the analysis carried out for the β-D-Glucosidase MIP and NIP. The rebinding studies show that the MIP which was imprinted with Acid Phosphatase had an affinity for the enzyme compared to the NIP; the activity assay results showed that whilst 72% of the Acid Phosphatase rebound to the MIP, only 52% could rebind on the NIP.

The generation of the next set of results was equally as exciting; the Acid Phosphatase retained high enzymatic activity levels whilst rebound to the MIP *versus* the significant lack of enzymatic activity for the enzyme bound to the NIP. The Acid Phosphatase rebound to the MIP retained 91% and the NIP 6%.

This correlates to the results observed with the β-D-Glucosidase enzyme with respect to what is occurring when the imprinted enzyme comes into contact with the MIP, but also with the NIP.

Binding to the NIP was lower that what was observed for the MIP, however the level of binding was still significant. After each rebinding stage of the experiment, and wash solution was applied to the samples. No active Acid Phosphatase was detected in the wash solutions, indicating that the Acid Phosphatase was fully bound within the columns.

The work conducted in this investigation also emulates other results observed in Chapter 6. When the activity assay was conducted on the bound enzyme, a dramatic drop in activity was observed in the NIP vs. the MIP, as was seen with the β-D-Glucosidase experiments. It is probable that the drop in activity level of the Acid Phosphatase bound to the NIP is related to the absence of template shaped binding sites in the NIP and likelihood that there have been K_2SO_4 binding sites generated instead.

9.4.4 Reproducibility

The conclusions in the previous sub-sections, and the results generated within this Chapter confirm that the approach applied throughout this investigation for the preparation and production of molecularly imprinted polymers, i.e., the use of PCMCs as the templates in molecular imprinting processes, is indeed a transferable method. The resultant polymeric material for all of the MIPs and NIPs produced was uniform with respect to the monoliths produced, and the resultant particle size post grinding and sieving (as discussed in Chapter 4).

The rebinding studies executed on the Myoglobin, β-D-Glucosidase and Acid Phosphatase MIPs show high levels of rebinding when applied in MISPE when compared with previous results where MISPE has been applied.[13, 14] This re-affirms

that molecularly imprinting of proteins utilising PCMCs as the template molecule is a viable method for imprinting proteins and the presence of the micro-crystal does not hinder the synthesis or resultant performance of the MIP.

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10.0 Working with a Biological Sample

10.1 Introduction

The selective removal of a protein or enzyme from a biological sample is generally referred to as protein purification.[1] A biological sample is also commonly referred to as a complex mixture.

Protein purification can be better defined as a series of processes used to isolate a protein from a complex mixture/biological sample. The protein extraction can then facilitate further analysis such as activity, characterisation and function studies.[1]

Work that has been carried out thus far in this investigation certifies that protein and enzyme molecularly imprinted polymers can specifically rebind their template molecule when utilised in molecularly imprinted solid-phase extraction (Chapter 5, 6 and 9).

Concerning β-Glucosidase, previous chapters have detailed the selective rebinding of β-D-Glucosidase onto a MIP versus a NIP with the retention of the enzymatic activity, however they have not delved into the possibility that β-D-Glucosidase can be extracted selectively from a mixture of proteins and enzymes or indeed from a biological sample. The next step of the investigation was to investigate such a possibility.

10.1.1 Cell Lysate

The term *cell lysate* refers to a mixture which contains lysed cells (a complex mixture). The term *lysed cells* refers to the process in which the cellular membrane and/or cell wall is ruptured by using either a mechanical or physical methodology, wherein mechanical and physical methods include sonication, pressure and agitation.[2]

For this investigation a pre-prepared sample of cell lysate was supplied by Dr Joseph Deere, University of Strathclyde.

10.1.2 Pichia pastoris Cell Lysate

The cells used to obtain the lysate were *Pichia pastoris (P. pastoris),* as seen in Figure 10.1.

Figure 10.1: Scanning Electron Micrograph of *Pichia pastoris,* Dennis Kunkel Microscopy, Inc[4]

P. pastoris is a methylotophic eukaryotic yeast.[3] It is an extremely common yeast species, readily used to produce proteins at laboratory scale for research purposes. Due to its widespread availability, expression technology for *P. pastoris* has been commercially available for some time.[5]

P. pastoris is a single-celled microorganism, thus exhibits a rapid growth rate and is can be manipulated in culture cost effectively, i.e., the cells can be grown in shake flasks within the laboratory.[5] Although the growth rate and the manipulation ability are attractive features, there is an additional feature which contributes to the use of *P. pastoris*. *P. pastoris* has the ability to use methanol as its only carbon source in the absence of glucose. Although the utilisation of methanol as the carbon source is an inexpensive and easy system to set up; it is not generally used as it can kill microorganisms.[6]

The *P. pastoris* cell lysate was prepared by Dr Deere using a mechanical technique. The cells were disrupted mechanically with the aid of a bead shaker; they were agitated in the presence of small polished silica beads, 200 μm in diameter. By

agitating the cells in the presence of the beads, the cell wall and membrane was disrupted, and the cell components were released. Once the disruption was completed, the cells were extracted. The glass beads were left to settle by gravitational methodology, and the lysate was easily removed.

10.1.3 β-D-Glucosidase Expression in Pichia pastoris

Cell based systems such as those from bacteria,[7] yeast,[8] baculovirus[9] and mammalian cells[10, 11] are commonly used to express proteins. Protein expression refers to a process wherein a protein of interest can be generated in a host cell. [12]

Pichia pastoris is a yeast and is commonly used to express proteins.[12] One of the advantages of using *Pichia pastoris* as an expression system is that *Pichia pastoris* produces minimal quantities of native proteins, [13] therefore making it more straightforward to extract the protein of interest i.e., the protein being expressed. For the purpose of this investigation the identity of native proteins expressed in *Pichia pastoris* is required, or at least, the knowledge that β-D-Glucosidase for almonds is or is or is not among the native proteins of *Pichia pastoris*.

β-D-Glucosidase belongs to a large family of β-Glucosidases. β-Glucosidases are found in all areas of living organisms.[14] There are however many different sources of β-Glucosidases such as bacteria, fungi, plants and yeasts.[15] Although the β-Glucosidases are from many different sources, they are classified as one entry i.e., they are allocated the same EC number. For β-Glucosidase this is EC 3.2.1.21.[15]

The β-Glucosidase used in this investigation is sourced from prunis dulcis which translates to the almond (plant). It has been reported that β-Glucosidase, EC 3.2.1.21 can be expressed in *Pichia pastoris*.[16, 17] No data could be found to verify that β-Glucosidase, EC 3.2.1.21 is or is not a native protein of *Pichia pastoris* so for the purpose of this investigation, the cell lysate was spiked with β-D-Glucosidase sourced from prunis dulcis.

10.2 Experimental Procedures

10.2.1 Chemicals

Ethanol (C_2H_6O)
Acetic Acid ($C_2H_4O_2$)
Sodium Citrate ($Na_3C_6H_5O_7$)
Citric Acid ($C_6H_8O_7$)
Deionised Water (dH_2O)
β-D-Glucosidase from almonds.
Methanol (CH_3OH)
TRIS-Glycine-SDS PAGE Buffer (10X Concentrate)
Protein Loading Buffer 2X Concentrate
4-20% Pre-Cast Gel
SigmaMarker, Wide Range Molecular Weight 6,500 Daltons – 200,000 Daltons
SilverQuest™ Silver Staining Kit

The β-D-Glucosidase from almonds, sodium citrate, methanol and citric acid were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, England). The β-D-Glucosidase was in the form of a lyophilised powder at ≥6 units/mg. The methanol, ethanol and acetic acid were HPLC grade and were ≥ 95% pure. The citric acid and sodium citrate were ≥ 99% pure. Also purchased from Sigma-Aldrich was the SigmaMarker, Wide Range Molecular Weight 6,500 Daltons – 200,000 Daltons.

The 4-20 % pre-cast 15 well gels were purchased from Bio-Rad (Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX). In addition to the pre-cast gels, the TRIS-Glycine-SDS PAGE Buffer (10X Concentrate) and protein loading buffer 2X Concentrate were purchased from BioRad. The SilverQuest™ Silver Staining Kit was purchased from Invitrogen (Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland, UK, PA4 9RF).

10.2.2 Rebinding on MIP and NIP

The MIP and NIP samples utilised were those prepared for work with β-D-Glucosidase, M_2 and N_2, respectively, as described in Chapter 4.

Using the procedure detailed in Chapter 6, Section 6.2.5, 5 mg of MIP and NIP sample were housed in four columns (Column type 2, as described in Chapter 2, Section 2.5), two containing MIP samples and two containing NIP samples for each gel. Two gels were required for this stage of the investigation.

The MIP and NIP samples were wetted prior to rebinding to allow access to the pores which had been generated during the molecular imprinting stage. This was accomplished by adding 2 mL of 10% CH_3OH in 10 mM citrate buffer solution. Each of the columns were sealed and rotated by means of a rotor at 24 rpm for 15 minutes. The temperature was 37 ºC.

After 15 minutes, the liquids were separated from the solids by means of centrifugation at 5000 rpm for 5 minutes.

2 mL of 10 mM citrate buffer was then added to the columns. Again, the columns were sealed and rotated for 15 minutes at 24 rpm and 37 ºC, before the buffer was eluted using the same centrifuge procedure described above.

The MIP and the NIP samples were now ready to have the load solutions applied.

The sample names utilised in this section of the investigation are $M_{2A,}$ M_{2B}, M_{2C} and M_{2D} for the MIP samples and N_{2A}, N_{2B}, N_{2C} and N_{2D} for the NIP samples.

The rebinding solutions and samples required for Gel 1 are shown in Table 10.1 and in Table 10.2 for Gel 2.

Sample	Rebinding Solution	Volume (mL)
M_{2A}	Cell Lysate spiked with 0.5 mg/mL β-D-Glucosidase	0.5
M_{2B}	Cell Lysate spiked with 0.5 mg/mL β-D-Glucosidase	0.5
N_{2A}	Cell Lysate spiked with 0.5 mg/mL β-D-Glucosidase	0.5
N_{2B}	Cell Lysate spiked with 0.5 mg/mL β-D-Glucosidase	0.5

Table 10.1: Rebinding Solutions and Samples Applied to β-D-Glucosidase MIP and NIP (M_2 and N_2) as Imaged on Gel 1

Sample	Rebinding Solution	Volume (mL)
M_{2A}	Cell Lysate spiked with 0.79 mg/mL β-D-Glucosidase	0.5
M_{2B}	Cell Lysate spiked with 0.79 mg/mL β-D-Glucosidase	0.5
N_{2A}	Cell Lysate spiked with 0.79 mg/mL β-D-Glucosidase	0.5
N_{2B}	Cell Lysate spiked with 0.79 mg/mL β-D-Glucosidase	0.5

Table 10.2: Rebinding Solutions and Samples Applied to β-D-Glucosidase MIP and NIP (M_2 and N_2) as Imaged on Gel 2

Each of the columns were sealed and rotated for 15 minutes at 24 rpm, 37ºC. Once this had been completed, the liquids were eluted by means of centrifugation at 5,000 rpm for 5 minutes. Each of the eluents were collected and transferred to an individual Eppendorf tubes for use as a sample in the electrophoresis stage of the experiment.

10.2.3 Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out in order to visualise the protein present in the biological samples (spiked and un-spiked) used in this section of the investigation. The procedure used is as detailed in Chapter 2, Section 2.5.

10.2.4 Sample Preparation

In accordance with the running order (Table 10.3 and Table 10.4) for both gels (Gel 1 and Gel 2), a selection of 250 μL Eppendorf tubes were labelled. To each Eppendorf tube, 10 μL of the protein loading buffer (Blue 2X Concentrate) was added.

Table 10.3 and Table 10.4 specify the running order and sample set for Gel 1 and Gel 2, respectively.

Eppendorf Tube Number	**Gel Lane Number**	**Sample**
1	2	Sigma Marker
2	3	Spiked Cell Lysate
2	4	Spiked Cell Lysate
3	6	Load Eluent M_{2A}
4	7	Load Eluent M_{2B}
5	8	Load Eluent N_{2A}
6	9	Load Eluent N_{2B}
7	10	Wash Eluent M_{2A}
8	11	Wash Eluent M_{2B}
9	12	Wash Eluent N_{2A}
10	13	Wash Eluent N_{2B}

Table 10.3: Sample and Lane Identification for Gel 1

Eppendorf Tube Number	Gel Lane Number	Sample
1	1	Load Eluent N_{2A}
2	2	Load Eluent N_{2B}
3	3	Wash Eluent N_{2A}
4	4	Wash Eluent N_{2B}
5	5	Load Eluent M_{2A}
6	6	Load Eluent M_{2B}
7	7	Wash Eluent M_{2A}
8	8	Wash Eluent M_{2B}

Table 10.4: Sample and Lane Identification for Gel 2

To the respective Eppendorf tubes, 10 µL of the appropriate eluent was added. In addition to this, the spiked lysate was also added to two Eppendorf tubes.

Once the Eppendorf tubes contained the protein blue 2X Concentrated and the required sample, they were mixed by means of a vortex for 1 minute, before being centrifuged for 30 seconds at 1,000 rpm.

All of the Eppendorf's were then heated using a heating block for ten minutes at 90 °C. Once the heating was completed, the Eppendorf's were again centrifuged for 30 seconds at 1000 rpm.

The samples were allowed to cool prior to being loaded into their respective gel wells.

The Sigma Marker was purchased as a dry powder. This was dissolved in 100 µL of dH_2O. The marker was not subjected to the sample preparation procedure; it was, however, centrifuged for 30 seconds at 1,000 rpm prior l to loading into the well of the gel.

10.2.5 Running the Gel

Each pre-cast gel cassette came complete from the manufacturer. Prior to loading the gel, the comb at the top of the gel was removed and the wells rinsed with dH_2O.

Starting from lane 2, 10 µL of the respective sample was loaded into the pre-determined well.

The gel was inserted into the pre-cast gel apparatus (Figure 10.2).

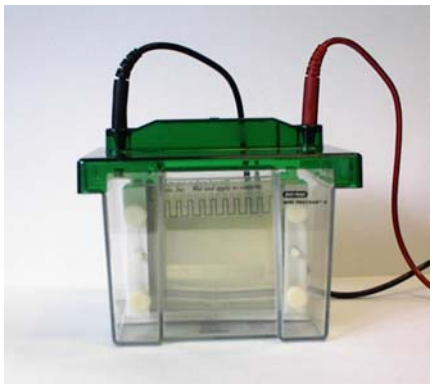

Figure10.2: Photographic Representation Bio-Rad Pre-Cast Gel Apparatus[18]

Once the gel was loaded, the mobile phase for the gel was added to the tank.

The mobile phase was prepared using TRIS-Glycine-SDS PAGE Buffer (10x). This was diluted for use; 50 mL of TRIS-Glycine-SDS PAGE Buffer (10x) was added to 450 mL dH_2O.

The apparatus was turned on. A voltage of 180 V was applied to the gel for 45 minutes. This was sufficient time to allow the molecules to be separated out. After 45 minutes, the pre-cast gel cassette was removed from the apparatus.

Taking care not to damage the gel, the cassette was opened and the gel transferred to a container suitable for the microwave. The gel was rinsed three times with dH_2O.

10.2.6 Staining

Upon completion of the electrophoresis, each gel was stained utilising the silver staining kit purchased from Invitrogen.

Table 10.5 details the reagents present within the SilverQuest™ Silver Staining Kit. Their function in the staining protocol is also described.

Reagent	**Function**
Sensitiser	Enhances the contrast and increases the sensitivity of the gel
Stainer	Allows the silver ions to bind to the protein present, thus forming an image
Developer	Reduces the silver ions bound to the protein to metallic silver. This results in protein bands being formed
Stopper	Complexes to any free silver to prevent any further reduction

Table 10.5: SilverQuest™ Silver Staining Kit Reagents and Functions[19]

In addition to the reagents listed in Table 10.5, a 30 % v/v aqueous ethanol solution was required. This was prepared by dissolving 30 mL of ethanol in 70 mL dH_2O.

Prior to staining, the comb separating each of the gel wells was removed and the gel was extracted from its cassette, taking care to ensure that no damage was inflicted upon the gel.

The gel was transferred to a microwaveable dish, where it was rinsed with dH_2O. Table 10.6 describes the protocol followed, detailing the length of time the gel was in the microwave following on from each stage of the protocol step, the volume of reagents necessary and the length of time the gel and reagent were agitated after each microwave stage was completed.

After each step, the reagent present in the microwave dish was decanted.

Protocol Step	Reagent Added	Volume (mL)	Microwave Time (seconds)	Length of Agitation (minutes)
1	Fixative	100	30	5
2	30 % Ethanol solution	100	30	5
3	Sensitiser	100	30	2
4	dH_2O	100	30	2
5	dH_2O	100	30	2
6	Stainer	100	30	5
7	dH_2O	100	N/A	0.75
8	Developer	100	N/A	5[†]
9	Stopper	10	N/A	10[‡]
10	dH_2O	100	N/A	10

Table 10.6: Silver Staining Protocol Utilised for Staining Gels 1 and 2 after Electrophoresis

[†] *The developer reagent was not removed from the microwave container after this step. The stopper reagent is added directly to the gel whilst it is in the presence of the developer reagent*.

[‡] *Once the stopper reagent has been added, a colour change was observed. When the colour changed from pink to clear, the gel development was completed.*

The gel was mounted onto a clear acetate sheet, with a further sheet being placed on top in order to secure and protect the gel. The gel was then scanned and stored electronically for analysis.

10.3 Results & Discussion

The analysis of Gel 1 and Gel 2 was completed visually.

The level of β-D-Glucosidase present in each of the sample eluents could not be quantified, however it was possible to approximate which eluent contained the highest quantity of β-D-Glucosidase by visual inspection. By comparing the relative intensity of each band, it was deduced which sample contained the highest level of β-D-Glucosidase; this was represented by the most intense band on the gel. Likewise, the band with the lowest intensity was representative of the sample containing the lowest level of β-D-Glucosidase.

10.3.1 Gel 1

Gel 1 can be seen in Figure 10.3.

Lanes 3 and 4 of the gel illustrate the spiked lysate sample. The band viewed at 65,000 Da is indicative of one of the sub-units present in β-D-Glucosidase.

Each sample (both load and wash eluents) loaded onto the gel contains this sub-unit. This is verified by the presence of the band at the relevant molecular weight point across the gel.

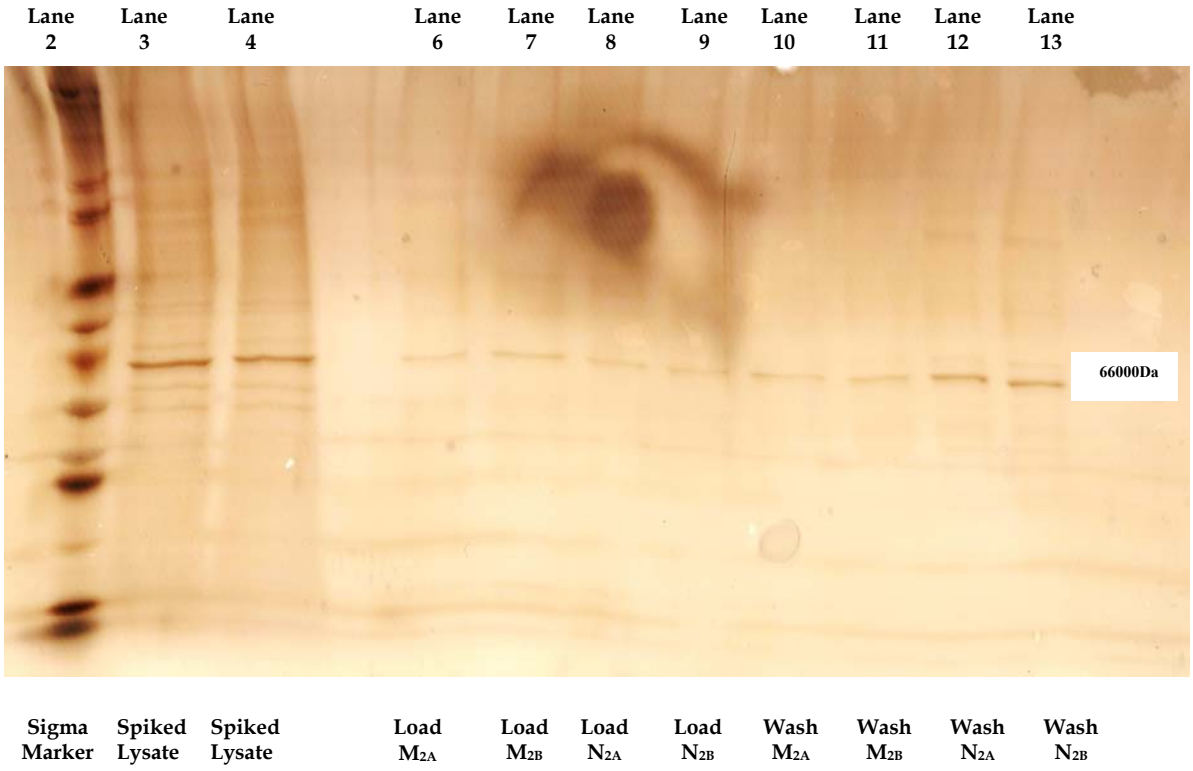

Figure 10.3: Image of Gel 1 Post Silver Staining

It can be observed that the intensity of the bands is similar, with the exception of lanes 12 and 13, where the intensity seems to increase slightly, indicative of a higher level of β-D-Glucosidase. Lanes 12 and 13 contain the wash eluents which were removed from the NIP samples.

10.3.2 Gel 2

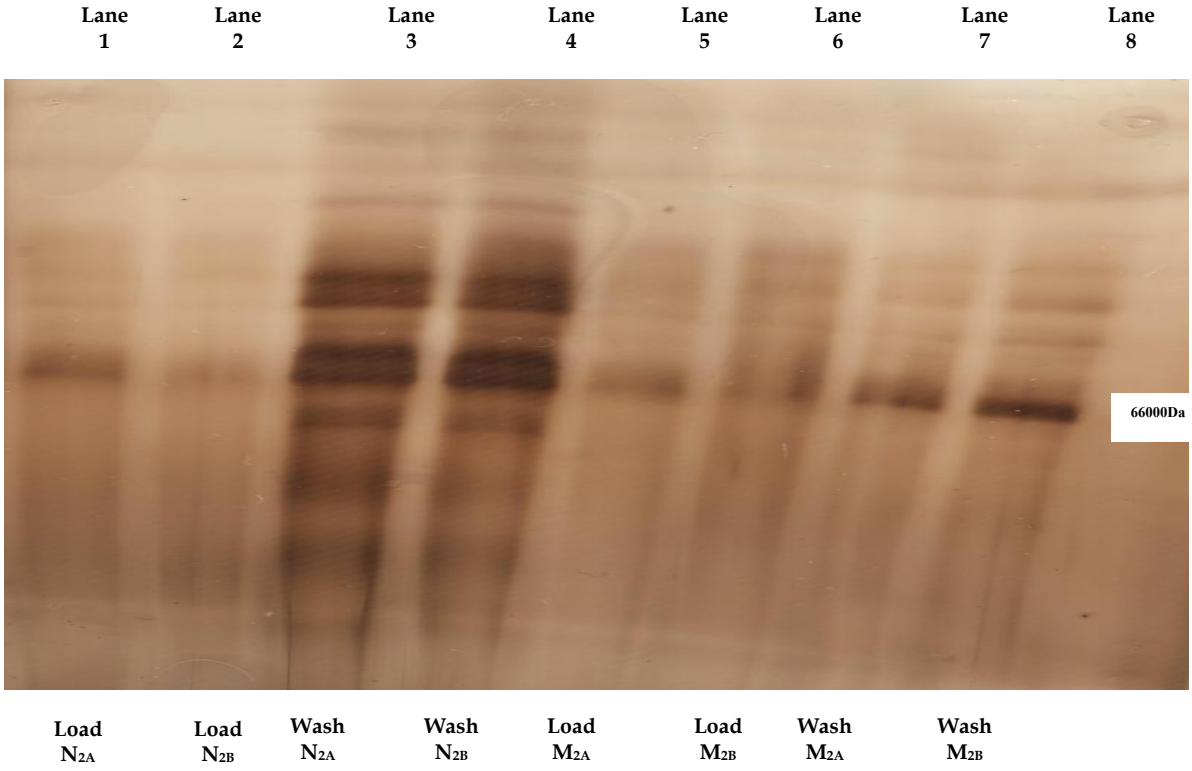

Figure 10.4: Image of Gel 2 Post Staining

Gels 2 provides a closer look at the load and wash eluents of the MIP and NIP samples. The concentration is higher, thus the potential β-D-Glucosidase content is higher and therefore the intensity of the bands are darker, allowing a more accurate assessment of the β-D-Glucosidase content.

Lanes 1 – 4 contain the eluents from the load and wash samples which were applied to the NIP. The bands which are visible in lanes 1 and 2 are evidently paler than those in lanes 3 and 4. This indicates that for the spiked lysate solution the β-D-Glucosidase present bound to the NIP samples. The bands which are visible in lanes 3 and 4, however, indicate that although the β-D-Glucosidase bound initially to the NIP the wash step applied was sufficient to elute the β-D-Glucosidase from the polymer. The bands from the wash steps are of a much higher intensity than those for the load steps. This result is both welcome and expected. It verifies further the results achieved in previous Chapters where the NIP is not selective towards β-D-Glucosidase, and is thus removed upon the application of a wash step.

Lanes 5 – 8 are concerned with the load and wash eluents from the MIP. As with the NIP load eluents, the overall intensity of the bands from the wash eluate is low. This indicates that rebinding to the MIP has occurred.

The wash eluents from the MIP samples tell a different story to those of the NIP however. The intensity of the two bands are as pale as those of the load eluates, indicating that after the loading and washing stages, the β-D-Glucosidase remains bound to the MIP. This result also verifies results from previous chapters; the MIP is selective towards its template molecule; β-D-Glucosidase. Most striking of all is the fact that the MIP binds β-D-Glucosidase, even when the latter is present in a biological sample, i.e., a cell lysate.

10.4 Conclusions

The results obtained from the gels indicate that the MIP has the ability to selectively extract its template molecule, β-D-Glucosidase even when the template is in the presence of a complex mixture such as a cell lysate.
The visual analysis conducted on the outputs from the two gels indicate that the MIP demonstrates an enhanced selectivity for the β-D-Glucosidase compared to that of the NIP; the MIP can retain β-D-Glucosidase after the wash solution has been applied, whereas the β-D-Glucosidase is eluted from the NIP upon application of the wash solution.

The investigation of the MIPs ability to extract its template molecule from a biological sample is in its preliminary stage. It would be recommended to complete further analysis, wherein the β-D-Glucosidase content of the eluents is quantified and the complete removal from the MIP and NIP assessed.

It would also be recommended to spike the lysate with additional proteins other than the template molecule, and to transfer the application to MIPs imprinted with different template molecules.

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11.0 General Conclusions & Further Work

The research findings in this investigation are very promising; however, there are various areas which require some further development work. Here, each chapter is considered separately, and an outline given of possible future research directions.

11.1 Chapter 3

Chapter 3 dealt solely with PCMC preparation.

The protein loading for the three PCMC sample sets was not varied, and in terms of molecular imprinting it would be interesting to ascertain the minimum protein loading required to deliver effective molecularly imprinted products.

Work to date in this investigation has not reported on the stability of the protein once immobilised on the crystal surface. Whilst optimising the conditions for PCMC preparation, the stability of the immobilised protein should also be measured. The stability of the protein to polymerisation conditions is also an area which merits further investigation, although protein instability does not seem to be a problem.

11.2 Chapter 4

This chapter was concerned with the preparation of MIPs. One of the obstacles presented throughout the preparation was the successful removal of the templated protein from the MIP in post-polymerisation extraction steps.

Although a MIP wash sequence was established, optimising the conditions for template removal would be extremely beneficial in terms of streamlining the entire process - reducing the time taken to remove the protein from the MIP and carry out the subsequent analysis would be beneficial in further investigations.

Assessing the way in which the MIP is prepared may allow the template to be extracted more easily, *e.g.*, changing the crosslinker used, altering the level of crosslinking in the imprinting products, initiating the polymerisation by different methods and at a lower temperature. Introducing a buffer into the polymerisation process may also facilitate the subsequent removal of template from the MIP.

Another area to consider would be to prepare a MIP with more than one template molecule, once the optimum MIP preparation conditions have been established.

11.3 Chapter 5

Although Chapter 5 provided exciting results with respect to rebinding and competitive rebinding, there is an opportunity to optimise these experiments further to realise their full potential. For example:

Efforts directed at further optimising the rebinding conditions to bind and elute the protein successfully (100%), whilst in the presence of a competing protein.

Selecting a suitable wash solution to do the above - allow the template protein to bind to the column whilst the competing protein is eluted from the column.

Investigate and optimise methods which will retain both the protein and the heme group during the rebinding process in terms of the myoglobin imprinted MIP

11.4 Chapter 6

The enzyme β-D-Glucosidase was used as the template molecule in this Chapter.

The rebinding experiments were carried out with a small quantity of β-D-Glucosidase present in order to accommodate the activity assay for β-D-Glucosidase.

Further work should focus on optimising the rebinding conditions for the β-D-Glucosidase, increasing the mass of β-D-Glucosidase and developing the activity assay. This would allow rebinding experiments to be conducted closer to the maximum binding capacity of the MIP.

As this Chapter was concerned only with rebinding the template molecule, a further step forward would be to carry out competitive rebinding experiments using the β-D-Glucosidase MIP, and thus assessing the activity and stability of the enzyme once it has been in the presence of a competing enzyme(s).

11.5 Chapter 7

The robustness of the MIP was analysed in this Chapter.

The suggestion to utilise the MIP no more than three times was reached on the basis of the wash protocol applied to the MIP.

Since being able to re-use a MIP for a large number of rebinding cycles is an important factor with respect to the capabilities and potential applications of a MIP, further investigations into optimising the wash protocol should be conducted. This would ensure that the regeneration of the MIP is complete in a short time period and may allow the re-evaluation of the re-usability of the MIP, *i.e.*, can the MIP be utilised more than three times for rebinding experiments.

11.6 Chapter 8

Analysis of the stability of the β-D-Glucosidase once rebound to the MIP was very promising. The analysis was carried out over a four week period, however a further, in-depth analysis may give a further indication as to what exactly is happening once the enzyme is rebound, *i.e.*, a day to day analysis.

In addition to this, temperature and time were the only two parameters looked at. Further parameters could be investigated in order to assess the overall enzyme stability, such as the effects of solvent or pH on the rebound enzyme.

11.9 Chapter 9

Chapter 9 related to the general applicability of the methods and procedures outlined in previous chapters, and described a different enzyme as the template molecule for molecular imprinting.

The outcome was successful, with Acid Phosphatase being imprinted successfully, subsequently rebound and the activity of the enzyme being maintained.

The transfer of the methods and procedures to alternative proteins and enzymes should be considered, in addition to broadening the scope further.

11.10 Chapter 10

Chapter 10 was concerned with the ability of MIPs to extract template molecules from biological samples.

The work in this Chapter was promising but preliminary, thus there is the potential for an extensive body of further work to be conducted.

Initially, it would be recommended to complete further analysis on the rebinding of the β-D-Glucosidase to the MIP. The β-D-Glucosidase content of the eluent could be quantified to establish the exact quantity rebound and then eluted from the MIP and NIP during the wash stage.

In addition to this, the complete removal of enzyme from the MIP and NIP should be accomplished; thus ensuring a suitable wash procedure is established. Again, this process should be quantified.