POLYANHYDRIDES AS DELIVERY SYSTEMS

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SUMMARY

Poly[(carboxyphenoxy) butane : sebacic acid] (P(CPB:SA)) 20:80 and 50:50 microspheres containing 10% w/w theoretical loading bovine serum albumin (BSA) were prepared by solvent evaporation / double emulsion, spray drying and solvent extraction / oil-in-oil techniques. This paper describes the characterisation of the microparticles obtained (morphology, particle size, drug content, *in vitro* drug release behaviour) and a comparison of the results (drug loading, drug release, size of the microspheres) obtained from different techniques used. The microspheres containing protein were general spherical, with diameters around 10 μm for double emulsion, around 2 μm for spray drying, and 50 μm for solvent extraction / oil-in-oil method. *In vitro* release of the protein into 0.1 M phosphate buffer at 37 °C from microspheres prepared by the three techniques showed that the BSA release rate from the microspheres prepared by solvent extraction / oil-in-oil technique was much quicker with large initial burst compared to the release from the solvent-evaporated and spray dried microspheres. The BSA release profiles for microspheres prepared by double emulsion method did not correlate with the degradation of the microspheres. After 24 hours degradation, most of the anhydride linkage was cleaved, while only around 10 % of the protein was released in six weeks *in vitro*, but in pH10 Na_2CO_3 / $NaHCO_3$ buffer nearly 70% of protein was released in two weeks. The IR spectra of the microspheres prepared by different methods, showed that some parts of the polymer began to degrade during the process of preparation, especial for microspheres prepared by spray drying and oil-in-oil methods.

Key words: Controlled release; drug delivery; double emulsion; spray drying; solvent extraction.

DEDICATION

I would like to dedicate this thesis to my family.

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1. INTRODUCTION

1.1 Controlled Delivery Systems for Peptides and Proteins

Compared with conventional drug compounds, peptides and proteins have unique requirements and restrictions for delivery to their site of action. Controlled delivery technologies of various types have been known for many years, but the field has experienced recent massive advancement in scope and sophistication with the thrust provided by the delivery needs of macromolecules.

In general, pharmaceutical proteins and peptides are administered parenterally, because of their poor bioavailability. This poor bioavailability can be the result of degradation in the gastrointestinal (GI) tract or the low permeability of epithelial barriers for high molecular weight molecules. Moreover, many proteins have a short plasma half-life *in vivo* (minutes to hours). Therefore, a frequent injection schedule or long-lasting infusions are necessary to obtain the desired therapeutic effect. Apart from the practical disadvantages of frequent administration, high concentrations resulting from bolus injections may have toxic side-effects, and effectively cancel out the therapeutic benefits (Crommelin, 1997).

The problems associated with protein and peptide drug administration have necessitated the development of drug delivery systems. These delivery systems may be designed to control the release rate of the proteins and peptides for a long time period to target to site of action or protect the drug from the harsh environment. Different classes of these delivery systems developed so far include:

- Liposomes;
- Biodegradable polymers, *i.e.,* co-polymers of lactide and glycolide (PLGA), polyanhydrides, and hydrogels based on cross-linked dextrans (Weert *et al.*, 1998).

1.1.1 Liposomes

A potential means of controlled delivery of peptides and proteins is by entrapment in liposomes. This has been shown to afford protection aginst enzymatic degradation for proteins (Weingarten *et al.*, 1985; Adrian & Huang, 1979).

In the early 1960s, it was noted that various phospholipids formed multilayered vesicles when dispersed in water. These cell-like structures become known as liposomes. In recent years, drug-containing lipsome systems have been developed for the delivery of drugs by various routes of administration including inhalation, ocular, injectable, dermal and oral (Ansel *et al.*, 1995). Advantages of liposomal systems from pharmaceutical aspects include:

- (a) the possibility of preparation under mild conditions;
- (b) they are especially suitable for hydrophilic substsances;
- (c) the ease of size control;
- (d) the convenience for chemical modification of their surface.

However, the major drawback of these systems is instability, both physical (*i.e.*, aggregation, relatively rapid leakage of entrapped substances, extraction of lipids from lipsomes by high-density lipoprotein, *etc.*) and biological (*i.e.*, rapid removal of liposomes from the bloodstream by the reticuloendothelial system (RES))(Okada, 1996). Two approaches to increase the physical stability of liposomes and provide slow

or pulsatile release patterns on liposomes are polymerised liposomes and microencapsulated liposomes (Okada, 1996).

An interesting application for polymerised liposomes is as an oral drug carrier. Lymphoid tissues, such as Peyer's patches, in the intestine are, by nature, responsible for the uptake of macromolecular antigens from the lumen and for processing them to confer immunity (Hanauer & Kraft, 1985). Macromolecules and small particles can be taken up by the tissues (Ebel, 1990), and this uptake is more efficient for more hydrophobic particles (Eldridge *et al.*, 1990). Therefore, it is possible that liposomes are absorbed efficiently because they are composed of phospholipid. Liposomes can be polymerised to prevent their destruction by bile acids. Over 3% uptake by Peyer's patches has been achieved using polymerised liposomes and this has been increased to 10% by covalently attaching ligands with an affinity for Peyer's patch M cells, such as *Ulex europaeus* agglutinin, to the liposome surface (Chen & Langer, 1996).

1.1.2 Biodegradable Polymers in the Controlled Delivery Systems of Peptides and Proteins

1.1.2.1 Biodegradable Polymers

A biodegradable polymer is ideal for immunisation purposes, for it can release a protein at the desired rate, and does not necessitate an additional surgical step for retrieval of the depleted system.

Linear polyesters are by far the most widely characterised and utilised group of biodegradable polymers, the most significant among them being PLGA (see section 1.1.2.2). Bioerodible hydrogels have been used for controlled release of the model

protein bovine serum albumin (BSA) (Sanders, 1990). At present, hydrogels are the only class of polymer that may be designed such that the peptide will be permeable through the continuum of the carrier. Therefore, a bioerodible hydrogel will have the combination of features of a diffusion-controlled release, possibly augmented by later erosional release, without the requirement of removal of the system on depletion (Sanders, 1990).

Maleic anhydride-co-methyl vinyl ester copolymers have been well studied for controlled-release applications, but have not been reported in the context of delivery of proteins or peptides. These polymers are belived to be surface eroding. In last decade, another group of surface-eroding polyanhydrides has been developed poly[bis (p-carboxyphenoxy) propane-co-sebacic acid anhydride] P(CPP:SA), and approved by the United States Food and Drug Administration for delivery of drugs to treat brain cancer.

1.1.2.2 Microspheres

Biodegradable polymers can be used in many different forms, depending on the application, with microspheres or microcapsules being the most popular form (Bhagat *et al.*, 1996). Concerning their structure, microcapsules can be defined as particles in which a (solid or liquid) core constituted by the drug is surrounded by a membrane (Figure 1.1a). The membrane is made up of one or more polymer and it is the rate-limiting element of drug release. These systems can be classified as reservoirs. Microspheres can be defined as micromatrices in which the drug is uniformally dispersed and / or dissolved in the polymeric network either as fine particles / agglomerates or in a molecular state (Figure 1.1b) (Giunchedi & Conte, 1995). Also in

this case, one or more polymers can make up the matrix structure. However, the term "microparticle" should be used because very often it is actually difficult to distinguish between microcapsules and microspheres. When the microparticles contain several drug crystals, it is a matter of semantics to decide if they are multinuclear microcapsules (Figure 1.1c) or hetergeneous microsphers (Figure 1.1d) (Aftabroushad & Doelker, 1994).

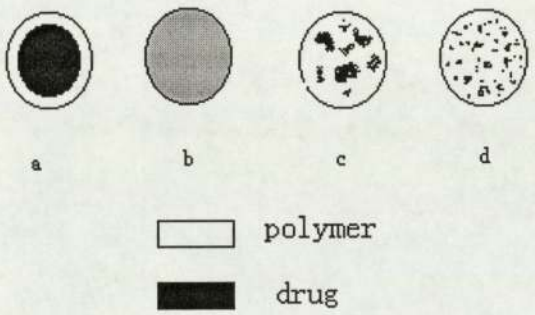


Figure 1.1 Microparticles: a. microcapsule; b. microsphere; c. multinulear microcapsule; d. heterogeneous microsphere (Giunchedi & Conte, 1995).

One of the greatest challenges facing formulation scientists is the oral delivery of macromolecules. One approach being studied for oral vaccine delivery is the development of small (≤ 5μm) microparticles that can be taken up by intestinal Peyer's patches and, to a lesser extent, enterocytes (Hitesh & Dalal, 1996). PLGA microspheres containing formalised staphylococcal enterotoxin B in a size range 1-10 μm were demonstrated as an effective delivery vehicle for the antigen, and its immunopotentiating action was concluded to be due to the protection of the labile antigen by the wall material during gastrointestinal transit and the efficient uptake of the microspheres by the Peyer's patches (Eldridge *et al.*, 1989, Eldridge *et al.*, 1990). Although many materials have been studied as delivery vehicles, there is usually a low uptake by Peyer's patches, less than 1% for polymers such as (PLGA) (Langer, 1998).

Lipophilic polymers led to increased uptake levels but most materials used are not degradable (Eldridge *et al.*,1990). Thus, polyanhydrides may be considered a good candidates for oral delivery owing to their hydrophobicity and biodegradability (see section 1.3).

1.1.2.3 Protein Release from Polyanhydride Microspheres

The incorporation and release of low molecular weight water-soluble species from microcapsules and microspheres of polyanhydrides P(CPP:SA), poly[1,3-bis(p-carboxyphenoxy)hexane-co-sebacic acid] [P(CPH:SA)] and poly(fatty acid dimer-co-sebacic acid) [P(FAD:SA)] is reasonably well understood (see section 1.10.2), but many difficulties are encountered when attempts are made to incorporate macromolecules such as bioactive proteins into polymeric systems (Mathiowitz *et al.*, 1990a; Tabata *et al.*, 1993). Organic solvents are usually used in the preparation of microspheres and contact of proteins with organic solvents can affect protein conformation (Uversky *et al.*,1997). Also, proteins and peptides often fail to yield a desired drug-release behaviour because of poor drug-release control or stability problems (Johnson *et al.*, 1991). Most of these derive from either reduced solubility and hydrolytic stability, or interaction between the polymer matrix and the incorporated macromolecule, *i.e.* 'polymer-protein' compatibility phenomena or the instability of biologically active macromolecules, unless in the crystalline state, to tolerate the solvents and temperatures involved in the fabrication of polymer-based delivery vehicles.

One approach to achieving oral delivery of complex molecules, including genes, has been the development of polyanhydride microspheres, which display strong adhesive

interactions with the intestinal mucosa and cell lining (Langer, 1998). The advantage of using biodegradable, thermoplastic polymer for oral drug delivery is based upon the fact that carboxylic acid groups are exposed on the surface during degradation and may interact with mucus glycoproteins to form secondary bonds between the polymer and the biological substrate. These adhesive bonds theoretically strengthen as the polymer hydrolytically degrades and exposes more carboxylic acid groups, increasing adhesion and delaying transit times through GI tract (Chickering *et al.*, 1996). A delay in transit time will most likely result in increased bioavailability of the loaded pharmaceutical agent (Harris & Robinson, 1990). Low molecular mass drugs such as dicumarol, as well as larger molecules such as insulin and even genes, can be taken up in animals using this type of approach, presumably because the microspheres maintain contact with the intestinal epithelium for longer times. Microscopic evidence has shown that the microspheres can actually penetrate the epithelium, through and between cells (Mathiowitz *et al.*, 1997). Although the extent of uptake is still an area of disagreement, it is generally accepted that intact particle uptake does occur for particles smaller than 10 μm in diameter.

1.1.2.4 Double-Walled Microspheres

Double-walled microspheres consist of two different polymer layers that allow polyanhydrides to be combined with other degradable polymers, such as poly (lactic acid). This might be useful for suppressing the burst release of proteins, or for generating pulsatile-release profiles (Peppas, 1993). For example, PLGA-poly(vinyl alcohol) (PVA) composite microspheres have been shown to release a model protein

(BSA) for up two months (Wang *et al.*, 1999). There are two methods by which such microspheres might be prepared. The first takes advantage of the partial or complete insolubility of polymers in one another. The cosolution of such polymers in organic solvents is dripped into aqueous solutions of PVA. On solvent evaporation the two polymers begin to separate. In their final state, they consist of an inner core made of one polymer and an outer wall that consists of the second polymer (Pekarek *et al.*, 1994). An alternative method by which double-walled microspheres can be prepared is by a modified double-emulsion technique. The polymer [*e.g.* poly(D,L-lactic acid)] (D,L-PLA) or PLGA is dissolved in an organic solvent, such as dichloromethane (DCM) or ethyl acetate, into which a small volume of aqueous phase, containing microspheres, is dispersed to form a water-in-oil emulsion. This emulsion is then dispersed into an aqueous solution of PVA in which new microspheres are spontaneously formed, which contain a core that consists of only one type of polymer and a coating that consists of a second type of polymer (Göpferich *et al.*, 1994).

1.2 Historical Development of Polyanhydrides

Polyanhydrides were first synthesised from aromatic monomers in 1909, by Bucher and Slade. In 1930, the first aliphatic polyanhydrides were synthesised as prospective raw materials for the manufacture of textile fibres. Further research was also pursued in vain during the 1950s to synthesise polyanhydrides with enhanced chemical stability, as polyanhydrides are not hydrolysis-resistant enough to serve as long lasting materials (Domb & Langer, 1987). It is this property, however, that renders polyanhydrides appealing for controlled release applications. In the early 1980s, polyanhydrides were

rediscovered in the search for fast-degrading polymers that could be used for erosion-controlled drug delivery (Rosen *et al.*, 1983). To simplify nomenclature, the monomer names are abbreviated as indicated. The copolymer poly [1,3-bis (p-carboxyphenoxy) propane-co-sebacic acid], with a monomer ratio of 20:80, for example, is abbreviated P(CPP:SA) 20:80. A homopolymer such as poly (sebacic acid) is abbreviated P(SA). This terminology will be used throughout this thesis.

1.3 Significance of Polyanhydrides as Biodegradable Polymers

Although much effort has been focused on utilising polymers that have a history of medical use and then adapting their microstructures to provide desired delivery rates, another approach is the intentional design of materials that solve specific drug-delivery problems (Langer, 1998). In fact, most biomaterial research in the 1960s and 1970s focused on utilising 'off-the shelf' polymers designed for consumer applications and adapting these polymers for medical purposes. For example, materials used in the artifical heart were originally components used to make women's girdles (Peppas & Langer, 1994). Most degradable polymers used in injectable drug-delivery systems display bulk erosion that causes the polymers to dissolve throughout the entire matrix. This makes constant release rates complex to achieve and creates the possibility of dosage dumping as the system eventually hydrolyses. One of the major goals in research on degradable polymers in medicine and pharmacy has been to obtain materials that allow drug release to be controlled by polymer erosion. To maximise control over the release progress, it is desirable to have a polymer system, which degrades only from the surface and deters the permeation of drug molecules (see section 1.8). Achieving

such a heterogeneous degradation requires the rate of hydrolytic degradation on the surface to be much faster than the rate of water penetration into the bulk.

The ideal polymer would have a hydrophobic backbone, but with a water-labile linkage. Many classes of polymers including polyesters, polyamides, polyurethanes, polyorthoesters, polyacrylonitriles, and polyphosphazenes, have been studied for controlled delivery applications, but few, except for polyorthoesters, have been designed with this consideration in mind. Polyorthoesters, however, erode from the surface only if additives are included in the matrix (Heller *et al.,* 1981). Taking advantage of the pH-dependence of the rate of orthoester cleavage, preferential hydrolysis at the surface is obtained by either addition of basic substances to suppress degradation in the bulk, or incorporation of acidic catalysts to promote degradation in the surface. In designing a biodegradable system that would erode in a controlled heterogeneous manner without requiring any additives, polyanhydrides may be a promising candidate due to the high liability of the anhydride linkage to water attack since carboxylic acid anhydrides are among the functional groups that hydrolyse the most rapidly. Table 1.1 reports a survey on the half lives of functional groups that are typical for degradable polymers. Carboxylic acid anhydrides and orthoesters are the most reactive bonds, which makes polyanhydrides and poly(ortho-esters) fast-degrading polymers (Park *et al.*, 1993).

Polyanhydrides have been reported to be surface-eroding (Langer & Vacanti, 1993), as they are composed of a hydrophobic polymer backbone joined by anhydride linkages that readily split in the presence of water to form two carboxylic acid end groups. Thus a polyanhydride delivery device is predicted to undergo surface front erosion, which can

be characterised by degradation on the surface that approaches the centre of the device as erosion continues (Mathiowitz *et al.*, 1993).

POLYMER CLASS	HYDROLYSIS RATE
Polyanhydride	0.1 hours
Polyketal	3 hours
Poly(ortho-ester)	4 hours
Polyacetal	0.8 years
Polyester	3.3 years
Polyurea	33 years
Polycarbonate	42,000 years
Polyurethane	42,000 years
Polyamide	83,000 years

Table 1.1 Half-lives of degradable polymers.

Polyanhydrides can be regarded as "designer polymers" in that they can be synthesised from a large pool of monomers, they can be manufactured with various degrees of crystallinity (Tamada & Langer, 1992), they allow control of degradation rates and water uptake (Domb & Maniar,1993), the hydrolytic degradation rates can be altered over 1000-fold by simple changes in the polymer backbone, and they can be manufactured with a branched structure (Maniar *et al.*, 1990), or they may be cross-linked (Domb *et al.*, 1991).

The hydrophilic anhydride linkage provides the basis for using a variety of backbones and yet ensuring biodegradability. In a preliminary study, a model polyanhydride, poly [bis (p-carboxyphenoxy) alkane anhydride] and its copolymers with SA, displayed near zero-order erosion and release kinetics (Chasin *et al.*, 1990).

The biocompatibility of polyanhydrides has been investigated extensively. When polyanhydrides are used for the parenteral administration of drugs, besides its

biodegradablity, another advantage is their biocompatibility (Brem *et al.*, 1989; Brem *et al.*, 1992) in combination with excellent drug-release control. Poyanhydrides, as well as their products after degradation (monomeric diacids), are highly biocompatible, as has been shown by tissue response and toxicological studies (Leong *et al.*,1986; Bakker *et al.*, 1988). Early studies assessed the biocompatibility of P(CPP), poly(terephthalic acid anhydride) [P(TA)], P(CPP:SA), and poly (terephthatic acid-co-sebacic acid) [P(TA:SA)] as well as the toxicity of their monomers (Leong *et al.*,1986). The monomers tested were non-mutagenic, non-toxic, and were found to have a low teratogenic potential *in vitro*. The polymers did not lead to inflammatory responses after 6 weeks of implantation into the rabbit cornea and there were no signs of inflammation after subcutaneous implantation into rats (Bakker *et al.*, 1988). The biocompatibility of P(CPP:SA) 20:80 has been tested subcutaneously in rats. The polymer showed excellent biocompatibility up to doses of 2,400 mg/kg in rats (Laurencin *et al.*, 1990). The biocompatibility of P(CPP:SA) 20:80 in the brain was first assessed in rodent models (Tamargo *et al.*, 1989; Brem *et al.*, 1989). There was a slight transient inflammatory response to the polymer, but it was comparable to the response provoked by Surgicel®, an oxidised, regenerated cellulose and established hemostatic agent used routinely in neurosurgery. Similar results were obtained for P(CPP:SA) 50:50 in rabbits. The polymer showed again no signs of toxicity and a tissue reaction comparable to Gelfoam®, a resorbable gelatin sponge. The brain compatibility of P(FAD:SA) was also assessed in rats. It showed an acute inflammatory response after 3-6 days comparable to P(CPP:SA) and Surgicel® (Brem *et al.*, 1992). Finally the brain biocompatibility was verified in a monkey model (Brem, 1990). In the same animal model, carmustine-loaded implants made of P(CPP:SA) 20:80 were found to be a safe dosage form.

Since the CPP:SA copolymer was designed to be used clinically to deliver an anticancer agent directly into the brain for the treatment of brain neoplasms, only *in vivo* safety elvaluations, subcutaneous implant and brain biocompatibility were assessed.

1.4 Polyanhydride Composition

1.4.1 Monomers

The general formula of polyanhydrides is shown in Figure 1.2. They are bifunctional carboxylic acids, which differ in the chemical groups R1 and R2, separating the carboxylic acid ends. Polyanhydrides can be synthesised as homopolymers (R1=R2), or as copolymers (R1≠R2). Some of the numerous monomers that have been used for the manufacture of polyanhydrides are shown in Figure1.3.

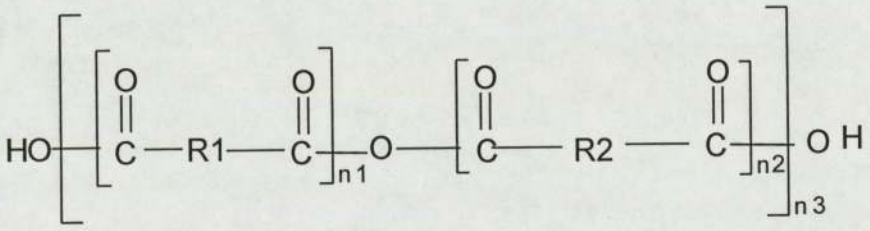
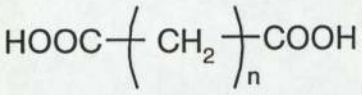


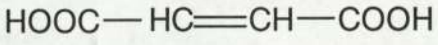
Figure 1.2 General polyanhydride structure

They can be manufactured as aliphatic or aromatic homopolymers and copolymers as well as cross-linked or branched polymers. Not all polyanhydrides made of the monomers shown in Figure 1.3 are ideal materials for the manufacture of drug delivery systems as some of the homopolymers have poor mechanical properties and an undesired stability or instability against degradation. For example, poly(sebacic

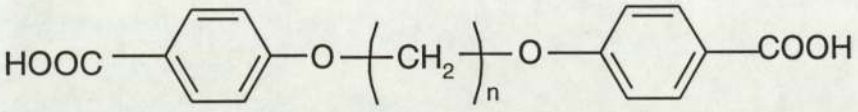
anhydride) (P (SA)), is highly crystalline, has poor mechanical properties, and erodes rapidly. Weight loss measurements, which were used to determine the rate of degradation of the polymers, revealed that 50% degradation was achieved after 24 h with blank microspheres (Mathiowitz *et al.*, 1990a). However, P(CPP) has been reported to be stable for years (Leong *et al.*, 1985) and it cannot be melt-processed as it has a high melting point at which it also begins thermal degradation. Furthermore, its solubility in common solvents is very low. This illustrates why tremendous efforts have been undertaken to improve the properties of polyanydrides by copolymerisation. By the appropriate choice of monomers, the polyanhydride matrix can degrade over periods ranging from one day to several months or any time in between (Leong *et al.*, 1985).



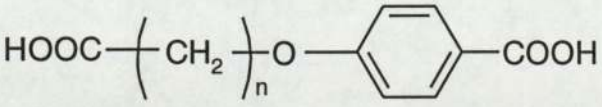
n=4 adipic acid (AA)
n=8 sebacic acid (SA)
n=10 dodecanoic acid (DA)



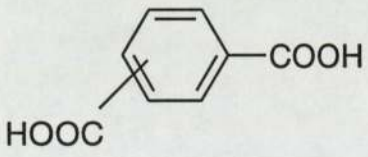
fumaric acid (FA)



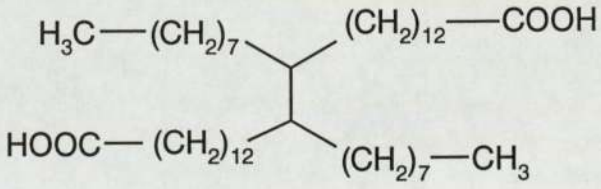
n=1 bis (p-carboxyphenoxy)methane (CPM)
n=3 1,2-bis(p-carboxyphenoxy)propane (CPP)
n=6 1,3-bis (p-carboxyphenoxy)hexane (CPH)



n=1 p-carboxyphenoxy acetic acid (CPA)
n=4 p-carboxyphenoxy valeric acid (CPV)
n=8 p-carboxyphenoxy octanotic acid (CPO)



meta: isophthalic acid (IPA)
para: terephthalic acid (TA)



erucic acid dimer (FAD)

Figure 1.3 Examples of monomers that have been used for the manufacture of polyanhydrides

1.4.2 Aliphatic Polyanhydrides

One class of aliphatic polyanhydrides that have proved to be useful for drug delivery purposes is P(FAD:SA) (Domb & Maniar, 1993). Many other aliphatic polyanhydrides, however, have properties that are not advantageous for the manufacture of drug delivery systems. For example, P(FAD) is a liquid and not well suited for the manufacture of solid drug delivery systems. Aliphatic polyanhydrides generally hydrolyse much more rapidly than aromatic polymers ones due to the better accessibility of the bonds to water. For example, the infrared (IR) spectrum for microspheres of the aliphatic P(FAD:SA) showed no anhydride bonds after about 120 hours incubation *in vitro*, suggesting complete microsphere degradation (Tabata & Langer, 1993a).

1.4.3 Aromatic Polyanhydrides

Aromatic polyanhydrides generally degrade and erode more slowly than aliphatic compounds (Leong *et al.*, 1985), due to their increased hydrophobicity and the hindered approach of water to the anhydride bond (Tamada & Langer, 1992). Some aromatic polyanhydrides, such as pure P(CPP), degrade in about 3 years (Chasin *et al.*, 1990). The erosion rate can be increased by copolymerisation with aliphatic monomers. Copolymerisation allows the adjustment of erosion rates and, therefore, the duration of drug release in drug delivery applications. For example, P(CPP:SA) erodes within weeks or months depending on the composition (Leong *et al.*, 1985). Examples of aromatic polyanhydrides that have been investigated for drug delivery applications are P(CPP) and poly[1,2-bis(p-carboxyphenoxy)propane-co-isophtalic acid] [P(CPP:IPA)].

1.4.4 Cross-linked and Branched Polyanhydrides

Unsaturated polyanhydrides of the structure $[-(OOC-CH=CH-CO)_x-(OOC-R-CO)_y-]_n$ have the advantage of being able to undergo secondary polymerisation of the double bonds to creat a crosslinked matrix. A series of unsaturated polyanhydrides were prepared by melt or solution polymerization of fumaric acid (FA), acetylenedicarboxylic acid (ACDA), and 4,4'-stilbendicarboxylic acid (STDA) (Domb *et al.*, 1991).

The unsaturated homopolymers were crystalline and insoluble in common organic solvents whereas copolymers with aliphatic diacids were less crystalline and were soluble in chlorinated hydrocarbons, and at same time the mechanical stability of polyanhydrides was increased (Domb *et al.*, 1991). This can be important for their use as load-bearing biomaterials in orthopedic applications.

Cross-linked polyanhydrides can be obtained after introducing double bonds into the polymer backbone. A monomer that has been used for that purpose is FA in combination with SA. When P(SA) was compared with branched P(SA), an impact on drug release was noticeable but there was little change in physical and mechanical properties (Maniar *et al.*, 1990). By increasing the amount of branching agent benzenetricarboxylic acid from 0 to 2 %, it was possible to reduce the release of morphine from approximately 70% to approximately 40% within 8 days.

1.5 Synthesis of Polyanhydrides

Concomitantly, tremendous efforts made to synthesise new polymers have led to a better understanding of polymer erosion in general and finally to new drug delivery

systems being introducted onto the market. For example, P(CPP:SA) polymers are used as a delivery system for the treatment of brain cancer in humans (Brem *et al.*, 1993; Domb & Ringel, 1994).

There are a number of ways to synthesise polyanhydrides from carboxylic acid monomers (Leong *et al.*, 1987). Polyanhydrides have been synthesised by melt condensation of activated diacids (Domb *et al.*, 1987), ring opening polymerisation, dehydrochlorination and dehydrative coupling agents (Leong *et al.*, 1987; Domb *et al.*, 1988). Solution polymerisation yields, in general, low molecular weight polymers. The most frequently used technique for the manufacture of linear polyanhydrides is melt polycondensation, but another common method of initiation of polycondensation is the activation of the carboxylic acids using acetic acid anhydride (Albertsson & Lundmark, 1990; Domb & Langer, 1987). For the manufacture of copolymers, all individual monomers are activated separately. The resulting mixed anhydrides, *i.e.*, the so-called prepolymers, are isolated and purified. They usually consist of a few monomers that are connected to one another *via* anhydride bonds and form a mixed carboxylic anhydride group with acetic acid at each end of molecule. For the actual polymerisation, these prepolymers are heated to 180°C under vacuum. During the polymerisation of the oligomers, acetic anhydride is formed as a side product, and removed by distillation and vacuum during the reaction. The advantage of polycondensation is that a high molecular weight product can be obtained. A disadvantage of the method is the thermal stress to which the monomers are subjected.

1.5.1 High Molecular Weight of Polyanhydrides

In previous studies, a molecular weight of 12,030 weight average (Mw) and 5,280 number average (Mn) was reported for a P(CPP:SA) 20:80 coplymer prepared by the melt-polycondensation method at 130°C, as determined by gel permeation chromatography (GPC) analysis (Domb & Langer, 1987). When the polymerisation was commenced at 175°C for 4 days, a tarry product whose soluble portion showed a Mw of 52,800 and Mn of 4,200, was obtained. Thus, although synthesis of polyanhydrides is well documented, high molecular weight polyanhydrides are essential, however, for applications where superior physicomechanical properties are required. In addition, by raising the molecular weight of polyanhydrides, even less hydrophobic polymers could exhibit film-forming properties. For example, it has been reported that increasing either the percent of CPP content in the P(CPP:SA) or the molecular weight, increases tensile strength (Domb & Langer, 1987). Decreasing the Mn of the films of the same CPP content (60%) from 12,100 to 6,400 resulted in lower tensile strength.

1.5.2 The Function of Catalysts in the Synthesis of Polyanhydrides

Since the reaction is an anhydride interchange that involves nucleophilic attack on a carbonyl carbon, a catalyst that will increase the electron deficiency of the carbonyl carbon will affect the polymerisation. Many effective coordination catalysts have been suggested for the transesterification polymerisation of polyesters, which is a reaction similar to the anhydride interchange. Also, similar catalysts have been found to be effective in ring-opening polymerisation of epoxides due to metal oxygen complexation. It is conceivable that these catalysts might be effective in producing polyanhydrides, so they have been used in the polymer synthesis to obtain polyanhydrides with high

molecular weight. There are many catalysts, which have been used in the polycondensation process to produce polyanhydrides, including metal salts, earth metal oxides, alkoxy metals, organometals and ferric compounds. Significantly higher molecular weights in shorter times were achieved by utilising cadmium acetate, earth metal oxides, and $ZnEt_2$-H_2O. The molecular weights ranged from 140,935 to 245,010 with catalysts, in comparison to 116,800 without catalysts (Domb & Langer, 1987).

1.6 The Composition of Copolymers of Polyanhydrides

Copolymers of polyanhydrides were first investigated for the randomness of the monomer distribution in the polymer backbone by nuclear magnetic resonance (NMR) spectroscopy (Ron *et al*, 1991). The results differentiate between randomly distributed monomers in the polymer backbone and a more block-like structure. In a copolymer with monomers A and B, there are three possible types of bonds AA, BB, and AB. The relative number of these bonds can be determined experimentally by using NMR and compared with predictions based on the random distribution of monomers (Tamada & Langer, 1992). There should be reasonable agreement between predicted and experimental values, in order to conclude that the copolymers are formed randomly. For copolymers made of SA, in combination with CPP or 1,3-bis(p-carboxyphenoxy)hexane (CPH), it was found that the monomers were mainly randomly distributed when the content of both monomers was equal (Ron *et al*., 1991). The extent of randomness in the distribution is important with respect to erosion. A block-like arrangement of the monomers inside the polymer chain might lead to the discontinuous

erosion of the material when the two blocks exhibit different resistance against degradation and erosion.

1.7 Characterisation of Polyanhydrides

To understand the characteristics of polyanhydride drug delivery systems, it is necessary to have a detailed knowledge of the properties of the polymers. Understanding the erosion mechanism is necessary for the successful application of these materials in drug delivery applications. For this reason polyanhydrides have been characterised extensively, which makes much physicochemical data available (Tamada & Langer, 1993; Mathiowitz *et al.*, 1993; Albertsson & Lundmark, 1990; Chasin *et al.*, 1990). One of the most important properties is crystallinity as it is the determining factor affecting degradation and dissolution of the polymer into its monomeric constituents (Santos *et al.*, 1999). The crystallinity of polyanhydride has been investigated by wide angle X-ray diffraction (WAXD). Some of the homopolymers such as P(SA), P(CPP), and P(FA) were found to be partially crystalline (Mathiowitz *et al.*, 1990c); others such as P(FAD) were found to be amorphous (Shieh *et al.*, 1994). Crystallinities as high as 60% have been recorded. The crystallinity of copolymers has been shown to depend on the monomer ratio. The lowest degree of crystallinity is reached at a copolymer composition of 1:1 for many polyanhydrides. Polyanhydrides derived from monomers such as FAD or CPH in combination with SA are an exemption. As P(CPH) is almost amorphous, the crystallinity of P(CPH:SA) increases only with increasing SA content (Mathiowitz *et al.*, 1990c). The same can be observed for P(FAD:SA). When copolymers are made of one crystallisable type of monomer such

as SA and one that does not form crystallites such as FAD, their crystallinity has also been calculated from heats of fusion data measured by differential scanning calorimetry (DSC). Important parameters for processing polyanhydrides to dosage forms are the glass transition temperature (Tg) and the melting point (Tm) (Tamada & Langer, 1992). Tg, the glass to rubber transition temperature, indicates a transition from a rigid to a flexible structure causing a change in heat capacity and hence a shift in the baseline of DSC. It influences polymer mechanical properties, polymer forming and processing characteristics, permeability and drug diffusion. Below the Tg, the polymer loses its flexible working behaviour, polymer molecules behave as rigid units. At the Tg, molecular vibrations / oscillations are large enough to overcome intermolecular force and the polymer may be deformed comparatively easily. Above the Tg, the thermal energy of molecules is so large that polymer exhibits rubbery behaviour, and eventually the polymer is converted to a liquid if the temperature is raised further. The Tm depends on the degree of crystallinity and is the crystalline melting temperature.

Besides the melting point and the heat of fusion, which are related to the crystalline phase of a polymer, the glass-transition temperature Tg of the amorphous phase also can be determined by DSC. Melting points have been found to be as high as 246°C for P(FA), 240°C for P(CPP), and 143°C for P(CPH) (Mathiowitz *et al.*, 1990c). The melting point drops substantially after copolymerisation. For all homopolymers made of SA, FA, CPP, and CPH and all copolymers made of SA in combination with FA, CPP, and CPH, Tg values ranged from 2°C to 60°C. Only P (CPP) is the exception with a Tg of 90°C. The lowest values of Tg were obtained for the copolymers with equal molar composition (Mathiowitz *et al.*, 1990c).

1.8 Characteristics of Polyanhydrides Degradation and Erosion

Erosion of the polymer bulk can be affected by a variety of parameters. Most important is the chemical degradation of bonds in the polymer chains. Polyanhydrides differ from many other polymers in the reactivity of the anhydride bond (Park *et al.*, 1993), since carboxylic acid anhydrides are among the functional groups that hydrolyse the most rapidly (see Table 1.1). The faster a polymer erodes, the greater its chances that drug release might be erosion-controlled. Polyanhydrides are, therefore, an ideal material for the manufacture of erosion-controlled drug-delivery systems.

1.8.1 Definition of Degradation and Erosion

Degradation, which is the process of chain cleavage, can be investigated by following the molecular weight change of a substance. The erosion of degradable polymers is a complicated process, in which various reaction and transport processes are involved. Erosion starts with the intrusion of water into the polymer bulk and triggers degradation. Degradation is the polymer chain scission process and is the most important part of erosion. Through degradation, oligomers and monomers are created that finally diffuse to the polymer surface, where they are released from the polymer bulk.

Erosion is the sum of all processes leading to the loss of mass from a polymer matrix. It should be kept in mind that degradation is not mandatory for a polymer matrix to erode. If the polymer is at least partially soluble in the erosion medium, for example, dissolution processes might contribute to erosion as well. Conversely, if the polymer has degraded completely, it does not necessarily erode.

1.8.2 Kinetics of Degradation

All degradable polymers consist of monomers that are connected to one another by functional groups that break down during degradation process. Hydrolysis is the major cause for degradation (Park *et al.*, 1993). The degradation velocity depends on the type of hydrolysable functional group from which the polymer is built and determines how a polymer erodes (Göpferich, 1996a). The fast degradation of polyanhydrides has consequences for the erosion mechanism.

The degradation of polyanhydrides, in general, varies with a number of factors. These factors include the nature and hydrophobicity of the monomers used to produce the polymer, the level of drug loading in the polymeric matrix, the pH of the surrounding medium (the higher the pH, the more rapidly the polymers degrade), the shape and geometry of the implant (degradation is a function of the surface area) and the accessibility of the implant to water (porous materials will degrade more rapidly than non-porous) (Domb *et al.*, 1997). When P(CPP:SA) 20:80 matrix discs were incubated in phosphate buffer (pH7.4) at 37°C, it was found that the molecular weight dropped exponentially during the first 24 hours (D'Emanuelle *et al.*, 1992). It is important that such investigations reveal the time scale on which degradation occurs, as the chemical degradation of bonds in the polymer chains is important among the variety of parameters affecting the erosion of the polymer bulk, therefore, yielding precious information on the expected time over which drugs may be released. However, the result of investigating large matrix discs does not allow the assessment of the degradation properties unequivocally. With increasing dimensions, the result depends on other processes in addition to degradation, such as the diffusion of water into the polymer bulk. If water diffusion is slow, the degradation of the polymer matrix disc is

affected because the lack of water prevents the degradation inside the polymer matrix. From the resulting molecular weight changes, it can be concluded that polyanhydrides degrade across their entire cross section for geometries of a reasonable size (Göpferich, 1996b).

When investigating the degradation behaviour of polyanhydride microspheres, the process is more complex. For example, after the complete cleavage of anhydride bonds occurred in the P(FAD:SA) microspheres, prepared by a double emulsion method, containing acid orange 63, acid red 8, or p-nitroaniline, oily water-insoluble FAD monomers were still left in the micrspheres (Tabata & Langer, 1993a). For P(FA:SA) microspheres made by the hot melt encapsulation process, it appears that although the P(FA:SA) material degrades very quickly (18 hr) in an aqueous environment, there is a plateau in the degradation and oligomeric material containing anhydride linkages remains stable for an extended period of time (Santos *et al.*, 1999).

Other aspects that have to be considered are autocatalytic effects that stem from the free monomers created during degradation. More recently, NMR investigations were performed to monitor the degradation of individual bonds in polyanhydride copolymers (Heatley *et al.*, 1998). Studies on P(CPP:SA) confirmed that bonds in which SA is involved are cleaved faster than bonds between CPP molecules.

The fast degradation of polyanhydrides is their strength and concomitantly their weakness. This is illustrated by experiments with polyanhydrides in solution. Even when dissolved in anhydrous chloroform, polyanhydrides have been reported to decrease in molecular weight. Poly(p-carboxyphenoxy-valeric acid) [P(CPV)] Mw 18,500 and poly(p-carboxyphenoxy-octanotic acid) [P(CPO)] Mw 25,950 lost 50% of

their molecular weight within approximately 1.5 hours (Domb & Langer, 1989). The examples illustrate that polyanhydrides have to be stored under anhydrous conditions. Polyanhydrides, in general, degrade more rapidly in basic media than in acidic media (Leong *et al.*, 1985). At pH 7.4, pure P(CPP) degrades in about 3 years. However, this rate increases markedly as the pH rises, and at pH 10.0, this material degrades in just over 100 days. At very acidic pH values, many of the polyanhydrides virtually do not degrade at all (Santos *et al.*, 1999). The solubility of CPP and SA depends on the pH of the release medium. As expected for carboxylic acids, the solubility of both compounds can be increased by increasing the pH (Göpferich & Langer, 1993).

1.8.3 The Importance of Erosion for Drug Release

The erosion mechanism has consequences for the release of drugs from degradable polymers. Drug release can be classified into diffusion-, swelling-, and erosion-controlled release (Langer, 1990). A degradable polymer might release drugs by all three mechanisms. The quickest mechanism, however, will dominate the drug release. If the three processes proceed at similar speed, drug release will be controlled by all three simultaneously. In order to have optimal control over drug release from degradable polymers, it is desirable that it could be mainly erosion-controlled. The only way that this can be achieved is by using fast-eroding polymers, which is the case for polyanhydrides.

The effect of different backbones on erosion rates was demonstrated in a study of the homologous poly [(p-carboxyphenoxy) alkane] series. As the number of methylene groups in the backbone increased from 1 to 6, thus decreasing the reactivity of the

anhydride linkage and rendering the polymer more hydrophobic, the erosion rates underwent a decrease of three orders of magnitude. (Leong *et al.*, 1985).

The erosion zones that are created during polyanhydride erosion may have some effect on the release of monomer. P(CPP:SA) and P(FAD:SA) can serve again as a good example. Comparing the release of SA from both polymers, the release is slightly faster from P(CPP:SA) compared to P(FAD:SA). Most likely the different nature of the erosion zones accounts for this effect. Whereas in P(FAD:SA), SA can diffuse through a network of pores, it has to pass through an amorphous lipid layer in the other, thus slowing its release. The impact of erosion zones on monomer release from P(CPP:SA) has also been illustrated by applying diffusion theory (Göpferich & Langer, 1995a). Under the assumption that a saturated solution of SA, which is in equilibrium with suspended SA, exists at the erosion front, a diffusion model simulates the release of SA through the porous and tortuous erosion zone. Assuming further that SA controls the solubility of the CPP, the sigmoidal release profile for CPP was confirmed by this modelling approach. Meanwhile, the poor solubility of the monomers is also reflected by the fast release of drugs from P(CPP:SA) matrix discs. Indometacin, for example, was found to be released faster than SA (Göpferich *et al.*, 1995).

The release of macromolecules from biodegradable microspheres is influenced both by the structure of the microparticles and properties of the biodegradable polymer itself. Most of the antigen-delivering microparticles exhibit a matrix-type internal, solid dispersion morphological structure (Kissel & Koneberg, 1996). The proteins are insoluble in the polymeric matrix, and the macromolecules are released by a mechanism that combines pore diffusion and polymer erosion (Kissel & Koneberg, 1996). Initially, water diffuses into the matrix, dissolving drug particles adjacent to the surface of the

device. The resulting osmotic pressure is relieved by forming a tortuous channel to the surface, releasing a defined amount of protein in the initial drug burst. This burst effect is controlled mainly by three factors: the protein / polymer ratio, the particle size of the dispersed protein, and the particles size of the microspheres (Kissel & Koneberg, 1996). The release of protein depends on the monomer composition of polyanhydrides used, which also affects the erosion of the microspheres. For example, no initial burst is observed during the release of BSA from P(FAD:SA) 25:75 microsphees at various protein loadings, and the protein is released for up to 3 weeks at a near-constant rate. The deposition of FAD monomer on the surface of the particles during erosion might be responsible for the changing release profiles (Tabata & Langer, 1993a).

1.8.4 Changes in Polyanhydride Properties During Erosion

1.8.4.1 Morphological Changes During Erosion

The erosion of a degradable polymer is perhaps the most crucial property with respect to its performance as a carrier material for drug delivery. When research on degradable polymers for drug delivery intensified, a basic classification was proposed for degradable polymers. Bulk-eroding or homogeneously-eroding polymers were distinguished from surface-eroding or heterogeneously-eroding ones (Langer & Peppas, 1983). The difference is illustrated in Figure 1.4. In the surface-eroding polymers, degradation is faster than the intrusion of water into the polymer bulk and, therefore, is confined to the polymer surface. Consequently, erosion also affects only the outermost polymer layers. Bulk-eroding polymers, in contrast, degrade slowly and, because of the

rapid intrusion of water into the bulk, throughout their cross-section. Therefore, erosion is not limited to the polymer surface.

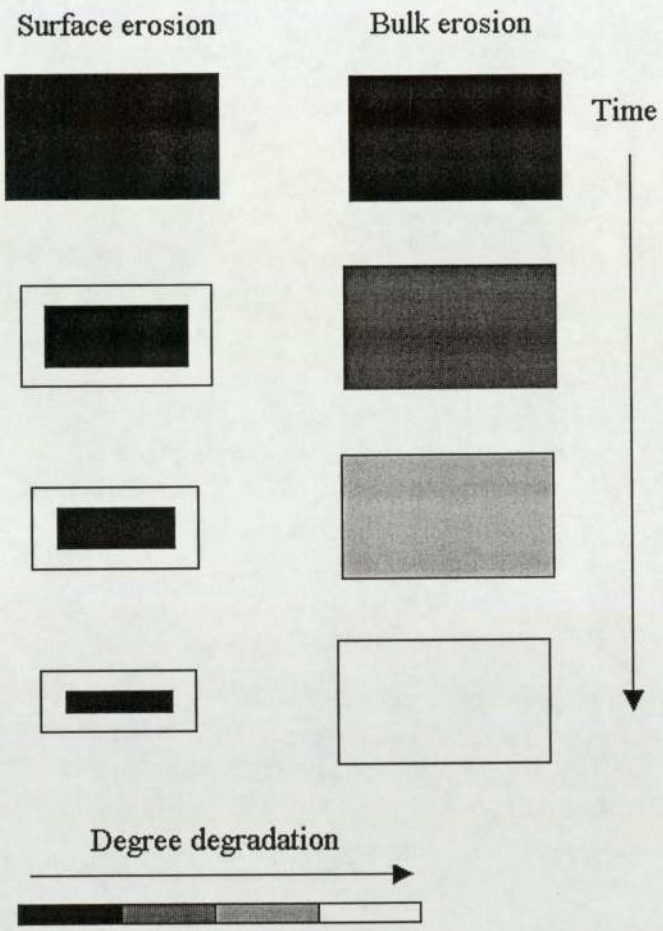


Figure 1.4 Schematic illustration of surface erosion and bulk erosion polymers.

An essential condition for a water-insoluble polymer to undergo surface erosion is the fast degradation of its polymer backbone (Brunner & Göpferich, 1996). Polymers containing reactive functional groups tend to degrade quickly and to be surface-eroding, whereas polymers containing less reactive functional groups tend to be bulk-eroding. It is not surprising that polyanhydrides and poly (ortho-esters) are among the few polymer

groups that have been reported to be surface eroding, since they are assembled from fast-hydrolysing functional groups. Polymers that have hydrophobic monomer units connected by water-labile bonds has the advantage of keeping water contacts with the matrix surface (Langer, 1998). Polymer matrices that display predominantly surface erosion have been created by synthesising hydrophobic polyanhydrides (Tamada & Langer, 1993). For example, polymer erosion and protein release over 1 month were achieved by increasing the percentage of the most hydrophobic monomer (CPP) in the polymer backbone (Chiba *et al.*, 1997).

However, surface erosion and bulk erosion are ideal cases. For most polymers, erosion has features of both mechanisms, which is also the case for most polyanhydrides. The crystalline parts of polyanhydrides degrade and erode too slowly to allow perfect surface erosion (Brunner & Göpferich, 1996). Crystalline erosion zones remain on the polymer surface. However because of the high porosity in the erosion zone, results from the disappearance of amorphous polymer and the remaining crystalline skeleton, P(CPP:SA) comes close to a perfect surface-eroding polymer. Erosion zones in P(FAD:SA) are different. This is because of the physical state of FAD, which is an oily liquid. Rather than building erosion zones, the FAD monomer created during erosion, sticks to the surface of the polymer, leading to the steady accumulation of an FAD film. Such FAD films might act as diffusion barriers and contribute substantially to the control of drug release from an eroding polymer (Shieh *et al.*, 1994). As a general rule it can be assumed that surface-eroding polymers erode faster than bulk-eroding ones. The erosion of most of the clinically relevant polyanhydrides has been investigated and revealed useful information for the manufacture of drug delivery systems. The erosion mechanism for polyanhydride discs, which creates porous erosion zones, has a marked

impact on the release of substances. Macropores are created immediately after P(CPP:SA) discs contact the erosion medium. They result from cracks on the surface of polyanhydride matrix discs (Göpferich & Langer, 1995b). Erosion designates the loss of material from the polymer bulk, which may be parts of the polymer or its degradation products, such as oligomers and monomers. The weight loss of polymer matrices is therefore an indicator of erosion (Göpferich, 1997). Originally erosion was followed by the determination of monomer release from the polymers (Tamada & Langer, 1993) and the mass loss of polymer matrices during erosion (Göpferich & Langer, 1993).

Although the mass loss of P(CPP:SA) matrix discs is almost linear, expected from a surface-eroding polymer, the release profiles of the two monomers do not follow the same kinetics. To solve this paradox, other factors having an impact on erosion, besides degradation, must be taken into account. The microstructure of the polymer discs and the monomer solubility are two major factors, that can influence the erosion of polymer matrix.

The microstructure of polymer discs and changes during erosion have to be assessed using physicochemical techniques as this information is essential to understand how polymers erode (Göpferich, 1997). For P(CPP:SA) the crystallinity changes have been investigated using DSC, WAXD, scanning electron microscopy (SEM) and solid state NMR (Göpferich & Langer, 1993; Mathiowitz *et al.*, 1993). It was found that these polymers do not erode according to a perfect surface-erosion mechanism. The amorphous polymer parts were found to erode substantially faster than the crystalline ones. As a consequence, erosion zones formed in which the amorphous polymer disappears first and is replaced by a network of pores that stretch through the crystalline

areas of noneroded polymer. The foremost line of eroded polymer, the erosion front, moves from the surface of the polymer matrix into the centre (Brem, 1990).

Despite well established manufacturing procedures (Donbrow, 1992), microspheres remain a very delicate and complicated drug delivery system. Their microstructure can change upon even slight variations of the manufacturing process (Schugens *et al.*, 1994). In a previous study, P(CPP:SA) microspheres made by melt microencapsulation displayed dominantly surface erosion (Mathiowitz & Langer, 1987). In the case of P(CPP:SA) microspheres prepared by solvent remolval method, the crystalline polymers precipitated to form a porous structure. In spite of the highly crystalline polymers, water penetrated through the pores of the polymer, causing rapid release (Mathiowitz *et al.*, 1990a). Some micropores were also gradually created by erosion and resulted from the faster erosion of amorphous polymer areas compared with crystalline ones as the porous microstructure allowed fluid to enter into the microspheres and led to a bulk erosion. Thus, the same microspheres prepared by different processes, illustrated different morphological changes during erosion due to different microstructures.

However for microspheres prepared from P(FAD:SA) by a solvent evaporation method using a double emulsion, the scanning electron microscopy (SEM) photograph of the microspheres cross-section after 44 h of degradation showed that only the microsphere surface was attacked. After 122 h of degradation the spherical shape of microspheres was no longer observed (Tabata & Langer, 1993a). In case of P(CPP:SA) 50:50 microspheres prepared by solvent removal, the microspheres with a smooth external surface, lost their integrity, occasionally leaving an empty shell after 12 h degradation (Mathiowitz *et al.*, 1988). Therefore, the erosion mechanism of microspheres also depends on the composition of the polymer matrices in addition to the microstructure.

1.8.4.2 Crystallinity Changes During Erosion

The faster erosion of amorphous polymers compared with crystalline ones, changes the overall crystallinity of polymer matrices during erosion. Changes in crystallinity can be followed by wide-angle X-ray diffraction (WAXD) and DSC. From DSC studies of P(CPP:SA), it was estimated that new crystalline matter may have been created by erosion, and the source of new crystallinity was identified to arise from the crystallisation of the monomers, SA and CPP. This observation was further confirmed in studies where the degradation of the polymer chains was faster than the diffusion of monomers to the matrix surface, possibly leading to monomer crystallisation inside the porous network of the devices (Göpferich & Langer, 1993). pH investigations have shown that the pH inside the porous layers is determined by the monomers which have a limited solubility. It cannot, however, be excluded that anhydride oligomers are present in these polymers. Thermal analysis of P(FA:SA) indicated formation of stable oligomeric material (Santos *et al.*, 1999). Therefore, these data suggest that these monomers have the tendency to crystallise inside the pores of the erosion zone.

1.8.4.3 pH Changes During Erosion

Investigation of the erosion mechanism of polyanhydrides has focused on the characterisation of the erosion zones and the chemical conditions that prevail within them. An issue of special interest is the question of pH within the eroding polyanhydrides, as the pH inside eroding microspheres might affect the solubility and the stability of incorporated drugs. As the polymer degrades into shorter pieces, the

nature of these products may strongly affect the internal environment of the microspheres and, hence, that of the protein. If the products of degradation are acidic, the pH can dramatically change within the polymer. It appears that all polyanhydride possess the ability for sequestration of acid. Therefore, the pH range that may be present in the microspheres must be considered as a potential cause of protein inactivation within biodegradable microspheres. Proteins are often stable in a narrow pH range and the rate of protein aggregation can be strongly affected by pH (Wang,1999). The acid environment produced by the degradation of the polyanhydride would be a substantial hurdle to overcome for delivery of proteins, because acid denaturation of some proteins may be rapid in this acid environment. For example, at pH 2.5 37°C, tetanus toxoid loses its antigenicity in less than 1 week (Jameela *et al.*, 1997). Human growth hormone, released after 30 days from (PLGA) microspheres prepared by a dispersion solvent-extraction method, is induced to degrade by a pronounced pH drop in the release medium and within polymer matrix, caused by hydrolysis of PLGA (Gombotz & Pettit, 1995; Xing *et al.*, 1996). The lowering of the pH, caused by hydrolysis of polymer, may affect protein conformation and enhance chemical degradation, such as hydrolysis and deamidation (Manning *et al.*, 1989). One approach that has been taken to counteract the acidity is the incorporation of buffering substances into the matrix during the device fabrication. The incorporation of buffering substances within the polymer does result in an increase of the pH inside the delivery system (Domb *et al.*, 1997). It has been postulated that the pH drop is much less pronounced *in vivo*, than *in vitro,* as the degradation products of the polymer that cause the pH drop will be rapidly cleared from the injection site *in vivo* (Park *et al.*, 1995).

P(SA) and P (CPP:SA) 20:80 exhibit a highly tortuous network of pores in the erosion zone, in contrast, P(CPP:SA) 50:50 is too amorphous to build up crystalline superstructures (Göpferich & Langer, 1993). Investigation by confocal fluorescence microscopy using pH-sensitive fluorescent probes revealed that the pH on the surface of P(CPP:SA) 20:80 matrices was one unit lower than in the surrounding pH 7.4 buffer (Göpferich & Langer, 1993). The pH within polymer pores may be even lower. In combination with findings by DSC and WAXD, which indicate that both monomers crystallise inside the erosion zone, it was concluded that the pH inside the erosion zone was about 5 (Göpferich & Langer, 1993). These assumptions were confirmed more recently by spectral spatial electron paramagnetic resonance imaging using pH-sensitive spin probes (Mäder *et al.*, 1997). The effect that this pH microclimate has on the release of substances can again be seen from the release profiles of the monomers. P(CPP:SA) 20:80 and 50:50 released the monomers in a similar way to each other but different from the homopolymers. A lag period during the first hours of erosion was visible which was also observed for weight loss. Between days 1 and 6, the release of SA was almost constant and fast. After one week, all SA had been released from copolymers. Compared to SA, the release of CPP was much slower and more complicated. During an initial period of 7 days, the release rate of CPP was almost linear. It then increased instantaneously, displaying a slightly sigmoidal profile. The release was triggered by examining the disappearance of SA. This discontinuity can be explained by the solubility of the monomers. It was found that both substances have a similar first pK_a value (pK_a = 4.8 for SA and pK_a = 4.5 for CPP), but the SA is five times more soluble than CPP. The pH will therefore, be determined mainly by SA. Whenever SA has left the device, the pH will rise and CPP will become more soluble. Two major factors

influence monomer release and will cause the slower release rate of CPP compared to that of SA. Firstly, because of CPP's higher molecular weight relative to that of SA, CPP will diffuse more slowly through the highly porous and tortuous eroded zone. Secondly, the lower solubility of CPP relative to SA affects its release rate. These results confirm that even though the mass loss kinetics appear to be simple, the individual processes of erosion can become quite complicated (Göpferich & Langer, 1993).

1.9 Preparation of Polyanhydride Microspheres

Polyanhydride microspheres have been prepared by four different methods: solvent evaporation, solvent removal, hot-melt encapsulation, and spray-drying (Brunner & Göpferich, 1996). In addition, two methods for the manufacture of dual-walled microspheres have been reported (see section 1.1.2.4) (Brunner & Göpferich, 1996).

1.9.1 Solvent Evaporation

For the preparation of microspheres by solvent evaporation, the polymer is first dissolved in an organic solvent, such as dichloromethane (DCM). This polymer solution is processed to an oil-in-water emulsion by dispersion into an aqueous solution of a surfactant, such as partially hydrolysed PVA. The emulsion is stirred, during which the organic solvent evaporates, leaving the hardened microspheres (Tabata & Langer, 1993a). With slight modifications, the solvent evaporation technique is suited to the encapsulation of hydrophilic substances. Firstly, a small amount of aqueous phase is dispersed in the organic polymer solution to from a water-in-oil emulsion, which is then

processed to form microspheres as just described. According to the multiple emulsion (w/o/w) that is created, the method is termed double emulsion technique.

Polyanhydride microspheres that are manufactured by solvent evaporation tend to be porous. The porosity, which increases drug release from microspheres, depends on the process parameters (Mathiowitz & Langer, 1992). The disadvantages of any kind of solvent evaporation technique include solvent residues in the polymer, the potential instability of proteins during microsphere preparation, and the risk of polymer degradation (Tabata *et al.*, 1993).

1.9.2 Solvent-Removal Technique

The solvent-removal technique uses only organic phases for the manufacture of microspheres, which has the advantage of reducing hydrolysis during microspheres preparation (Mathiowitz & Langer, 1992). The method for the preparation of bioerodible polyanhydride microspheres in silicone oil is a modification of organic phase precipitation, but it offers significant advantages: the preparation is carried out at room temperature and totally in organic solvents.

In this method, the drug is dispersed or dissolved in a polymer solution of a volatile organic solvent, such as DCM for P(CPP:SA). This mixture is suspended in a mixture of organic oil (silicone oil for P(CPP:SA)), DCM, and a surfactant, such as Span 85. The organic solvent is extracted into the oil, creating microspheres. The microspheres are hardened by adding a nonsolvent, such as petroleum ether, to the suspension. The microspheres are isolated by filtration, washed with petroleum ether, dried overnight in a lyophiliser and stored in a freezer. The microspheres obtained by solvent extraction

are porous. A potential problem might be the use of organic solvents and the danger of organic oil residues in the microspheres. Compared with holt-melt technique, this method permits the preparation of microspheres from polymers with various melting points, and the encapsulation of drugs, which lose biological activity at high temperature (Mathiowitz *et al.*, 1988).

1.9.3 Hot-Melt Encapsulation

An interesting approach to reduce organic solvent residues in polyanhydride microspheres is the formation of microspheres from melted polymer (Brunner & Göpferich, 1996). For this hot-melt encapsulation procedure, polyanhydride was melted, and drugs were dispersed in the melt as solid particles. The mixture was suspended in a nonmiscible solvent that was heated to 5 °C above the melting point of the polymer and stirred continuously. The microspheres solidify on cooling. The solvent used in this process was silicone or olive oil. In some cases the drug can be used without sieving but, in general, a particle size of less than 50 μm was found to be optimal and substantially improved the drug distribution within the microspheres. After cooling, the microspheres were washed by decantation with petroleum ether to give a free-flowing powder. They were then sieved, dried, and stored in a freezer. Size distribution can be controlled by the stirring rate. Microspheres made by hot-melt encapsulation have a smooth surface and are less porous than by double emulsion (Mathiowitz & Langer, 1987). However, the temperature to which polymer and drug are exposed limit the broad application of the method, and the size of the microspheres is not suitable for oral delivery (Brunner & Göpferich, 1996).

1.9.4 Spray Drying

Spray drying is a widely used technology in the pharmaceutical and biochemical fields and in the food industry. The main applications of spray drying in the pharmaceutical field are: drying processes, of plant extracts for example, and of heat sensitive materials; improvement of the flow properties of pharmaceutical powders and their excipient production, such as spray dried lactose; granulation; preparation of solid dispersions with water-soluble polymers and complexation with cyclodextrins, to improve the dissolution rate of drugs that are poorly soluble in water; alteration of the polymorphism of a drug; preparation dry powder for aerosols; encapsulation volatile products; encapsulation for taste masking and protection from oxidation (Giunchedi & Conte,1995).

The process consists of the transformation of a liquid (solution), which is normally called the feed, into a solid (powder). The spray drying process involves the following four sequential stages (Broadhead *et al*., 1992)

-atomisation of the feed materials into a spray nozzle,

-spray-air contact,

-drying of the sprayed droplets,

-collection of the solid product (spray dried microparticles) obtained.

Due to the rapid evaporation of the solvent, the temperature of the droplets can be kept far below the drying air temperature (Masters, 1990) and for this reason, spray drying can be applicable to heat-sensitive materials.

For the manufacture of microspheres by spray drying, polyanhydride polymers were dissolved in DCM and were spray-dried with the drug suspended therein. Microspheres made by spray drying tend to have an irregular shape and high porosities that may cause the fast release of drugs (Mathiowitz *et al.*, 1992).

The main advantages compared with the other microencapsulation methods in the production of microsparticle systems are:

-general applicability, both concerning the drugs (heat-sensitive materials can be used) and concerning the polymers (hydrophilic and hydrophobic);

-continuous in operation: it is one stage process;

-adaptable on the industrial scale.

A possible disadvantages of spray-drying can be the loss of product, the low yields, the use of organic solvent and cost of the spraying equipment (Giunchedi & Conte,1995).

1.10 Characterisation of Microspheres

Microspheres have a distinct microstructure that depends strongly on the process parameters, physicochemical properties of payload and polymer used. Their properties can change upon even slight variations in the manufacturing process, resulting in a different microstructure (Schugens *et al.*, 1994). The microstructure, in return, affects the stability and release of drugs. The problems in drug release and drug stability can be controlled by a careful investigation of the microstructural characterisation of microspheres. There are numerous physicochemical methods by which microspheres might be characterised, such as WAXD (Mathiowitz *et al.*, 1990c), DSC (Tabata & Langer, 1993), scanning electron microscopy (SEM) (Mathiowitz *et al.*, 1990b),

transmission electron spectroscopy (Pekarek *et al.*, 1994), gel-permeation chromatography (GPC) (Mathiowitz et al., 1987), in terms of obtaining detailed information on the degradation, erosion, microstructure and morphology of microspheres.

1.10.1 Scanning Electron Microscopy (SEM)

Use of SEM is one of the standard techniques for microsphere characterisation, as it offers, compared with light microscopy, a much higher resolution. In contrast with scanning transmission electron microscopy (STEM), the sample preparation is simple, as particles do not have to be cut with a microtome, which cannot be easily achieved for brittle polymers or double-walled microparticles. SEM allows investigation of microsphere surfaces and, after particles are cut, their cross-sections. It has also proved useful for the investigation of multiple-walled microspheres made of polyanhydrides and PLGA microspheres. After cutting such systems, the internal structure can be revealed.

1.10.2 Drug Release

Drug release from degradable microspheres might yield information on the microstructure of particles and the mechanism of erosion. The release of low molecular weight compounds from polyanhydride microspheres has been studied extensively and has revealed some of the polymer properties. The release of acid orange from P(SA), for example, has been observed to be very rapid. This is because that aliphatic polyanhydrides erode faster than aromatic ones (Mathiowitz *et al.*, 1990a). In addition,

surface cracking after contact with water is much more pronounced for P(SA) than it is for P(CPP:SA) copolymers. The slower release of drugs from copolymers containing aromatic monomers has been proven by releasing acid orange from P(CPH:SA) 50:50, which is substantially slower than from p (SA). The decreased release rate is due to the slower degradation of the polymer and the increased hydrophobicity of the matrix owing to the content of aromatic CPH monomer.

An interesting observation is the release of protein from P(FAD:SA). No initial burst was observed in the release profiles of BSA from P(FAD:SA), irrespective of the protein loading. The protein was released for up to 3 weeks at a near-constant rate. The release rate of protein depends on the monomer composition of polyanhydrides used (Tabata *et al.*, 1993).

1.11 Applications of Polyanhydrides

Compared with the relatively short period during which they have been synthesised as drug carriers, polyanhydrides have been applied very successfully. Polyanhydride matrices have been used to locally deliver chemotherapeutic drugs such as carmustine (BCNU) to treat brain cancer (Brem *et al.*, 1995). In this case, the surgeon removes as much of the tumour as possible at the time of operation, but also places up to eight small polymer-drug wafers at the tumour site. The drug is slowly released from the polymer for 1 month to kill remaining tumour cells. Because the drug is delivered locally, harmful side effects that normally occur from systemic chemotherapy are minimised. One recent clinical trial showed that after 2 years, 31% of the treated patients were alive whereas only 6% in the control group survived (Valtonen, 1997). In

1996, the United States Food and Drug Administration approved this treatment for patients with recurrent glioblastoma, the first new brain cancer therapy (Gliadel®) approved in over 20 years (Domb & Ringel, 1994). Using a similar approach, Septacin® implant has been used to locally release gentamicin to treat chronic bone infection (Brem *et al.*, 1995) in humans after promising clinical trials (Brem *et al.*, 1993).

In the past few years, investigations have expanded to newer polymers and other drugs such as 4-hydroperoxy cyclophosphamide (4HC), cisplatin, carboplatin, taxol and several alkaloid drugs in an effort to develop a better system for treating brain tumors (Brem *et al.*, 1994; Olivi *et al.*, 1996; Judy *et al.*, 1995). Carboplatin incorporated in P(FAD-SA), prepared by mixing the drug in the melted polymer has been evaluated for the treatment of brain tumours in laboratory animals with promising results (Olivi *et al.*, 1996).

The effect of long term glutamic acid stimulation of trigeminal motoneurons, using poly(FAD:SA) microspheres has also been explored. This study was undertaken to determine the role of glutamate in possible growth disorders of the craniofacial skeleton. Pronounced skeletal changes in the snout region were observed in rats that received glutamate showing that sustained release of glutamic acid *in vivo* can effect the development of skeletal tissue in growing rats (Hamilton-Byrd *et al.*, 1992). Local anaesthetics have also been successfully delivered from polyanhydride cylinders in close proximity to the sciatic nerve to produce a neural block for several days (Masters *et al.*, 1993a; Masters *et al.*, 1993b).

Some results of studies suggest that bioadhesion of polymers lacking chain flexibility could largely be a result of secondary bond formation, such as hydrogen bonding

between mucin and free carboxyl groups after polyanhydride hydrolysis. Rapid degradation of P(FA:SA) polymers may enhance their bioadhesive nature through production of carboxylic acid and increase in surface roughness (Peppas & Sahlin, 1996; Mathiowitz *et al.*, 1997). Therefore, bioerodible, bioadhesive polyanhydrides may be excellent candidates for the development of orally administered drug delivery systems.

1.12.Objectives of This Study

This study extends previous work on microparticulate systems fabricated from the biodegradable aliphatic and aromatic copolymer P (CPP:SA) and release of bovine serum albumin (BSA).

During these studies, polyanhydride microspheres are prepared from a new polymer, which is more hydrophobic than P(CPP:SA), poly [bis (p-carboxyphenoxy) butane: sebacic acid] P(CPB:SA), CPB copolymerised with sebacic acid SA with molar ratios of 20:80 and 50:50. Three preparation methods are applied in this study, double emulsion (w/o/w), spraying drying (SD), and oil-in-oil (o/o) microencapusulation.

All the polymers and the resulting microspheres were characterised as follows: polymer or microspheres were examined by infrared (IR) spectroscopy, differential scanning calorimetry (DSC), and nuclear magnetic resonance spectra (NMR). The morphology was examined by scanning electron microscopy (SEM).

In addition to monitoring microsphere size distribution, surface morphology, encapsulation efficiency, the characterisation and the result of release studies using

bovine serum albumin (BSA), the effect of molecular weight of polymer used on the release of BSA *in vitro* has also been determined.

2. EXPERIMENTAL

2.1 Materials

Reagents of analytical grade and double distilled water were used throughout this study. Poly (vinyl alcohol) (PVA) with 13,000-23,000 average molecular weight and 87-89% hydrolysed, dichloromethane (DCM) (HPLC grade used without further purification), silicone oil, sodium azide (NaN_3), 1 μm polystyrene latex, Span 85, potassium bromide (KBr, 99% FI-IR grade) and buffered saline tablets were all purchased from Aldrich Chemical Co., Gillingham, Dorset, UK. Sodium dodecyl sulphate (SDS) were supplied by Avocado, Research Chemicals Ltd. All other solvents used were of analytical grade. Sebacic acid was recrystallised two times from dry methanol. Bis (p-carboxy-phenoxy) butane was synthesised by reaction in sodium hydroxide solution (Macromolecular Syntheses, Volume2, 1977). Bicinchoninic acid (BCA, 4,4`-dicarboxy-2, 2`-biquinoline, sodium salt) protein assay reagent and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., Poole, UK. Solid particles of BSA (Aldrich) were sieved to a size lower than 50 μm.

2.2 Polymer Synthesis

Polyanhydrides were synthesised by melt polycondensation (Figure 2.1). Briefly, 50 g SA monomer was recrystallisd twice from 150 ml dry methanol, and the excess water in purified SA was removed by heating under vacuum. After purification, purified SA was stored in a sealed flask filled with N_2. The acetic anhydride (AA) was distilled at 138 °C and was also stored in sealed flasks before use. About 10 g purified SA was converted

to the anhydride by refluxing in 100 ml purified acetic anhydride for about 30 minutes in an oil bath (see Figure 2.1 eq 1). After cooling to room temperature, excess acetic anhydride was removed under vaccum at 20 ~ 30 °C. Following cooling of the resulting mixture in a freezer, the white crude prepolymer sebacic acid anhydride (SAA) was precipitated, and it was immersed in a 1:1 mixture of dry petroleum ether (dried by 4A molecular sieves) and dry ethyl ether over-night at 4 °C to extract the acetic anhydride. The pure aliphatic prepolymer was washed with petroleum and diethyl ether, filtrated under a N_2 environment, and dried under vaccum.

Aromatic prepolymer was synthesised according to the method applied by Conix (Conix, 1977). In a three-necked flask equipped with a stirrer, a condenser, and a dropping funnel, a solution of 138 g (1.0 mole) of p-hydroxybenzoic acid and 80 g (2 moles) sodium hydroxide in 400 ml water, was placed. Through the funnel, 108 g (0.5 moles) of 1,4-dibromobutante was added over a period of one hour, while the contents of the flask were stirred and kept at the reflux temperature (see Figure 2.1 eq 2). After the addition, the reaction mixture was refluxed for 3.5 hours. Then 20 g (0.5 mole) of solid sodium hydroxide was added to the mixture, and the reflux continued for another 2 hours. The reaction mixture was cooled at room temperature overnight. The disodium salt precipitate was isolated by filtration and washed with methanol. The wet precipitate was dissolved in 250 ml distilled water and while the solution was heated to 60 ~70 °C, 6 N sulphuric acid was added untill the pH value of the solution was about 1 (tested with pH paper). The dibasic acid precipitated in acid was filtrated and dried in a vaccum oven at 80 °C.

In a 200 ml three-necked flask, equipped with a stirrer, a condenser, and a gas-inlet tube, 10 g (0.03 moles) of aromatic monomer 1,4-bis(p-carboxyphenoxy) butane and

100 ml of purified acetic anhydride was placed. A slow stream of dry argon, passed through $CaCl_2$, was bubbled through the mixture while it was refluxed for approximately 2 hours (see Figure 2.1 eq 3). Then the unreacted diacid was removed by filtration. The slightly yellow-coloured filtrate was concentrated to a volume of about 25 ml by distilling acetic anhydride under vaccum at a temperature not higher than 65°C. Aromatic prepolymers were isolated by crystallisation from the concentrated acetic anhydride solution, purified with dry diethyl ether, and dried in a vaccum oven at 70°C.

Both of the prepolymers were characterised by ^{1}H-NMR and stored in a vaccum desiccator before further application. The prepolymers were then subjected to melt-polycondensation as follows: in a typical reaction, CPBA [1,4-bis(p-carboxyphenoxy) butane prepolymer] (2.0 g, 5 mmols for ratio 50:50, or 0.4 g, 1 mmol for ratio 20:80) was mixed with SAA (sebacic acid prepolymer) (1.15 g, 5 mmols) and 2 molar percent catalyst, cadmium acetate, in a mortar, and placed in a glass tube (2 x 20 cm) with a top arm equipped with a capillary nitrogen inlet, passed through the $CaCl_2$. The tube was immersed in an oil bath at 180°C. After the prepolymer was melted, a high vacuum (10^{-4} mm Hg) was applied through the side arm (see Figure. 2.1 eq 4). The condensation product was collected in an acetone/dry ice trap. During the polymerisation, a strong nitrogen sweep, with vigorous agitation of the melt, was performed for 30 seconds every 15 mins. After 30 mins, the reaction was stopped. After cooling at room temperature, the mixture of polymer and catalyst was dissolved in dry DCM. Catalysts were removed from the polymer DCM solution by filtration. The crude polymer was purified by precipitation in dry petroleum ether from the DCM solution. The precipate was then extracted with anhydrous ether for several hours at room temperature. The

purified polymer was characterised by ^{1}H-NMR, IR, GPC, and stored in a vacuum desiccator for further use.

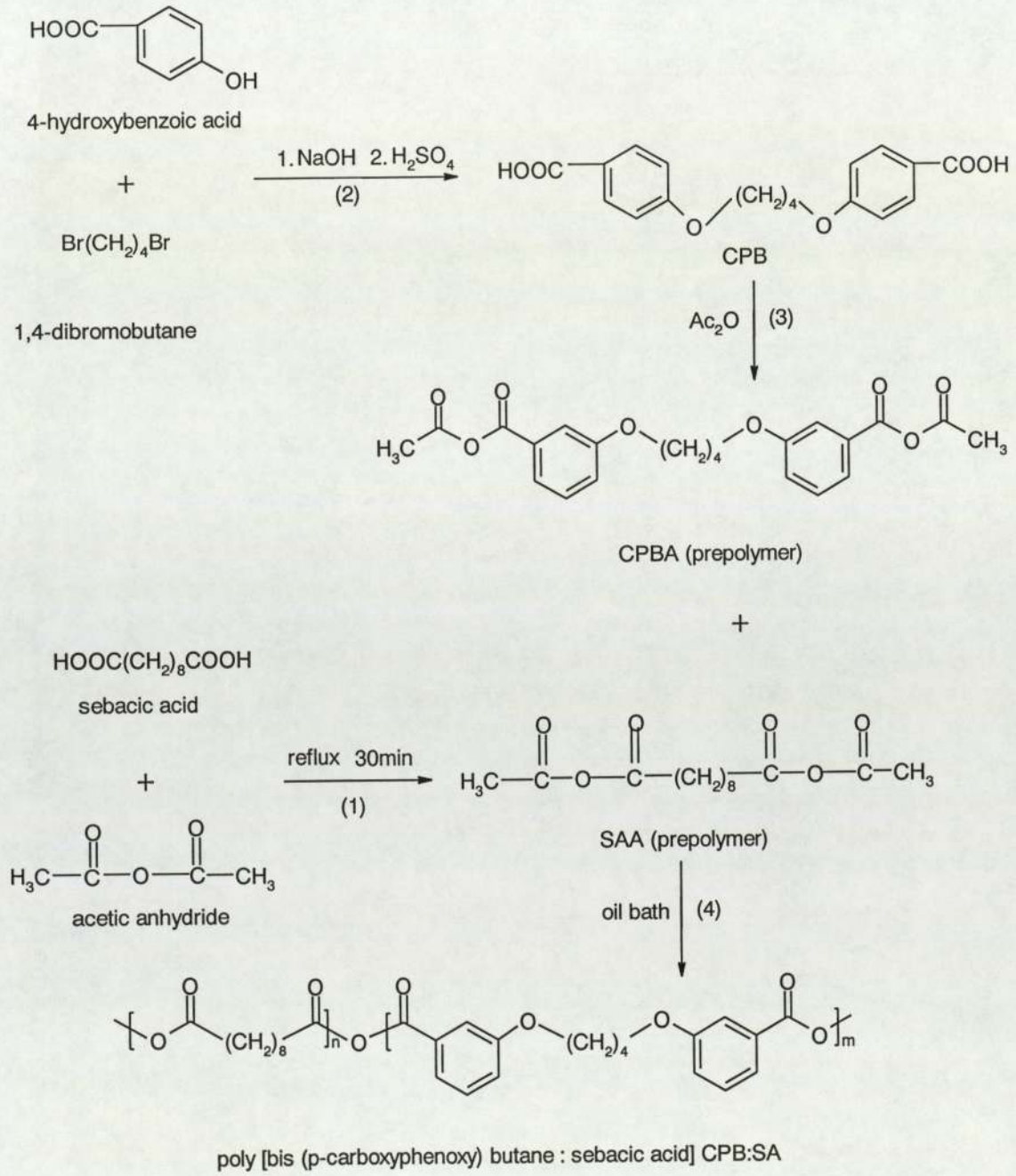


Figure 2.1 Synthesis of copolymer P(CPB:SA)

2.3 Determination of Polymer Composition

The composition of P(CPB:SA) copolymer was determined by ^{1}H-NMR (Bruker AC 250 NMR Spectrometer using QNP probe head) from the ratio of the peak integration at 1.3 ppm (8H, SA) and 6.9 ~ 8.2 ppm (8H, CPB) using win –NMR version 3 software. The following copolymer characteristics can also be studied by ^{1}H NMR (Ron *et al.*, 1991): the degree of randomness that suggests whether the polyanhydride is a random or block copolymer; the average length of sequence (Ln) and the frequency of occurrence of specific comonomer sequences.The protons on the aromatic ring close to the anhydride groups experience a lower density of shielding electrons and absorb at lower frequency. On the other hand, the protons next to aliphatic comonomers, absorb at higher frequency. Accordingly, the CPB-CPB and CPB-SA diads were represented by peaks at 8.1 ppm and 8.0 ppm respectively, and the triplets at 2.6 ppm and 2.4 ppm represent the SA-CPB and SA-SA respectively. By integration of the 1H-NMR spectra of P(CPB:SA), the degree of randomness, average block length, and the probability of finding the diacid SA-SA or SA-CPB were calculated (see section 3.1).

2.4 Microsphere Preparation

2.4.1 Preparation of Microspheres by Oil-in-Oil Method (o/o)

P(CPB:SA) 20:80 microspheres were prepared as follows: 0.1 g polymer (Mw 19kDa or 34kDa) was dissolved in 1 ml DCM, BSA powder (diameter less then 50 μm after sieving) was suspended in the solution and dropped into the external oil phase made up of 40 ml silicone oil, 20 ml DCM and 4 ml of Span 85 under stirring using an overhead stirrer (Heidolph) with a three-blade impeller, at 200rpm. The oil-in-oil emulsion was

immediately poured into 180 ml petroleum ether and the agitation was continued using a stirring bar for two hours to harden the microspheres. The microspheres were isolated by filtration through a 0.4 μm cellulose acetate filter, washed with petroleum ether, freeze-dried (Edwards Modulyo Freeze-drier), and stored in a desiccator in a refrigerator at 4°C.

When trying to apply the same method to P(CPB:SA) of ratio 50:50, the above process resulted in rod formation rather than microspheres owing to its higher viscosity and quicker precipitation than 20:80 (see section 3.3). In this case a different concentration of polymer solution was used: 0.05 g polymer (Mw 19.0kDa) was dissolved in 1ml DCM. The ratio between silicone oil and DCM was 1:1, but all additional procedures were as above.

2.4.2 Preparation of Microspheres by Double Emulsion Method (w/o/w)

2% w/v and 0.1% w/v polyvinyl alcohol (PVA) solution was perpared by dissolution of 0.2 g and 0.1 g PVA in 10 ml and 100 ml double distilled water. 500 μl of aqueous solution containing 2% (w/w) BSA was emulsified into 5ml DCM containing 0.1g P(CPB:SA) (Mw 3.5kDa, 19.0kDa, and 34.4kDa for polymer of CPB:SA 20:80, 7.3kDa and 19.0kDa for 50:50) by probe sonication (Soniprep 150), output 50w for 3 minutes on ice, to form the primary emulsion. The organic solution of P(CPB:SA) in DCM, 2% and 0.1% PVA solution were cooled in an ice bath for one hour before use. The primary was poured into 10 ml of 2% (w/v) PVA aqueous solution pre-saturated with DCM and mixed vigorously on a vortex mixer for 1 minute to form the double emulsion. The resultant double emulsion was added into 100ml 0.1% (w/w) PVA solution and stirred

at room temperature for 4 hours on a magnetic stirring plate, to allow the DCM to evaporate completely and to harden the microspheres. The hardened microspheres were collected by centrifugation at 10000rpm for 35 min (JA-14 rotor, Beckman Centrifuge U.K.). The microspheres was washed with double-distilled water after each centrifugation and then freeze-dried. The free-flowing powder was stored in a desiccator in a refrigerator at 4°C.

2.4.3 Preparation of Microspheres by Spray Drying Method (SD)

Batch	Ratio of CPB:SA	Inlet air Temp.$_i$	Outlet Temp.$_i$	Aspirator setting mm WC[a]	Pump setting ml/min	Spray flow Nl/h[b]	Mw of Polymer[c] kDa
1	50:50	49	43	150	8	350	19.0
2	50:50	49-50	43	150	5	400	46.8
3	20:80	47-48	39-40	150	5	400	34.4
4	20:80	47-48	39-40	170	3	550	34.4
5	20:80	47	42	150	8	400	34.4

Table 2.1 Spray drying conditions employed for different batches
[a] WC: water column.
[b] Nl/h: normliter/h
[c] Mw of polymer was determined by GPC.

Microspheres were prepared using a Büchi 190 mini spray dryer (see Figure 2.2, supplied by SmithKline Beecham Pharmaceuticals). 0.4 ml 10% (w/v) BSA aqueous solution was emulsified into 20 ml 2% (w/v) polyanhydride P(CPB:SA) in DCM solution, using probe sonication at output 70 to 80 W for 4 minutes on ice, until an emulsion was formed. Microspheres were then obtained by spray drying the polymer-drug emulsion through a 0.7 mm nozzle. The emulsion was stirred on ice before feeding to prevent droplet coalescence. Process parameters were investigated as detailed in

Table 2.1. The yield was calculated from the ratio of the weight of microspheres obtained to the total amount of drug and polymer used in the preparation.

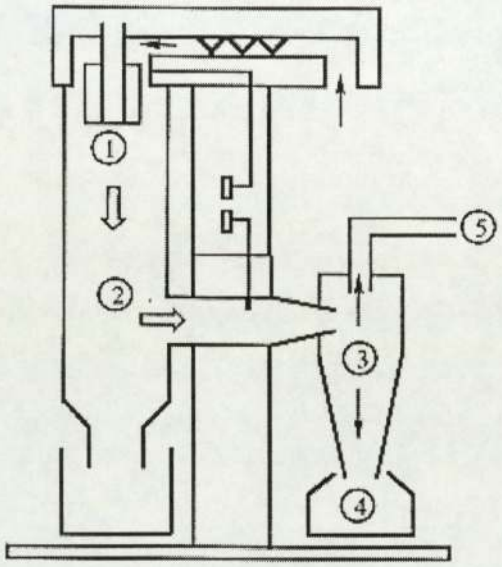


Figure 2.2 Schematic representation of Mini Büchi Spray dryer apparatus: (1) 0.7mm nozzle; (2) spray chamber; (3) cyclone; (4) collector; (5) aspirator (adapted from Conte *et al.*, 1994).

2.5 Bovine Serum Albumin Entrapment in P(CPB:SA) Microspheres

The method used to determine protein entrapment was adapted from literature (Hora *et al.* 1990). Briefly, 2-3 milligrams of the freeze-dried microparticles, accurately weighed, were incubated in 1ml of 1M NaOH. Sodium hydroxide catalyses the hydrolysis of the polymer. Extraction of the protein occurred after degradation of the polymer for 4 hours shaking in a 37°C water bath. The sample was centrifuged and the BSA concentration determined using the bicinchoninic acid (BCA) assay (see section 2.6). From this result, the percentage (w/w) of BSA entrapped *per* dry weight of microspheres was determined. Each sample was assayed in triplicate. The percentage

entrapment efficiency was expressed by relating the actual BSA entrapment to the theoretical BSA entrapment.

$$\text{Encapsulation efficiency\%} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100\%$$

2.6 Release Studies

Release experiments from P(CPB:SA) microspheres prepared by different methods were carried out using ~ 2-3 mg microspheres in an ependorff tubes at 37°C (n=3). The microsphere suspensions were kept shaking thoroughout the experiment duration. The release media used were 1 ml phosphate buffer saline (PBS), pH 10.06 Na_2CO_3 / $NaHCO_3$ buffer (Merck Index, 1989), 0.1% w/v Na_2CO_3 (pH 11.1) solution containing 2% w/v SDS. NaN_3 was added at a concentration of 0.02 mg / ml as an antibacterial agent for all release media. The PBS was prepared by dissolving 1 phosphate buffered saline tablet in 200 ml of double distilled water to obtain 0.1 M phosphate buffer, pH7.4 at 25°C. 100 µl of sample was removed at predetermined time following centrifugation at 21000rpm for 10 min (Micro Centaur Bench top centrifuge), 100 µl of the appropritate fresh buffer was added to the samples. Dilution and discarding of material of the dissolution medium was corrected in all calculations. Each experiment was performed in triplicate and results were the mean of three samples.

The concentration of BSA in the release samples was monitored using the BCA method of protein determination (Smith *et al.*, 1985). The water-soluble sodium salt, BCA is sensitive, stable and highly specific for the Cu (I) ion forming an intense purple complex at 60°C in an alkaline environment (see Figure. 2.3). This colour generation

forms the basis of the analytical method, capable of monitoring the amount of Cu (I) ion produced when the peptide bonds of a protein, complex with the alkaline Cu (II) ion (Biuret reaction). The absorbance of the purple complex at room temperature at 572 nm increases proportionally over a broad range of protein concentrations (0.5-1200 ug / ml). 200µl of the working reagent, consisting of 50 parts of BCA and 1 part 4% $CuSO_4$, was added to 10µl of the protein sample on a 96 well microtitre plate (Fisons, Loughborough, U.K.). The solution was incubated at 60°C for one hour, cooled to room temperature and the absorbance was read using an MRX microplate reader (Dynex, Technologies) at 570nm. Each absorbance is the average of at least 4 readings. A standard calibration was carried out each time from 10 µg/ml to 250 µg/ml (see Figure 2.4). The calibration curve was constructed by plotting the absorbance of a series of protein standards subjected to the same conditions as the samples.

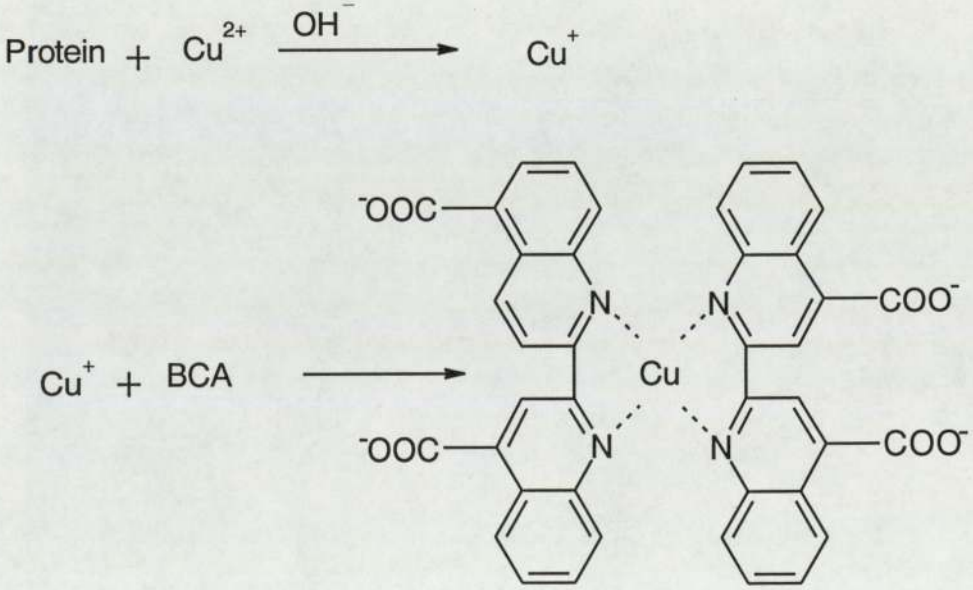


Figure 2.3 Formation of Cu (I) ion purple complex.

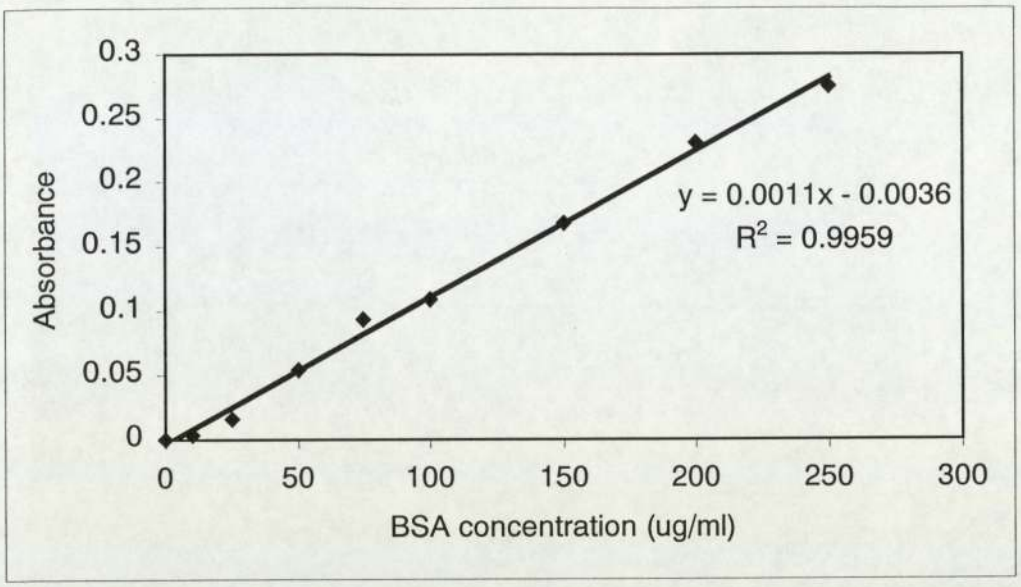


Figure 2.4 A typical calibration curve of the series of standard protein in 0.1 M pH7.4 PBS.

2.7 Hydrophobic Interaction Chromatography (HIC)

HIC was applied to characterise the microspheres based on the fact that there are hydrophilic and hydrophobic areas in polymer structure. HIC makes use of the affinity of the hydrophobic domains in the polymer for the hydrophobic agarose derivatives. A range of hydrophobic matrices are available based on the chain length of the derivative. Using an adaptation of procedures developed for determining the hydrophobic surface characteristics of bacteria (Mozes & Rouxhet, 1987; Smyth *et al.*, 1978), it is possible to study the hydrophobicity of microsphere surfaces. The main work on determination of microparticle surface hydrophobicity has been carried out by Muller (1991). The procedure employed was based on the method of Alpar & Almeida (1994) using a series of agarose derivatives to distinguish between batches of slightly differing hydrophobicities. The stationary phase used was propyl-agarose. These neutral gels have been manufactured to minimise any electrostatic interactions, maximising the

contribution of hydrophobic interactions (Hjerten *et al.*, 1974). Before use, the gels were centrifuged at 10000rpm for 5 minutes, washed with double-distilled water for three times, suspended in 0.6M NaCl, and then stored in refrigerator until used.

HIC columns were prepared by layering 25mm of the processed gel onto a glass wool sinter in a Pasteur pipette (Figure 2.5). The columns were washed with double-distilled water and 0.6M NaCl solution separately. 1ml suspensions of microspheres or 1 μm polystyrene latex used as reference, which were adjusted to OD 600 of about 0.5 (Cecil CE292 Digital ultraviolet spectrophotometer, series 2) before use, were loaded onto the columns simultaneously, and then the loaded columns were washed with 2 x 1ml of 0.6M NaCl and 2 x1ml of 0.1% Triton X-100 (Sigma) in double-distilled water. The five 1ml fractions of the eluent were collected in 1ml plastic semi-micro cuvettes and the OD600 was compared with the OD600 of the original suspension. Three identical columns were run for each microsphere sample on the agarose gel used. The result was expressed as the total percentage eluted from the column according to the decrease in the OD600 values. The hydrophobicity of the particles was compared to surfactant-free uniform polystyrene particles, with diameter of 1μm, 2 wt% dispersion in water stablilised with 0.05% w/v sodium azide. All results were obtained under the same conditions.

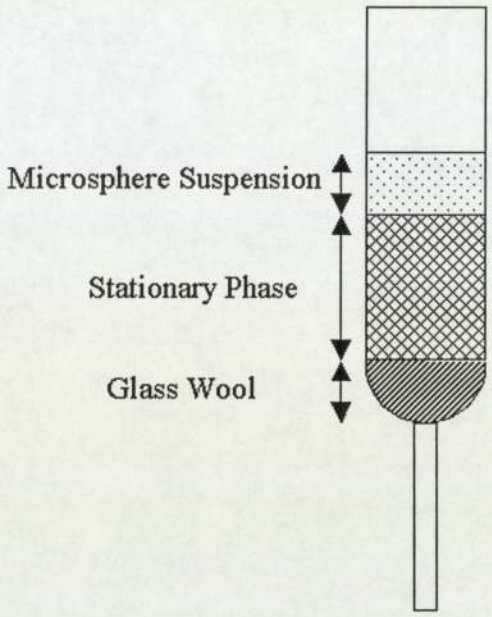


Figure 2.5 Diagram of a HIC column adapted from a glass Pasteur pipette.

2.8 Degradation of Microspheres

Degradation experiments were conducted in the same environments as release studies. Polyanhydride microspheres 20 mg were suspended in 20 ml PBS. As the microspheres degraded and lost their morphology rapidly when incubating in pH 10.06 Na_2CO_3 / $NaHCO_3$ buffer, or 0.1% Na_2CO_3 (pH 11.1) solution, it was difficult to obtain the solid particles of microspheres after degradation in these release medium, so the degradation was carried out in PBS only. Every day 3 ml of the suspension was collected, centrifuged at 21000 rpm for 10 minutes, freeze-dried (Edwards Modylo freeze drier) and stored in a desiccator at room temperature for SEM, GPC, and IR analyses.

2.8.1 Gel Permeation Chromatography (GPC)

The molecular weight of the polymers before and after microsphere preparation and during degradation was followed by GPC. An adjustable flow rate pump (Altex model 110 A) preceded by a sintered metal frit was used to pump HPLC grade chloroform at 1 ml/minute around the system. Two 300 x 7.5 mm, 500Å pore size, 5μm mixed pore highly cross-linked spherical macroporous polystyrene-divinylbenzene matrix (PLGel) columns (Polymer Laboratories Ltd, Shropshire, U.K.) were used in series and were protected by a 50 x 7.5 mm 10μm mixed pore guard column (PLGel) (Polymer Laboratories Ltd, Shropshire, U.K.). A Pye Unicam LC3 UV detector at a wavelength of 254nm was used for sample detection (Domb & Langer, 1987). Samples were dissolved in chloroform, filtered and injected using a 100μl sample size through a Rheodyne injector valve (Waters, CA, U.S.A).

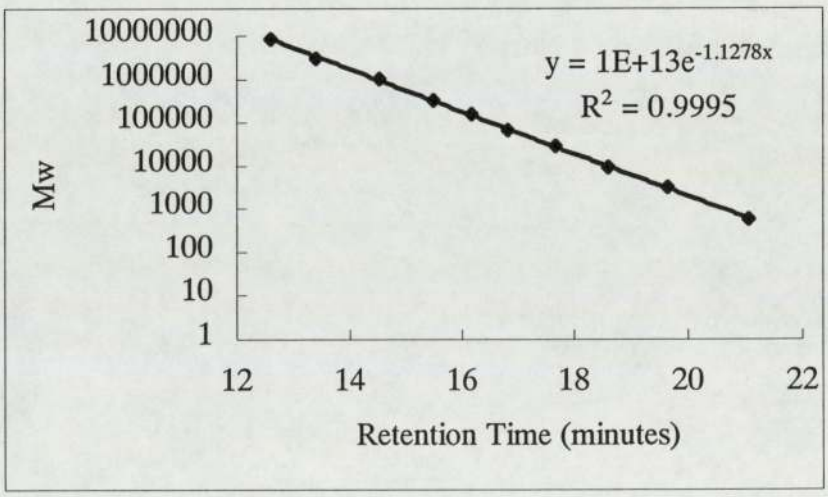


Figure 2.6 A calibration curve for estimation of molecular weight by GPC. Molecular weight refers to weight average molecular weight.

Standardisation of the GPC system was obtained by narrow-MW polystyrene standards (Easical, Polymer Laboratories Ltd, Shropshire UK). Inert PTFE strips coated with polystyrene (~5 mg) were immersed in 5 ml of chloroform. There were two types of strips each representing Mw values of 580, 9200, 66000, 330000, 3040000 and 3250, 28500, 156000, 1030000, 8500000 respectively. The calibration curve of retention time was obtained under the same conditions used for the polymer and microspheres (see Figure 2.6).

2.8.2 Surface Morphology of Microspheres

The surface morphology of microspheres was studied, after preparation and after degradation, *via* scanning electron microscopy (SEM) (Cambridge Instruments, ISI Model DS-130). Samples for SEM were thoroughly dried, mounted on aluminium stubs using adhesive tabs, and sputter-coated in an argon atmosphere with gold-palladium. The surface morphology of at least twenty-five randomly selected microspheres for each polymer was monitored using a Cambridge Instruments Stereoscan 5150 scanning electron microscope equipped with a photographic facility. The diameter of microspheres was determined using the scale bar on electron micrographs.

2.8.3 FTIR

Infrared spectroscopy was performed on a FTIR spectrophotometer (Mattson Galaxy 3020 FTIR Spectrphotometer, Unicam). Polymer and microsphere samples were impressed into potassium bromide (KBr) discs. The spectrum was analysed using Mattson First fourier infrared software tools.

Area calculations for the carboxylic acid and anhydride carbonyl peaks were determined utilising the established baseline and the peak boundaries (1810 cm^{-1} for the aliphatic anhydride bonds, 1780 cm^{-1} for the aromatic anhydride bonds and 1700 cm^{-1} for the carboxylic acid groups). The anhydride area was taken to be only the area under the first (1810 cm^{-1}) of the double carbonyl peaks, as the second carbonyl peak(1720-1740 cm^{-1}) was often overlapped by the acid peak at 1700 cm^{-1}.

2.9 Thermal Analysis

The crystallinity of polymer and microspheres before and after degradation was investigated by differential scanning calorimetry (Pekin Elmer DSC-4). Samples of about 5mg were sealed into aluminium sample pans. The instrument was manipulated with empty aluminium pans under the same condition, and the measurements were carried out from -40℃ to 400℃ under nitrogen at a scan rate of 10℃/min for heating and at 320°C for cooling (Perkin Elmer system 4, Thermal analysis microprocessor controller). The rate of heating and cooling was controlled by the computer, and the thermograms were analysed by thermal analysis computer software. The melting point was taken as the maxpoint of the endotherm peak. Tg was taken as the midpoint of the transition curve.

3. RESULTS AND DISCUSSION

3.1 Polymer Characterisation

The ^{1}H NMR spectrum of polymer P(CPB:SA) 50:50 after synthesis is shown in Figure 3.1. Copolymer composition was verified by ^{1}H NMR by the integration ratio of the peaks at 1.3 ppm (8 protons of sebacic) and the peaks at 6.9-8.2 ppm (8 protons of CPB). The copolymer composition in the polymer (by ^{1}H NMR) was identical to the comonomer ratio in the feed (see Table 3.1).

In order to correlate the NMR spectra to the composition and the frequency of occurrence of specific comonomer sequences and to determine the degree of randomness and the number-average sequences, modified mathematical models were applied (Ron *et al.*, 1991). If the copolymer is not strictly alternating or blocklike, a randomly selected pair of comonomer units (diad) in the polymer chain may be represented as follows: SA-SA, SA-CPB (or CPB-SA), and CPB-CPB. An examination of the ^{1}H NMR spectrum of P(CPB:SA) 50:50 revealed two doublets at 8.1 and 8.0 ppm and two triplets at 2.6 and 2.4 ppm. The downfield doublets at 8.1 and 8.0 ppm were the diads, CPB-CPB and CPB-SA, respectively. Similarly, the upfield triplets at 2.6 and 2.4 ppm were the diads SA-CPB and SA-SA, respectively. By integration of the ^{1}H NMR spectra of P(CPB:SA) 20:80 and 50:50, the degree of randomness, average block length, and the probability of finding the diad SA-SA or SA-CPB were calculated (see Table 3.1).

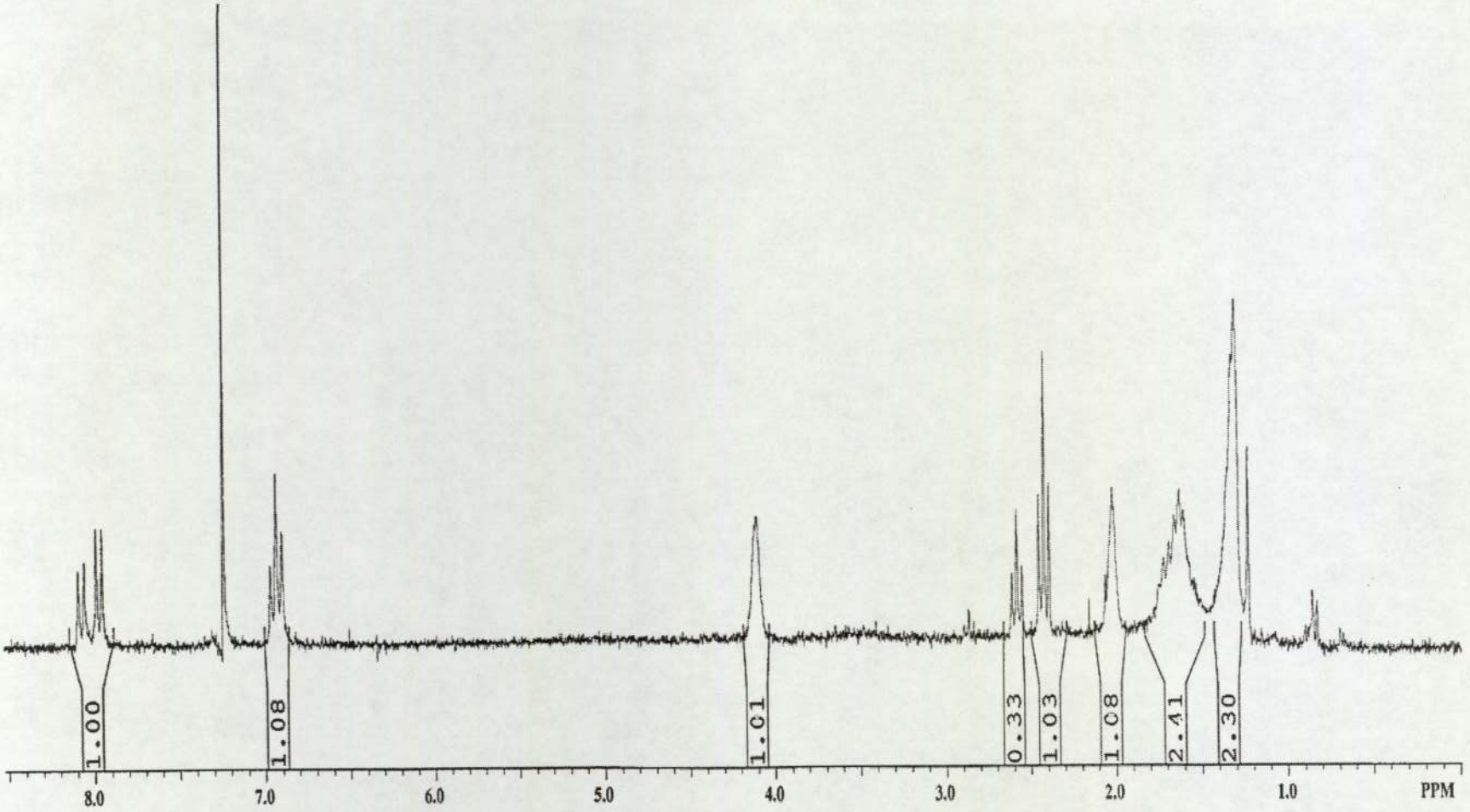


Figure 3.1 ^{1}H NMR spectrum of polymer P(CPB:SA) 50:50.

mole ratio of CPB:SA in the feed[a]	20:80	50:50
mole ratio of SA-CPB in the polymer, p(SA)[b]	0.77	0.53
probability of finding the diad SA-SA, p(SA-SA)	0.41	0.47
probability of finding the diad SA-CPB, p(SA-CPB)	0.12	0.08
average block length L(SA)	4.3	2.1
average block length L(CPB)	1.3	1.9
degree of randomness (H)	0.62	0.68

Table 3.1 Comonomer sequence distribution of the P(CPB:SA) 20:80 and 50:50.
[a]presynthesis ratio
[b] integration ratio of the peaks in NMR

These were determined from the integration ratios of the diads: SA-SA / SA-CPB (peaks at 2.4 and 2.6 ppm, respectively) and CPB-CPB / CPB-SA (peaks at 8.1 and 8.0 ppm, respectively). From these probabilities and the feed ratios the degree of randomness (H) can be calculated:

$$H = p(CPB\text{-}SA) / p(SA)\, p(CPB)$$

$H < 1$ indicates block characteristics within the copolymer (Ron *et al.*, 1991). The data in Table 3.1 show that both P(CPB:SA) 20:80 and 50:50 exhibit block characteristics, and the number-average sequence length of sebacic acid (L(SA)) is changed from 4.3 to 2.1 when the mole ratio changes from 20:80 to 50:50. Therefore, the monomers were not distributed randomly in the chain of copolymer P(CPB:SA). Large regions of aliphatic anhydrides could be created for P(CPB:SA) 20:80 due to the presence of long blocks of SA. Large block regions in the copolymer can lead to uneven hydrolysis in sensitive spots during degradation (see section 3.6).

The physiochemical properties of the polymers determined in this study are summarised in Table 3.2. These polymers were chosen for two reasons:

to investigate the effect of CPB content on microsphere release rates;

to investigate the effect of changing polymer properties (*i.e.*, molecular weight) on microsphere performance.

Weight average molecular weights (Mw) of the various polymers were determined and are listed in Table 3.2. They ranged from 3.5kDa to 34.4kDa for P(CPB:SA) 20:80 and 7.3kDa to 46.8kDa for P(CPB:SA) 50:50. The molecular weight of P(CPP:SA) prepared by a similar method ranged from 140kDa to 245kDa with catalysts, in comparison to 116kDa without catalysts for P(CPP:SA) 20:80, and 37kDa without catalysts for P(CPP:SA) 50:50 (Domb & Langer, 1987).The Mw of P(CPB:SA) prepared using catalysts is therefore lower than the reported values for P(CPP:SA). Critical factors affecting the polymer molecular weight are monomer purity, temperature of reaction, time of reaction, and the removal of condensation product (Domb & Langer, 1987).

The acetic mixed anhydride prepolymers were prepared by heating diacids in acetic anhydride during the synthesis. Operating under these conditions can result in oligomerisation. Aromatic oligomers with polydispersity > 4 are not suitable for further polymerisation due to their high melting points (mp > 260 °C) (Domb & Langer, 1987). However, during the melt-polycondensation, the mixture of prepolymer may not melt totally, and may stick on the wall of the glass tube following immersion in the oil bath at 180 °C. By raising the reaction temperature to 220 °C and increasing the reaction time to 120 minutes, a rubbery gel was produced which did not dissolve in DCM. It is proposed that the purity of the aromatic prepolymer CPB, with long oligomers, resulted in lower yields (30%-50%) and a lower molecular weight of resultant polymer.

Since using long oligomers in the synthesis of copolymers would create large regions of aliphatic (SA) or aromatic (CPB) anhydrides, this would preclude a fine distribution of the repeating unit for provision of a uniform hydrolytic degradation. Therefore, a

monomeric prepolymer could be obtained by reducing the reaction time or lowering the reaction temperature, and evaporation of excess acetic anhydride at room temperature. Table 3.2 also summarises the heat of fusion, molecular weight (Mw), melting point (Tm) and IR absorption for the polymers synthesised. The polymers had no glass transition point detectable in the range of controlled temperature using the DSC system available. The IR peaks of copolymers P (CPB-SA) 20:80 at 1810 and 1740 cm^{-1}, indicate a low percentage of pure aromatic anhydride bonds. The chain of the copolymer P(CPB:SA) 20:80 is composed mostly of the aliphatic anhydride, which is separated by less aromatic anhydrides than P(CPB:SA) 50:50. In the P (CPB-SA) 50:50 polymer, three maximal absorptions appear at 1810, 1770, 1740cm^{-1}, which are typical for aliphatic and aromatic polyanhydrides. It indicates a fine distribution of aromatic anhydride and aliphatic anhydrides and hence provides uniform hydrolysis. These results are consistent with the copolymer composition obtained by NMR analysis.

Ratio CPB:SA	Yield %	Mw kDa	Tm °C n=3	Heat of fusion cal/gram n=3	IR cm^{-1}
20:80	52	3.5	78.9	47.1	1810, 1740
	63	19.0	71.4	45.4	
	57	34.4	69.5	37.1	
50:50	34	7.3	60.9	25.1	1810, 1780, 1740
	53	19.0	55.3	10.2	
	37	46.8	54.4	10.1	

Table 3.2 Characterisation of polymer by DSC, GPC and IR.
Mw: GPC (Gel Permeation Chromatography)
Tm: DSC (Differential Scanning Calorimetry)

The melting point and the heat of fusion are related to the crystalline area of the polymer. DSC indicates that both the melting point and the heat of fusion for the polymers falls with increasing Mw. The Tm and heat of fusion dropped from 60.9°C to

54.4°C and 25.1 cal/gram to 10.1 cal/gram respectively when the Mw of P(CPB:SA)50:50 increased from 7.3kDa to 46.8kDa. It seems that as the Mw increases, the crystallinity is reduced. This is due to a relatively homogeneous monomer distribution, which undergoes coupling to yield a high molecular weight fraction, and hence less thermal energy was needed to overcome the intermolecular forces.

The lowest degree of crystallinity is reached at a copolymer composition of CPB:SA 50:50 (Tm 55.3°C, heat of fusion 10.2 cal/gram) compared to P(CPB:SA) 20:80 (Tm 71.4°C, heat of fusion 45.4 cal/gram) with a similar Mw. Another possibility is that the decrease in crystallinity is a direct result of the random presence of the CPB units in the P(CPB:SA) chain. When two monomers -one forming a crystalline homopolymer and another forming an amorphous homopolymer - are copolymerised, the degree of copolymer crystallinity decreases as the second constituent is added to either homopolymer (Shieh *et al.*, 1994). Although the heat of fusion indicates changes in crystallinity when the amount of CPB in the copolymer increases from 20% to 50%, further investigation is required to obtain data regarding the crystallinity of P(CPB) by a combination of X-ray diffraction and DSC in order to correlate with the rate of degradation and erosion of the polymer. The degree of crystallinity may play an important role in preventing water diffusion into the polymer bulk and thus preventing bulk erosion. Another important property in preventing bulk erosion is hydrophobicity. In this case, the less hydrophobic polymer (20:80) has the higher crystallinity, probably due to crystalline regions of P(SA) units. The more hydrophobic polymer 50:50 ratio is less crystalline.

3.2 Loading and Encapsulation Efficiency of Microspheres

Microsphere[a]	Mw[b] (kDa)	Actual loading (w/w) (%)	Encapsulation efficiency (%)	Size[c] (μm)	Morphology of microspheres	Yield %
20:80 w/o/w	3.5	6.4	70.3	10	smooth with holes	75-85
20:80 w/o/w	19.0	6.9	74.8	10	smooth with polymer fragments	
20:80 w/o/w	34.4	7.2	78.7	10	rough	
50:50 w/o/w	7.3	7.8	85.1	10	porous	
50:50 w/o/w	19.0	8.8	95.8	10	porous	
20:80 SD	34.4	5.8	63.3	2	smooth	35-45
20:80 SD	34.4	6.1	65.6	2	smooth with aggregation	
50:50 SD	19.0	5.1	55.8	2	aggregated	
50:50 SD	46.8	4.6	49.9	2	aggregated as robs	
20:80 o/o	19.0	5.8	65.2	50	smooth	55-70
20:80 o/o	34.4	6.5	71.3	50	smooth	
50:50 o/o	19.0	6.5	70.7	50	rough	

Table 3.3 Characteristics of the different batches of microspheres prepared by double emulsion (w/o/w), spray drying (SD) and oil-in-oil (o/o) methods
[a] 20:80, microspheres made from polymer P(CPB:SA) 20:80;
50:50, microspheres made from polymer P(CPB:SA) 50:50;
[b] Mw, molecular weight of polymer before microencapsulation
[c] Size, determined by electron microscopy.

The characteristics of actual drug content, encapsulation efficiencies and particle sizes of the batches of microspheres are reported in Table 3.3. An increase in the Mw of P(CPB:SA) used led to a increase in the BSA encapsulation efficiency for microspheres prepared by the double emulsion technique. When Mw of P (CPB:SA) used in the oil phase increased from 3.5kDa to 34.4kDa for P(CPB:SA) 20:80 and 7.3 to 19.0 for 50:50, the microspheres prepared by the double emulsion method were more spherical and no polymer fragments could be seen under SEM. In addition, the Mw increase also led to an increase in the BSA encapsulation efficiency from 70% to 79% for P(CPB:SA) 20:80 and from 85% to 96% for 50:50.

The molecular weight of the polymer plays an important role among the factors related to the mechanical stability of polyanhydrides. Polymers with higher molecular weights have improved mechanical and film-forming properties, which can affect the stability of microspheres during preparation (Youan *et al.*, 1999). The more stable process, the more protein can be encapsulated. Furthermore, it was assumed that increasing the Mw of the polyanhydride P (CPB:SA) led to an increase in viscosity of organic phase, which may reduce protein diffusion into the external aqueous phase before microparticle hardening.

On comparing the preparation techniques, the batches obtained by the double emulsion method were characterised by the highest encapsulation efficiencies, both in the case of microspheres of P(CPB:SA) 20:80 and in the case of microspheres of P(CPB:SA) 50:50 (Table 3.3). The comparison between the oil-in-oil and spray drying method shows that the oil-in-oil method is more effective in achieving high drug loading when compared with the spray drying, particularly in the case of P(CPB:SA) 50:50 microspheres. This could be related to the solid protein particles used (*i.e.* BSA particles < 50 μm used for o/o method, 10% BSA aqueous solution for spray drying) and the higher concentration of the polymer solution (*i.e.* 10% or 5% for o/o method, 2% for spray drying). All these factors reduce the chance of loss of drug during the initial stages of microparticle formation prior to polymer precipitation. The possibility of protein adsorption rather than encapsulation can not be excluded in the case of a high burst effect due to the location of drug islands close to the matrix surface for microspheres prepared by oil-in-oil method (see section 3.7.1).

Low BSA incorporation levels achieved with the spray drying method were probably due to the high concentration of BSA aqueous solution used as the inner water phase,

and the instability of the emulsion before spray drying. If large droplets formed before feeding, the spray nozzle could split the emulsion into its individual components, which may stick on the cylinder due to the higher boiling point of water. Thus, it is possible that some microspheres harvested in the product collector could have little or no drug content due to a loss of products in the drying chamber. It could be shown by further investigation of actual drug loading of microspheres harvested from the spray dryer cylinder and from the harvested collector. This problem may be avoided by decreasing the spray flow to increase the microsphere size or decreasing the concentration of BSA solution as it has been reported that the best encapsulation efficiencies for spray drying are always obtained with the lowest amount of drug added to the polymer (Pavanetto *et al.*, 1993).

3.3 Microsphere Preparation using Oil-in-Oil Method

The yield of microspheres prepared by this method was in the range of 55-70%, the size was around 50 μm, almost no precipitation on the stirrer was observed. Successful microsphere preparation by solvent removal depends on two factors, the rate of precipitation of the polymer and the rate of DCM diffusion into the silicone oil. For microspheres prepared from polymer P(CPB:SA) 20:80 (Mw 19.0kDa and 34.4kDa), it was possible to obtain a stable suspension of polymer in silicone oil for longer periods than P(CPB:SA) 50:50. The solubility of P(CPB:SA) 20:80 in DCM was higher than P(CPB:SA) 50:50, and therefore the polymer solution was mixed into oil efficiently before precipitation occurred. The surfactant Span 85, which is immiscible with silicone oil, was introduced to prevent coagulation of the polymer phase. Mixing this emulsifier

with silicone oil resulted in a binary system consisting of large droplets of surfactant dispersed in silicone oil.

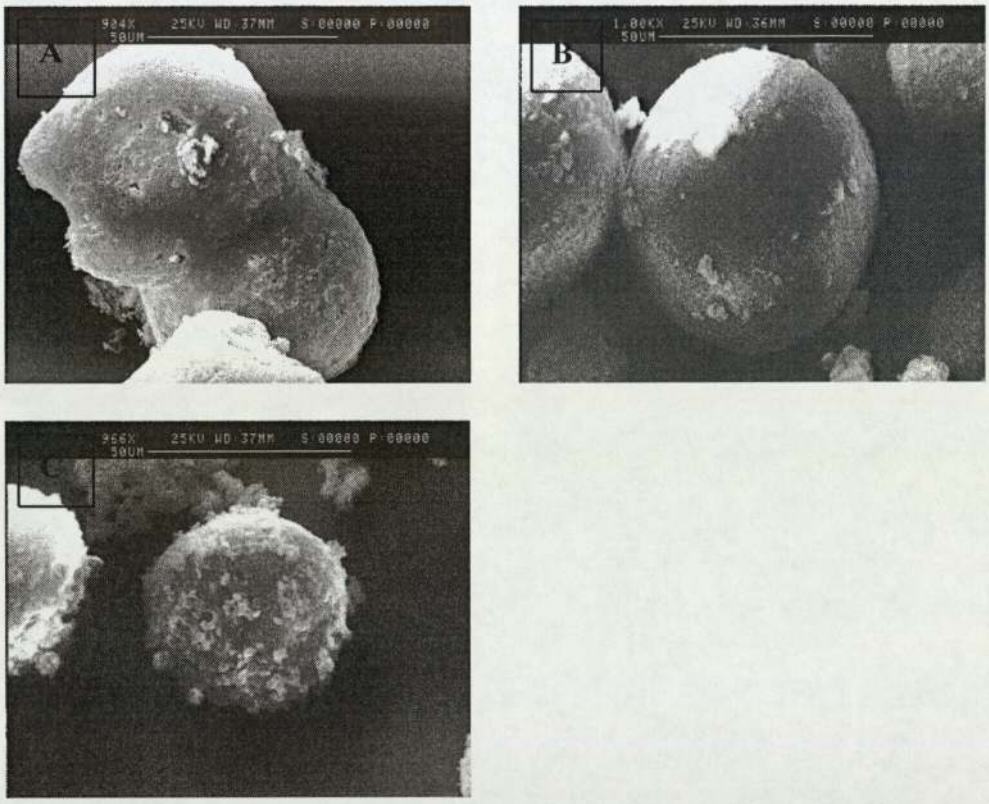


Figure 3.2 SEM of P(CPB:SA) microspheres prepared by o/o method: (A) P(CPB:SA) 50:50 microspheres aggregated as rod; (B) the dense external structure of P(CPB:SA) 20:80 5.8% (w/w) BSA-loaded microspheres; (C) the dense external structure of P(CPB:SA) 50:50 6.5% (w/w) BSA-loaded microspheres (Mw 19.0kDa).

During the preparation, the emulsion was checked using microscopy (x 400). All the microspheres of P(CPB:SA) 20:80 were engulfed by the surfactant droplets. There were also many surfactant droplets devoid of microspheres. The use of such an immisicible surfactant achieved the isolation of the P(CPB:SA) 20:80 microspheres, but for P(CPB:SA) 50:50 microspheres, some of the particles were aggregated (see Figure 3.2 A). The reason may be that the polymer precipitated rapidly before the particles were engulfed by the surfactant droplets. The process can be controlled by increasing the

amount of DCM in the oil; this was verified with P(CPB:SA) 50:50 where decreasing the concentration of the polymer solution and increasing the concentration of DCM improved the microencapsulation process of BSA particles by yielding microspheres rather than rods.

After precipitation of polymer begins, the process of microencapsulation is more complicated, involving diffusion both in the solution and in the already precipitated polymer. The first precipitation occurs in the external area and this layer slows subsequent diffusion of DCM into the oil phase. The removal of the DCM was accelerated by adding petroleum ether. For P(CPB:SA) 50:50 (Mw 19.0kDa), it was impossible to obtain a stable suspension for a longer time than P(CPB:SA) 20:80 without the addition of increased volumes of DCM, as the polymer precipitation occurred so quickly that the polymer solution was not efficiently dispersed. Several modifications of the procedure were required: (1) more DCM was added to the oil in order to slow diffusion of solvent from the polymer phase, at least, for the first stage when the suspension is formed; and (2) petroleum ether was added immediately upon obtaining the suspension of the polymer solution in the oil. The surfaces of P(CPB:SA) 50:50 (see Figure 3.2 C) microspheres obtained *via* this method were not as smooth as P(CPB:SA) 20:80 (see Figure 3.2 B). The rate of precipitation may also be a key to understanding the type of microspheres obtained. The rough surface of the P(CPB:SA) 50:50 may be due to the faster precipitation of P(CPB:SA) 50:50 than P(CPB:SA) 20:80. Thus precipitation occurred in the P(CPB:SA) 50:50 microspheres before a stable emulsion could form. In contrast, in slower precipitating systems P(CPB:SA) 20:80, first an emulsion formed and then precipitation occurred. This 'two-step' process

of microencapsulation can be further controlled by using different surface-active compounds to stabilise the emulsion.

The dense external structure is typical of the most of the microspheres produced by this method (see Figures 3.2 B and C). A closer inspection of the physical events occurring while these microspheres were formed may help in understanding their performance. A schematic representation of the process is shown in Figure 3.3 and a proposed description of the process taking place is as follows:

The process of microencapsulation is diffusion-controlled, at least in the first stages where the difference in concentration of DCM between the two phases is significant when the polmer solution was introduced into the oil phase. As the DCM diffuses quickly into the oil phase, the concentration of the polymer near the surface is high, which explains why precipitation of the outer shell occurs first, leaving a high concentration of polymer dissolved in DCM inside the core. This organic solvent can be later removed by adding a non-solvent or applying high vacuum. Thus, this dense external structure for microspheres of both polymers was presumably obtained because during precipitation of the microspheres, the external surface precipitates first, thus making it difficult for the microspheres to shrink.

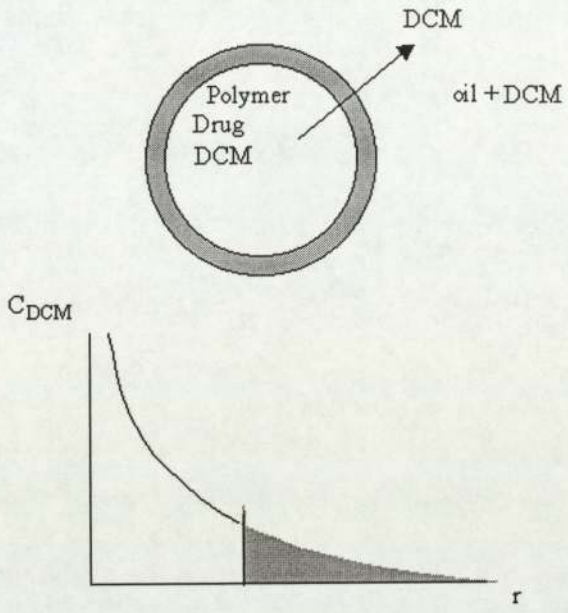


Figure 3.3 Schematic representation of solvent removal process.
r = distance from center of the microspheres; C_{DCM} = concentration of DCM inside microspheres during solidification process. (adapted from Mathiowitz *et al.*, 1990a)

Figures 3.4 and 3.5 show the morphology changes for microspheres P(CPB:SA) 50:50 and 20:80 at different stages during one week degradation *in vitro* in PBS. After day 1, microspheres lost their dense external structure, and a lot of visible pores were formed on the surface for microspheres of P(CPB:SA) 50:50 (Figure 3.4 A). During the following days, the size of the pores increased notably, and the large pores developed into deep holes stretching into the core of the microspheres (Figure 3.4 B, C, D). After 7 days, the structure of microspheres although dramatically altered and the fragments of the microspheres after degradation sometimes can be found (Figure 3.4 D), but most of the microspheres still reserved their spherical shape.

The morphology changes in P(CPB:SA) 20:80 microspheres during degradation (Figure 3.5) were different from the P(CPB:SA) 50:50. After day 1, microspheres still exhibited

a dense external structure, and only a few visible pores were formed on the surface and this condition lasted up to three days (Figures 3.5 A and B). At the 4th day, the microsphere structure came apart and internal drug pockets were visible (Figure 3.5 C). After 7 days, microspheres lost their spherical shape, but fragments with dense external surface were visible (Figure 3.5 D,E).

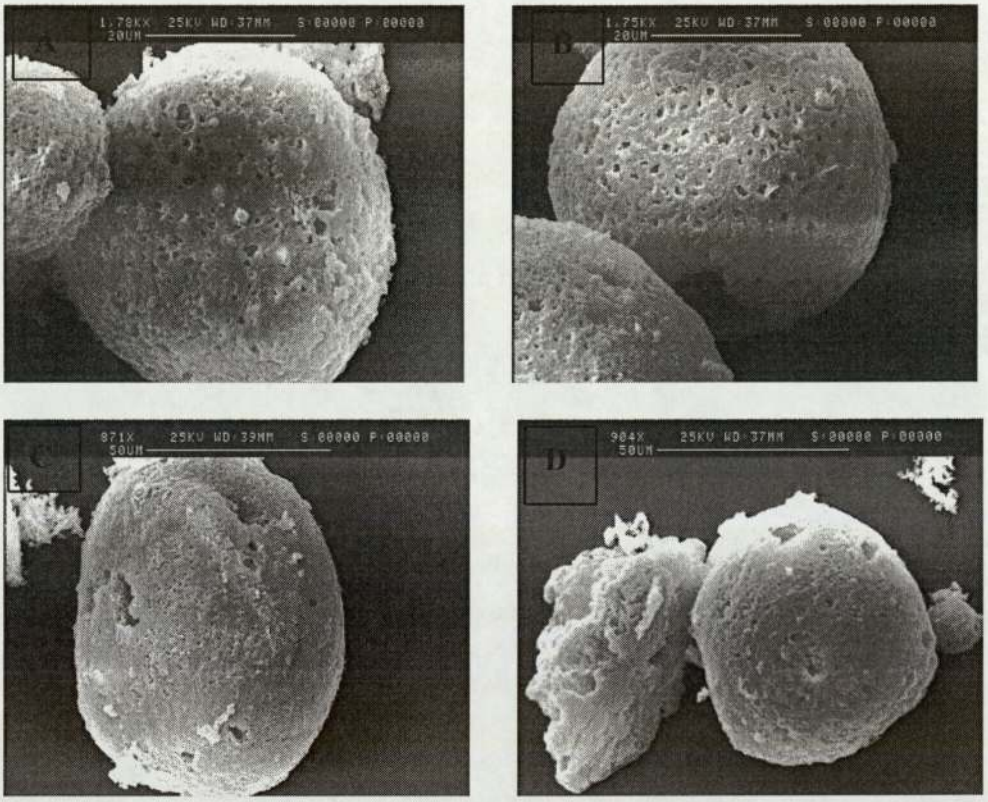


Figure 3.4 SEM of P(CPB:SA) 50:50 6.50% (w/w) BSA-loaded microspheres (Mw 19.0kDa), prepared by o/o method, at different degradation stages *in vitro*: (A) day 1; (B) day 3; (C) day 5; (D) day 7.

The differences during degradation may be attributed to the different crystallinity and concentration of the two polymers used in microspheres preparation, 10% w/w for P(CPB:SA) 20:80 and 5% w/w for 50:50. At the beginning of the degradation, release medium infiltrated into the surface of the microspheres and porous surface structure was formed for both microspheres. Owing to the lower concentration and crystallinity of

P(CPB:SA) 50:50, the shell of the microspheres is more easily attacked by release medium. The BSA particles trapped inside the microspheres were swollen after contact with the release medium through the formed pores during the degradation, and resulted in the breaking apart of P(CPB:SA) 20:80 microspheres due to its high crystallinity and brittle characteristics.

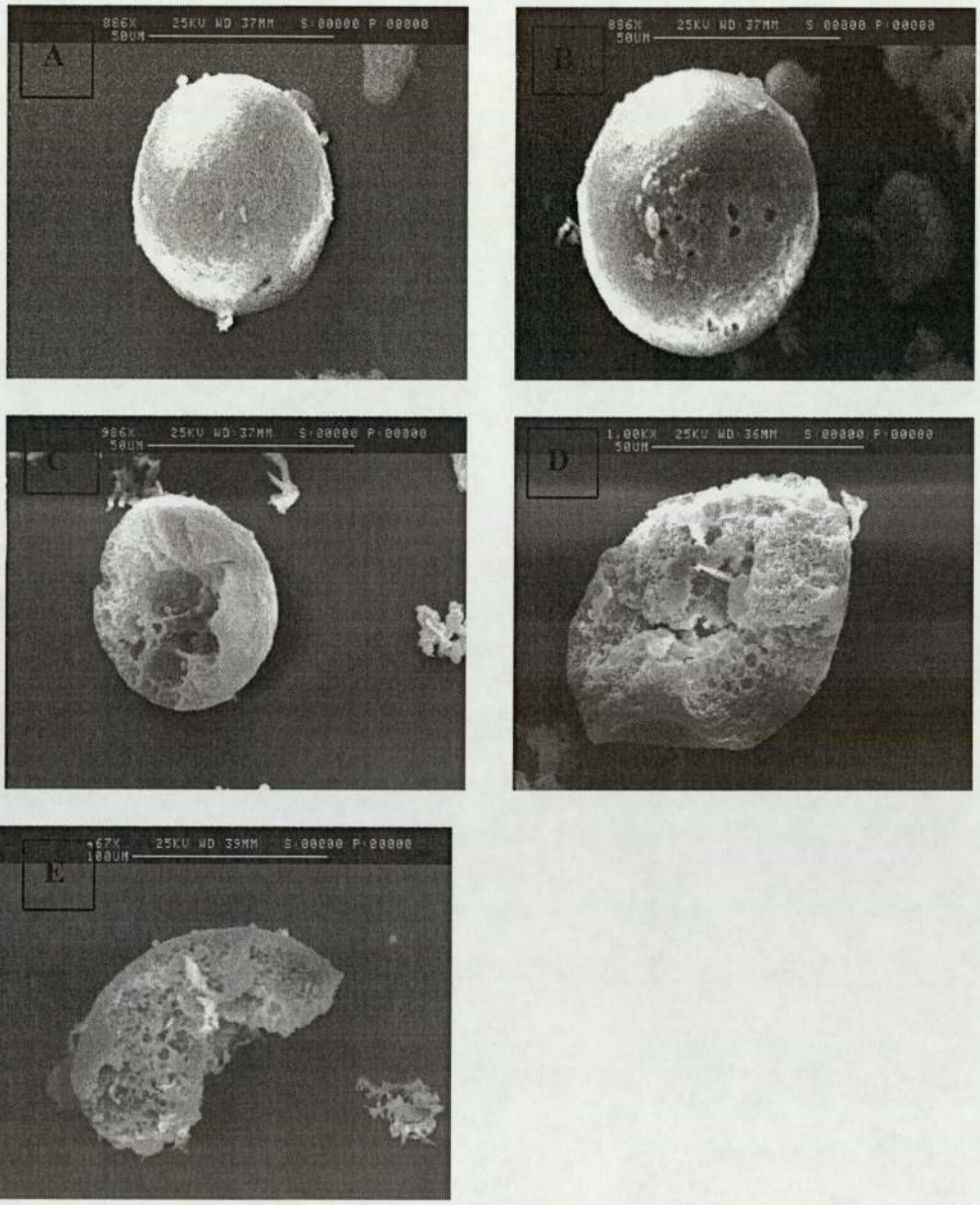


Figure 3.5 SEM of P(CPB:SA) 20:80 5.80% (w/w) BSA-loaded microspheres (Mw 19.0kDa), prepared by o/o method, at different degradation stages *in vitro*: (A) day 1; (B) day 3; (C) day 4; (D) day 5; (E) day 7.

3.4 Microsphere Preparation by Spray Drying

The yield of microspheres prepared by this method was in the range of 35-45% and the size of the microspheres was around 2 μm. In order to create a delivery system using spray drying, it is essential to dissolve the polymer matrix in a volatile liquid. Polyanhydrides degrade in aqueous solution and it is preferable to process them in organic solvents. Volatile solvents, such as DCM, make it possible to encapsulate various heat sensitive drugs, including proteins at low temperatures.

For this particular study, copolymer P(CPB:SA) 20:80 (Mw 34.4kDa) and 50:50 (Mw 19.0kDa and 46.8kDa) were used. Since the spray dryer nozzle was 0.7 mm, it was important to obtain very fine droplets of the initial emulsion. This was achieved by maintenance of the emulsion by stirring on ice throughout the feeding process to increase the viscosity of polymer phase. Throughout this work, the same organic solvent (DCM) and the same polymer concentration (2 % w/w) were used. This allows comparison of the properties of microspheres of P(CPB:SA) 20:80 and 50:50.

In general, it was possible to spray dry the copolymer P(CPB:SA). However, each batch of microspheres appeared different (see Figures 3.6 and 3.7) due to the different composition of the polymer and the change in spray drying conditions (see section 2.4.3). Scanning electron micrographs of microspheres made of P(CPB:SA) 50:50 are shown in Figure 3.6. The external surface of P(CPB:SA) 50:50 (Mw 19.0 and 46.8kDa) microspheres loaded with 5.1% and 4.6% BSA was smooth and dense. However, the microspheres tended to fuse with each other before the final drying step. The same phenomenon was observed with P(CPP:SA)50:50 microspheres (Mathiowitz *et al.*, 1992). Lowering the concentration of the polymer solution can prevent the aggregation. It has been found that the main reason for the high degree of fusion during spray drying

is the low glass transitions of these polymers (Mathiowitz *et al.*, 1992). However, in this study no Tg was detected with the DSC conditions employed. The aggregation could also be the result of the high viscosity of this polymer solution as well as its low melting point (see Table 3.2). It has been reported that where polymer concentration in the starting solution is maintained constant, the spray globule size increases with polymer Mw due to enhanced intermolecular forces between polymer chains, until a limiting Mw is reached, where threads, not microspheres, result due to insufficient force to break up the stream of feed solution (Bain *et al.*, 1999). In this study, more fusion was also observed for microspheres prepared from polymer Mw 46.8 kDa than from Mw 19.0 kDa (see Figure 3.6).

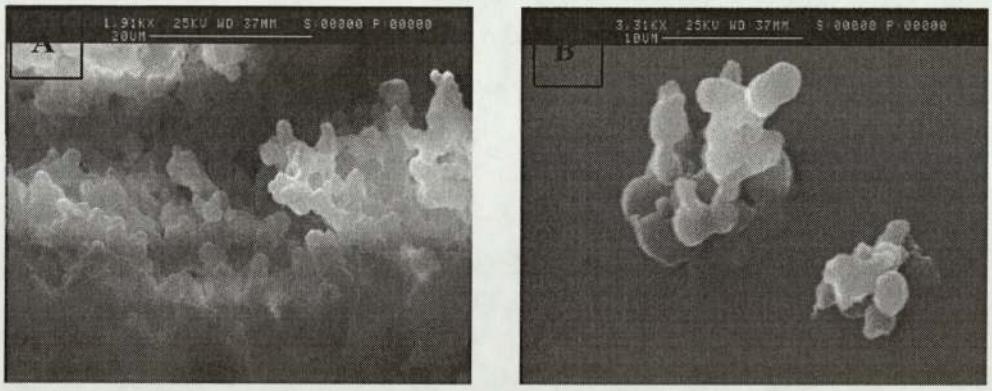


Figure 3.6 SEM of P(CPB:SA) 50:50 microspheres prepared by spray drying method: (A) Mw 19.0kDa, pump setting 8 ml/min (5.1% BSA actual loading); (B) Mw 46.8kDa, pump setting 5 ml/min (4.6% BSA loading).

Scanning electron micrographs of P(CPB:SA) 20:80 BSA-loaded microspheres immediately following preparation and during degradation are shown in Figure 3.7. The microspheres displayed a smooth external surface with size around 2 μm. They were spherical in shape with few agglomerates when the outlet temperature was lower than 40˚C (see Figure 3.7 A). The aggregation was reduced with P(CPB:SA) 20:80 (Tm 69˚C) (see Figure 3.7 B) compared to P(CPB:SA) 50:50 (Tm 54˚C), owing to the

different melting points of the two polymers.

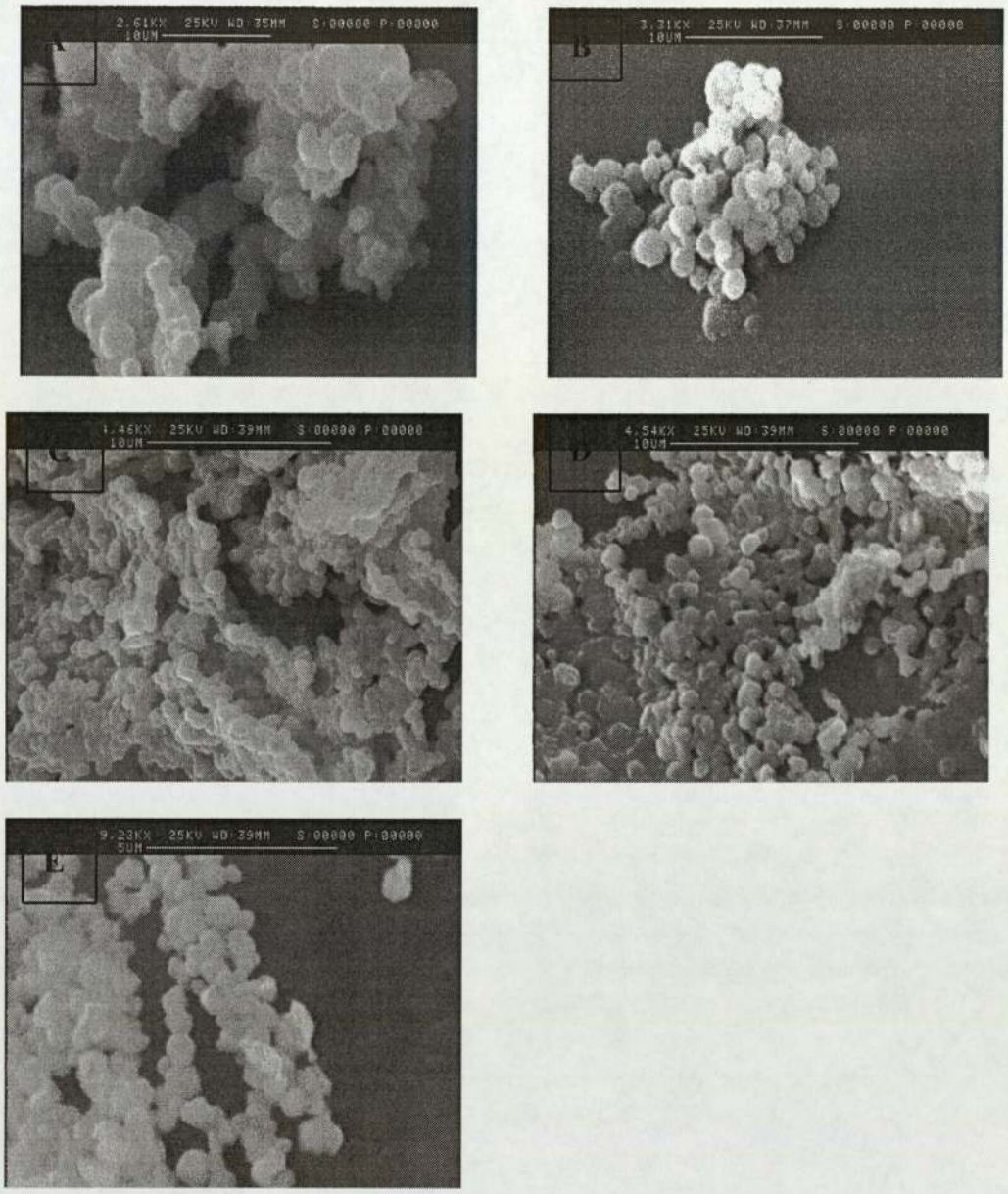


Figure 3.7 SEM of P(CPB:SA) 20:80 (Mw 34.4kDa) BSA-loaded microspheres, prepared by spray drying method after preparation (A, B) and at different degradation stages *in vitro* (C, D, E) for B: (A) 6.1% loading, outlet T. 39-40˚C; (B) 5.8% loading, outlet T. 42˚C, day 0; (C) day 1; (D) day 3; (E) day 7.

During microsphere preparation, some of the spheres accumulated in the spray drier trap, and this is related to the aggregation of the microspheres. When the surface concentration of the sprayed droplets reaches saturation point, crusts will form. If the

crust is sufficiently dry or hard, there is no change in appearance and the dried particles are spherical. If the crust is not dry or hard enough, the microspheres will appear deformed or adhere to each other in the spraying chamber and result in aggregation. The aggregation during the evaporation process prevented some of the microspheres from reaching the final collecting tube and accumulated in the trap of the spray drier. This is also responsible for the lower yields in spray drying.

Scanning electron micrographs of microspheres made of P(CPB:SA) 20:80 during degradation are shown in Figure 3.7 C, D, E. No changes were visible during the degradation. The microsphere remained spherical, with no visible pores, softening or fusion. This may be due to slow penetration of water into the polymer matrix owing to the more hydrophobic nature of P(CPB:SA) than P(CPP:SA). Furthermore, due to the low permeation of hydrophilic low molecular weight ions of the phosphate buffer through the polymer matrix, degradation products cannot leave the microspheres immediately. Therefore, no clear erosion signs were observed after 7 days degradation.

3.5 Microsphere Preparation by Double Emulsion Method

Polyanhydride P(CPB:SA) microspheres, prepared by the modified solvent evaporation method using a double emulsion, were spherical (Figures 3.8 and 3.10). The yield was in the range of 75-85%. The diameter of microspheres was around 10μm confirmed by the scale bar on electron micrographs. It has been found that when the inner emulsion was prepared by vortex mixing, the resulting microspheres were larger (50-185 μm) with a larger inner emulsion than the ones prepared by probe sonication, for which a microfine inner emulsion was formed and the overall microspheres were much smaller

(Tabata *et al.*, 1993). Since the microsphere size depended mainly on the mixing rate used during the inner emulsion preparation, it was very important to control the time and power used to form the initial emulsion, so the same sonication conditions were maintained (see section 2.4.2).

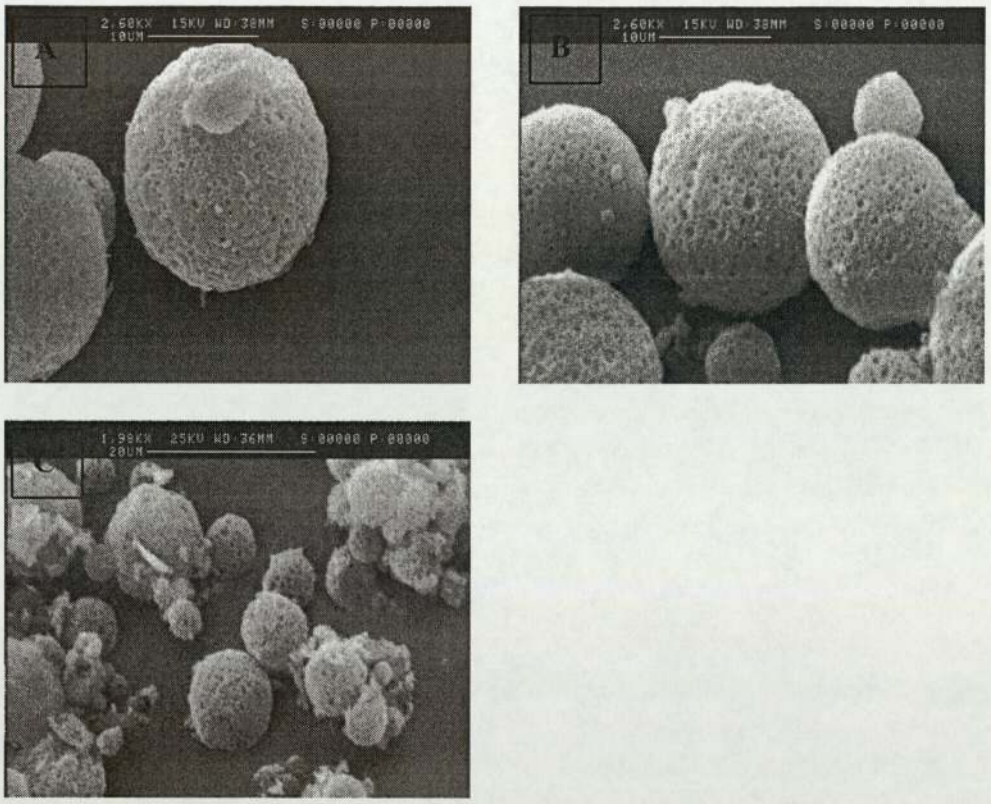


Figure 3.8 SEM of P(CPB:SA) 50:50 BSA-loaded microspheres, prepared by double emulsion after preparation and degradation *in vitro*: (A) 7.8% loading, Mw 7.3kDa; (B) 8.8% loading, Mw 19.0kDa, day 0; (C) day 7.

There was no change in appearance of P(CPB:SA) 50:50 microspheres with different Mw (see Figures 3.8 A and B) under SEM and the particles were porous and spherical. The higher porosity of BSA-loaded microspheres made from P(CPB:SA) 50:50 (Mw 7.3kDa or 19.0kDa) could be explained by areas of rapid evaporation of the water-immiscible DCM before particles hardened. For unloaded microspheres, they appear smooth (see Figure 3.9 A). It could be related to the processing conditions, such as the

speed of stiring during particle hardening. Thus, further investigation should be made to optimise the conditions for double emulsion preparation.

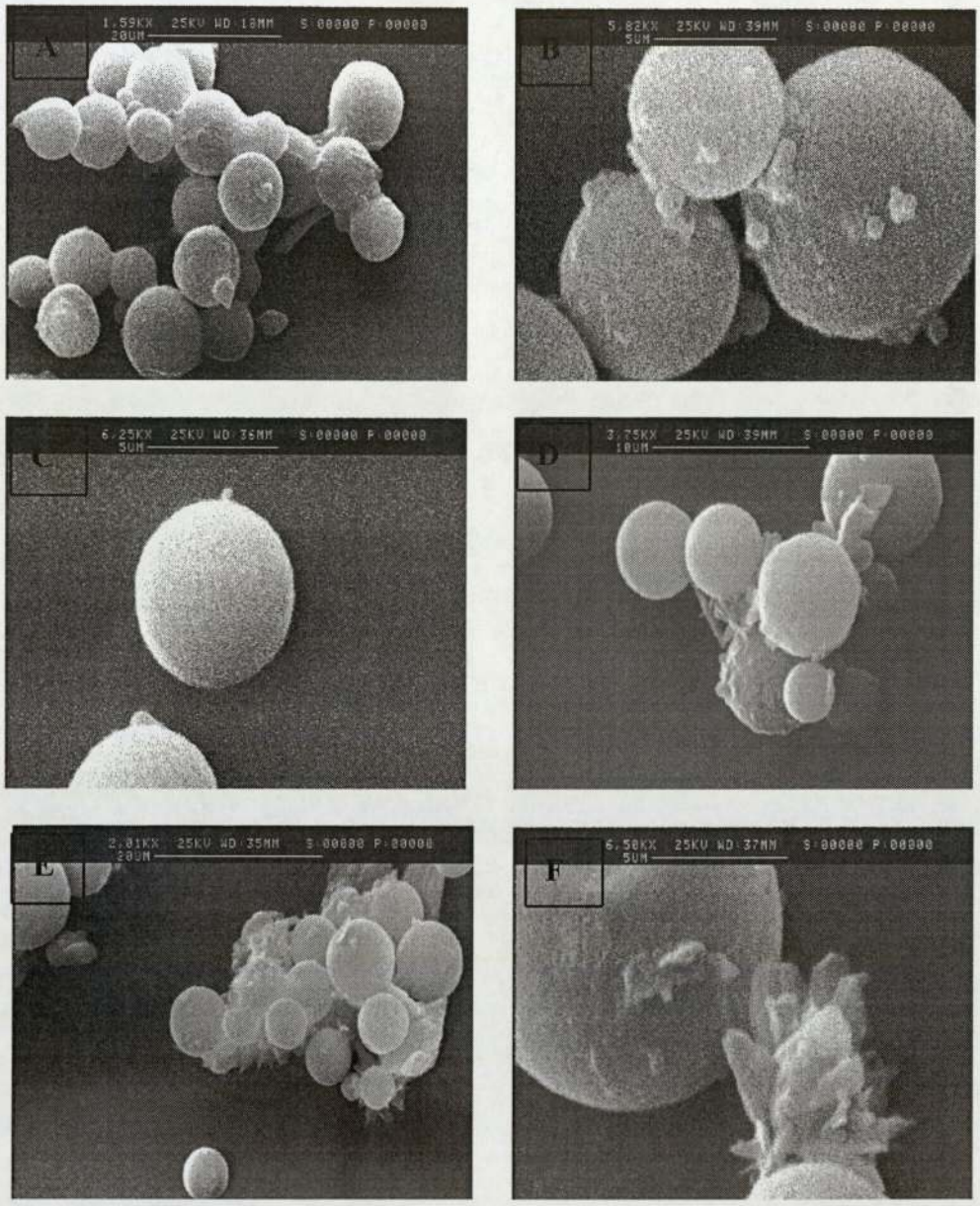


Figure 3.9 SEM of P(CPB:SA) 50:50 (Mw 19.0kDa) unloaded microspheres, prepared by double emulsion, after preparation and at different degradation stages *in vitro*: (A) day 0; (B) day 1; (C) day 3; (D) day 4; (E) day 5; (F) day 7.

Figure 3.9 shows scanning electron micrographs of P(CPB:SA) 50:50 unloaded microspheres at different degradation stages. Immediately after preparation, the

microspheres are spherical in shape with a dense and smooth surface structure, and this surface characteristic is maintained till the 5th day of degradation. After 5 days, a number of the particles appeared to have collapsed and fragmented while others appeared unchanged. However, for BSA-loaded microspheres made from the same polymer, no changes were visible after 1 week (see Figure 3.8 C). Presumably, loaded and unloaded microspheres would have different internal structures, which could lead to different degradation mechanisms.

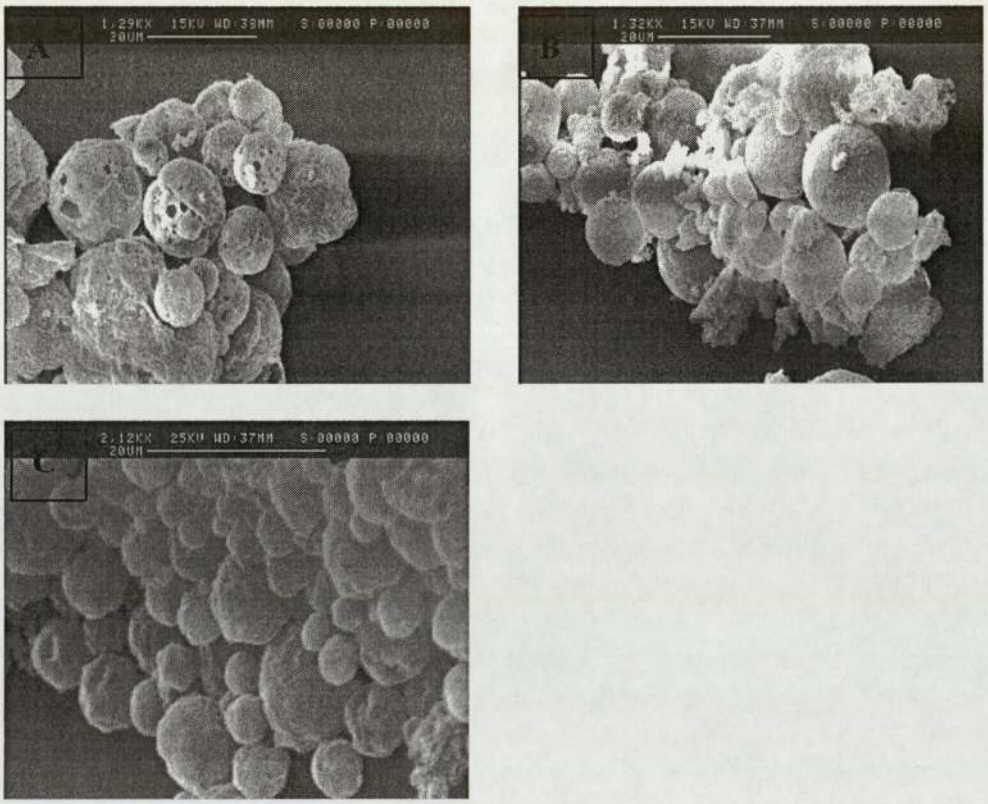


Figure 3.10 SEM of P(CPB:SA) 20:80 BSA-loaded microspheres, prepared by double emulsion after preparation: (A) Mw 3.5kDa, loading 6.4%; (B) Mw 19.0kDa, loading 6.9%; (C) Mw 34.4kDa, loading 7.2%.

Figure 3.10 shows the P(CPB:SA) 20:80 microspheres prepared by double emulsion method using a range of molecular weights. Three different Mws of P(CPB:SA) 20:80 were investigated from 3.5kDa to 34.4kDa. As can be seen in Figure 3.10, more

polymer fragments were visible at low Mw. The molecular weight of the polymer is related to the mechanical stability of polyanhydrides. Increasing the molecular weight increases the tensile strength and the intrinsic viscosity of polyanhydride in organic solvent (Domb & Langer, 1987; Domb *et al.*, 1997). Polymers with higher molecular weight will have improved mechanical and film-forming properties, which can affect stability of the microspheres during preparation (Youan *et al.*, 1999). Thus, application of a polymer with a relatively high molecular weight is essential for microspheres production using high shear stress such as this.

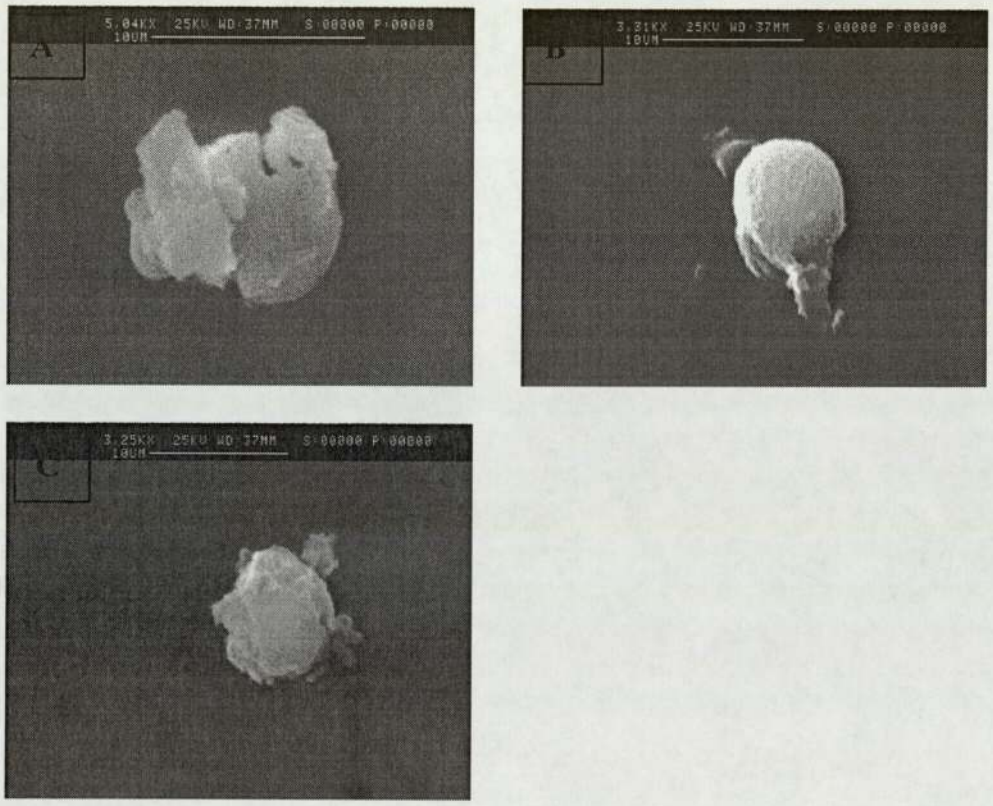


Figure 3.11 SEM of P(CPB:SA) 20:80 (Mw 34.4kDa) 7.2% BSA-loaded microspheres, prepared by w/o/w, at different degradation stages *in vitro*: (A) day 2; (B) day 5; (C) day 6.

Figure 3.11 shows the SEM micrographs of the BSA-loaded microspheres made from P(CPB:SA) 20:80. Generally, after 4 days degradation, the microspheres lost their

spherical shape, while the morphology of the single microspheres during degradation is more complicated. At different time intervals, some microspheres showed separation of the bulk (see Figure 3.11 A) or outer-layer (see Figure 3.11 B) from the microspheres, whereas some looked rough on the surface (see Figure 3.11 C). This could be related to the distribution of monomer inside copolymer chains investigated by NMR (disscused in section 3.1). Whether monomers are randomly distributed in the polymer backbone or have a more block-like structure is a very important factor in degradation and erosion. For copolymers made of SA in a combination with CPP or CPH, it was found that the monomers were mainly randomly distributed when the content of both monomers was equal (Ron *et al.*, 1991). A block-like arrangement of the monomers inside the polymer chain might lead to the discontinuous erosion of the material when the two blocks exhibit different resistance against degradation and erosion. From this result combined with the analysis of NMR results, the erosion of P(CPB:SA) microspheres seems more like a combination of surface and bulk erosion.

3.6 Degradation of Polyanhydride P(CPB:SA)

Polyanhydrides are composed of a hydrophobic polymer backbone joined by anhydride linkages that readily split in the presence of water to form two carboxylic acid end groups. This structure has been proposed to cause the polyanhydride device to undergo surface front erosion, which is characterised by degradation on the surface that approaches the center of the device as erosion continues (Mathiowitz *et al.*, 1993). The degradation of polyanhydride microspheres is more complicated due to the effects of various external and internal structures formed by different preparation methods.

The molecular weight of the polymer forming the BSA-loaded microspheres was measured immediately after preparation and following one month storage in a desiccator. There was indicated no significant change in Mw. However, compared to the original Mw of polymer used, there was a dramatic decrease in Mw on manufacture, such as Mw droped from 34.4kDa to 8.2kDa, 19.0 to 4.5 for P(CPB:SA) 20:80, from 19.0 to 7.0, 7.3 to 4.8 for P(CPB:SA) 50:50 after preparation using double emulsion method. The scope of the decrease varied with the preparation method and the polymer used, but it seems that the higher the Mw of the polymer used, the more the Mw of the microspheres droped. However, the same trend Mw change of microspheres on degradation was observed irrespective of the type of the method used for preparation. The data for degradation of microspheres prepared by double emulsion are included here as a example. Results of molecular weight measurements of degraded P(CPB:SA) microspheres prepared by double emulsion method are presented in Figure 3.12. A sharp decrease in molecular weight was observed during the first 24 h, followed by a slow degradation phase which kept the Mw at 3,000 ~ 2,000 for another 6 days for P(CPB:SA) 50:50. The molecular weight distribution of polymer and microspheres at all times was unimodal and relatively narrow, but shoulders corresponding to low or intermediate molecular weight fragments appeared and were maintained through the degradation (see Figure 3.13). This is a strong indication of formation of stable oligomeric chains that are more resistant to hydrolytic degradation than larger copolymer chains. Due to the low solubility of P(CPB:SA) 20:80 microspheres after degradation in chloroform or DCM, the sensitivity of GPC was limited at low concentrations.

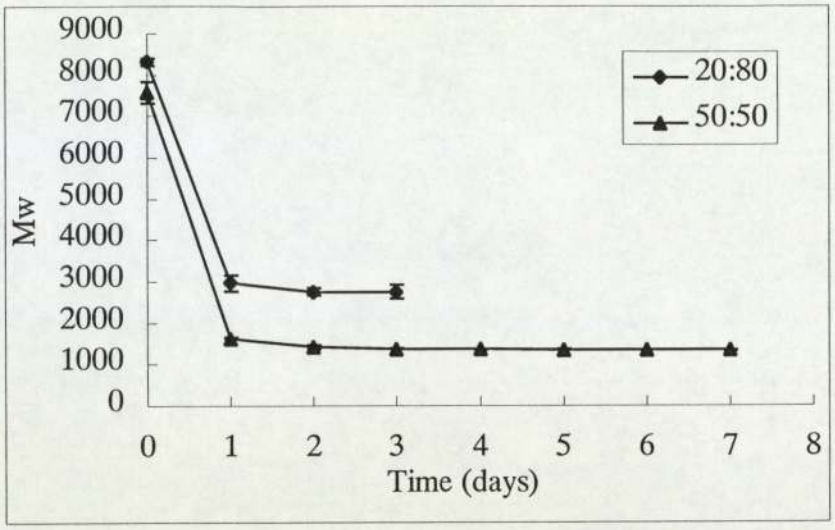


Figure 3.12 The Mw change of microspheres prepared by the double emulsion method following degradation in 0.1 M phosphate buffer at 37°C (n=3; mean ± s.d.)

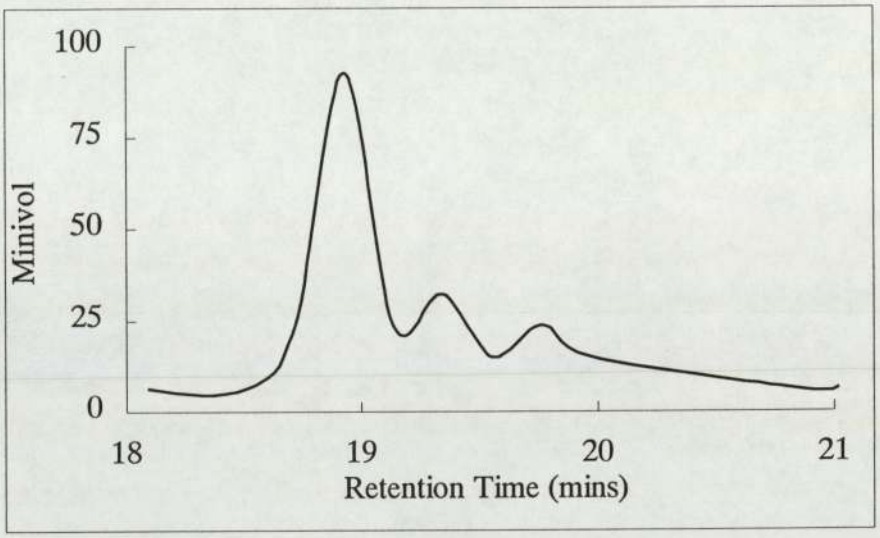


Figure 3.13 Molecular weight distribution of P(CPB:SA) 20:80 microspheres during degradation (GPC output).

To observe the microstructure of the microspheres during degradation, degraded polymer samples were freeze-dried and visulised by SEM. These results have been discussed in sections 3.2 to 3.4.

In addition, changes in the IR spectrum for polymers P(CPB:SA) 50:50 and 20:80 before and after microsphere preparation suggest that polymer degradation took place

during micrsphere preparation (see Figures 3.14 and 3.15). The emergence of the carboxylic acid peak at 1700 cm^{-1} is obvious and becomes stronger for microspheres prepared by spray drying and oil-in-oil methods, at same time the aliphatic anhydride peak at 1810 cm^{-1} decreases. It can be concluded that the aliphatic anhydrides are easily to be attacked during preparation. The proposed reduced degradation for microspheres prepared by oil-in-oil method was not achieved according to the IR results. However, results obtained by GPC indicated that all of the three methods resulted in reduced Mw, and there were no dramatical differences in Mw change between the microspheres prepared by different methods as indicated by IR results. Hydrolysis of the anhydride bonds caused by water attack is only one of the factors attributed to the degradation of the polyanhydrides. It has been reported that ultrasound can enhance the polymer degradation (Liu *et al.*,1992) and polyanhydrides lost 50% of their molecular weight within approximately 1.5 hours even in anhydrous chloroform (Domb & Langer, 1989). The degradation during preparation using the oil-in-oil method could be attributed to the sonication and the high concentration of the polymer solution in the core, for which a long hardening time was needed. Thus, further investigation should be taken to optimize the condition of the preparation.

Figures 3.16 and 3.17 show the IR spectra of P(CPB:SA) microspheres prepared by a double emulsion method at different degradation stages as well as the spectrum for the original copolymer. Due to time limits, only microspheres prepared by the double emulsion method were tested. The relative intensity of the doublet at 1810 and 1740 cm^{-1}, attributed to be carboxylic anhydride bonds, became weaker as the microspheres degraded. Instead, a band appeared near 1700cm^{-1} region, indicating the emergence of carboxylic acid groups due to the hydrolysis of the anhydride linkage. The spectra for

the P(CPB:SA) 20:80 microspheres after degradation 24 hours in PBS (Figure 3.17) showed the emergence of the carboxylic acid peak at 1700cm^{-1} and the disappearance of the aliphatic carboxylic acid groups peak at 1810cm^{-1} just 24 hours after incubation, and this was maintained till the end of the experiment. However for P(CPB:SA) 50:50, the aromatic anhydride bonds around 1770cm^{-1} can still be detected after seven days degradation in pH 7.4 phosphate buffer (Figure 3.16). This result may be explained in terms of the higher hydrophobicity of CPB than SA. It is possible that the hydrophobic nature of the polymer inhibits intial water entrance and therefore slows degradation. Another reason could be that the anhydride of SA is much easier to be attacked by the water than that of CPB.

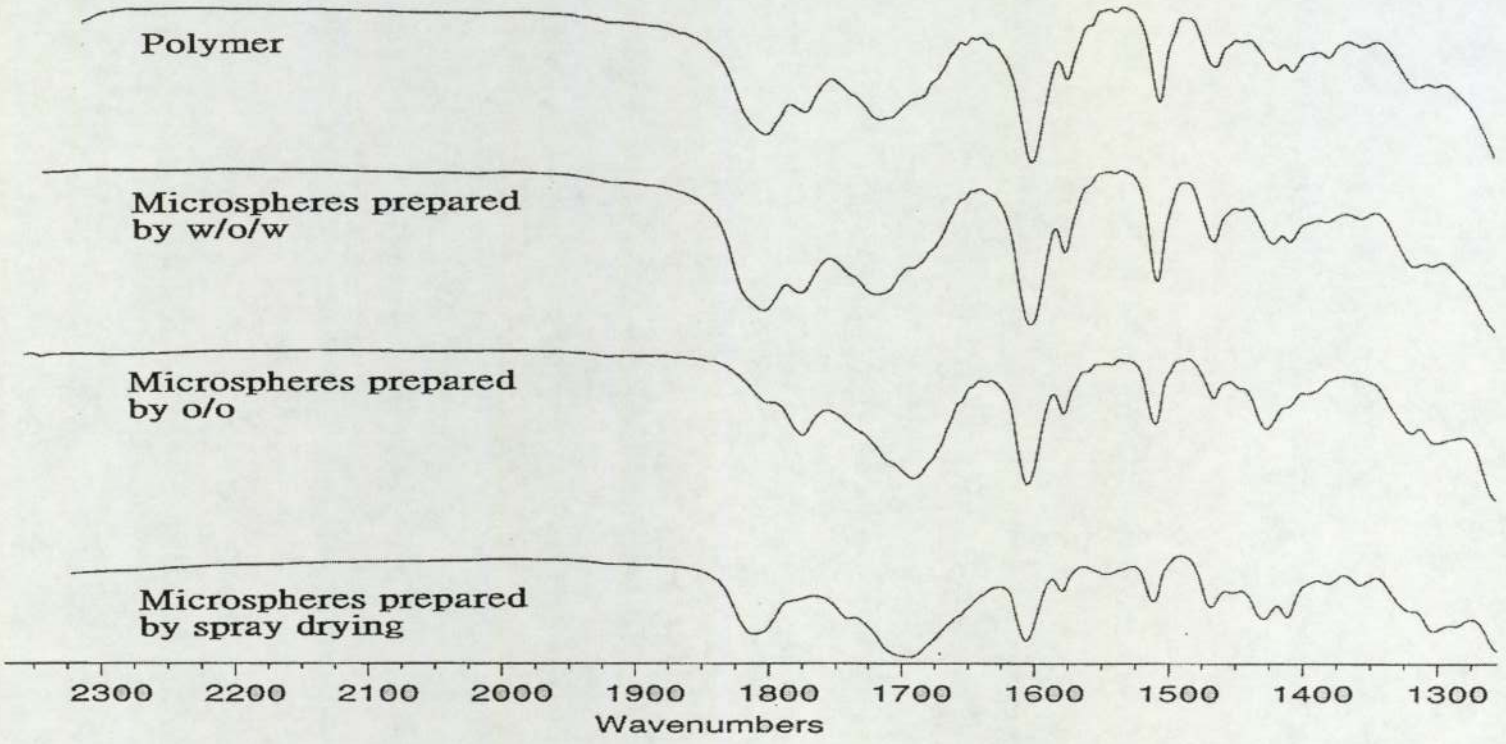


Figure 3.14 IR spectra of polymer P(CPB:SA) 50:50 (Mw 19.0kDa) and microspheres prepared by different methods

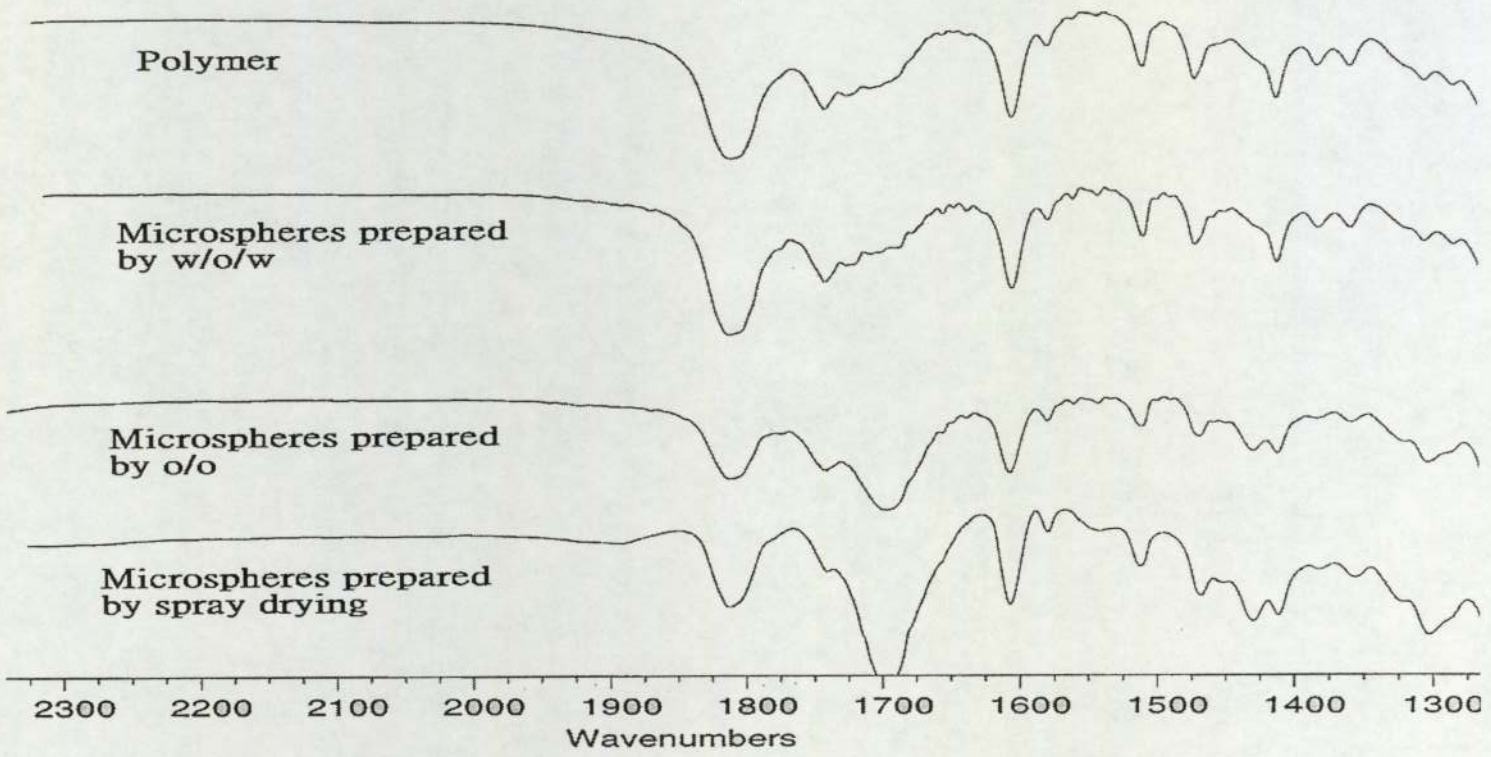


Figure 3.15 IR spectra of polymer P(CPB:SA) 20:80 (Mw 34.4kDa) and microspheres prepared by different methods

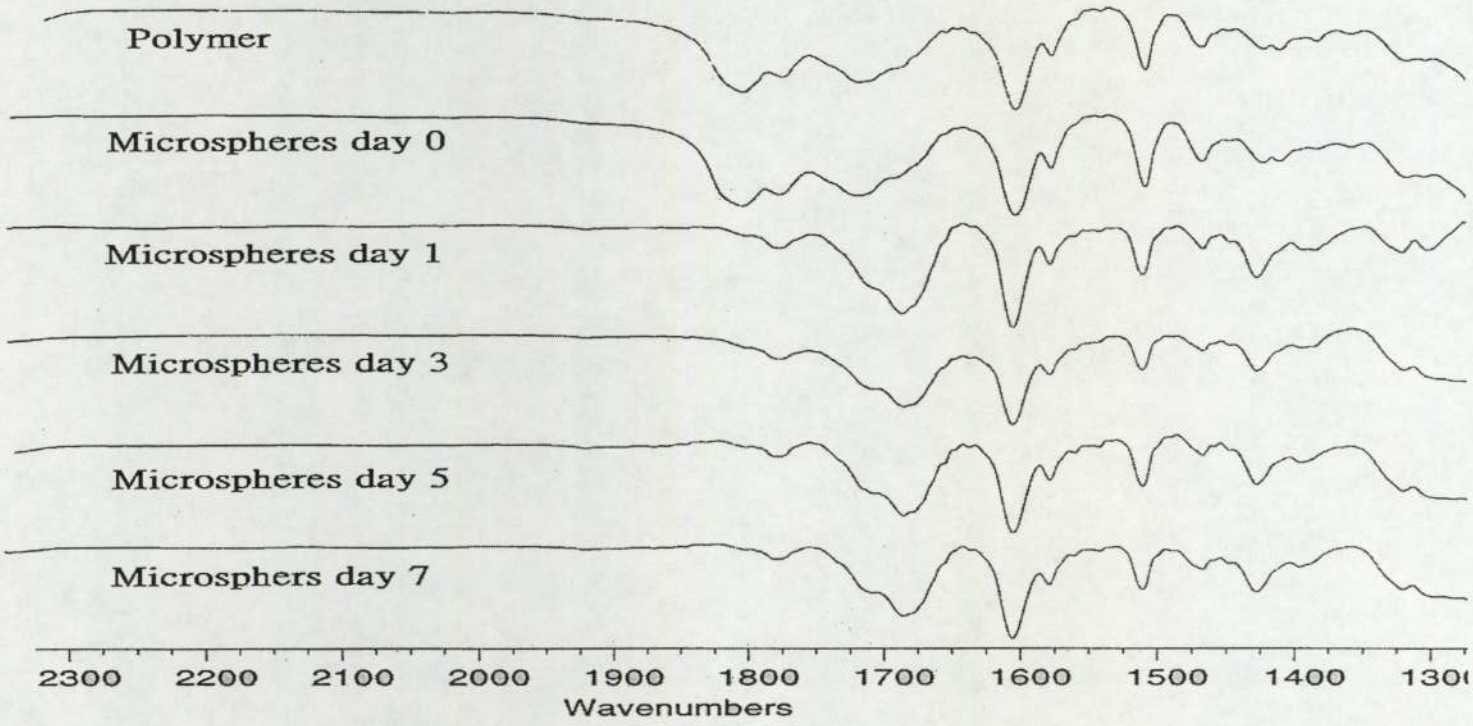


Figure 3.16 IR spectra of P(CPB:SA) 50:50 (Mw 19.0kDa) BSA-loaded microspheres (w/o/w) during degradation.

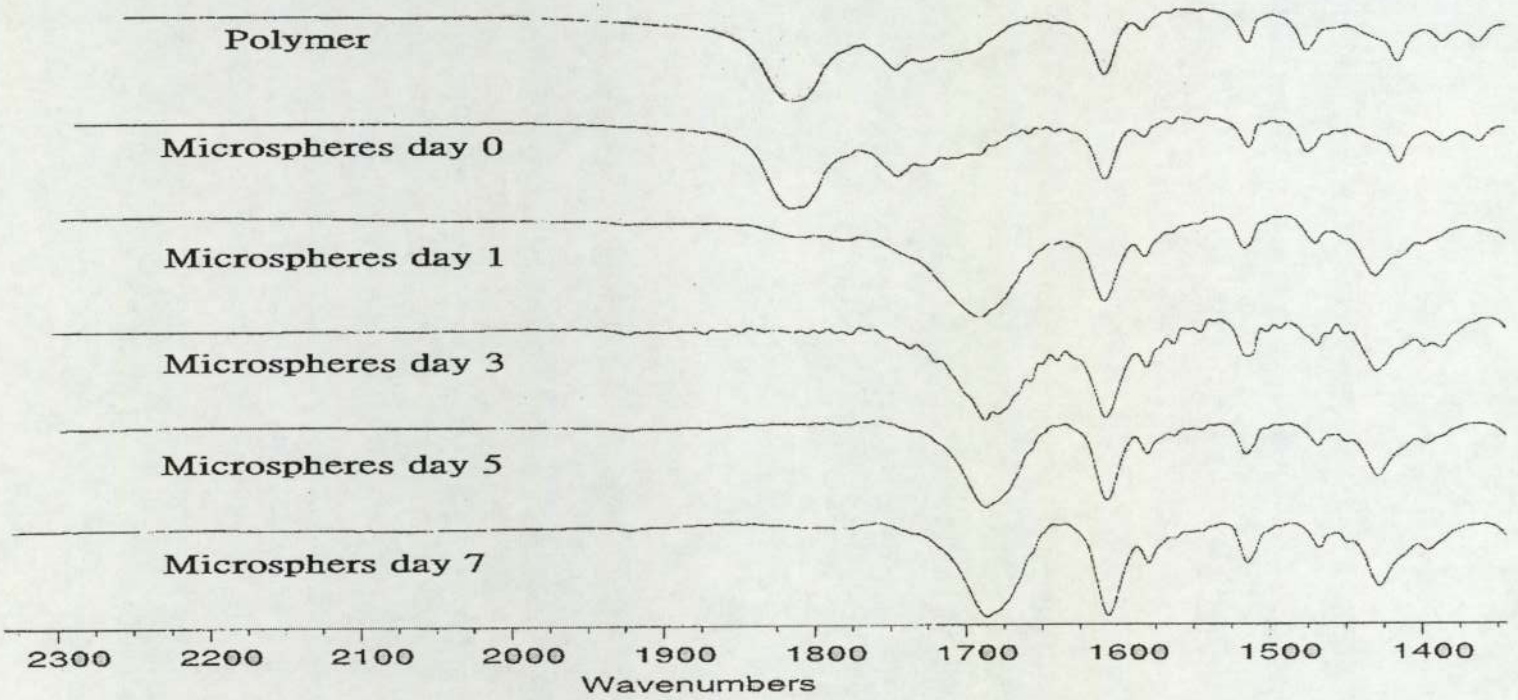


Figure 3.17 IR spectra of P(CPB:SA) 20:80 (Mw 34.4kDa) BSA-loaded microspheres (w/o/w) during degradation.

3.7 Release of BSA from Microspheres

3.7.1 Release in PBS

The drug release behavior depends on both the choice of polymer and on the formulation procedure. During *in vitro* release studies, SDS was incorporated in some studies to reduce BSA adsorption onto the polymer surface (Figure 3.18). The cumulative release value for microspheres prepared by double emulsion and spray drying methods may be perceived to fall (see Figures 3.19, 3.20 and 3.21). The BSA could be adsorbed on the microspheres surface again after it is released out from the microspheres into the release medium. This is confirmed by the cumulative release of BSA increased from 4% to 12% due to the complete solubilisation of the protein during the polymer hydrolysis in the presence of SDS in release medium.

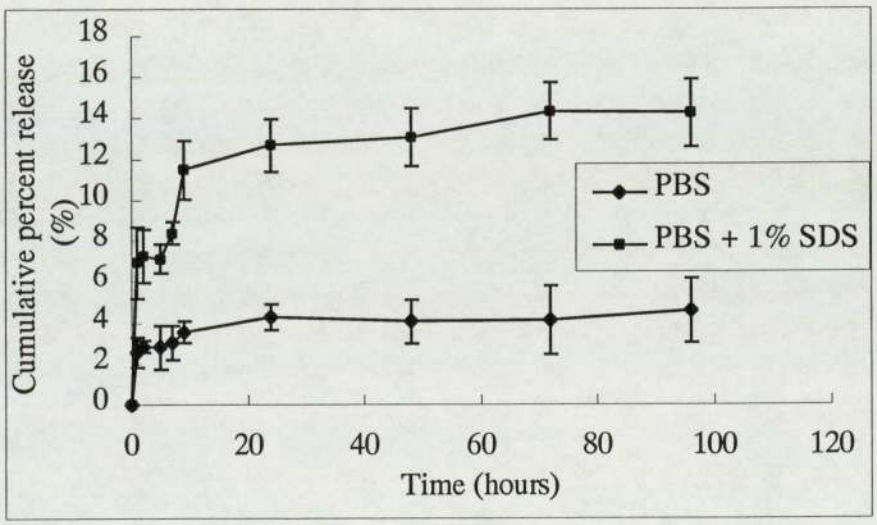


Figure 3.18 Release profiles of 7.2% w/w BSA from 10 μm P(CPB:SA) 20:80 microspheres prepared by w/o/w method (Mw of polymer 34.4kDa) in PBS and PBS containing 1% w/v SDS (n=3; mean ± s.d.)

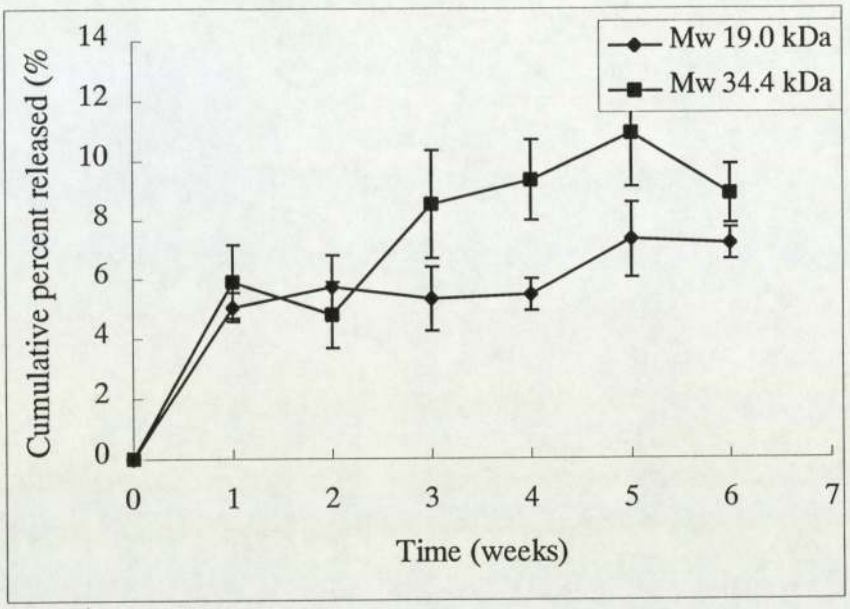


Figure 3.19 The effect of polymer molecular weight on BSA release from P(CPB:SA) 20:80 microspheres prepared using w/o/w technique (loading is 6.9% w/w for Mw 19.0kDa & 7.2% w/w for Mw 34.4kDa) (n=3; mean ± s.d.)

Characteristic release curves for BSA-loaded microspheres prepared from P(CPB:SA) 20:80 with different molecular weights using w/o/w method are shown in Figure 3.19. The initial burst and the release curve before the second day are similar. After the second day, the release rate for microspheres with the higher molecular weight increases. This could be due to the higher loading (6.90% for Mw 19.0kDa, 7.19% for Mw 34.4kDa) of this preparation.

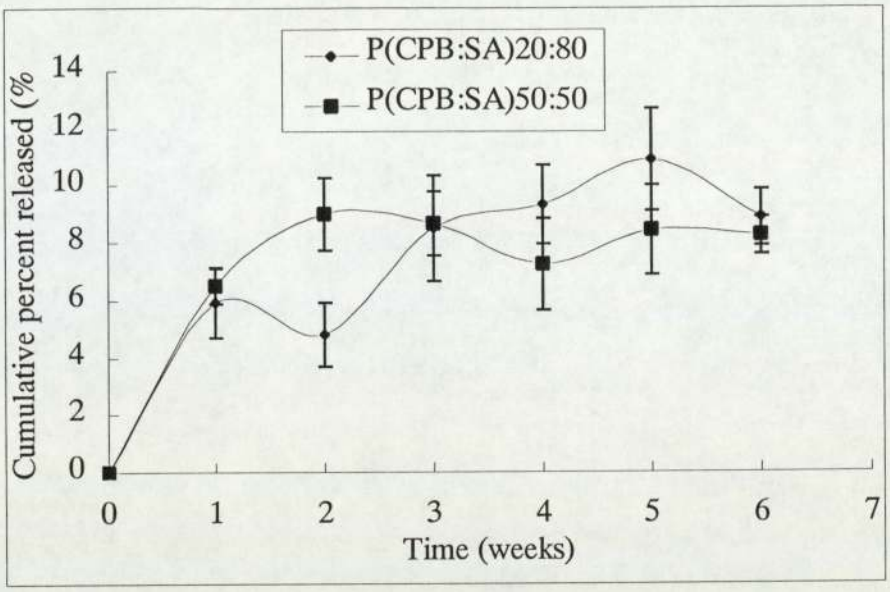


Figure 3.20 Release of BSA from P(CPB:SA) 50:50 and P(CPB:SA) 20:80 microspheres prepared by w/o/w in PBS (n=3; mean ± s.d.)

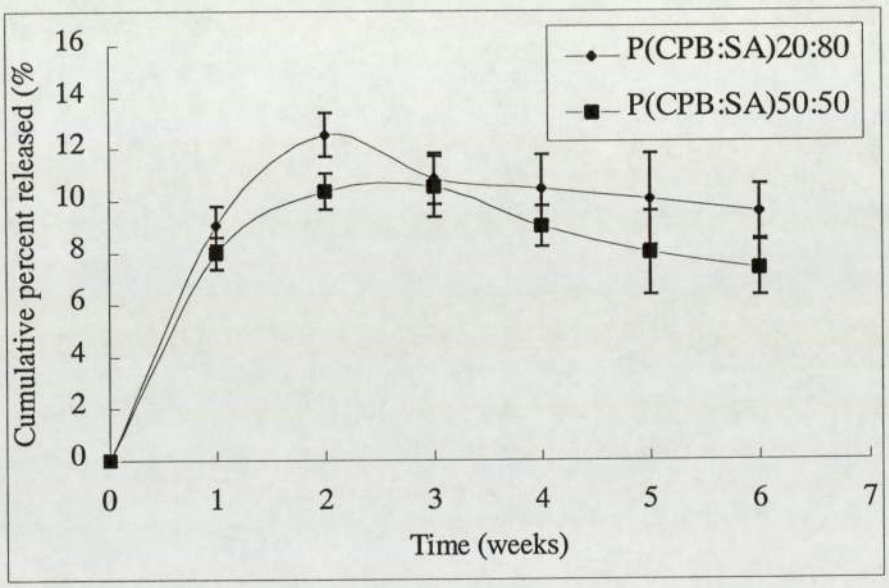


Figure 3.21 Release profiles of BSA from P(CPB:SA) 50:50 and 20:80 microspheres prepared by spray drying in PBS (n=3; mean ± s.d.)

In Figures 3.20 and 3.21, BSA release from different polyanhydride microspheres is shown. There is little difference between the release from P(CPB:SA) 20:80 and P(CPB:SA) 50:50 by double emulsion and spray drying methods. This could be due to a

number of reasons. One is P(CPB:SA) 20:80 is a fast-degrading polymer due to the rapid hydrolysis of the aliphatic polyanhydride bond (see section 3.6). However, compared to P(CPB:SA) 50:50, microspheres are more crystalline as shown by DSC (see section 3.1), which may prevent water uptake by the polymer. On the other hand, although P(CPB:SA) 50:50 is more amorphous, the hydrophobicity of this polymer could still be the main barrier for water uptake. Overall, there may be little difference in release rates.

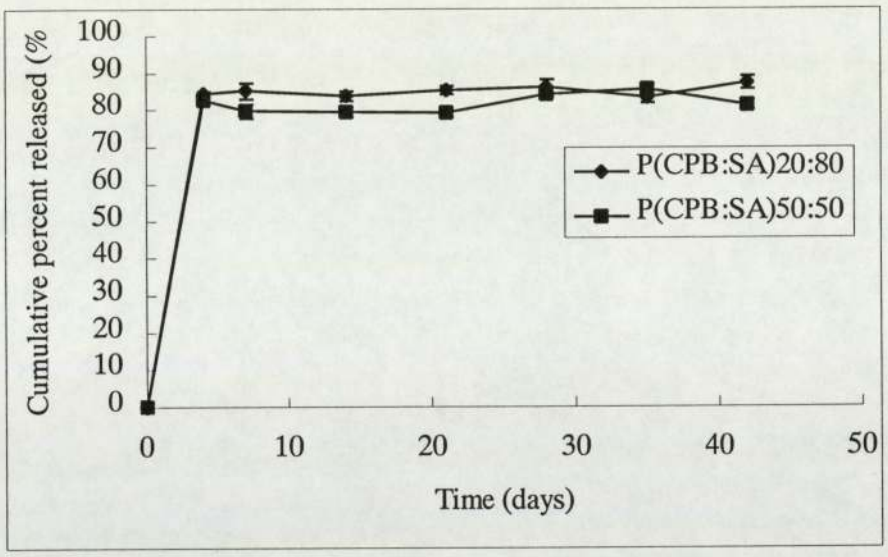


Figure 3.22 Release profiles of BSA from P(CPB:SA) 50:50 and 20:80 microspheres prepared by o/o method in PBS (n=3; mean ± s.d.).

BSA release from P(CPB:SA) microspheres prepared by the oil-in-oil method is shown in Figure 3.22. During the first week *in vitro*, cumulative drug release is in the range 80% for microspheres prepared by this method, but only 6-8% for the spray dried and double emulsion preparations. The release of the incorporated material can occur *via* two independent processes. The first is diffusion of the drug through fluid-filled pores, formed by the dissolution of the incorporated drug particles; the second is *via* erosion of the polymer matrix as the anhydride bonds are hydrolysed. The total release of drug will

be the sum of these two release rates. Two main ways to encapsulate hydrophilic material in hydrophobic polymers have been described here. These processes are based on the dispersion of the material as a powder or as an aqueous solution into the organic solution containing the polymer. It has been reported that the percentage of protein released is higher and less consistent for microspheres prepared by the powder dispersion procedure (Alonso *et al.*, 1993). This may also explain the leaching-out of the protein located at the surface of the microspheres and, thereby, the formation of large pores and interconnecting cavities through which the encapsulated protein can diffuse. Thus, in this case, both the porous structure and the dispersion of BSA incorporated inside the microspheres could result in a fast overall release.

Microspheres prepared by the double emulsion method and spray drying exhibited relatively low burst effects and a slow release was achieved (see Figures 3.19, 3.20 and 3.21). These results are contrary to those obtained with BSA-loaded P(FAD-SA) 25:75, prepared by double emulsion with 2% BSA-loading, diameter 50-150 μm, for which a near constant release rate up to 800 hrs was obtained (Tabata *et al.*, 1993). The result may be explained by the different characteristics of P(FAD:SA). P(FAD-SA) microspheres are degraded to form oily, poorly water-soluble FAD monomers (Tabata *et al.*, 1993). It is also possible that the hydrophobic nature of the polymer inhibits initial water entrance or the CPB component slows diffusion of SA from the polymer. Obviously, due to the relatively high hydrophobicity of P(CPB:SA), the burst effect was generally considerably lower than literature values. For example, it has been reported that the burst release from P(CPP:SA) 50:50 microspheres containing 7% BSA is around 40% *in vitro* (Chiba *et al.*, 1997). For P(CPB:SA) microspheres prepared by double emulsion and spray drying methods, it is only around 6% - 12%, even with SDS

incorporated in the release medium.

In fact, a burst effect is seen when the drug islands located close to the matrix surface quickly dissolve after being immersed in solution, like microspheres prepared by oil-in-oil method (see Figure 3.22). Release rates are also affected by the pattern of drug inside microspheres. If the drug is located as fine solid crystals in the microsphere matrix and are not in contact with each other, the drug molecules or the solvent must diffuse through the matrix before release can occur and a slow release is caused. If the drug exists as larger particles inside the microspheres, the trapped drug will form a network or a fine dispersion in high loaded particles. Once release is initiated, channels may form inside the matrix. The residual protein particles can diffuse through these channels by an osmotically driven mechanism, thus a burst effect and rapid release would occur. This could be one explanation for the release profiles obtained using the oil-in-oil method. In most cases, this burst effect is undesirable because it releases an uncontrollable significant portion of the drug immediately at the beginning of the release period. This burst leaves smaller amounts of drug available for release over the entire release period (Tabata *et al*. 1993). Therefore, the relatively low burst effect (around 10%), found with microspheres prepared by double emulsion or spray drying, could be advantageous. However, in both cases, there was not a significant subsequent continuous release of the protein over the following 42 days (< 20%) in PBS. This would require further investigation.

In all cases, the *in vitro* release of the drug is characterised by a bimodal behavior: an initial 'burst effect' occurs, the intensity of which ranges from 5% for double emulsion method to 80% for oil-in-oil method and is dependent on the method employed to produce microspheres. This burst effect is followed by a 'plateau' that lasts a few

weeks. The bimodal behavior could be explained by the presence of part of the drug in the external surface of the microspheres (burst effect) and part of the drug entrapped in the polymeric network ('plateau'), which requires significant polymer erosion prior to drug release.

3.7.2 Release in Basic Medium

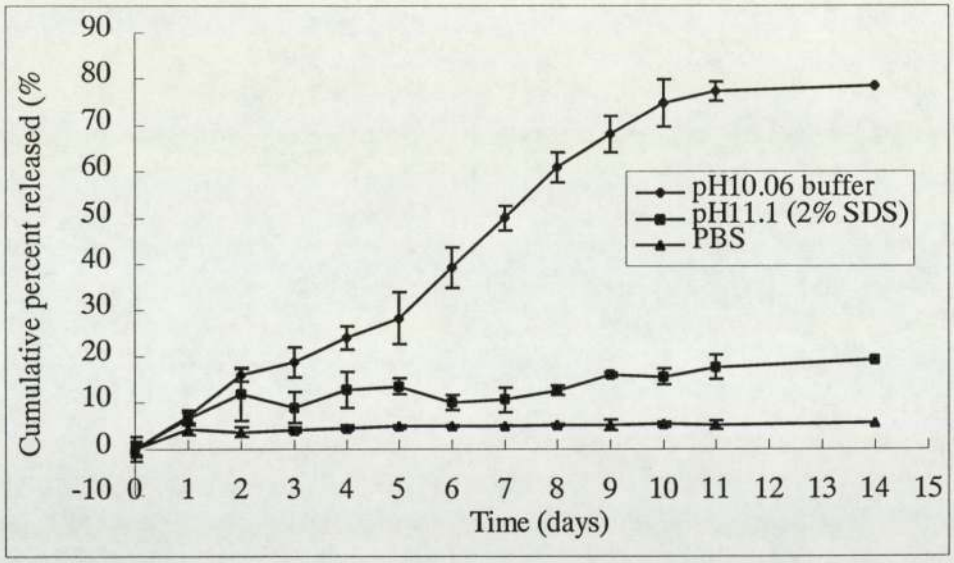


Figure 3.23 Release profiles of 8.8% w/w BSA from 10 μm P(CPB:SA) 50:50 microspheres prepared by w/o/w method (Mw 19.0kDa) in pH10.06 Na_2CO_3 and $NaHCO_3$ buffer, pH11.1 0.1% w/v Na_2CO_3 and 2% w/v SDS solution, and PBS (n=3; mean ± s.d.)

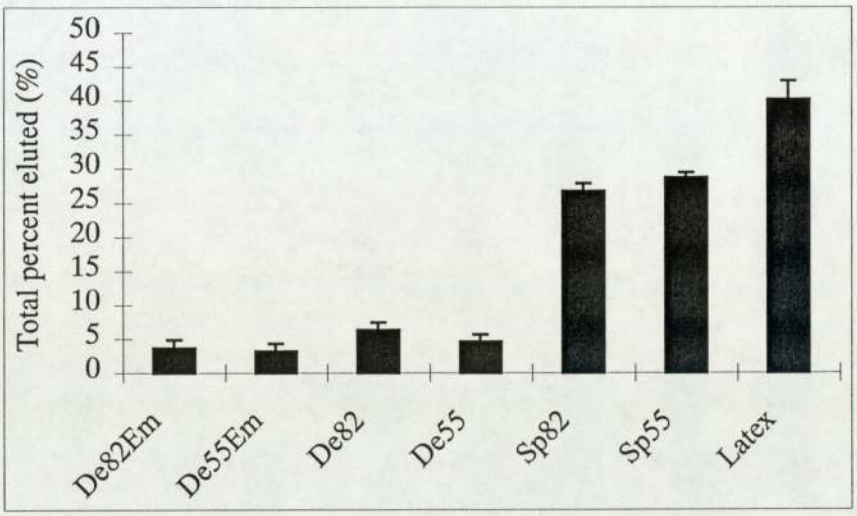
The release rates were found to be pH dependent. The release from P(CPB:SA) microspheres increased significantly in basic release medium (see Figure 3.23). Comparing the BSA release rate in pH 10.06 buffer and pH11.1 Na_2CO_3 with 2% (w/v) SDS solution, neutralisation of the acid degradation product was found to be very important for the release of protein. It could be related to the increased solubility of the monomers in basic medium. The pH value of the release medium PBS was found to fall

to around 5-6 during the study in one week. During degradation, the anhydride linkages split in the presence of water to form two carboxylic acid end groups. If the resultant acid affects the buffer pH value, a saturated condition may be formed inside the microspheres due to the decreased solubility of the SA or CPB in this acid environment. It has been reported that the anhydride bonds are more labile in high pH conditions (Park *et al.*, 1996), and polyanhydrides degraded very quickly in basic buffer (Santos *et al.*, 1999). Thus, BSA release rates from P(CPB:SA) were significantly reduced at low pH and enhanced under basic conditions. The 'stability' of P(CPB:SA) microspheres at low pH could be an advantage for oral delivery of vaccines when mucosal immunity is desired, since microspheres less than 10 μm in diameter are known to be taken up from the intestine into Peyer's patches (Eldridge *et al.*, 1990).

3.8 Hydrophobicity of P(CPB:SA) Microspheres

Hydrophobic Interaction Chromatography (HIC) is a column chromatographic method, which separates substances or particles on the basis of differences in their hydrophobic interaction with a hydrophobic gel matrix (Müller, 1991). The separation achieved is dependent on the hydrophobicity of the solute and polymer and interactions with and between the solvent water molecules. Therefore, as described in Section 2.6, 0.6 M NaCl solution was used throughout the experiments for elution of the microspheres, and non-ionic surfactant (Triton X – 100) was used to facilitate further elution by binding to the stationary phase causing displacement of the adhered particles (Müller, 1991). 1.0 μm latex microspheres, having distinctly more hydrophobic surfaces than biodegradable particles (Cartensen *et al.*, 1991), were used as a reference for studying the

hydrophobicity of microspheres. Only one size of the latex was utilised during the experiment due to the limited time.



De82Em: Empty microspheres prepared by w/o/w from P(CPB:SA) 20:80
De55Em: Empty microspheres prepared by w/o/w from P(CPB:SA) 50:50
De82: BSA microspheres prepared by w/o/w from P(CPB:SA) 20:80
De55: BSA microspheres prepared by w/o/w from P(CPB:SA) 50:50
Sp82: BSA microspheres prepared by spray-drying from P(CPB:SA) 20:80
Sp55: BSA microspheres prepared by spray-drying from P(CPB:SA) 50:50

Figure 3.24 The total percent eluted from propyl-agarose columns for microspheres prepared by w/o/w or spray-drying from P(CPB:SA)20:80 Mw 34.4kDa and 50:50 Mw 19.0kDa (n=3; mean ± s.d.).

Figure 3.24 shows the total percentage eluted from columns for loaded and unloaded microspheres formed by double emulsion and loaded microspheres by spray drying methods. The P(CPB:SA) microspheres prepared by spray drying were washed from the columns more readily. A series of standard latexes , size range from 0.2 μm to 1.0 μm, had been tested and the result showed that large particles passed the column more readily (Conway, 1996). Although microspheres (2 μm) prepared by spray drying method are smaller than those (10 μm) prepared by the double emulsion method, and

could be more readily eluted, it may be possible that the surface of the spray dried microspheres is less hydrophobic than those prepared using double emulsion method. There are not many differences between the hydrophobicity of loaded and unloaded microspheres prepared by double emulsion. Generally, P(CPB:SA) microspheres are more hydrophobic than latex particles. This is also confirmed by the BSA release from the microspheres prepared by the double emulsion and spray drying methods. The burst release of microspheres prepared by these two methods is very low, only around 8%. It could be that the more hydrophobic surface of microspheres reduced the penetration of the dissolution medium and resulted in a reduced burst effect.

4. CONCLUSION

Throughout this study, microspheres were successfully produced using a number of methods. The microspheres did not crumble, nor were there any visible cracks in the preparations and degradation, expect for P(CPB:SA) 20:80 microspheres prepared by oil-in-oil method. This may be related to its relatively large size and the uneven distribution of BSA particles within it. Its surface became porous after 24 hours, owing to the penetration of water and initial degradation. Further more, after 4 days, some microspheres crumbled into small pieces for P(CPB:SA) 20:80 microspheres. However, the microsphere surfaces remained non-porous during the degradation for the microspheres prepared by spray drying and double emulsion methods. As it is generally accepted that intact particle uptake following oral delivery does occur for particles smaller than 10 μm in diameter, P(CPB:SA) microspheres (50μm) prepared by oil-in-oil method would not be suitable for oral vaccination.

The method of solvent evaporation has been used extensively in the preparation of microspheres. However, the presence of an aqueous phase used in this method was predicted to initiate polymer hydrolysis. Therefore microencapsulation by solvent removal or spray drying might provide a new approach for capsule formation of polyanhydrides. However, the results indicated that some of the anhydride bonds, especially for the aliphatic anhydrides, were attacked during microsphere preparation by an oil-in-oil method. Thus double emulsion and spray drying are effective as preparation methods for polyanhydride microspheres containing BSA (protein). Double emulsion yields more acceptable products with high loading and encapsulation efficiency. Spray drying is rapid and easy to scale-up, albeit having the lowest yield of

production. This problem may be overcome by increasing the microsphere batch size to industrial scale. The atomisation of larger quantities of polymer using conventional nozzle designs is known to result in an increase in particle yield (Bittner & Kissel, 1999). Due to the lower melting point of P(CPB:SA)50:50, it can be considered a satisfactory choice of polymer for double emulsion preparations rather than spray drying.

Based on the study of Eldridge *et al.* (1990), it was assumed that such particles would be particularly suitable for use in oral immunisation due to the high hydrophobicity of P(CPB:SA). The fact that the increased BSA release was achieved in more alkaline medium and the hydrophobicity of the polymer make this micropsheres ideal for targeted delivery to the small intestine. Before reaching the Peyer's patches the protein antigen is thought to be protected from the hazardous environment in the stomach and intestines (*e.g.* low pH and digestive enzymes) by the polymer matrix surrounding it (Chiba *et al.*, 1997). Polyanhydrides may be particularly well suited for oral delivery because they are known to adhere to the mucosal lining in the intestine, thereby increasing their residence time in GI tract (Chiba *et al.*, 1997).

5. OUTLOOK FOR FUTURE STUDIES

Polymers based on poorly soluble long chain diacids (7 –10 methylenes), lost 20 % of their weight within 48 h, while the short aliphatic chain polymers (4 – 6 methylenes) lost 70 % of their weight during the same period (Domb & Nudelman, 1995). In the short aliphatic chain polymers, 70 % of anhydride bonds were converted to acids within 48 h, while only 20 % conversion was obtained for the long chain polymers. After one week, the long chain polymers still possessed 50-70 % anhydride bonds, while the short chain polymers underwent total hydrolysis. It is clearly seen that the more soluble the monomer, the faster the degradation and erosion of the respective polymer. So finding the weight change during the degradation is very important to understand the correlation between polymer degradation and microspheres erosion. In this study, degradation and erosion of microspheres was investigated according to the change in Mw, anhydride bond and morphology, and each of these methods alone gives only partial and limited information on the total erosion process. In combination with the investigation of the weight change during erosion, quantitative data could be obtained to understand the process in future studies.

From the IR spectra before and after degradation, it is found that the anhydride bonds were almost completely broken for P(CPB:SA) 20:80 with slightly more anhydride bonds left for P(CPB:SA) 50:50. However, the morphology of microspheres under SEM showed no obvious changes for microspheres prepared by double emulsion and spray drying, which could be explained by the different microstructures involved. The changes in porosity and crystallinity during erosion could be investigated by SEM and polarized light microscopy. Another reason for the slow erosion could be that the

solubility of SA decreases after polymerisation with CPB. Further research could concentrate on the release of monomers during degradation and erosion.

Process parameters for spray drying such as temperature, air-flow, and spraying rate need to be optimised in order to improve the yield. A spray-air movement through the dryer chamber should be created which prevents the deposition of partially dried product on the walls. Further work is necessary to improve yields and reduce the loss of polymer. Product deposition on the chamber walls can result from semi-wet particles or from sticky deposits caused by the nature of the polymer. The temperature in the drying chamber had to be kept below the softening temperature of the polymer and the spray drying conditions could be investigated in the future study.

The future research on microspheres will focus on a more precise characterisation of the microspheres properties that may affect the extent of uptake from GI tract, including the particle size, surface charge, hydrophobicity, and attachment of ligands.

6. REFERENCES

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