SYNTHESIS AND DEGRADATION OF BIODEGRADABLE POLYANHYDRIDE MICROSPHERES FOR ENCAPSULATION OF PROTEINS

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DEDICATION

I would like to dedicate this thesis to my family, especially my parents, with love and thanks for all their support, guidance and encouragement.

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The University of Aston in Birmingham

Synthesis and degradation of biodegradable polyanhydride microspheres for encapsulation of proteins.

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SUMMARY

A new biodegradable polyanhydride copolymer, poly[bis(p-carboxyphenoxy)butane-sebacic acid] [poly(CPB:SA)], was synthesised using modified melt-polycondensation. Microspheres made using the new polyanhydride copolymers were prepared using a modified double emulsion, solvent evaporation technique using a model protein, bovine serum albumin (BSA) at a 10% w/w theoretical load. The degradation studies of the polymers and microspheres were carried out in PBS, with shaking, at 37°C and monitored using gel permeation chromatography (GPC), IR spectroscopy and scanning electron microscopy (SEM). Water penetration and anhydride bond cleavage (polymer degradation) occurred rapidly (< 5 days) compared to the time scale of overall microsphere erosion (weeks to months) with different polymer compositions. Subsequent to bond cleavage, the ultimate erosion of the microsphere and release of entrapped BSA was due mainly to the slow dissolution of the individual hydrophobic monomers (CPB and SA) from the microsphere surface. This surface erosion mechanism leads to predictable drug release rates which may be appropriate for the delivery of many protein therapeutics, especially vaccine antigens. Protein release rates could be adjusted by changing monomer composition ratios. Due to the fast degradation of anhydride bonds relative to microsphere erosion, initial polymer molecular weight did not have a significant effect on macromolecule release rates. Instead, protein release rates from polymers of identical composition could be varied by changing the amount of protein encapsulated.

KEYWORDS: Polyanhydride; Microsphere; Biodegradable; Protein delivery.

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Chapter 1

Introduction

1.1 Problems in Protein Therapeutics and Delivery

With the recent development of recombinant DNA technology, large quantities of proteins with pharmacological activity are produced, and development of protein therapeutics has become a long-term focus of the biotechnology industry. Currently, there are large numbers of proteins which are marketed or are undergoing clinical testing, including monoclonal antibodies, growth factors, cytokines, soluble receptors, hormones, and proteins for treating and preventing a variety of infectious diseases. Because many of the cellular functions are carried out by proteins, and because the discovery of new proteins is increasing, it is possible that protein therapeutics will be developed rapidly, and it is possible that protein drugs will eventually constitute a significant part of the pharmacopoeia.

Although proteins have many attractive properties, and many protein therapeutics have been approved or are in advanced clinical testing, the development of more advanced delivery systems for this kind of therapeutic agents is still slow. Proteins also have the following disadvantages that may limit their widespread use and acceptance by patients and physicians:

(a) short *in vivo* half-lives

rapid plasma clearance

(b) physical and chemical instability

denaturation

deactivation

immunogenicity

(c) low oral bioavailability

susceptibility to proteolytic enzymes

(d) high molecular weight

Although oral is the most convenient route for the systemic delivery of pharmaceuticals, because protein and peptide drugs have the above chemical and physical properties, delivery of large molecular weight protein and peptide drugs orally has not been widely successful. Proteins are susceptible to hydrolysis, affected by gastric pH levels and can be degraded in the small intestine. This is because of the protein's susceptibility to breakdown by gastric acid and the proteolytic enzymes in the gastrointestinal tract. Bioavailability *via* this route is poor for molecules with molecular mass greater than several hundred daltons. Because high-molecular-weight substances like peptides and proteins do not easily cross the intestinal mucosa, therefore, the oral bioavailabilities of most peptides and proteins are very low.

Other routes of administration may be used to overcome these problems and a number of alternative routes such as nasal, rectal and transdermal are developing, but these routes have also a low and variable bioavailability and none of these offers a general solution for all proteins.

Consequently, for systemic delivery of peptide and protein drugs, parenteral administration is currently almost universally required in order to achieve consistent therapeutic activities.

Of the parenteral routes, intravenous (i.v.) administration and subcutaneous administration are usually efficient to deliver protein and peptide drugs to the systemic circulation. For example,

optimal blood levels of γ-globulin can be achieved by the intravenous route (Buckley, 1982), insulin can be efficiently administered by subcutaneous injections (Nora *et al.*, 1964; Koivisto and Felig, 1978).

While most peptide and protein drugs can be efficiently delivered to the systemic circulation by parenteral injections, because of rapid plasma clearance mechanisms *in vivo*, the therapeutic applications of many of these proteins are limited. In order to maintain the drug concentration at a therapeutic level, multiple or high dosing is often required. Such frequent injections are not only unpleasant to the patients, but also can lead to usual complications such as thrombophlebitis and tissue necrosis.

During attempts to reduce multiple or high dosing, a variety of technologies have been examined for the sustained and controlled release of drugs *in vivo*. For example, the drug substances can be encapsulated in some manner such as liposomes for prolonging the release of drug. A second example is to increase the biological half-life of the protein by either application of a soluble protein-carrier conjugate or by the covalent modification of the protein to decrease the susceptibility and / or clearance rates.

An attractive way to overcome these problems would be to create a dosage form that delivers the protein continuously and maintains the concentration within the therapeutic window for an extended period. Such formulations would offer numerous advantages including protection of the protein from degradation or elimination; the ability to deliver the protein locally to a particular

site or body compartment, thereby lowering overall systemic exposure; and increased patient comfort, convenience, and compliance.

Vaccination as an important part of protein therapeutics has significantly reduced the incidence of many diseases worldwide. It is the most effective method for human beings to prevent infectious diseases today. For example, if vaccines which are currently developed to prevent diarrhoeal disease, acute respiratory infections and malaria are used successfully, it is estimated that infant and childhood deaths could be reduced by up to 50% by immunisation (Chiba *et al.*, 1997).

Currently, children have been immunised against a variety of diseases for several years. Many of these vaccines require repeated immunisations to achieve complete protection. Unfortunately, children in developing countries and children in rural areas often do not receive the required booster immunisations. The main reason for this failure to receive complete immunisation includes poor or limited access to medical care, lack of patient education regarding the importance of booster vaccinations, cultural or societal misconceptions about vaccines and inconvenience of repeated immunisations.

A single-administration vaccine which can offer the equivalent of repeated administrations automatically may provide the best solution to this growing problem. Several different approaches have been used to achieve controlled release of a vaccine antigen—liposomes, unilamellar vesicles, emulsions and polymers have all been tested as single-immunisation vehicles. Because of the instability of liposomes, lipid vesicles and emulsions *in vivo*, these

systems yield only an initial exposure to the antigen, and a booster immunisation is usually required to achieve protection against disease.

Polymer systems for controlled-release vaccine delivery have been studied for over 20 years and have shown promise as single-administration vaccines. One approach is the development of injectable controlled-release microsphere polymeric formulations containing the vaccine antigen that is released as a pulse 1-6 months after injection. The time of the pulse is dependent on the rate of polymer degradation, which is effected by the polymer's composition. This controlled-release system may provide complete and long-lasting protection against disease after a single administration. Other advantages of this 'perfect' vaccine technology would include:

(1) stability at high temperatures, to reduce the need for refrigeration;

(2) low production costs to allow widespread use;

(3) the general applicability of the technology to most vaccines.

1.2 Biodegradable Polymeric Drug Delivery Systems

Biodegradable polymeric drug delivery systems have several advantages compared to conventional drug therapeutics, including improved patient compliance, avoidance of the peaks and valleys of drug plasma levels associated with conventional injections, localised delivery of the drug to a particular body compartment or cell type, thereby lowering the systemic drug level, protection of drugs that are rapidly degraded in the body, and improved drug efficacy. The obvious advantage of biodegradable polymers for drug delivery over non-degradable systems is that they do not have to be removed from the patient after administration.

1.2.1 Potential Advantages of Biodegradable Systems

In addition to the above advantages, the biodegradable systems have several potential advantages compared to non-biodegradable systems:

1. The drug release rate is more dependent on the polymer matrix system which plays an important role in controlling the drug release. The drug release rate is mainly controlled by such parameters like polymer degradation rate and composition. For example, varying copolymer composition can change its degradation rate which determines the release rate of contained drug. Therefore although drug solutes might be trapped in some erosion-controlled systems, a complete release can always be achieved in a biodegradable system after the polymer matrix system is degraded completely.

2. The release rate may be more stable with time compared to conventional drug delivery systems. A biodegradable system may yield constant release even with a simple monolithic device if the matrix degradation can be *via* a constant surface degradation, and drug diffusion is minimal, the release rate will also be constant. However, this means the release can also be erratic if the matrix degradation is unpredictable. For instance, there is the danger of dose dumping if the matrix suddenly disintegrates.

3. Biodegradable systems may be more suitable to the delivery of unstable drugs. This point is particularly important to molecular biology and genetic engineering which lead to many new bio-macromolecules like proteins. For a non-biodegradable matrix, the steps leading to

release are water diffusion into the matrix, dissolution of the drug solutes, and out-diffusion of the solutes. Therefore, the residence time of drug particles existing in solution state is longer for a non-biodegradable matrix. It is conceivable that a fraction of the drug can be decomposed inside the non-biodegradable matrix before it can be released. Some could then aggregate and reprecipitate, clogging the channels for diffusion.

1.2.2 Polymer Degradation Mechanisms

The different mechanisms of biodegradation have been classified into discrete mechanisms by several polymer investigators (Langer, 1990; Heller, 1980; Langer *et al.*, 1981). Understanding these mechanisms is important in the design of a degradable drug delivery system and can have a profound effect on the release kinetics. Figure 1.1 is a schematic representation of the different types of polymer degradation mechanisms. In the first example, there is an unstable (biodegradable) bond in the polymer backbone. Cleavage of the bond converts a water-insoluble polymer into water-soluble, low molecular weight polymer fragments. Hydrolysis of the unstable bond can be induced both chemically and enzymatically. Polymers that degrade by this mechanism include polyesters, polyanhydrides, poly(ortho esters). In the second example, the polymer exists as a cross-linked network, and cleavage of unstable linkages in the cross-links releases soluble polymer fragments. The size of these fragments depends on the density of the hydrolyzable bonds in the cross-linked network. Cross-linked polymers such as calcium alginate degrade by this mechanism. The third example is polymer solubilisation. With this mechanism the polymer itself does not disintegrate and its molecular weight remains essentially unchanged. In the simplest type of solubilisation, water diffuses into the polymers, leading to the formation

of a swollen system which ultimately dissolves. Polymer gels made of poly(ethylene oxide), or poly(vinyl alcohol) (PVA) degrade by this mechanism.

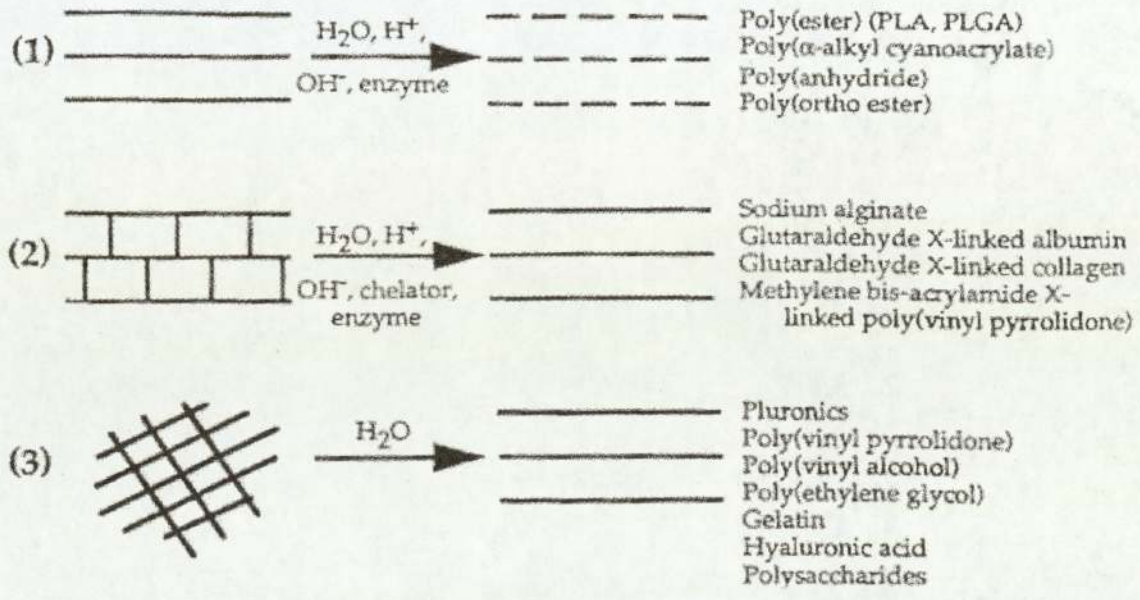


Figure 1.1 Schematic representation of different polymer degradation mechanisms:

(1) Hydrolysis of the polymer backbone may occur *via* acid, base, or enzymatic mechanisms. The degradation byproducts are low molecular weight and are generally water soluble, which allows the contained protein or peptide to be released.

(2) Hydrolysis of a cross-linked polymer network is catalysed *via* acid, base, or metal ion chelator, or enzymatically. Broken cross-links allow protein or peptide release.

(3) Diffusion of water into the polymers, leading to the formation of a swollen system which dissolves. Solubilisation of a polymer matrix allows the out-diffusion of proteins or peptides.

Polymer degradation may also be described in physical terms and may be either homogeneous or heterogeneous. In homogeneous degradation or bulk erosion, hydrolysis of a device occurs at an even rate throughout the polymer matrix. Polymers like polyesters degrade in this way. In heterogeneous degradation or surface erosion, the delivery system degrades only at its surface. Polymers degrading by this mechanism include polyanhydrides, and the drug release kinetics from this type of system are more predictable.

1.2.3 Degradable Polymer Erosion

Polymer erosion is started by degradation. Degradation produces oligomers and monomers which leave the polymer bulk by releasing into the degradation medium. Among the processes that finally lead to erosion, there are two that compete with each other and define how degradable polymers erode: the diffusion of water into the polymer and the degradation of polymer bonds (Heller, 1986). The ratio of the velocity of both processes determines how degradable polymers erode. If the diffusion of water into the polymer is faster than degradation, as shown in Fig. 1.2, the polymer may swell prior to erosion (case a) which may be a major factor in the erosion of a degradable polymer (Lee, 1992). If polymer degradation is faster than water uptake, polymer swelling may be less important (case b). Depending on which process is the major factor, polymer erosion can be classified as a surface- (or heterogeneous-) or a bulk- (or homogenous) erosion (Langer *et al.*, 1983). In the first case, degradation is faster than water diffusion. Therefore, thus degradation and erosion are surface phenomena. In the case of bulk erosion, water uptake is fast and degradation and erosion affects all the polymer bulk. This distinction has been observed from the appearance of the polymer matrix during erosion. Fig. 1.3 shows cross-

sections of surface eroding, and bulk eroding polymer matrices. Because of the loss of material from the surface, the surface erosion polymer shrinks in its dimensions. The advantage of surface erosion is the predictability of erosion (Göpferich *et al.*, 1995) as well as the predictability of drug release, which is related to the erosion rate of the surface eroding polymer (Langer *et al.*, 1995). Polyanhydrides have been reported to display surface erosion (Mathiowitz *et al.*, 1993). The matrix geometry of bulk erosion polymers usually does not change for a substantial length of time (Langer *et al.*, 1990). Erosion and degradation are hard to predict for these polymers. Examples of bulk-erosion polymers are poly(lactic acid) and poly(lactic-co-glycolic acid). Surface and bulk erosion are extreme cases and erosions of most polymers are determined by a combination of these (Mathiowitz *et al.*, 1993).

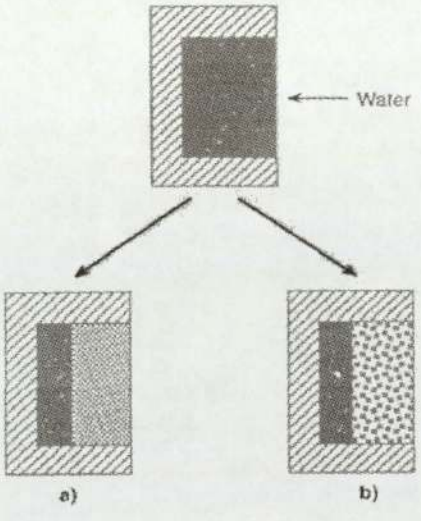


Figure 1.2. Illustration of water diffusion and degradation in degradable polymers:
a) Water diffusion is faster than degradation.
b) Degradation is faster than water diffusion into the polymer.

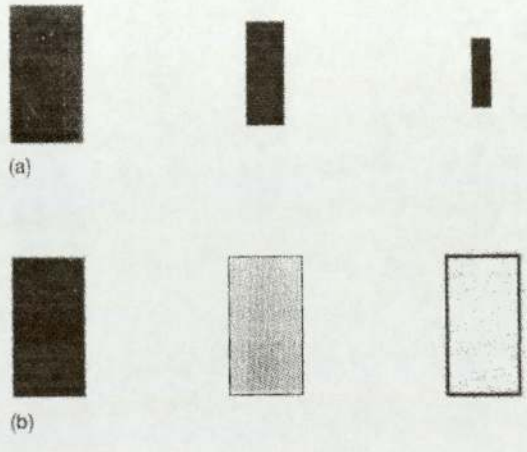


Figure 1.3. Schematic illustration of surface and bulk erosion
(a) surface erosion
(b) bulk erosion

If the drug release is intended to be erosion-controlled, the relationship between erosion velocity and other important steps have to be considered. It is the relationship between three processes that determines how drug release is controlled: water diffusion, polymer swelling and polymer erosion. Fig. 1.4 shows the three general cases that can be distinguished: if erosion is slower than the diffusion process, the polymer will control drug release by diffusion. If the diffusion of water into the polymer is faster than erosion but slower than polymer swelling, the polymer will control release by swelling. Only if erosion is the fastest process, may drug release be erosion controlled. Therefore, not all degradable polymers control drug release through erosion only. Degradable hydrogels may be a good example because water diffusion is often substantially faster than polymer erosion, making drug release diffusion- or swelling- but not erosion-controlled. Release kinetics can become very complicated and hard to predict. For many polymers, the three processes have similar velocities and each of them affects drug release. Many systems made of degradable polymers are not truly erosion-controlled drug delivery systems, and their only advantage is the disappearance of the drug carrier through degradation.

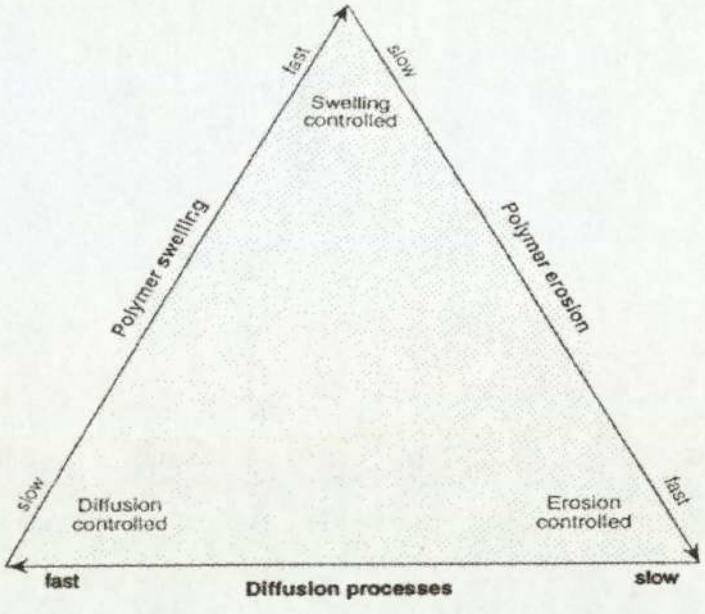


Figure 1.4 Possible mechanisms of drug release from degradable polymers

1.2.4 The Application of Degradable Polymers for Drug Delivery

There are many potential applications for degradable polymers. In drug delivery, degradable polymers are used to manufacture controlled-release systems as carriers. For example, parenteral depot formulations for antigens have met with increasing interest because of several aspects, such as protection of sensitive proteins from degradation, prolonged or modified release of the antigen, and pulsatile release patterns. In addition to these, they might provide a promising platform for the development of single-shot vaccines. From a formulation point of view the systems described in the following sections can be distinguished:

1.2.4.1 Implants

Implants may be rod- or disk-shaped devices for subcutaneous application. These devices can be manufactured by compression molding, injection molding and screw extrusion. Sizes of 1-1.5 mm in diameter and 1-2 cm in length can be applied subcutaneously using a trocar. Disk- or tablet-shaped implants require a small surgical incision for application.

Implants have the advantage that they can be designed and manufactured easily and much more uniformly compared to other dosage forms made of biodegradable polymers such as microspheres. A major disadvantage is the need for surgery to apply the devices or at least a painful injection for their application, and their sizes may not be tolerated when they are applied subcutaneously. In addition, there are also safety concerns when they carry large amounts of drug. Autocatalytically accelarated degradation may increase the release rates that could induce toxic blood levels.

Despite these disadvantages, implants are useful systems for long-term systemic and local drug delivery. Zoladex® as one of the marketed implant systems made of poly(lactic-acid) is used for the treatment of prostate cancer using LH-RH agonists. Local drug delivery is another promising field for the use of implants. The major advantage is a decreased risk of side effects associated with the high local drug concentrations that are attained. For example, poly(D,L lactic-acid)-based (Stricker *et al.*, 1991) and polyanhydride-based (Brem *et al.*, 1993) implants have been used effectively for local tumour therapy. The local chemotherapy of brain tumours showed that high local concentrations of cytostatics can be attained. This is impossible to achieve *via*

systemic therapy due to the low permeability of the blood brain barrier to cytostatics (Tamargo *et al.*, 1992). However, the systemic toxicity of the cytostatics is significantly decreased compared to systemic chemotherapy (Olivi *et al.*, 1991). Areas of current and future research are the design of injectable implants made of degradable polymers for sensitive drugs such as proteins by simple mixing and can be injected *via* syringes (Heller *et al.*, 1990; Heller, 1994).

Although the design and manufacture of implants are very easy and may be applied to a wide range of biodegradable polymers, the high temperature required by melt-processing techniques becomes a major limiting factor for many sensitive drugs. For example, lactide-glycolide copolymers require temperatures of close to 80-100°C for screw extrusion and 140-180°C for injection molding. Many antigens are unstable at such high temperatures; therefore, it is necessary to develop other delivery systems

1.2.4.2 Microparticles

A host of microencapsulation techniques has been developed in the past 60-70 years. According to definition, the size range of microparticles is between 1 and 1000 μm. The drug substance in gaseous, liquid, or solid form is encapsulated in a polymeric material, Fig. 1.5 shows two types of structures of microparticles, reservoir type and matrix type. Fig. 1.5A is a true microcapsule containing a core of the drug substance surrounded by a coat of polymer. Microspheres that contain the drug dispersed or dissolved in a polymeric matrix were shown in Fig. 1.5B and Fig. 1.5C. The term microcapsule should be used to describe reservoir type devices, whereas microspheres are matrix-type microparticles.

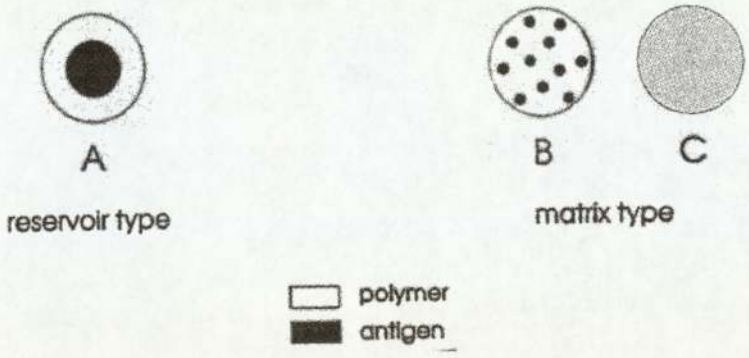


Figure 1.5 Typical structures of microparticles

In recent years, microspheres have emerged as one of the most popular controlled release dosage forms for parenteral use. Compared to large implants, microspheres have advantages, including injectability and allowing the incorporation of sensitive drugs such as proteins and peptides (Vrancken, 1970). Now there are two microsphere-based products for the treatment of prostate cancer on the market, and they are Enantone® and Decapeptyl Depot®. Both of them are made of poly(lactic-co-glycolic acid).

1.2.4.3 Nanoparticles

Nanoparticles have a size range between 1 and 1000 nm, which allows them to be used intravenously. Like microparticles, nanoparticles also have two types of structure, a matrix-type nanoparticle with the drug substance dispersed or dissolved in the polymeric matrix and nanocapsules with a reservoir-type structure, consisting of a solid shell and an inner liquid core (Allémann *et al.*, 1993).

The major goal of nanoparticles in drug delivery applications is to modify the pharmacokinetics and biodistribution of incorporated drugs, for example, to increase the drug half-life and to improve drug targeting on specific body sites or cell types. Nanospheres were originally made of non-degradable polymers such as polystyrene (Shahar *et al.*, 1986). To overcome the problem of accumulating polymer degradation products in the body, degradable polymers were used for the manufacture of nanoparticles since the early 1980s. Since then, materials such as poly(lactic acid) (Gurny *et al.*, 1981), and poly(lactic-co-glycolic acid) have been used extensively. The major shortcoming of nanoparticles is their rapid plasma clearance, which is non-specific and characteristic for all colloidal drug carriers.

1.2.5 Marketed Protein Drug Delivery Systems made from PLGA Copolymers

Three protein drug delivery systems made from PLGA copolymers that have been approved and are available as marketed products. They are Lupron Depot, Zoladex, and Decapeptyl ^{SR}, and each of them releases peptide analogues of Luteinizing Hormone-Releasing Hormone (LHRH).

Zoladex is a cylindrical implant approximately 1 mm in diameter and 3-6 mm in length. The device is made from a 50:50 PLGA copolymer and contains 3.6 mg of drug which is homogeneously dispersed throughout the matrix. After subdermal injection in the abdominal wall, the drug is released over 28 days. Release of the peptide is initially controlled by a dissolution/diffusion mechanism from the surface of the device. At later times, the degradation of the polymer leads to the generation of microporosity and increased water uptake by the system, which ultimately results in further release of the drug.

Lupron Depot is a biodegradable PLGA microsphere delivery system, and it is also designed to release the LHRH analog, leuprolide acetate, over 1 month. The microspheres are prepared by a double emulsion technique using a PLGA (75:25) copolymer with a molecular weight of approximately 14000. Gelatin is added to the inner water phase of the system together with the peptide in order to increase the viscosity in the inner phase of the emulsion. This increased viscosity results in complete incorporation of the drug in the microspheres, and loadings of 10-20% by weight can be obtained. The release of peptide from this system is described as biphasic. After a small initial burst, a diffusion-controlled release occurs followed by polymer degradation and further erosion-controlled release. This product is administered by subcutaneous or intramuscular injection.

In 1986, Decapeptyl® was first developed and introduced to the market as an injectable one-month controlled release formulation for LHRH. Today, Decapeptyl® is prepared *via* a microgranulation technique. The microgranules are obtained by cryogenic grinding of extruded polymer containing the dispersed peptide during a process that does not involve the use of organic solvents. This process results in particles of amorphous shape, which are sieved. The microgranules are then lyophilised into individual vials. For administration, the microgranules of Decapepty® are suspended in an aqueous injection vehicle and injected intramuscularly. Decapepty® is made from PLGA, which contains triptorelin as pamoate, an insoluble salt of the LHRH analog. The rate of release of the peptide from the microgranules is controlled mainly by the speed of the biodegradation of the polymer matrix. After injection, the sex hormone testosterone is measured, an initial plasma peak is observed during 3 hours, then triptorelin plasma levels fall below the castration levels and remain below this level for at least 4 weeks.

1.2.6 Biodegradable Microspheres for Vaccines

1.2.6.1 The Use of PLA and PLGA

A key factor in the design of injectable vaccine delivery systems is the choice of an appropriate polymer. Biodegradable polymers are preferred because surgical removal of the spent device is unnecessary. The role of the polymeric material in the design of parenteral delivery systems exceeds that of an inert excipient. This integral component influences not only the biodegradation kinetics, but also the mode and rate of antigen release, toxicity, and tissue compatibility, as well as antigen stability under *in vitro* and *in vivo* conditions. Mechanical and physiochemical properties also are important to selection of an appropriate microencapsulation technology.

A variety of synthetic and natural polymers have been studied over the past 30 years (Chasin *et al.*, 1990), and polyesters have been used widely. That is because thermoplastic polyesters of poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactic-co-glycolic acid) (PLGA) have many advantages, including an excellent record for biocompatibility and lack of toxicity. These materials have been used as synthetic resorbable sutures, and have been approved by regulatory authorities as polymeric excipients for microparticles. These polymers are used in drug delivery because they are regarded as less costly and more straightforward than with new polymers, not requiring new registration.

Homo- and copolymers of lactic acid and glycolic acid are synthesised by a ring-opening polymerisation of the cyclic dimers, lactide and glycolide (Lewis, 1990). Direct condensation of lactic acid and glycolic acid yields homo- or copolymers with low molecular weight (Mw) in the

range of 10-15 kDa (Yamamoto *et al.*, 1986). Because of its additional methyl group, PLA is more hydrophobic than PGA. Both PLA and PLGA are soluble in organic solvents, such as chloroform, dichloromethane, and acetone. The degradation rate and drug release rate are mainly dependent on copolymer composition and molecular weight. Copolymerisation of lactic acid and glycolic acid is a very effective method to manipulate biodegradation and antigen release of microspheres by controlling copolymer composition, and polymer molecular weight (Lewis, 1990).

There is an unstable (biodegradable) bond, ester bond, in the polyester backbone. Degradation of polyesters occurs by a random, non-enzymatic hydrolytic cleavage of ester linkages. The polyesters have been reported to display bulk erosion. The degradation products of PLGA, lactic acid and glycolic acid, are physiologically occurring metabolites.

Rate of hydrolysis depends mainly on comonomer ratio and molecular weight of PLGA. As shown in Table 1.1, many investigators have studied the degradation kinetics of polyesters both under *in vitro* and *in vivo* conditions (Miller *et al.*, 1977; Visscher *et al.*, 1985). The half-life of PLGA with a comonomer composition of 50% mol LA and 50% mol GA, frequently abbreviated in the literatures as PLGA (50:50), is about 14 days, leading to a complete resorption of the microspheres within 50-60 days (Lewis, 1990). A shift of comonomer ratio toward either component leads to a significant decrease in biodegradation rate. The well-known biodegradation and biocompatibility properties of PLGA have favoured this type of biomaterial considerably and they are used widely. On the other hand, PLGA also has some inherent shortcomings. It is quite hydrophobic compared with most of the antigens to be microencapsulated. A lack of antigen-

polymer compatibility may lead to stability problems of the antigen during storage or under *in vivo* release conditions. Because both hydration and degradation of PLGA are key factors for the release of the antigen during bioerosion phase, the proteins need to be sufficiently stable at low pH over an extended period (1-12 months) at body temperature. Very little information on antigen-polymer compatibility and stability is now available from the literature. In addition, polyesters degrade by bulk erosion, so the antigen release from polyester systems is unpredictable.

Polymer	Approximate Time for Biodegradation (months)
Poly(L-lactide)	18-24
Poly(glycolide)	12-16
Poly(lactide-*co*-glycolide) 85:15	5
Poly(lactide-*co*-glycolide) 50:50	2

Table 1.1 *In Vivo* Biodegradation Time of Poly(lactide-*co*-glycolide)

Source: Adapted from Lewis, (1990).

1.2.6.2 Antigen Release from Microspheres

The release of macromolecules from biodegradable microspheres is influenced both by the structure, or micromorphology of the microparticles and properties of the biodegradable polymer itself. True microcapsules consist of a solid or liquid core and a biodegradable coating. The

antigen is incorporated in the inner core. The biodegradable coating controls release by an osmotically driven burst mechanism. The protein could not permeate the biodegradable coating of the microcapsules, but water could slowly diffuse into the core, creating sufficient osmotic pressure to rupture the coating. The release of the antigen would follow instantaneously, leading to a pulsatile *in vitro* and *in vivo* release profile, as outlined in Fig. 1.6. Therefore, reservoir microcapsules might be able to mimic conventional injection schemes for vaccines, which require two to three separate injections.

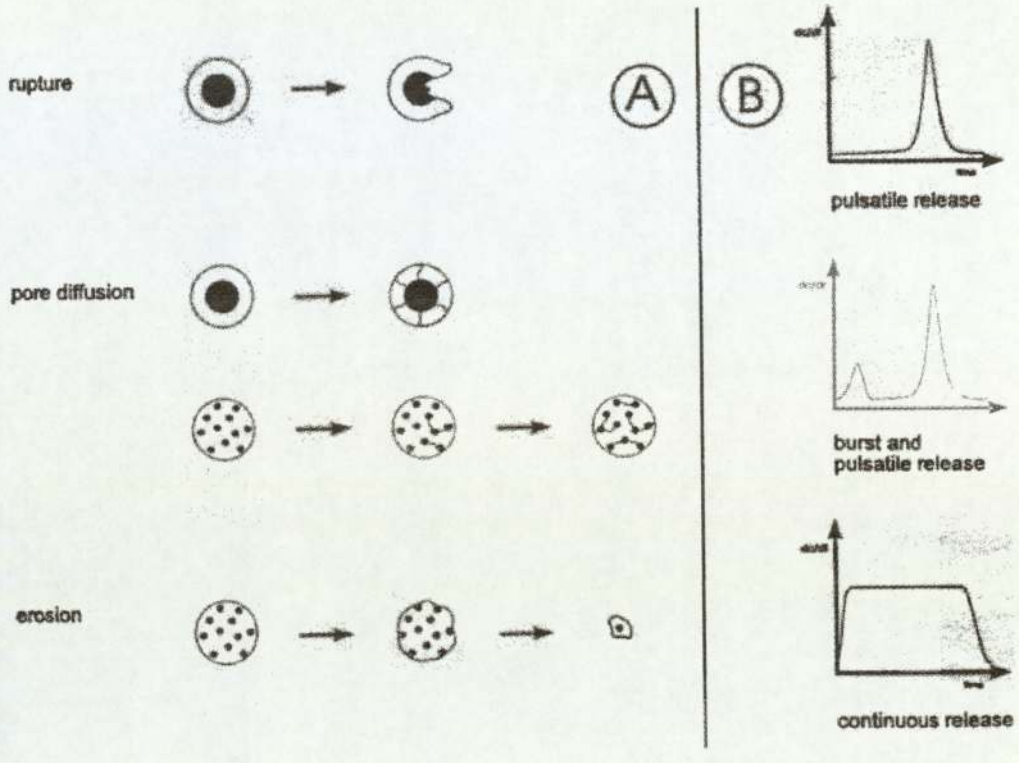


Figure 1.6 Release of antigens from microspheres:

(A) mechanisms (B) release profiles

This type of microparticle has not yet been realised. Most of the antigen delivery microparticles exist as matrix-type microspheres. The antigen is dispersed in the polymeric matrix. The proteins are insoluble in the polymeric matrix, and the macromolecules are released by both pore diffusion and polymer erosion mechanisms. 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. Through the channel, a defined amount of antigen is released in the initial drug burst phase. This burst effect is controlled mainly by three factors: the

protein/polymer ratio, the particle size of the dispersed protein, and the particle size of the microspheres. With the continuous penetration of water, a network of water-filled pores is created. Water begins to diffuse in the direction of the microparticulate core, the dispersed protein particles are dissolved and released. Hence, this process is named as pore-diffusion mechanism (Bodmer *et al.*, 1992). PLGA microspheres release macromolecules by this mechanism in the initial phase. Additional factors, such as swelling phenomena and osmotic effects have to be considered to describe the release behavior of PLGA microspheres within the first 3-10 days. The *in vitro* and *in vivo* release rate decreases considerably, reaching very low levels (see Fig. 1.6). When the polymer degradation has reached a stage of rapid mass loss, protein release is more dependent on the composition and molecular weight of the PLGA. This phase is controlled by the degradation of the polymer, leading to a degradation or erosion of the matrix. The erosion may occur by hydrolysis of the polymer in the bulk of the microspheres, as observed with biodegradable polyesters of the PLGA type. Physicochemical properties of protein and polymer, as well as the drug loading, will determine the length and depth of the "valley" between the initial drug burst and the antigen release as a consequence of matrix erosion. Continuous release of macromolecules from PLGA microspheres (see Fig. 1.6) requires combination of both the pore-diffusion and polymer-erosion mechanisms. For surface-erosion controlled polyanhydride microspheres, the degradation of polymer is faster than the diffusion of water into the polymer matrix. The degradation and erosion of polyanhydride microspheres begins at the surface of microspheres. With the continuous degradation and erosion, dispersed protein from surface to centre is released. A continuous and stable release profile can be obtained, as can be seen from Fig. 1.6. Theoretically, continuous antigen release for an extended period with a single administration can be achieved automatically by using this type of microspheres.

1.3 Polyanhydrides

A large number of biodegradable polymers have been investigated for use as drug delivery systems. To maximise control over the release process, it has generally been considered desirable to have a polymeric system which degrades only from the surface. In order to achieve such a heterogeneous degradation, it is essential that the rate of hydrolytic degradation at the surface of the polymeric system is much faster than the rate of water penetration into the bulk of the matrix. Such a feature may also aid in the delivery of water-labile drugs like proteins because it is more difficult for water to interact with these substances until they are released. In order to design a biodegradable system that would erode in a controlled heterogeneous manner without requiring any additives, many investigators have suggested that due to the high lability of the anhydride bond, polyanhydrides may be promising candidates.

1.3.1 Historical and Present Development of Polyanhydrides

The earliest report on the synthesis of polyanhydrides was by Bucher and Slade in 1909. Years later, in 1930s Hill and Carothers had synthesised polymers based on aliphatic diacid monomers for textile applications. During the 1950s and 1960s, Conix and Yoda synthesised over a 100 new polyanhydrides based on aromatic and heterocyclic diacid monomers. Polyanhydrides have recently been reviewed by Domb *et al.*, (1997). In 1980, Langer proposed the use of polyanhydrides as biodegradable carriers for controlled drug delivery systems (Rosen, 1983). As a result, there are two implantable devices for human use (i.e. Gliadel™ implant for the treatment of brain tumors and Septacin™ implant for treating chronic bone infections) that had been marketed rapidly (Brem *et al.*, 1995; Domb *et al.*, 1994).

Since the discovery of polyanhydrides in 1909, hundreds of polymer structures have been reported. A representative list of polymers is shown in Table 1.2.

Today, polyanhydrides can be regarded as "designer polymers" for many reasons. They can be synthesised from a wide range of monomers. They allow control of degradation rates and water uptake (Domb *et al.*, 1993), can be manufactured with a branched structure (Maniar *et al.*, 1990), or they may be cross-linked (Domb *et al.*, 1991). Probably, their most important advantage is their biocompatibility (Brem *et al.*, 1989; Brem *et al.*, 1992) in combination with excellent drug-release control (Ron *et al.*, 1993).

Compared with the relatively short period during which they have been synthesised as drug carriers, polyanhydrides have been very successful. At present, p(CPP-SA) polymers are used for the treatment of brain cancer in humans after promising clinical testing (Brem *et al.*, 1993). The p(FAD-SA) polymers are being evaluated for the same type of therapy, with microspheres because of their injectability (Painbeni *et al.*, 1994).

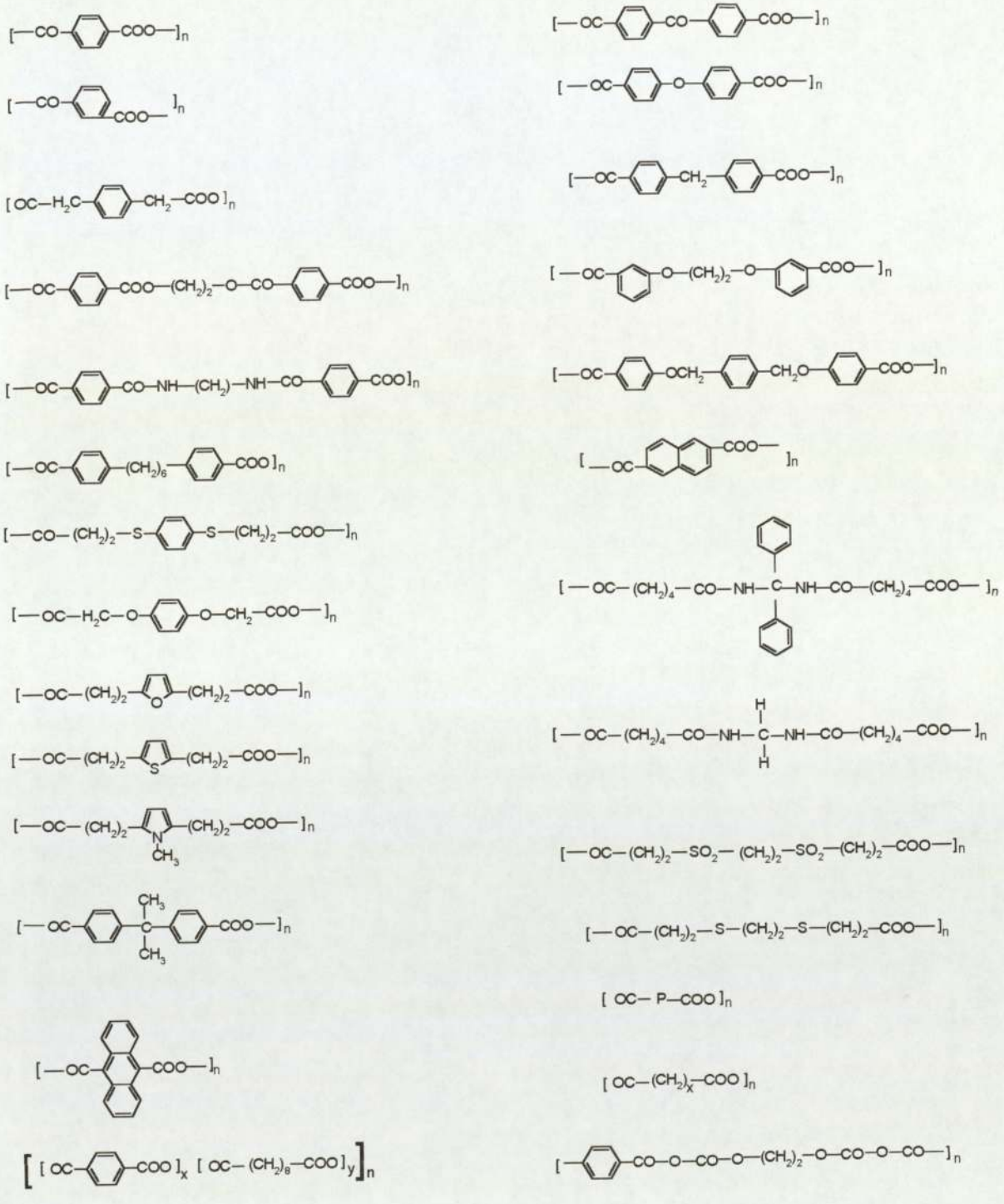


Table 1.2 Representative polyanhydrides synthesised during the years 1909-1980

1.3.2 The Characteristics of Polyanhydride Erosion

An understanding of polyanhydride degradation and erosion mechanisms is essential to understand the properties of degradation and erosion of polyanhydride microspheres.

1.3.2.1 The Definitions of Degradation and Erosion

The erosion of degradable polymers is a complicated process, including various reactions and transport processes. Erosion starts with the intrusion of water into the polymer bulk and triggers degradation. Degradation is the most important part of erosion and is the chain scission process that breaks polymer chains down to oligomers and finally into monomers. Through degradation, oligomers and monomers are created that finally diffuse to the polymer surface, where they are released from the polymer bulk to the release medium. The term "biodegradable" is used for materials, where degradation is mediated at least partially by a biological system (Vert *et al.*, 1992). Degradation leads finally to erosion, which is the process of material loss from the polymer bulk. Such material can be monomers, oligomers, parts of the polymer backbone or even parts of the polymer bulk. The term "bioerodible" indicates again that a biological system is involved in the process. With such definitions, degradation is part of the erosion process. Among the many phenomena involved in erosion, such as water uptake and mass loss, degradation is probably the most important one. Erosion is the sum of all these processes that finally lead to the loss of mass from the polymer bulk.

Despite the clear definition, of the terms "degradable" and "non-degradable", the actual phenomena can not be easily distinguished. Actually, all polymers degrade. Only the time they

require for degradation is different. For polyanhydrides, only several hours are required, however, many years for polyamides (Park *et al.*, 1993). It is the time-scale that is used to measure degradation to make us distinguish between degradable and non-degradable polymers (Reiner, 1964). Besides our human lifetime, the time-scale of the application can be used as a measurement to distinguish degradable from non-degradable polymers. Usually a polymer is described as degradable when the time-scale of degradation has the same order of magnitude as the time-scale of the application.

1.3.2.2 The Importance of Erosion for Drug Release

Polyanhydrides differ from other polymers by the reactivity of the anhydride bond (Park *et al.*, 1993). The half-lives of the typical functional groups for degradable polymers are represented in Table. 1.3. Carboxylic acid anhydrides are the most reactive bonds, which makes polyanhydrides fast-degrading polymers.

Polymer class	Hydrolysis rate
Polyanhydride	0.1 h
Polyketal	3 h
Poly(*ortho*-ester)	4 h
Polyacetal	0.8 yr
Polyester	3.3 yr
Polyurea	33 yr
Polycarbonate	42000 yr
Polyurethane	42000 yr
Polyamide	83000 yr

Table 1.3 Half-Lives of Degradable Polymers

Source: Adapted from Park *et al.*, (1993).

The fast degradation of polyanhydrides is determined by the erosion mechanism. For degradable polymers, two different erosion mechanisms have been reported: surface or heterogeneous, and bulk or homogeneous erosion (Langer *et al.*, 1983). The difference is illustrated in Fig. 1.3 (see section 1.2.3). In the surface erosion 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. In the bulk erosion polymers, in contrast, intrusion of water into the bulk throughout their cross-section is faster than polymer degradation. Therefore, erosion is not limited to the polymer surface. Polymers containing reactive functional

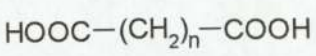
groups like polyanhydrides tend to degrade rapidly and to exhibit surface erosion, whereas polymers containing less reactive functional groups like polyesters tend exhibit bulk erosion.

The erosion mechanism affects the release of drugs from degradable polymers significantly. Drug release has been classified into diffusion-, swelling-, and erosion-controlled release (Langer 1990). The release of drugs from degradable polymer might be controlled by all three mechanisms. The quickest mechanism, however, will dominate the drug release (see Fig. 1.4 in section 1.2.3). 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.

1.3.3 The General Formula for Polyanhydrides

Numerous polyanhydrides have now been synthesised, and most of them were synthesised by melt polycondensation, which is the standard method of synthesis (Leong *et al.*, 1987), although other methods might be also used (Leong *et al.*, 1989). The general formula for polyanhydrides is shown in Fig. 1.7A. The monomers are bifunctional carboxylic acids, which differ in the chemical groups R_1 and R_2, separating the carboxylic acid ends. Polyanhydrides can be synthesised as homopolymers ($R_1=R_2$), or as copolymers ($R_1\neq R_2$). Some monomers that have been used for the manufacture of polyanhydrides are shown in Fig. 1.7B.

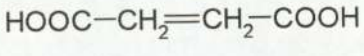
(A)



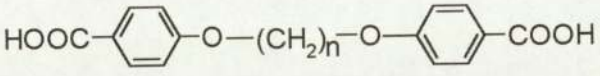
n = 4 adipic acid (AA)

n = 8 sebacic acid (SA)

n = 10 dodecanoic acid (DA)



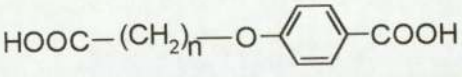
fumaric acid (FA)



n=1 bis(p-carboxyphenoxy)methane (CPM)

n=3 1,3-bis(p-carboxyphenoxy)propane (CPP)

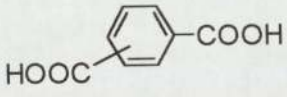
n=6 1,3-bis(p-carboxyphenoxy)hexane (CPH)



n=1 p-carboxyphenoxy acetic acid (CPA)

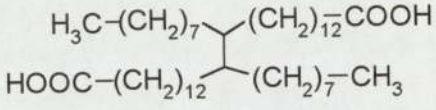
n=4 p-carboxyphenoxy valeric acid (CPV)

n=8 p-carboxyphenoxy octanoic acid



meta: isophtalic acid

para: terephtalic acid



erucic acid dimer (FAD)

(B)

Figure 1.7 (A) General formula of polyanhydride polymers

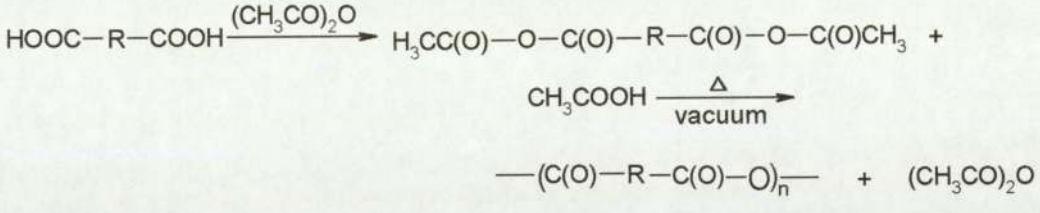
(B) Monomers used for the synthesis of polyanhydrides

1.3.4 The Synthesis of Polyanhydrides

1.3.4.1 Melt- Polycondensation

Briefly, the diacids were refluxed in excess acetic anhydride. The reflux lasted between 30 min and 1 hr. The rest of the solvent was evaporated in a rotary vacuum evaporator, and the residue was recrystallised in a mixed solvent of ethyl ether and petroleum ether. The condensation of the obtained prepolymer was carried out in a glass tube with a side arm equipped with nitrogen at a pressure of 10^{-4} mmHg and at various temperatures (Leong *et al.*, 1987). (see Eq 1.1).

Eq 1.1

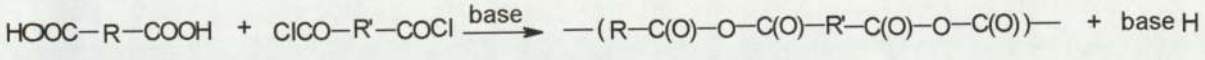


However this method has some limitations. For example, the reversible thermal depolymerisation may limit the highest molecular weight obtainable, and the acetic anhydride reflux may be unsuitable for many heat-sensitive monomers. Milder reaction conditions are therefore desirable.

1.3.4.2 Dehydrochlorination

Polyanhydrides can be synthesised at room temperature by a dehydrochlorination between a diacid chloride and a dicarboxylic acid. For a typical solution polymerisation, 20 mmoles of the diacids and 40 mmoles of base were dissolved in 20 ml of solvent (*e.g.*, dichloromethane or chloroform). While the mixture was stirred, 20 mmoles of the acyl chloride was added in 200 ml of the above solvent. The reaction flask was stoppered by a moisture guard tube containing calcium chloride. After 2h of reaction at room temperature, the mixture was quenched in 1l of petroleum ether under agitation. This would also trap the triethylamine hydrochloride salt. For this reason the yield was only estimated. The polymers can be cleaned by shaking the reaction mixture in 50 ml of cold methanol before quenching (Leong *et al.*, 1987). (see Eq 1.2).

Eq 1.2



It is essentially a Schotten-Baumann condensation, a reaction extensively studied for polyamide, polyester, and polycarbonate synthesis. However, only Yoda *et al.* (Yoda *et al.*, 1959) and Windholz (Windholz, 1965) have used this reaction for obtaining polyanhydrides. Since the carboxylic hydrogen is less reactive than that of an amine alcohol, or mercaptan, the condensation is expected to be less efficient. Nevertheless it was hoped that under optimal conditions the reaction may still be able to yield useful polyanhydrides.

1.3.4.3 Dehydrative Coupling

An alternative synthetic route is considered to synthesise polyanhydrides. By using this route, sensitive monomers do not have to be subjected to the acylation conversion. In a typical reaction, the diacid (10 mmol in 25 ml of solvent) dissolved in the presence of acid acceptor (20 mmol) was added in a single portion to a magnetically stirred solution containing the coupling agent (5 mmol in 5 ml of solvent). The reaction was conducted at room temperature in a stoppered flask. The following procedures were used: W1, the resulting suspension was filtered, the solid was washed with 50 ml of chloroform, and the filtrate was vacuum-evaporated; W2, the reaction mixture was directly quenched into petroleum ether; W3, the reaction mixture was extracted with 25 ml of cold dilute HCl, and the organic phase was then quenched into petroleum ether (Leong *et al.*, 1987). (see Eq 1.3).

Eq 1.3

$$ClP(O)R_2 + -R'-COOH \xrightarrow{base}$$

$$-R'-C(O)-O-P(O)R_2 + base\cdot HCl \xrightarrow{+\,-R'-COOH\cdot base}$$

$$-(C(O)-R'-C(O)-O)_n- + base\cdot HOP(O)R_2$$

The reaction has been used by Cabre-Castellvi (Cabre-Castellvi *et al.*, 1981) and Mestres (Mestres *et al.*, 1981) for obtaining monomeric anhydrides.

1.3.5 Safety Evaluation of Polyanhydrides

For drug delivery systems, it is of great importance to assure that they and their degradation products are safe and biocompatible. The biocompatibility and safety of polyanhydrides were established following the 1986 guidelines by the Food and Drug Administration (FDA) for testing and evaluating new biomaterials. Several accepted tests to evaluate new biomedical materials were used to assess the safety of polyanhydrides (Leong *et al.*, 1986b; Braun *et al.*, 1982; Laurencin *et al.*, 1990). Among these polyanhydrides, poly[bis (p-carboxyphenoxy) propane anhydride (PCPP) and its copolymers with sebacic acid, p(CPP-SA), have been tested. As the results of mutation assays evaluation, neither mutagenicity nor cytotoxicity or teratogenicity was associated with the polymers and their degradation products (Rock *et al.*, 1991). The tissue response to these polyanhydrides was studied by subcutaneous implantation in rats and in the cornea of rabbits. The polymers did not induce inflammatory responses in the tissues over a six-week implantation period. Histological evaluation indicated relatively minimal tissue irritation without evidence of local or systemic toxicity (Laurencin *et al.*, 1990). Systemic response to the polymer was evaluated by determining blood chemistry and haematological values, and by comprehensive examination of organ tissues. Both methods revealed no significant response to the polymer.

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 tumors, *in vivo* safety evaluations and brain biocompatibility were assessed in rats (Tamargo *et al.*, 1989), rabbits (Brem *et al.*, 1989), and monkeys (Brem *et al.*, 1988). In the rat brain study, the tissue reaction of the polymer (CPP-SA

20:80) was compared to the reaction observed with two standard materials used in surgery, which have been extensively studied namely, Gelfoam® (absorbable gelatin sponge), and Surgicel® (oxidized cellulose absorbable hemostas commonly used in brain surgery). Histological evaluation of the tissue demonstrated a small rim of necrosis around the implant, and a mild to marked cellular inflammatory reaction limited to the area immediately adjacent to the implantation site. The pathological response associated with poly(CPP-SA) copolymer was slightly more pronounced than Surgicel® at the earlier time points, but noticeably less marked than Surgicel® at the later times. The reaction to Gelfoam® was essentially equivalent to that observed in control rats. In a similar brain biocompatibility study carried out in monkeys, no tissue abnormalities were noted either in Computed Tomography (CT) and Magnetic Resonance Imaging (MRI). Furthermore, no abnormalities were observed either in the blood chemistry or haematology evaluations (Brem *et al.*, 1988). There appeared to be no adverse systemic effects due to the implants as assessed by the histological evaluation of tissue tested. Overall, no unexpected or untoward reaction to the treatment was observed. Copolymers of sebacic acid with several aliphatic comonomers such as dimer of erucic acid (FAD), fumaric acid and isophthalic acid were also tested subcutaneously and in the rat brain were found to be biocompatible (Rock *et al.*, 1991).

The hydrolysis and elimination processes of polyanhydrides have been studied using a series of polyanhydrides derived from different linear aliphatic diacids (Domb *et al.*, 1995b). These polymers degrade into their monomer or oligomer units at about the same rate but differ in the water solubility of their degradation products. Polymers based on natural diacids of the general structure -[OOC-$(CH_2)_X$-CO]- where x is between 4 and 12, were implanted subcutaneously in

rats and the elimination of the polymers from the implantation site studied. The *in vitro* hydrolysis of this polymer series was studied by monitoring the weight loss, release of monomer degradation products and the changes in the content of anhydride bonds in the polymer as a function of time. It was observed that, both *in vitro* and *in vivo* the rate of polymer elimination was a function of monomer solubility. The elimination time for polymers based on soluble monomers (x =4-8) was 7-14 days, while the polymers based on monomers with lower solubility (x =10-12) were eliminated only after 8 weeks. All polymers were found to be biocompatible and useful as carriers for drug delivery.

The elimination of the biodegradable polymer poly(CPP-SA) based implant (Gliadel^{TM}), which is currently in clinical use for the treatment of brain cancer, was studied in rabbit and rat brains using radioactive polymer and drug (Domb *et al.*, 1994d, 1995c). The implant was composed of N, N-bis (2-chloroethyl)-N-nitrosourea (BCNU) dispersed in a copolyanhydride matrix of CPP and sebacic acid (SA). Four groups of rabbits were implanted with wafers loaded with BCNU, one in a ^{14}C-SA-labeled polymer, another in a ^{14}C-CPP-labeled polymer, and two groups with ^{14}C-BCNU in a non-labeled polymer, one for BCNU elimination study and one for residual drug study. In the rabbits implanted with the ^{14}C-SA-labeled polymer, approximately 10% of the radioactivity was found in the urine and 2% in the faeces, and about 10% remained in the device seven days after implantation. In contrast, only 4% of the radioactivity associated with the ^{14}C-CPP labeled polymer was found in urine and faeces during this period. However, a drastic increase in the CPP excretion was found after 9 days, and at 21 days during which 64% of the implanted ^{14}C-CPP was recovered in the urine and faeces, and 29% was still in the recovered wafers. Studies with radiolabeled BCNU in rabbit brain revealed that approximately 50% of the

BCNU in the wafers was released in 3 days, and over 95% was released after 6 days in the rabbit brain. Excretion of this polymer after implantation in the rat brain using radiolabeled polymers showed that over 70% of the sebacic acid comonomer was excreted in seven days with about 40% of the sebacic acid metabolised to CO_2 (Domb *et al.*, 1995c).

The elimination of poly(FAD-SA) rods loaded with 0, 10 and 20 weight % of gentamicin sulphate after implantation in the femoral muscle and bone of dogs was studied as part of the preclinical studies for Septacin™ -bone implant (Domb and Amselem, 1994). Most of the polymer implant was gradually eliminated from bone and muscle within 4 to 8 weeks post implantation with the elimination from bone being faster; leading to new bone formation in the implant site without any polymer entrapment. The elimination rate was dependent mainly on the amount of polymer implanted. Gentamicin was released for a period of about 3 weeks with no residual drug detected in the polymer remnants 8 weeks post implantation. In all experiments, no local or systemic toxicity was observed.

1.3.6 Manufacture of Polyanhydride Microspheres

Polyanhydride microspheres have been manufactured by four different methods: solvent evaporation, solvent removal, hot-melt encapsulation, and spray drying (Brunner *et al.*, 1995). In addition, two methods for the manufacture of double-walled microspheres have been developed.

1.3.6.1 Solvent Evaporation

This process has also been termed a double-emulsion technique. As shown in Fig. 1.8, the drug substance in an aqueous solution is emulsified with the non-miscible organic solution of the polymer to form a water-in-oil (W1/O) emulsion. The organic solvent dichloromethane is widely used, and the homogenisation step is carried out by using either high-speed homogenisers, ultrasound, or vortex mixing. This primary (W1/O) emulsion is then rapidly transferred to a vast excess of an aqueous medium, containing a stabiliser, usually poly(vinyl alcohol). Again homogenisation or intensive stirring is used to initially form a triple emulsion of W1/O/W2. The drug containing W1 phase is separated from the continuous W2 phase by the organic polymer solution (O). The solvent dichloromethane is only slightly water-soluble (ca. 1%), but through the use of the large excess of water, the organic solvent is rapidly extracted from the O-phase, yielding solid microparticles that contain drug substance in a polymeric matrix. In the hardening step, residual solvent is extracted and evaporated (solvent extraction or solvent evaporation). Model proteins, such as Bovine Serum Albumin (BSA) and Ovalbumin (OVA) have been shown to retain their integrity by using this technique to encapsulate.

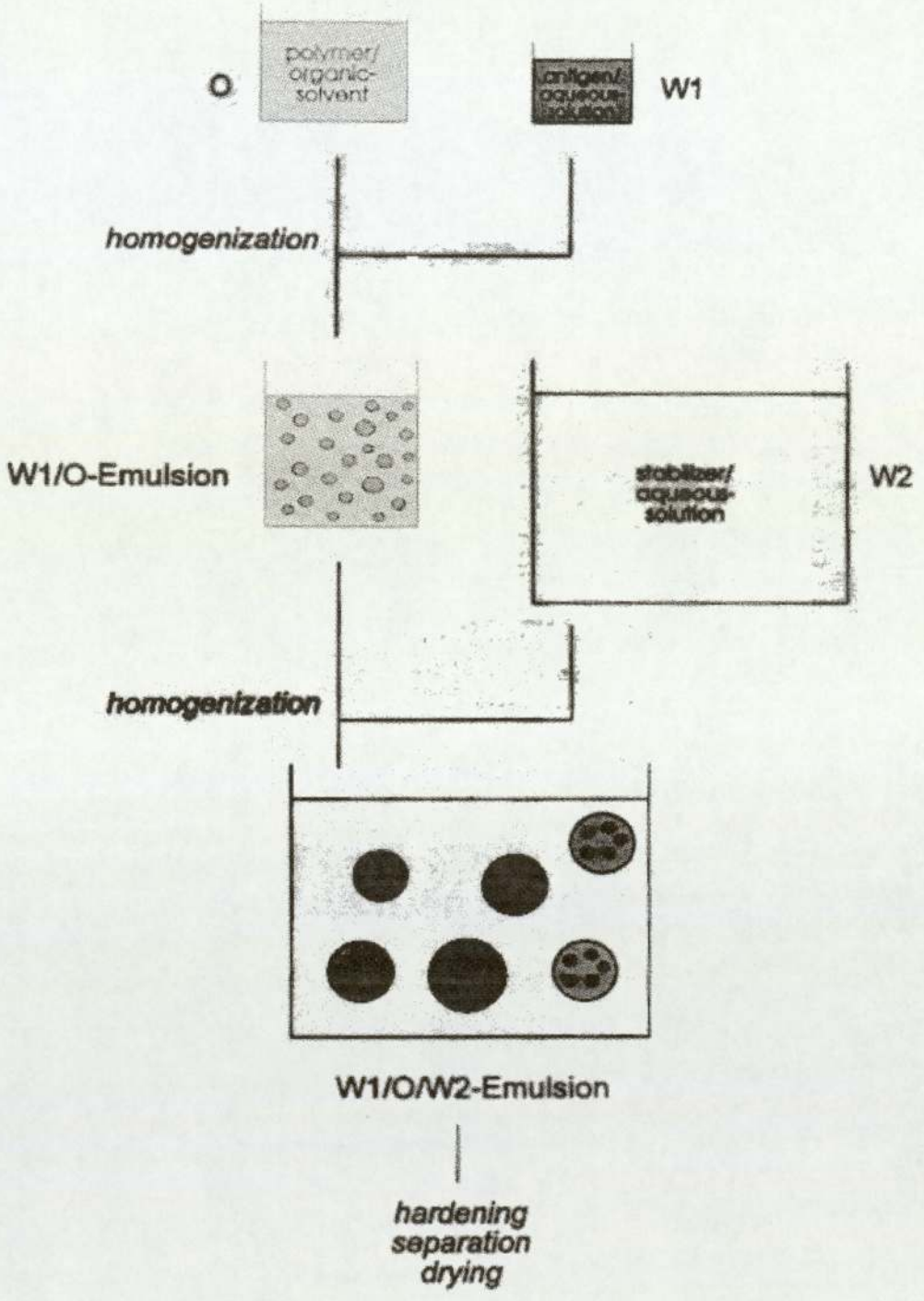


Figure 1.8 Manufacturing of microspheres: W/O/W double-emulsion method

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

1.3.6.2 Solvent-Removal Technique

The solvent-removal technique uses only organic phases to prepare microspheres, which has the advantage of preventing hydrolysis during the microsphere preparation procedure (Mathiowitz *et al.*, 1992). The drug substance, such as peptide or protein in solid form, is dispersed into a solution of polymer that is dissolved in an organic solvent such as dichloromethane (Fig. 1.9A). Alternatively, emulsions can also be used (Fig. 1.9B). The dispersion containing drug substance and polymer in organic solvent is again dispersed in a mixture of silicone oil, methylene chloride, and a surfactant, such as Span 85. The microspheres are hardened by adding a non-solvent, such as petroleum ether, to the suspension. These microspheres obtained by solvent extraction are porous. A problem might be the use of organic solvents and the residual content of silicone oil in the microspheres.

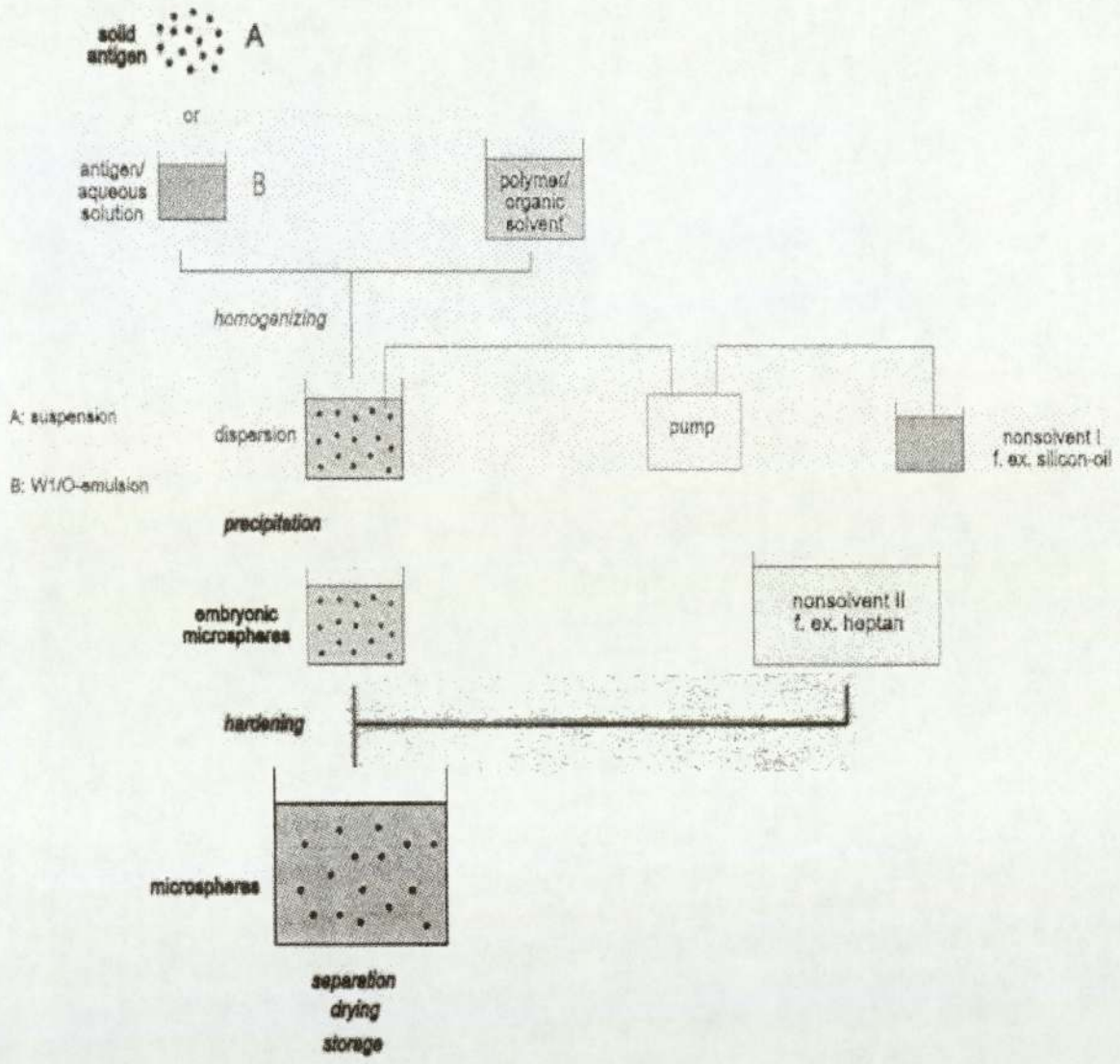


Figure 1.9 Manufacturing of microspheres: solvent-removal technique

The solvent-removal technique is used to fabricate Decapeptyl®, the parenteral depot form of a LH-RH agonist (Csernus *et al.*, 1990; Redding *et al.*, 1984).

1.3.6.3 Hot-Melt Encapsulation

An interesting approach to reduce the organic solvent residues in polyanhydride micropheres is to prepare microspheres from melted polymer. For this hot-melt encapsulation procedure polyanhydrides are melted, and drugs in solid form are dispersed in the melted polymer. This suspension is then transformed to microspheres by dispersion into a non-solvent, such as silicone or olive oil, at 5°C above the melting point of the polymer. The microspheres are solidified on cooling and washed with petroleum ether. Microspheres made by hot-melt encapsulation have smooth surfaces and are less porous than the microspheres made by other methods (Mathiowitz *et al.*, 1987). However, the high temperatures limit the wide application of the method, especially to heat sensitive polymers and drugs.

1.3.6.4 Spray-drying

In typical experiments, the biodegradable polymer is dissolved in a volatile organic solvent, such as dichloromethane or acetone, the drug in solid form is dispersed in the polymer solution by high-speed homogenisation, and this dispersion is atomised in a stream of heated air. With the formation of droplets, the solvent evaporates instantaneously, yielding microspheres in typical size ranges from 1 to 100 μm, depending on the atomising conditions. The microspheres are collected from the airstream by a cyclone separator. Residual solvents are removed by vacuum drying. The process can be operated under aseptic conditions, and in closed loop configurations, spray-drying in a nitrogen atmosphere is technically feasible (Masters, 1979). The process scheme is outlined in Fig. 1.10. The important advantages of the spray-drying technique over

other encapsulation techniques are, its reliability under production conditions, the proven reproducibility, and the well-defined control of particle size, as well as drug release properties of the resulting microspheres. Disadvantages are high investment for technique and residual organic solvents, a feature shared by all microencapsulation processes that use biodegradable polymers. Thermal stress to the drug substance is usually not a limiting factor, because the product is typically under 40-60°C temperature for only a short time. Proteins that are encapsulated by using this technique are lyophilised before the dispersion and homogenisation in the organic polymer solution. These processing conditions are likely to induce aggregation and denaturation to sensitive antigens. Therefore, stability of the microencapsulated antigen during processing, release, and storage becomes a major concern.

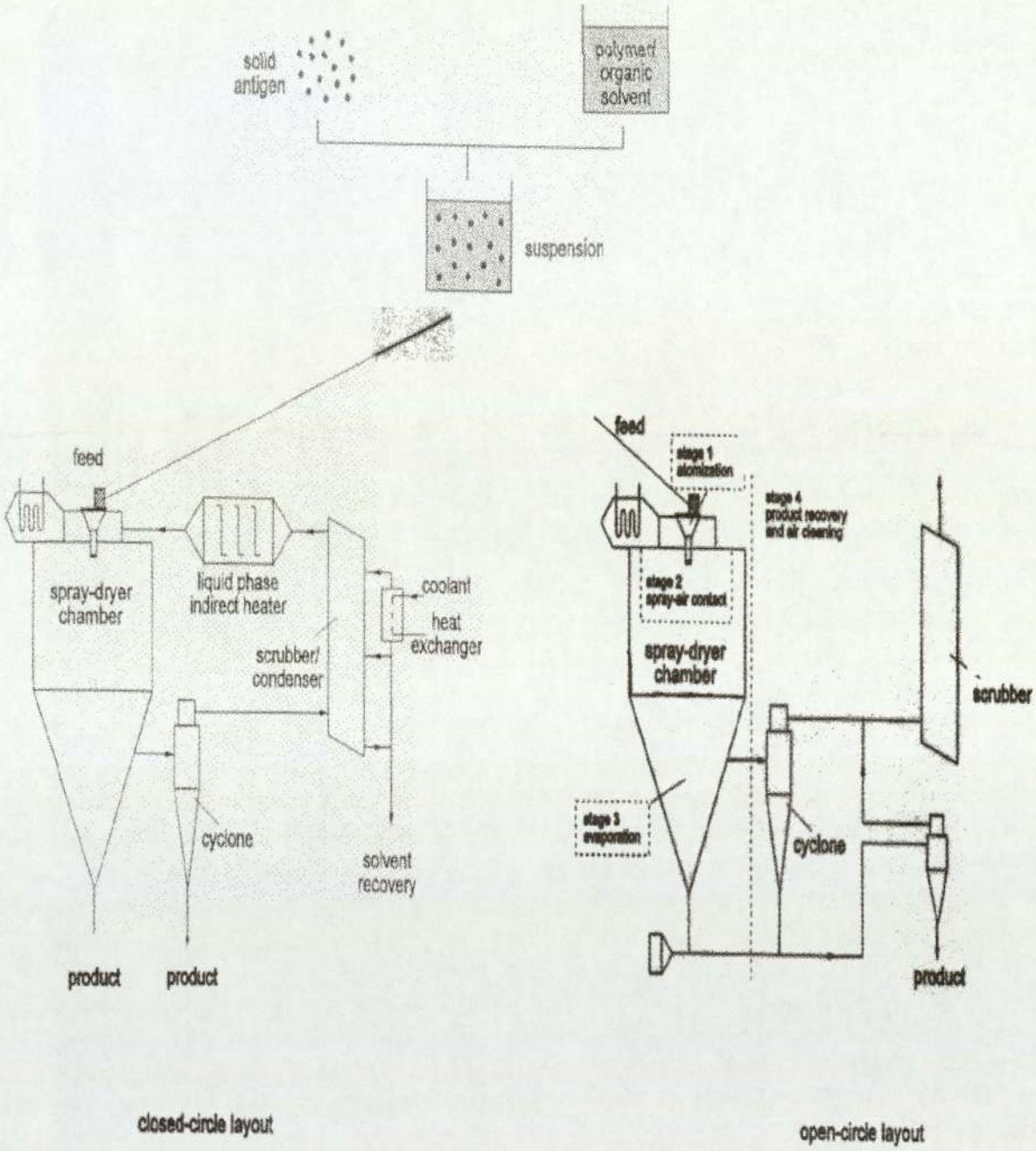


Figure 1.10 Manufacturing of microspheres: spray drying

Microspheres made by spray-drying tend to have an irregular shape and a high porosity that may cause the fast release of drugs (Mathiowitz *et al.*, 1992). The spray-drying method has been used to manufacture bromocriptine mesylate (Parlodel LAR), a parenteral microsphere formulation recently marketed (Kissel *et al.*, 1990; Kissel *et al.*, 1991).

1.3.6.5 Double-Walled Microspheres

Double-walled microspheres consist of two different polymer layers-polyanhydrides and another combined degradable polymer, such as poly(lactic acid). They might be useful for suppressing the burst release of drugs, or for generating pulsatile-release profiles (Peppas, 1993). There are two methods to prepare such microspheres. The first involves the co-dissolution in organic solvents of the two mutually inmiscible polymers. The cosolution with such polymers is dripped into aqueous solutions of poly(vinyl alcohol). With the evaporation of organic solvent, the two polymers begin to separate. In their final state, they form microspheres with an inner core made of one polymer and an outer wall that consists of the second polymer (Pekarek *et al.*, 1994). Another method to prepare double-walled microspheres is a modified double-emulsion technique that is shown in Fig. 1.11. The polymer is dissolved in an organic solvent, such as methylene chloride or ethyl acetate, and a small volume of aqueous phase containing microspheres that are prepared using another polymer, is dispersed into the solution to form a water-in-oil emulsion. This emulsion is then dispersed into an aqueous solution of poly(vinyl alcohol). After the organic solvent is evaporated completely, new microspheres are formed, which contain a core that consists of one type of polymer and a coating that consists of a second type of polymer.

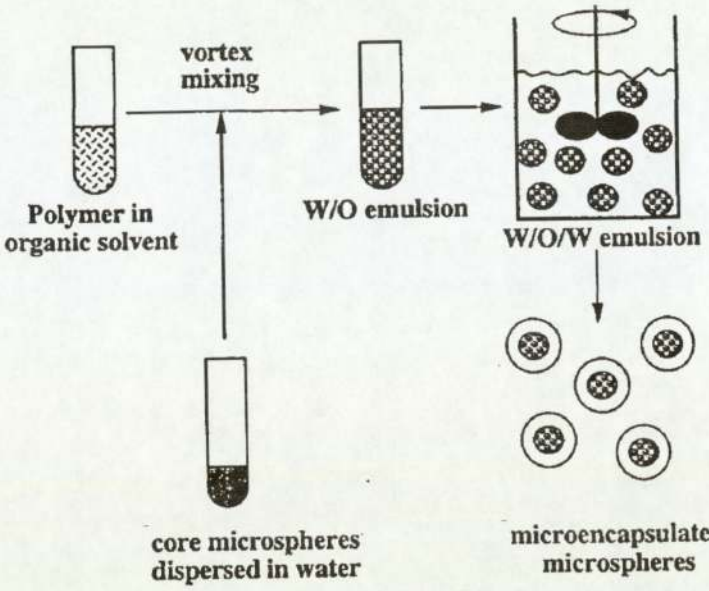


Figure 1.11 Manufacturing of double-walled microspheres by a modified double-emulsion technique

Chapter 2

Materials and Instrumentation

2.1 Materials

Sebacic acid (BDH Chemicals Ltd. Poole England)

Acetic anhydride (Acros Organics)

p-Hydroxybenzoic acid (Sigma-Aldrich)

1,4-Bis (p-carboxyphenoxy)butane (Sigma-Aldrich)

Cadmium acetate (BDH Chemicals Ltd. Poole England)

Phosphate-buffer saline tablet (Sigma-Aldrich)

Bovine serum albumin (Sigma-Aldrich)

Poly(vinyl alcohol) (Aldrich)

Bicinchoninic acid protein assay reagent (Sigma-Aldrich)

Toluene (Fisher Scientific)

Petroleum ether (Fisher Scientific)

Dichloromethane (Fisher Scientific)

HPLC grade chloroform (Fisher Scientific)

HPLC grade dichloromethane (Fisher Scientific)

Dry ethyl ether (Fisher Scientific)

Dry methanol (Fisher Scientific)

Sodium hydroxide (Rectapur™, made in CE-EMB)

Sulphate acid (Sigma-Aldrich)

2.2 Instrumentation

Gel Permeation Chromatography (GPC) systems

PERKIN-ELMER DSC-4 Differential Scanning Calorimeter

2020 Galaxy FT-IR Spectrometer

Bruker NMR AC250 Spectrometer

Cambridge Instruments Stereoscan S90B

Malvern Mastersizer/E Laser Diffraction Analyser

Soniprep 150

Dynatech Plate Reader

Chapter 3

Synthesis of Polyanhydrides

3.1 Introduction

Research on polyanhydride drug-carriers has continued at a strong pace in the past several years. Significant progress has been made in synthesis, structural identification, stability determination, and potential and clinical applications. A minor drawback of the polyanhydrides synthesised in the past has been the relatively low molecular weight. Recent studies show that by optimising the melt-polycondensation conditions such as prepolymer purity, reaction time and temperature, and removal of the condensation by-product, polyanhydrides with high molecular weight can be obtained (Domb *et al.*, 1987). To facilitate the anhydride interchange in the polymerisation, coordination catalysts have been used to enhance the nucleophilicity of the carbonyl carbon. In the synthesis of the copolyanhydrides of bis(p-carboxyphenoxy)propane and sebacic acid (CPP-SA), significantly higher molecular weights in shorter times are achieved by using cadmium acetate, earth metal oxides, and zinc-etherate as catalysts. The catalysts are also found to be effective in increasing the molecular weights of other polyanhydrides (Domb, 1992).

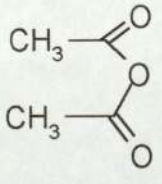
With the intention of preparing the polymer in an even milder reaction conditions, a single-step, one-pot synthesis in which a dicarboxylic acid monomer can be directly converted into the polyanhydride at room temperature has been reported. Limited success is obtained with the use of organophosphorus compounds as dehydrative coupling agents (Leong *et al.*, 1987). A disadvantage of the use of these coupling agents is the difficulty in isolating and purifying the final products without evoking hydrolytic decomposition. To circumvent this difficulty during work-up, as well as to improve the molecular weight, a one-step polymerisation using diacyl chloride, phosgene, or diphosgene as coupling agents was developed (Domb *et al.*, 1988). The

study examines ways to remove the acid acceptor-hydrochloride salt from the polymerisation mixture by either using an insoluble acid acceptor (e.g., cross-linked polyamides, inorganic bases) or using solvents that dissolve exclusively either the polymer or the salt. In either case, the by-product or the precipitating polymer is isolated by simple filtration. For instance, good yields and reasonable molecular weights were obtained for poly(sebacic acid) (PSA) when the reaction of sebacoyl chloride and sebacic acid is conducted in DMF or toluene using cross-linked poly(4-vinyl-pyridine) (PVP) or triethylamine (TEA) (Leong *et al.*, 1987).

3.2 Experimental

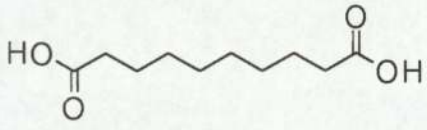
3.2.1 Purification of Starting Materials

3.2.1.1 Acetic Anhydride (AA)



Acetic anhydride was distilled in a round bottom flask with anti-bumping granules. The distilled liquid was collected at the temperature 138-140°C. Purified acetic anhydride was stored in sealed flasks until required.

3.2.1.2 Sebacic Acid (SA)



Firstly, sebacic acid was recrystallized twice from dry methanol (Domb *et al.*, 1987) and dried under vacuum. After that, in the flask the sebacic acid was heated at 140°C to melt. Under vacuum the heating was lasting for 20 min in order to remove water. Finally, the heating was stopped, and the purified sebacic acid was cooled and solidified in the flask.

3.2.1.3 Dry Petroleum Ether

This confusing name is used for mixtures of aliphatic hydrocarbons containing smaller amounts of aromatic compounds. It is generally supplied as several fractions each having a 20°C boiling range (40-60°C, 60-80°C etc.). The petroleum ether with 60-80°C boiling range was used in the experiment. This solvent was dried by distilling, or over 4A molecular sieves (Leonard *et al.*, 1990).

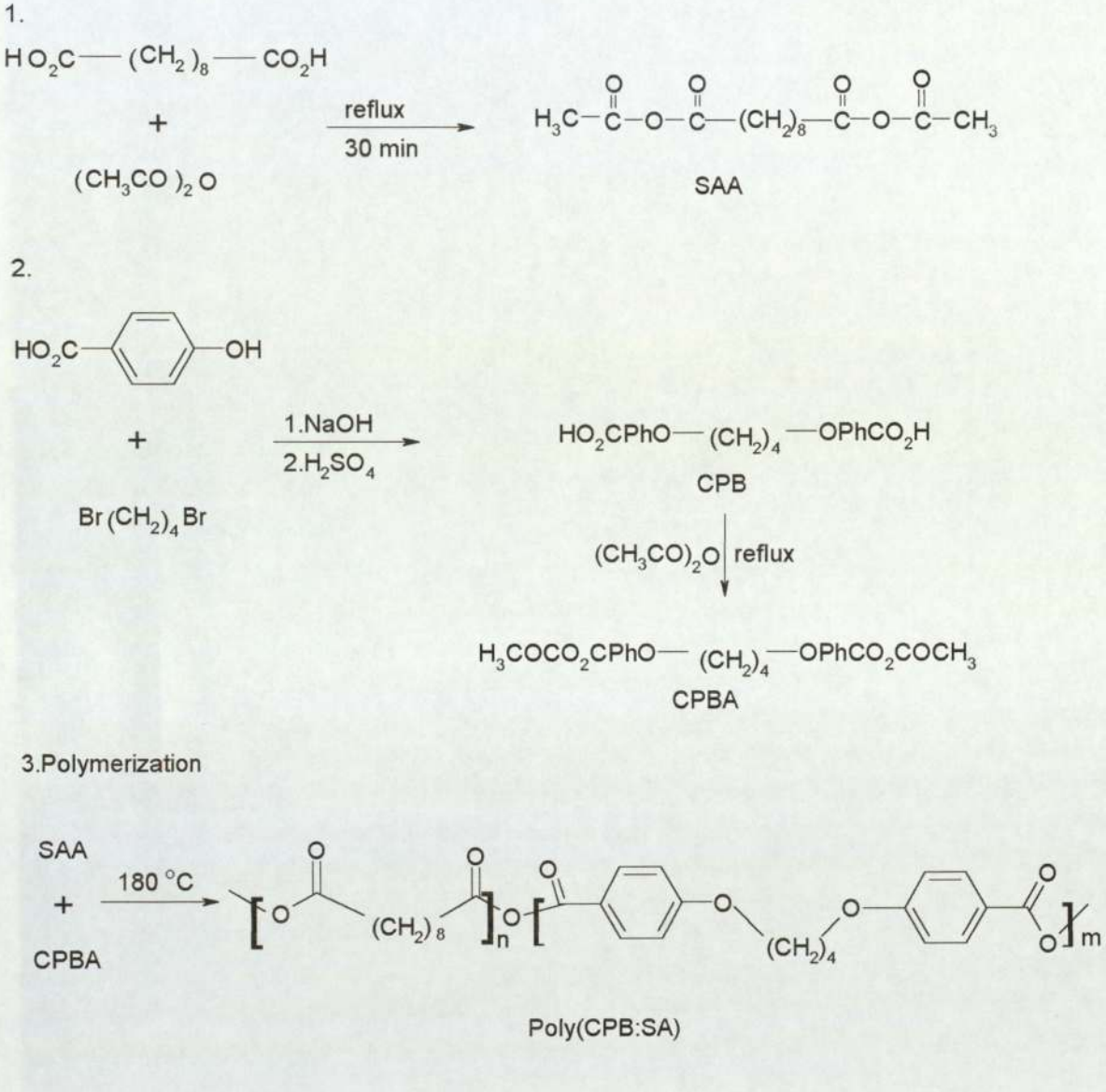
3.2.1.4 Dry Dichloromethane

Dichloromethane was refluxed over calcium hydride (5% w/v) and distilled onto 4A molecular sieves. Purified dichloromethane was stored in the dark and sealed flasks (Leonard *et al.*, 1990).

3.2.1.5 Dry Toluene

Toluene was distilled over calcium hydride (3% w/v) before use (Leonard *et al.*, 1990), and stored over 4A sieves in sealed flasks.

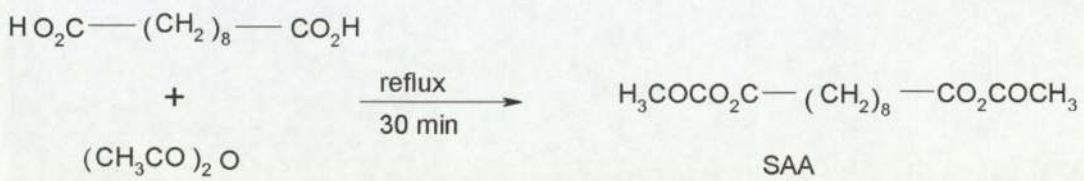
3.2.2 Polymerisation



Scheme 3.1

3.2.2.1 Synthesis of prepolymers

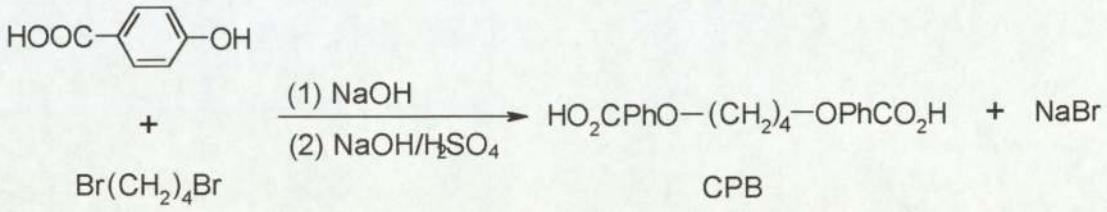
(a) Sebacic acid anhydride (SAA)



Scheme 3.2

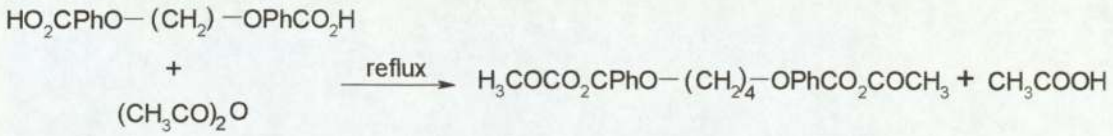
SAA prepolymer was prepared by refluxing the dicarboxylic acid monomer, and purified sebacic acid (8 g, 40 mmol) in purified acetic anhydride (100 ml) for 30 min. The reaction mixture was cooled to room temperature. Then the excess acetic anhydride was removed to dryness under vacuum at 20-30°C. The reaction mixture was kept in refrigerator overnight. The white crude prepolymer was recrystallised once from dry toluene. The crystals were then immersed in a 1:1 mixture of dry petroleum ether (50ml) and dry ethyl ether (50ml) overnight to extract traces of acetic anhydride and toluene. After filtration, the pure crystals were dried under vacuum (Leong *et al.*, 1985).

(b) 1,4-Bis(p-carboxyphenoxy)butane anhydride (CPBA)



Scheme 3.3

A solution of p-hydroxybenzoic acid (34.5g, 0.25mole) and sodium hydroxide (20g, 0.5mole) in 100 ml distilled water was refluxed and stirred in a 200 ml two-necked flask equipped with a condenser and a dropping funnel. While the mixture was stirred and kept at reflux temperature, 1,4-dibromobutane (15 ml, 0.125mole) was added over a period of 1 hour through the dropping funnel. After the addition of the 1,4-dibromobutane, the reaction mixture was refluxed for 3.5 hours. Then solid sodium hydroxide (5g, 0.125mole) was added to the mixture, which was refluxed for 2 hours again. Heating was stopped, and the reaction mixture was left standing overnight. The fine, powdery, white precipitate of the disodium salt was isolated by filtration and washed with 100ml methanol. The still wet precipitate was dissolved in 250ml distilled water. The solution was warmed at 60-70°C, while it was acidified with 6N sulfuric acid to pH≈1. The still warm mixture was filtered and dried in a vacuum oven at 80°C (Conix *et al.*, 1994).

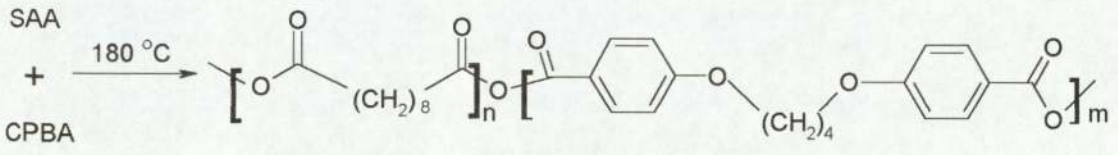


CPBA

Scheme 3.4

A solution of 1,4-bis (p-carboxyphenoxy) butane (12g, 0.038mole) and 130 ml purified acetic anhydride was refluxed and stirred in a 200 ml two-necked flask fitted with a condenser and a gas inlet tube. A slow stream of dry nitrogen was bubbled through the reaction mixture which was refluxed. Nitrogen was dried through over calcium chloride before entering the flask. After 30 minutes almost all the dibasic acid was dissolved. The mixture was filtered when it was still hot, and the yellow-colored filtrate was concentrated by distilling excess acetic anhydride under vacuum at room temperature. The reaction mixture was kept in a refrigerator overnight. The white crystals were filtered, washed with dry ethyl ether, and dried in a vacuum oven at 70°C (Conix *et al.*, 1994).

3.2.2.2 Synthesis of copolymer



Poly(CPB:SA)

Scheme 3.5

CPBA and SAA prepolymers were mixed in a mortar and pestle, according to the different mole ratios of 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. Then the mixture was put into a glass tube with a side arm equipped with nitrogen inlet. 2 molar percent cadmium acetate, as a known catalyst in polyanhydride synthesis, was mixed with the prepolymers prior to polymerisation. The tube was immersed in an oil bath at 180°C. After the prepolymers were melted, a high vacuum (0.1 mmHg) was applied through the side arm. The condensation product (acetic anhydride) 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 s every 15min. Nitrogen was dried over calcium chloride before passing the tube. The reaction lasted for 30 min. After cooling at room temperature, the crude copolymer was dissolved in dry dichloromethane. The catalysts were removed from the solution by filtration, and the crude copolymer was purified by precipitation in dry petroleum ether from the dichloromethane solution. The precipitate was filtered and extracted with dry ethyl ether for several hours at room temperature. After filtration, the pure copolymer was dried under vacuum (Domb *et al.*, 1987).

3.3 Results

Initially eleven types of polyanhydrides were synthesised by melt-polycondensation (see Table 3.1).

Polymer	yield % (w/w)
Poly(CPB:SA 10:90)	73.8
Poly(CPB:SA 20:80)	75.9
Poly(CPB:SA 30:70)	68.5
Poly(CPB:SA 40:60)	68.9
Poly(CPB:SA 50:50)	52.3
Poly(CPB:SA 60:40)	61.2
Poly(CPB:SA 70:30)	60.9
Poly(CPB:SA 80:20)	67.6
Poly(CPB:SA 90:10)	18.6
Poly(SA)	75.9
Poly(CPB)	41.6

Table 3.1 Yields of synthesised polymers

Because of susceptibility of the polyanhydrides to moisture, the polyanhydrides were stored in sealed flasks filled with nitrogen in order to avoid hydrolysis after synthesis.

Chapter 4

Polyanhydride Characterisation

Polyanhydrides with greater than 50% CPB in their backbone have limited solubility in dichloromethane and therefore are not good candidates for microencapsulation by solvent-evaporation processes. So only poly(CPB:SA 10:90), poly(CPB:SA 20:80), poly(CPB:SA 30:70), poly(CPB:SA 40:60) and poly(CPB:SA 50:50) were studied in the following experiments.

4.1 GPC Analysis

4.1.1 Introduction

GPC is in simplest terms a mechanism of solute separation with molecular size as the discriminating factor. Sample molecules permeate the stationary phase to different degrees and are thus retained within the column for periods of time proportional to their molecular size. Columns are tightly packed with a gel and completely filled with solvent (the mobile phase). Within the column the pore size of the packing particles determines the molecular size range within which separation occurs.

Traditionally, GPC has been used for the analysis of molecular weight distributions of synthetic polymers. Data treatment can involve the calculation of molecular weight averages imparting information about chain length and the extent of cross-linking within a polymer. The molecular weight averages (Mn, Mw) indicate the number and length (or weight) of the polymeric chains formed during manufacture. Mn is the number-average molecular weight, which is the molecular weight of the average chain length in a polymer sample. Mw refers to the molecular weight equal to the modal molecular weight of polymer chains, known as the weight average molecular weight. As Mn represents the molecular weight of the average chain length in a polymer sample,

and Mw refers to the molecular weight equal to the modal molecular weight of the polymer chains the value of Mw is always larger than Mn except in the case of a truly monodisperse system where the values are identical. Polydispersity index is a measure of the breath of molecular mass distribution and its definition is:

$$\textbf{P.I.} = \overline{\textbf{Mw}}/\overline{\textbf{Mn}}$$

There is quite a large range of P.I. of synthetic polymers, but a perfectly monodispersed polymer would have a P.I. value of 1.0. Using selected procedures it is possible to synthesise polymers with narrow distributions. Theoretically, it can be expected that a narrow distribution will result if the following conditions are met:

- Each polymer molecule grows exclusively by consecutive addition of monomer to terminal group.
- All active terminal species must be equally reactive throughout the polymerisation.
- Each step of the reaction is kinetically irreversible.
- No chain transfer, or termination process can occur.

4.1.2 Calibration

Standardisation of the GPC system was achieved using narrow-MW polystyrene standards (EasiCal, Polymer Laboratories Ltd, Shropshire UK) inert PTFE strips coated with polystyrene (~5 mg) were immersed in 50 ml of chloroform to give a polystyrene concentration of 0.010% w/v. The kit contained two types of strips (A and B) each representing Mw values of 580, 9200, 66000, 330000, 3040000 and 3250, 28500, 156000, 1030000, 8500000 respectively. About 100µl

aliquots were injected into the GPC system to elucidate the retention time for each Mw value and a calibration curve was obtained (see figure 4.1). To ensure accuracy, each retention time is the average of at least 3 readings.

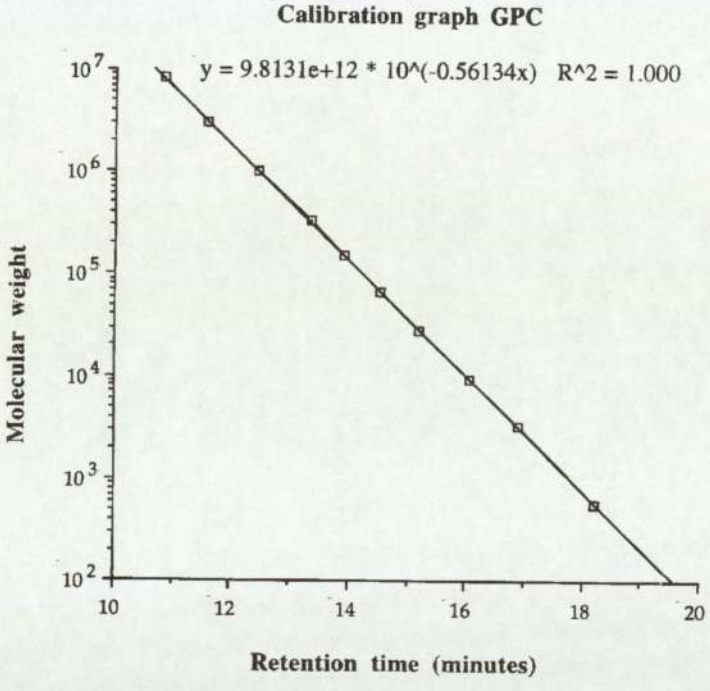


Figure 4.1 Calibration curve for GPC (n=3)

4.1.3 Results and Discussion

Polymer	Mw
Poly(CPB:SA 10:90)	27000
Poly(CPB:SA 20:80)	33781
Poly(CPB:SA 30:70)	23422
Poly(CPB:SA 40:60)	25202
Poly(CPB:SA 50:50)	23727

Table 4.1 Molecular weight of polyanhydrides

It was found that polymerisation in the presence of a catalyst resulted in higher molecular weight polymers in same reaction time (30 min) (see Table 4.2). Cadmium acetate, a known catalyst in polyanhydride synthesis, was therefore used during the synthesis of polyanhydrides.

Polymer	Mw	
	no catalyst	with catalyst
Poly(CPB:SA 20:80)	14027	33781
Poly(CPB:SA 50:50)	7256	23727

Table 4.2 Effect of catalyst on molecular weight (polymerisation time 30 min)

4.2 Thermal Analysis

4.2.1 Introduction

Thermal analysis is defined by ICTA (the International Confederation for Thermal Analysis) as a " term covering a group of techniques in which a physical property of a substance and/or its reaction products is measured as a function of temperature". Differential scanning calorimetry (DSC) is currently one of the most widely used methods of thermal analysis in polymer science. DSC curves reflect changes in the energy of the system under investigation – changes that may be chemical or physical in origin. The technique is therefore particularly useful for polymers because polymerisation or structural changes are almost invariably accompanied by energetic effects so that crystallisation and melting, curing and other reactions, and the glass transition all show characteristic DSC curves. Small samples (a few mg) and rapid experimentation (heating, q (+), or cooling, q (-), rates of up to 10-320 $°Cmin^{-1}$ are common) mean that thermal analysis finds applications in both research laboratories and routine quality control.

4.2.2 Procedure

DSC analysis was performed on a Perkin-Elmer DSC-4 Differential Scanning Calorimeter. 2-10 mg of polyanhydride was weighed into aluminium pans, and the lid was crimped into position carefully. The sample pan was placed in left-hand cup on DSC machine. The results were analysed using the system software.

The following parameters were used:

T final 400 °C

T min –40 °C

T incr 44 °C

T initial –40 °C

Y range 50

Heat rate 10°C/min

Cooling rate 320°C/min

4.2.3 Results and Discussion

Tg and Tm of the polymers used in this study were determined using DSC measurement (see Table 4.3). The polymers used in this study were amorphous because of the lack of crystallinity and Tc that were due to the random distribution of two monomers throughout the polymer backbone. This randomness made crystallisation more difficult especially when the monomers have very different structures (Chiba *et al.*, 1997).

Related to expected results, Tg for poly(SA) was reported as 60°C and Tm was 86°C. They should decrease as CPB is added up to 50:50, and then increase again (Mathiowitz 1990). For all these polymers used in this study, they were in rubbery state at room temperature.

CPB:SA (mol %)	Tg (°C)	Tm (°C)
10:90	*	72.83
20:80	2.58	*
30:70	3.7	62.61
50:50	*	78.64
60:40	0.63	86.26
80:20	-1.4	*

* not detectable

Table 4.3 Thermal analysis of poly(CPB:SA)

4.3 IR Analysis

4.3.1 Introduction

The energy of most molecular vibrations corresponds to that of the infrared region of the electromagnetic spectrum. Molecular vibrations may be detected and measured either in an infrared spectrum or indirectly in a Raman spectrum. The most useful vibrations, from the point of view of the organic chemist, occur in the narrower range of 2.5-16 μm (1 μm = 10^{-4}cm). The position of an absorption band in the spectrum may be expressed in microns (μm), or very commonly in terms of the reciprocal of the wavelength, cm^{-1}. The usual range of an infrared spectrum is, therefore, between 4000 cm^{-1} at the high frequency end and 625 cm^{-1} at low frequency end.

Functional groups have vibration frequencies, characteristic of that functional group, within well-defined regions of this range. The fact that many functional groups can be identified by their characteristic vibration frequencies makes the infrared spectrum the simplest, most rapid and often most reliable means for assigning a compound to its class.

4.3.2 Results

Infrared spectroscopy was performed on a 2020 Galaxy FT-IR spectrometer. Polymeric samples were pressed into KBr pellets before analysis.

ν_{max} (KBr): 1810 (anhydride peak), 1740, 1700, 1605, 1500, 1460, 1250, 1030 cm^{-1}.

4.4 NMR Analysis

4.4.1 Introduction

The NMR phenomenon (first observed in 1946) is observable because certain nuclei behave like bar magnets. Most important among such nuclei are ^{1}H, ^{13}C, ^{19}F and ^{31}P, all having a nuclear spin (I) of ½; those with nuclear spin of 1 include deuterium (^{2}H) and ^{14}N. Certain other nuclei which are important in organic chemistry have a nuclear spin of zero and therefore give no nuclear resonance signals; these include ^{12}C and ^{16}O.

To observe NMR signals, we require a radio frequency (r.f.) transmitter, a homogeneous magnetic field and a r.f. receiver. The sample may be heated or cooled by a stream of hot or cold gas, allowing spectra to be obtained at various temperatures. Subsequently, it is seen that

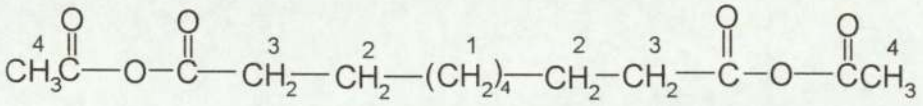
magnetic nuclei in a molecule normally have different electronic environments, and hence resonate at slightly different field strengths.

4.4.2 Results

4.4.2.1 NMR Characterisation of Prepolymers

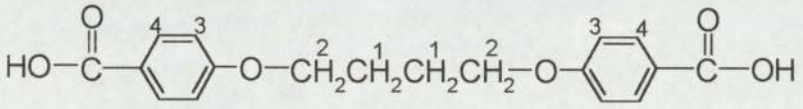
^{1}H NMR spectra were obtained on a Bruker NMR AC 250 Spectrometer, using deuterated chloroform as a solvent.

Sebacic acid anhydride prepolymer (SAA prepolymer)



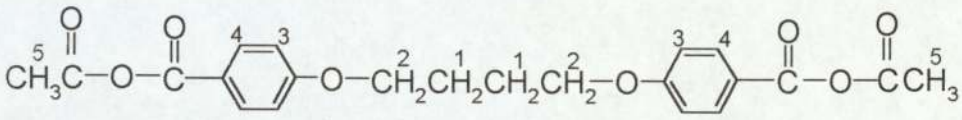
^{1}H NMR ($CDCl_3$) δ 2.47 (t, 4, J= 7.5 Hz, H-3), 2.24 (s, 6, H-4), 1.70-1.65 (m, 4, H-2), 1.34 (m, 8, H-1) (see Appendix 1).

1,4-bis (p-carboxyphenoxy) butane prepolymer (CPB prepolymer)



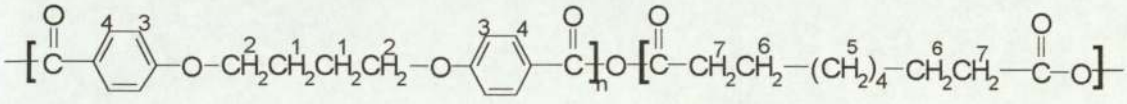
^{1}H NMR ($CDCl_3$) δ 7.86 (d, 4, J= 7.5 Hz, H-4), 7.00 (d, 4, J= 7.5 Hz, H-3), 4.10 (t, 4, H-2), 1.88 (m, 4, H-1) (see Appendix 2).

1,4-bis (p-carboxyphenoxy) butane anhydride prepolymer (CPBA prepolymer)



^{1}H NMR ($CDCl_3$) δ 7.98 (d, 4, J= 7.5 Hz, H-4), 6.92 (d, 4, J= 7.5 Hz, H-3), 4.10 (t, 4, H-2), 2.35 (s, 6, H-5), 2.02 (m, 4, H-1) (see Appendix 3).

4.4.2.2 NMR Characterisation of Poly(CPB:SA)



^{1}H NMR ($CDCl_3$) δ 7.97 (d, 4, J= 7.5 Hz, H-4), 6.92 (d, 4, J= 7.5 Hz, H-3), 4.11 (t, 4, H-2), 2.43 (t, 4, J= 7.5 Hz, H-7), 2.02 (m, 4, H-1), 1.63 (m, 4, H-6), 1.30 (m, 8, H-5) (see Appendix 4).

From the ratio of NMR peak's integration, practical mole ratios of CPB-SA in the poly(CPB:SA) were calculated (see Table 4.4). It was found that there were no significant differences between theoretical mole ratios and practical mole ratios.

Theoretical mole ratios of CPB-SA in the poly(CPB:SA)	Practical mole ratios of CPB-SA in the poly(CPB:SA)
10:90	7:93
20:80	13:87
30:70	28:72
40:60	43:57
50:50	69:31

Table 4.4 Practical mole ratios of CPB-SA in the poly(CPB:SA) according to NMR integration analysis

Chapter 5

Studies of Pharmaceutical Aspects of Polyanhydrides

5.1 Polyanhydride degradation studies

5.1.1 Degradation of polyanhydride in PBS

5.1.1.1 Experimental

Polymer samples (~ 20 mg) were incubated in 20 ml buffer (0.01 M phosphate-buffered saline pH 7.4) on a shaker at 37°C. Samples of the degrading polymer (3ml) were removed daily for 7 days. The degraded samples were collected by centrifugation and dried under vacuum prior to analysis. The degraded samples were pressed into KBr discs before IR analysis, and the molecular weight of degrading polymers was studied by GPC.

5.1.1.2 Results and Discussion

In general, degradation occurred more rapidly in polyanhydrides with low percentages of CPB, the more hydrophobic monomer. After 5 days in release buffer, the molecular weights of polyanhydrides decreased to the monomer molecular weight, indicating that the polyanhydrides were completely degraded at this point. As expected, degradation of poly(CPB:SA 50:50) occurred most slowly because of the highest percentage of CPB in its backbone (see Figure 5.1).

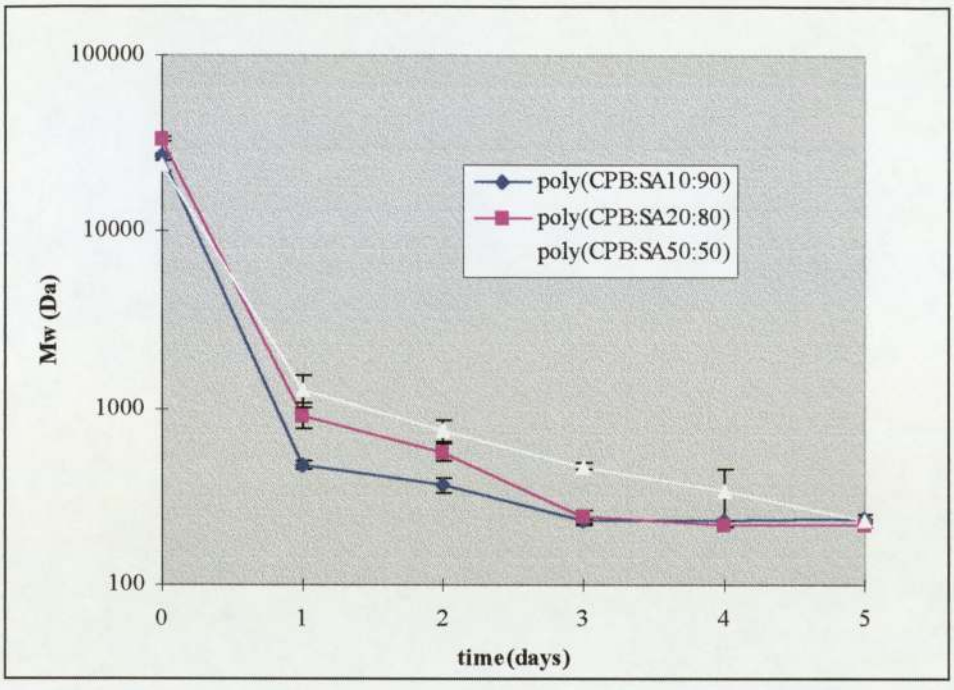


Figure 5.1 GPC analysis of degradation of polymers in PBS (represented by Mw decrease) (n=3, mean±s.d.)

The degradation rates of polyanhydrides were also confirmed by infrared (IR) spectroscopy. Complete disappearance of the characteristic anhydride IR peak at 1810cm^{-1} was observed after approximately 5 days in release medium at 37°C (see Appendix 5, 6 and 7).

5.1.2 Effect of pH on Degradation of Polyanhydrides

5.1.2.1 Experimental

(a) Buffer Preparation

1. KCl / HCl solution pH 1

7.455 g KCl was added into 500 ml distilled water to make 0.2 N KCl solution.

8.28 ml HCl (37%, d=1.19) was added into distilled water to make 0.2 N HCl solution 500ml.

25 ml KCl solution, 54.2 ml HCl solution and 20.8 ml distilled water were mixed to make pH 1 KCl / HCl solution 100 ml.

2. Phosphate buffer pH 8

4.5365g KH_2PO_4 was added into 500 ml distilled water to make 1/15 mol/L solution.

5.935 g $Na_2HPO_4 \cdot 2H_2O$ was added into 500 ml distilled water to make 1/15 mol/L solution.

3.7 ml KH_2PO_4 solution and 96.3 ml Na_2HPO_4 solution were mixed to make pH 8 phosphate solution 100 ml.

(b) Incubation

Polymer samples (~ 20 mg) were incubated in 20 ml of each buffer on a shaker at 37°C. The degraded samples were collected periodically by centrifugation and dried under vacuum prior to IR and GPC analysis.

5.1.2.2 Results and Discussion

The effect of pH on polyanhydride degradation was also tested, and it was found that pH does not have a significant effect on the degradation rate of polyanhydride (see Figure 5.2).

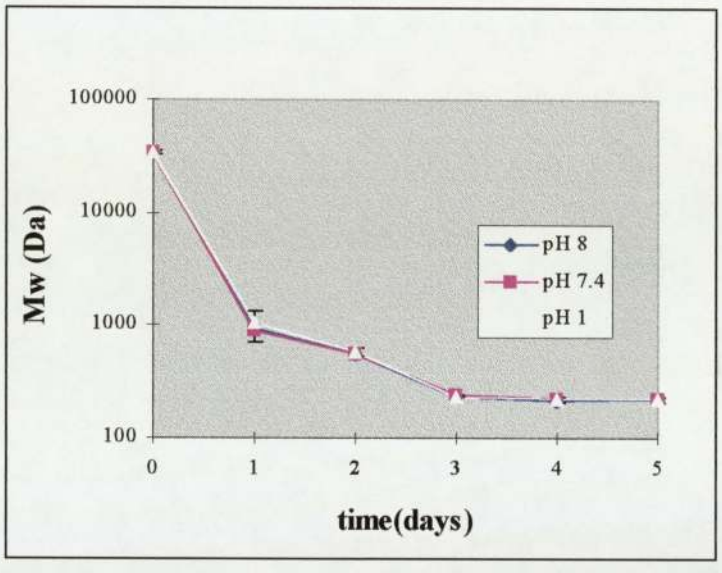


Figure 5.2 Effect of buffer pH on poly(CPB:SA 20:80) degradation (n=3, mean±s.d.)

Degradation is water uptake accompanied by hydrolysis of anhydride bond. Due to the fast degradation, the effect of pH levels on the degradation could be ignored in this range. During the degradation, the decrease in the molecular weight of polyanhydrides occurred until molecular weights of polyanhydrides decreased to the monomer molecular weights (Hanes *et al.*, 1997).

5.2 Microsphere Preparation

Poly (CPB: SA) microspheres were prepared by a modified solvent evaporation method using a double emulsion (Hanes *et al.*, 1997). 500 µl of an aqueous BSA solution (2% w/v) was emulsified into 5 ml methylene chloride, containing 100 mg dissolved poly (CPB: SA), by probe sonication at output 50 W for 3 min on ice. To this primary emulsion, 10 ml of aqueous 2% w/v PVA solution saturated with methylene chloride was added, followed by vigorous mixing with a vortex mixer for 1 min to form a double emulsion. The resulting double emulsion was poured into 100 ml 0.1% w/v PVA solution and stirred at room temperature to allow the methylene chloride to completely evaporate. The polymer precipitated as the methylene chloride evaporated, thereby trapping the internal water droplets containing the protein. The hardened microspheres were subsequently collected by centrifugation, washed twice with double-distilled water and freeze-dried into a free-flowing powder consisting of antigen dispersed in small drug pockets throughout the polymer microspheres.

During the procedure, poly(CPB:SA 10:90) with initial Mw 27000, poly(CPB:SA 50:50) with initial Mw 23727 and poly(CPB:SA 20:80) with initial Mw 33781, 14027 and 8096 were used to make microspheres. In order to obtain microspheres with different protein loading, 1% w/v aqueous BSA solution was also used.

5.3 Studies of Microsphere Properties

5.3.1 Particle Size Analysis

Microspheres were suspended in double-distilled water and sonicated. The particle size distribution was determined using a Malvern Mastersizer/E laser diffraction analyser.

Mirospheres made of poly(CPB:SA) with BSA	Volume mean
10:90 (7.0% w/w BSA)	24.46±14.689μm
20:80 (7.2% w/w BSA)	19.98±14.806μm

Table 5.1 Particle size of microspheres

Size distribution measurements showed that the microspheres also had a Gaussian distribution of sizes (see Figure 5.3). More than 82% of the microspheres had diameters ranging from 1 to 35 μm. It was possible to control microsphere size from a few micrometers to several millimetres, for example, by varying the intensity of mixing during the formation of the second emulsion. In this study, the second emulsion was prepared by vortex mixing and microsphere size depended instead on the mixing method used in the inner emulsion preparation. When the inner emulsion was prepared by vortex mixing, the resulting microspheres were larger than the microspheres when the inner emulsion was prepared by probe sonication (Chiba *et al.*, 1997).

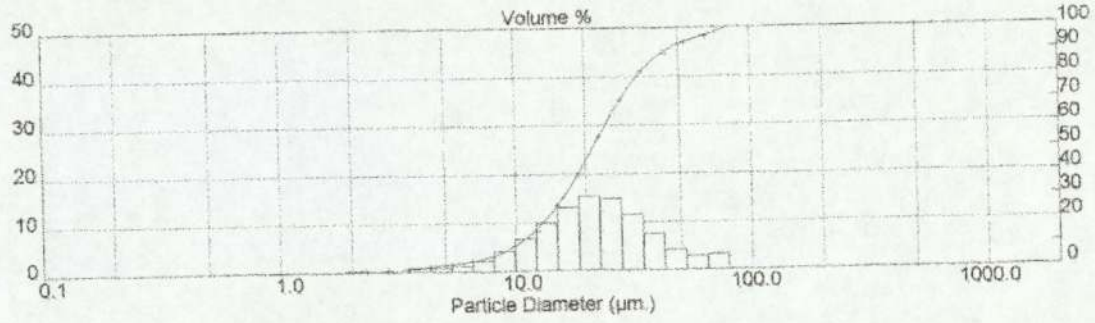


Figure 5.3 Distribution of Particle Size

5.3.2 Protein Loading

5.3.2.1 BCA protein assay method

For accurate determinations of low protein content, a bicinchoninic acid (BCA) assay procedure was followed (Smith *et al.*, 1985). Proteins react with alkaline copper II to produce copper I. Two molecules of the BCA reagent (see Figure 5.4) react with Cu^{1+} to form a copper/peptide chelate. The product of the reaction (see Figure 5.5) is water-soluble and has an intense purple colour.

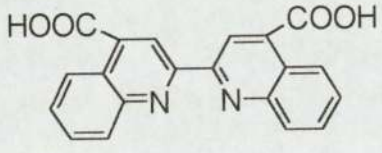


Figure 5.4 Structure of bicinchoninic acid

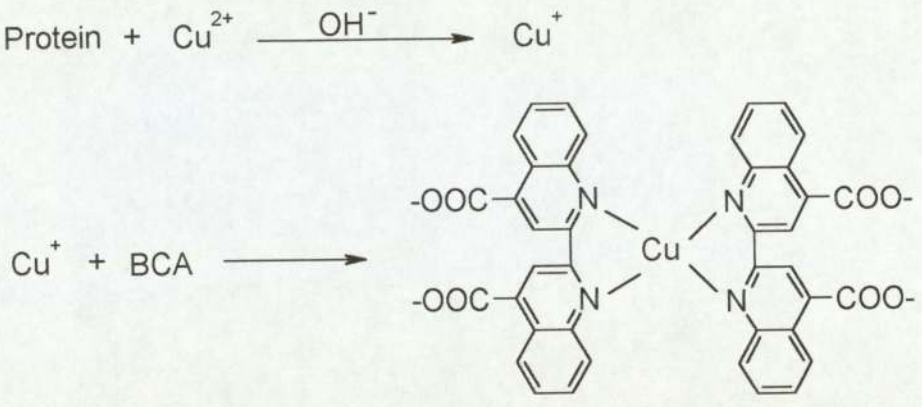


Figure 5.5 Formation of purple complex with BCA and cuprous ion

Reagent A was purchased from Sigma-Aldrich (see table 5.2), and reagent B consisted of 4%w/v $CuSO_4 \cdot 5H_2O$. These reagents are stable for up to six months at room temperature. To prepare the working reagent 50 parts of reagent A were added to 1 part of reagent B. This solution was initially apple green but develops a purple colour on standing. 200 µl of the working reagent was added to 10 µl of the protein sample on a 96 well microtitre plate. The solution was mixed before incubation at 60°C for one hour. The sensitivity of the assay was heightened by extending the incubation period from 30 minutes recommended to one hour (Smith *et al.*, 1985).

Ingredient	Quantity
BCA disodium salt	5.00g
$Na_2CO_3 \cdot H_2O$	10.00g
Na_2 tartrate	0.80g
NaOH	2g
$NaHCO_3$	4.75g
Double-distilled water	to 500ml

Table 5.2 Formula for BCA reagent A

5.3.2.2 Calibration

A series of protein standards was prepared in 1 M NaOH/HCl solution. The concentrations of protein used were in the range of 10-200 μg/ml (see Table 5.3).

Concentrations of protein (μg/ml)	2 mg/ml BSA solution (μl)	1 M NaOH/HCl solution (μl)
10	5	995
20	10	990
25	12.5	987.5
50	25	975
100	50	950
150	75	925
200	100	900

Table 5.3 A series of protein standards (protein in 1M NaOH/HCl solution)

200 μl of the working reagent was added to 10 μl of the protein standard sample on a 96 well microtitre plate. The solutions were mixed, and incubated at 60°C for one hour. After cooling to room temperature, the absorbances were read using a Dynatech Plate Reader at 570 nm. Blanks were run under the same conditions and these values were subtracted from the standard or the unknown. Calibration curves were constructed by plotting net absorbance at 570 nm *versus* protein concentration and the concentrations of the unknowns were determined. To ensure accuracy at such low protein levels, each absorbance was the average of at least 4 readings.

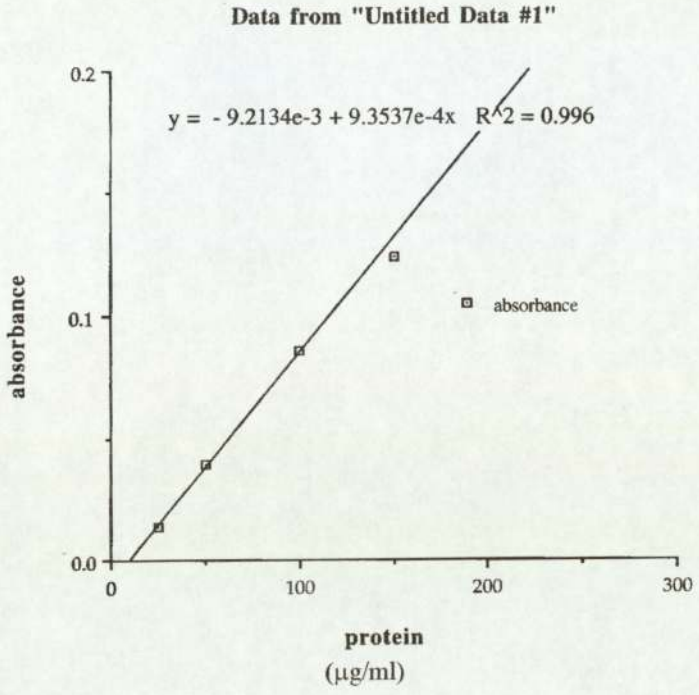


Figure 5.6 Typical calibration curve for protein loading

5.3.2.3 Experimental

The amount of protein encapsulated in polymer microspheres (expressed as %w/w) was determined by completely digesting a known weight of microspheres in 1 M NaOH.

Microspheres (~5 mg) which were weighed accurately were incubated in 2 ml 1 M NaOH on a shaker at 37°C until solution went completely clear. 0.2 ml of 10 M HCl solution was added to neutralise the NaOH solution. Then 10 μl solution was taken out to determining the protein content by bicinchoninic acid protein assay (BCA) method (n=4).

5.3.2.4 Results and Discussion

Poly(CPB:SA)	Mw of polymer	Theoretical protein loading (%w/w)	Actual protein loading (%w/w)	Loading efficiency
20:80	33781	5	2.6	52%
20:80	33781	10	7.2	72%
20:80	14027	10	6.7	67%
20:80	8096	10	7.1	71%
50:50	23727	10	7.3	73%
10:90	27000	10	7.0	70%

Table 5.4 Protein loading of microspheres made from different compositions and initial Mw of polymers

At higher loading, migration of the inner water phase to the outer water phase increased because the particles of the inner water phase easily aggregated owing to changes in the surface properties, and their inner water phase were connected with each other from the surface into the core (Ogawa *et al.*, 1988). Related to expected results, the loading efficiency decreased as the loading percentage increased. Perhaps due to the loss of protein during the encapsulation procedure, the ideal results were not obtained in this study.

5.3.3 Molecular Weight

Molecular weights of the empty microspheres were determined by GPC. It was found that the molecular weights of empty microspheres decreased due to the encapsulation procedure, compared with the molecular weight of the used polyanhydrides. This could be reduced by encapsulation using non-aqueous solvents (see Table 5.5).

Polyanhydrides	Mw of the polyanhydride	Mw of the empty microspheres
Poly(CPB:SA 20:80)	33781	6487
Poly(CPB:SA 50:50)	23727	5651

Table 5.5 Mw of the empty microspheres compared with the polymers prior to encapsulation

5.3.4 SEM Analysis

Microsphere morphology was observed by Scanning Electron Microscopy (SEM) using Cambridge Instruments Stereoscan S90B. Microspheres were mounted on metal stubs on carbon and coated with gold prior to observation.

SEM studies showed that poly(CPB:SA) microspheres were spherical and had smooth external surfaces without visible pores (see Figure 5.7a and 5.7b). However, the microspheres had a high

degree of internal porosity owing to large drug-containing pockets (see Figure 5.8). The large drug pockets formed may correspond to the first emulsion (w/o phase) of the microsphere preparation process.

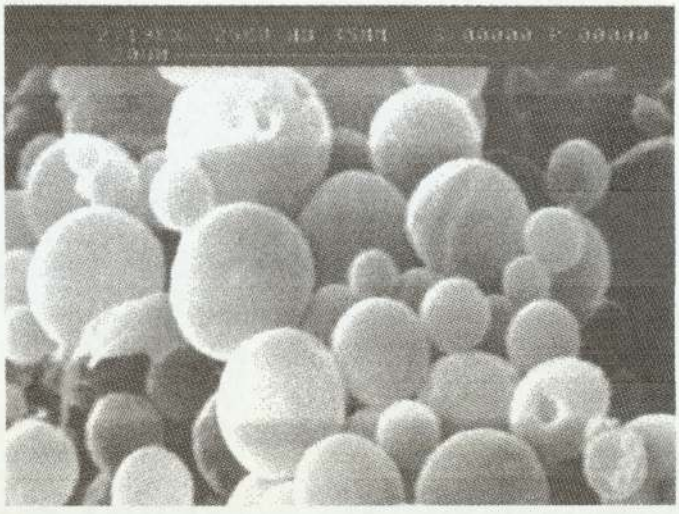


Figure 5.7a. A typical batch of poly(CPB:SA 20:80) microspheres containing 7.1%w/w BSA

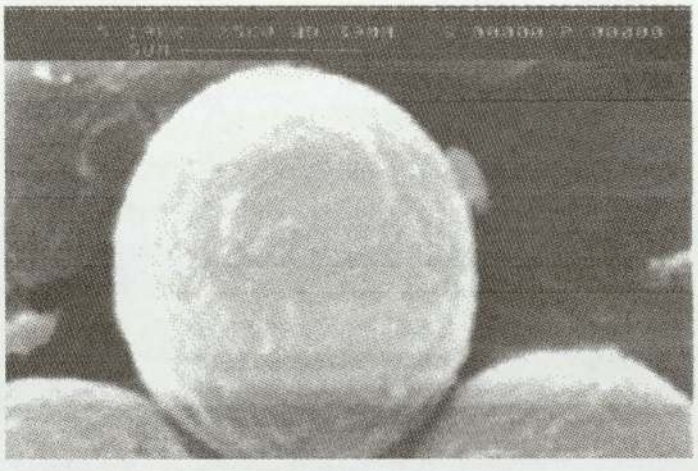


Figure 5.7b A close-up of a single poly(CPB:SA 20:80) microsphere containing 7.1%w/w BSA

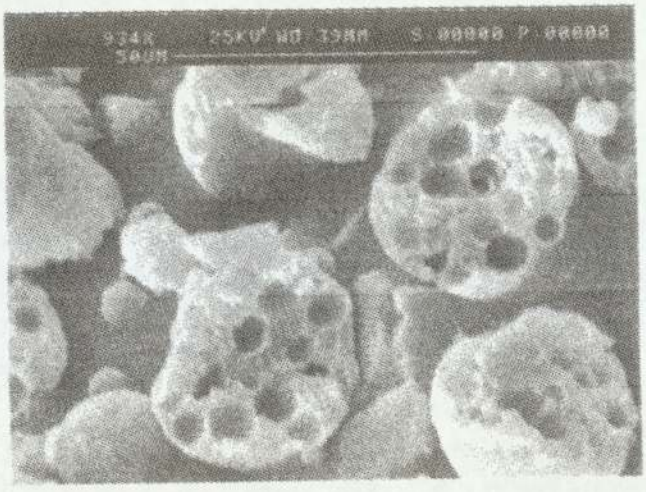


Figure 5.8 The porous internal microsphere morphology
protein are trapped within the internal drug pockets
poly(CPB:SA 20:80) microspheres containing 7.1%w/w BSA

5.4 Degradation of Microspheres

5.4.1 Degradation Studies of Empty Microsphere

Empty microspheres (~20mg) were incubated in 20 ml release buffer (0.01 M phosphate-buffered saline pH 7.4), KCl/HCl buffer (pH 1) and phosphate buffer (pH 8) on the shaker at 37°C. The degraded microspheres were collected everyday for a week by centrifugation and dried prior to IR and GPC analysis.

The degradation of empty microspheres in release buffer was shown in Figure 5.9. The result of empty microspheres degradation in release buffer (0.01 M phosphate-buffer solution pH 7.4) and

the effect of pH on empty microspheres degradation were similar to the results of polyanhydride degradation (see Figure 5.9 and Figure 5.10). The results were also confirmed by IR analysis (see Appendix 8, 9).

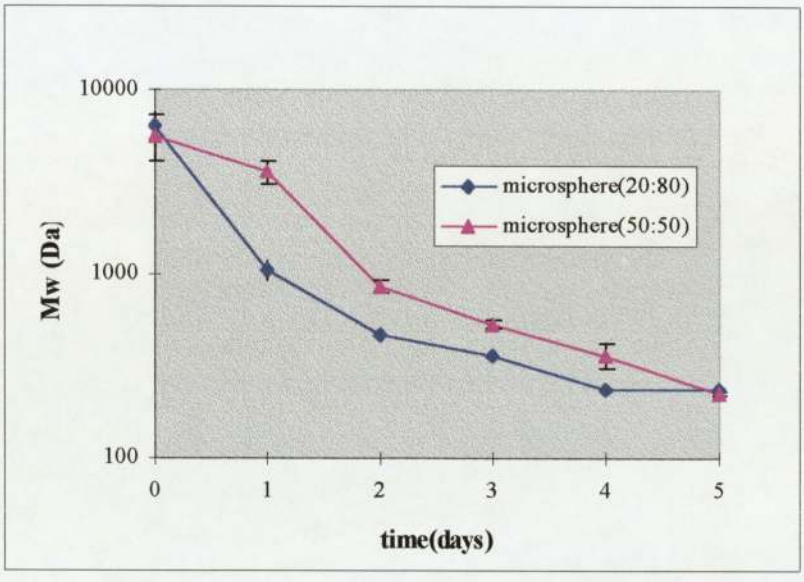


Figure 5.9 GPC analysis of degradation of microspheres in PBS (represented by Mw decrease) (n=3, mean±s.d.)

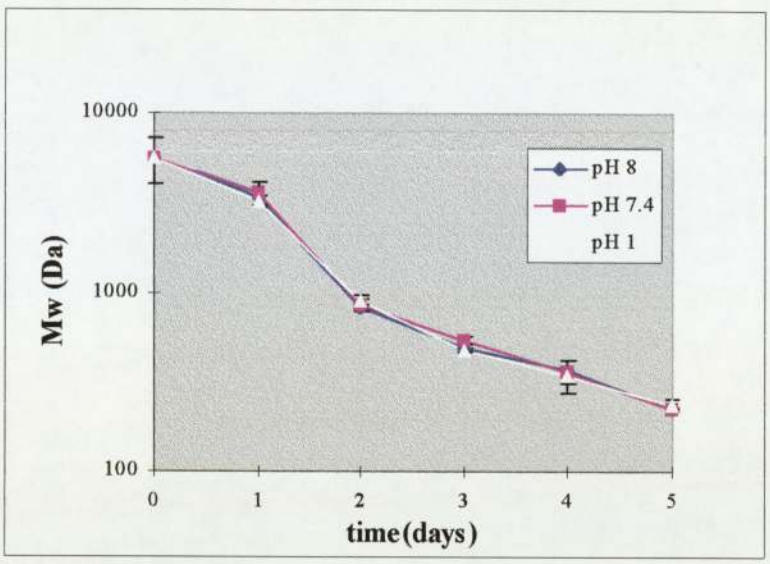
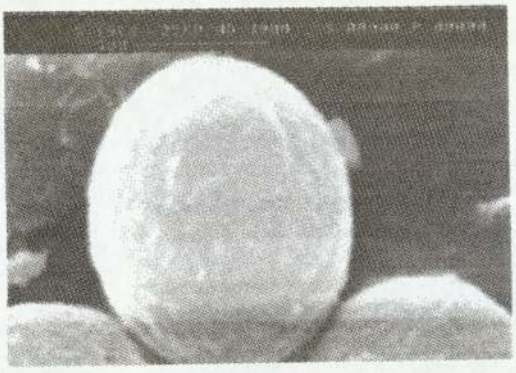


Figure 5.10 Effect of buffer pH on microspheres (50:50) degradation (n=3, mean±s.d.)

5.4.2 Microsphere Morphology Study during the Degradation

Microsphere morphology at specific times during degradation in PBS was observed by Scanning Electron Microscopy (SEM).

Morphological studies of poly(CPB:SA 20:80) microspheres during degradation were carried out by SEM. It was observed that after 2 days in buffer, the microspheres had degraded significantly, however, they still maintained some structural integrity indicating that the polymer had not been completely hydrolysed. However, after 5 days in buffer, the microspheres appeared to have lost structural integrity, indicating that the polymer was in large part degraded at this point (see Figure 5.11).



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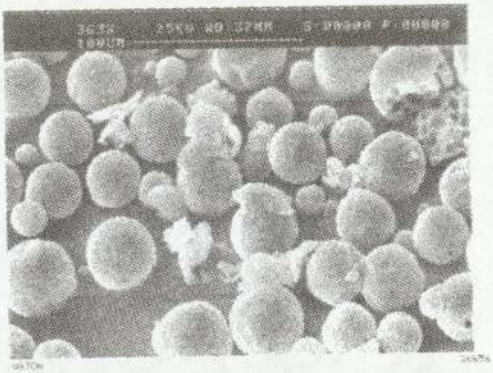

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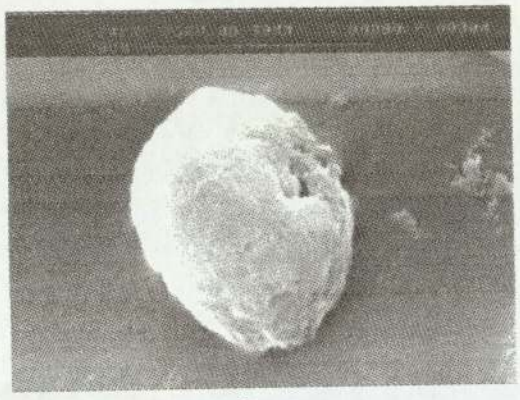

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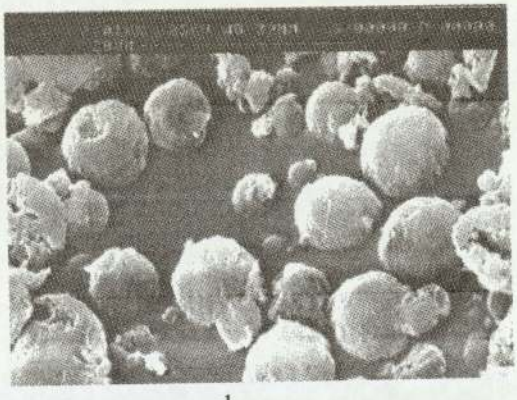

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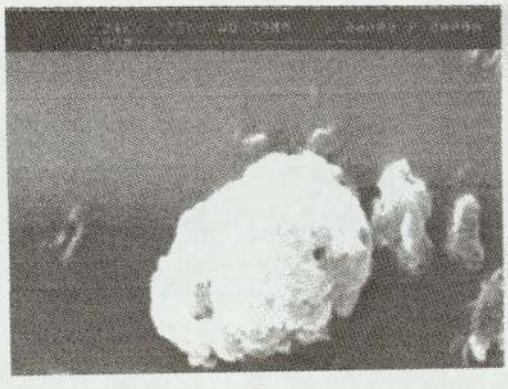

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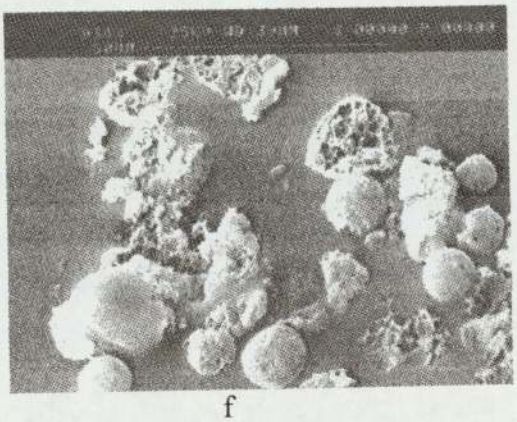


f

Figure 5.11 SEM micrographs of poly(CPB:SA 20:80) microspheres before and after *in vitro* degradation.

Figure 5.11a. close-up of a single microsphere before degradation

Figure 5.11b. batch of microspheres before degradation

Figure 5.11c. close-up of a single microsphere after 2 days of degradation

Figure 5.11d. batch of microspheres after 2 days of degradation

Figure 5.11e. close-up of a single microsphere after 5 days of degradation

Figure 5.11f. batch of microspheres after 5 days of degradation

5.5 Protein Release Studies

5.5.1 Experimental

5.5.1.1 Calibration

A series of protein standards was prepared in PBS. The concentrations of protein used were in the range of 10-200 μg/ml (see table 5.6).

Concentrations of protein (μg/ml)	2 mg/ml BSA solution (μl)	PBS (μl)
10	5	995
20	10	990
25	12.5	987.5
50	25	975
100	50	950
150	75	925
200	100	900

Table 5.6 A series of protein standards (protein in PBS)

Calibration curves were obtained by using bicinchoninic acid protein assay (BCA) method.

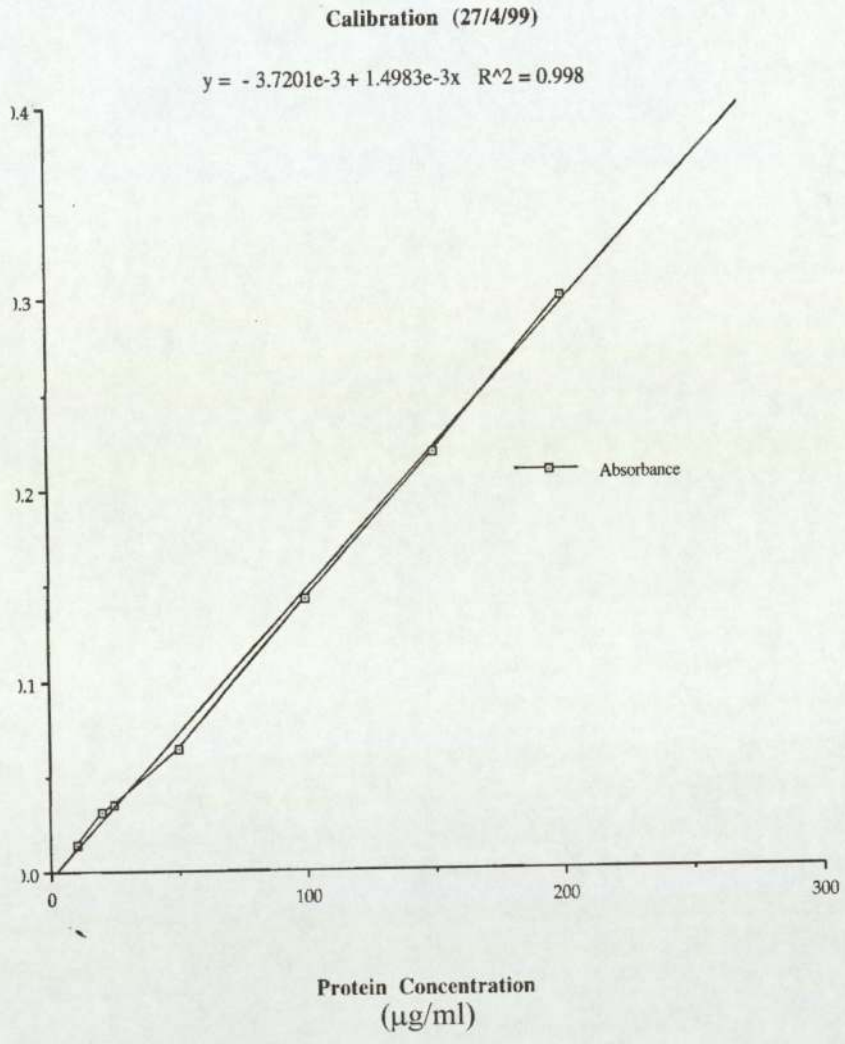


Figure 5.12 Calibration curve for protein release

5.5.1.2 Incubation

The microspheres formed using a range of copolymer ratios, the microspheres with different BSA loading and the microspheres made of different initial molecular weights of polyanhydride were suspended in release buffer (0.01 M phosphate-buffered saline pH 7.4) and incubated at 37°C on an orbital shaker. At various times the sample tubes were removed from the incubation, centrifuged at 13000 rpm for 10 min and the PBS solution (100μl) was collected and replaced with fresh PBS. The protein in the samples was determined by bicinchoninic acid protein assay (BCA) method.

5.5.2 Results and Discussion

5.5.2.1 Release of Different BSA Loaded Microspheres

Changing the protein load of the microspheres can be used to achieve different total BSA release rates. Figure 5.13 shows that the rate of BSA released from microspheres during a given length of time is observed as increasing with the initial BSA load.

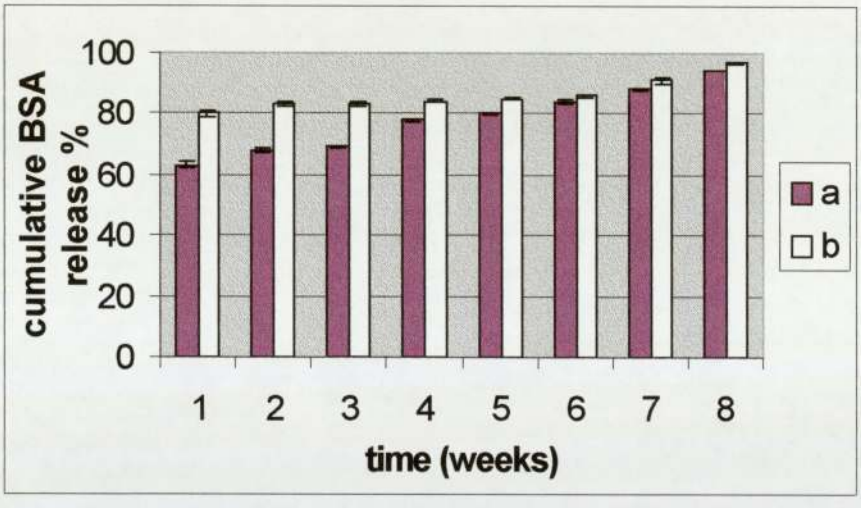


Figure 5.13 Release of different initial BSA loaded microspheres made of poly(CPB:SA 20:80) (n=3, mean±s.d.).

a. poly(CPB:SA 20:80) microspheres containing 2.6%w/w BSA;

b. poly(CPB:SA 20:80) microspheres containing 7.1%w/w BSA.

5.5.2.2 Release of Drug From the Microspheres of Different Polymer Composition

Polymer composition is the most important tool by which to achieve vastly different macromolecule release rates and release times from poly(CPB:SA) microspheres. The overall BSA release rate, as well as the amount of protein released during the initial release phase, increased with increasing amounts of SA monomer in the polymer backbone (see Figure 5.14). As a result, polymers with high SA contents, demonstrated increased rates of protein release. It is theoretically possible to deliver drugs for periods ranging from hours to months just by changing the ratio of SA to CPB in the polymer.

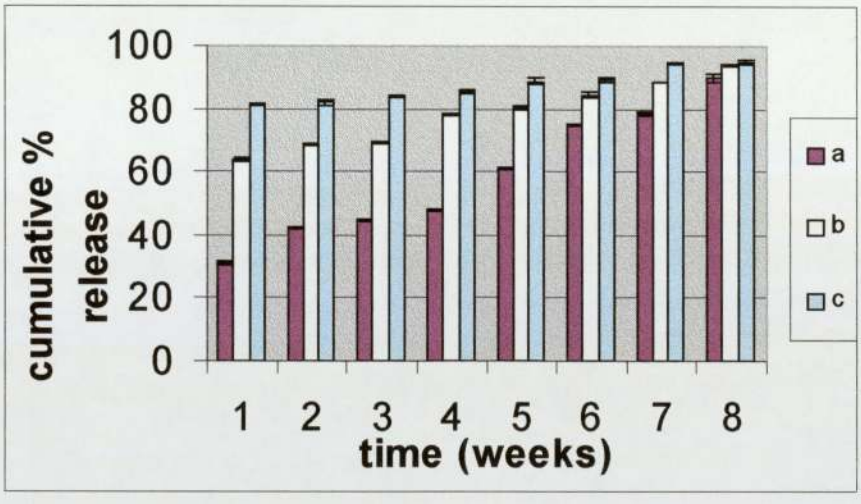


Figure 5.14 Cumulative percentage BSA released from microspheres made from a range polymers (n=3, mean±s.d.).

a. poly(CPB:SA 50:50) microspheres containing 7.3%w/w BSA;

b. poly(CPB:SA 20:80) microspheres containing 7.2%w/w BSA;

c. poly(CPB:SA 10:90) microspheres containing 7.0%w/w BSA.

5.5.2.3 Release of Different Initial Mw Microspheres

To determine whether polymer molecular weight could have an effect on BSA release rates from poly(CPB:SA) microspheres, polymers with molecular weight ranging from 7256 to 33781 were used to encapsulate and the BSA release rates were determined. It was found that initial molecular weight of polymer had no significant effect on protein release rates (see Figure 5.15). That may be because microspheres made of poly(CPB:SA 20:80) were degraded by hydrolysis which plays the most important role to degradation and release, role of initial molecular weight can be ignored.

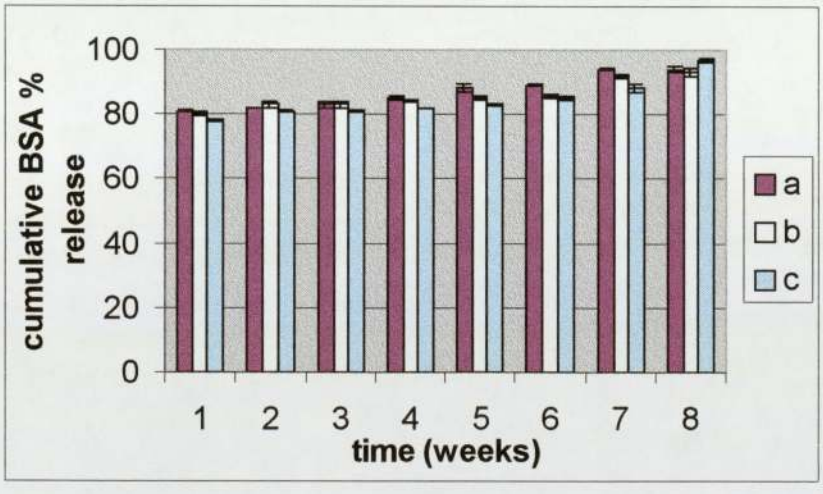


Figure 5.15 Release of different initial Mw microspheres made of poly(CPB:SA 20:80) (n=3, mean±s.d.).

a.microspheres made of poly(CPB:SA 20:80) Mw 33781 containing 7.2%w/w BSA;
b.microspheres made of poly(CPB:SA 20:80) Mw 14027 containing 6.7%w/w BSA;
c.microspheres made of poly(CPB:SA 20:80) Mw 8096 containing 7.1%w/w BSA.

Chapter 6

Conclusions

The use of catalyst was effective in the synthesis of polyanhydrides. The polymerisation in the presence of cadmium acetate, a known catalyst in polyanhydride synthesis, resulted in polymers with higher molecular weight in a shorter reaction time.

Using the ratios of NMR peak's integration, practical mole ratios of CPB-SA in the poly(CPB:SA) were calculated, and there were no significant differences between theoretical mole ratios and practical mole ratios. Polyanhydrides could be synthesised by melt-polycondensation successfully.

During degradation, the decrease in the molecular weight of polyanhydrides occurred until the molecular weight of polyanhydrides decreased to the monomer molecular weights. Because CPB was more hydrophobic monomer, degradation occurred more rapidly in polyanhydrides with low percentages of CPB.

Degradation is defined as water uptake accompanied by hydrolysis of anhydride bond, so the pH levels did not have a significant effect on the degradation rate of polyanhydrides.

Microspheres were made using the double emulsion solvent evaporation process. The microspheres were spherical with smooth surfaces and encapsulated about 70 % of the protein.

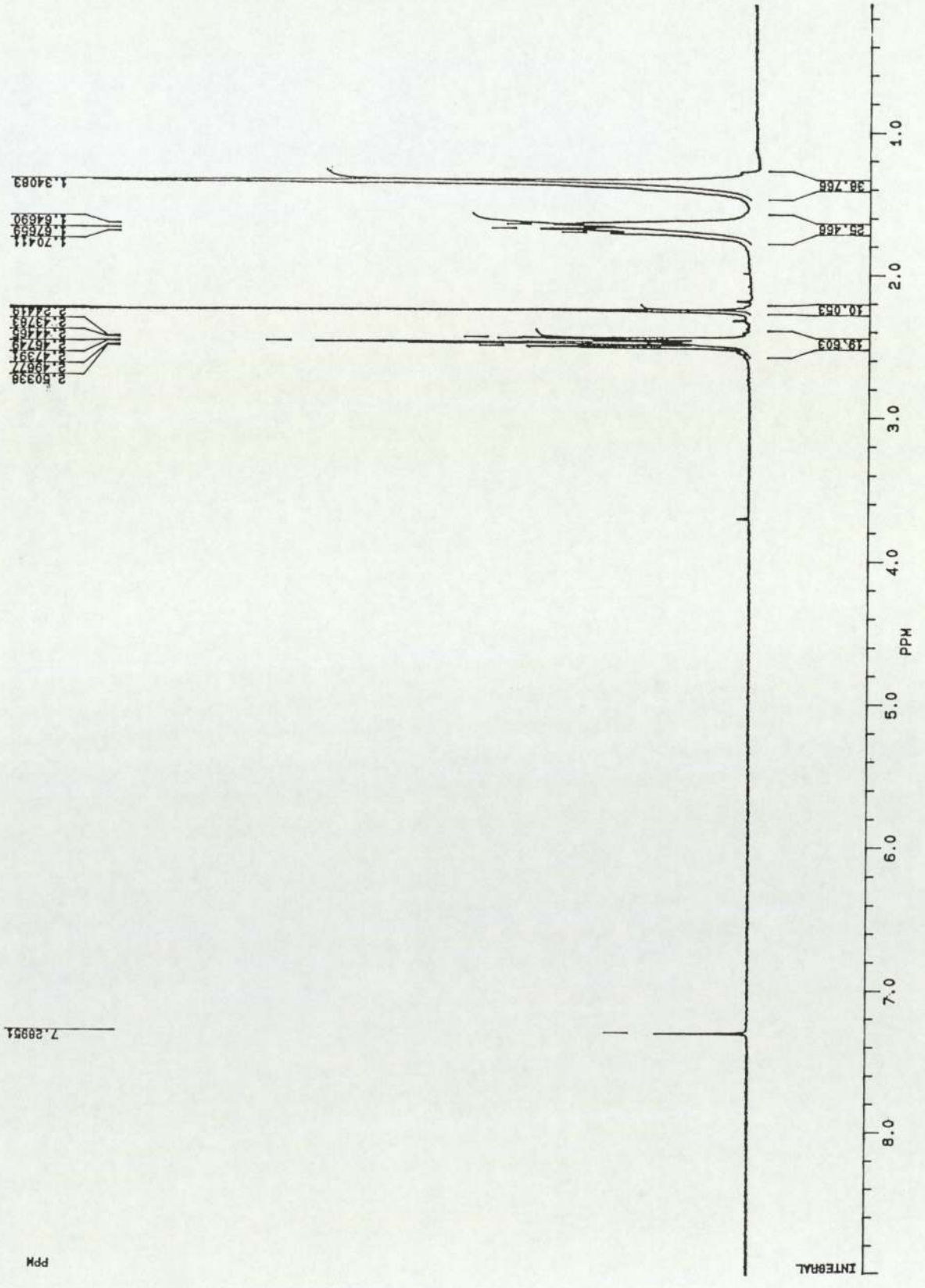
The rates of BSA released from microspheres during a given length of time increased with the initial BSA load.

Due to the fast degradation of polyanhydrides, role of initial molecular weight could be ignored. The initial Mw of polymer had no significant effect on protein release rates.

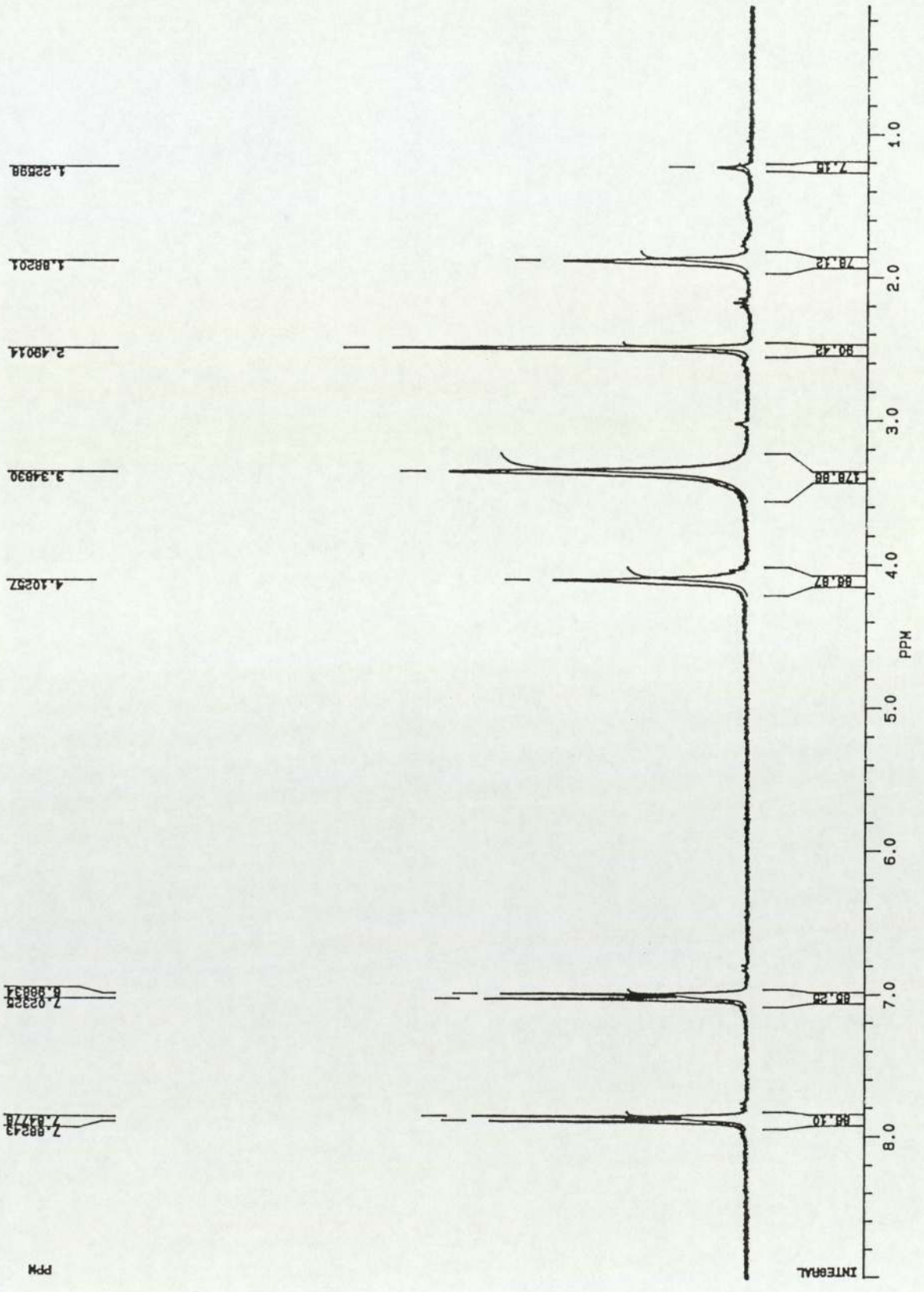
Polymer composition played an important role in the release of protein. The protein release rates increased with increasing percentage of SA monomer in the polymer backbone, due to the hydrophilicity of SA. It is theoretically possible to deliver protein for period ranging from hours to months just by changing the ratio of SA to CPB in the polymer.

Appendices

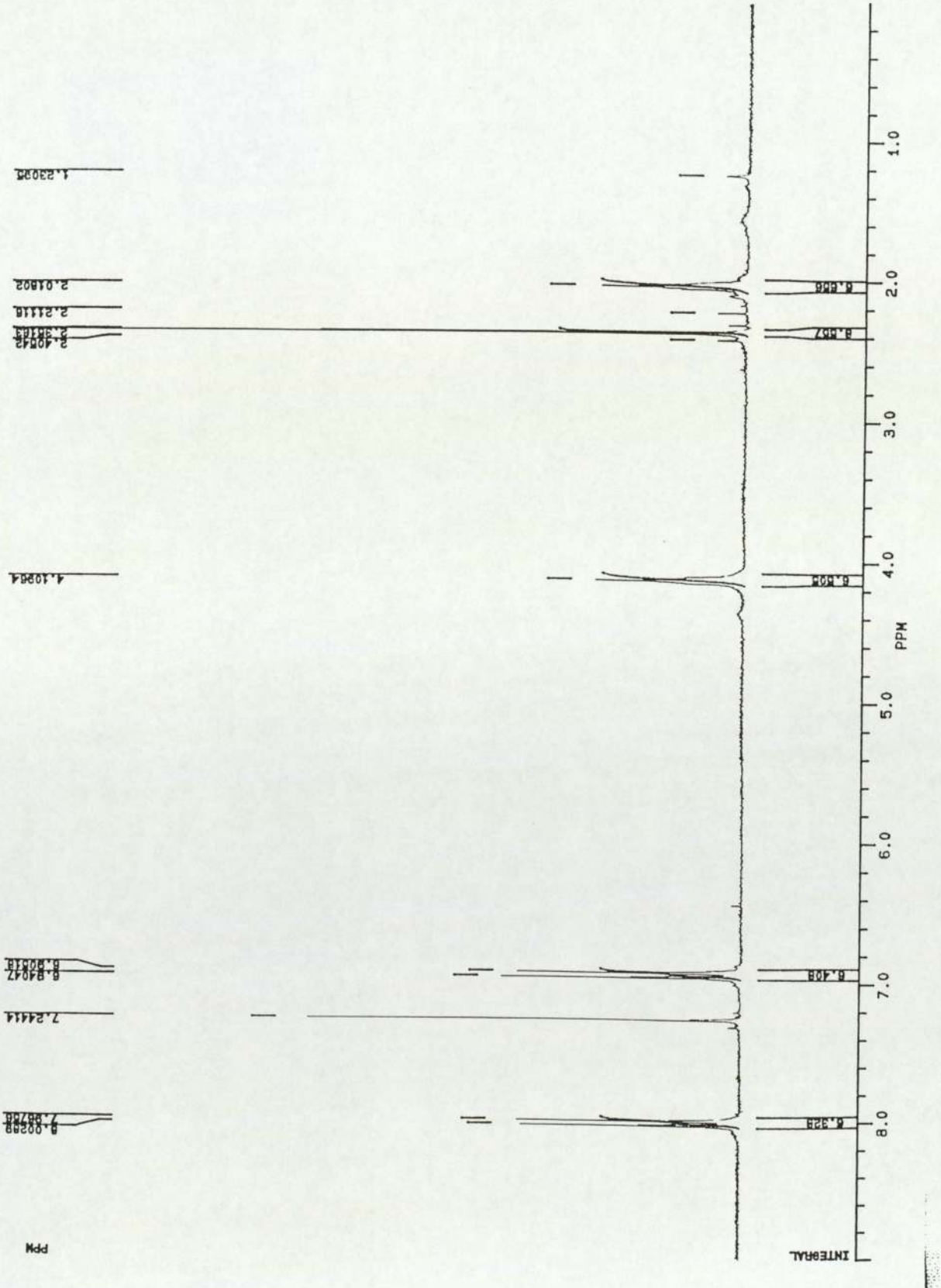
Appendix 1 NMR Characterisation of Sebacic Acid Anhydride Prepolymer (SAA prepolymer)



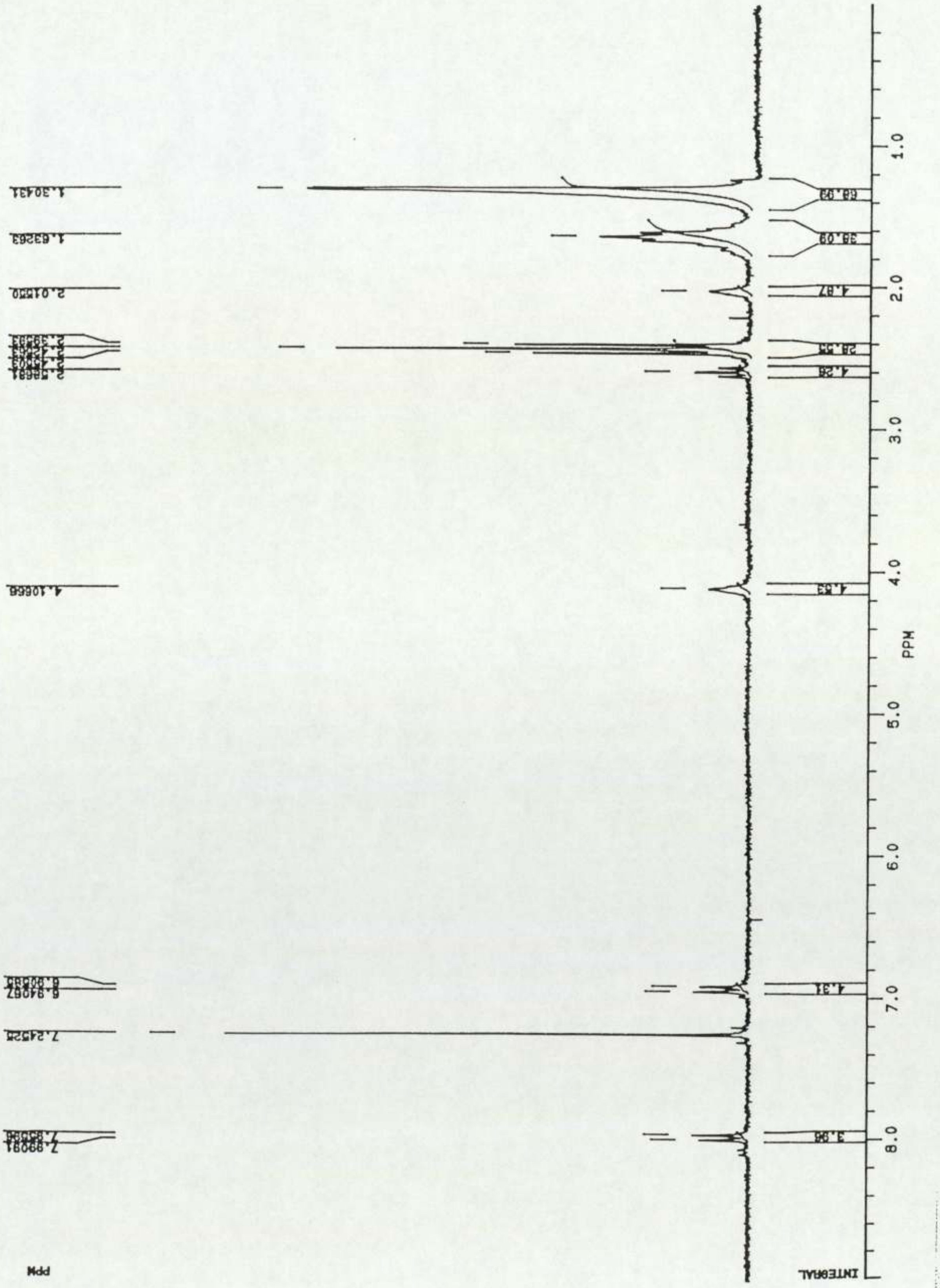
Appendix 2 NMR Characterisation of 1,4-Bis (p-carboxyphenoxy) butane Prepolymer (CPB prepolymer)



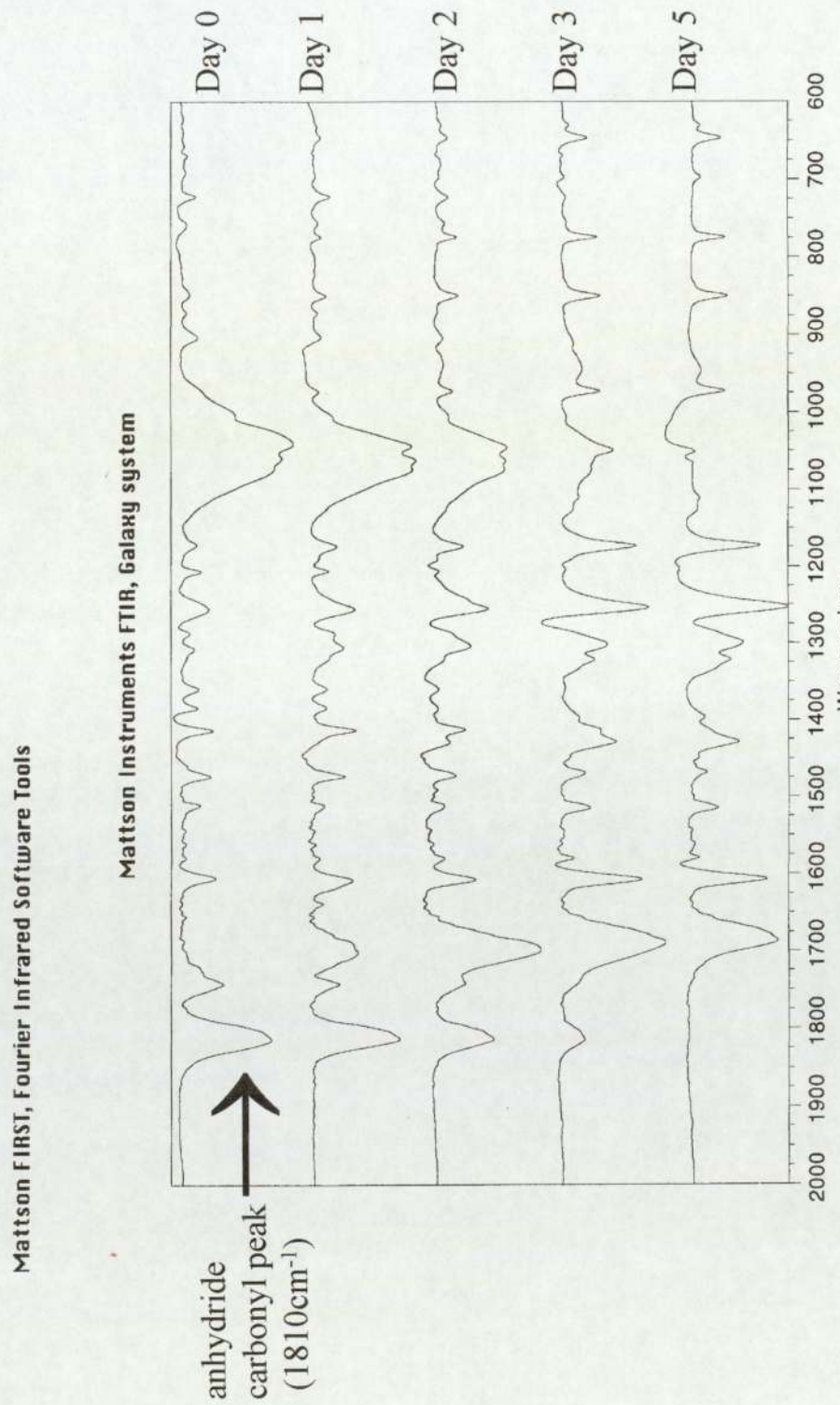
Appendix 3 NMR Characterisation of 1,4-Bis (p-carboxyphenoxy) butane Anhydride Prepolymer (CPBA prepolymer)



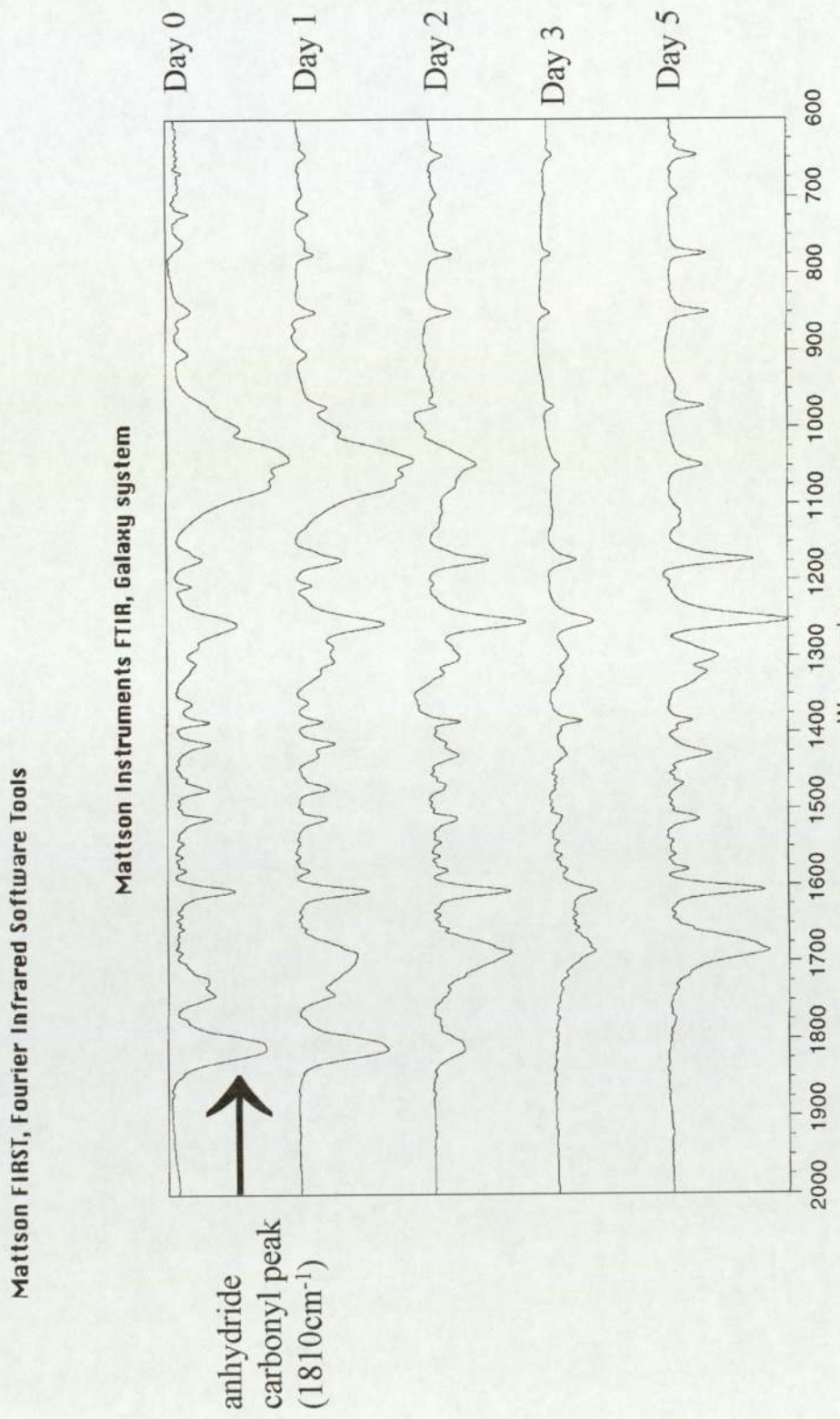
Appendix 4 NMR Characterisation of Poly (CPB:SA)



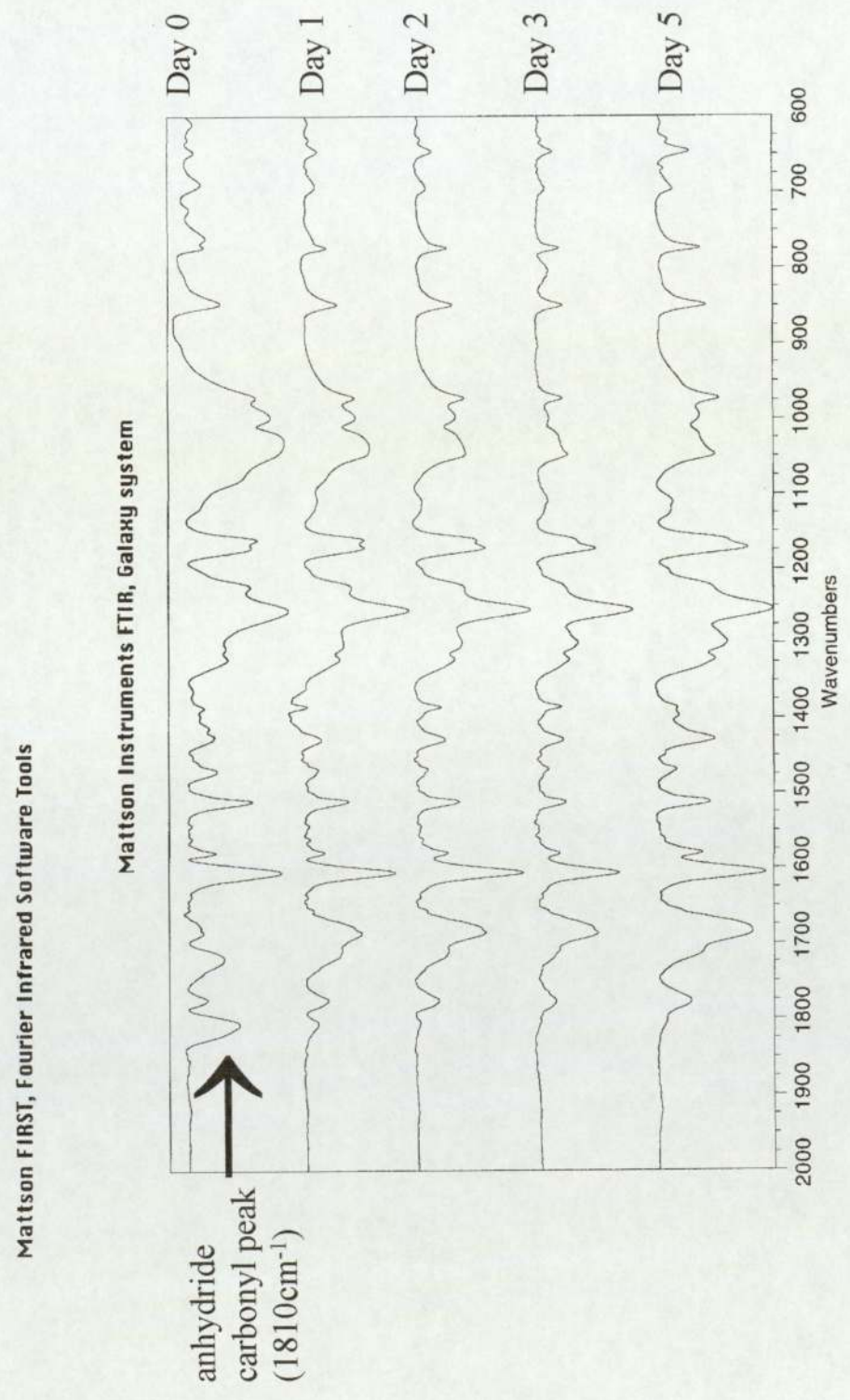
Appendix 5 IR spectra showing degradation of poly(CPB:SA 50:50) over time in PBS



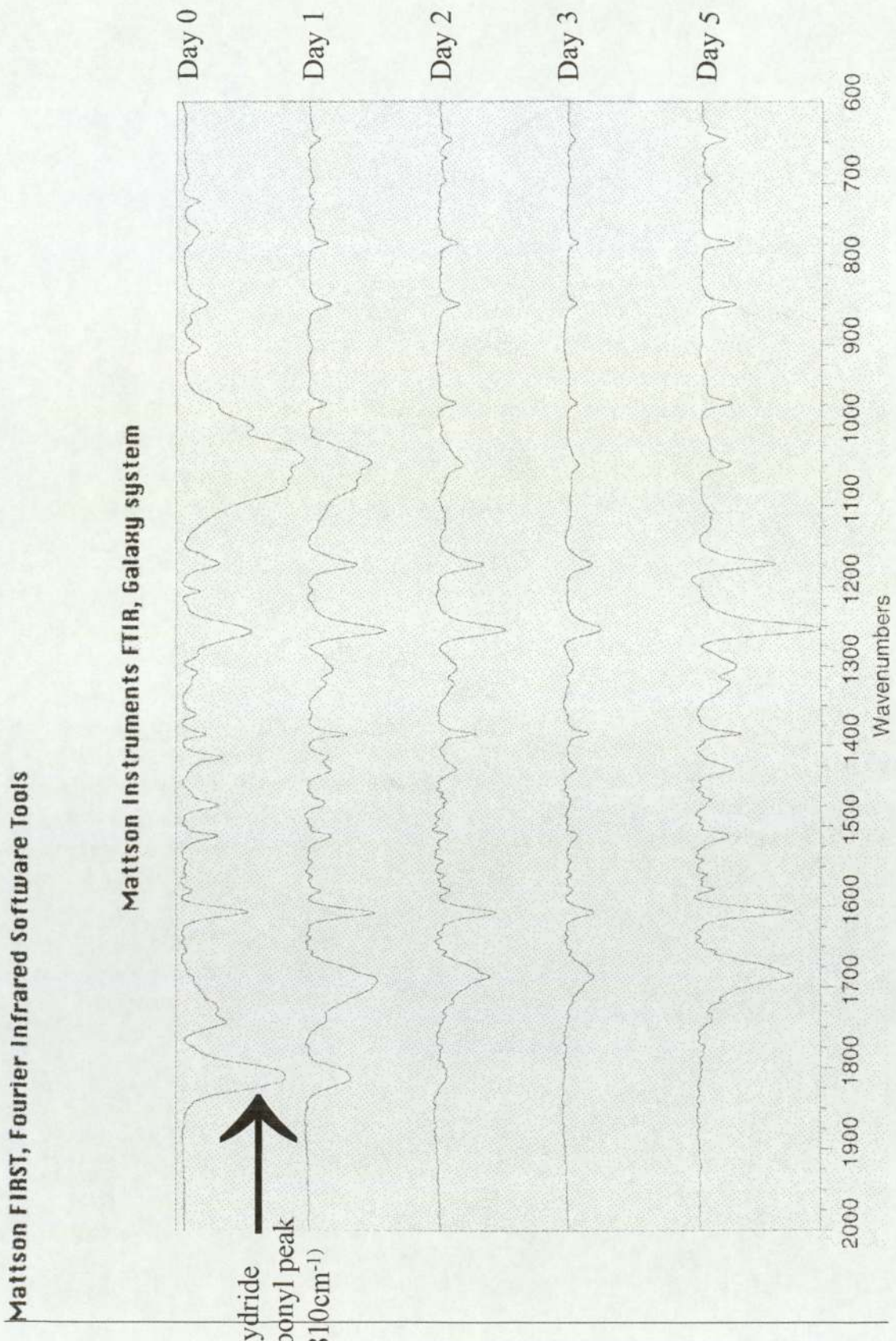
Appendix 6 IR spectra showing degradation of poly(CPB:SA 20:80) over time in PBS

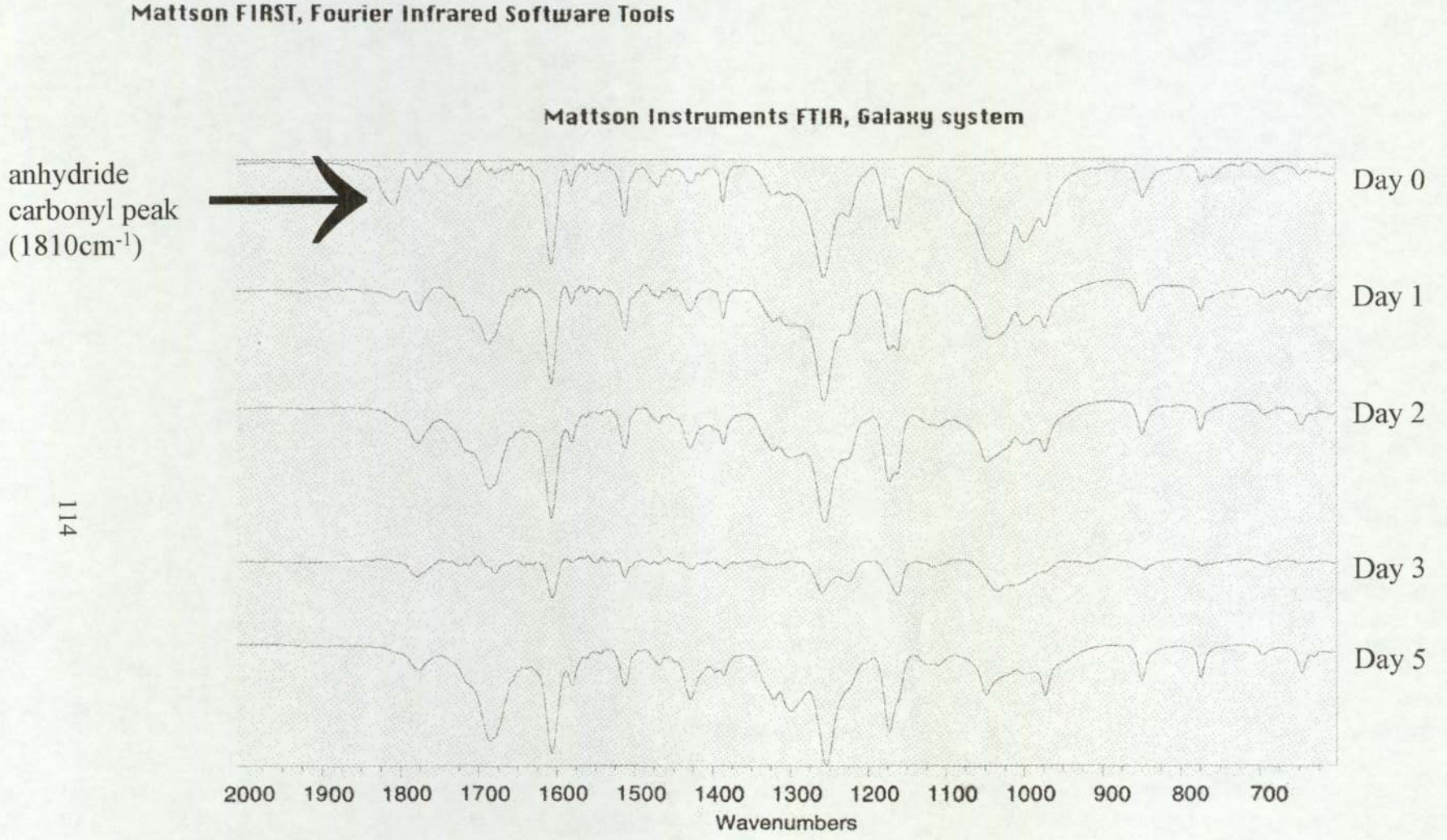


Appendix 7 IR spectra showing degradation of poly(CPB:SA 10:90) over time in PBS



Appendix 8 IR spectra showing degradation of poly(CPB:SA 50:50) microspheres over time in PBS





Appendix 9 IR spectra showing degradation of poly(CPB:SA 20:80) microspheres over time in PBS

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