IN THE NAME OF ALLAH, THE MOST BENEFICENT, THE MOST MERCIFUL.

BIODEGRADABLE MICROSPHERES FOR DRUG DELIVERY

A THESIS

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DEDICATION

This thesis is dedicated to my parents, for their kindness and continuos encouragement, my husband Asaad for his support and assistance and to my son Ahmed.

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ABSTRACT

The use of microspheres in drug delivery systems is one of the strategies that represents technologies which have made improvements in the pharmacokinetic and pharmacodynamics of therapeutic agents. This research is aimed at the preparation and characterization of three different types of biodegradable microsphere: poly (d,l lactide-co-glycolide) (PLGA), hyaluronic acid (HA) and cross-linked HA microspheres. Dexamethasone was the drug used in this study with its potential application in delivery to the brain and colon. The method used for the microsphere preparation was an emulsification solvent evaporation technique. PLGA and HA microspheres were characterized in terms of particle size, drug content and *in vitro* drug release. The results of particle size analysis showed an average size range of 2-5μm for PLGA microspheres. HA microspheres were in the range of 21-23μm. The difference in particle size range for PLGA and HA microspheres was due to the different mixing speed and polymer viscosity. Light microscopy results showed spherical microspheres for PLGA and HA, consistent with the laser diffractometry results. The level of dexamethasone in HA and PLGA microsphere was 73.5% and 15.5%, respectively. The *in-vitro* release pattern in phosphate buffer saline at 37°C over 200hrs showed a burst release followed by a slower sustained release of 30% (PLGA microspheres) and 12% (HA microspheres). HA microspheres swelled in water due to hydration. The last part of the project involved modification of HA microspheres using cystine methyl ester and carbodiimide as cross linking agents in order to improve HA microsphere stability. Clear, spherical, cross linked HA microspheres were obtained with a size range of 60-70μm which appeared to be more stable.

CONTENTS

CHAPTER ONE

1 INTRODUCTION

1.1 The concept of controlled drug delivery and drug targeting

Controlled drug delivery is the use of systems and techniques for altering and controlling the pharmacokinetic and pharmacodynamic properties of pharmacologically active agents which aims to optimize the therapeutic effects of a drug and to minimize its toxic effects (Juliano 1980).

1.2 Characteristics of an ideal Drug Delivery System (DDS)

There are different types of DDS including colloidal particles, microspheres, nanoparticles, liposomes, soluble macromolecules and monoclonal antibodies. Certain criteria are necessary for an ideal drug delivery system to achieve the goal of optimized drug delivery. Widder *et al.* (1979) described the characteristics of an ideal targetable drug delivery system as follows:

1-The DDS should be able to restrict drug distribution to the desired target tissues;

2-The DDS should be able to deliver a therapeutically effective dose to the target site(s), and the dose incorporated in or associated with the DDS must provide a controllable and predictable rate of drug release;

3-The DDS should be able to minimize leakage of drug from the delivery system during transit, and minimize drug metabolism or distribution into non- host tissues;

4-The DDS should exhibit biocompatible surface properties and negligible antigenicity;

5-The DDS should undergo biodegradation with prompt elimination and minimal toxicity of the breakdown products;

6-The DDS should be capable of carrying a wide range of therapeutic agents;

7-The formulation must make practical sense both in terms of production and administration;

8-The DDS must protect the drug from inactivation by plasma enzymes;

9-The DDS must have a suitable size and proper shape to allow carrier system passage to the target site without causing toxicity to the body (Illum *et al.* 1982).

1.3 Drug targeting theory

The delivery of drugs to specific cells within the body has been one of the most attractive goals in therapeutics ever since Paul Ehrlich (1906) foresaw the "use of bodies which possessed a particular affinity for certain organs as carriers by which to bring therapeutically active groups to the organ in question". In recent decades, the availability of new drugs with short biological half lives (e.g. peptides) and very potent drugs with strong side effects (e.g. tumour necrosis factor) has led to increasing interest in the possibility of delivering drugs to their desired site of action (drug targeting). Such drugs demand new formulations which protect both the drug and the host and are able to direct the drug to the desired tissues (Muller 1991a). In short, drug targeting is the administration of a drug to a particular anatomical site in

order to avoid or reduce toxicity by optimising the amount and persistence of the drug in the vicinity of the "target cell" while reducing the drug exposure of the "non target cell".

The degree of targeting of drug delivery systems can be classified into three orders based on the level of selectivity of the delivery systems (Widder *et al.* 1979)

1st order Targeting

Targeting to the organ (organ targeting)

2nd order targeting

Targeting to a specific cell type within an organ (cellular targeting).

3rd order Targeting

Targeting to a particular subcellular organelle (subcellular targeting).

1.4 Methods of controlled drug delivery and targeting

In the treatment of a disease with a drug, it is usually desirable to maintain a drug concentration in the target tissue(s) which is both constant and within the therapeutically effective dose range. Thus it has long been the goal of researchers to find an ideal drug delivery system that will target active molecules to specific sites in the body. To achieve these goals, several systems have been proposed, including

Liposomes (Fendler and Romero 1977), Monoclonal antibodies (Tyle and Ram 1990), Prodrugs (Gardner 1983), Soluble macromolecules (Lloyd *et al.* 1986), Nanoparticles (Oppenheim 1981) and Niosomes (Baillie *et al.* 1985). During the last decade microspheres and microcapsules have been extensively studied for their ability to direct the drug bearing package to the specific target (Whateley *et al.* 1993). However, the major disadvantage of particulate drug carriers is that they are recognized by the body as foreign particles. Consequently, they are removed from the blood circulation by the macrophages of the Reticuloendothelial System (RES). About 60 to 90% of the particles will be phagocytosed by the macrophages in the liver (Kupffer cells) and spleen (Splenic macrophages). This is called "RES clearance" which is the greatest obstacle for the utilisation of particulate systems in controlled drug delivery. This obstacle has been taken as an advantage and was used to passively target drugs for treatment of some parasite diseases (e.g. leishminiasis) which can be specifically targeted by using a particulate carrier (Muller 1991a).

The capture of colloidal particles by RES macrophages is mediated by a process of opsonisation. In opsonisation, certain plasma components are adsorbed onto the particle surface soon after it has been administered, followed by the recognition of the adsorbed factors that potentiate the adhesion and engulfment of `particles by the Kupffer cells (Illum and Davis 1984; Allen and Chonn 1987). Illum (1989) reported the possibility of minimizing both opsonisation and adhesion between particles and Kupffer cells by coating the particles with high molecular weight non-ionic polymeric material that would give the particles a hydrophilic surface which provides a barrier for opsonisation, hence, allowing these drug loaded carriers to remain in the circulatory system for longer. The block copolymers, poloxamers and poloxamines,

can be used to modify the surface of the particle as the hydrophobic part of the polymer attaches onto the surface of the particle while the hydrophilic moiety extends into the surrounding area reducing uptake by liver cells.

The modification of the surface properties of biodegradable (PLGA) nanospheres by coating with poly (lactide)-poly(ethylene glycol) (PLA:PEG) copolymer was carried out by Stolink *et al.* (1994). The biological behavior of these PLGA nanospheres was studied in terms of *in vitro* characterization methods followed by *in vivo* studies of nanosphere biodistrubtion after intravenous injection into rats. The results showed that the PLA:PEG copolymer can be exploited as a coating for biodegradable PLGA nanospheres to direct intravenously administered colloidal drug carrier particles away from the reticulo-endothelial system as compared to the naked PLGA nanospheres, so they are able to reach the desired target sites. Surface modification of the colloidal drug carrier results in avoiding opsonisation and in improving the ability of the colloidal carrier systems to target drugs to particular organs.

Susan *et al.* (1994) studied the effect of different densities of poly (ethylene glycol-2000) (PEG-2000) at the particle surface of polystyrene-poly (ethylene glycol-2000) (PS-PEG-2000) particles. The *in vitro* studies showed a decrease in the interaction of the particles with non-parenchymal liver cells as the surface density of PEG increased. The *in vivo* biodistribution after intravenous injection of PS-PEG into the rat showed a significant decrease in uptake of particles by the liver as the amount of PEG increased. The researcher concluded that there is a direct relationship between the particle surface density of PEG and particle cell interaction for a polymer colloidal particle system both *in vitro* and *in vivo*.

As the work of this project involves microspheres, so the focus will be on nanoparticles and microspheres.

1.5 Nanoparticles

Kreuter (1994) has defined nanoparticles as colloidal particles ranging in size from 10 to 1000nm to which drugs are bound by sorption, incorporation or chemical binding. According to the process used for the preparation of the nanoparticles, nanospheres or nanocapsules can be formed.

Nanospheres are monolithic matrix systems in which the drug is dispersed throughout the particles while nanocapsules are vesicular type systems in which the drug is confined to a cavity enclosed by a polymeric membrane (Couvreur *et al.* 1995).

One of the most promising applications of nanoparticles is their use as carriers for anticancer drugs. Couvreur *et al.* (1982), studied the possibility of reducing the toxicity of the anticancer drug Doxorubicin by fixing it onto polyisobutylcyanoacrylate nanoparticles. Bound and free drug were given intravenously to female mice. The results showed a significant decrease of bound doxorubicin toxicity in comparison to free drug. This decrease corresponds to both a diminution in weight loss and to a higher survival rate in mice after administration of bound doxorubicin for various doses and administration schedules. In addition, the

cardiotoxicity of doxorubicin was decreased and this was also indicated by the absence of doxorubicin fluorescence in cardiac muscle when the drug was adsorbed on the nanoparticles. An experimental study of polyalkylcyanoacrylate nanoparticles as an anticancer drug-carrier was carried out on Lewis lung carcinoma-bearing mice by Grislain *et al.* (1983). It showed rapid clearance of nanoparticles from the blood stream, with its main concentration in the reticuloendothelial system and an intense pulmonary concentration in lung metastases. This was explained by a change in the structure of the capillary wall due to the inflammatory process at the tumor site. The previous study also showed that following subcutaneous injection, accumulation of nanoparticles occurs in the gut wall. Kante *et al.* (1980) studied the influence of the adsorption of ^{3}[H] actinomycin D onto polybutylcyanoacrylate nanoparticles upon the tissue distribution of the drug in rats. The results, 24hr after administration, showed the tissue concentration with polybutylcyanoacrylate nanoparticles injected rats to be over 64 fold higher for the liver, 44 fold higher for the spleen and 56 fold higher for the muscle, compared to free drug injected rats. Furthermore the urinary excretion of ^{3}[H] actinomycin D was diminished when the drug is bound to polybutylcyanoacrylate nanoparticles. These results provide evidence that nanoparticles of polycyanoacrylate can be successfully used to modify the tissue distribution of actinomycin D. The results clearly demonstrate that the use of nanoparticles as a drug carrier can reduce considerably the inherent toxicity and side effects of a cytotoxic drug and could be useful in cancer chemotherapy.

1.6 Polymers for drug delivery

According to Fildes *et al.* (1991) polymers can be divided into two groups:

(1) Non-biodegradable polymers:

These are polymers which are not broken down *in vivo* by either hydrolytic or enzymatic means. Examples include:

cross-linked poly(vinyl alcohol) hydrogel,

ethylene/vinyl acetate copolymer and

silicone elastomers.

(2) Biodegradable polymers:

These can be defined as synthetic or natural polymers which are degradable *in vivo*, either enzymatically or non-enzymatically, to produce biocompatible or non-toxic by-products. These can be further metabolized or excreted via normal physiological pathways.

A range of biodegradable polymers have been considered in the sustained release of drugs including:

poly(alkyl-α-cyanoacrylate),

poly(ortho-esters),

poly(hydroxybutyrate)

poly(lactic acid) (poly lactide),

poly(glycolic acid) (poly glycolide),

poly(lactic acid-co-glycolic acid) (poly lactide-co-glycolide) (PLGA).

Biodegradable polymeric systems are prefered to non- biodegradable systems which have to be surgically removed after the drug has been released. Other advantages of biodegradable polymeric systems include minimising adverse reaction as the polymer erodes and complete release of the drug, which may not always happen in non-biodegradable systems (Yolles & Sartori 1980).

The biodegradable polymers are the material of choice to be used as a matrix in drug delivery system, since the specific requirements for a polymeric material to be used as a matrix include safety, biocompatibility and lack of toxicity of the polymer and its degradation products (Whateley 1993).

Our study includes the preparation of biodegradable microspheres using PLGA as a synthetic polymer and hyaluronic acid (HA) as a natural polymer.

1.6.1 Biodegradable Poly(D,L-lactic-co-glycolic acid) (PLGA)

The copolymers of PLGA are used in a variety of medical applications, and have been

extensively utilized in the pharmaceutical field as a drug delivery device for various drugs. They degrade slowly by bulk hydrolysis, undergoing random, non-enzymatic chain scission of the ester linkage. The degradation leads to the formation of the lactic acid and glycolic acid which are normal body metabolites (Muller 1991b).

PLA is more resistant to hydrophilic attack as compared to PLG due to the presence of an additional methyl group which causes an increased hydrophobicity as shown in (Fig. 1).

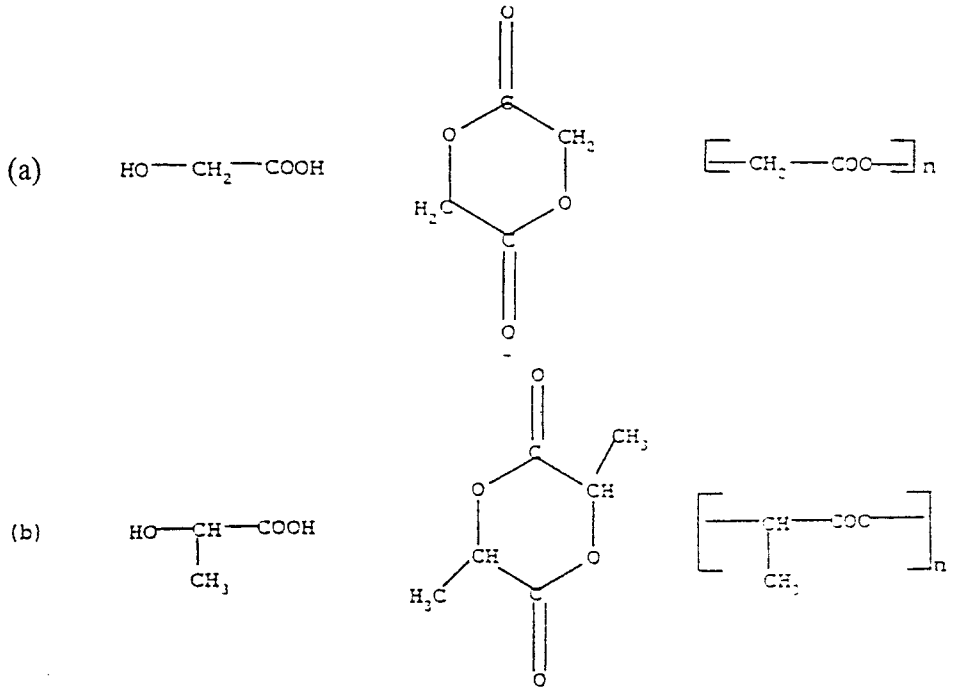


Fig. 1 Structures of (a) glycolic acid (b) lactic acid, their cyclic dimers (glycolide and lactide) and their homopolymers.

The poly(glycolic acid) polymer is very crystalline and insoluble in solvents other than fluorinated ones. The poly(L-lactic acid) polymer is more crystalline than

poly(D,L-lactic acid) material where the chains can not pack so well, crystallinity decreases as glycolic acid is introduced into the poly(lactic acid) system (Whateley 1993). The crystallinity of the PLGA polymer depends upon the molar ratio of the two monomer components in the copolymer chain (Gilding and Reed 1979).

Many studies using homo- and co-polymers of lactic and glycolic acids have shown that these materials are inert and biocompatible in the physiological environment and degrade to toxicologically acceptable products (Kalkarni 1966; Wise *et al.* 1979). Meni *et al.* (1993) studied the biodegradation and biocompatibilty of stereotactically implanted PLGA (50:50) microspheres in rat brain tissue 1-4 months following implantation. The animals survived the experiment without any behavioural changes or neurological deficit indicating the non-toxicity of PLGA. In both *in vitro* and *in vivo*, PLGA microspheres became heterogeneous at 10 days and were irregular vacuolar in appearance at 1 month. The study also showed that the degradation was faster *in vivo* than *in vitro* following the observation at 2 months of several fragments *in vitro* in comparison to few fragments in brain tissues. This was explained by the presence of biological compounds in the tissues. The inflammatory reaction produced by PLGA microspheres in brain tissue was found to be similar to that in control rats. This reaction was noted to decrease significantly after 1 month before almost ending at 2 months when the microspheres were totally biodegraded.

Histological studies on the liver tissue have shown a moderate inflammatory response to microspheres of PLGA (75:25) during the degradation of microspheres (Splenhauer *et al.* 1989). Moreover, no signs of scar formation were observed in the

hepatic tissues after complete disappearance of microspheres, which illustrated the good biocompatability of PLGA.

Drug release from PLGA copolymer devices is generally "biphasic", or "triphasic" with an initial surface release which is known as the "burst release" followed by a continuous, sustained release until drug depletion of the matrix takes place, In some cases, the sustained release phase may also be followed by rapid release due to the collapse of the matrix following hydrolytic degradation (the triphasic release). By altering the ratio of lactide to glycolide in the PLGA matrix, along with varying the molecular weight of PLGA chosen, the time for the sustained release can be modified by weeks and often months. Consequently, many different drugs including (anticancer drugs, steroids, antibiotics) have been incorporated in these biodegradable polymers, mainly as implants or injectable microparticles, microspheres or nanoparticles (Brophy and Deasy 1990).

1.6.1.1 Preparation of PLGA copolymer

The preferred method for producing the higher molecular weight polymers required for pharmaceutical application is the ring-opening polymerization of the cyclic dimer, lactide or glycolide, using tetraphenyl tin or stannous octanoate as a catalyst (Wise *et al.* 1979).

Copolymers with varying ranges of lactide to glycolide can be synthesized. Deasy *et*

al. (1989) described the production and synthesis of a range of PLA, PGA, and copolymers of the two from lactide and glycolide. They studied the effect of synthetic parameters (lactide type, reaction and temperature time, and the catalyst concentration) on the properties of the polymers produced, which was characterized by technique of Gel Permeation Chromatography (GPC), Laser Light Scattering and Differential Scanning Calorimetry (DSC) among others. The result of this study showed the significant effect of the choice of monomers and reaction conditions on the properties of the polymer including appearance, Mw, and crystallinity which have an important role in the selection of these materials for pharmaceutical application.

1.6.1.2 *In vivo* and *in vitro* degradation of PLGA

The degradation and factors that affect the degradation rate of the biodegradable polymers are important, especially when developing biodegradable drug loaded polymers for sustained release studies. Degradation of these polymers has been characterized in terms of molecular weight and its distribution, weight loss, water uptake and morphology of the hydrated and degraded polymer. Degradation of the polymer *in vitro* in buffer at pH 7.4 results in progressive changes in the molecular weight and molecular weight distribution. Under these conditions degradation is not enzyme mediated and must occur by simple hydrolytic cleavage of ester groups. High molecular weight polymers degrade to lower molecular weight fragments, as measured by viscosity, their water insolubility, weight loss occur after prolonged time of degradation. Polymers with very low molecular weight can degrade with

immediate weight loss (Hutchinson *et al.* 1987).

Vert *et al.* (1991) outlined the factors which affect the polymer degradation including chemical structure and composition, Mw, Mw distribution, sterilizing process, morphology and physical factors including shape and size changes. Consideration of these factors provide guidelines, which have a role in the choice of PL/GA type degradation matrix with respect to the control of drug delivery. They discussed the influence of drug load on polymer degradation rate. They concluded that the chemical and physicochemical properties of the drug play an important role on the degradation of the matrix. They explained that hydrophobic substances prevent water uptake into the polymer and hence cause a decrease in the degradation rate while hydrophilic substances will permit water to penetrate into the polymer matrix and hence accelerate the degradation of the matrix. Park *et al.* (1994) examined the degradation behaviour of two poly(D,L-lactic acid) microspheres, prepared from two different molecular weights (17000 and 41000). The results showed that the lower molecular weight polymeric microspheres degraded more rapidly than the high molecular weight polymeric microspheres. This was confirmed by GPC, which showed a gradual decrease in Mw distribution of the low Mw microspheres, in comparison to the late changes (53 days of study) seen in the case of high Mw microspheres. Makio *et al.* (1985) studied the effect of certain parameters including pH on the degradation of PLA microcapsules. Their results showed that there was an increase in the rate of hydrolysis at high and low values of pH, typical of acid-base catalysed ester hydrolysis.

There are three major variables which affect the properties of the co-polymer, i.e.

molecular weight, ratio of lactic to glycolic acid and γ- irradiation. Each of these parameters has an effect on the degradation rate, which in many cases is the major factor controlling the rate of drug release (Whateley 1993).

An increase in molecular weight of PLA decreases the rate of degradation whilst an increasing content of glycolic acid (up to 60-70%) causes an increase in the rate of degradation (Kaetsu *et al.* 1987). The rate of degradation decreases again as the percentage of glycolic acid approaches 100%, due to the greater crystallinity of this material.

The effect of γ irradiation (often used for terminal sterilisation of PLGA system) on the polymer molecular weight (as indicated by the intrinsic viscosity), shows a near-linear decrease in molecular weight with increase in irradiation dose. Thus, γ irradiation sterilisation has the effect of reducing the molecular weight of the polymer, with consequent effect on the rate of degradation. These factors allow control of the rate of degradation of PLGA polymers and consequently on the rate of drug delivery (Whateley 1993).

1.6.1.3 Therapeutic and experimental uses of PLGA

Early experimental studies on PLGA polymers showed its biocompatability and biodegradability in tissues and led to the first useful application of copolymers as absorbable sutures in surgery (Kalkarni 1966). This was followed by their use for

surgical clips and other surgical implants of different shapes and sizes which retain their structural and strength properties at the site of implant (Wise *et al.* 1979). The use of lactide/glycolide copolymer in marketed products was approved by the regulatory authorities following the confirmation of its biocompatability and the availability of its extensive toxicological documentation. The first product to be developed successfully was Zoladex®, a once-a-month subcutaneous implant to deliver the polypeptide, goserelin based on the biodegradable poly (lactic-co-glycolic acid) polymers. Zoladex® depot formulations are approximately 1cm long, 1mm diameter, administered subcutaneously (S/C) in the abdomen wall with a 16guage needle and release 3.6 mg of drug over 28 days. It is used in the treatment of prostate cancer in men and advanced breast cancer in premenopausal women (Fildes *et al.* 1991). The other product which is on the market is Prostap SR®. This product consists of microspheres of mean size 20μm prepared from a polymer (PLGA) of molecular weight 14,000. The injection vehicle for the microspheres contains carboxymethyl cellulose (CMC) in order to increase the viscosity of the medium and to ensure that the microspheres remain in suspension during administration. A 23 gauge needle can be used for the once-a-month subcutaneous injection of these microspheres. The other examples of peptides which are microencapsulated successfully in poly (D,L lactide-co-glycolide) is nafarelin acetate, a potent analogue of luteinizing hormone-releasing hormone (Sanders *et al.* 1984; 1986), [D-TRP6]-LHRH (Decapeptyl). It delivers 3.75mg of [D-TRP6]-LHRH over a month and is indicated for the treatment of testosterone-dependent prostate cancer (Redding *et al.* 1984) and leuprolide acetate (Ogawa *et al.* 1988; Okada *et al.* 1989).

Parlodel was the second microsphere product delivering bromocriptine for a one

month peroid (Kissel *et al.* 1988). Bromocriptine is a dopamine agonist used in the treatment of galactorrhea, pituitary adenoma and Parkinson's disease. It acts by reducing serum prolactin concentration.

Ike *et al.* (1988; 1990) studied the local anticancer effect of adriamycin (ADR)-containg poly(L-lactic acid) microspheres (ADR-MS) with an average diameter of 50 μm. The microspheres released all of the contained ADR in 20 days *in vitro.* The *in-vivo* studies after instillation of ADR-MS into the pleural cavity of patients with pleuritis carcinomatosa showed that the amount of drained ADR was less than a few percent of the administered dose indicating that almost all the ADR-MS was concentrated in the pleural cavity. The intraperitoneal administration of ADR-MS for the treatment of mice bearing P815 tumour cells resulted in a higher survival rate than with free ADR

Thus the researchers concluded that the local effect of ADR-MS resulted in a maximum anti-cancer effect with less side effects.

The concept underlying hepatic arterial chemotherapy has two main benefits, the first is the possibility that the intra-arterial (i.a) route can produce a greater total tumour exposure to the drug, thus raising the therapeutic index. The second is that drug extraction or metabolism in the organ may reduce the amount of chemotherapeutic agent reaching the systemic circulation, thereby reducing overall toxicity (Hu and Howell 1983).

Mitomycin C (MMC), an effective broad spectrum anti-cancer drug, has been in use for around 20 years. It is currently used for the treatment of a range of tumours

including, breast, liver and colonic cancer, and administered by the hepatic arteriac route in the form of ethyl cellulose coated microspheres to treat hepatic metastasis. Particles in the range, 20-200μm are trapped in the capillary bed of the liver following intra-arterial administration, a process known as a chemoembolisation (Eley *et al.* 1992). Second order targeting (i.e. to the tumour rather than to the whole organ) can be achieved by the concurrent administration of angiotension II (AT II), a vasoconstrictor, which restricts arterial flow to the normal liver but doesnot affect the capillary blood flow network of the tumour.

Microspheres loaded with MMC in this manner have 3 advantages:

(1) Systemic levels are low, with consequent reduction in side effects.

(2) The sustained release of MMC from the trapped microspheres extends the time scale of exposure of the tumour cells to the cytotoxic drug. The embolic effect of the microspheres increases retention of the drug at the site of release.

(3) In the case of a cytotoxic drug such as MMC, which requires bioreduction for activation, the reduced oxygen levels resulting from embolism by the microspheres may enhance the therapeutic efficacy of MMC.

Goldberg *et al.* (1991) and Eley *et al.* (1992) have shown that the peak plasma levels with MMC microencapsulated with ethyl cellulose could be reduced from 812 +/-(423)ng/ml for free MMC in solution to 80 +/-(75) ng/ml for microencapsulated MMC administered as a bolus via the hepatic artery. A phase I clinical trial showed that the dose of microencapsulated MMC could be increased to 40 mg without toxicity (Eley *et al.* 1992).

Diphtheria toxoid, which is an important vaccine in the expanded programme of

immunization in developing countries has been microencapsulated using poly (D,L-lactide) by Singh *et al.* (1991). After injection in BALB/c mice, the antibody units determined using ELISA until day 75 were comparable to those obtained in a group receiving a 3-dose injection of diphtheria toxoid with calcium phosphate as an adjuvant. Thus, microspheres may be useful for increasing the immunogenicity of antigen, thereby allowing safer and more effective immunization.

1.6.2 Hyaluronic acid (HA)

HA is a naturally occurring biodegradable polymer. It is classed as a member of a group of structurally and functionally related polysaccharides known as the glycosaminoglycans. HA was first named by Meyer and Palmer (1934) following its identification as a polysaccharide in the vitreous humour of the bovine eye, hence the name hyaluronic (Hyal) originating from the Greek word hyaloid which is vitreous. HA has been known as hyaluronate in its salt form, and the term hyaluronan has also been suggested as a general term for this polysaccharide (Balazs *et al.* 1986).

In this report, the raw material used was sodium hyaluronate and as hyaluronic acid is present in tissues as the ion, the abbreviation used is HA.

The chemical structure of HA was determined by Meyers and co-workers in 1950s who established it as an unbranched linear polymer built from repeating disaccharide units consisting of D-glucuronic acid and N-acetyl -D-glucosamine linked by β(1-3) and β(1-4) glycosidic linkages, respectively (Fig. 2).

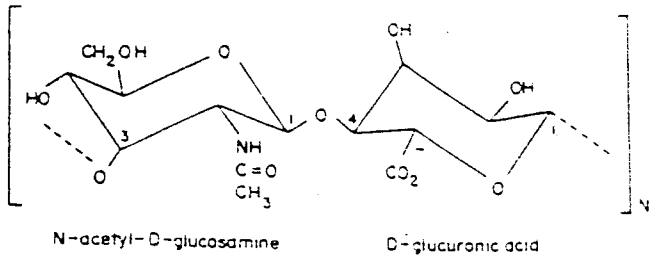


Fig. 2 Structure of hyaluronic acid

1.6.2.1 Distribution

HA is widely distributed in the body. It occurs in both prokaryotes and eukaryotes. HA was first isolated from bovine vitreous humour at a yield of 0.7g/100 eyes (Meyer and Palmer 1934) and from umbilical cord (Meyer and Palmer 1936). It has also been isolated from synovial fluid, dermis and aqueous humour (Whistler and Smart 1953; Laurent and Fraser 1986) and rooster comb (Swann 1968).

HA is a high-molecular weight polysaccharide, present in varying amounts in all tissues and body fluids of higher animals and most abundantly in soft connective tissues. The highest concentration of HA were found in the umbilical cord, skin, vitreous humour and synovial fluid, reflecting the important physiological roles for this polysaccharide in these tissues. (Whistler and Smart 1953) (Table 1).

The concentration of HA in human skin during ageing was found to be reduced from newborn to infancy but remain stationary during middle age before dropping further during old age (Fleischmajer *et al.* 1972).

Tissue or fluid	Concentration (mg/l)
Rooster comb	7500
Human umbilical cord	4100
Human synovial fluid	1420 – 3600
Bovine nasal cartilage	1200
Human vitreous body	140 – 338
Human dermis	200
Rabbit brain	65
Human thoracic lymph	8.5 – 18
Human urine	0.1 – 0.5
Human serum	0.01 – 0.1

Table 1. Concentration of hyaluronan in tissues and body fluids (Laurent and Fraser 1992).

The analytical techniques for determination of HA concentration in different tissues are being continuously refined. In the first determination, 100μg of hyaluronan was required to measure the amounts of glucuronic acid and N-acetyl glucosamine using a modified carbazole reaction (Bitter and Muir 1962). The limit was improved to 1μg by the discovery of specific microbial enzymes that degrade hyaluronan into unsaturated disaccharides. The third generation techniques appeared in 1980, which involved the use of proteins with a specific affinity for hyaluronan to detect as little as 1ng of the polysaccharide (Tengblad 1980).

The molecular weight of HA ranges from 10^4 to 10^7 depending on the source, isolation procedure and method of determination (Shimada and Matsumura 1975; Laurent 1970) (Table 2).

Tissue	Molecular weight	technique
Human umbilical cord	3.4×10^6	Light- scattering
Bovine vitreous body	7.71×10^6 1.7×10^6	Light-scattering Sedimentation diffusion
Bovine synovial fluid	14×10^6	Light-scattering
Human synovial fluid Normal Rheumatoid	 6×10^6 $(2.7\text{-}4.5) \times 10^6$	 Light-scattering Light scattering
Rooster comb (main fraction)	1.2×10^6	Ultracentrifugation
Streptococcal cultures	0.115×10^6 0.93×10^6	Sedimentation & Viscosity

Table 2. Molecular weights of HA from different tissues (Laurent 1970).

The degree of polymerization varies with both species and different body tissues and fluids. The extraction method used for HA and degradation during fractionation of the tissue extract has shown different molecular values in certain experimental studies (Cleland 1970). There are a variety of techniques used for the molecular weight determination including light scattering, sedimentation, diffusion and

viscometry. The physical properties of HA are dependent on the Mwt and this is an important factor in its uses, with different Mwt products being used for different purposes (Biotechnology, 1986).

1.6.2.2 Sources of HA

HA can obtained by two methods: tissue extraction and bacterial fermentation.

A) Tissue extraction

HA is extracted mainly from rooster comb, human umbilical cords and bovine vitreous where its concentration is high. HA can be easily extracted from tissues in high amounts by aqueous extraction using water or salt solutions then using precipitation from the solution using water-miscible solvent such as ethanol or acetone or quaternary ammonium compound such as cetyl pyridium chloride (CPC) (Scott 1960; Laurent 1970; Fleishchmajer, 1973; Shimada and Matsumura 1975). The precipitates are then washed and dried or freeze-dried.

The isolated HA has been always associated with proteins and other polysaccharides. Sandson and Hammerman (1962) described the isolation of hyaluronate from the synovial fluid by either ultrafiltration or zone electrophoresis. They showed that hyaluronate consistently contained about 2% protein. Swann (1968) prepared the polysaccharide from rooster comb by CPC precipitation and found the protein content to be 0.35%. Despite the various methods employed for removal, such as

fractional precipitation (Scott 1960), chromatography on ion-exchangers and adsorbent (Swann 1968; Laurent 1970), small amounts of protein are still present in the extract and even trace amounts of these contaminants could affect the polysaccharide properties. In addition, large amounts of the material are required, as in the case of rooster comb, 5 kg are required to produce only 4g of purified HA (Biotech. 1986). Since the tissue extraction method is complex, expensive and time consuming, biotechnological techniques have been developed to provide an alternative source for protein-free HA by bacterial fermentation.

B) Bacterial fermentation

Group A and C Streptococci have been discovered to have a mucopolysaccharide in their capsule identical to HA and found to produce high levels of HA in cultures (Maclennan 1956; Woolcock 1974).

Bacterial HA is structurally identical to that derived from animal tissues (Wessels *et al.* 1991; Liesegang 1990) HA is one of only a few polysaccharides occurring naturally in both animals and bacteria (Liesegang 1990). Although most strains of group C streptococci produce both HA and hyaluronidase (the enzyme which degrades it), a small number of strains only form HA (Maclennan 1956). Streptococcus equi has been shown to yield useful amounts of HA and no hyaluronidase (Woolcock 1974). HA can be produced by microbial fermentation either by continuous bacterial fermentation or batch fermentation. Fermentech (Edinburgh, UK), who supplied the material used in this thesis, produces HA by

bacterial fermentation by continuous rather than a batch process. By altering the growing conditions of the culture in continuous fermentation, HA of different molecular weights can be produced (Liesegang 1990).

1.6.2.3 Depolymerization

HA from various animal tissues has been depolymerised by several methods.

Bothner *et al.* (1988) prepared HA fractions of low molecular weight by heat degradation, which involved exposure of solutions of polysaccharides at neutral pH to the high temperature (128°C) of an autoclave (Bothner *et al.* 1988). Exposure of HA to ultrasonic irradiation has also resulted in depolymerisation. High molecular weight cock's comb sodium hyaluronate solutions were reduced to about a half of their original molecular weight in about five minutes (Chabrecek *et al.* 1990). In the living body, HA is known to be degraded by either the specific enzyme hyaluronidase or by hydroxyl radicals.

During the first stage of the inflammation process, an enhancement of the capillary permeability permits the accumulation of polymorphonuclear leukocytes and other phagocytic cells at the inflammation site, resulting in activation of phagocytic cells by immune complexes and other inflammation generating compounds which result in the production of hydroxyl radicals. Production of hydroxyl radical has been implicated in the damage of organs, tissues and in the degradation of macromolecules such as HA. This explains the mechanism of HA degradation in acute inflammatory processes as in the inflamed joint fluid in rheumatoid arthritis,

which is probably due to the generation of hydroxyl radicals (Yui *et al.* 1992). There are three different types of hyaluronidase: leech, testicular and bacterial (Meyer 1971). Leech hyaluronidase can only affect HA or HA oligosaccharides.

1.6.2.4 Physiological roles of HA

At physiological pH, the molecule is fully charged with one negative residue per disaccharide unit (Laurent and Reed 1991). The total amount of HA in an adult man of 70kg, has been estimated to be 11-17g (Laurent and Reed 1991). HA has a half life of 2-6 min. in man. Its serum level is in the range of 10-100μg/l and the turnover is 10-100mg/day (Lebel 1991).

The level of HA varies during the day. There is an obvious increase in the level of HA in some diseases; 10-100 times the normal concentration has been reported in inflammatory diseases (rheumatoid arthritis) and liver diseases (primary biliary cirrhosis) which is due either to impaired hepatic uptake or to increased production (Laurent and Fraser 1986).

HA has been found attached to the cell membrane as part of a large aggregate with proteoglycans in cartilage or in its free state in fluids such as synovial fluid and vitreous humour (Laurent and Fraser 1986).

HA is synthesized both by mammalian cells and bacteria. Prehm (1984) has shown that HA is synthesized in the plasma membrane in contrast to other glycosaminoglycous which are synthesized in the cell's Golgi apparatus.

In bacteria, HA synthesis takes place in the protoplast membrane of streptococci

(Stoolmiller and Dorfman 1970).

Fraser *et al.* (1988) presented evidence that the lymph nodes are the main site of turnover of hyaluronan and additional degradation takes place in the liver (Smedsrød *et al.* 1991). The work of Laurent & Reed (1991) has furthermore shown that the polysaccharide can be endocytosed and degraded locally in tissues.

In cartilage, HA is bound in large aggregates with two other components, protoglycan monomers and link proteins.

The HA content of cartilage increases with age but with a decrease in aggregate size (Holmes *et al.* 1988) where its molecular weight becomes lower (Strachan *et al.* 1990).

HA is considered as a jelly–like matrix holding cells together and as a viscous substance it has a role in the tissue defense mechanism against invading bacteria and in restricting diffusion of injected poisons (Whistler 1953). HA acts in tissues as a barrier against rapid changes in water content due to its high resistance against water flow (Laurant and Fraser 1995). HA also binds water and acts as an osmotic buffer which gives it a role in tissue water homeostasis (Laurant 1970; Laurent and Fraser 1995). Due to its rheological properties and elastic behavior, it acts as a lubricant and shock-absorber in joints (Gibbs *et al.* 1968). Moreover, HA has a physiological role in cell proliferation and mitosis by facilitating cell detachment from the substrate, hence HA synthesis is found to be increased at this stage (Brecht *et al.* 1986). It has also been reported that HA plays an important role in many physiological functions *in vivo* including controlled permeation of molecules, cells, and cell differentiation (Yui *et al.* 1992) in addition to stimulation of phagocytosis (Laurent and Fraser 1986).

1.6.2.5 Cross-linked HA

Despite the use of HA in some medical fields, it has been precluded in many clinical applications because HA has a limited shelf life, and is rapidly degraded on administration.

Hylans are a family of chemically modified hyaluronans including hylan fluids, gels, micropartcles and membranes (sheets, coating) (Larsen and Balazs 1991). Through chemical modification of HA, a series of novel biopolymers can be formed with physico-chemical properties that are significantly different from those of HA, but they retain the desired biological properties of HA and have prolonged residence time on application.

HA esters can be prepared using standard methods for the esterification of carboxylic acids, such as the treatment of free HA with the desired alcohol in the presence of an appropriate catalyst. HA esters represent a new class of biomaterials, which are biocompatable, biodegradable, non-immunogenic and able to release HA *in situ* by enzymatic and/or chemical bond cleavage (Ghezzo *et al.* 1992).

Benedetti (1994) discussed the biocompatability of different HA esters, by examining the *in-vitro* and *in-vivo* behavoiur of HA ester after subcutaneous and intraperitoneal implantation in rats. The results showed the presence of very poor polymorphonuclear cell infiltration into samples removed four and seven days after implantation, which confirms the biocompatabilty of these esters. Moreover, it has been noticed that the degree of resorption is correlated to the degree of esterification.

HA esters can be processed into a number of physical forms where the esters proved to have prolonged residence times on application and extended shelf lifes.

Furthermore, the biodegradation rates of these polymers may be controlled, and as a consequence, the residence time in the body may be adjusted to meet specific clinical needs (Benedetti 1994). Therapeutic uses of cross-linked HA will be discussed in the following section.

1.6.2.6 Therapeutic and experimental uses of HA

HA is naturally occurring, biocompatable and biodegradable. Highly purified HA is a non-inflammatory, non-pyrogenic, non-cytotoxic and non-immunogenic polymer, and can be formulated in a variety of ways to enhance the natural functional activities in the body (Shah and Barnett 1992). HA has been initially developed for use in eye surgery. Its use was expanded to include a variety of medical applications including orthopedic surgery, rheumatology, plastic surgery and drug delivery vehicles. The use of HA and similar polymers in ophthalmology has been reviewed by Liesegang (1990). Tissue-extracted HA is routinely used in ophthalmic surgery, anterior segment surgery and, in particular, cataract removal and intraocular lens implantation. It has also been used as a solution to protect the cornea from mechanical damage following corneal transplantation. Furthermore, HA was used as an injection into the anterior chamber of the eye during cataract surgery serving to maintain anterior chamber space and to protect endothelial cell layers from mechanical damage by instruments and the inserted plastic implant (Balazs 1989; Liesegang 1990).

Sodium hyaluronate (Healon) was the first viscoelastic substance introduced

commercially (Liesegang 1990). It has been successfully used as a tear replacement for treatment of severe keratitis sicca (dry eye) (Deluise *et al.* 1984). HA is used in the treatment of arthritic joints where intra-articular injection into the joints of lame-horses was found to improve function and diminish symptoms (Weis and Balazs 1984). In humans, joints treated with HA showed improvement (Weis and Balazs, 1984). Pietrogrande *et al.* (1991) compared the activity of HA with 6 methyl prednisolone acetate (6-MPA) after the intra-articular administration to patients suffering from osteoarthritis of the knee. The results showed the effective role of HA to reduce pain symptoms and improve joint function. Moreover, HA was found to be superior to the standard treatment of 6-MPA injection, especially on a long term basis.

HA has also been found to be beneficial in wound healing, and is being investigated as a potential agent for this purpose (King *et al.* 1991). In foetal rabbit wounds, HA has been shown to heal without scar formation in a process resembling regeneration of tissues (Mast *et al.* 1991). HA is also being used as a component of cosmetics, and was included as an ingredient in several products including a softening lotion, moisturizing lotion and Estee Lauder's "Night repair" cream (Balazs and Band 1984). HA is increasingly being investigated as a drug delivery vehicle in the form of the polymer, esters or solutions. The bioavailabilty of pilocarpine in the eye was increased when a solution of HA was used as the vehicle (Camber and Edman 1989). This effect was shown to be dependent on both the concentration and molecular weight of HA and, consequently, the solution viscosity. Solutions of high molecular weight HA significantly increased the bioavilabilty of pilocarpine, while low molecular weight HA solutions gave no increase in bioavailabilty as compared to

control (HA-free pilocarpine) (Saettone *et al.* 1991).

Different HA-ester preparations have been investigated. In particular, microspheres have been evaluated as a drug delivery device to protect, carry and release many drugs including steroids and protein drugs (Ghezzo 1992; Benedetti 1994). The microspheres were perpared by two methods, first from simple esters of hyaluronic acid with hydrocortisone physically incorporated and second, from HA esterified by hydrocortisone. The release of hydrocortisone from HA microspheres was studied and it was found that physically entrapped hydrocortisone was released in about ten minutes while the release rate was slower when the drug was covalently bound to the polymer (>100 hours in some cases) (Benedetti *et al.* 1990).

The study of Benedetti (1994) included the investigation of microspheres derived from HA esters as a possible delivery system for vaginal administration of salmon calcitonin (SCT). Vaginal absorption of SCT was compared to that incorporated in microspheres, which were dispersed in a lipid base, and administered to rats and sheep. The results showed an enhanced absorption when the drug was incorporated into microspheres.

Films prepared from HA ester have been used to study the release of methylprednisolone (Kyyronen *et al.* 1992). Release was again found to be slower when the drug was covalently bound to the polymer than when physically incorporated (Kyyronen *et al.* 1992). HA coated polyvinylacetal (PVA) disks, loaded with serotonin, were found to improve drug delivery properties when compared to uncoated PVA disks as control (Larsen and Balazs 1991). In all these cases, the drug release rate is altered by the presence of HA, which provides a means of modifying and controlling release when present as a simple vehicle. Altering the

concentration and/or MWt gives different release rates (Camber and Edman 1989; Saettone *et al.* 1991), while as a carrier, changing the means of drug incorporation gives some control over release (Kyyronen *et al.* 1992).

The discovery of methods of determining HA concentrations in blood by simple techniques made HA play a role in the diagnostic field. It was reported that HA can be used as a diagnostic marker in many diseases such as a rheumatoid arthritis (Engstrom-Laurent & Laurent 1989), cirrhosis and in the detection of early graft rejection following liver transplant (Adams *et al.* 1989). HA has also been found in large quantities in bronchial lavage fluid of inflammatory pulmonary diseases (Nettelbladt *et al.*1989).

1.7 Microcapsules and microspheres for drug delivery

Microcapsules and microspheres are small particles (in the 1-500μm size range) which are used as carriers of drugs and other therapeutic agents.

The term "microcapsule" is used to define systems having a definite coating or shell encapsulating the contents. Depending on the manufacturing process, various types of microcapsule structures can be obtained. The term "microsphere" describes a monolithic spherical structure with the drug or therapeutic agent distributed throughout the matrix either as a molecular dispersion or as a dispersion of particles (Whateley 1993).

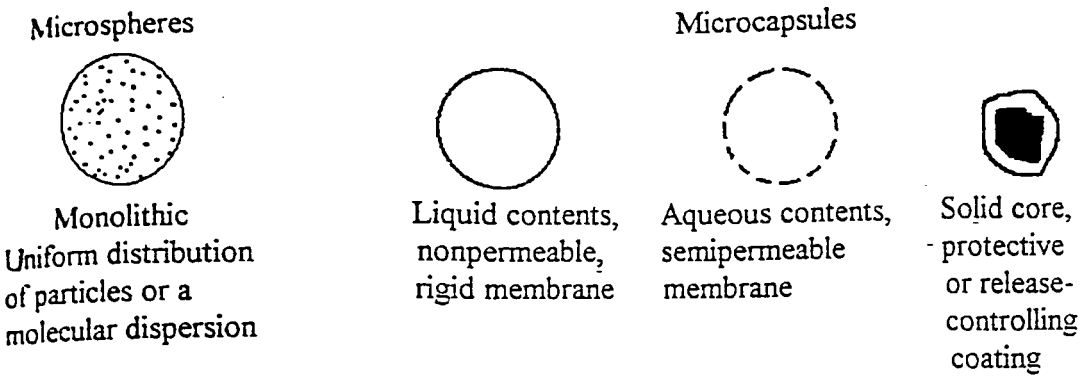


Fig. 3 Types of microspheres and microcapsules (Whateley 1996)

At present, there is no universally accepted size range that particles must have in order to be classified as microcapsules. However, researchers classify capsules smaller than 1μm as nanocapsules and those larger than 1000μm as macrocapsules. Commercial microcapsules typically have a diameter between 3 and 800μm and contain 10-90 wt. percent core.

The principle of using microspheres as drug delivery systems is based on the following:

1-Targeting: They provide 1st and 2nd order targeting by using the appropriate route of administration.

2-Sustained release of drug: They can prevent the release of drug during transit therefore reducing systemic toxicity, and the sustained release at the target site will improve therapeutic efficiency.

Microencapsulation involves a series of techniques for the entrapment of solids or liquids within polymers matrices. These processes have been utilized in many industries such as

food additives, cosmetics, adhesives and agricultural materials. Microencapsulation has been used in the pharmaceutical industry for the conversion of liquids to solids, improvement of drug stability, taste-masking, providing sustained release, reduction of gastric irritation and for producing targetable drug carriers (Bakan 1994; Thies 1992). The first pharmaceutical product using microencapsulation was a controlled release aspirin product (Bell 1966), to avoid the side effects of gastric irritation and haemorrhage.

The concept of preparing small particles that carry a core material trapped within a shell material dates back, at least, to spray-drying work carried out in the 1930s. The first commercial product that utilized such particles was carbonless copy paper (Thies 1996). Many techniques of production have been utilized pharmaceutically for both nano- and microparticles including emulsification-solvent evaporation, coacervation-phase separation, and spray drying (Tice 1989; Couvreur and Puisieux 1993).

The choice of the appropriate technique depends upon many factors, including their proposed function, desired size, physicochemical properties of the drug and polymer, compatability of the process conditions and the ability to produce the highest ratio of drug: polymer to minimize the mass administered to the patient without compromising release kinetics (Tice 1989; Zimmer and Kreuter 1995).

As the preparation of microspheres in this thesis was carried out using the emulsification-solvent evaporation technique so the focus will be on this method.

1.7.1 Emulsification-solvent evaporation method

The emulsification-solvent evaporation technique was first developed by Beck *et al.* (1979). Since then numerous studies have been carried out on this method. Watts *et al.* (1990) summarized this method into 2 main stages: initially, the emulsification of a polymer solution containing the drug (either dissolved or dispersed) into a second, immiscible liquid phase containing an emulsifier to form a dispersion of drug-polymer-solvent droplet. In the following step, the solvent is removed from the dispersed droplets by application of heat, vacuum or by allowing evaporation at room temperature, to leave a suspension of drug-containing polymer microspheres which are then separated by filtration or centrifugation, washed and dried (Fig. 4).

Although the solvent evaporation technique is conceptually simple, the physicochemical phenomena governing this process are very complex. This system is characterized by the presence of several interfaces through which mass transfer occurs during particle formation (Thies 1992; Benoit *et al.* 1996).

The solvent evaporation technique works best for water-insoluble drugs. There are 3 main systems for this technique

1.7.1A Oil-in-water emulsion system (o/w)

In this system, the polymer is dissolved in an organic solvent such as methylene chloride or chloroform. The active agent is dissolved or dispersed in the same medium. The mixture is then emulsified in an aqueous solution containing an

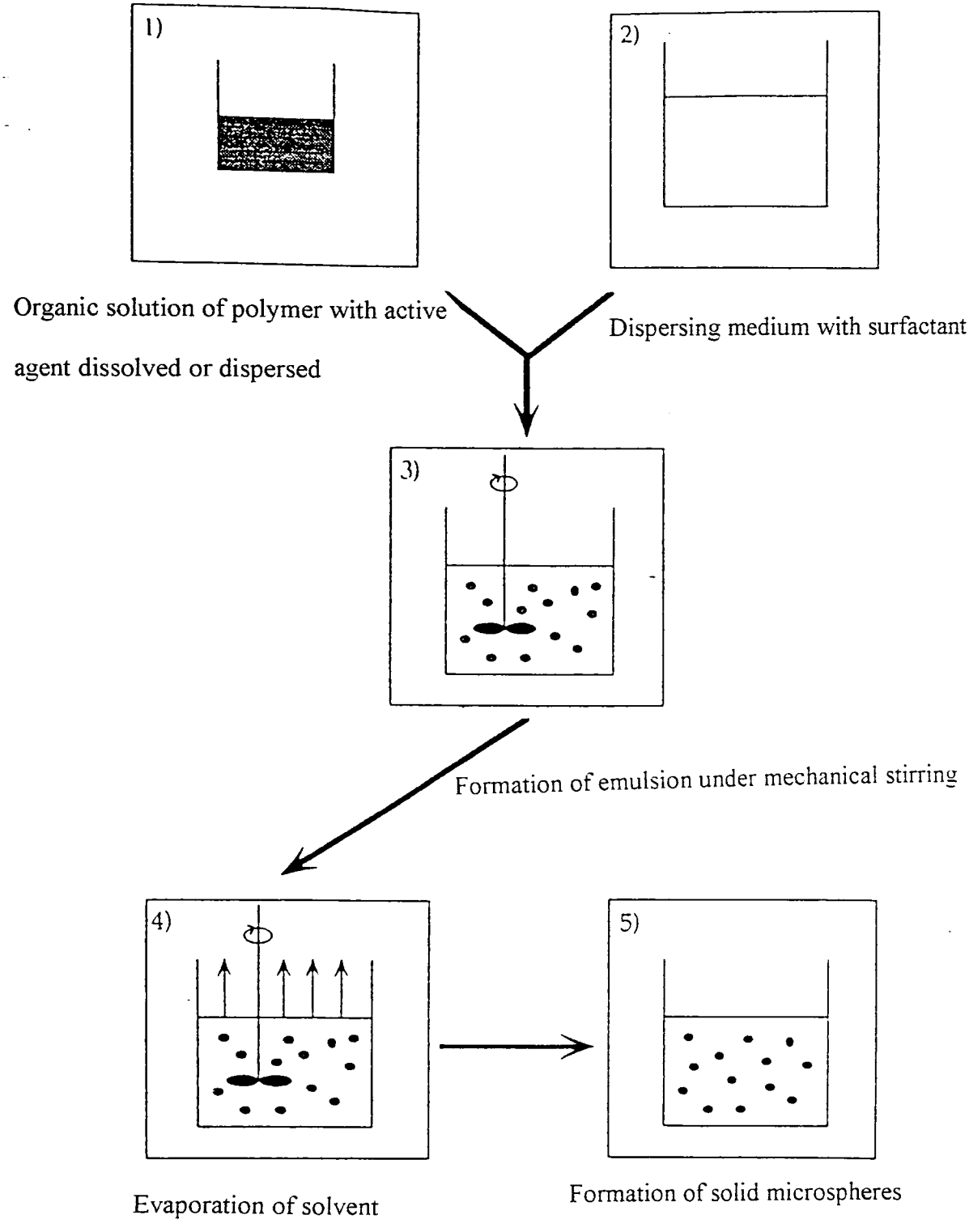


Fig.4 The principle of the preparation of microspheres using solvent evaporation technique (Benoit *et al.* 1996)

appropriate surfactant. This technique has been widely used for the encapsulation of different classes of drugs with PLGA (Bodmerier and McGinity 1987; Jeffery *et al.* 1991; Crossan and Whateley 1994). There are many advantages of this technique, including the simplicity of the process and easy clean-up requirements for the final product (Huang and Gheber-Sellassie 1989). In addition, this technique can be tailored to produce microspheres over a wide range of sizes from large (100μm) to nanoparticle size (<1μm), by controlling the stirring rate and process conditions. The produced microspheres have hydrophilic surface properties which allow ready re-suspension without aggregation (Whateley 1993). However, the major disadvantage of this technique is in encapsulating highly water soluble drugs, as the drug will rapidly partition from the more hydrophobic polymer-solution phase into the aqueous surrounding phase thus contributing to a substantial lowering of the yield. However, strategies permitting a reduction in the loss of water-soluble drugs have been proposed to increase microencapsulation yield, such as saturation of the continuous phase with the therapeutic agent (Spenlehauer *et al.* 1988) or adjustment of the pH of the aqueous phase to minimize drug solubility, as in the case of ionizable drugs (Bodmeier and McGinity 1987).

1.7.1B Oil-in-oil emulsion system (o/o)

This technique solves the problem for highly water soluble drugs using the solvent evaporation technique: the polymer and drug, contained in a polar solvent, (e.g. acetonitrile) are emulsified into an immisible lipophilic phase (e.g. liquid paraffin)

containing the surfactant. Water soluble drugs that have been encapsulated by this method including mitomycin C (Tsai *et al.* 1986) and the moderately water soluble phenobarbitone using both poly (L- lactic acid) and poly (DL-lactic acid) by Jalil and Nixon (1990).

However, serious drawbacks of the o/o method are the difficulty in obtaining small microspheres (<50μm) (Whateley 1993) and the problems in the final washing of the microparticles (Jones and Pearce 1995).

1.7.1C Water-in-oil-in-water (w/o/w)

For drugs which are very soluble in water a multiple emulsion method has proved to be very effective. In this method (Benoit *et al.* 1996), the active principle is incorporated in an aqueous solution, which is poured into an organic solution of the polymer to form an emulsion of the type water-in-oil (w/o). This primary emulsion is itself emulsified in an external aqueous phase leading to a multiple emulsion of the type water-in-oil-in-water (w/o/w). The organic phase acts as a barrier between the two aqueous compartments preventing the diffusion of the water soluble drug into the external aqueous phase; the subsequent evaporation of the organic solvent leaves an aqueous suspension of microspheres that can then be filtered and dried. This technique has been used successfully for the encapsulation of the highly soluble luteinizing hormone releasing hormone (LHRH) analogue, leuprolide acetate (Ogawa *et al.* 1988; Okada *et al.* 1989).

1.7.2 Microsphere characterization

1.7.2.1 Size and morphology

1.7.2.1A Size determination

Light scattering is the most commonly used routine technique for assessing the size and size distribution of microspheres. Size is one of the dominant factors determining the targeting ability of microspheres and their subsequent distribution *in vivo*. The microspheres in this project were analysed by laser diffraction using a Malvern Particle Sizer series 2600c. The principle of laser diffractometery was described by Muller (1991) and Washington (1992) and uses Fraunhofer diffraction of laser light scattered from particles in suspension to calculate a size distribution. The particles cause diffraction of the laser light through different angles and make a diffraction pattern of light rings of varying radii. The diffraction ring patterns are detected on a detector and used to calculate the size distribution.

The laser diffractometer (Fig. 6) consist of laser as a light source which is almost invariably a helium neon laser with a wavelength of 632.8μm and a power from 2 to 10 mW. It also has a beam expander which is used to produce a uniform parallel beam of 5-10mm in diameter to allow a useful sample volume to be illuminated. A Fourier transform lens (receiver lens) for the focusing of the scattered light onto the detector, a small measuring cell with an integral stirrer which contains the particle suspension are located at a certain distance from the receiver lens. The great advantage of laser diffractometers is that any transparent dispersion medium, either

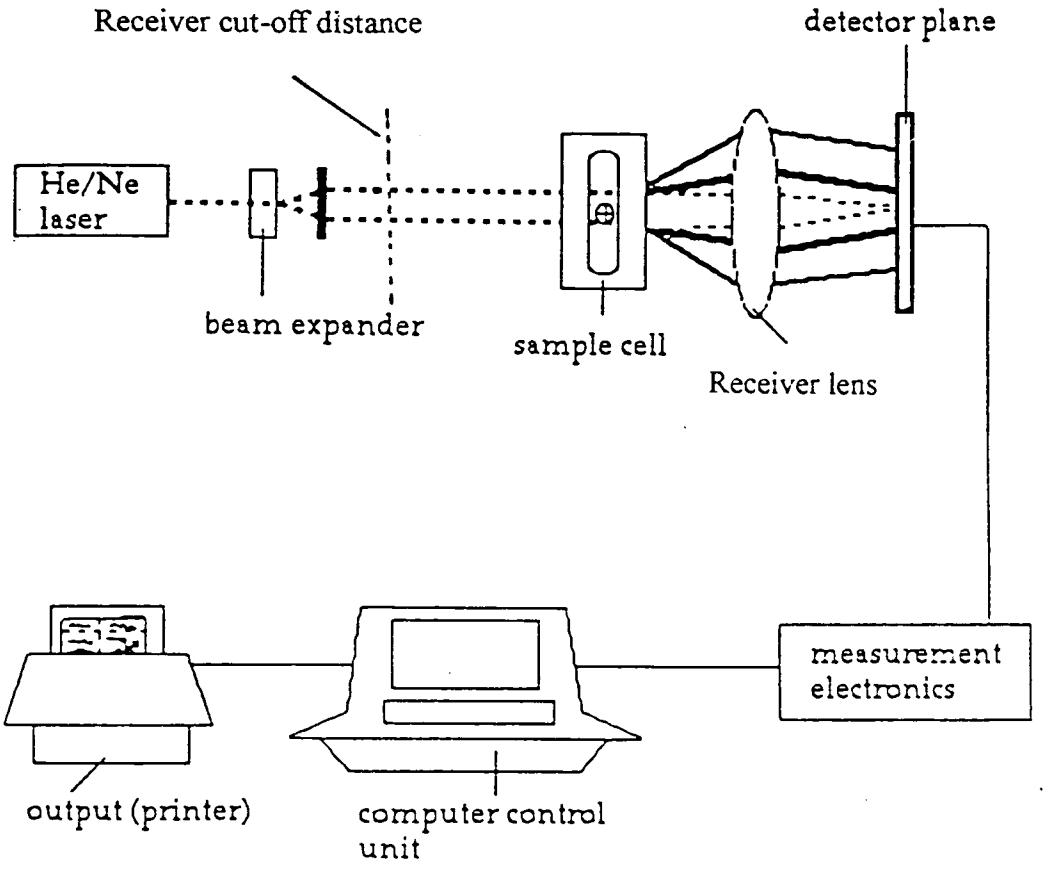


Fig. 5 Laser diffraction experimental for particle sizing (adpated from Malvern Instruments Particle Sizer Reference Manual 1993)

aqueous, organic or air can be used.

In general, the diameter of microspheres depend on the size of the microdroplets that are formed in the emulsion. There are several factors which control or influence the size of microspheres including the physicochemical properties of the incorporated drug, the viscosity of the organic and aqueous phase, the polymer and surfactant concentration and the stirring rate (Tice 1985; Muller 1991b; Jeffery *et al.* 1991).

1.7.2.1B Optical method of particle size analysis

The optical method of particle size analysis used in this project is light microscopy examination. This method is useful for assessing microsphere shape, general distribution and the presence of aggregates (Watts *et al.* 1991). The main advantage of the microscopy technique is that the particles are visible, and an exact observation of their nature can be made.

1.7.2.2 Drug loading

Drug loading is one of the parameters that can be adjusted to control the characteristics of the microspheres. The extent of incorporation of a drug into a microspherical system depends on both the input ratio of the core material to the wall material that is dissolved in the organic phase and on the solubility of the core material in the aqueous phase. If the drug is too soluble in aqueous medium, it will

be extracted from the oil microdroplets into the aqueous phase before the microcapsule walls have a chance to form. As a result, the core loading will be lower than expected. Moreover, the physical and chemical properties of the core material and polymeric wall material have a role in obtaining high drug loadings (as high as 80-90 wt % can be obtained). In turn, this affects the release process (Tice 1985).

1.7.2.3 Drug release

The rate at which the drug or therapeutic active agent appears in the systemic circulation or is made available to the target site is an important determinant of the drug pharmacodynamic effect.

The mechanism of drug release depends upon the physicochemical properties of the drug and polymer used in the manufacture of the device including type of matrix material, physicochemical nature of the incorporated drug, polymer molecular weight and drug payload (Heya *et al.* 1991; Couvreur and Puisieux 1993).

Controlled drug release systems for parentral administration are based on either physical (dissolution, diffusion and desorption) or chemical principles (chemical or biological degradation) or both.

Langer (1993) summarized the mechanisms of drug release from polymeric controlled release systems (microspheres). He explained that the diffusion of the drug through the polymer matrix is the rate limiting step for the release of the drug. The drug molecule diffuses by a random movement through the matrix down a

concentration gradient and the release rate can be determined by applying Fick's first law of diffusion

$$J = - D \, dc/dx$$

Where J is the flux, D is the diffusion coefficient and dc/dx is the drug concentration gradient.

As drug diffusion occurs in a random manner through pores in the polymeric matrix, Siegel (1990) remarked that the random nature of the pore network renders the mathematical modeling of drug release from these systems difficult, not only because of the changes in pore structure as diffusion progresses but also due to the fact that the polymer matrix may swell as the release study continues.

Avogoutakis and Nixon (1993) described the water uptake by PLGA when immersed in an aqueous medium. They noticed the water uptake by PLGA matrices increased steadily with time, causing the matrix to become more hydrophilic. They explained that as water penetrates the matrix, chain scission occurs, generating hydrophilic groups (hydroxyl and carboxyl end groups) which cause increases in matrix hydrophilicty. The researchers concluded that the continuous water uptake and considerable swelling of the polymer matrix affected drug release from PLGA devices.

If a drug is insoluble or poorly soluble in a polymer matrix, then the drug is likely to be dispersed as small particles throughout the polymer device. Higuchi (1963) described the kinetics of drug release in this particular case, and the Higuchi equation relies on Fick's first law of diffusion described above

$$Q = [CsDs \, (2A-Cs) \, t]^{1/2}$$

Where Q is the amount of drug released at time t, Cs is the solubility of the drug in the polymer, A is the amount of the drug present at the beginning of the experiment and Ds is the diffusion coefficient.

The microsphere manufacturing process attempts to achieve a balance between microsphere biodegradability (so as to permit drug to be released and repeated dosing to occur) and microsphere stability (so as to achieve adequate product presentation and prolonged half-lives).

1.8 AIM OF RESEARCH

The aim of this research was to prepare biodegradable microspheres using both the synthetic polymer, PLGA, and a natural polymer, hyaluronic acid (HA), using the emulsification-solvent evaporation technique.

The physicochemical properties of both microspheres such as particle size, incorporation and *in vitro* release studies of the anti-inflammatory drug, dexamethasone, from PLGA and HA microspheres were to be studied. Dexamethasone was chosen because of its potential applications for sustained delivery in medicine and also because it has been used previously in our laboratories for controlled delivery to the brain for the treatment of cerebral oedema associated with brain tumours.

HA microspheres were also to be chemically modified by esterification using carbodiimide and cysteine methyl ester as cross linking agents to improve the microsphere stability.

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Core material

Dexamethasone powder (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

2.1.2 Polymers

Poly(d,l-lactide-co-glycolide) (85:15) (PLGA), molecular weight (MW) 62,000 (Medisorb, Cincinnati, Ohio, USA)

Solid sodium hyaluronate (HA), MW 1.64×10^6 was provided by Fermentech Medical Limited, Edinburgh, Scotland, UK.

2.1.3 Emulsifying agents

Polyoxyethylene (20) sorbitan monooleate (Tween 80) (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

Sorbitan monosterate (Span 60) (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

2.1.4 Cross-linking agents

1-Ethyl-3-(3-dimethylamine-propyl) carbodiimide hydrochloride(EDAC), $C_8H_{17}N_3.HCl$ (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

L-cysteine methyl ester hydrochloride, $C_4H_9NO_2.HCl$ (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

2.1.5 Additional materials

Dichloromethane (DCM) AnalaR grade (Merck Ltd., Poole, England, UK)

Phosphate buffer saline tablets (pH=7) (PBS) (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

Cottonseed oil (Sigma-Aldrich Company Ltd., Gillingham, England, UK)

Hydrochloric acid (0.1M) diluted from stock solution

Ethanol (J Mills Limited, Liverpool, England, UK)

Methanol HPLC grade (Rathburn Chemicals Limited, UK)

2.2 Methods

2.2.1 Preparation of blank microspheres of poly(lactic-co-glycolic acid)

PLGA blank microspheres were prepared by using the oil-in-water (o/w) emulsion solvent evaporation technique adapted from the process described by Beck *et al.* (1979). 300 mg of PLGA (85:15) was dissolved in 15ml of DCM to form the oil phase. The aqueous phase was prepared by dissolving 2g of Tween 80, which acts as an emulsion stabilizer, in 100 ml distilled water. The aqueous solution was mixed for 2 hrs using a magnetic stirrer to ensure complete dissolution. For microsphere preparation, a laboratory emulsifier (Silverson machines Ltd., Chesham, Bucks., England, UK) was used. The aqueous phase was placed in an ice bath and the speed of the mixer was gradually increased to avoid foam formation. The oil phase was added dropwise to the aqueous phase. The speed was increased to 6000 rpm and the mixing was continued for 10 mins. The emulsion was then stirred overnight at room temperature using a magnetic stirrer to ensure solvent evaporation and microsphere formation. The emulsion was centrifuged at 3000 rpm for 20 mins using a BTL Bench Centrifuge. The supernatant was discarded. The pellet was washed by centrifugation a further twice in fresh distilled water. The microspheres were re-suspended in distilled water.

2.2.2 Incorporation of dexamethasone into PLGA microsphere

Using the o/w emulsion solvent evaporation technique, 240mg PLGA (85:15) and 60mg dexamethasone were mixed together and mixed in 15ml DCM to achieve a drug loading of 20%. Sonication was carried out in an ultrasonic bath to ensure uniform distribution of dexamethasone throughout the oil phase. The aqueous phase was prepared as described previously by dissolving 2g of Tween 80 in 100ml distilled water followed by stirring for 2 hrs. The Silverson stirrer was used with a gradual increase in speed to avoid foam formation until 6000 rpm was reached, when the oil phase was added dropwise to the aqueous phase. The mixing was continued for 10 mins and the emulsion stirred overnight using a magnetic stirrer to allow solvent evaporation and microsphere formation. The microspheres were isolated by centrifugation for 20 mins at 3000 rpm. The supernatant was discarded, and the microspheres were washed by centrifugation a further twice in fresh distilled water. Sonication of the pellet suspension was carried out for particle de-aggregation and efficient washing. The microspheres were re-suspended in distilled water.

2.2.3 Preparation of hyaluronic acid blank microspheres

Using the water in oil (w/o) emulsion solvent evaporation technique, 100mg of HA (MW 1.64×10^6) was hydrated in 10ml of distilled water to form a 1% HA solution. The sample was left overnight for complete hydration, to form the aqueous phase. The

oil phase was prepared by dissolving 1g of Span 60, which acts as an emulsion stabilizer, in 100ml cottonseed oil and heated at 70°C to ensure complete dissolution. Mixing of the microsphere preparation was performed by using a Silverson laboratory emulsifier. The speed of the mixer emulsifier was gradually increased until it reached 3700 rpm. The aqueous phase was then added dropwise to the oil phase. Mixing was continued for 3hrs; heat generated from mixing raised the temperature to 60-70°C. The emulsion was left overnight and centrifuged at 3000 rpm for 30mins using a BTL Bench centrifuge. The supernatant was discarded, and the microspheres were washed a further three times with absolute alcohol. Sonication was carried out for 10mins during the washing process. The microspheres were re-suspended in absolute alcohol.

2.2.4 Incorporation of dexamethasone into HA microspheres

Using the w/o emulsion solvent evaporation method, 20mg of dexamethasone were dispersed in 10ml of distilled water. Sonication was carried out for 5 mins to ensure particle de-aggregation. 100mg of HA was added to the dexamethasone dispersion to form the aqueous phase. This was left overnight for complete hydration (the vial was placed on a magnetic stirrer for 10 mins to ensure uniform distribution). The oil phase preparation, mixing, isolation, washing and resuspension of the microspheres were as described in section 2.2.3.

2.2.5 Characterisation of PLGA and HA microspheres

2.2.5.1 Particle size measurements

Particle size and particle distribution of the microspheres were studied using the Malvern Particle Sizer series 2600c (Malvern Instruments, Malvern, UK) with a 63mm focal length lens. A small amount of the microsphere suspension was diluted either with distilled water or absolute alcohol, for PLGA or HA microsphere, respectively, to achieve an appropriate concentration in the cell. This was indicated by an obscuration in the effective range (0.15 - 0.3). Background and sample measurements were processed by computer and the 50% size average was reported.

The PLGA microsphere suspension was pre-frozen in a bath of liquid nitrogen and left overnight in a freeze-drier (Modulyo Freeze Dryer, Edwards, UK). A small amount of the freeze-dried microspheres were hydrated with distilled water and sized by laser diffractometry.

The HA microsphere suspension was poured in a petri-dish and left overnight for drying. A small amount of the dried microspheres were dispersed with absolute alcohol and their particle size was assayed using Malvern Particle Sizer.

2.2.5.2 Morphological examination

Morphological studies of the different types of microspheres were performed under the light microscope using the Polyvar (Reichert-Jung, Austria).

A small amount of dried microspheres was placed on a glass slide. A drop of water or absolute alcohol, for PLGA or HA microspheres, respectively, was added. The particles were observed under x250, x100.

2.2.5.3 Drug loading

A) Standard curve preparation

2.5mg of dexamethasone powder was accurately weighed and dissolved in 100ml PBS (pH7) to produce a solution of 0.025mg/ml. Serial dilution of this stock solution in PBS were carried out to prepare dexamethasone standards in the range of 0.00078mg/ml to 0.025mg/ml. The samples were analysed using a Milton Roy Spectronic 1201 spectrophotometer at 240nm and absorbance was plotted against dexamethasone concentration.

B) Procedure for drug loading determination

(i) PLGA microspheres

20mg of PLGA-dexamethasone microspheres was dissoved in 1ml acetonitrile. 9ml of methanol was added to precipitate the polymer. The sample was centrifuged at 3000 rpm for 15 mins and 1ml of the supernatant was diluted to 100ml with water:methanol solution (80:20 v/v) which was then analysed by UV spectrophotometer at 240nm against a blank of water/methanol solution (80:20v/v).

The Dexamethasone concentration was then calculated from the standard curve and hence the dexamethasone content of the sample of microspheres.

(ii) HA microspheres

The same procedure was used as for PLGA microspheres; i.e. 5mg of HA-dexamethasone microspheres were disolved in 1ml acetonitrile, 9ml methanol was added. This system resulted in a clear solution. 1ml of this solution was taken and diluted to 100ml with water:methanol (80:20v/v) and the absorbance was determined at 240nm.

2.2.5.4 Rate of drug release

The *in vitro* release of dexamethasone from microspheres was studied as follows: 20mg of PLGA-drug microspheres or 7mg of dexamethasone loaded HA microspheres were added to 30ml PBS (pH7) in a conical flask and shaken at 37°C using an orbital shaker incubator (LH Engineering Co. Ltd. MKV, England, UK) at 100 rpm. At specific times, a 10ml sample was removed and replaced by an equivalent volume of fresh PBS. The removed sample was centrifuged at 3000 rpm for 15 mins and the absorbance of dexamethasone in the supernatant was determined spectrophotometrically at 240nm.

% of drug release was calculated according to the following equation:

$$\% \text{ release} = \frac{\text{mass released}}{\text{actual drug loading}} \times 100$$

2.2.5.5 Stability of HA microspheres

A) Blank microspheres

The stability of the size of HA microspheres was examined with respect to time as follows: 5 mg of HA microspheres was dispersed into 6 ml of either distilled water or PBS. Particle size analysis was carried out at different time intervals. The sample was left shaking between measurements.

Light microscopy examination was performed at similar time intervals.

B) Loaded microspheres

After 2 weeks of release studies of loaded HA microspheres, a 10 ml sample was used for particle size analysis. The same procedure was repeated after 27 and 31 days.

2.2.6 Cross-linking of HA microspheres

Cross-linking of HA microspheres was carried out according to the method of Courtney and Eccleston (Courtney, 1993 PhD thesis). Using the w/o solvent evaporation technique, 100 mg of HA was hydrated into 10 ml of distilled water to form a 1% HA solution and left overnight for complete hydration. A 1% of carbodiimide and 1% cysteine methyl ester were prepared in distilled water. Carbodiimide and cysteine methyl ester act as cross-linking agents. The pH of HA solution was adjusted to 4.70±0.05 by the addition of 0.1M hydrochloric acid dropwise. 150ml of cottonseed oil was used as the oil phase which was placed in a metal beaker. The beaker was fixed into another metal beaker containing water and the system was placed on a hot plate (Hot Plate, Gallenkump, England, UK). For the cross-linked microsphere preparation, a Paddle Stirrer (Stuart Scientific Co. Ltd., UK) was used at 500 rpm. Equal (1.5ml) volumes of HA, carbodiimide and cysteine methyl ester solutions were well mixed to form 4.5ml of the aqueous phase. The aqueous phase was immediately added to the oil

temperature was gradually increased. It was kept constant at 60-70°C and mixing was continued for 3 hrs. During the 3 hr stirring, examination of the formation of cross-linked microspheres was carried out using a light microscope. After microsphere preparation, the oil was left at room temperature for cooling. The cooled oil was centrifuged at 3000 rpm for 20 mins using a bench centrifuge. The supernatant was discarded and the pellet was washed by centrifugation a further 3 times in cyclohexane to remove excess oil. Light microscopy examination of the cross-linked microspheres was performed before and after washing. The microspheres were re-suspended with absolute alcohol and particle size analysis was carried out (either in absolute alcohol or water) using the Malvern Particle Sizer.

In a different experiment, washing of the HA cross-linked microspheres was carried out using either cyclohexane or absolute alcohol for comparison purposes.

CHAPTER THREE

3 RESULTS AND DISCUSSION

The development of a procedure for the microencapsulation of pharmaceuticals using the emulsification solvent evaporation technique involves the selection of the two liquid phases: the dispersed phase and the continuous phase. The dispersed phase solvent should have the ability to dissolve the polymer and ideally to dissolve the drug. In addition, it must have a lower boiling point than the continuous phase solvent. It is essential that the continuous phase is immiscible with the dispersed phase solvent and unable to dissolve the polymer, in addition to its low solubility for the drug (Watts *et al.* 1990).

According to the above factors, the o/w emulsion solvent evaporation method was chosen as a suitable technique for the preparation of PLGA microspheres whereas the w/o emulsion solvent evaporation technique was used for the preparation of HA microspheres as described in Materials and Methods.

3.1 PLGA microspheres

Numerous natural and synthetic materials have been used as matrices for microspheres. One of the most commonly employed matrices is poly(lactic-co-glycolic acid) since it degrades slowly by hydrolysis to lactic and glycolic acid which are safe biocompatables and non toxic body metabolites (Muller, 1991b).

Blank and dexamethasone loaded PLGA microspheres were prepared using the oil in water emulsion solvent evaporation technique. DCM was used as an organic solvent because of its high volatility that facilitates easy removal by evaporation and it has good solubility towards a range of encapsulating polymers (Watts *et. al.* 1990).

However, using the Silverson mixer in this technique has the disadvantage of generating heat during emulsification. To overcome this problem, both a cooling system and short emulsification time were used.

In loaded PLGA microspheres, sonication was necessary to achieve de-aggregation and a uniform dispersion of the drug throughout the oil phase.

3.1.1 Particle size measurements

Size and size distribution measurements are important aspects of the characterization of microspheres since they play a dominant role in the determination of the release and targeting ability of microspheres and their subsequent distribution *in vivo*.

Blank and loaded PLGA microspheres, which were prepared by emulsification at 6000 rpm, showed a narrow size of distribution. A relatively small particle size was obtained with both blank microspheres [mean diameter of 2.74 and 2.78μm; Fig. 1 (a and b)] and loaded PLGA microspheres [mean diameter of 2.72, 4.13, 2.74 and 4.96μm; Fig. 2(a and b) and 3(a and b)] as measured by the Malvern Particle Sizer.

Comparing particle size distribution of the different PLGA microspheres before and after drying, it is clear that there is a slight change within the same batch of

microspheres (Fig. 1a, 2a and 3a versus 1b, 2b and 3b, respectively). The observed tail after drying could be due to the presence of water residue and thus particle aggregation. Therefore, over-night freeze-drying may not be enough to completely dry the sample. The results also showed that there is no obvious difference in the average size of the microspheres, whether they were non-loaded or drug-loaded (Fig. 1-3); thus, it can be seen that the presence of the drug does not affect the size of the microspheres.

3.1.2 Morphological examination

Preparation of microspheres using the oil in water emulsifying technique typically produced PLGA microspheres that have a round, spherical geometry either with or without the incorporation of dexamethasone. However, sonication of the re-suspended freeze-dried microspheres was necessary to de-aggregate the particles before carrying out the morphological examination. Most of the microspheres, examined under the light microscope, were in the range of 2-5μm in the case of blank microspheres (Fig.4) and of 4-10μm in loaded microspheres (Fig. 5 and 6) which were consistent with the Malvern results (Fig. 1, 2 and 3).

3.1.3 λ_{max} of dexamethasone

A sample of dexamethasone was scanned using UV spectrophotometer to determine its $\lambda_{max.}$ (Fig. 7) which was 241nm. The result is similar to that quoted in the B.P. (1993).

Moreover, no interference was obtained between dexamethasone and water or phosphate buffered saline.

3.1.4 Calibration curve of dexamethasone

Dexamethasone concentrations and their respective absorbances at 240nm are represented as shown in Fig. 8. A linear relationship was obtained with a correlation coefficient of 0.9998 and a slope of 37.9. A good linear relationship was obtained with the line going through the origin.

3.1.5 Drug loading and *in vitro* release studies

The results of drug loading and *in vitro* release are shown as the mean of two experiments. Drug loading was calculated both from the theoretical drug loading and actual drug loading resulting from the total content studies. The level of drug loading achieved was 15.5% with an average entrapment efficiency of 77.5%.

The release of dexamethasone from PLGA microspheres was examined by collecting a sample at different time intervals and analysed spectrophotometrically at 240nm as described in Materials and Methods. Fig. 9 shows an initial rapid release known as the "burst release" of about 25% which took place during the first 3 hrs. The initial fast release is due to rapid dissolution of the drug particles on or near the surface of the microspheres. A slower release phase was followed caused by the time required for

water to penetrate into the polymer matrix and diffusion of drug through the polymer system to the external fluid. Only about 30% of the loaded dexamethasone was released over the 200hrs of the experiment. The remainder of dexamethasone is assumed to be in the interior of the microspheres which is not accessible to the external phase. This material would only be released when the microsphere structure collapses following hydrolytic degradation of the PLGA.

MALVERN Series 2600 SB.0D Easy Mode Tue 3 Dec 1996 5:05 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 0.34
				36.3	0.0	31.4	100	6.18	0.0	5.33	99.7	
				31.4	0.0	27.1	100	5.33	0.0	4.60	99.7	D[4,3]
				27.1	0.0	23.3	100	4.60	0.0	3.97	99.7	2.68µm
				23.3	0.0	20.2	100	3.97	0.0	3.42	99.7	
118	0.0	102	100	20.2	0.0	17.3	100	3.42	10.7	2.95	89.0	D[3,2]
102	0.0	88.2	100	17.3	0.0	15.0	100	2.95	73.2	2.55	15.8	2.62µm
88.2	0.0	76.0	100	15.0	0.0	12.9	100	2.55	0.0	2.19	15.8	
76.0	0.0	65.6	100	12.9	0.0	11.1	100	2.19	11.1	1.90	4.7	D[v,0.9]
65.6	0.0	56.6	100	11.1	0.1	9.63	99.9	1.90	4.7	1.64	0.0	2.97µm
56.6	0.0	48.8	100	9.63	0.1	8.31	99.8	1.64	0.0	1.41	0.0	
48.8	0.0	42.1	100	8.31	0.1	7.16	99.8	1.41	0.0	1.22	0.0	D[v,0.1]
42.1	0.0	36.3	100	7.16	0.0	6.18	99.7	1.22	0.0	0.32	0.0	2.03µm

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5]
	Log. Diff. = 6.330		2.74µm
Focal length = 63 mm	Obscuration = 0.2611	Volume Conc. = 0.0016%	
Presentation = pil	Volume distribution	Sp.S.A 2.2910 m²/cc.	Shape OFF

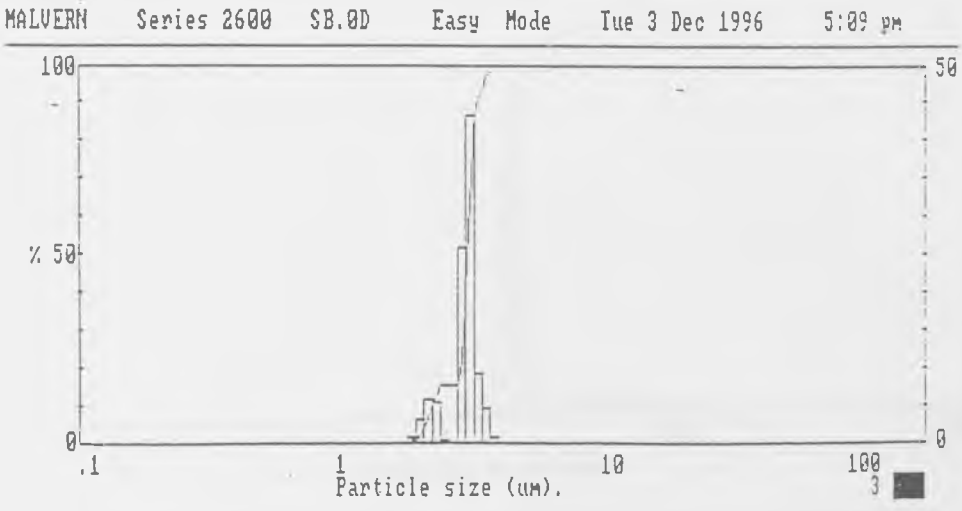


Fig.1a. Size distribution histogram of PLGA(85:15) microspheres before drying.

MALVERN Series 2600 SB.0D Easy Mode Mon 18 Nov 1996 5:45 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 0.80
				36.3	0.0	31.4	100	6.18	2.1	5.33	91.2	
				31.4	0.0	27.1	100	5.33	1.5	4.60	89.7	D[4,3] 3.23µm
				27.1	0.0	23.3	100	4.60	1.7	3.97	88.0	
				23.3	0.0	20.2	100	3.97	3.2	3.42	84.8	
118	0.0	102	100	20.2	0.0	17.3	100	3.42	11.5	2.95	73.3	D[3,2] 2.94µm
102	0.0	88.2	100	17.3	0.0	15.0	100	2.95	59.1	2.55	14.2	
88.2	0.0	76.0	100	15.0	0.0	12.9	100	2.55	14.1	2.19	0.0	
76.0	0.0	65.6	100	12.9	0.1	11.1	99.8	2.19	0.0	1.90	0.0	D[v,0.9] 4.75µm
65.6	0.0	56.6	100	11.1	0.4	9.63	99.4	1.90	0.0	1.64	0.0	
56.6	0.0	48.8	100	9.63	1.2	8.31	98.2	1.64	0.0	1.41	0.0	
48.8	0.0	42.1	100	8.31	2.3	7.16	95.9	1.41	0.0	1.22	0.0	D[v,0.1] 2.51µm
42.1	0.0	36.3	100	7.16	2.6	6.18	93.3	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 2.78µm
	Log. Diff. = 6.471		
Focal length = 63 mm	Obscuration = 0.1893	Volume Conc. = 0.0014%	
Presentation = pil	Volume distribution	Sp.S.A 2.0380 m²/cc.	Shape OFF

MALVERN Series 2600 SB.0D Easy Mode Mon 18 Nov 1996 5:46 pm

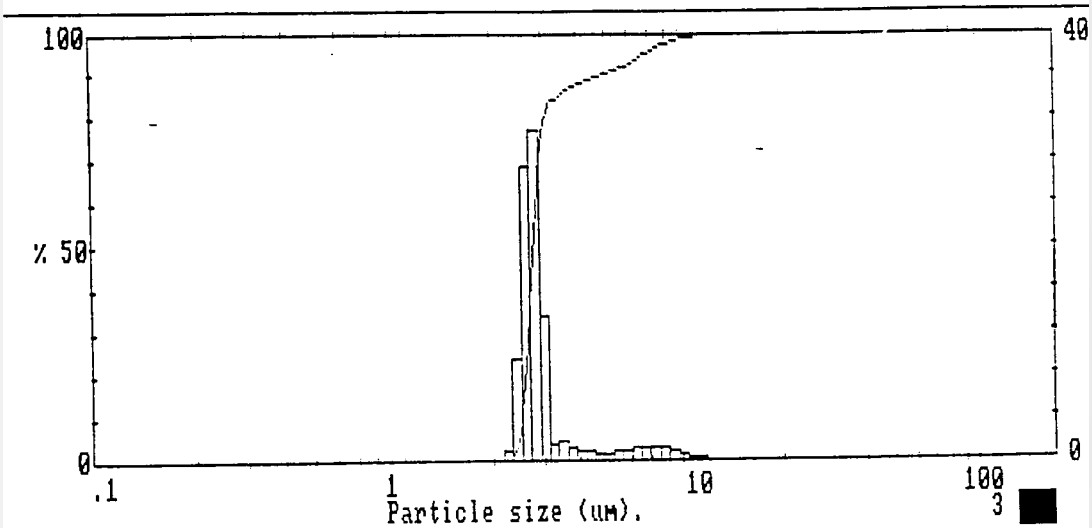


Fig.1b. Size distribution histogram of PLGA(85:15) microspheres after drying.

MALVERN Series 2600 SB.0D Easy Mode Tue 3 Dec 1996 4:22 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 0.39
				36.3	0.0	31.4	100	6.18	0.0	5.33	100	
				31.4	0.0	27.1	100	5.33	0.0	4.60	100	D[4,3] 2.59µm
				27.1	0.0	23.3	100	4.60	0.0	3.97	100	
				23.3	0.0	20.2	100	3.97	0.0	3.42	100	
118	0.0	102	100	20.2	0.0	17.3	100	3.42	11.3	2.95	88.7	D[3,2] 2.51µm
102	0.0	88.2	100	17.3	0.0	15.0	100	2.95	62.6	2.55	26.2	
88.2	0.0	76.0	100	15.0	0.0	12.9	100	2.55	0.0	2.19	26.2	
76.0	0.0	65.6	100	12.9	0.0	11.1	100	2.19	18.3	1.90	7.8	D[v,0.9] 2.98µm
65.6	0.0	56.6	100	11.1	0.0	9.63	100	1.90	7.8	1.64	0.0	
56.6	0.0	48.8	100	9.63	0.0	8.31	100	1.64	0.0	1.41	0.0	
48.8	0.0	42.1	100	8.31	0.0	7.16	100	1.41	0.0	1.22	0.0	D[v,0.1] 1.93µm
42.1	0.0	36.3	100	7.16	0.0	6.18	100	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 2.72µm
	Log. Diff. = 6.266		
Focal length = 63 mm	Obscuration = 0.2533	Volume Conc. = 0.0014%	
Presentation = pil	Volume distribution	Sp.S.A 2.3861 m²/cc.	Shape OFF

MALVERN Series 2600 SB.0D Easy Mode Tue 3 Dec 1996 4:24 pm

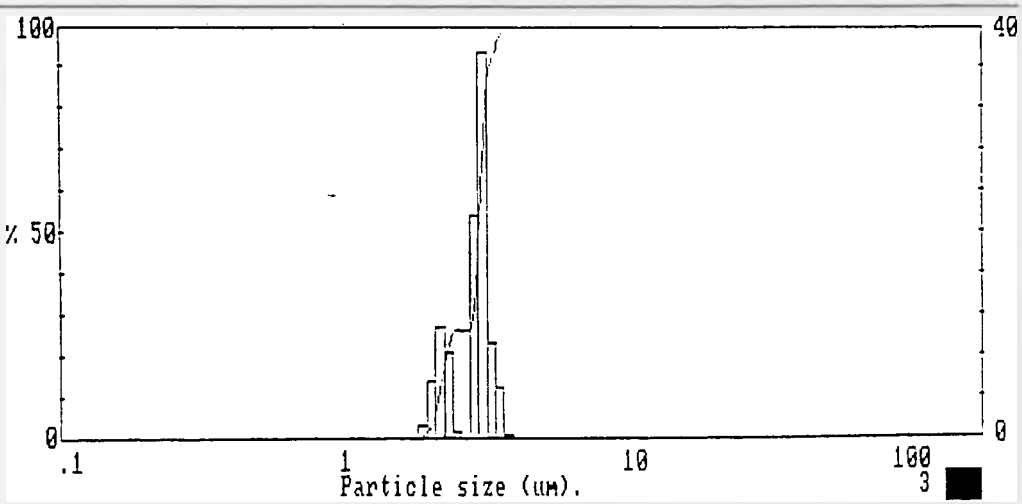


Fig.2a. Size distribution histogram of 16.78% dexamethasone loaded PLGA(85:15) microspheres before drying.

MALVERN Series 2600 SB.0D Easy Mode Mon 9 Dec 1996 2:10 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 1.62
				36.3	0.0	31.4	100	6.18	7.0	5.33	61.7	
				31.4	0.0	27.1	100	5.33	6.9	4.60	54.8	D[4,3] 5.20µm
				27.1	0.0	23.3	100	4.60	6.5	3.97	48.3	
				23.3	0.0	20.2	100	3.97	6.4	3.42	41.9	
118	0.0	102	100	20.2	0.0	17.3	100	3.42	15.4	2.95	26.5	D[3,2] 4.10µm
102	0.0	88.2	100	17.3	0.1	15.0	99.9	2.95	22.2	2.55	4.3	
88.2	0.0	76.0	100	15.0	1.1	12.9	98.8	2.55	4.3	2.19	0.0	
76.0	0.0	65.6	100	12.9	2.8	11.1	96.0	2.19	0.0	1.90	0.0	D[v,0.9] 9.38µm
65.6	0.0	56.6	100	11.1	4.9	9.63	91.1	1.90	0.0	1.64	0.0	
56.6	0.0	48.8	100	9.63	6.9	8.31	84.2	1.64	0.0	1.41	0.0	
48.8	0.0	42.1	100	8.31	7.8	7.16	76.4	1.41	0.0	1.22	0.0	D[v,0.1] 2.69µm
42.1	0.0	36.3	100	7.16	7.6	6.18	68.7	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 4.13µm
	Log. Diff. = 6.516		
Focal length = 63 mm	Obscuration = 0.2462	Volume Conc. = 0.0027%	
Presentation = pil	Volume distribution	Sp.S.A 1.4617 m²/cc.	Shape OFF

MALVERN Series 2600 SB.0D Easy Mode Mon 9 Dec 1996 2:11 pm

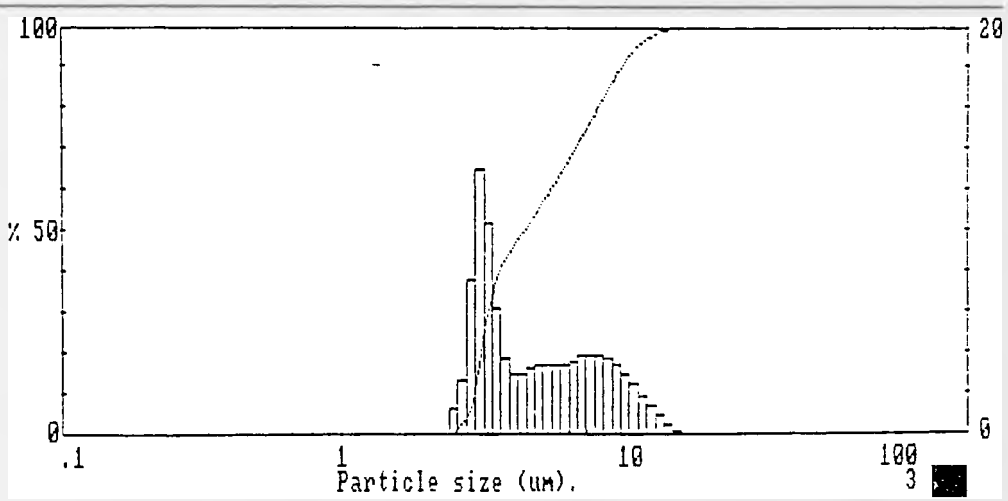


Fig.2b. Size distribution histogram of 16.78% dexamethasone loaded PLGA(85:15) microspheres after drying.

MALVERN Series 2600 SB.0D Easy Mode Tue 7 Jan 1997 2:53 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 0.15
				36.3	0.0	31.4	100	6.18	0.0	5.33	99.8	
				31.4	0.0	27.1	100	5.33	0.0	4.60	99.8	D[4,3] 2.73µm
				27.1	0.0	23.3	100	4.60	0.0	3.97	99.8	
				23.3	0.0	20.2	100	3.97	0.0	3.42	99.8	
118	0.0	102	100	20.2	0.0	17.3	100	3.42	8.4	2.95	91.5	D[3,2] 2.68µm
102	0.0	88.2	100	17.3	0.0	15.0	99.9	2.95	80.7	2.55	10.8	
88.2	0.0	76.0	100	15.0	0.0	12.9	99.9	2.55	3.6	2.19	7.1	
76.0	0.0	65.6	100	12.9	0.0	11.1	99.9	2.19	5.0	1.90	2.1	D[v,0.9] 2.94µm
65.6	0.0	56.6	100	11.1	0.0	9.63	99.9	1.90	2.1	1.64	0.0	
56.6	0.0	48.8	100	9.63	0.0	8.31	99.9	1.64	0.0	1.41	0.0	
48.8	0.0	42.1	100	8.31	0.0	7.16	99.8	1.41	0.0	1.22	0.0	D[v,0.1] 2.53µm
42.1	0.0	36.3	100	7.16	0.0	6.18	99.8	1.22	0.0	0.32	0.0	

Source =	:Sample	Beam length = 14.3 mm	Model indp
		Log. Diff. = 6.418	
Focal length =	63 mm	Obscuration = 0.3212	Volume Conc. = 0.0023%
Presentation =	pil	Volume distribution	Sp.S.A 2.2352 m²/cc.

D[v,0.5] 2.74µm

Shape OFF

MALVERN Series 2600 SB.0D Easy Mode Tue 7 Jan 1997 2:53 pm

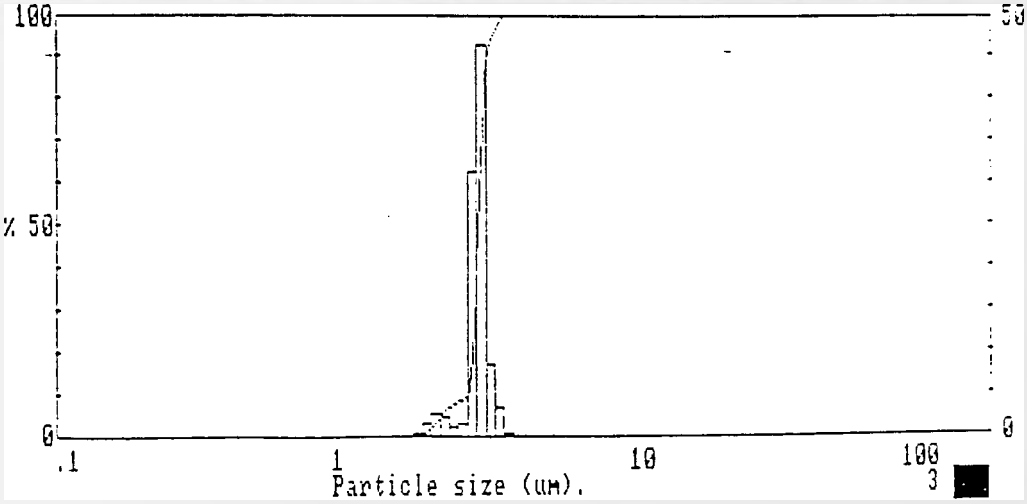


Fig.3a. Size distribution histogram of 13.6% dexamethasone loaded PLGA(85:15) microspheres before drying.

MALVERN Series 2600 SB.0D Easy Mode Tue 14 Jan 1997 2:26 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 3.07
				36.3	1.1	31.4	96.1	6.18	4.8	5.33	51.9	
				31.4	1.2	27.1	94.9	5.33	3.8	4.60	48.1	D[4,3] 8.59µm
				27.1	1.4	23.3	93.4	4.60	4.1	3.97	44.0	
				23.3	1.6	20.2	91.8	3.97	6.8	3.42	37.3	
118	0.0	102	100	20.2	2.4	17.3	89.4	3.42	9.6	2.95	27.7	D[3,2] 4.63µm
102	0.0	88.2	100	17.3	3.2	15.0	86.3	2.95	22.7	2.55	5.1	
88.2	0.1	76.0	99.9	15.0	4.1	12.9	82.2	2.55	5.1	2.19	0.0	
76.0	0.2	65.6	99.7	12.9	4.6	11.1	77.6	2.19	0.0	1.90	0.0	D[v,0.9] 17.88µm
65.6	0.4	56.6	99.4	11.1	4.9	9.63	72.7	1.90	0.0	1.64	0.0	
56.6	0.6	48.8	98.8	9.63	5.3	8.31	67.4	1.64	0.0	1.41	0.0	
48.8	0.8	42.1	98.1	8.31	5.4	7.16	61.9	1.41	0.0	1.22	0.0	D[v,0.1] 2.64µm
42.1	0.9	36.3	97.1	7.16	5.2	6.18	56.7	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 4.96µm
	Log. Diff. = 6.502		
Focal length = 63 mm	Obscuration = 0.1769	Volume Conc. = 0.0021%	
Presentation = pil	Volume distribution	Sp.S.A 1.2972 m²/cc.	Shape OFF

MALVERN Series 2600 SB.0D Easy Mode Tue 14 Jan 1997 2:27 pm

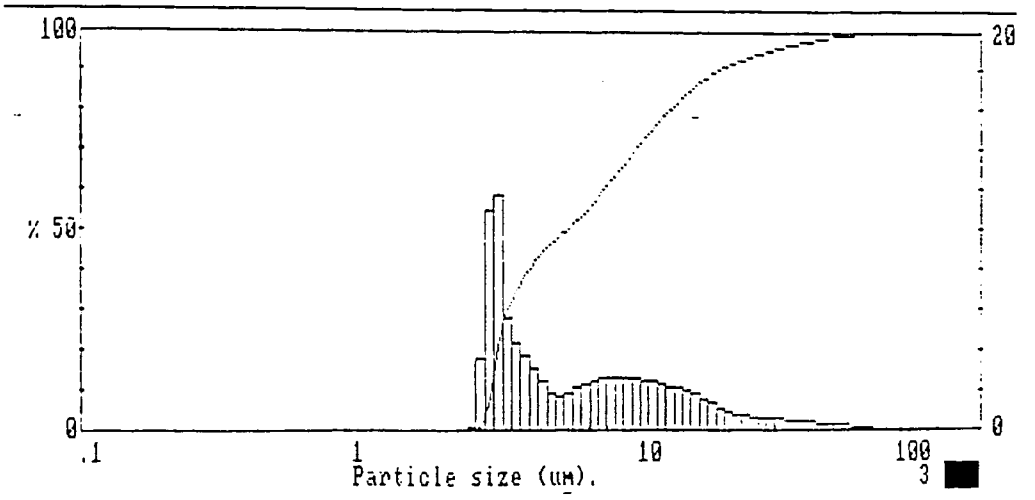


Fig.3b. Size distribution histogram of 13.6% dexamethasone loaded PLGA(85:15) microspheres after drying.

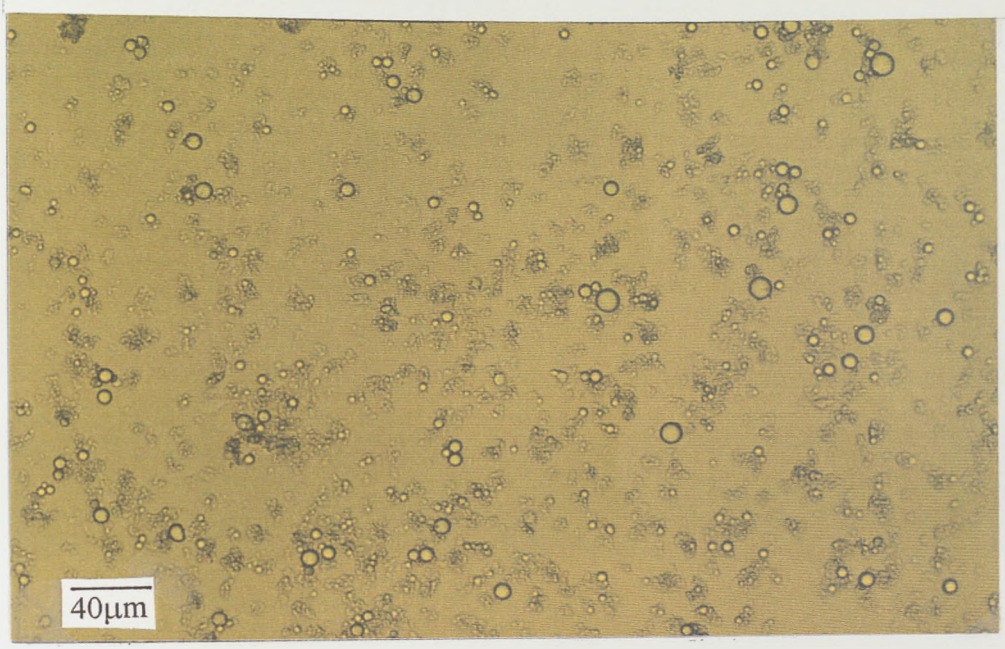


Fig. 4. Optical microscopic photograph (x250) of PLGA blank microspheres.

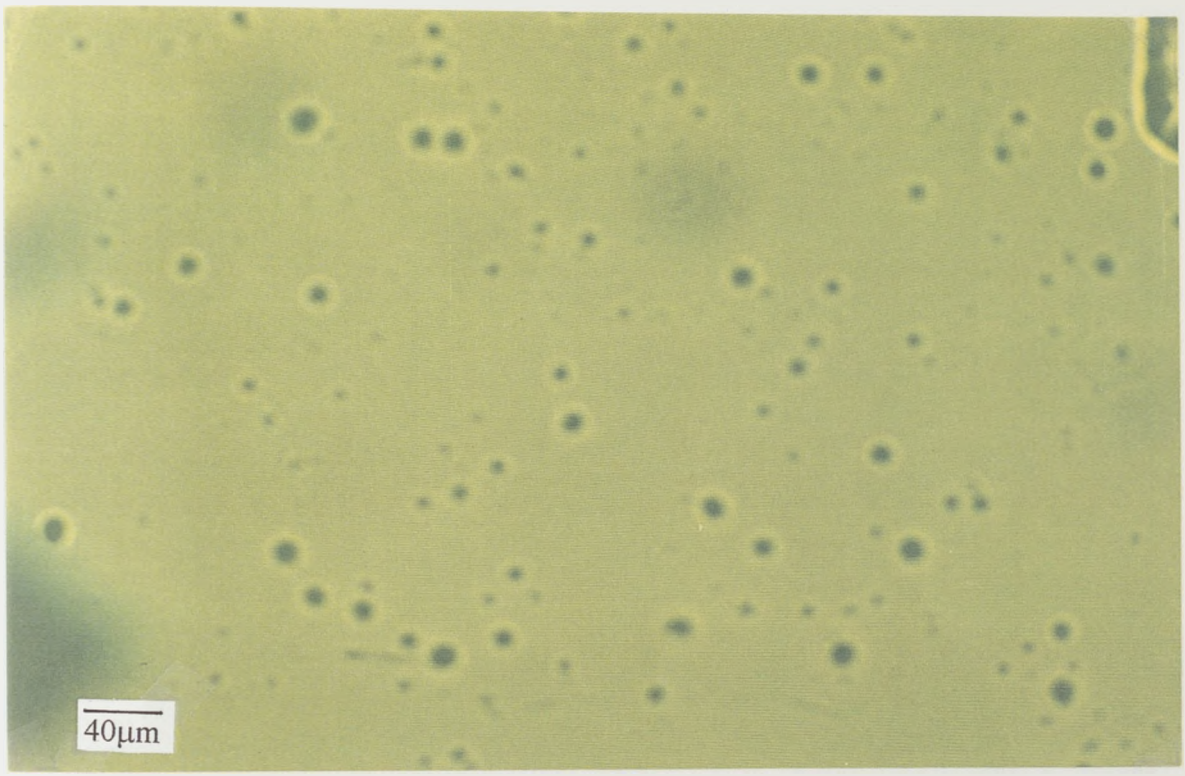


Fig.5. Optical microscopic photograph (x250) of 16.78% dexamethasone loaded (85:15) PLGA microspheres.

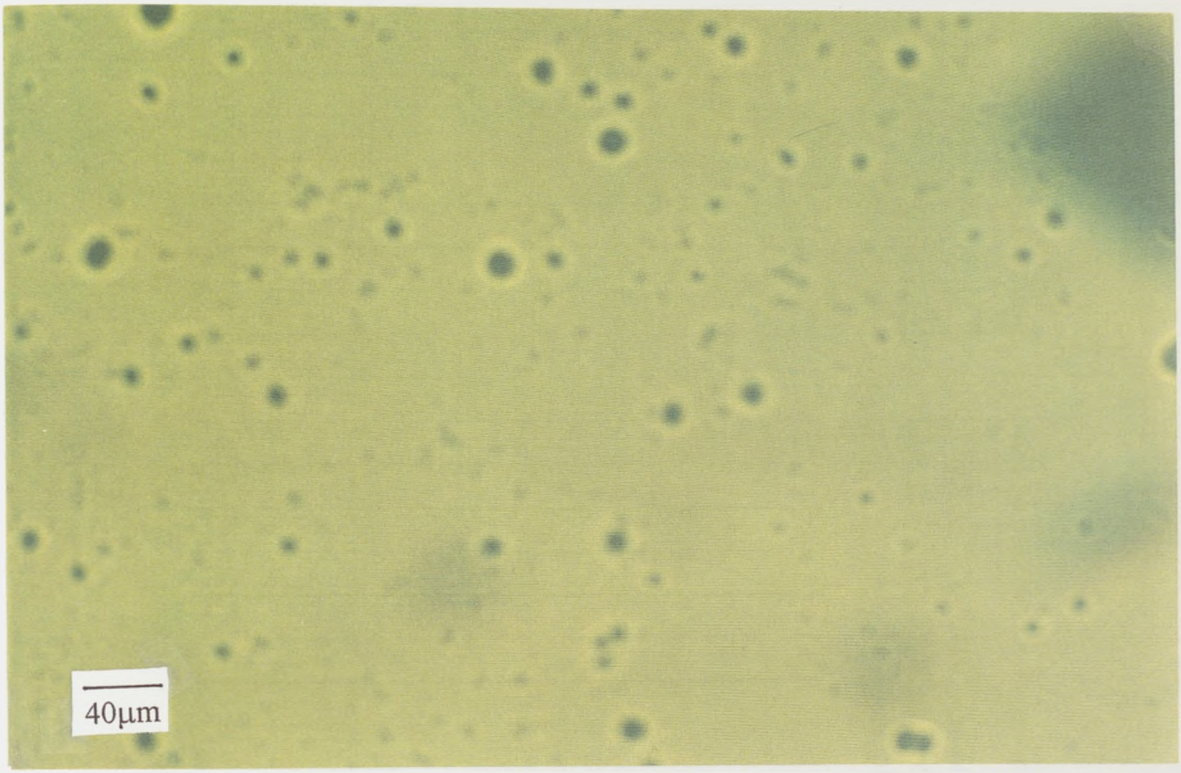
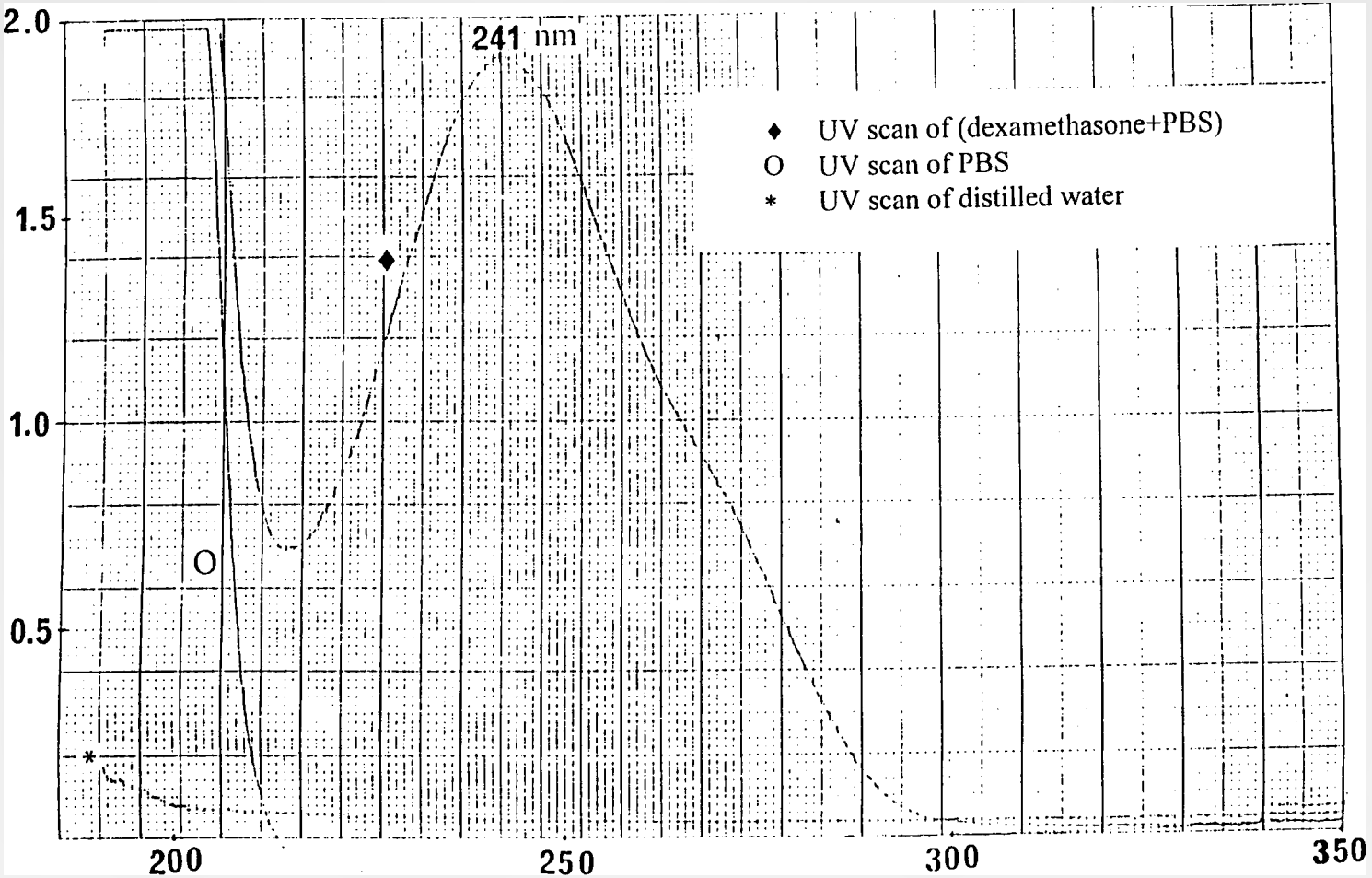


Fig.6. Optical microscopic photograph (x250) of 13.62% dexamethasone loaded (85:15) PLGA microspheres.

Fig. 7. UV scan of dexamethasone in PBS



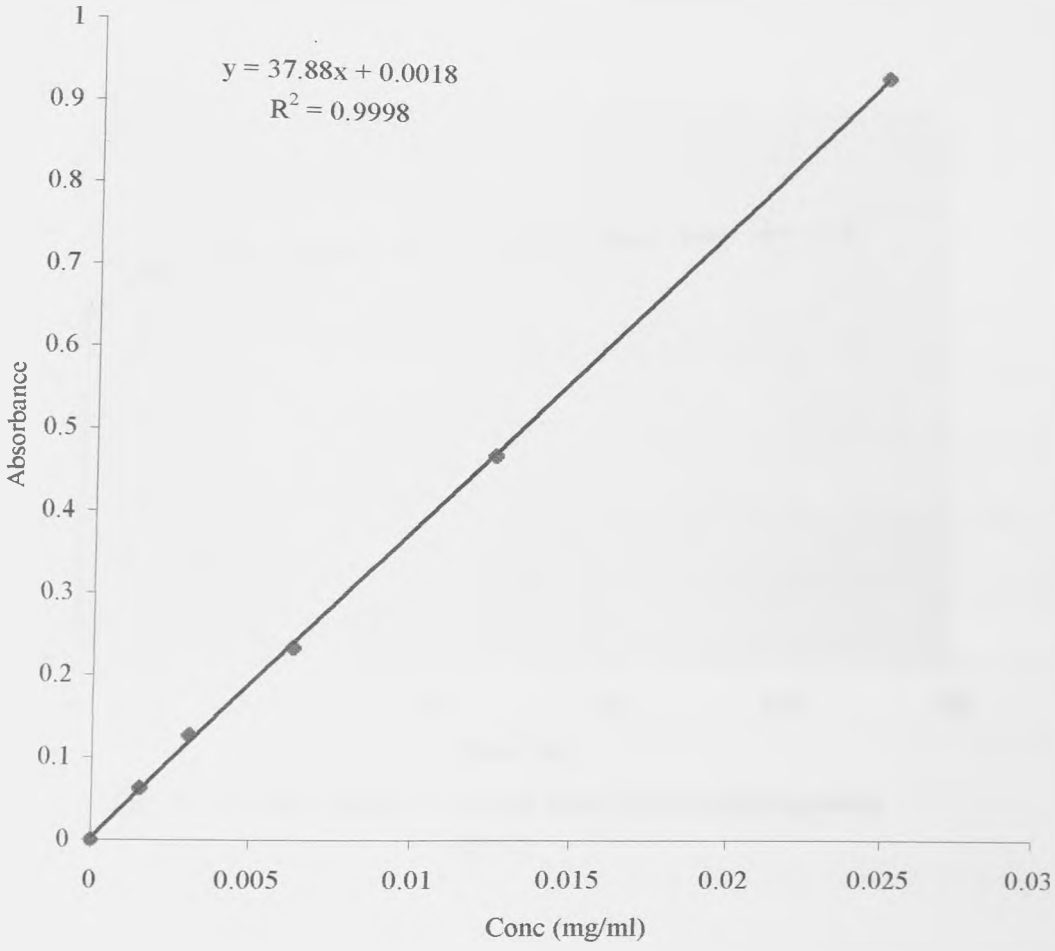


Fig.8. Calibration curve of dexamethaone in PBS.

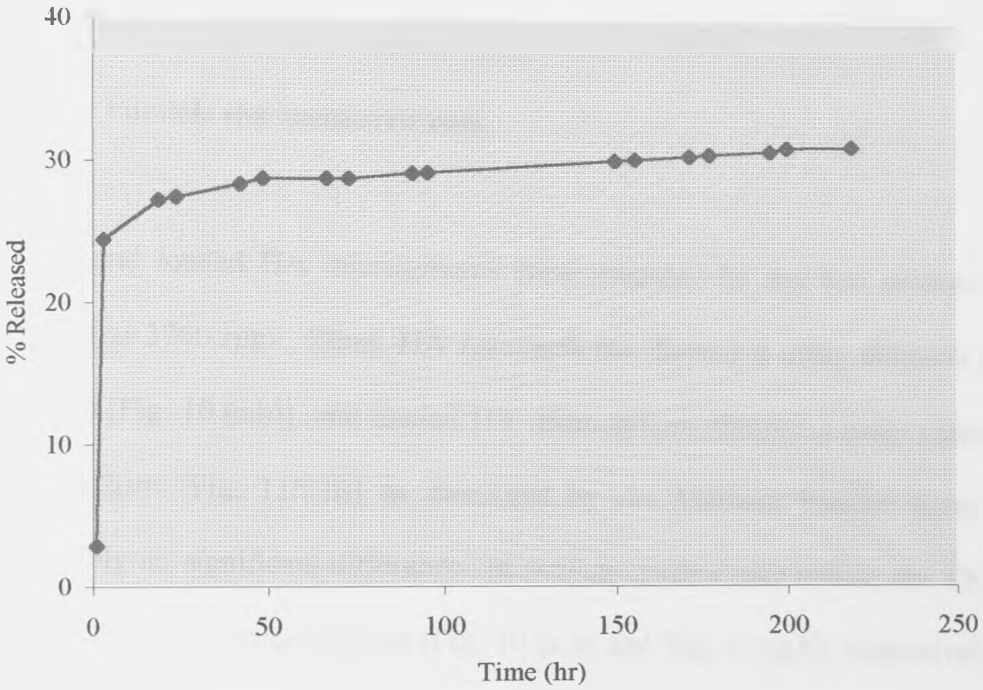


Fig. 9. Dexamethasone release from PLGA microspheres (n=2).

3.2 HA microspheres

HA is a natural, biodegradable polymer and has been used as a matrix for microspheres due to its biological and chemical properties which qualify this macromolecule as a prospective carrier of drugs, particularly for local application and/ or targeting to the lymphatic system. It is immunologically inert and safely degraded in lysosomes of many cells (Dorbnik 1991).

3.2.1 Particle size measurements

Blank and loaded HA microspheres were prepared by the w/o solvent evaporation method at 3700 rpm. Blank HA microspheres showed a mean diameter of 28.4 and 23.6μm [Fig. 10 (a,b)], and loaded HA microspheres showed a mean diameter of 23.5 and 21.2μm [Fig. 11(a,b)] as measured by the Malvern Particle Sizer. There is essentially no significant differences in average particle size before and after drying of the same batch of microspheres (Fig. 10 (a,b) and Fig. 11 (a,b), respectively) although the dried blank microspheres have a less smooth size distribution. Moreover, there is no significant difference between non-loaded and drug-loaded particles (Fig. 10b and 11b respectively).

3.2.2 Morphological examination

Examination of HA microspheres under the light microscope showed consistency with

the Malvern results. Most of the microspheres were in the range of 18-28μm in the case of blank microspheres (Fig.12) and of 10-25μm for loaded microspheres (Fig.13).

3.2.3 Drug loading and *in vitro* release of dexamethasone from HA microspheres

The high viscosity of HA, due to its high molecular weight (MW $1.64X10^6$) and the poor solubilty of dexamethasone in water, resulted in agglomeration during dexamethasone-HA microsphere preparation. However, sonication of these particles resulted in a hard mass. This affected the yield of the loaded microspheres which was only 18mg.

The dexamethasone content of 5mg of microspheres was 3.7mg. The rest, 1.3mg, should be hyaluronic acid polymer. In theory, the expected drug loading and polymer content is 0.83 and 4.17mg, respectively, for a 5mg sample. This showed that a high amount of HA was lost in the preparative procedure. Consequently, the dexamethasone loading, 3.7mg, was high.

The release of dexamethasone from HA microspheres was examined using the sampling and separation method. Fig.14 shows a burst release of about 9% after 1hr of release, followed by a slow release phase. Only about 12% of the loaded dexamethasone was released over 200hrs of the experiment. This slow release probably reflects the low solubility of dexamethasone and the larger average particle size of HA microspheres, 21-23μm, in comparison to PLGA 2-5μm average size range.

3.2.4 Stability of HA microspheres

A) Blank microspheres

The stability of the size of HA microspheres in water was performed by using the Malvern Particle Sizer at different time intervals. Fig. 15 shows an increase in particle size of the microspheres with time after incubation in water. This is probably due to water uptake and swelling of the microspheres.

Light microscopy examination was carried out by dispersing HA microspheres in PBS. Figs. 16(a-f) show an obvious increase in size with time, which confirmed the above results. After 28hrs and 46hrs, it is clear that the microspheres are aggregating as the HA becomes hydrated.

B) Loaded microspheres

Particle size analysis on loaded microspheres at 15, 27 and 31days after release was carried out using the Malvern Particle Sizer. Fig.17 shows an increase in particle size with time. However, these results could be reflecting the presence of dexamethasone particles, perhaps with some hydrated HA.

MALVERN Series 2600 SB.0D Easy Mode Thu 9 Jan 1997 5:54 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 1.38
				36.3	12.6	31.4	59.2	6.18	0.7	5.33	4.2	
				31.4	13.2	27.1	45.5	5.33	0.6	4.60	3.6	D[4,3] 31.15µm
				27.1	12.5	23.3	33.0	4.60	0.6	3.97	3.0	
				23.3	8.8	20.2	24.2	3.97	0.7	3.42	2.3	
118	1.2	102	98.8	20.2	5.9	17.3	18.3	3.42	1.0	2.95	1.3	D[3,2] 19.09µm
102	1.3	88.2	97.5	17.3	3.2	15.0	15.1	2.95	1.1	2.55	0.2	
88.2	1.4	76.0	96.1	15.0	2.2	12.9	12.9	2.55	0.2	2.19	0.0	
76.0	1.5	65.6	94.6	12.9	2.1	11.1	10.8	2.19	0.0	1.90	0.0	D[v,0.9] 49.71µm
65.6	1.7	56.6	92.9	11.1	2.0	9.63	8.8	1.90	0.0	1.64	0.0	
56.6	3.5	48.8	89.4	9.63	1.8	8.31	7.1	1.64	0.0	1.41	0.0	
48.8	7.2	42.1	82.2	8.31	1.3	7.16	5.8	1.41	0.0	1.22	0.0	D[v,0.1] 10.51µm
42.1	10.3	36.3	71.9	7.16	0.9	6.18	4.9	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 28.44µm
	Log. Diff. = 6.748		
Focal length = 63 mm	Obscuration = 0.2325	Volume Conc. = 0.0118%	
Presentation = pil	Volume distribution	Sp.S.A 0.3143 m²/cc.	Shape OFF

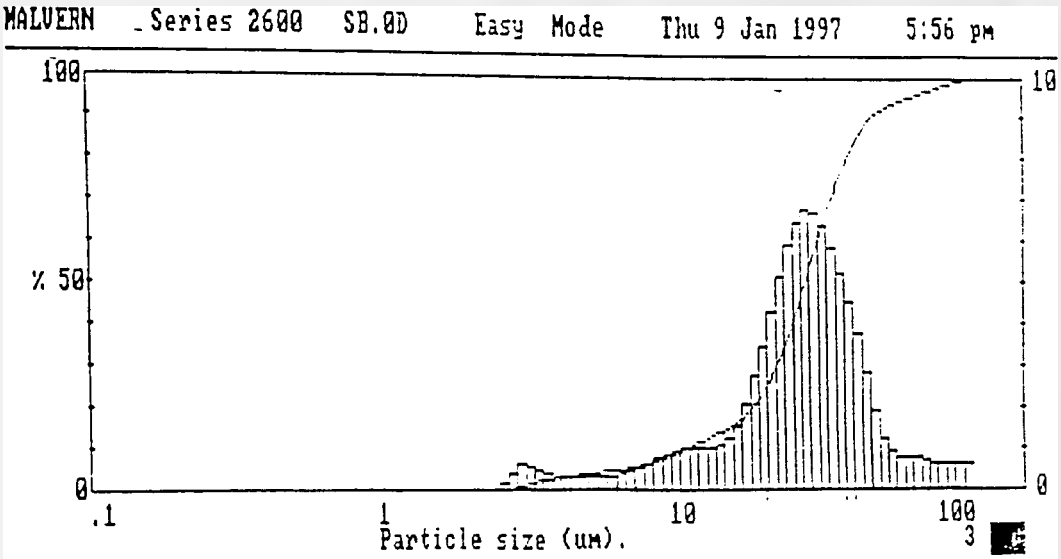


Fig.10a. Size distribution histogram of HA microspheres before drying.

MALVERN Series 2600 SB.0D Easy Mode Fri 10 Jan 1997 1:15 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 1.79
				36.3	8.9	31.4	67.3	6.18	1.4	5.33	10.0	
				31.4	9.1	27.1	58.2	5.33	1.0	4.60	9.0	D[4,3] 26.71µm
				27.1	9.1	23.3	49.1	4.60	1.3	3.97	7.8	
				23.3	8.2	20.2	41.0	3.97	2.7	3.42	5.1	
118	1.1	102	98.9	20.2	6.7	17.3	34.3	3.42	3.1	2.95	2.0	D[3,2] 13.33µm
102	1.1	88.2	97.8	17.3	4.1	15.0	30.2	2.95	1.8	2.55	0.2	
88.2	1.2	76.0	96.7	15.0	3.4	12.9	26.8	2.55	0.2	2.19	0.0	
76.0	1.3	65.6	95.4	12.9	3.6	11.1	23.1	2.19	0.0	1.90	0.0	D[v,0.9] 47.66µm
65.6	1.5	56.6	93.9	11.1	3.9	9.63	19.3	1.90	0.0	1.64	0.0	
56.6	3.1	48.8	90.8	9.63	3.7	8.31	15.6	1.64	0.0	1.41	0.0	
48.8	6.3	42.1	84.5	8.31	2.5	7.16	13.1	1.41	0.0	1.22	0.0	D[v,0.1] 5.32µm
42.1	8.3	36.3	76.3	7.16	1.7	6.18	11.4	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 23.65µm
Focal length = 63 mm	Log. Diff. = 6.698		
Presentation = pil	Obscuration = 0.1906	Volume Conc. = 0.0066%	Shape OFF
	Volume distribution	Sp.S.A 0.4500 m²/cc.	

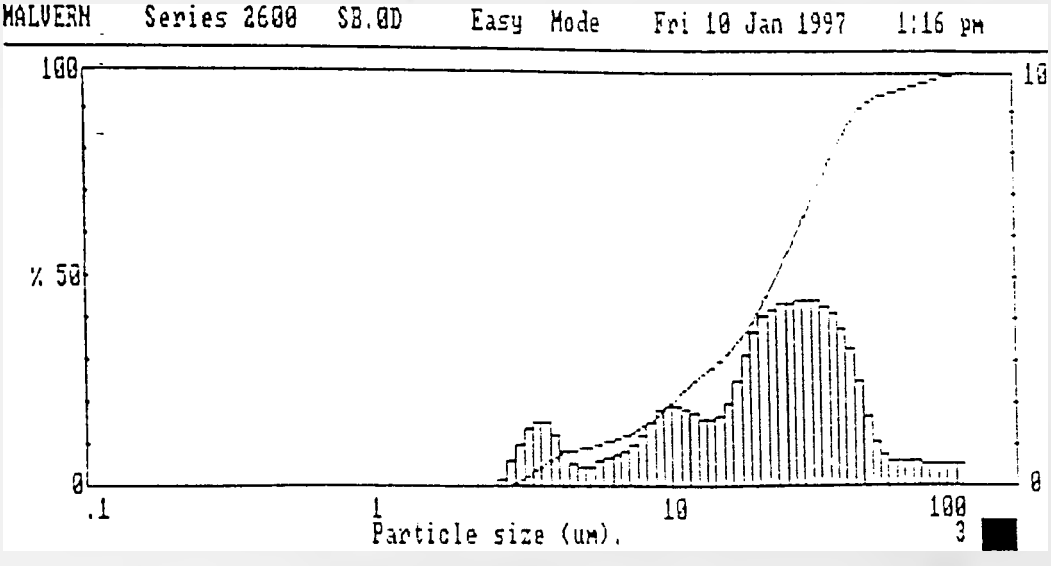


Fig.10b. Size distribution histogram of HA microspheres after drying.

MALVERN Series 2600 SB.0D Easy Mode Fri 14 Feb 1997 4:17 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 1.85
				36.3	8.4	31.4	69.7	6.18	1.4	5.33	10.3	
				31.4	9.6	27.1	60.0	5.33	1.2	4.60	9.1	D[4,3] 27.37µm
				27.1	10.6	23.3	49.4	4.60	1.2	3.97	7.9	
				23.3	10.4	20.2	39.0	3.97	1.4	3.42	6.5	
118	1.3	102	98.7	20.2	9.3	17.3	29.7	3.42	2.6	2.95	3.9	D[3,2] 13.53µm
102	1.3	88.2	97.4	17.3	6.2	15.0	23.6	2.95	3.2	2.55	0.7	
88.2	1.4	76.0	96.0	15.0	4.0	12.9	19.6	2.55	0.7	2.19	0.0	
76.0	1.5	65.6	94.5	12.9	2.1	11.1	17.5	2.19	0.0	1.90	0.0	D[v,0.9] 48.66µm
65.6	1.7	56.6	92.8	11.1	1.4	9.63	16.1	1.90	0.0	1.64	0.0	
56.6	2.7	48.8	90.1	9.63	1.4	8.31	14.7	1.64	0.0	1.41	0.0	
48.8	5.1	42.1	85.0	8.31	1.5	7.16	13.2	1.41	0.0	1.22	0.0	D[v,0.1] 5.15µm
42.1	7.0	36.3	78.1	7.16	1.5	6.18	11.6	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 23.50µm
Focal length = 63 mm	Log. Diff. = 6.652		
Presentation = pil	Obscuration = 0.2117	Volume Conc. = 0.0075%	Shape OFF
	Volume distribution	Sp.S.A 0.4436 m²/cc.	

MALVERN Series 2600 SB.0D Easy Mode Fri 14 Feb 1997 4:20 pm

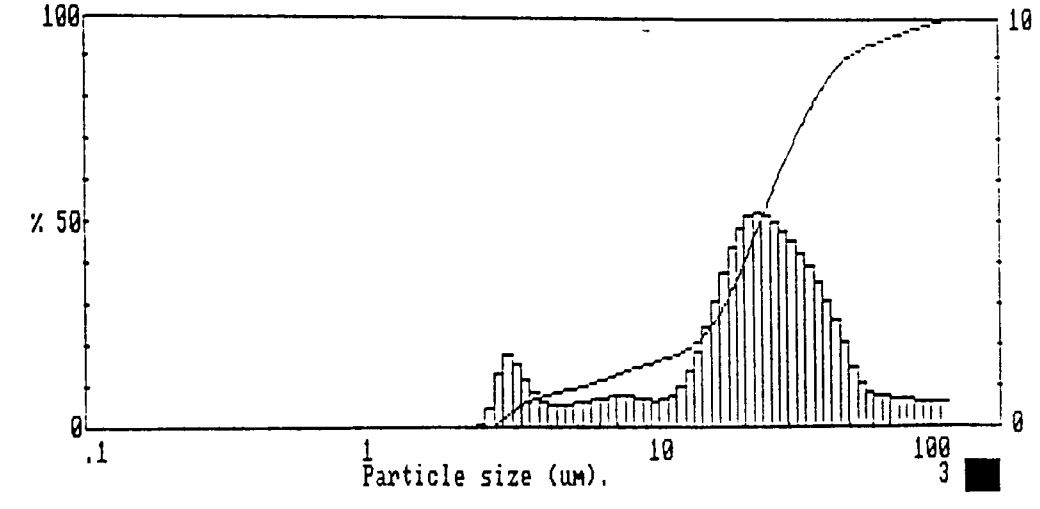


Fig.11a. Size distribution histogram of dexamethasone loaded HA microspheres before drying.

MALVERN Series 2600 SB.0D Easy Mode Mon 17 Feb 1997 4:40 pm

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 1.83
				36.3	7.4	31.4	75.5	6.18	1.4	5.33	11.1	
				31.4	8.7	27.1	66.8	5.33	1.4	4.60	9.8	D[4,3] 24.51µm
				27.1	10.0	23.3	56.8	4.60	1.2	3.97	8.5	
				23.3	10.4	20.2	46.4	3.97	1.3	3.42	7.2	
118	0.8	102	99.2	20.2	10.2	17.3	36.1	3.42	2.7	2.95	4.5	D[3,2] 12.57µm
102	0.9	88.2	98.3	17.3	7.9	15.0	28.3	2.95	3.7	2.55	0.8	
88.2	0.9	76.0	97.4	15.0	5.9	12.9	22.3	2.55	0.8	2.19	0.0	
76.0	1.0	65.6	96.4	12.9	3.6	11.1	18.8	2.19	0.0	1.90	0.0	D[v,0.9] 43.53µm
65.6	1.1	56.6	95.2	11.1	2.1	9.63	16.7	1.90	0.0	1.64	0.0	
56.6	2.1	48.8	93.1	9.63	1.4	8.31	15.3	1.64	0.0	1.41	0.0	
48.8	4.3	42.1	88.9	8.31	1.3	7.16	13.9	1.41	0.0	1.22	0.0	D[v,0.1] 4.73µm
42.1	6.0	36.3	82.8	7.16	1.4	6.18	12.6	1.22	0.0	0.32	0.0	

Source = :Sample	Beam length = 14.3 mm	Model indp	D[v,0.5] 21.20µm
Focal length = 63 mm	Log. Diff. = 6.646		
Presentation = pil	Obscuration = 0.2022	Volume Conc. = 0.2066%	
	Volume distribution	Sp.S.A 0.4773 m²/cc.	Shape OFF

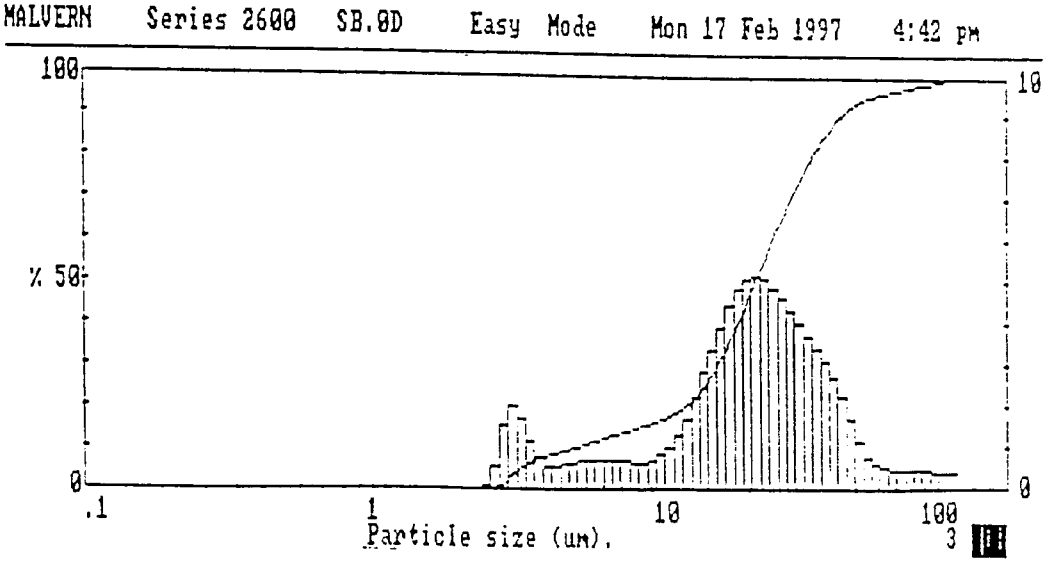


Fig.11b. Size distribution histogram of dexamethasone loaded HA microspheres after drying.

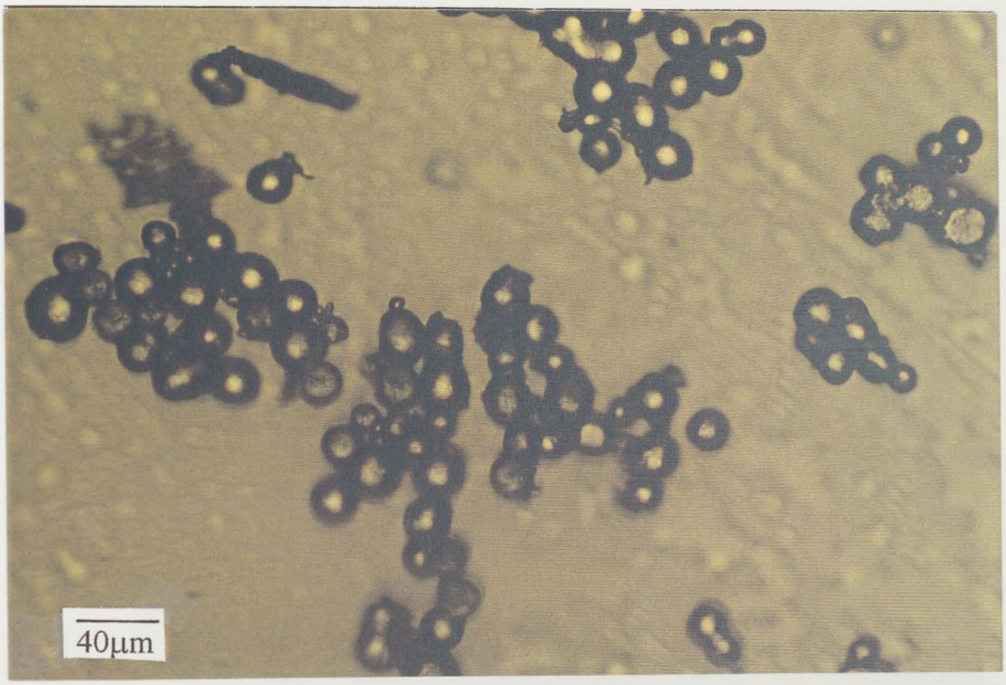


Fig.12. Optical microscopic photograph(x250) of HA blank microspheres.

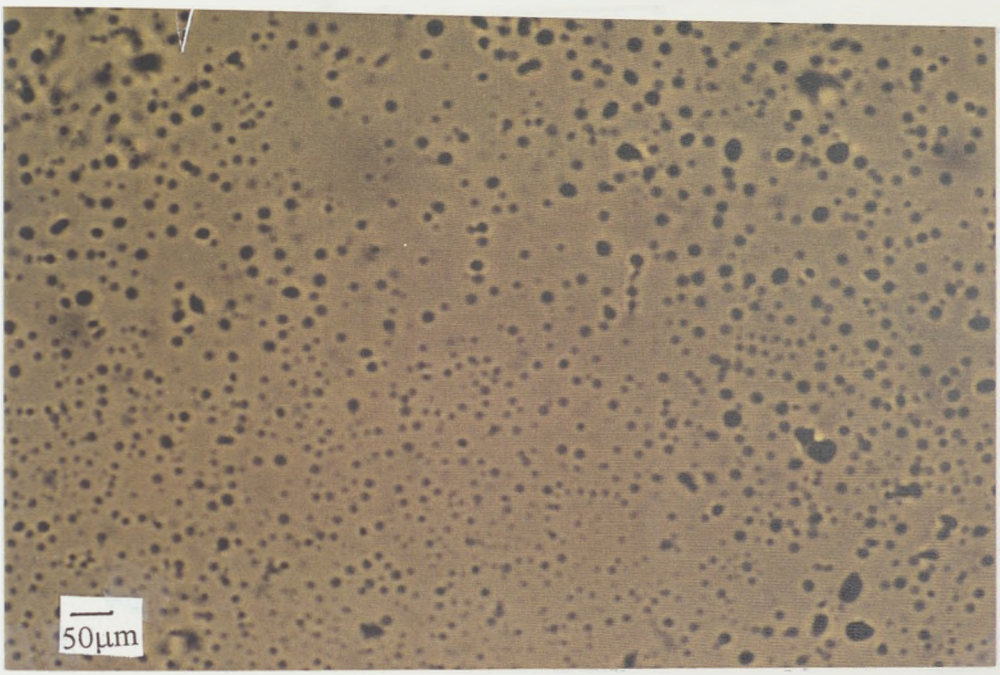


Fig. 13. Optical microscopic photograph (x100) of dexamethasone loaded HA microspheres.

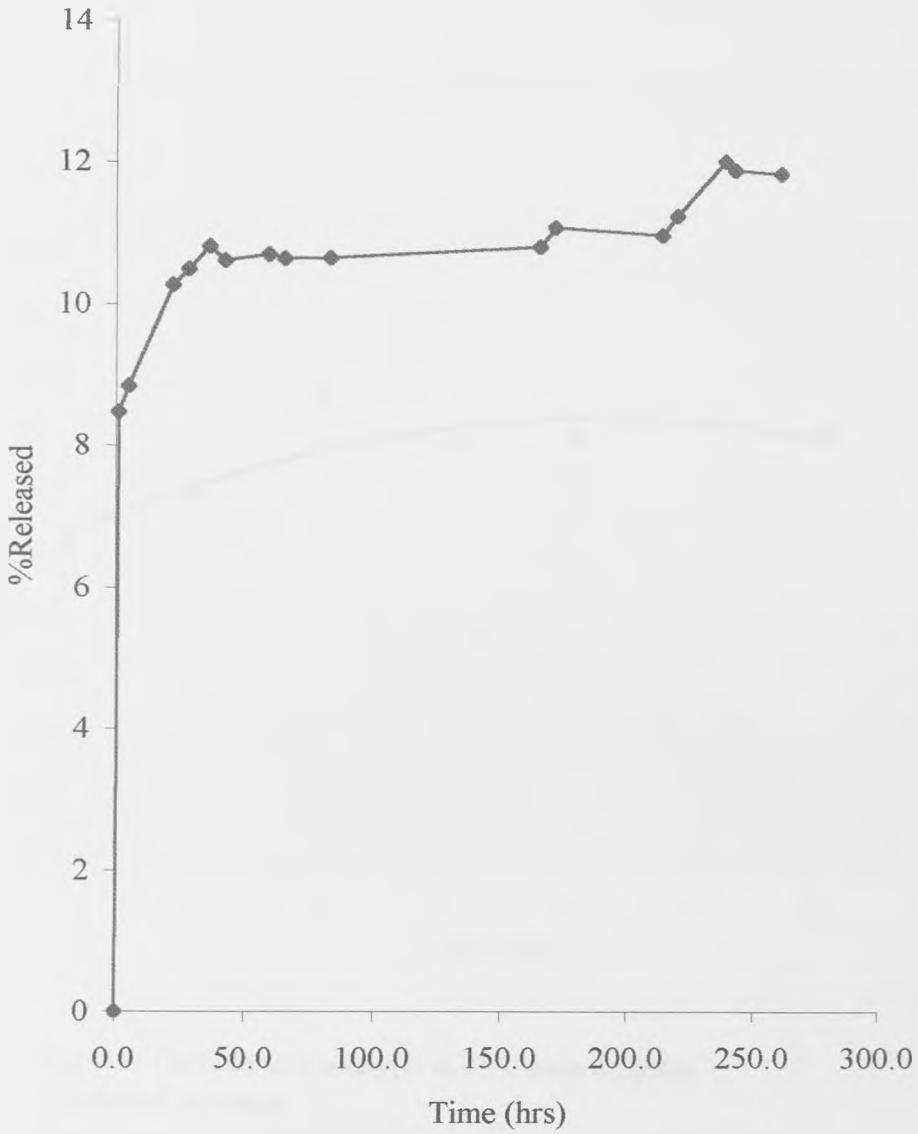


Fig. 14. Dexamethasone release from HA microspheres

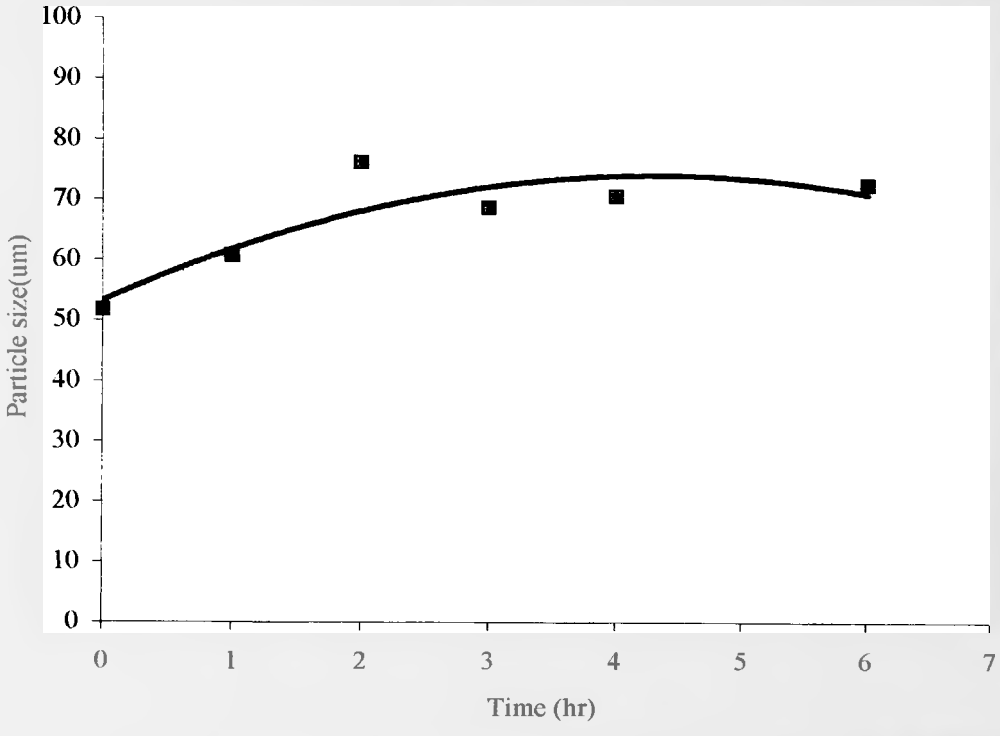


Fig. 15. Particle size analysis of HA microspheres incubated in water.

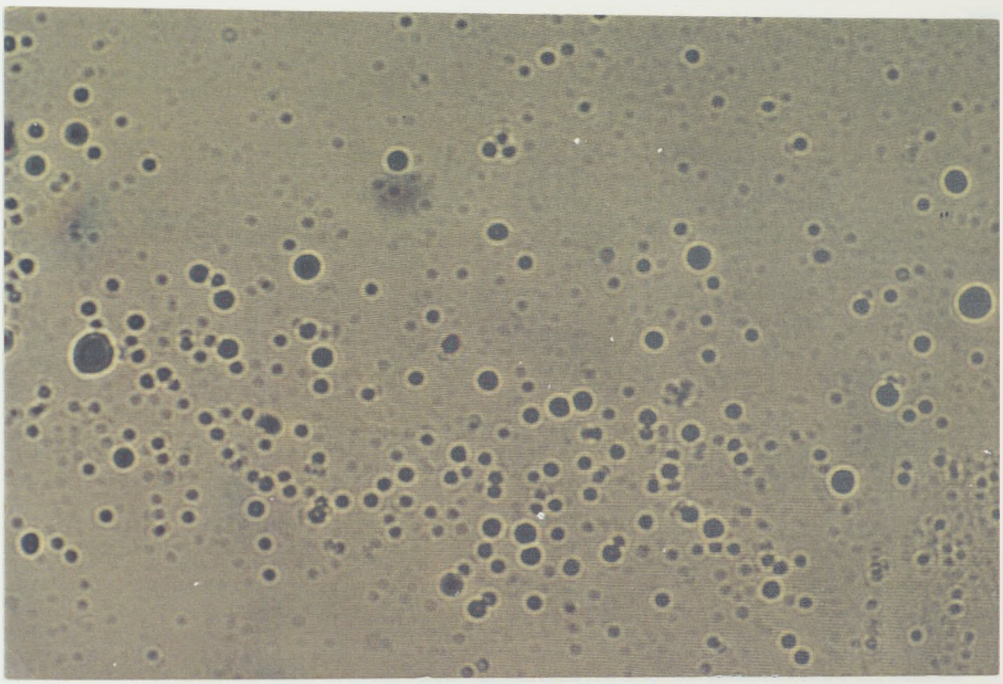


Fig.16a. Optical microscopic photograph (x250) of HA microspheres in PBS at 0 hour.

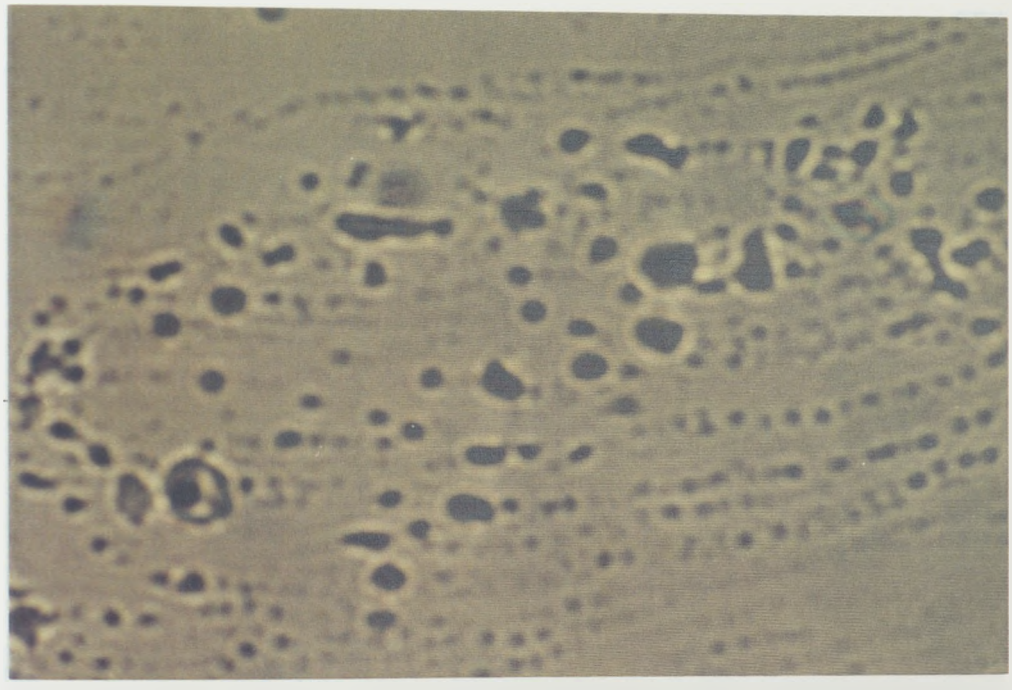


Fig.16b. Optical microscopic photograph (x250) of HA microspheres in PBS after 1 hour.

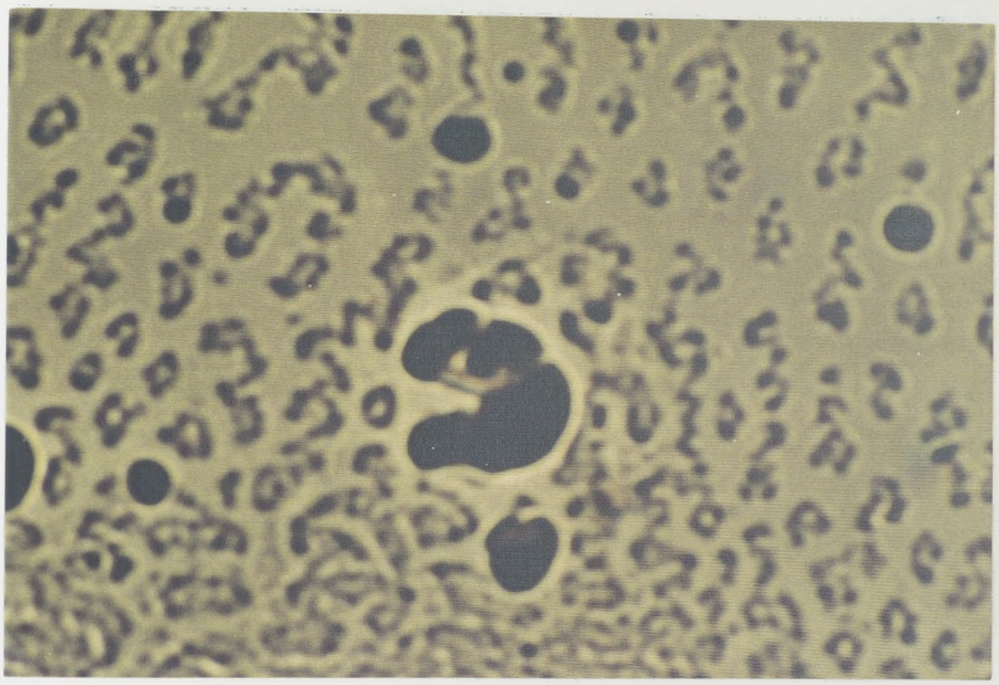


Fig.16c. Optical microscopic photograph (x250) of HA microspheres in PBS after 4 hours.

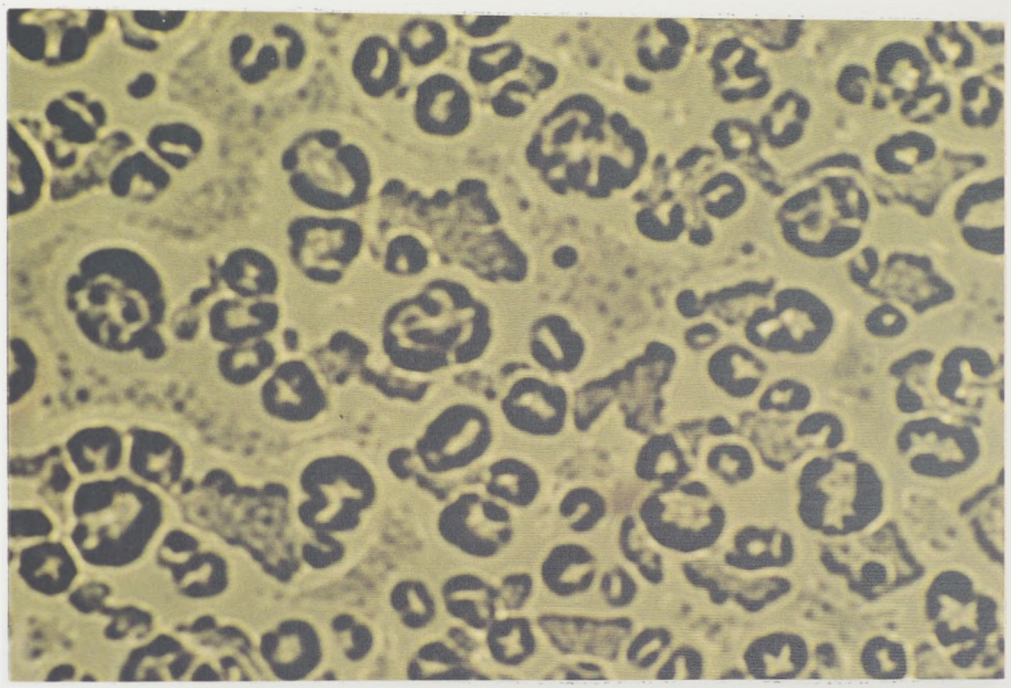


Fig.16d. Optical microscopic photograph (x250) of HA microspheres in PBS after 6 hours.

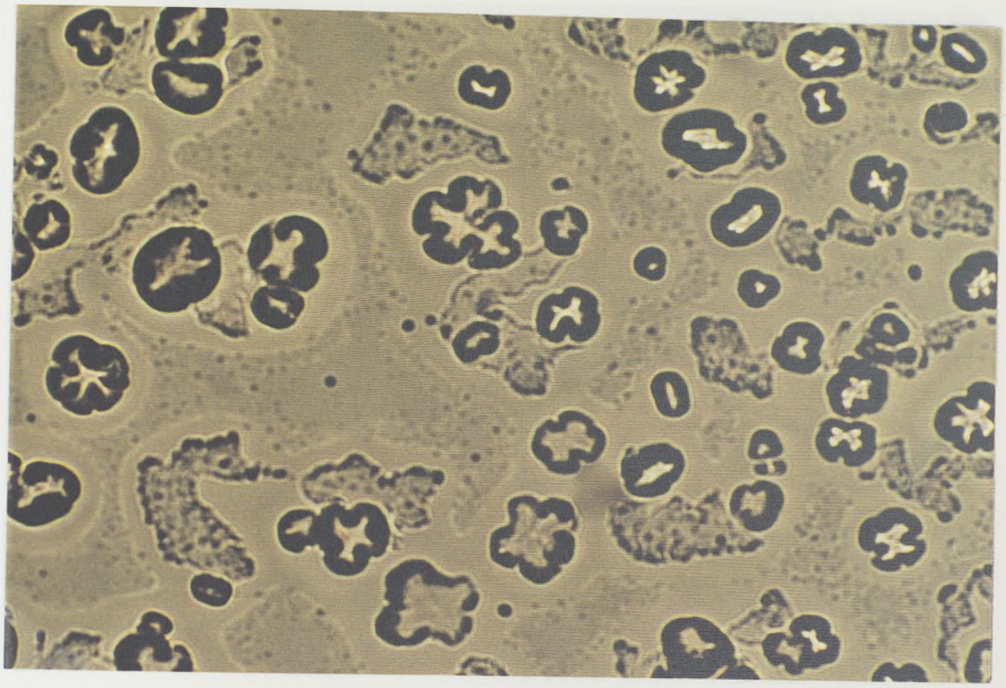


Fig.16e. Optical microscopic photograph (x250) of HA microspheres in PBS after 28 hours.

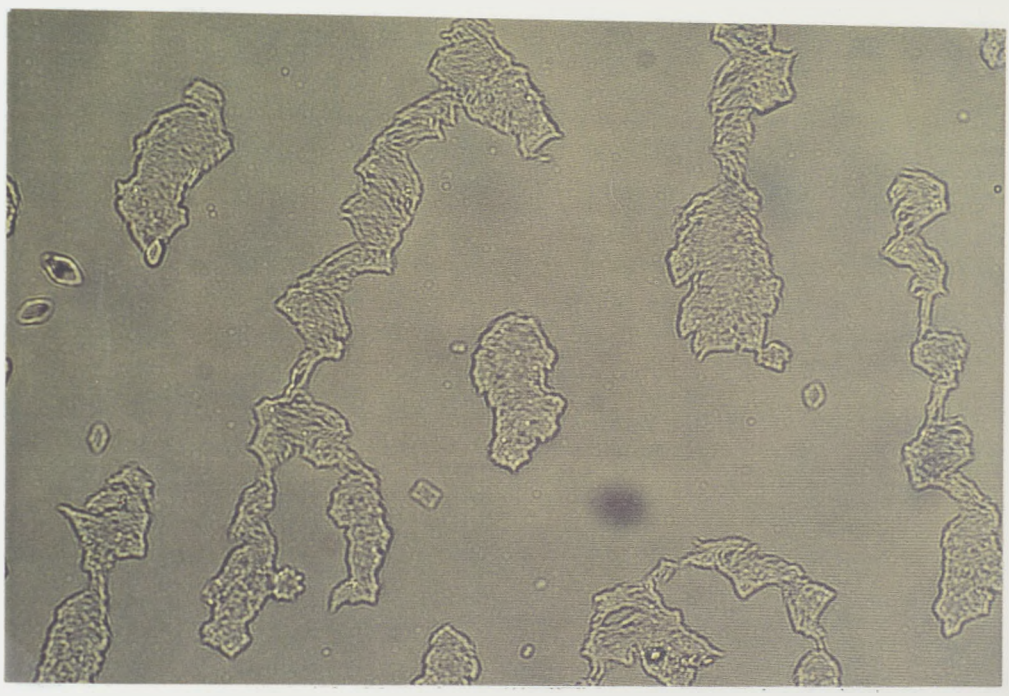


Fig.16f. Optical microscopic photograph (x250) of HA microspheres in PBS after 46 hours.

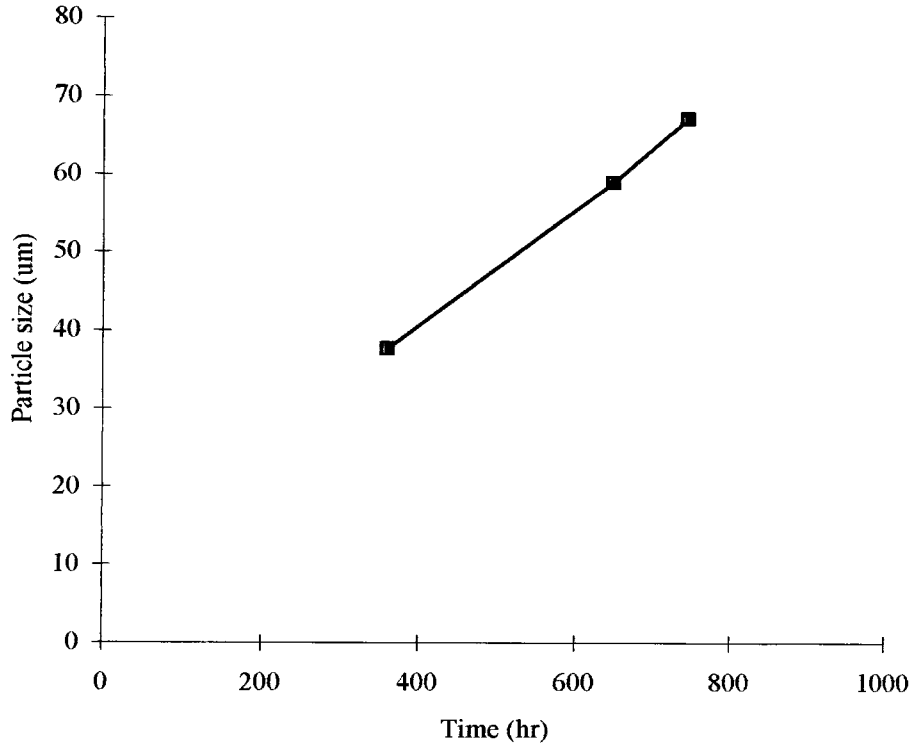


Fig. 17. Particle size analysis of loaded HA microspheres after release studies.

3.2.5 Cross-linked HA microsphere

Cross-linked HA microspheres were prepared by the w/o solvent evaporation technique using a paddle stirrer at 500rpm. Three stages can be considered during the formation of cross-linked microspheres. Firstly, the preparation of the emulsion; secondly, the stability of the emulsion droplets, and finally, removing the microsphere droplets from the continuous phase, washing and re-suspending as described in section 2.2.6. The procedure was first attempted using HA at room temperature, pH 7.7, which resulted in the formation of fragile microspheres. It was difficult to isolate these fragile microspheres. Since 4.75 is the optimum pH for the action of Carbodiimide, the next step was to change the pH of the HA from 7.7 to 4.7 in order to prepare more rigid microspheres. Every 30mins, one drop of the emulsion was taken and examined under the microscope. The examination was continued for 6hrs. Heating of the emulsion at 60-70°C was allowed in order to help the evaporation of the dispersed phase. Spherical droplets were seen within 3hrs, which was a sign of microsphere formation. The advantage of using the paddle stirrer in this technique was in order to obtain large microspheres, which were easier to isolate and study.

3.2.5.1 Particle size measurements

Particle size analysis of the cross linked microspheres was carried out using the Malvern Particle Sizer 2600. A small aliquot of the suspended microspheres were diluted in absolute alcohol. Fig. 18 shows that 50% of the particles had a mean

diluted in absolute alcohol. Fig. 18 shows that 50% of the particles had a mean diameter below 40μm. Another small aliquot of the suspended microspheres was examined in water and the result showed 50% of the particles with a mean diameter of below 8.4μm (Fig. 19) although there was a wide particle size distribution.

3.2.5.2 Morphological examination

Examination of the microspheres was carried out under the optical microscope before washing. Most of the microspheres were in the size range of 60-70μm as shown in Fig. 20. Washing was performed either with cyclohexane (Fig. 21) or absolute alcohol (Fig. 22). It is clear from the comparison between Fig. 21 and Fig. 22 that absolute alcohol is a better solvent for washing the microspheres than cyclohexane although there is evidence of aggregation and adhesion in both cases. Fig. 20 clearly shows that HA cross-linked microspheres have been formed.

MALVERN Series 2600 SB.0D Easy Mode Fri 9 May 1997 9:10 am

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 11.90
				173	0.1	150	57.5	29.5	6.5	25.4	35.2	
				150	0.3	129	57.2	25.4	7.6	21.9	27.6	D[4,3] 194.57µm
				129	0.3	111	56.9	21.9	7.5	18.9	20.1	
				111	0.4	96.0	56.5	18.9	6.0	16.3	14.1	
564	11.5	487	88.5	96.0	0.5	82.5	56.0	16.3	4.2	14.1	9.9	D[3,2] 27.85µm
487	10.7	420	77.8	82.5	0.5	71.5	55.5	14.1	2.7	12.1	7.2	
420	9.2	362	68.6	71.5	0.9	61.5	54.6	12.1	1.6	10.4	5.6	
362	6.7	313	61.9	61.5	1.3	53.0	53.3	10.4	0.8	9.05	4.8	D[v,0.9] 496.22µm
313	3.5	270	58.4	53.0	1.6	45.8	51.7	9.05	0.6	7.80	4.1	
270	0.6	233	57.8	45.8	2.1	39.5	49.6	7.80	0.9	6.70	3.3	
233	0.2	201	57.7	39.5	3.2	34.1	46.4	6.70	1.0	5.80	2.3	D[v,0.1] 14.09µm
201	0.1	173	57.6	34.1	4.7	29.5	41.7	5.80	2.3	1.50	0.0	

Source = :Sample	Beam length = 2.2 mm	Model indp	D[v,0.5] 40.52µm
Focal length = 300 mm	Log. Diff. = 3.423		
Presentation = pil	Obscuration = 0.2047	Volume Conc. = 0.0964%	Shape OFF
	Volume distribution	Sp.S.A 0.2155 m²/cc.	

MALVERN Series 2600 SB.0D Easy Mode Fri 9 May 1997 9:12 am

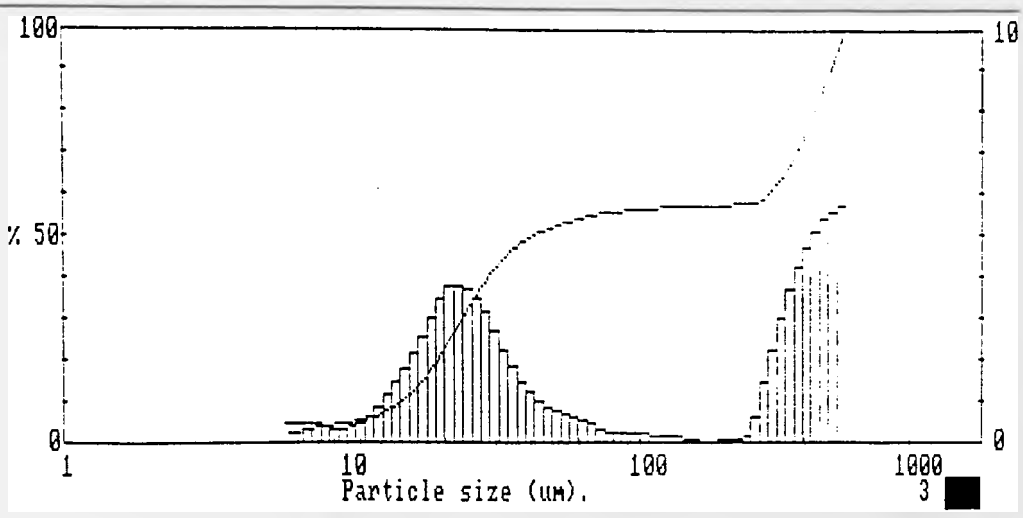


Fig.18. Size distribution histogram of cross-linked HA microspheres in absolute alcohol.

MALVERN Series 2600 SB.0D Easy Mode Mon 12 May 1997 9:57 am

Upper	in	Lower	Under	Upper	in	Lower	Under	Upper	in	Lower	Under	Span 2.94
				173	0.0	150	100	29.5	2.3	25.4	88.5	
				150	0.0	129	100	25.4	4.2	21.9	84.3	D[4,3] 12.90µm
				129	0.0	111	100	21.9	5.0	18.9	79.4	
				111	0.0	96.0	100	18.9	3.4	16.3	76.0	
564	0.0	487	100	96.0	0.0	82.5	100	16.3	3.2	14.1	72.8	D[3,2] 5.83µm
487	0.0	420	100	82.5	0.0	71.5	100	14.1	4.8	12.1	68.0	
420	0.0	362	100	71.5	1.2	61.5	98.8	12.1	6.8	10.4	61.2	
362	0.0	313	100	61.5	3.1	53.0	95.7	10.4	7.4	9.05	53.8	D[v,0.9] 27.51µm
313	0.0	270	100	53.0	2.2	45.8	93.5	9.05	7.5	7.80	46.4	
270	0.0	233	100	45.8	0.8	39.5	92.6	7.80	6.6	6.70	39.7	
233	0.0	201	100	39.5	0.6	34.1	92.0	6.70	5.4	5.80	34.3	D[v,0.1] 2.85µm
201	0.0	173	100	34.1	1.1	29.5	90.9	5.80	34.3	1.50	0.0	
Source = :Sample				Beam length = 2.2 mm				Model indp				D[v,0.5] 8.40µm
				Log. Diff. = 3.598								
Focal length = 300 mm				Obscuration = 0.1833				Volume Conc. = 0.0179%				Shape OFF
Presentation = pil				Volume distribution				Sp.S.A 1.0287 m²/cc.				

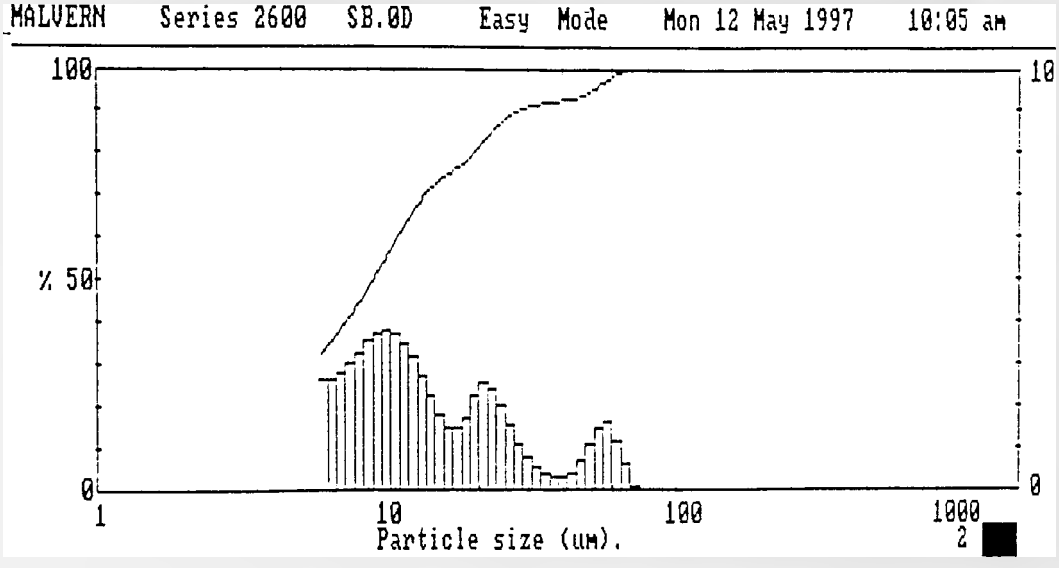


Fig.19. Size distribution histogram of cross-linked HA microspheres in water.

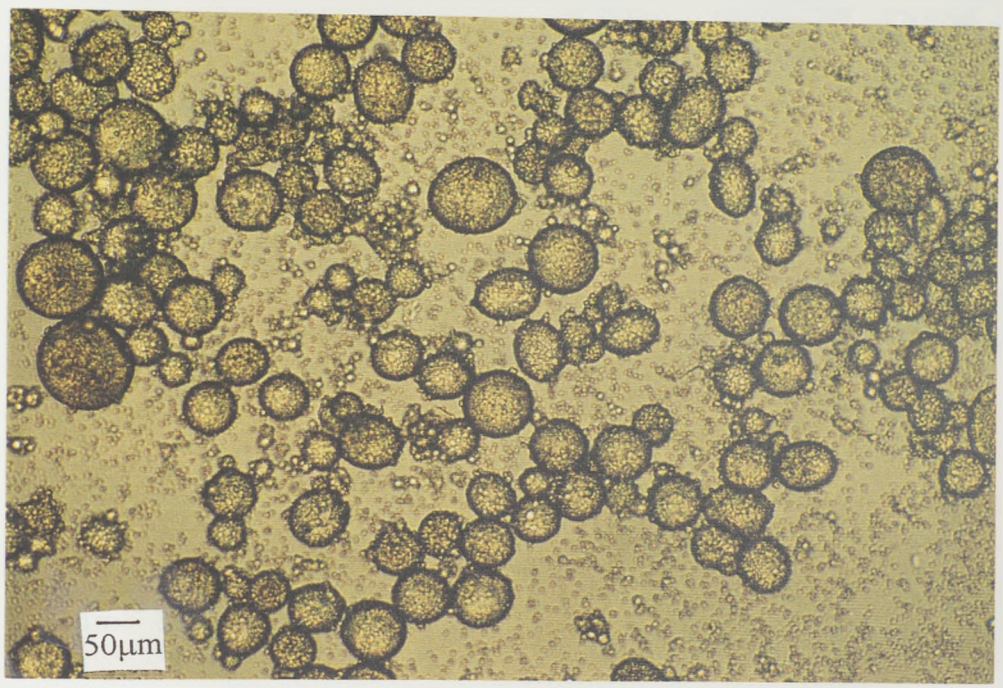


Fig.20. Optical microscopic photograph (x100) of cross-linked HA microspheres before washing.

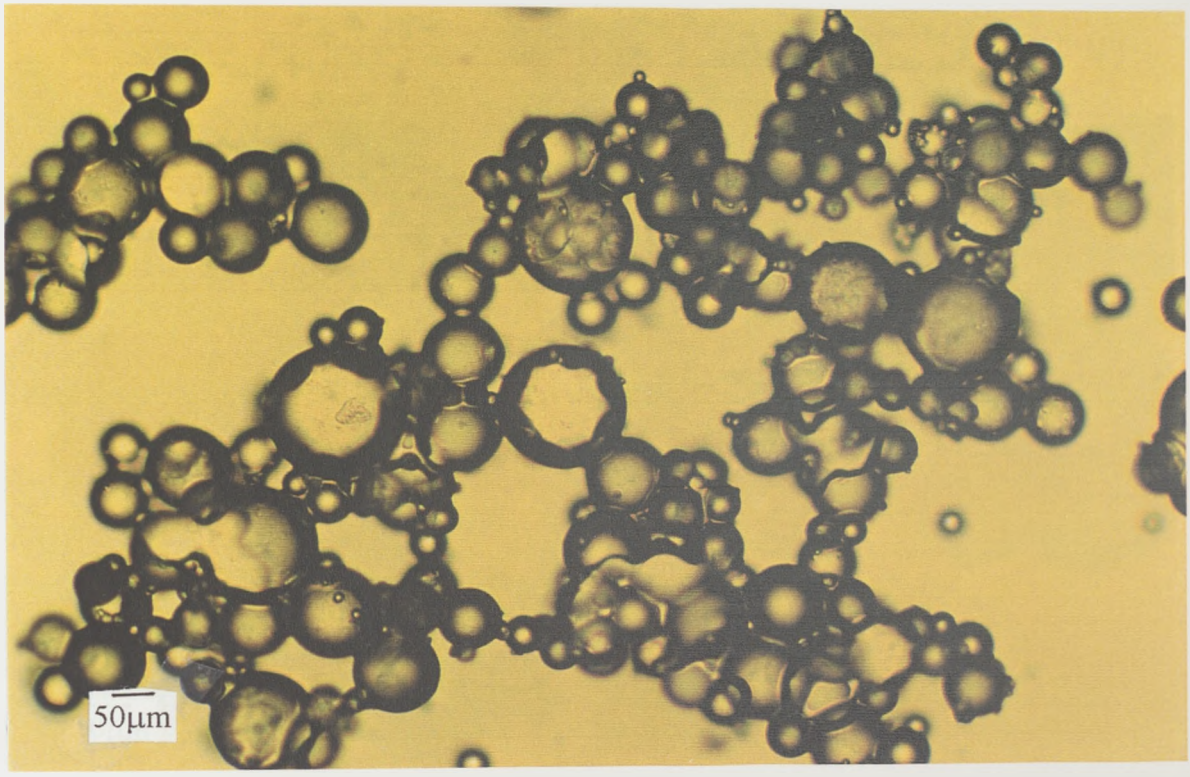


Fig.21. Optical microscopic photograph (x100) of cross-linked HA microspheres washed with absolute alcohol.

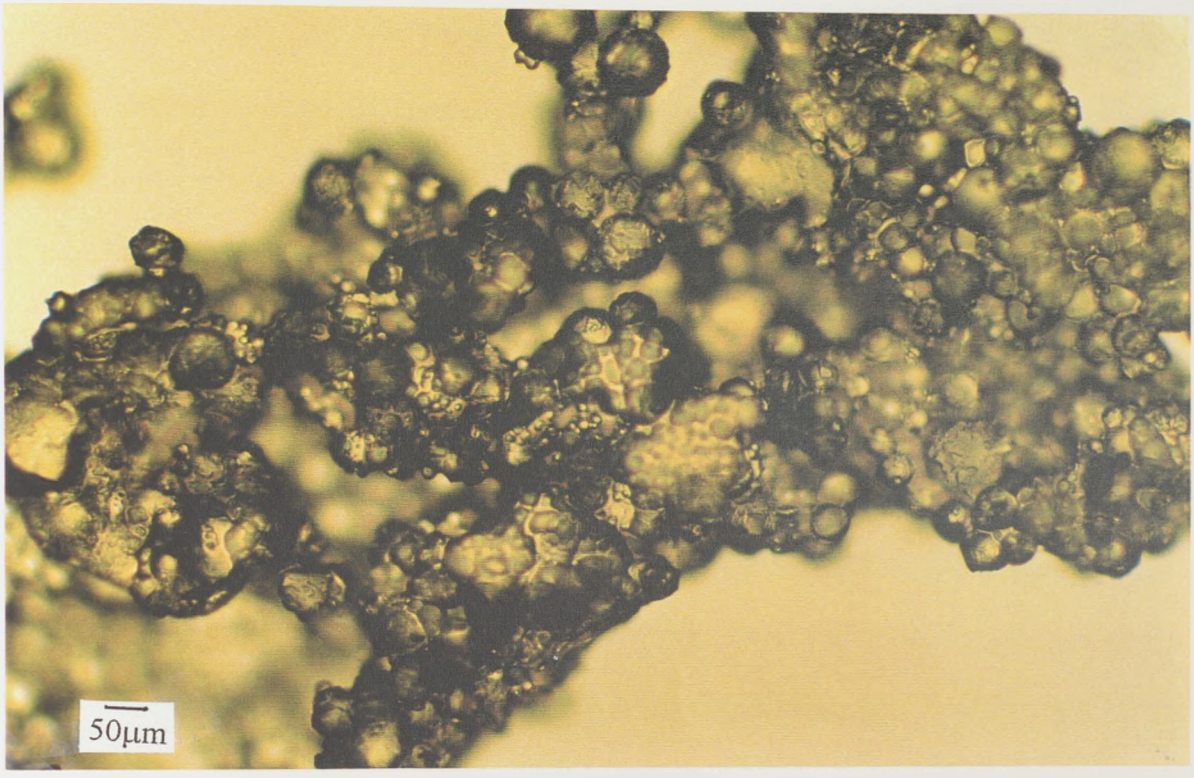


Fig.22. Optical microscopic photograph (x100) of cross-linked HA microspheres washed with cyclohexane.

CHAPTER FOUR

4 OVERALL DISCUSSION

PLGA microspheres have been studied experimentally and clinically by many scientists and clinicians and have been widely used for drug delivery following the confirmation of their biocompatability and biodegradability to non-toxic products (Beck *et al.*, 1979; Jalil and Nixon, 1990 and Whateley, 1993). A literature search has shown no studies on the natural polymer, hyaluronic acid, as microspheres. Hence our study aimed at the preparation and characterization of novel HA microspheres and comparing them to conventional PLGA and cross-linked HA microspheres.

The physico-chemical properties of both the drug and polymer play a significant role in the choice of an appropriate technique for the preparation of microspheres. In this research, the oil-in-water (o/w) emulsion solvent evaporation method was chosen for PLGA microspheres due to the hydrophobicity of PLGA. On the other hand, a water-in-oil (w/o) emulsion solvent evaporation technique was used for the preparation of HA and cross-linked HA microspheres due to its aqueous hydration.

The production of microspheres of different particle sizes was dependent on the mixing speed used. The use of mixing speed of 6000 rpm resulted in a small particle size for PLGA microspheres in the range of 2-5 μm with a narrow size distribution. Meanwhile, reducing the mixing speed to 3700 rpm in case of HA and 500rpm for cross linked-HA has resulted in the formation of a larger particles in the range of 21-23μm and 60-70μm, respectively. This might be explained by the reduction in the mixing speed and the indirect effect of HA viscosity, which may have led to the overall increase in the size of the microspheres. The findings and conclusions of previous studies by Jeffrey *et al.* (1991) and Tice and Gilley (1985) support this explanation.

Light microscopy showed spherical PLGA, HA and cross-linked HA microspheres which is considered the ideal shape for passage of the carrier system to the target site without causing side effects (Illum *et al.* 1982). Light microscopy and laser diffractometery results were similar for PLGA and HA microsphere preparation.

The high molecular weight HA ($1.64X10^{6}$), as compared to that of PLGA (62,000), resulted in a highly viscous HA solution. Consequently, agglomeration of HA polymer appeared during the mixing procedure, which led to high loss of the natural polymer. This explains the relatively high drug loading (73.5%), which was obtained with HA microspheres, but not with PLGA microspheres (15.5%).

The *in vitro* release of dexamethasone from PLGA and HA microspheres showed a biphasic release over the 200 hrs. The burst release of 25% dexamethasone from PLGA microsphere within the first 3 hrs was followed by a sustained release resulting in a total of 30% release. The sustained second release phase was due to the time required for the solvent to penetrate into the polymer matrix causing hydrolysis of the matrix and diffusion of the drug through the polymer system to the external fluid (Bodmeier and McGinity, 1987). On the other hand HA microspheres showed 9% of dexamethasone released within the first hour, followed by a slower sustained release to a total of 12%. Although a high content of dexamehasone was recorded with HA microspheres, the sustained release was lower than PLGA microspheres, this is due to the PBS uptake by polymer which resulted in swelling rather than degradation, leading to increased particle size, in addition to the low solubility of dexamethasone.

To improve the stability of HA microspheres we attempted to cross-link HA by

interacting it with cystine methyl ester in the presence of 1-ethyl-3-(3 dimethyl amino propyl) carbodiiamide (EDC) which was used to affect modification by activation of the COOH group on free HA. This cross-linking produced microspheres of greater stability than the non cross-linked HA, but not of sufficient stability for application. Further work requires to be done on this procedure.

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