BIODEGRADABLE POLYANHYDRIDES AS DRUG DELIVERY SYSTEMS

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Abstract: Polyanhydrides are useful biodegradable vehicles for controlled drug delivery. In aqueous media the breaking of the anhydride bonds resulting in gradually polymer fragments collapse and release drugs in a controlled manner. In this study, two new biodegradable polyanhydrides copolymers were synthesised using a melt-polycondensation method. The first is poly (bis (*p*-carboxyphenoxy)-2-butene-co-sebacic acid) (CP2B: SA), which has double bonds along the polymer backbone. The second is crosslinked poly (glutamic acid-sebacic acid-co-sebacic acid) (GluSA: SA), where the conjugated unit of glutamic acid with sebacic acid (glutamic acid-SA) acted as a crosslinking fragment in producing the crosslinking polymer. The two polymers were applied in preparation of microspheres with bovine serum albumin (BSA) as a model protein, using both double emulsion solvent evaporation and spray drying methods. The characterisation of the microspheres, morphology, particle size, and drug loading, was studied. The *in vitro* hydrolytic degradation of polymers and blank microspheres was monitored using IR, GPC, and DSC. *In vitro* drug release behaviour was also studied. Though the studies showed cleavages of anhydride bonds occurred rapidly (<5 days), bulks of the polymer microspheres could be observed after a few weeks to a month; and only around 10-35 % of the protein was detectable in a four-week period *in vitro*. We found the pH of the medium exerts a large impact on the release of the protein from the microspheres. The higher the pH, the faster the release. Therefore the release of the protein from the polyanhydride microspheres was pH-sensitive due mainly to the dissolution of monomers from the microspheres.

Keywords: biodegradable polyanhydrides, drug delivery, controlled release, microspheres, double emulsion, spray drying.

To my family, especially to Mum and Dad

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Chapter 1 Introduction

1.1 The Delivery of Proteins, Anticancer and Antibiotic Therapies

Since the 1970s', when genetic engineering emerged to be a practical method for application of significant quantities of proteins and peptides drugs, the therapeutic potential of proteins and peptides for treatment of diseases has been attracting biomedical research. In contrast to synthetic small molecule drugs, proteins generally have a high molecular weight and are susceptible to proteolysis, chemical modification, and denaturalisation during storage and administration. Their typically poor bioavailability can result from their degradation in the gastrointestinal tract or low permeability of epithelial barriers for high molecular weight molecules. Although 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. Thus, 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 (Tabata *et al.*, 1993).

There are also a number of restrictions in the delivery of anticancer drugs and antibiotics. The traditional methods for delivery of anticancer agents and antibiotics to the pathological site are mainly through intravenous perfusion, and the drug is transported throughout the body. Associated drawbacks of this can be systemic toxicity characterised by delayed haematopoietic depression, cytotoxic effects on kidney, liver and central nervous system, and the short exposure time of the drug to

the targeted organ (Taylor *et al*, 1990). For example, in osteomyelitis treatment, it is difficult to treat satisfactorily by systemic administration due to the short-life of the osteomyelitis and poor circulation to the infected area. However, a high systemic toxicity would result from employing high doses of antibiotic for long periods of time by a combination of routes. A commercial implant product (Septopal®) comprising poly (methyl methacrylate), (PMMA), beads loaded with gentamicin has been approved for use in Europe (Seligson and Henry, 1991) to treat osteomyelitis. Although, the implant can resolve some problems such as decreasing the systemic toxicity, this delivery system has the disadvantage that it cannot degrade and must be removed at a later date.

The problems associated with protein, anticancer drug and antibiotic administration have necessitated the development of drug delivery systems. Scientists have undertaken intensive research for the design of more reliable and effective drug delivery systems, including biodegradable polymeric drug delivery systems. They can be designed to deliver the drug continuously and maintain the concentration within the therapeutic window for an extended period or in a pulsate fashion to target site of action. Additionally, there is no need for surgical removal of the device. Furthermore, this system would protect sensitive drugs from decomposition or elimination before release.

1.2 The Advantages of Biodegradable Polymer Drug Delivery Systems

Applications that take advantage of polymeric materials that degrade at an increased rate in a biological environment have emerged over the last 20 years. Polymer degradation has advantages for all those cases where the post-treatment removal of the materials is inconvenient or even impossible. Degradable polymers were first used as biomaterials for manufacturing resorbable surgical sutures e.g., poly (glycolic acid) PGA and poly (lactic-co-glycolic acid) PLGA sutures (Herrmann *et al.*, 1970; Miller and Williams, 1985), and orthopaedic fixture materials e.g., poly (L-lactide) PLA as plates and screws for internal fracture fixation (Tschakaloff *et al.*, 1994). Since then, tremendous efforts have been undertaken to apply these biomaterials in areas such as drug delivery (Langer, 1990) or scaffolds for tissue engineering (Langer and Vacanti, 1993). For the manufacture of pharmaceutical preparations, synthetic biodegradable polymers were first introduced in the field to serve as drug carriers, diffusion barriers or protective coatings.

Biodegradable polymeric drug delivery systems have several advantages compared to conventional drug therapies. These include improved patient compliance, avoidance of the peaks and valleys of drug plasma levels associated with conventional injections, localised delivery of drug to the targeted organ, thereby lowering the systemic drug level, protection of drugs that are rapidly degraded in the body, and improved drug efficacy. In addition, they do not need to be removed from the body, which is an obvious advantage over non-degradable systems. Moreover, because drug delivery is

controlled primarily through the properties of the polymer, the release of both conventional low-molecular-weight drugs and the release of macromolecular drugs including hormones e.g., insulin, growth hormone, polysaccharides e.g., heparin, antibodies, antigens, and enzymes, is possible (Tamada and Langer, 1992). Furthermore, biodegradable polymeric systems may be suitable for the delivery of unstable drugs. This is because for a non-degradable matrix, the steps leading to drug release are water diffusion into matrix, dissolution of drug solutions, and out-diffusion of the solute. The mean residence time of drug particles existing in solution state is therefore longer for a non-degradable than for a biodegradable matrix, since a long passage through the channel for a biodegradable matrix may not be required. It is conceivable that a fraction of drug is decomposed inside the non-degradable matrix before it can be released (Leong *et al.*, 1985).

1.3 Degradation and Erosion of Biodegradable Polymers

The degradation and erosion behaviour is the most important characteristic of biodegradable polymers. A better understanding of polymer degradation and erosion processes is essential for a better understanding of the properties of biodegradable polymers as drug delivery systems, and solving some questions. These include stability of proteins and peptide drugs in a constantly changing environment when polymer degrades or erodes mechanical stability of polymers during erosion, and the impact of polymer erosion on drug release.

1.3.1 The Definition of Degradation and Erosion

Degradation can be based on enzymatic or hydrolytic breakdown of polymers. Enzymatic degradation is mainly relevant to natural polymers such as proteins, polysaccharides or poly (β-hydroxy esters), where specific enzymes exist (Park *et al.*, 1993). Hydrolysis is by far the most important degradation mechanism for synthetic polymers, since for most of them, no specific enzymes exist (Göpferich, 1996a). The term "biodegradable" is used for materials, where degradation is mediated at least partially by a biological system (Helder *et al.*, 1990).

Polymer degradation is the chain scission process that breaks polymer chains down to oligomers and finally into monomers, and is the most important part of erosion. Through degradation, oligomers and monomers are created that finally diffuse to the polymer surface, where they release from the polymer bulk. Erosion is the sum of all these processes that finally lead to the loss of mass from the polymer bulk, and is a complicated process that involves various reaction and transport processes (Brunner and Göpferich, 1996).

The distinction between degradable and non-degradable polymers is not clean-cut and is in fact arbitrary, as all polymers degrade. Only the time they require for degradation is different, for example, polyanhydrides require hours, but in case of poly (ethylene vinyl acetate) it is many years. It is the time-scale of polymer applications that seems to distinguish degradable from non-degradable polymers. Degradable polymers degrade during their application or immediately after it. Non-degradable polymers

require a substantially longer time to degrade than the duration of their application (Göpferich, 1996a).

1.3.2 Factors Affecting Polymer Degradation

There are several factors that influence the velocity of polymer degradation. The types of chemical bonds by which the polymer is built, pH of the degradation medium and copolymer composition are the most important (Göpferich, 1996b).

1.3.2.1 The Type of Polymer Bond

Among the factors that affect polymer degradation, the types of chemical bonds in polymer chains are the most important. The structures and the half-life of functional groups that are typical for degradable polymers are given in ***Table 1.1***:

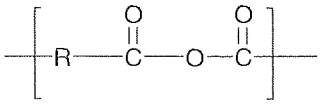
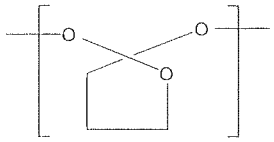
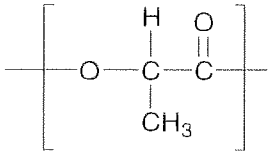
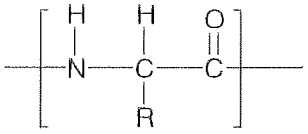
Structural features	Polymer class	Half-life
	Polyanhydrides	0.1 hour
	Polyorthoesters	4 hours
	Polyesters	3.3 years
	Polyamides	83,000 years

Table 1.1 The structures and the half-life of functional groups of typical degradable polymers

Redrawn from Göpferich (1996)

It can be seen that carboxylic acid anhydrides and orthoesters are the most reactive bonds, which makes them fast degrading polymers (Brunner and Göpferich, 1996).

1.3.2.2 The pH of Degradation Medium

For most synthetic degradable polymers, bond hydrolysis was found to depend markedly on the pH of the degradation medium *in vitro*. The hydrolysis of functional groups can be catalysed by acid or base (Göpferich *et al.*, 1995). For example, for polyanhydrides, the hydrolysis rate of carboxylic anhydride bonds increased

significantly from pH 7.4 to pH 10 in buffer. Conversely, polyorthoesters degrade faster at acidic pH compared to neutral pH (Heller, 1985). By employing acidic or basic additives, the rate of polymer hydrolysis can be varied in a controlled way. For example, taking advantage of the pH dependence of orthoester hydrolysis, preferential hydrolysis from the surface is obtained by either addition of basic substances to suppress degradation in the bulk, or incorporation of acidic catalysts to promote degradation on the surface (Leong *et al.*, 1985).

Conversely, the degradation products of polymers can affect the pH of the degradation medium. The pH of buffer medium decreased during the period when polyanhydrides and polylactides degraded, as the degradation products are acidic. In the case of polyanhydrides p (bis-(p-carboxyphenoxy) propane: sebacic acid) (CPP: SA), after one-day degradation, the pH of the buffer medium dropped from 7.4 to 6.6 (Göpferich, 1997). The shorter the half-life of polymer bonds, the faster monomers are created upon degradation causing a rapid decrease in pH (Göpferich, 1997). Additionally, changes in the internal pH during degradation have been investigated of as well. The pH inside eroding poly (lactic acid) and poly (lactic-co-glycolic acid) rods can be as low as 2, even when exposed to a pH 7.4 buffer (Martin *et al.*, 1996).

1.3.2.3 Copolymer Composition

In addition to incorporating pH-regulating substances, changing the polymer matrix structure is also a useful method to control polymers degradation. There are two principal methods of achieving this: copolymerisation and polymer blending. In the

first case, poly (lactic-co-glycolic acid) is a good sample. Example as with increasing glycolic acid content, poly (lactic-co-glycolic acid) degrades faster as the ester bond is more accessible to water (Pitt *et al.*, 1981). An example of polymeric blending is the introducing 9,10-dihydroxystearic acid, a hydrophilic monomer, into the back bone of poly (orthoesters) (Heller, 1990) or by blending poly (vinyl alcohol) and poly (lactic-co-glycolic acid) (Pitt *et al.*, 1981). Both these were found to increase degradation rate compared to non-blended polymers.

Other factors that depend on the copolymer composition, including the glass transition temperature and the crystallinity of copolymers, can affect the polymers degradation. Generally, the degradation rates of polymers depend on the prevailing type of bond (Göpferich, 1996b).

1.3.3 Polymer Erosion

Polymer degradation finally leads to erosion and erosion is the release rate-controlling process. The useful lifetime of the biodegradable polymers devices *in vivo* depend on their erosion duration (Tamada and Langer, 1993).

There are two factors that compete with each other and define how degradable polymers erode: the diffusion of water into the polymer bulk and the degradation of polymer bond (Heller, 1984). If the diffusion of water into the polymer is faster than polymer degradation, the polymer may swell prior to erosion and that may be a major factor controlling the performance of a degradable polymer (Langer, 1990; Brunner and Göpferich, 1996). If polymer degradation is faster than water ingress, polymer

swelling may be of minor importance. Depending on which process prevails, two different erosion mechanisms have been proposed: surface or heterogeneous erosion, and bulk or homogeneous erosion as shown in ***Figure 1.1***.

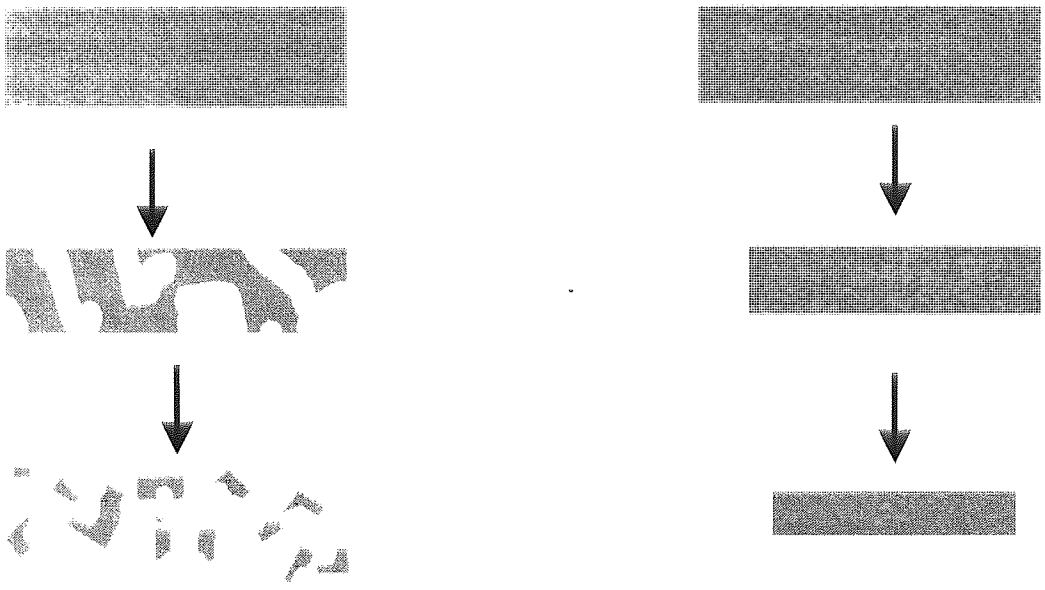


Figure 1.1 Schematic figure depicting the surface and bulk erosion, the mechanisms by which biodegradable polymers erode

Redrawn from Park *et al.* (1996)

In surface erosion, polymer degradation is faster than the water intrusion into the polymer bulk and, therefore, is confined to the polymer surface. As a result, erosion also affects only the outermost polymer layers. In contrast, bulk erosion polymers degrade slowly and, because of the fast water diffusion into the bulk, throughout their cross section. Herein, therefore, erosion cannot be limited to the polymer surface. Polymers containing reactive functional groups tend to degrade rapidly and to exhibit

surface erosion, whereas polymers built by less reactive bonds tend exhibit bulk erosion (Brunner and Göpferich, 1996).

Typical examples of bulk eroding polymers are poly (lactic acid) and poly (lactic-co-glycolic acid) (Göpferich, 1996a). In bulk erosion, water penetrates into the bulk of the polymer, resulting in degradation occurring through out the polymer matrix at the same time that leads to pores and channels in the matrix. As a result, it is complex and difficult to accurately control the matrix erosion and drug release, and it enables the possibility of dosage dumping as the system eventually hydrolyses (Pitt and Schindler 1979; Parks *et al.*, 1996).

As materials are lost mainly from the surface, the surface eroding polymers decrease in their dimensions. The advantage of surface erosion is that it is predictable, and drug release is related to the erosion rate (Göpferich *et al.*, 1995). Polyorthoesters and polyanhydrides have been reported to display surface erosion (Mathiowitz *et al.*, 1993).

Polymer erosion is far more complex than degradation, because it depends on many other processes, such as degradation, swelling, the dissolution and diffusion of oligomers and monomers, and morphological changes (Göpferich, 1996a). Moreover, degradation is not mandatory for a polymer matrix to erode. If the polymer is at least partially soluble in the erosion medium, for example, dissolution processes might contribute to the erosion. Conversely, if the polymer has degraded completely, it does not necessarily erode (Göpferich, 1996b). In the case of polyanhydrides, the molecular weight can decrease substantially during the first 12 hours, while there is almost no loss of mass and no change in geometry (Göpferich and Langer, 1993).

1.3.4 Polymer Erosion and Other Factors Determining Drug Release

If the drug release is intended to be erosion-controlled, the relationship between erosion velocity and other kinetically important steps has to be considered. It is the relationship between the timescales of three processes that determines how drug release is controlled: (1) drug and water diffusivity; (2) polymer swelling; (3) polymer erosion (Göpferich, 1996). A degradable polymer might release drugs by all three mechanisms. The quickest mechanism, however, will dominate (***Figure 1.2***). The faster a polymer erodes, the greater its chances that drug release might be erosion-controlled. It is obvious that polyanhydrides are an ideal material for producing erosion-controlled drug delivery systems.

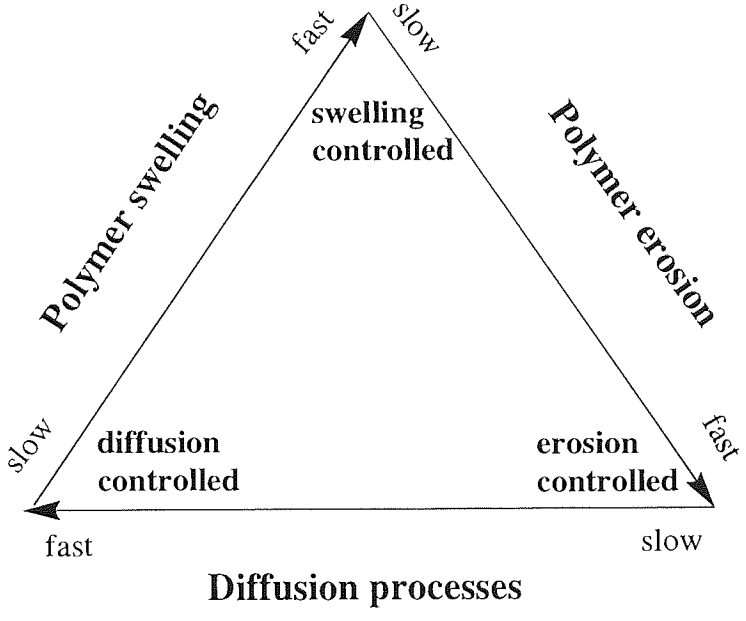


Figure 1.2 Possible mechanisms of drug release from degradable polymers

Redrawn from Brunner and Göpferich, (1996)

1.4 Biodegradable Polymers for Drug Delivery Systems

During attempts to achieve biodegradable polymeric drug delivery systems, varieties of biodegradable polymers have been studied and have shown their unique attractions.

Both natural and synthetic biodegradable polymers have been studied for drug delivery. The former includes polypeptides and proteins (e.g., albumin, fibrinogen, gelatin, and collagen), polysaccharides (e.g., hyaluronic acid, starch, and chitosan, virus envelopes) and living cells (e.g., erythrocytes, fibroblasts, and myoblasts). Synthetic polymers including aliphatic polyesters of hydroxy acids [PLA, poly (glycolic acid) (PGA), PLGA, poly (hydroxybutyric acid) (PHBA), poly (ε-caprolatone)], poly (orthoesters), poly (alkycarbonates), poly (amino acids), polyanhydrides, polyacrylamides and poly (alkyl-α-cyanoacrylates) have been investigated.

Natural polymers such as protein and polysaccharides usually vary in purity and often require crosslinking in the formulation process, which can lead to denaturalisation of the polymers and the embedded drug. On the contrary, the synthetic polymers can dissolve in organic solvents in which a lipophilic drug can be dissolved, and a hydrophilic drug can be suspended or emulsified as an aqueous solution to prepare the pharmaceutical formulation. Therefore, synthetic polymers are preferable for the development of commercial products (Piskin, 1995).

Lactide/glycolide homo- and copolymers (PLA/GA) and polyanhydrides (poly (bis-(p-carboxyphenoxy) propane: sebacic acid) (CPP: SA) and poly (fatty acid dimer:

sebacic acid) (FAD: SA)) are the only two classes of degradable polymers with FDA-approved controlled release products (Hanes *et al.*, 1998).

1.4.1 Biodegradable Polyesters

Poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymers (PLGA) are the most significant among the linear polyesters, and remain a popular choice as the biodegradable drug carriers.

Homo- and copolymers of lactic and glycolic acids are synthesised by a ring-opening polymerisation of the cyclic dimers, lactide and glycolide (***Figure 1.3***). Direct condensation of lactic acid and glycolic acid yields homo- or copolymers with low molecular weight in the range of 10-15 kDa (Deasy *et al.*, 1989). The ring opening method with a catalyst, such as dialkyl zinc, trialkyl aluminium, and tetraalkyl tin in which the lactide and/or glycolide rings form a cyclic dimer, produces high molecular weight polymers. Therefore, its polymers consist of L-, D-, and D, L-lactic acid in which the L- or D-polymers have a crystalline form, and the D-, L-polymers are amorphous and more rapidly degradable. Because of its additional of methyl group, PLA is more hydrophobic than PGA. Both PLA and PLGA are soluble in organic solvents, such as chloroform, dichloromethane, acetone, and ethyl acetate, to a variable extent, depending on copolymers composition and molecular weight (Kissel and Koneberg, 1996).

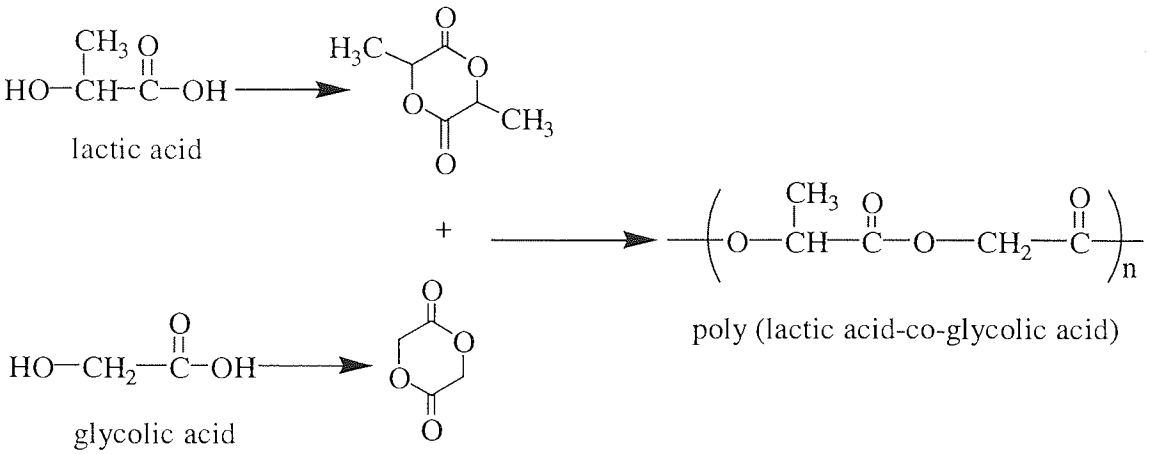


Figure 1.3 Poly (lactic acid-co-glycolic acid) (PLGA)

The degradation products of PLGA, lactic and glycolic acids are physiologically occurring metabolites. Craig *et al.* (1975) originally demonstrated the biocompatibilities of PLA and PLGA using sutures. These polymer sutures when implanted into rats induced a mild local inflammatory reaction. The well known biodegradation and biocompatibility profiles of PLGA have led to this material being widely used.

PLA and PLGA have been approved by regulatory authorities for drug delivery systems and there are drug delivery systems made from PLGA copolymers available as marketed products, such as Zoladex®, Enantone® and Decapeptyl®, and each of them releasing peptide analogues of LHRH. At the same time, an increasing number of researchers have focused on designing new systems loaded with anticancer drugs such as Taxol and Camptothecin (Wang *et al.*, 1996; Ertl *et al.*, 1999).

On the other hand, PLGA has some inherent shortcomings. It is relatively hydrophobic, which may lead to stability problems of antigen during storage or under

in vivo release conditions (Kissel and Koneberg, 1996). Additionally, degradation of aliphatic polyesters occurs by random, non-enzymatic hydrolytic cleavage of ester linkages, usually referred as bulk erosion mechanism compared with surface erosion. As a result, a burst effect is often seen, and this burst effect is undesirable because it release an uncontrolled significant portion of the drug immediately or at sometime over the entire release period.

1.4.2 Biodegradable Polyamides

Polyglutamates with various ester content, and with chemically or enzymatically degradable bonds, have been evaluated as drug-carriers. The poly [(ter-butyloxycarbonylmethyl) glutamates] are obtained by partial esterification of poly (glutamic acid) with ter-butyl bromoacetete as shown in ***Figure 1.4***.

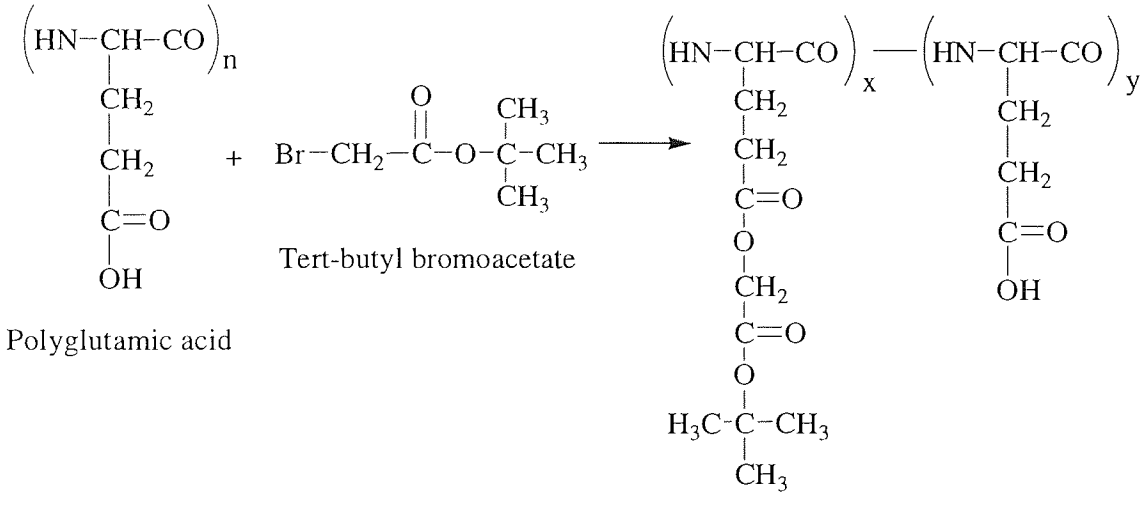


Figure 1.4 Poly [(ter-butyloxycarbonylmethyl) glutamates]

These polymers are amorphous and extricable at low temperatures. The degradation proceeds by cleavage of the tert-butanol side chain, leading to a soluble polymer,

followed by cleavage of the backbone by leucine aminopeptidase. The hydrophobicity of the polymer can be increased by the ester content. Good tissue biocompatibility is reported for the progesterone-impregnated polymers in rat implantation studies (Leong and Langer, 1988). However, due to the long time of degradation, the use of polyamide in drug delivery is limited.

1.4.3 Biodegradable Polyorthoesters

Polyorthoesters can be prepared by the addition of diols to diketene acetals. When these polymers are placed into an aqueous environment, an initial hydrolysis to a diol and γ-butyrolactone takes place. The γ-butyrolactone then rapidly hydrolyses to γ-hydroxybutyric acid (***Figure 1.5***). This hydrolysis is an autocatalytic process because the γ-hydroxybutyric acid degradation product accelerates hydrolysis of the acid-sensitive polymer. As a result, in order to prevent a catastrophic disintegration, a basic compound such as Na_2CO_3 must be added to neutralize the γ-hydroxybutyric acid. This polymer system is now marketed as Alzamer® and has been investigated as biodegradable inserts for the delivery of the narcotic antagonist, naltrexone, and for the delivery of the contraceptive steroid norethisterone (Heller, 1990). However, the steroidal implant was found to cause local tissue irritation in human clinical trials, and therefore further work with the formulation is still required.

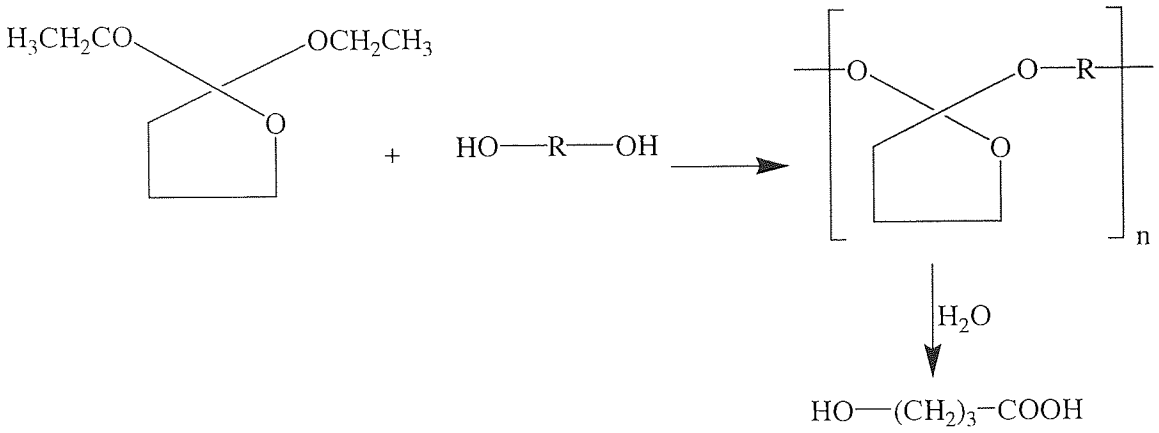


Figure 1.5 Poly (orthoester) I and its hydrolysis product

Moreover, a crosslinked polyorthoester (***Figure 1.6***) was prepared and has been investigated to release the LH-RH analogue Nafarelin® (Heller *et al.*, 1987).

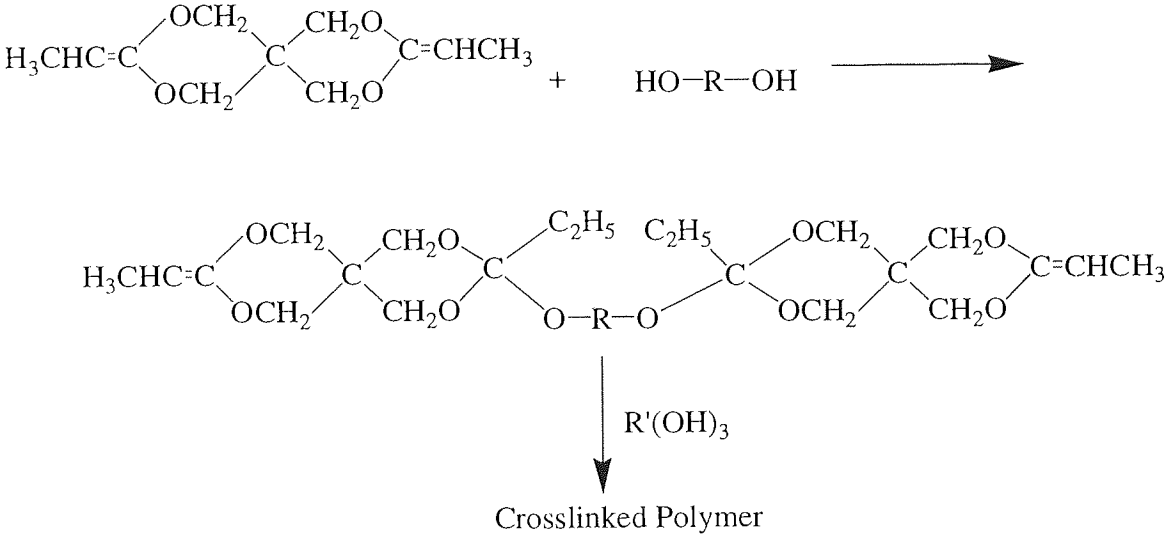


Figure 1.6 Poly (orthoester) II

A new generation of polyorthoesters (***Figure 1.7***) was developed as an ointment. These polymers have highly flexible chains so that they have a semi-solid consistency at room temperature. Therefore, the therapeutic agents can be mixed into polymer

matrix simply at room temperature without the use of solvents (Heller *et al.*, 1990). The polymers were under investigation for 5-fluorouracil (5-FU) and mitomycin C (MMC) release as an adjunct for use after glaucoma-filtering surgery (Bernatchez *et al.*, 1994).

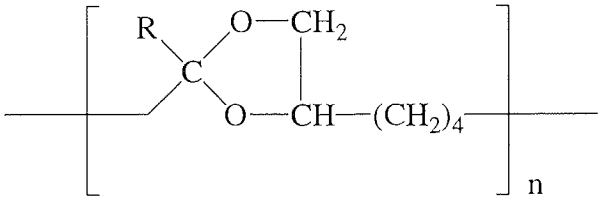
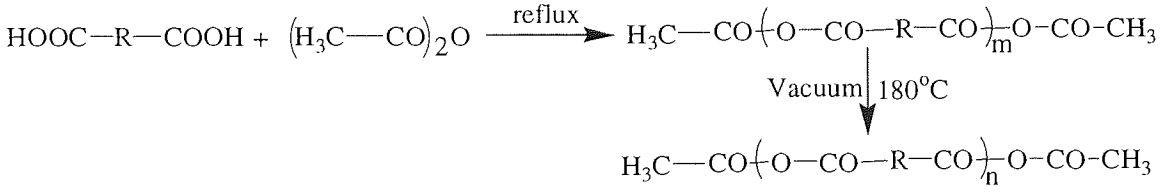


Figure 1.7 Poly (orthoester) III

1.4.4 Biodegradable Polyanhydrides

In last decade, biodegradable polyanhydrides, which erode in a controlled heterogeneous manner without requiring any additives, have been developed. Polyanhydrides have been synthesised widely by melt condensation of dicarboxylic acids treated with acetic anhydride (Domb *et al.*, 1995):



m =1-20; n =100-1000

Figure 1.8 Synthesis of polyanhydrides by melt-polycondesation

1.5 Formulation of Biodegradable Polymers for Controlled Drug Delivery Systems

In drug delivery systems, biodegradable polymers are used as carriers. From a formulation point of view the following systems can be distinguished (Kissel and Koneberg, 1996):

- Implants
- Microspheres
- Nanoparticles

1.5.1 Implants

In implant systems, the drug is incorporated into a biodegradable polymer. The polymer-drug mixture is formulated into devices such as rod- or disk-shapes suitable for implantation into body. These devices have been 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 (Kissel and Koneberg, 1996).

The implanted devices erode upon contact with body fluids, releasing the drug to the body. Implanted controlled release systems provide several advantages over conventional oral or injectable drug formulations. Delivery can be localised to the site of implantation, which lowers the drug dosage, thereby reducing potential systemic side effects. Drug delivery rates are steady and controlled, which can better maintain

the drug concentration within its therapeutic window. Moreover, implants can be designed and manufactured easily and much more uniformly compared to other dosage forms of biodegradable polymers (Göpferich, 1996). Local drug delivery using implants has already been used effectively for the release of anesthetics for the local tumour therapy (Masters *et al.*, 1993) and local antibiotic therapy (Stephens *et al.*, 2000). 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.

For the therapy of bacterial infections or cancer, the continuous administration of drugs over long time may cause a loss of sensitivity against antibiotics or cytostatics. To avoid such a loss of sensitivity, several chemotherapeutic agents are used one after another. For vaccines, the discontinuous administration of antigen may increase immunity. An interesting implantable drug delivery system made of a combination of polyanhydride and poly (D, L-Lactic acid), could meet these requirements. The programmable release implant was made of a second drug-loaded polyanhydride core and a first-drug loaded polyanhydride mantle. In order to avoid the premature release of the second drug, slow eroding poly (D, L-Lactic acid) was used as a layer. Such implant could be beneficial for the local treatment of cancer because it allows release of two drugs one after another or for vaccinations releasing antigens twice during a month (Göpferich, 1999).

However, surgery or painful injection is required for implant application, and the size of implants may not be tolerated when applied subcutaneously. Additionally, there are also safety concerns when they carry a large drug load. Autocatalytically accelerated

degradation may increase the release rate that could produce toxic blood levels (Göpferich, 1996a).

1.5.2 Microspheres

During the last 20 years, biodegradable polymer microspheres have emerged as a popular controlled release dosage form (Brunner and Göpferich, 1996). However, because it is actually difficult to distinguish between microcapsules and microspheres, the term "microparticles" was used. According to definition, the diameter of microspheres range between 1 and 1000 μm. ***Figure 1.9*** shows three types of structures of microparticles. ***Figure 1.9A*** shows a "true microcapsule", where drug (solid or liquid) is sourrounded by polymer matrix in a mononuclear state. This system can be classified as a reservoir. Microspheres, in which the drug is homogenously dispersed (see ***Figure 1.9B***) or dissolved (***Figure 1.9C***) in the polymeric matrix, are matrix-type microparticles. Hence, the term microcapsule should be reserved for reservoir type devices, whereas microspheres are monolithic or matrix-type microparticles (Kissel and Koneberg, 1996).

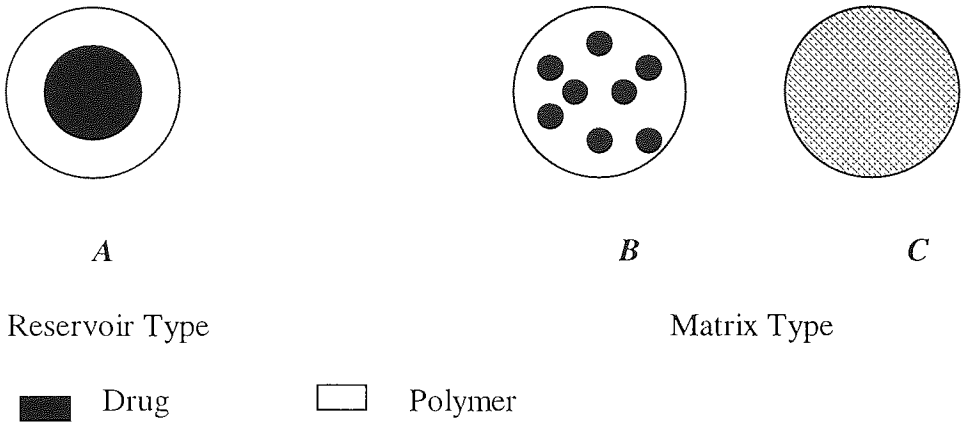


Figure 1.9 Typical structures of microparticles

Redrawn from Kissel and Koneberg (1996)

Microspheres offer advantages over large implants, since they can be injected in suspension. Microspheres also allow the encapsulation of drugs in polymer matrix, which can improve the stability of sensitive drugs such as vaccines and peptides, by protecting them form oxidative and hydrolytic degradation, thus increasing their therapeutic efficacies (Tabata *et al.*, 1993). Also, the drug loaded in a microsphere remains separated from that in other microspheres, so a further advantage is that the potential to administer multiple drugs in a single injection, that for compatibility reasons would otherwise need to be separated (Kipper *et al.*, 2002). For example, in 1989, the USA Food and Drug Administration (FDA) approved the first system to slowly release a peptide, Decapetyl®. It contains polymer microspheres that entrap an LHRH analogue (Ogawa *et al.*, 1985). This peptide, if given orally or injected in unencapsulated form, is rapidly destroyed. However, when placed in a polymer matrix, release can be sustained for 3 months (Okada *et al.*, 1994).

Microspheres, however, remain a very delicate and complicated drug delivery system. Even slight variations in the manufacturing process can change the properties of

microspheres and can cause problems in their particle morphology, microstructure, release behaviour and mechanisms (Göpferich, 1996b).

Common problems with microspheres are the burst release of drug and instability of sensitive protein and peptide drugs during the manufacturing procedure (Johnson *et al*, 1991; Göpferich, 1999). The problems can be solved by choosing the appropriate manufacturing procedures (Tabata *et al.*, 1993), or by using the appropriate polymers (Tabata *et al.*, 1993; Park *et al.*, 1996). For example, by employing water–in-oil (W/O) emulsion method, a water-soluble drug can be homogeneously distributed throughout the polymer matrix, which results in a great reduction of initial burst effect (Tabata *et al.*, 1993).

1.5.3 Nanoparticles

Nanoparticles have a diameter of less 1000 nm, which allows their intravenous application. Nanoparticles can also have a reservoir-like structure consisting of solid shell and an inner liquid core, or a matrix-type structure (Allémann *et al.*, 1993). Nanoparticles modify the pharmacokinetics and biodistribution of incorporated drugs, for example to increase the drug half-life and to improve drug targeting to a specific site action (Göpferich, 1996a). Poly (lactic acid) and poly (lactic acid-co-glycolic acid) have been used extensively. The major problem with nanoparticles is their rapid clearance from the bloodstream, which is non-specific and characteristic for all colloidal drug carriers (Göpferich, 1996a).

1.6 Polyanhydrides

As a class of biodegradable polymers, polyanhydrides were developed specifically for controlled release drug delivery applications about 20 years ago. Due to a highly water labile anhydride linkage and a hydrophobic backbone, polyanhydrides were proposed as a promising candidate that would erode in a heterogeneous manner without requiring any additives (Leong *et al.*, 1985).

1.6.1 Historical Perspective, Significance and Present Uses of Polyanhydrides as Biodegradable Polymers

Bucher and Slade reported synthesis of polyanhydrides for the first time in the 1930's, and Hill and Carothers prepared a series of aliphatic polyanhydrides for textile applications. During 1950s-1960s, more than 100 new polyanhydrides based on aromatic and heterocyclic diacid monomers were synthesised. However, despite further development, polyanhydrides were never commercialised for textile use because of their poor resistance to hydrolysis. In the early 1980's, the scientists at Massachusetts Institute of Technology proposed the use of polyanhydrides as biodegradable carriers for controlled drug delivery systems. They also observed that the degradation characteristics of polyanhydrides, which are not desirable for textiles, render them suitable as materials in biodegradable drug delivery systems. This revitalised the development of polyanhydrides.

Polyanhydrides were proposed as a promising candidate for drug delivery system, because polyanhydrides undergo "surface erosion" (Park *et al.*, 1996). The anhydride

linkage is water labile, and, by rational selection of monomer units, the polyanhydrides could be made sufficiently hydrophobic to discourage water penetration (Leong *et al.*, 1985), which make the polymers erode like a bar of soap from the outside to the inside and exclude water penetration into the bulk of the matrix. Ideal surface erosion provides release at a rate proportional to the surface area and aids in the delivery of water–labile drugs by minimising water interaction with the drug prior to release (Tamada and Langer, 1992).

Polyanhydrides are regarded as "designer polymers" because:

- They can be prepared from a large pool of monomers;
- They can be manufactured with various degrees of crystallinity (Tamada and Langer, 1992);
- They allow control of degradation rates and water uptake by varying the molar ratio of the monomers (Domb and Maniar, 1993);
- They can be synthesised with a branched structure (Maniar *et al.*, 1990), or they may be cross-linked (Domb *et al.*, 1991).

In addition, the hydrophobic nature of polymer, such as poly (bis-(p-carboxyphenoxy) propane: sebacic acid) (CPP: SA), would provide the encapsulated drug with some protection from degradation before release (Fleming and Saltzman, 2002).

The most important advantage for polyanhydrides is their biocompatibility in combination with drug release control (Brunner and Göpferich, 1996). The biocompatibility and safety of polyanhydrides were established by the 1986 guidelines of the Food and Drug Administration (FDA) for testing and evaluating new biomaterials. Several accepted criteria and tests to evaluate new biomedical materials

were applied to assess the safety of polyanhydrides (Leong *et al.*, 1986; Laurencin *et al.*, 1990). In their study, poly (bis-(p-carboxyphenoxy) propane) anhydride (CPP) and its copolymers with sebacic acid, poly (CPP: SA) (***Figure 1.10***), were tested. Neither nutagenicity nor cytotoxicity or teratogenicity was associated with polymers and their degradation products, as evaluated by mutation assays. The polymers did not induce inflammatory responses in the tissues over a six-week implantation period in rats and in the cornea of rabbits. Histological evaluation indicated minimal tissue irritation without evidence of local or systemic toxicity (Laurencin *et al.*, 1990). Systemic responses to the polymer were evaluated by determining blood chemistry and haematological values, and by comprehensive examination of organ tissues. Both methods revealed no significant response to the polymer (Domb *et al.*, 1997). Copolymers of sebacic acid (SA) with several aliphatic comonomers such as dimer of erucic acid (FAD) poly (fatty acid dimer: sebacic acid) (FAD: SA) (***Figure 1.11***), fumaric acid and isophthalic acid were also tested subcutaneously and in the rat brain were found to be biocompatible as well (Rosen *et al.*, 1983).

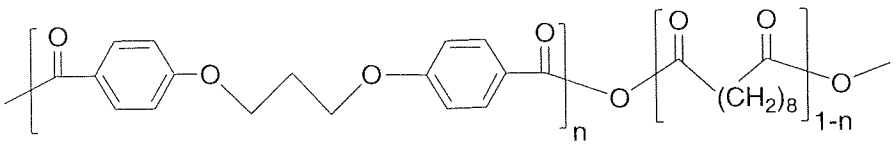


Figure 1.10 Poly (CPP: SA)

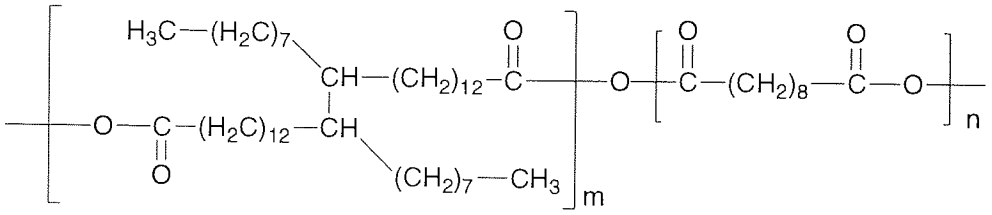


Figure 1.11 Poly (FAD: SA)

In the past ten years, several drug delivery applications have been realised using polyanhydrides. For example, the anticancer agent, 1,3-bis-(2-chloroethyl)-1-nitrosourea) (Carmustine), has been incorporated into poly (CPP: SA, 20:80) wafers (Gliadel®) by a compression molding of a spray dried polymer-Carmustine powder. This preparation has been approved by FDA for the site-specific chemotherapy for the treatment of brain tumours (Domb *et al.*, 1999), and is used for treating second surgery patients with glioma multiforma. This marks the first time in over 20 years that a new brain cancer treatment was approved and the first time ever that controlled release polymer-based chemotherapy was approved. In 2001, Gliadel® has received regulatory approved in 22 counties (Brem and Gabikian, 2001). At the same time, a number of investigations were made to new drugs, such as 4-hydroperoxy cyclophosphamide (4HC), cisplatin, carboplatin, Taxol and several alkaloid drugs, in an effort to develop a better system for treating brain tumours (Brem *et al.*, 1994; Olivil *et al.*, 1996; Judy *et al.*, 1995). Also, local anaesthetics were successfully delivered from polyanhydride cylinders in close proximity to the sciatic nerve to produce a neural block for several days (Masters *et al.*, 1993). Carboplatin incorporated in poly (FAD: SA), prepared by mixing the drug in the melted polymer

was evaluated for the treatment of brain tumours in laboratory animals with promising results (Olivil *et al.*, 1996). Poly (FAD: SA) has also been used to develop a delivery system (Septacin®). Septacin® is a product for the treatment of osteomyelitis. It is a controlled release implant that consisting of gentamicin sulfate dispersed into a biodegradable poly (FAD: SA, 1:1) matrix. Septacin® also serves as a useful "model system" for the release of a hydrophilic drug from an extremely hydrophobic polyanhydride matrix (Stephens *et al.*, 2000).

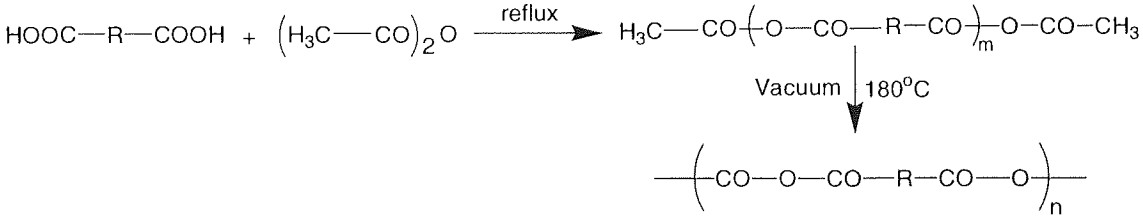
1.6.2 Synthesis of Polyanhydrides

Polyanhydrides have been synthesised by both melt-condensation of activated diacids and solution methods (Domb *et al.*, 1987). The solution methods include dehydrochloronation involving the reaction of acyl chloride with carboxylic acids (Leong *et al.*, 1987), dehydrative coupling using bis-(2-oxo-3-oxazolidinyl) phosphinic chloride and phenyl N-phenyl phosphoroamidochloridate (Leong *et al.*, 1987; Domb *et al.*, 1988), and a one-step polymerisation using phosgene or diphosgene coupling agents (Domb *et al.*, 1988). Solution polymerisation yielded generally low molecular weight polymers. The most commonly used method is the melt-condensation of two dicarboxylic acid anhydrides under high vacuum and at high temperature. The improved melt-polycondensation method adopted a catalyst to promote the formation of high molecular weight polyanhydrides (Domb and Langer, 1987).

High molecular weight of polyanhydrides is very essential for applications where superior physical and mechanical properties are required. In addition, by raising the molecular weight of polyanhydrides, even less hydrophobic polymers could exhibit film-forming properties. In the study of poly (CPP: SA), it was reported that increasing either percent CPP or the molecular weight increased tensile strength. Decreasing the Mn of films of the same CPP content (60%) from 12,100 to 6,400 resulted in lower tensile strength (Domb and Langer, 1987).

1.6.2.1 Melt-polycondensation

In 1932 Carothers and Hill prepared a prepolymer first by converting the carboxyl group to a mixed anhydride with acetic acid before subjecting the prepolymer to melt-condensation :



m =1-20; n =100-1000

Figure 1.12 Melt-polycondensation method

Melt polycondensation involves a series of steps. Prepolymers are synthesised by reflux of the diacid in excess acetic anhydride for several hours. This forms the mixed anhydride prepolymer. The crude prepolymer is purified by recrystallisation from dry

toluene, and then immersed in a 1:1 mixture of dry petroleum ether and diethyl ether to extract traces of acetic anhydride and toluene. This purificate step for prepolymers is found to be critical to further synthesis into high molecular weight polymers. The purified prepolymers are placed into a temperature-controlled vessel under high vacuum, where polymerisation takes place. The acetic anhydride, which is produced by the polymerisation reaction, is removed by vacuum. The optimum temperature for polymerisation was determined to be 180°C to synthesise polyanhydrides with high molecular weight. After polymerisation is completed, the polymer is purified by precipitation in dry petroleum ether from a dichloromethane solution. The precipitate is then extracted with dry diethyl ether for several hours at room temperature. The polymers are stored under dry nitrogen at -20 °C (Domb and Langer, 1987).

Since the polymerisation reaction is an anhydride interchange that involves nucleophilic attack on a carbonyl carbon, a catalyst that will increase the electron deficiency under the carbonyl carbon will affect the polymerisation (Chasin *et al.*, 1990). A number of effective coordination catalysts, including metal salts (cadmium acetate and zinc acetate), earth metal oxide (calcium oxide, barium oxide and calcium carbonate), alkoxy metals (Ti-isopropoxide and Al-isopropoxide), organometals ($ZnEt_2$) and ferric compounds, have been used in polycondensation to produce high molecular weight polyanhydrides. These catalysts are suggested for the transesterification polymerisation of polyesters, which is a reaction similar to the anhydride interchange. Also similar catalysts have been found to be effective in ring-opening polymerisation of epoxides due to metal oxygen complexation. Significantly higher molecular weights in shorter times were achieved by utilising cadmium acetate, earth metal oxides, and $ZnEt_2$-H_2O. The molecular weight ranged from

140,935 to 245,010 with catalysts, in comparison with 116,800 without catalysts. When a catalyst is used in synthesising polyanhydrides with high molecular weight, 2 molar percent catalyst is mixed with prepolymers prior to polymerisation (Domb and Langer, 1987).

However, this method suffers from certain limitations: the reversible thermal depolymerisation may limit the high molecular weight obtainable, and the acetic anhydride reflux may be not suitable for many heat-sensitive monomers (Leong *et al.*, 1987).

1.6.2.2 Dehydrochloronation

Polyanhydride formation can be effected under a milder reaction condition (at room temperature) by a dehydrochloronation between a diacid chloride and a dicarboxylic acid:

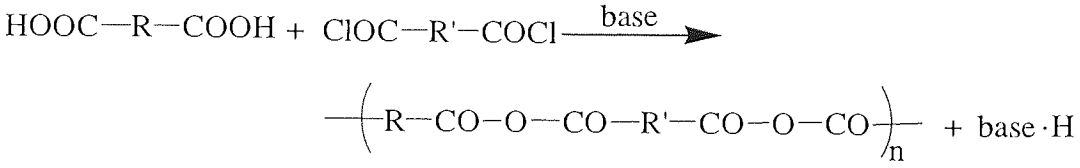


Figure 1.13 Dehydrochloronation method

It is an essential Schotten-Baumann condensation, a reaction extensively studied for polyamide, polyester, and polycarbonate synthesis. For a typical solution polymerisation, a defined amount of a diacid and a base is dissolved in solvent (*e.g.*, dichloromethane or chloroform). Acyl chloride is added to the magnetically stirred

mixture, and reaction flask stoppered by a moisture guard tube containing calcium chloride. After two hours of reaction at room temperature, the mixture is quenched in petroleum ether under agitation. This will also trap the triethylamine hydrochloride salt. For this reason, the yield was only estimated. Although the polymer can be cleaned by shaking the reaction mixture in cold methanol before quenching, the polymer would be slightly degraded. This has been shown by IR spectra (Leong *et al.*, 1987). However, only a few scientists have used this reaction for synthesising polyanhydrides, because the carboxylic hydrogen is less reactive than that of an amine alcohol, or mercaptan, the condensation is expected to be less effective. Nevertheless it was hoped that under optimal conditions that reaction might still be able to yield useful polyanhydrides (Leong *et al.*, 1987).

1.6.2.3 Dehydrative Coupling

Alternative synthetic routes, where sensitive monomers do not have to be subjected to the acylation conversion, have been considered. Powerful dehydrative coupling reagents were applied in the reaction due to their function in mild reaction conditions. One class of reagents that appears particularly promising is the organophosphorus compounds. In this reaction, the diacid dissolved in the presence of acid acceptor, was added in a single portion to a magnetically stirred solution containing the coupling agent. The reaction was conducted at room temperature in a stoppered flask. The following workup procedures were used: W1, the resulting suspension was filtered, the solid was washed with chloroform, and the filtrate was vacuum evaporated; W2, the reaction mixture was directly quenched into petroleum ether; W3, the reaction

mixture was extracted with cold dilute HCl, and the organic phase was then quenched into petroleum ether. This reaction was only used to prepare monomeric anhydrides (Leong *et al.*, 1987).

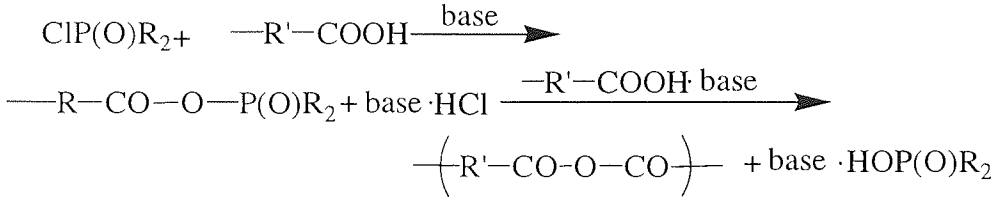


Figure 1.14 Dehydrative coupling method

1.6.2.4 One-step Polymerisation

In this method, the following reagents were used: dicarboxylic acid chloride (*e.g.*, sebacoyl chloride), phosgene (dichloroformate), or diphosgene (trichloromethyl chloroformate) as coupling agents, and a removable acid acceptor that effect a one-step polymerisation of dicarboxylic acids. These coupling agents are suitable for one-step polymerisation whereby the only by-product formed is a hydrochloric acid-acid acceptor salt. This acid acceptor is typically an amine base or potassium carbonate. This salt can be removed from the polymerisation mixture by either (1) using an insoluble acid acceptor (*e.g.*, crosslinked polyamides, inorganic bases) or (2) using solvents that dissolve exclusively either the polyanhydrides or the hydrochloric acid-acid acceptor salt. On the basis of the mechanism proposed for the reaction of phosgene and diphosgene, the suggested polymerisation mechanism is shown in ***Figure 1.15***. One-step polymerisation is a method to synthesis polyanhydrides at ambient temperature in solution. However, the coupling agents employed in this

reaction are hard to handle. For example, phosgene is a reactive gas and its vapour toxicity limits its applicability. Moreover, this solvent polymerisation results in lower molecular weight polyanhydrides compared to melt-polycondensation (Domb *et al.*, 1988).

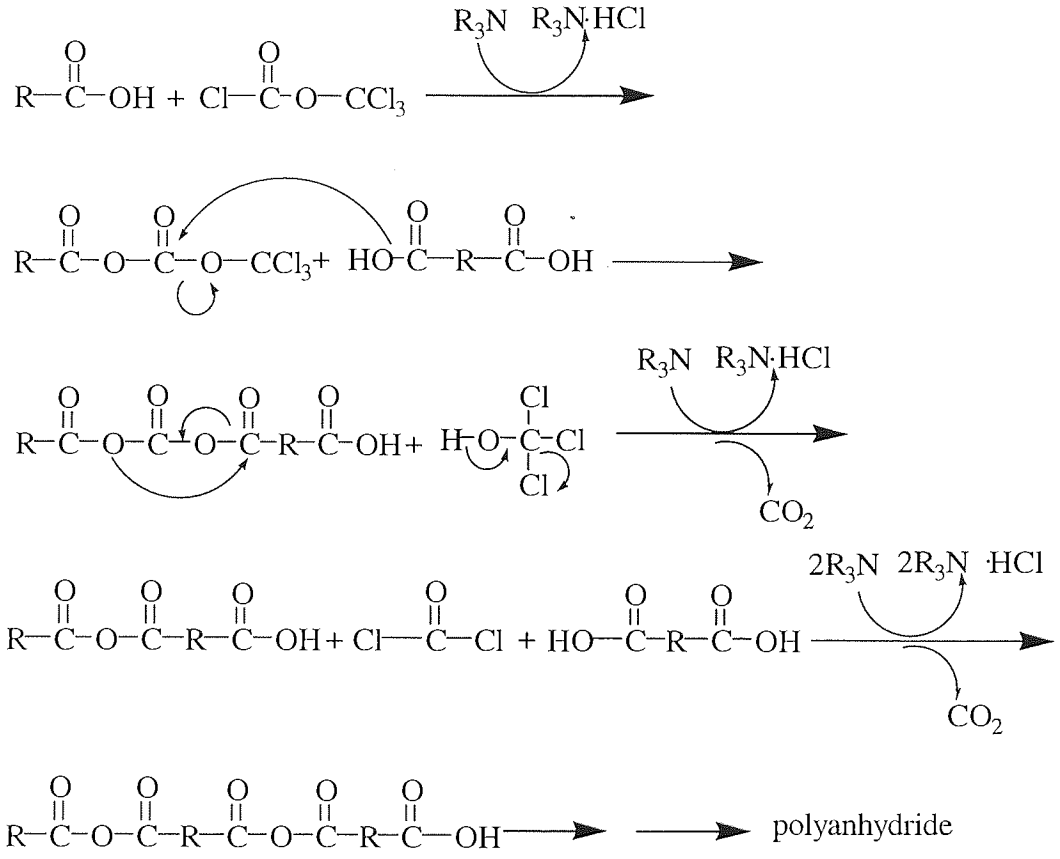


Figure 1.15 One-step polymerisation

1.6.3 Classes of Polyanhydrides

Since the discovery of polyanhydrides in 1909, hundreds of polymers structures have been reported. The monomers used for the synthesis of polyanhydrides are bifunctional with at least two carboxylic acid groups per molecule. General polyanhydrides structure is shown in ***Figure 1.16***.

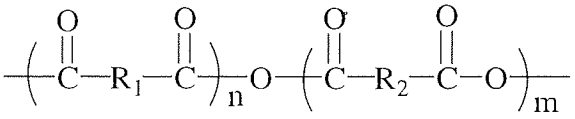
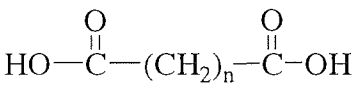


Figure 1.16 General polyanhydrides structure

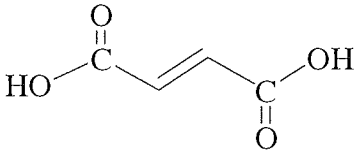
1.6.3.1 Monomers

The structures of the monomers determine the properties of the polymer. As a result, careful selection of the monomers is a crucial element in the development of polyanhydrides (Tamada and Langer, 1992). ***Figure1.17*** lists some of monomers used for preparation of polyanhydrides:

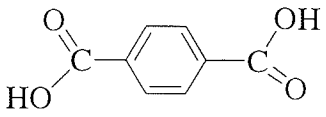


n = 8 sebacic acid (SA)

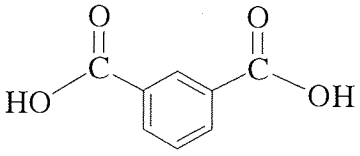
n =10 dodecandioic acid (DA)



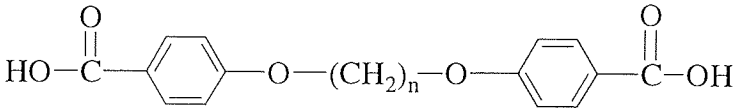
fumaric acid (FA)



isophthalic



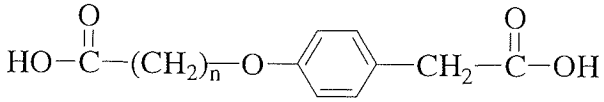
terephthalic



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

n = 2 1,3-bis(*p*-carboxyphenoxy)propane (CPP)

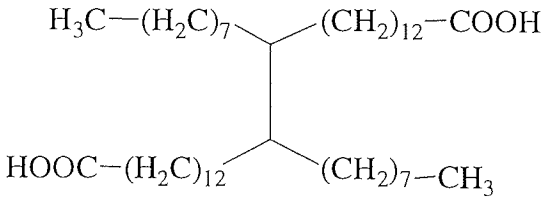
n = 6 1,6-bis(*p*-carboxyphenoxy)hexane (CPH)



n = 1 *p*-carboxyphenoxy acetic acid (CPA)

n = 4 *p*-carboxyphenoxy valeric acid (CPV)

n = 8 *p*-carboxyphenoxy octanoic acid (CPO)



erucic acid dimer (FAD)

Figure 1.17 Some of monomers used for preparation of polyanhydrides

Redrawn from Göpferich (1999)

However, not all polyanhydrides formed from by the monomers listed above are ideal for the preparation of microspheres. For example, poly (SA) is highly crystalline, which erodes too fast, whereas poly (CPP) erodes too slowly.

1.6.3.2 Aliphatic Polyanhydrides

Aliphatic polyanhydrides degrade in a few days due to the better accessibility of the bonds to water than aromatic ones, and most of them are not suitable for manufacture of drug delivery systems. For example, poly (sebacic acid) is very brittle, while poly (erucic acid dimer) is a liquid and not suitable for the manufacture of solid drug delivery systems. One class of aliphatic polyanhdride, poly (FAD: SA), was proved useful in drug delivery (Göpferich 1999). Some advantages of the poly (FAD: SA) copolymer are: (1) it is simpler and less expensive to synthesise than poly (CPP: SA); (2) it has some suitable physical properties for fabrication: more flexible than poly (CPP: SA), low melting point, high solubility in some organic solvents, high mechanical strength (Domb and Maniar, 1993); (3) it can be easily processed and shaped into desired delivery devices such as bead, slab, film and rod (Shieh *et al.*, 1994).

1.6.3.3 Aromatic Polyanhydrides

The first polyanhydride successfully applied to the concept of drug delivery was poly (bis(*p*-carboxyphenoxy)methane), poly (CPM). This initial study successfully

demonstrated the feasibility of controlled release from a polyanhydride matrix (Rosen *et al.*, 1983). However, poly (CPM) lacked the capacity to provide release of drug in a specific range of rates and duration. Furthermore, aromatic polyanhydrides have low solubility in common organic solvents and have high melting points (Leong, 1985); therefore, they cannot be easily fabricated into films or microspheres using solvent or melt techniques (Domb *et al.*, 1989). Fully aromatic polyanhydrides that are soluble in chlorinated hydrocarbons and melt at temperatures below 100°C were obtained by copolymerisation of aromatic diacids such as isophthalic acid (IPA), terephthalic acid (TA), 1,3-bis-(*p*-carboxyphenoxy) propane (CPP) or 1,6-bis (*p*-carboxyphenoxy) hexane (CPH) (Domb *et al.*, 1997).

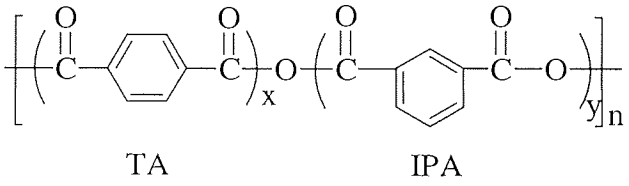


Figure 1.18 Copolyanhydrides of terephthalic acid and isophthalic acid

1.6.3.4 Aliphatic-Aromatic Homopolymers

Polyanhydrides based on aliphatic and aromatic diacids, poly (*p*-carboxyphenoxy alkanoic anhydride), were synthesised (Domb *et al.*, 1989). These polymers generally have low melting points and can be dissolved in common organic solvents, thus they are suitable for hot-melt microencapsulation, injection molding formulation, and solution formulation. Their degradation is dictated by the length of alkanoic chain

wherein, an increasing degradation time is observed with an increasing chain length (Domb *et al.*, 1989).

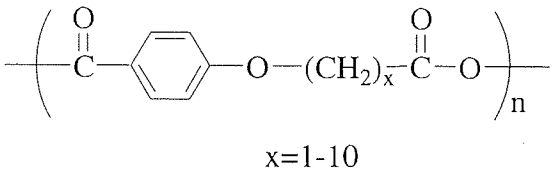


Figure 1.19 Aliphatic-aromatic homopolymer

1.6.3.5 Aliphatic-Aromatic Copolymers

In order to achieve maximum flexibility in polymer erosion profiles, two different kinds of monomers can be used to formulate copolymers. Aromatic polyanhydrides erode slowly due to their high hydrophobicity and the hindered approach of water to the anhydride bond (Tamada and Langer, 1992). The erosion rate can be increased by copolymerisation with aliphatic monomers. Each particular polymer composition will affect drug release rate and duration. Poly (CPP: SA) serves as a successful example. Poly (CPP: SA) erodes from days to months depending on the composition (Leong *et al.*, 1985). At pH 7.4, pure poly (CPP) degrades about 3 years. However, the molecular weight of poly (CPP: SA) 20:80 dropped exponentially for the first 24 hours when it was incubated in pH 7.4 buffer (Santos *et al.*, 1999).

1.6.3.6 Modified Polyanhydrides

The physical and mechanical properties of polyanhydrides can be altered by modification of the polymer structure. Several modifications include the formation of branched polymers and crosslinked polymers (Domb *et al.*, 1997). However, at present, there are numerous studies needed in both fields, such as how to obtain optimum conditions for synthesising these materials and how to assess the *in vitro* and the *in vivo* responses to these polyanhydride networks from an engineering, chemical, and biological standpoint (Domb *et al.*, 2000).

Branched polyanhydrides were synthesised in the reaction of diacid monomers with tri- or polycaboxylic acid branching monomers (Maniar *et al.,* 1990). Sebacic acid was polymerised with 1,3,5 benzenetricarboxylic acid (BTC) and poly (acrylic acid) (PAA) to yield random and graft-type branched polyanhydrides as shown in ***Figures 1.20*** and ***1.21***. The molecular weights of the branched polymers were significantly higher (mol. wt. 250,000) than the molecular weight of respective linear polymer (mol. wt. 80,000). The specific viscosities of the branched polymers were lower than linear polyanhydrides with similar molecular weights. Except for the difference in molecular weights, there were no noticeable changes in the physicochemical or thermal properties of branched polyanhydrides and the linear ones. Release of drug was faster from the branched polymers as compared to the respective linear polymer of a comparable molecular weight (Domb *et al.*, 1997).

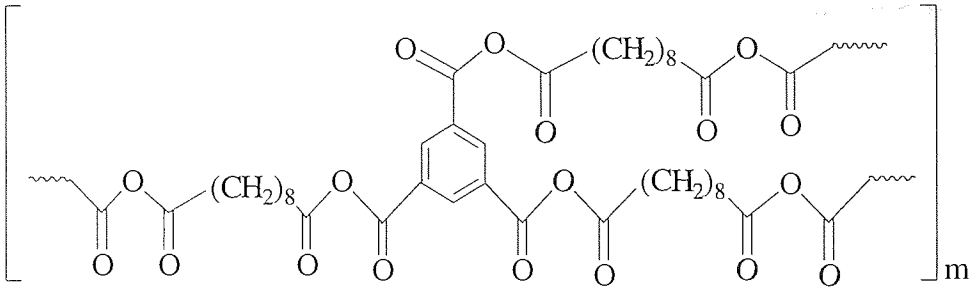


Figure 1.20 Poly (sebacic anhydride) branched with 1,3,5 benzenetricarboxylic acid (BTC)

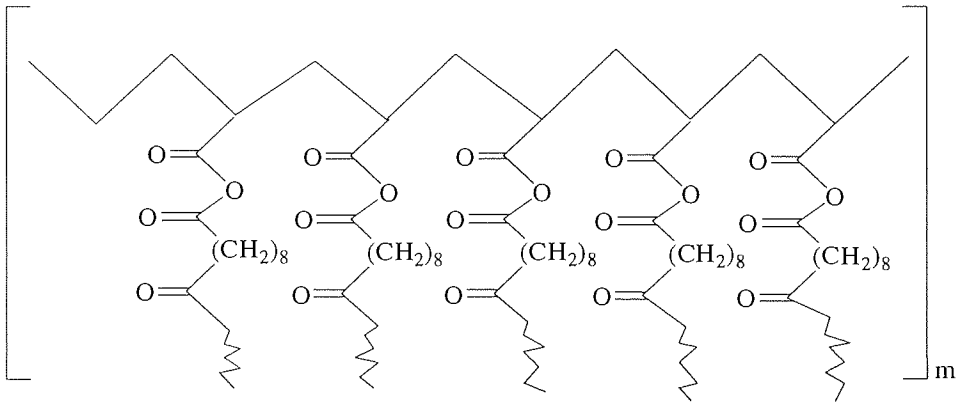


Figure 1.21 Poly (sebacic anhydride) branched with poly (acrylic acid) (PAA)

Redrawn from Maniar *et al.* (1990)

A class of unsaturated polyanhydrides, which have double bonds along the polymer backbone available for secondary polymerisation, may be used to form a crosslinked polymer (Domb *et al.*, 1991). A copolymer of (fumaric-co-sebacic acid) (***Figure 1.22***) was explored as a crosslinked polymer since fumaric acid retains its double bond in this copolymer. Chemical crosslinking was conducted both in bulk and in solution. For solution crosslinking, poly (FA: SA) was dissolved in tetrahydrofuran. Styrene or

methyl methacrylate was added, along with benzoyl peroxide or 2-butanone peroxide, and dimethyltoluidine as an accelerator. Alternatively, azobisbutyronitrile and divinylbenzene were also tested as solution crosslinking agents. Bulk crosslinking was accomplished by mixing p (FA: SA) 50:50 copolymer with equi-molar amounts of either styrene or methyl methacrylate, accompanied with the addition of either benzoyl peroxide or 2-butanone peroxide, and also accompanied with the addition of an accelerator, dimethyltolidine, either with or without cobalt naphtonate. These preliminary studies exhibited difficulties in yield and molecular weight of the crosslinked materials, as the crosslinked polymer formed by these methods was insoluble in common solvents. Nonetheless, it may be possible to develop crosslinked unsaturated polyanhydrides in some applications such as orthopaedics (Tamada and Langer, 1992).

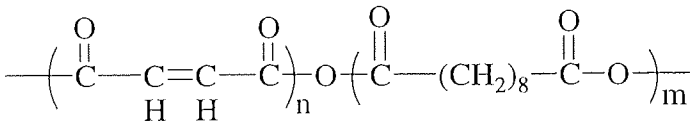


Figure 1.22 Poly (FA: SA)

Another class of crosslinked polyanhydride can be also obtained by copolymerising the monomer with a difuctional crosslinking agent (Albertsson and Eklund, 1996). This reaction can be described as a copolymerisation between the cyclic anhydride and the epoxide to give a polyester structure as shown in ***Figure 1.23***. The degradation profile slows markedly after the rapid hydrolysis of the polyanhydride structure and before the degradation of the crosslinked polyester-related network. The extent of this plateau region depends on the crosslinking density of the network. The

results show that the synthetic route to crosslinked aliphatic polyanhydrides seems feasible (Albertsson and Eklund, 1996).

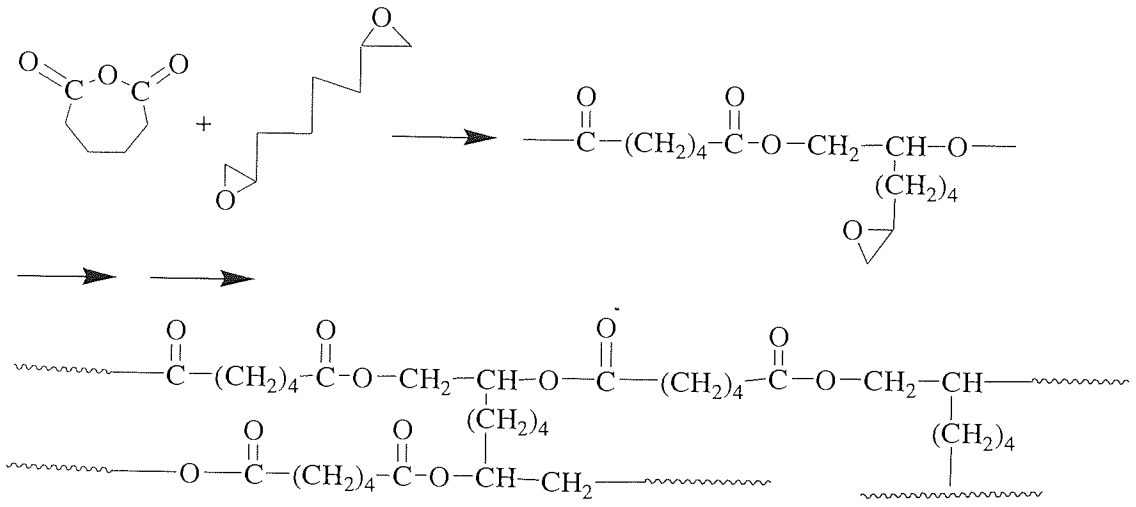


Figure 1.23 Schematic illustration of the formation and structure of crosslinked poly (adipic anhydride) with 1,2,7,8-di-epoxyoctane (DEO) [Redrawn from Albertsson and Eklund (1996)]

Following development of a photopolymerisation technique, photocrosslinked polyanhydrides have been studied widely for orthopaedic applications such as bone cements. ***Figure 1.24*** shows the monomer structures, polymer network formation, and final degradation products. The selection for monomers was based upon a class of FDA approved linear polyanhydrides (CPP and SA) used for drug delivery. The resulting polymers degrade from the surface inward, and the degradation rate can be changed simply by altering the overall hydrophobicity in the polymer network. Further, preliminary studies in rats showed good compatibility of the materials in subcutaneous tissue (Burkoth and Anseth, 2000).

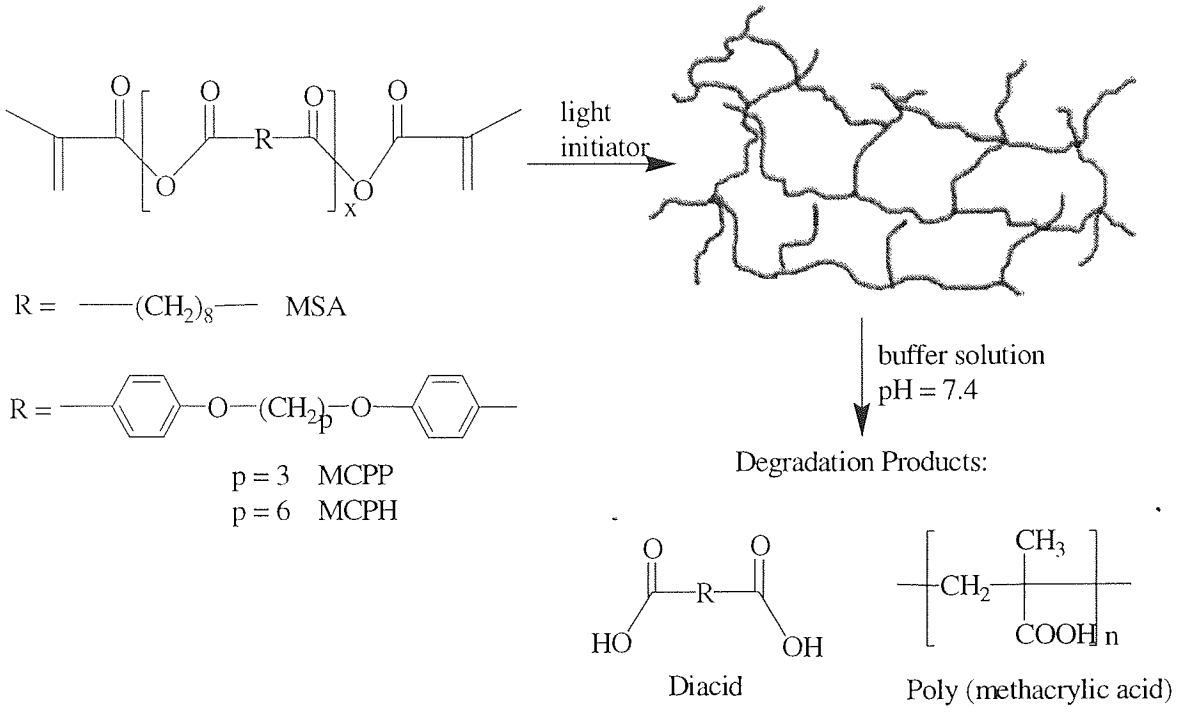


Figure 1.24 Dimethacrylated anhydride monomers, methacrylated sebacic acid (MSA), methacrylated 1,3-bis (*p*-carboxyphenoxy) propane (MCPP) and methacrylated 1,6-bis ((*p*-carboxyphenoxy) hexane (MCPH), as wall as a general polymerisation and degradation scheme [Redrawn from Burkoth and Anseth (2000)]

1.6.3.7Amino Acid Based Polyanhydrides

Amino acids were converted into dicarboxylic acids by derivatisation of their amino terminus with trimellitic anhydride. The resulting dicarboxylic acid can be converted into mixed anhydride prepolymer by refluxing in acetic anhydride. The amino acid prepolymer can be mixed with conventional prepolymers of sebacic acid and subjected to melt polymerisation (Staubi *et al.*, 1990) as shown in ***Figure 1.25***.

At the same time, TMA-Tyr (trimellitylimido-L-tyrosine): SA: CPP anhydride-co-imide terpolymer (shown in ***Figure 1.26***) has been studied (Hanes *et al.*, 1996), and its porous microspheres made by double emulsion solvent evaporation have also been investigated (Hanes *et al.*, 1998). In this study, bovine serum albumin (BSA) was entrapped into microspheres. The result of the biphasic polymer erosion pattern (fast initially as SA and TMA-Tyr erode and slow thereafter as CPP erodes) is a biphasic protein release profile. The higher the percentage of SA and TMA-Tyr, the more significant the initial protein release phase (first several days). Conversely, polymers with higher CPP levels release fewer drugs initially, leading to a more protracted release profile. Moreover, TMA-Tyr:SA:CPP copolymers for vaccine delivery may not require the addition of a soluble adjuvant (an immunostimulatory molecule producing maximal effect in initiating a protective immune response), since the copolymers have an adjuvant, L-tyrosine, built into their backbone. L-tyrosine and many of its derivatives are known to stimulate a potent immune response to absorbed antigens In addition, because polymer erosion and antigen release occur simultaneously, the tyrosine derivative and the antigen are presented to the immune system together. The co-delivery of adjuvant and antigen for several days may lead to enhanced levels of immunity against a variety of infections (Hanes *et al.*, 1998).

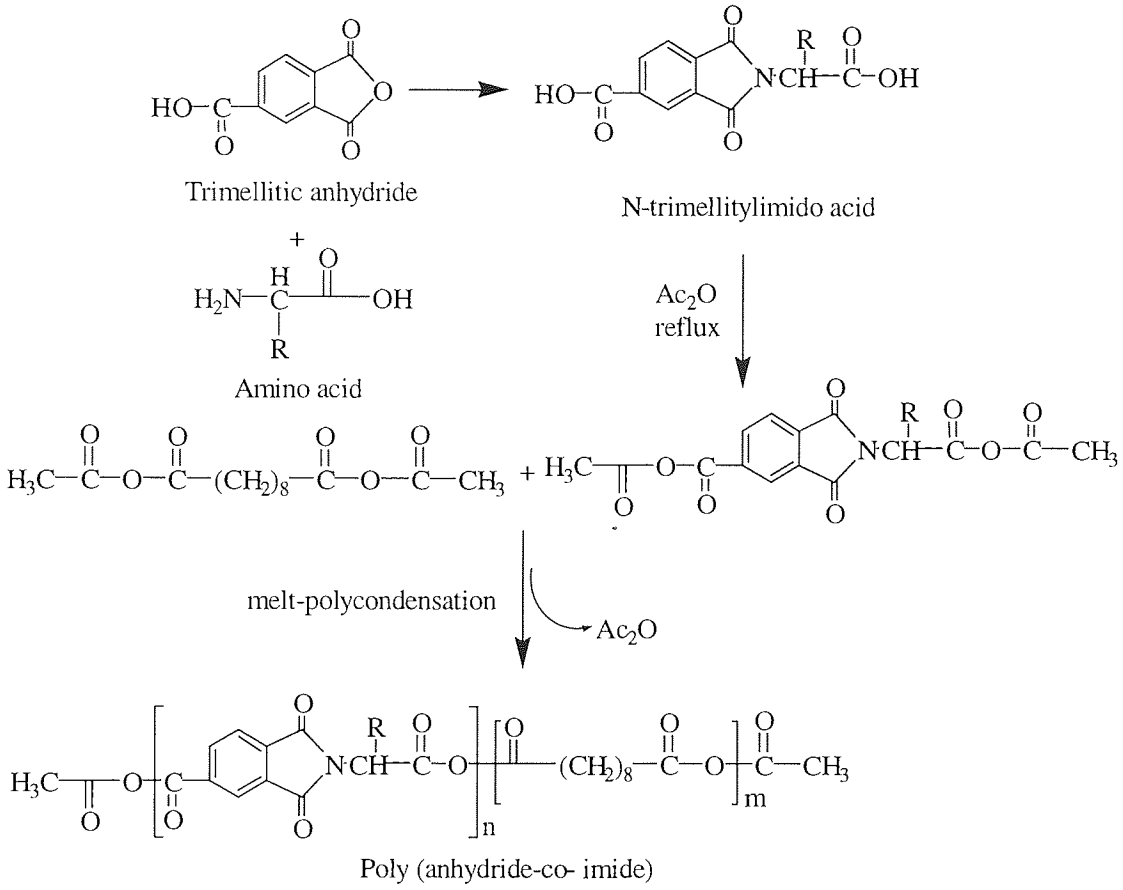


Figure 1.25 Reaction scheme for synthesis of poly (anhydride-co-imide)

Redrawn from Staubi *et al.* (1990)

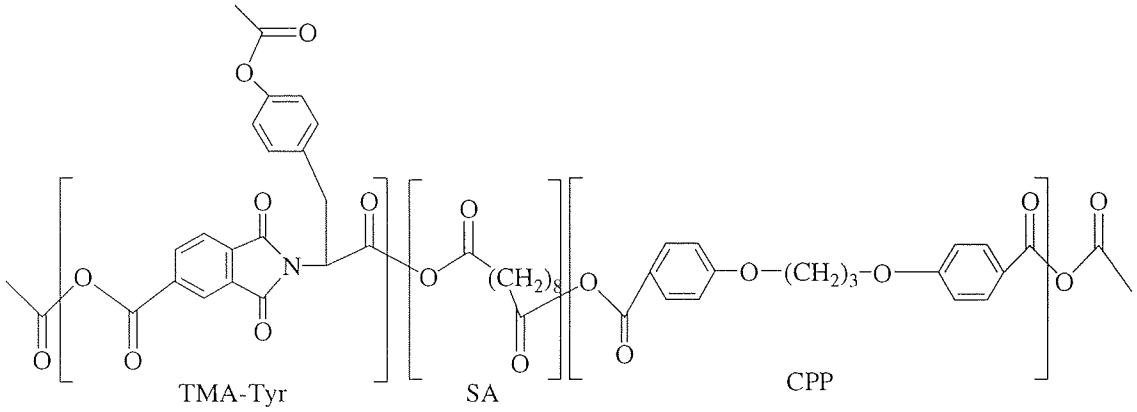


Figure 1.26 TMA-Tyr:SA:CPP anhydride-co-imide terpolymer

Redrawn from Hanes *et al.* (1996)

Dicarboxylic acid derivatives of amino acids were also prepared by amidation of the amino group with cyclic anhydride or by coupling two amino acids with a diacid chloride. The polymers were synthesised by melt or solution polymerisation; however, they had a low molecular weight ranging from 2200 to 12,400 (Domb, 1990). This approach may be useful in the synthesis of biodegradable polymeric drugs, and possibly in the design of polymers with improved mechanical strength and biocompatibility (Domb *et al.*, 1997). As examples, β-alanyl N-succinamide polymers and β-alanine reacting with sebacoyl chlorides are shown in ***Figures 1.27 and 1.28***.

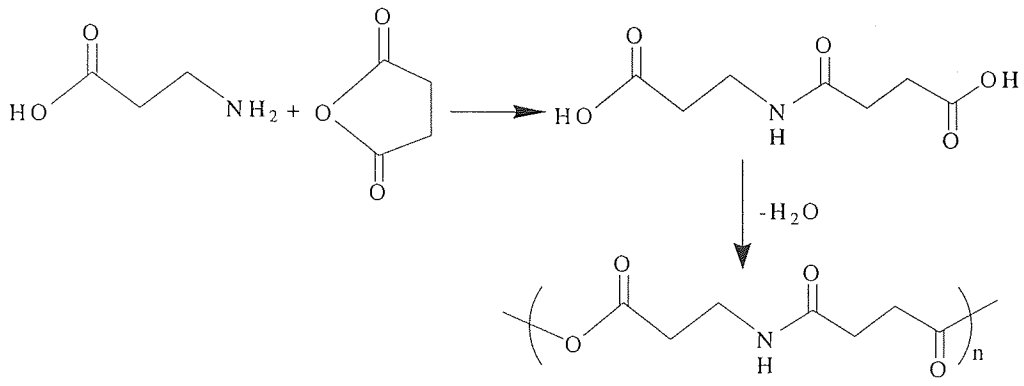


Figure 1.27 β-alanyl N-succinamide polymer

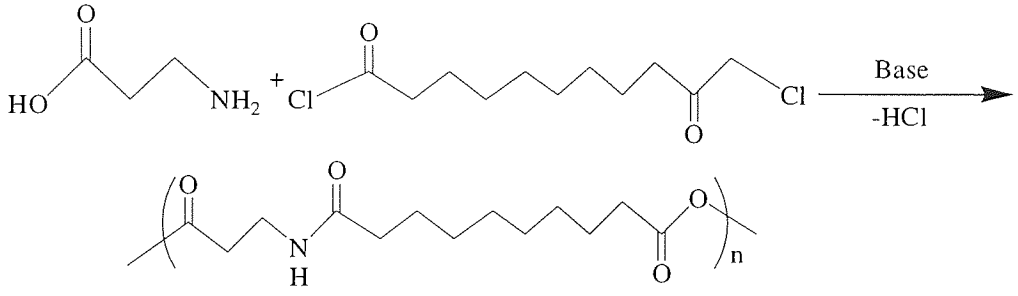


Figure 1.28 Reaction of β-alanine with sebacoyl chloride

Redrawn from Domb (1990)

1.6.4 Polyanhydride Degradation and Erosion

Polyanhydrides are composed of monomer units connected by anhydride bonds. The anhydride bond is hydrolytically labile and breaks down into carboxylic acid groups (*Figure 1.29*).

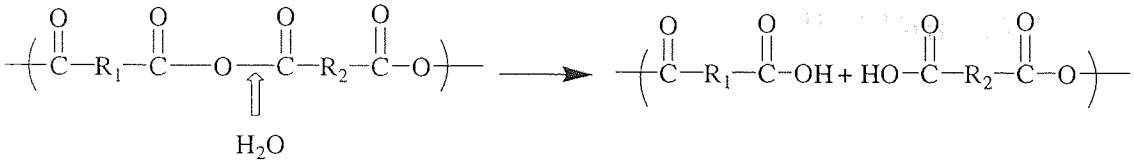


Figure 1.29 Hydrolysis of polyanhydrides

Redrawn from Tamada and Langer (1992)

Thus, degradation of polyanhydrides occurs by backbone chain scission across the anhydride bond. The initial long-chain polymer, which is water insoluble, is cleaved into shorter and more hydrophilic fragments, which are soluble and can be absorbed by the body (Tamada and Langer, 1992).

The degradation and erosion of polyanhydrides varies with a number of factors, such as the chemical nature of the monomers; the pH of the surrounding environment (the higher pH, the faster the polymers degrade); the shape and geometry of the implant and its porosity, which are determined by fabrication methods, (porous materials will degrade more rapidly than non-porous). For poly (CPP: SA) and poly (FAD: SA), a higher polymer erosion rate and hence higher drug release rate can be achieved by increasing the content of sebacic acid in the copolymer due to increasing the hydrophilicity of the copolymer. A ten fold increase in drug release rate was obtained by altering the ratio of monomers in poly (CPP: SA) and poly (FAD: SA) series copolymers, therefore, both polymers can be used to delivery drugs over a wide range of release rate (Domb *et al.*, 1997). Hence, careful selection of the monomers is a crucial element in development of biodegradable polyanhydrides.

1.7 Formulation of Polyanhydrde as Controlled Drug Delivery Systems

There are several approaches to formulate a polymer-drug mixture as a drug delivery system. The choice of method depends on properties of the drug, the polymer, and the desired drug release profile. Drug properties that affect the choice of formulation method consist of hydrophobicity, diffusivity, stability, and tendency to interact with the functional groups on the polymer. Polymer properties include melting point, crystallinity, or brittleness, dictate the conditions for fabrication (Tamada and Langer, 1991).

1.7.1 Compression Molding

Compression molding is a simple and flexible method of fabrication, and remains the most popular formulation method for polymers. The polymer-drug mixture is ground or spray dried into a fine powder, placed in a piston-type mold, and compressed into a flat wafer with a hydraulic press. This method is always done 5-10°C above the glass transition temperature of the polymer, which allows low temperature fabrication of devices of certain polymer composition, such as poly (CPP: SA, 20:80). Compression molding makes it possible to fabricate devices at room temperature for some polymers, which may alleviate problems of polymer-drug interaction. On the other hand, compression molding limits manufacture to flat devices with the mold geometry (Tamada and Langer, 1992).

1.7.2 Melt Molding or Injection Molding

Heating polymers above their melting temperature gives a viscous liquid, which can beused to mold the polymer-drug mixture into the desired geometry. Injection molding involves simply molding the melt under low pressure in a conventional mold, and gives a dense and uniform polymeric matrix. In the case of gentamicin-loaded poly (FAD: SA, 50:50) beads, after incorporation of gentamicin sulfate in the polymer by melted-mixing, the drug polymer blend is injection molded into a bead form (12mm×3mm) that is suitable for use (Stephens *et al.*, 2000). However, these methods are not suitable for the heat-sensitive drugs, e.g., high temperature can case protein denaturalisation.

1.7.3 Solvent Casting

Most polyanhydrides are sufficiently soluble in chloroform and methylene chloride to allow the formulation of solvent casting film (Tamada and Langer, 1992). In solvent casting methods, the polymer is dissolved at about 10% (w/v) in the solvent with the drugs that can be codissolved in the solvent. Solvent insoluble drug can be added as a fine powder. This solvent is allowed to evaporate slowly from the device, usually at -20 °C, producing a thin, flat film. A major disadvantage is that solvent casting can be difficult to control, and often results in fragile and porous, non-uniform films. In addition, there is a potential that the drug particles will settle to the bottom of the solution, producing films with more drug on one side of the device than on the other (Tamada and Langer, 1992).

1.7.4 Microsphere Fabrication Techniques

Four different methods have been developed to manufacture polyanhydride microspheres: hot-melt microencapsulation, solvent removal, solvent evaporation, and spray drying (Brunner and Göpferich, 1995).

1.7.4.1 Hot-melt Microencapsulation

In hot-melt microencapsulation, the polyanhydrides are melted, and drugs are dispersed in the melted polymer as solid particles. This suspension is added to a polymer immiscible solvent, such as silicone or olive oil, at 5 °C above the melting point of the polymer. The liquid is cooled until polymer solidifies into microspheres that are washed with petroleum ether. Microspheres made by hot-melt encapsulation have smooth surfaces and are less porous than those made using other methods (Mathiowitz and Langer, 1987). Since the hot-melt encapsulation process is analogous to the melt molding process, they have similar limitations in applications.

1.7.4.2 Solvent Removal

The solvent removal technique uses only organic solvents for the manufacture of microspheres, which can prevent hydrolysis during microsphere preparation (Mathiowitz and Langer, 1992). The polymer is dissolved in an organic solvent, such as methylene chloride, and is 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. Successful microsphere preparation by this method depends on two factors: the rate of precipitation of the polymer and the rate of methylene chloride diffusion into the silicone oil (Mathiowitz *et al.*, 1988). The resultant microspheres are porous. The restrictions associated with this method include the use of organic solvent and the danger of silicone oil residues in the microspheres (Mathiowitz and Langer, 1992).

1.7.4.3 Solvent Evaporation

The polymer is first dissolved in organic solvent, such as methylene chloride. This polymer solution is processed to an oil-in-water emulsion by dispersion into an aqueous solution containing a surfactant, such as hydrolysed poly (vinyl acetate). The emulsion is stirred till the organic solvent evaporates, leaving the hardened microspheres (Tabata and Langer, 1993). With modification, the solvent evaporation method can be adapted to encapsulate hydrophilic substances. Firstly a small volume of aqueous phase is dispersed in the polymer organic solvent to form a water-in-oil emulsion, which then processed into microspheres as above. The multiple (w/o/w) emulsion is created by modified solvent evaporation method termed double-emulsion technique. Polyanhydride microspheres prepared by this method tend to be porous, which may increase drug release from microspheres (Mathiowitz and Langer, 1992). The solvent evaporation method may result in solvent residues in the polymer and the risk of polymer degradation.

1.7.4.4 Spray Drying

Spray drying is a reproducible, rapid, and easy to scale up method for preparing microspheres. In this method, the polymer is dissolved in a solvent such as chloroform or methylene chloride along with the drug, either in a dissolved, emulsion, or dispersed form. The mixture is sprayed through an atomiser. As the particles fall toward the bottom of the spray dryer, they are simultaneously dried by an up-wards flow of nitrogen. Some polyanhydrides have been used to prepare microspheres using spray drying. The amorphous polymers, such as poly (CPP: SA, 50:50), poly (CPH: SA, 50:50) and poly (CPH), were not amenable to spray drying, and produced aggregates and uneven particle morphologies. It was proposed that the low glass transition temperatures of the polymers allowed them to fuse together during spray drying. On the other hand, poly (SA), poly (CPP: SA, 20:80), and poly (FA: SA, 20:80) were tested to form microspheres with 1-10 μm in diameter. The morphologies of these polymer microspheres made by spray drying varied from dense to porous, and smooth to rough, with the type of drug incorporated (Mathiowitz *et al.*, 1990 and 1992). The limits of spray drying could be low yield and the use of organic solvents (Giunchedi and Conte, 1995).

1.8 Objectives of This Study

Crosslinked polyanhydrides are in the form of three-dimensional network, and the network is stronger than the linear polyanhydrides. The photo-crosslinked polyanhydrides have high mechanical strength and are used as degradable orthopaedic

fixation devices, for example pins and screws, resorbable fillers for bone augmention and regeneration, bone cement, etc. (Muggli *et al.*, 1999). However, only branched polyanhydrides were studied for drug delivery (Domb *et al.*, 1997). None of crosslinked polyanhydrides has been reported for drug delivery applications, because these crosslinked polyanhydrides were found not to dissolve in common organic solvents. Towards the overall goal of synthesising novel crosslinked polyanhydrides as a potential drug delivery carrier in a form of microspheres, two novel kinds of polyanhydrides were synthesised by a modified melt-polycondensation method during this study. One of them is an unsaturated polyanhydride as shown in ***Figure 1.30***, where double bonds are introduced into this polymer backbone as a potential site for crosslinking and this was tested. In the other polyanhydride as shown in ***figure 1.31***, an amino acid, glutamic acid, was used to achieve a class of novel crosslinked polyanhydrides. All these polymers can be dissolved in chlorinated solvents, such chloroform ($CHCl_3$) and dichloromethane (DCM), which made these polymers suitable for solvent removal, solvent evaporation, and spray drying formulation methods. Polyanhydride microspheres were prepared by double emulsion (w/o/w) and spray drying methods, and bovine serum albumin (BSA) was entrapped as a model protein. The synthesis of the two novel polymers, the physicochemical properties of polymers, the degradation studies of polymers, the characteristics of resulting microspheres and the investigation of BSA release from microspheres are presented in this study.

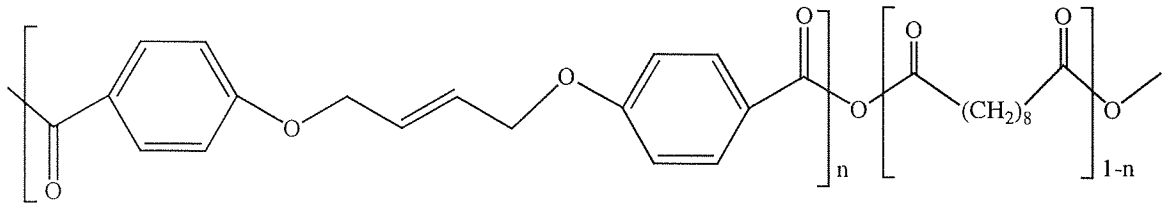


Figure 1.30 Poly (bis (*p*- carboxyphenoxy)-2-butene-co-sebacic acid) (CP2B: SA)

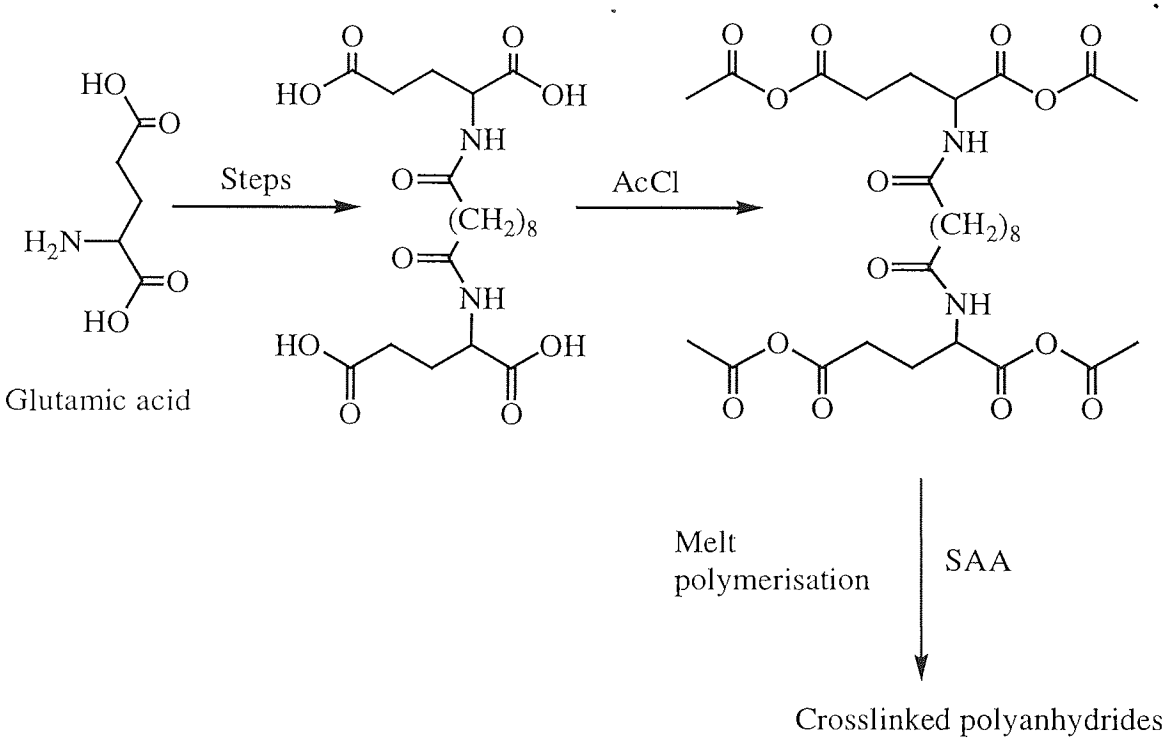


Figure 1.31 Poly (glutamic acid-sebacic acid-co-sebacic acid) (GluSA: SA)

Chapter 2 Synthesis and Characterisation of Unsaturated Polyanhydride for Crosslinking

2.1 Introduction

A series of unsaturated polyanhydrides were prepared by melt or solution polymerisation of fumaric acid (FA), acetylenediacarboxylic acid (ACDA), and 4,4'-stilbendicarboxylic acid (STDA) (Domb *et al.*, 1991). The double bonds remain intact throughout the polymerisation process and are available for a secondary reaction to form a crosslinked matrix. In this study, the anhydride monomer used is 1,4-bis- (*p*-carboxyphenoxy)-2-butene (CP2B) as shown in ***Figure 2.1*** made from *p*-hydroxybenzoic acid and 1,4-dibromo-2-butene.

Figure 2.1 1,4-bis- (*p*-carboxyphenoxy)-2-butene (CP2B)

Since aromatic homopolyanhydrides are normally insoluble in common organic solvents and melt at high temperatures above 200°C (Domb *et al.*, 1997), they cannot be fabricated into films or microspheres using solvent or melt techniques. However, employing two different kinds of monomers to formulate copolymers will change the polymer physicochemical properties, including solubility in organic solvents and erosion rates (Tamada and Langer, 1991). Incorporating saturated aliphatic monomers into a copolymer will increase the polymer solubility and decrease melting point, because these monomers are crystalline, melt at temperatures below 100°C, and are soluble in chlorinated hydrocarbons (Leong *et al.*, 1985). Currently, the main aliphatic monomer used in drug delivery applications is sebacic aid (SA). In this study,

copolyanhydrides were synthesised by copolymerisation of 1,4-bis- (*p*-carboxyphenoxy)-2-butene and sebacic acid.

In order to understand polyanhydride drug delivery systems, it is necessary to have a detailed knowledge of the properties of polyanhydrides. The characterisation of polyanhydrides includes their chemical composition, structure, crystallinity and thermal properties, mechanical properties and thermodynamic and hydrolytic stability (Domb *et al.*, 1997). In addition to confirming the structure of the polyanhydrides, ^{1}H-NMR (nuclear magnetic resonance) spectroscopy has been used to determine the degree of the comonomer sequence distribution of polyanhydride copolymers (Ron *et al.*, 1991). NMR has been also employed to suggest whether the polyanhydrides are block or random copolymers.

Infrared spectroscopy readily confirms the identity of the polymer as a polyanhydride. A doublet occurring between 1670 and 1800 cm^{-1} is characteristic of a carboxylic anhydride (Leong *et al.*, 1985). The significance of this analysis is that it measures the degradation of the anhydride bonds and not the dissolution of the degradation products that is dependent on the solubility of the degradation products (Domb *et al.*, 1997).

The molecular weight of polyanhydrides was measured by gel permeation chromatography (GPC) relative to polystyrene standards. The weight average molecular weights (Mw) of polyanhydrides were in the range 5,000-300,000 generally, and the intrinsic viscosities (η) increase with an increase in Mw (Domb *et al.*, 1997).

Differential scanning calorimetry (DSC) is used to characterise the thermal properties of polyanhydrides, which include glass transition (Tg) and melting (Tm) temperatures and heats of fusion. Tg and Tm are important parameters because they dictate which device fabrication methods are practical for a given system. Tg determines the temperature necessary for compression molding, and Tm determines the temperature necessary for injection molding or melt pressing (Tamada and Langer 1992).

Crystallinity is an important factor in controlling polymer erosion. Crystalline regions erode more slowly than amorphous regions (Mendelkern, 1964; Ward, 1982). Crystallinity has been characterised by wide-angle X-ray diffraction (WAXD) and X-ray diffraction and differential scanning calorimetry (DSC). Some of homopolyanhydrides of aromatic and aliphatic diacids, such as poly (SA), poly (CPP) and poly (FA) are crystalline (>50% crystallinity) (Mathiowitz *et al.*, 1990); wherein poly (FAD) and poly (CPH) were found to be amorphous (Shieh *et al.*, 1994). The crystallinity of copolymers has been shown to depend on the monomer ratio (Mathiowitz *et al.*, 1990). For example, as the composition shifts toward equimolar content of the monomers, crystallinity decreased, with only 5-10% crystallinity for poly (CPP: SA) 50:50 (Tamada and Langer, 1992). The heat of fusion values for the polymers demonstrated a sharp decrease as CPP was added to SA. After adding one monomer, a decreasing trend in crystallinity appeared in the results of X-ray and DSC analysis that was a direct result of the random presence of other units in the polymer chain (Domb, *et al.*, 1997).

2.2 Experimental

2.2.1 Materials

Sebacic acid (SA) (Sigma-Aldrich)

p-Hydroxybenzoic acid (Sigma-Aldrich)

1,4-Dibromo-2-butene (Sigma-Aldrich)

Tetrabutyl ammonium bisulphate (Sigma-Aldrich)

Dry acetic anhydride (Acros Organics)

Cadmium acetate (BDH Chemicals Ltd. Poole England)

Potassium bromide (KBr, 99% FI-IR grade) (Sigma-Aldrich)

NaOH (Sigma-Aldrich)

K_2CO_3 (Sigma-Aldrich)

H_2SO_4 (Sigma-Aldrich)

Dry methanol (Fisher scientific)

Dry ethyl ether (Fisher scientific)

Dry dichloromethane (DCM) (Fisher scientific)

All other solvents of analytical grade (Fisher scientific)

2.2.2 Instrumentation

Bruker NMR AC250 Spectrometer

2020 Galaxy FT-IR Spectrometer

Soniprep 150

PERKIN-ELMER Gel Permeation Chromatography (GPC) System

PERKIN-ELMER DSC-4 Differential Scanning Calorimeter

2.2.3 Purification of Starting Materials

2.2.3.1 Sebacic Acid

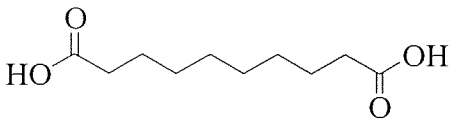


Figure 2.2 Sebacic acid

Sebacic acid was recrystallised twice from dry methanol (Domb *et al.*, 1987) and dried under vacuum. The purified sebacic acid was stored in a sealed flask until required.

2.2.3.2. Acetic Anhydride (AA)

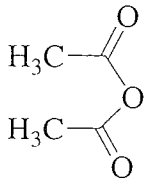


Figure 2.3 Acetic anhydride (AA)

Acetic anhydride was distilled in a round bottom flask with anti-bumping granules. The distilled liquid was not collected until the temperature reached 138-140°C. Purified acetic anhydride was stored over 4A molecular sieves in a sealed flask.

2.2.3.3 Dry Petroleum Ether

Petroleum ether with a boiling range of 60-80 °C was used in this experiment. This solvent was dried over 4A molecular sieves (Leonard *et al.*, 1990).

2.2.4 Preparation of Prepolymers

2.2.4.1 Sebacic Acid Anhydride (SAA)

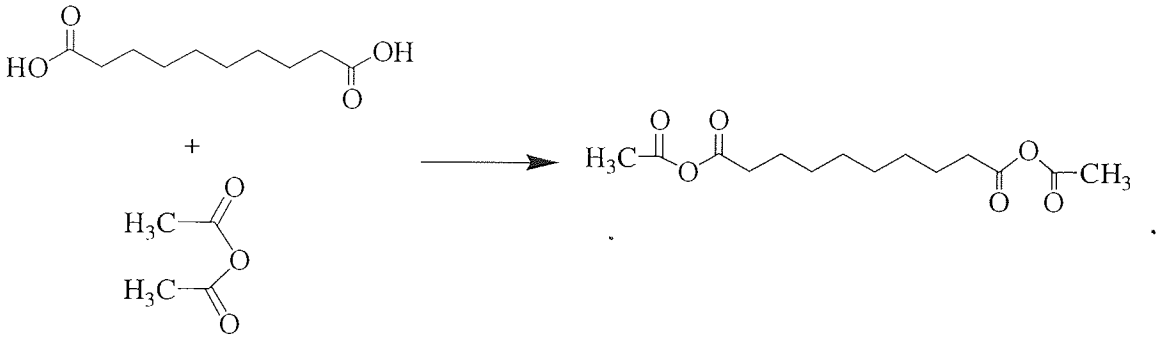


Figure 2.4 Synthesis of SAA

8 g (40 mmol) of purified sebacic acid was added into a 100 ml refluxing solution of purified acetic anhydride for 30 minutes. Sebacic acid was completely dissolved within 5 minutes and the excess acetic acid anhydride was removed by evaporation at 20-30 °C. The oily residue material was kept in a refrigerator overnight. The crude prepolymer was immersed a mixture of diethyl ether (50 ml) and petroleum ether (50 ml) overnight to extract traces of acetic anhydride. The white crystals were separated by filtration and dried in a $CaCl_2$ desiccator under vacuum (Domb *et al.*, 1988).

2.2.4.2 1,4-bis- (*p*-carboxyphenoxy)-2-butene (CP2B)

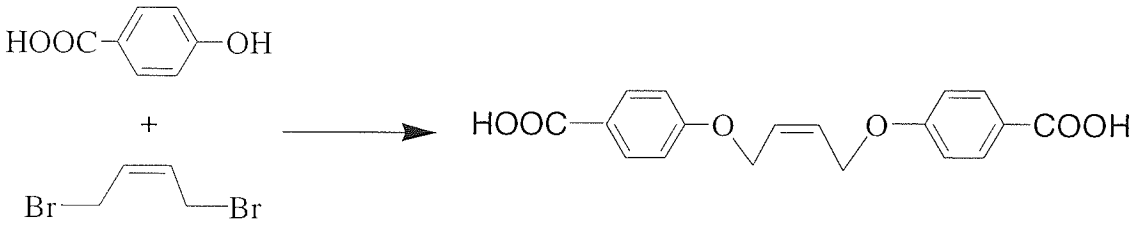


Figure 2.5 Synthesis of CP2B

3.45 g (0.025 mmol) of *p*-hydroxybenzoic acid, 7 g (0.175 mol, ground into fine powder) of sodium hydroxide, 4 g (0.03 mol) of anhydrous K_2CO_3 and 0.085 g of tetrabutyl ammonium bisulphate were stirred in 35 ml of anhydrous DMSO. 2.675 g (0.0125 mmol) of 1,4-dibromo-2-butene was added to the well-stirred suspension, and it was kept stirring at room temperature overnight. The mixture was poured into 100 ml of 60-70 °C warm 6N sulphuric acid, and the white precipitate was washed with 100 ml of methanol and filtered. The product was dried in a vacuum oven at 60°C.

2.2.4.3 1,4-bis- (*p*-carboxyphenoxy)-2-butene Anhydride (CP2BA)

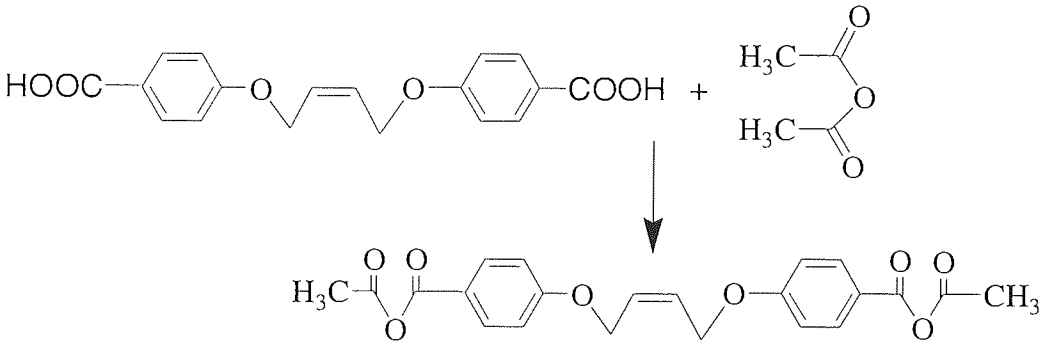


Figure 2.6 Synthesis of CP2BA

5.5 g (0.0168 mol) of 1,4-bis- (*p*-carboxyphenoxy)-2-butene was added to 60 ml of boiling purified acetic anhydride under dry N_2. Reflux was stopped at 1 hour, and then the solution was filtered while it was hot. About 5-10% of unreacted CP2B was separated. The filtrate was concentrated by evaporating excess acetic anhydride at 60 °C. The reaction mixture was left at 0 °C to crystallise overnight. The crystals were separated by filtration and transferred to 25 ml of anhydrous diethyl ether and allowed to wash for several hours at room temperature. The crystals were filtered and dried in a vacuum oven at 60 °C (Domb *et al.*, 1988).

2.2.5 Synthesis of Copolymers

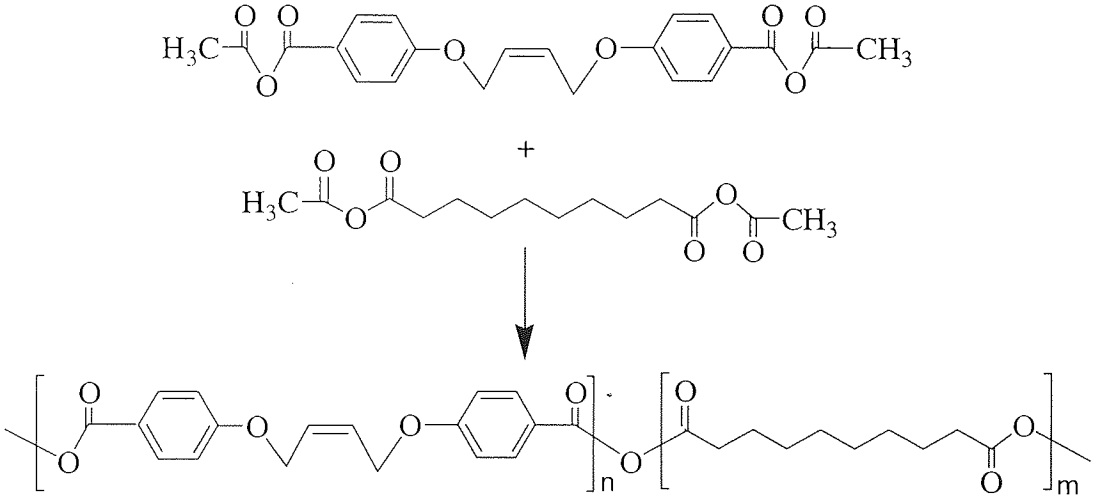


Figure 2.7 Synthesis of copolymers

CP2B and SAA prepolymers and 2 molar percent catalyst, cadmium acetate, were mixed in a mortar and pestle, according to the different mole ratio of 20:80 and 50:50. Then the mixture was put into a glass tube with a side arm equipped with a capillary nitrogen inlet. The tube was immersed in an oil bath at 180 °C. After the prepolymers were melted, about 2 minutes, high vacuum (2-3 mm Hg) was applied through the side arm. The condensation product, acetic acid, was collected in an acetone/dry ice trap. During the polymerisation, a strong nitrogen sweep with vigorous agitation of the melt was performed for 30 seconds every 15 minutes. After 30 minutes, the reaction was stopped. After cooling down to room temperature, crude polymer was dissolved in dry dichloromethane. The solution was filtered and dripped into dry petroleum ether with 6 times the volume of the solution. After several hours stirring at room temperature, the polymer precipitated in dry petroleum ether from DCM. After filtration, the precipitate was extracted with dry diethyl ether for several hours at room

temperature. The ether was decanted off and the ether residue was removed by evaporation under high vacuum (Domb *et al.*, 1988).

2.2.6 Synthesis of Crosslinked Polymers

2.2.6.1 Attempted Opening of Double Bond

The proposed method for synthesis of a crosslinked polyanhydride was to produce a tetra-acid by opening the double bond as shown in ***Figure 2.8***. It was hoped that through the tetra-acid acting like a crosslinking-bridge, a crosslinked polyanhydride would be obtained.

The reaction was carried out according to the method as reported by Malanga *et al.*(1998). A solution of 20 mmol of CP2B in 20 ml of CCl_4 was cooled to -20 °C in a dry ice-acetone bath, and one equivalent of bromine in 10 ml of CCl_4 was added slowly. After 30 minutes, 2.0 g of anhydrous Na_2CO_3 was added into the mixture. It was kept stirring for 15 minutes, the reaction was stopped, 50 ml of petroleum ether was added and, the white precipitate was isolated by filtration. ^{1}H-NMR analysis of the resulting product showed the double bond still remaining at 6.06 ppm and it to be a mixture of starting materials. The above reaction conditions were modified as following: A solution of 20 mmol of CP2B in 20 ml of CCl_4 was cooled to -20 °C in a dry ice-acetone bath, and one equivalent of bromine in 10 ml of CCl_4 was added slowly. The dry ice-acetone bath was replaced with an ice bath, and 20 ml of dry ethanol was added over a period of 20 minutes to the reaction mixtures. After 25

minutes at 0 °C, the solution was refluxed for 10 minutes and, after cooling, was neutralised by adding anhydrous Na_2CO_3. After filtration, the precipitate was studied by ^{1}H-NMR. ^{1}H-NMR analysis showed the double bond still remaining (6.07 ppm) and it to be a mixture of starting materials.

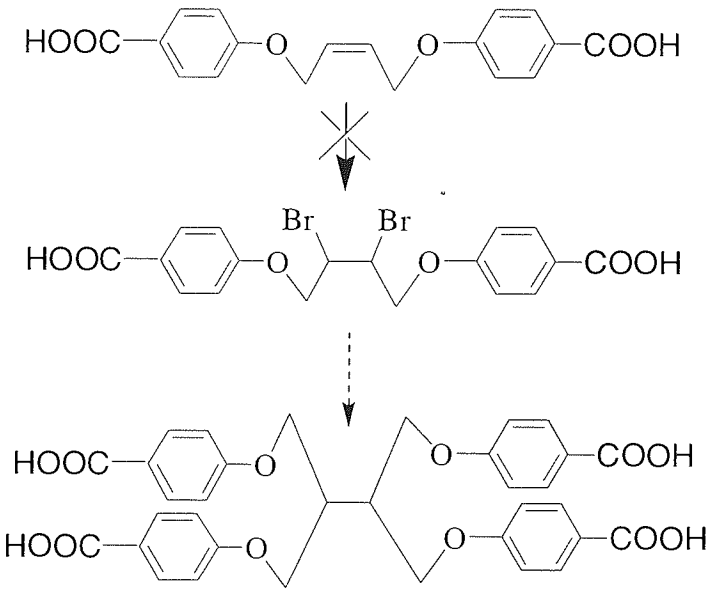


Figure 2.8 An Attempted Synthesis of a Tetra-acid

2.2.6.2 Attempted Synthesis of an Epoxide

In addition to reacting a prepolymer with a crosslinking agent, a crosslinked polymer network can be achieved by copolymerising the monomer with a difunctional crosslinking agent such as an epoxide (Witold, 1998). It was hypothesised that a crosslinked polyanhydride could be obtained from SAA by reaction with a difunctional epoxide as shown in ***Figure 2.9***. In fact, the reaction would give a polyester structure.

Generally, epoxides are easy to prepare via the reaction with a peroxy acid, and the process is known as epoxidation. A commonly used peroxy acid is 3-chloroperbenzoic acid (mCPBA). Epoxidation reactions are often carried out in an inert solvent ($CHCl_3$, CCl_4, $(C_2H_5)_2O$ *etc.*), but these reactions can tolerate a variety of solvents. According to the method developed by Malinovkii (1965), in an effect to achieve an epoxide, the reaction was carried out in dry $CHCl_3$. The solution of mCPBA in dry $CHCl_3$ was cooled to 0 °C, and was added with continuous stirring to the CP2B in dry $CHCl_3$, which was also cooled to 0 °C. Thus was kept stirring for 12 hours, the $CHCl_3$ was evaporated, and the residue was washd with an alkali ($NaHCO_3$), dried and analysed by ^{1}H-NMR. ^{1}H-NMR analysis indicated the presence of double bond. Extending the reaction from 12 hours to 24 hours, or increasing the reaction temperature to room temperature or even 45 °C (under reflux), however, the ^{1}H-NMR analysis always indicated the presence of a double bond. In some cases, the epoxides can be produced by the reaction of unsaturated compounds with hydrogen peroxide (H_2O_2). A solution of CP2B in alcohol was mixed with 15 % of H_2O_2 and 4 N of sodium hydroxide. The temperature of solution was maintained above 45 °C for

20 hours, but appearance of the mixture did not change and after evaporating the solvent and H_2O, ^{1}H-NMR analysis indicated the presence of a double bond (6.07 ppm).

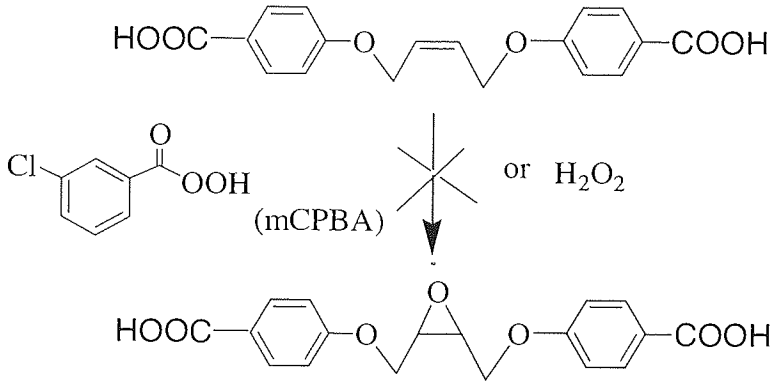


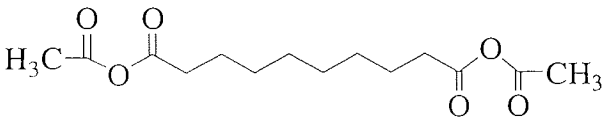
Figure 2.9 Planned Synthesis of an Epoxide

2.3 Polyanhydride Characterisation

2.3.1 ^{1}H-NMR Analysis

The ^{1}H-NMR data of prepolymers and the ^{1}H-NMR spectrum of polymer p (CP2B: SA, 20:80) (***Figure 2.11***) are presented in this section, using deuterated chloroform ($CDCl_3$) or deuterated methyl sulfoxide (DMSO) as solvents.

Sebacic Acid Anhydride (SAA)

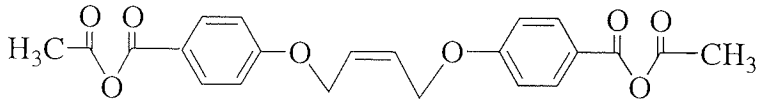


^{1}H-NMR ($CDCl_3$) δ 1.29 (8H, s, (C<u>H</u>$_2$)$_4$), 1.60-1.66 (4H, t, C<u>H</u>$_2$-(CH$_2$)$_4$), 2.40-2.47 (4H, t, C<u>H</u>$_2$CO), 2.24 (6H, s, C<u>H</u>$_3$CO).

1,4-bis- (*p*-carboxyphenoxy)-2-butene (CP2B)

^{1}H-NMR (DMSO) δ 6.06 (2H, s, C<u>H</u>-CH$_2$), 4.66 (4H, s, C<u>H</u>$_2$O), 7.00-7.03 (4H, d, Ar-<u>H</u>), 7.83-7.88 (4H, d, Ar-<u>H</u>).

1,4-bis-(*p*-carboxyphenoxy)-2-butene Anhydride (CP2BA)



^{1}H-NMR ($CDCl_3$) δ 2.34 (6H s, C$\underline{H}_3$O), 6.09 (2H, s, C$\underline{H}$-CH_2), 4.65-4.66 (4H, s, C$\underline{H}_2$O), 6.93-6.96 (4H, d, Ar-$\underline{H}$), 7.97-8.00 (4H, d, Ar-$\underline{H}$).

2.3.2 IR Analysis

Anhydrides present characteristic peaks using infrared spectroscopy (IR). In general, aliphatic polymers absorb at 1740 and 1810 cm^{-1}, and aromatic polymers at 1720-1780 cm^{-1} (Leong *et al.*, 1985). The presence of carboxylic acid groups in the polymer can be determined from the presence of a peak at 1700 cm^{-1}. Examination of the ratio between the anhydride peak at 1810 and 1700 cm^{-1} using IR can follow the degradation of polyanhydrides.

In this study, infrared spectra were recorded on a 3020 Galaxy FT-IR spectrometer. Poly (CP2B: SA) and microsphere samples were pressed into potassium bromide (KBr) discs, and analysis plus infrared data manager software (Perkin Elmer).

ν max (KBr): 1802 (anhydride peak), 1780, 1745, 1700, 1606, 1500, 1460 cm^{-1}.

2.3.3 Thermal Analysis

Differential scanning calorimetry (DSC) is a widely used method of thermal analysis in polymer science. The DSC curves reflect changes in the energy of the system under investigation-changes that may be chemical or physical in origin. Small samples (2-10 mg) and rapid experimental (heating or cooling, rates up to 10-320 $^{o}C\ min^{-1}$) mean that thermal analysis finds applications both research laboratories and routine quality control.

DSC analysis was performed on a Perkin Elmer system 4 and thermal analysis microprocessor controller. About 2-10 mg of polymer sample was sealed into aluminium sample pans. The instrument was manipulated with empty aluminium sample pans under same condition, and the measurements were carried out from - 40 ^{o}C to 200 ^{o}C under nitrogen at a scan rate of $10^{o}C\ min^{-1}$ as heating rate and at $320^{o}C$ min^{-1} as cooling rate. The thermograms were analysed by thermal analysis computer software. The melting point (Tm) was taken as the max-point of the endothermic peak, and glass transition temperature (Tg) was taken as the midpoint of the transition curve.

2.3.4 GPC Analysis

The molecular weight of polymers before or after preparation of microspheres, and during degradation was studied by gel permeation chromatography (GPC). An Altex model 110A adjustable flow rate pump preceded by a sintered metal frit was used to

pump GPC grade chloroform at 1 ml/min around the system. Two 300×7.5 mm, 500 Å pore size, 5μm mixed pore highly crosslinked spherical macroporous polystyrene-divinylbenzene matrix (PLGel) columns (Polymer laboratories Ltd, Shropshire, UK) were used in series and were protected by a 50×7.5 mm 10 μm mixed pore guard column (PLGel) (Polymer laboratories Ltd, Shropshire, UK). A Pye Unican LC3 UV detector at a wavelength of 254 nm was used for sample detection (Domb and Langer, 1987). Samples were dissolved in dichloromethane, filtered and injected using a 100μl sample size through a Rheodyne injector valve.

GPC is simply a mechanism of solute separation with molecular size as the discriminating factor. Sample molecules permeate the stationary phase to different degree and are thus retained within the column for periods of time proportional to their molecular size. Columns are tightly packed with a gel or some other porous material and completely filled with solvent (the mobile phase). Traditionally, GPC has been used for the analysis of molecular weight distribution of synthetic polymers. The molecular weight averages (Mn, Mw) indicate the number and length (or weight) of the polymer 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 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 expect in the case of a truly monodisperse system where the values are identical.

Standardisation of the GPC system was achieved using narrow Mw polystyrene standards (Easical, Polymer Laboratories Ltd, Shropshire UK). Inert PTFE strips coated with polystyrene (~5mg) were immersed in 5 ml of dichloromethane. There were two types of strip each representing Mw values of 580, 9200, 66000, 330000,3040000 and 3250, 28500, 156000, 1030000, 8500000 respectively. The calibration curve was obtained (***Figure 2.10***), and each retention time was the average of three readings.

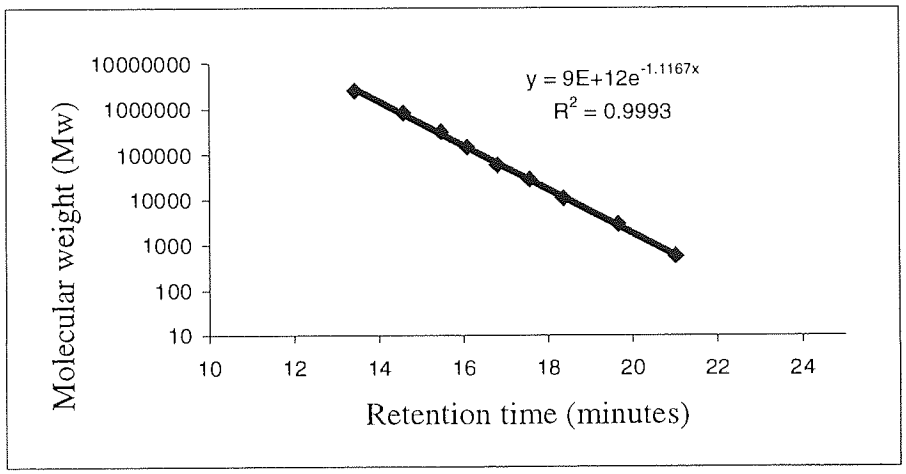


Figure 2.10 Calibration curve for estimation of molecular weight (Mw) by GPC (n=3)

2.4 Results and Discussions

During polymer synthesis, the acetic mixed anhydride prepolymers were prepared by heating diacids in acetic anhydride. Operating under a heating condition resulted in oligomerisation, which occurred in both steps of preparation, the reaction with acetic anhydride and the isolation of the product, when excess acetic anhydride was removed at high temperature (>70 °C). In order to avoid extensive oligomerisation during the isolation step, excess acetic anhydride was removed at a moderate temperature (40-50 °C). Prolong the reaction time for heating diacids in acetic anhydride resulted in high molecular weight oligomers. As using long oligomers in the synthesis of copolymers would create large regions of aliphatic anhydrides, it is advantageous to use shorter oligomers, which would enable a fine distribution of the repeating unit and hence provide uniform hydrolytic degradation as opposed to degradation in sensitive spots in the polymer.

According to the preparation of high molecular weight polyanhydrides (Domb and Langer, 1987), the highest molecular weight polymers were achieved using pure isolated prepolymers and carrying out the reaction under optimised conditions (temperature of 180 °C, vacuum of 10^{-4} mmHg with a dry ice/ acetone trap). Increasing the reaction temperature to 210 °C for 30 min, or increasing the reaction time from 30 min to 45 min at 180 °C, yielded a rubbery gel, which swells extensively in dichloromethane. This gel was partially soluble in dichloromethane. There could be

a depolymerisation process during excessive heating that yields entangled cyclic macromers, which are partially soluble (Domb and Langer, 1987).

In the attempts to produce a crosslinked polyanhydrides by opening the double bond, it is frustrating, but the reaction failed repeatedly. ^{1}H NMR analysis indicated the resulting residue composed of unreacted starting materials. It could be due to the two benzene rings in the polymer backbone. Further study is necessary.

It was found that polymerisation in the presence of a catalyst resulted in higher molecular weight polymers under the same reaction conditions. In this study, cadmium acetate, a well-known catalyst in polyanhydride synthesis, was used. The effect of catalyst on polymer molecular weight is shown in ***Table 2.1***. Since the reaction is an anhydride interchange that involves a nucleophilic attack on a carbonyl carbon, a catalyst that will increase the electron deficiency under the carbonyl carbon should be effective for the polymerisation. Additionally, increasing the aliphatic ratio (SA) resulted in a higher molecular weight, which is similar to the reported value of poly (CPP: SA) (Domb and Langer, 1987).

Polymer	Molecular weight (Mw) (kDa)	
	No catalyst	With catalyst
Poly (CP2B: SA, 20:80)	15.9	35.1
Poly (CP2B: SA, 50:50)	13.3	22.4

Table 2.1 Effect of catalyst inclusion on molecular weight poly (CP2B: SA), 20:80 and 50:50

The ^{1}H NMR spectrum of copolymer (CP2B: SA, 20:80) is shown in ***Figure 2.11.*** The composition of poly (CP2B: SA) was determined by ^{1}H NMR studies from the ratio of the peak integration at 1.3 ppm (8H, SA) and 6.9-8.2 ppm (8H, CP2B), and the practical mole ratios of CP2B: SA in the copolymers are shown in ***Table 2.2***. Additionally, NMR analysis can be used to determine if the polymer is block-like (*i.e.* -A-A-A-B-B-B-), alternating (*i.e.* –A-B-A-B-A-B-), random (*i.e.* probability that A or B is next to a given monomer is equal to its mole fraction in the polymer) or some combination of these (Tamada *et al.*, 1992). If the copolymer is not strictly block-like or alternating, a randomly selected pair of comonomer units (diad) in the polymer chain may be represented as following: SA-SA, SA-CP2B (or CP2B-SA), and CP2B-CP2B. Proton NMR spectra of p (CP2B: SA) in deuterated chloroform revealed two double doublets at 8.1 and 7.9 ppm, and two triplets at 2.6 and 2.4 ppm. The homopolymer p (SA) has only one triplet at 2.4 ppm, and the homopolymer p (CP2B) has only one doublet at 8.1 pp. Therefore, the downfield doublets at 8.1 and 7.9 ppm represent CP2B-CP2B and CP2B-SA respectively. The upfield triplets at 2.6 and 2.4 ppm are SA-CP2B and SA-SA, respectively. If the probability of SA reacting with SA is the same as the probability that CP2B reacting with SA and vice versa, then the portions of SA-SA, SA-CP2B, or CP2B-CP2B in the final polymer could be obtained from the integrations of the correspondent peaks in their ^{1}H NMR spectra. Furthermore, from these data, the degree of randomness (H) of the polymers and the number-average sequence length of a monomer (L) can be calculated (***Table 2.2***):

H= p (CP2B-SA)/ p (CP2B) p (SA)

L_{SA}= 1/p (CP2B)

L_{CP2B}=1/p (SA)

When H=0, this indicates either a completely block copolymer or a mixture of homopolymers; H<1 means block character of the copolymer; H=1 means the polymer takes a random distribution; H>1 means alternating tendency; and H=2 means a completely alternating copolymer (e.g., ...-ABAB-...) (Ron *et al.*, 1991). The sequence distribution of monomers in the copolymer can help explain erosion behaviour. If the different types of bonds have different reactivities, then the appearance of monomers relative to each other would be affected. This has been reported with more rapid erosion of SA than CPP in a copolymer device that occurred as the SA-CPP and SA-SA bonds were broken, leaving the CPP-CPP bonds behind (Tamada and Langer, 1992).

The data in ***Table 2.2*** showed that the copolymers, both p (CP2B: SA, 20:80) and p (CP2B: SA, 50:50), exhibited block character, and the number-average sequence length of SA was changed from 5.7 to 2.4 for the poly (CP2B: SA) 20:80 to 50:50 respectively. Therefore, the monomers were not distributed randomly in the chain of copolymers. The long block length of SA that was a high fraction of monomer in poly (CP2B: SA, 20: 80) created a large region of aliphatic anhydride.

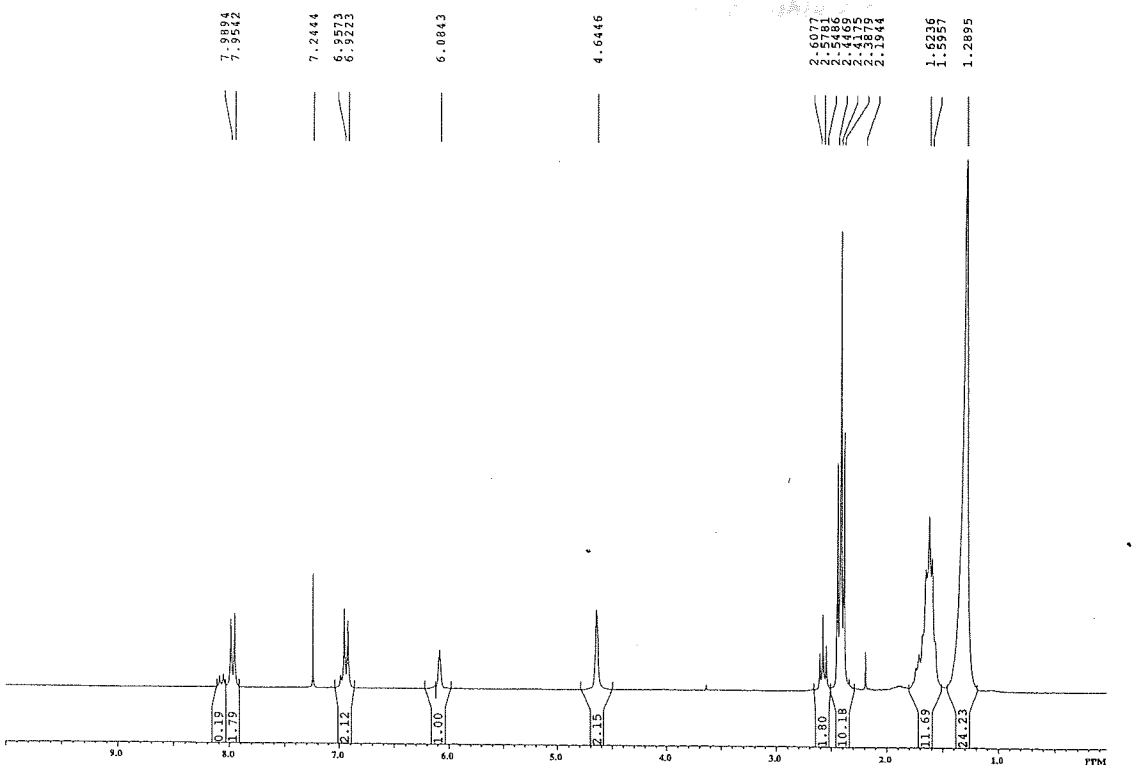


Figure 2.11 ^{1}H NMR spectrum of p (CP2B: SA) 20:80

Mole ratio of CP2B-SA in the feed (theoretical)	20:80	50:50
Mole ratio of CP2B-SA in the polymer, p (SA)	0.83	0.59
Probability of finding the diad SA-SA, p (SA: SA)	0.67	0.32
Probability of finding the diad SA-CP2B, p (SA: CP2B)	0.24	0.59
Average block length L_{SA}	5.7	2.4
Average block length L_{CP2B}	1.2	1.7
Degree of randomness (H)	0.64	0.87

Table 2.2 Comonomer sequence distribution of poly (CP2B: SA) 20:80 and 50:50

Data from IR analysis and DSC analysis are summarised in ***Table 2.3***.

Polymer	IR (KBr) (cm^{-1})	Tm (^{o}C)	Heat of fusion (J/ Gram)
P (CP2B: SA, 20:80)	1810,1740	70 ±1.6	60.2 ± 4.1
P (CP2B: SA, 50:50)	1810, 1780, 1740	62 ± 2.0	40.1 ± 4.4

Table 2.3 IR analysis and DSC analysis of Poly (CP2B: SA) 20:80 and 50:50 (n=3, mean ± s.d.)

A typical IR spectrum of aliphatic and aromatic polymer that contains aliphatic and aromatic anhydride bonds may present three distinct peaks, where the aliphatic peak is shown at 1810 cm^{-1}, the aromatic peak is shown at 1780 cm^{-1} and the peaks at 1720-1740 cm^{-1} in general overlap (Domb *et al.*, 1997). The IR analysis of poly (CP2B: SA, 20:80) showed a low percentage of the aromatic peak (1780 cm^{-1}), and a strong absorb at 1805 cm^{-1}, indicating that the polymer chain was composed mostly of SA. However, poly (CP2B: SA, 50:50) had typical absorptions at 1802, 1780 and 1740 cm^{-1}, which indicated there was a fine distribution of CP2B and SA in polymer.

DSC indicated that both the melting point and the heat of fusion of polymers fell with increasing percentage of CP2B. The heat of fusion values for the polymers demonstrated a decrease as CP2B was added to SA or vice versa. This could be due to a relatively homogeneous monomer distribution, which undergoes coupling to yield a high molecular weight fraction, and hence less thermal energy is needed to overcome the intermolecular forces. After adding one monomer, a decreasing trend in crystallinity is shown, which is a direct result of the random presence of other units in

the polymer chain. When two monomers - one forming a crystalline homopolymer (*e.g.* poly (SA)), and another forming an amorphous homopolymer (*e.g.* poly (CPH)) - are copolymerised, the degree of copolymer crystallinity decreases as the second constituent is added to either homopolymer (Shieh *et al.*, 1994). The poly (CP2B: SA, 20:80) contains more hydrolytic aliphatic acid SA, and has a higher heating fusion and higher crystallinity, probably due to crystalline regions of poly (SA) units.

Chapter 3 Synthesis and Characterisation of Glutamic Acid Based Polyanhydride

3.1 Introduction

A variety of polymeric structures containing amino acids have been synthesised, principally polypeptides which have been used in structural, immunological, and enzymological studies as well as in biomaterials, such as sutures , skin substitutes and drug delivery systems. These synthetic polypeptides have a significant capacity to be both biocompatible and to biodegrade to natural-occurring biological products. However, there are two obstacles commonly limiting the suitability of polypeptides for drug delivery applications: their biological degradation and potential toxicity Sanders and Kon, 1991). The catalytic activity of native enzymes is usually relied upon for polypeptide degradation (Pytela *et al.*, 1990), but the stability of the amide backbone towards hydrolysis. Incorporating labile chemical bonds into the polypeptides backbone can introduce hydrolytic instability to the polymer chain. As a result, the biological degradation of polypeptides will reduce, and the degradation will reply on the amide backbone hydrolysis mainly. To this end, the synthesis of copolymers of amino with anhydrides has been studied (Domb, 1990). Because amide linkages can strengthen the polymer by intermolecular attractive forces (hydrogen bonding), mechanical properties of polyanhydrides could be improved by integration amino acid (Andrea, *et al.*, 1990). In this study, the amino acid Glutamic acid (Glu) was incorporated into the polymeric backbone *via* hydrolytically labile anhydride linkages.

Soybean protein and its derivatives were a focus of polymer research in the 1930s and 1940s (Tadros *et al.*, 1999). As a major component of oil-seed protein, glutamic acid is easy to obtain at a low cost. In this study, glutamic acid was selected to produce a

prepolymer (GluSA) with sebacic acid (SA), and then synthesise copolymers with SA. Glutamic acid is an α-amino acid containing two carboxylic acids and one amino group as shown in ***Figure 3.1***, and poly (L-glutamic acid) is a biodegradable material (Anderson *et al.*, 1974). As a result, the degradation products of polymer (GluSA: SA) should be biocompatible.

The monomer (GluSA) produced by glutamic acid and SA (***Figure 3.2***), not only provides a protected amino acid for the next reaction, but also yields four carboxylic acid groups. Such a monomer could act like a crosslinking agent in polymerisation to prepare crosslinked polymers. Polymers (GluSA: SA) with different mole ratios of 0: 100, 5: 95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30 80:20, 90:10 and 100:0, were synthesised and studied. In order to extend the study the effect of GluSA in the polymers, copolymer (CPP: SA: GluSA, 20:70:10) was studied.

The characterisation of polyanhydrides is also presented in this chapter.

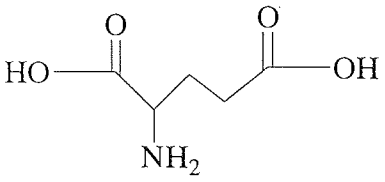


Figure 3.1 The structure of Glutamic acid

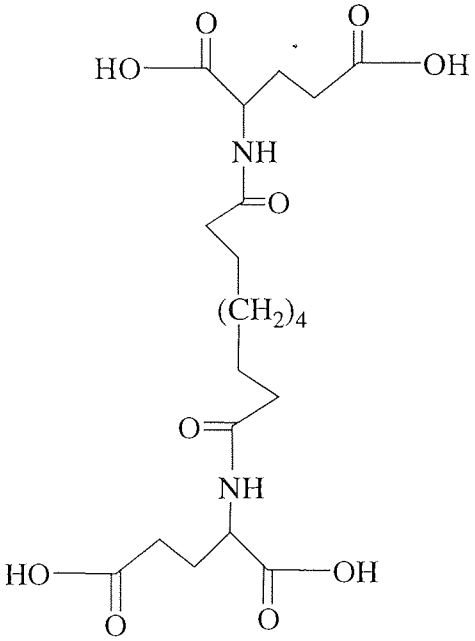


Figure 3.2 Glutamic acid –SA monomer (GluSA)

3.2 Experimental

3.2.1 Materials

Sebacic acid (SA) (Sigma-Aldrich)

1,3-bis (p-carboxyphenoxy) propane (Sigma-Aldrich)

p-hydroxybenzoic acid (Sigma-Aldrich)

Dry acetic anhydride (Acros Organics)

Glutamic acid (Acros Organics)

N-hydroxysuccinimide (Sigma-Aldrich)

1,3- dicyclohexylcarbodiimide (DCC) (Avocado Research Chemicals Ltd)

Thionyl chloride (Sigma-Aldrich)

Trietheylamine (TEA) (Avocado Research Chemicals Ltd)

4-dimethylaminopyride (DMAP) (Avocado Research Chemicals Ltd)

Acetyl Chloride (Sigma-Aldrich)

4-methylmorpholine (Sigma-Aldrich)

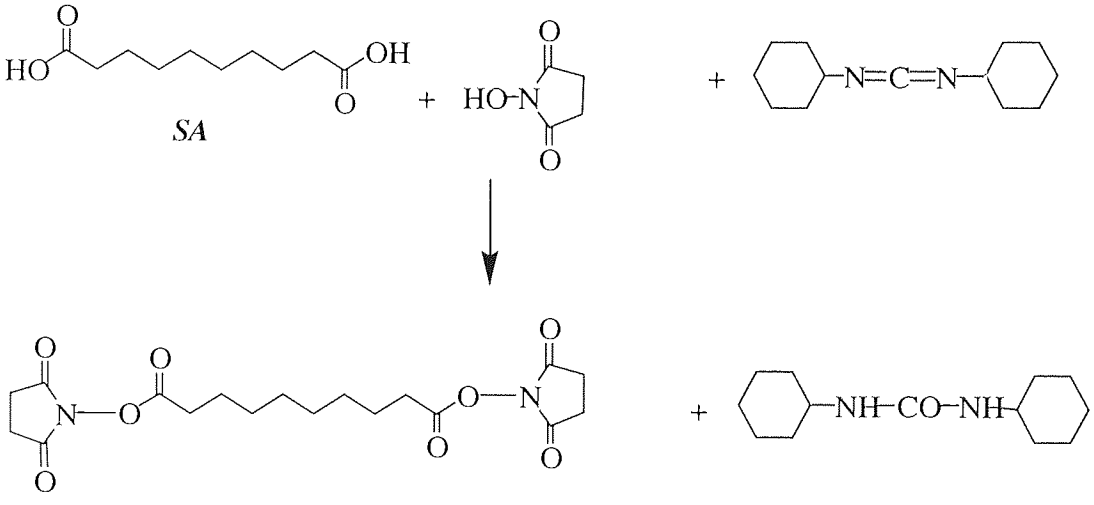
All other solvents of analytical grade (Fisher Scientific)

3.2.2 Preparation of Glutamic acid –SA monomer (GluSA)

3.2.2.1 Active Ester Method

Most coupling reagents simply cause the activation of a carboxyl group converting it into an active ester (Bodanzky, 1994). In this study, sebacic acid was converted into an active ester by employing N-hydroxysuccinimide as a coupling reagent (***step 1***). This active ester was then reacted with glutamic acid with removal of the coupling reagent (***step 2***) to achieve glutamic acid-SA (GluSA) monomer.

Step 1:



N-hydroxysuccinimide Ester

Figure 3.3 Synthesis of N-hydroxysuccinimide Ester

A solution of sebacic acid (2.0225 g, 10 mmol) and N-hydroxysuccinimide (2.3018 g, 20 mmol) in dry dimethylformamide (DMF) was cooled in an ice-water bath and 1,3-dicyclohexylcarbodiimide (4.1266 g, 20 mmol) was added with stirring. After 30 minutes, the mixture was kept stirring at room temperature overnight. The white precipitate N, N'-dicyclohexylurea was removed by filtration. The filtrate was poured into saturated $NaHCO_3$ solution to result in a white precipitate. The crude product was washed with saturated $NaHCO_3$, 1 N HCl and distilled water 3 times in turn, and dried in vacuum.

Step 2:

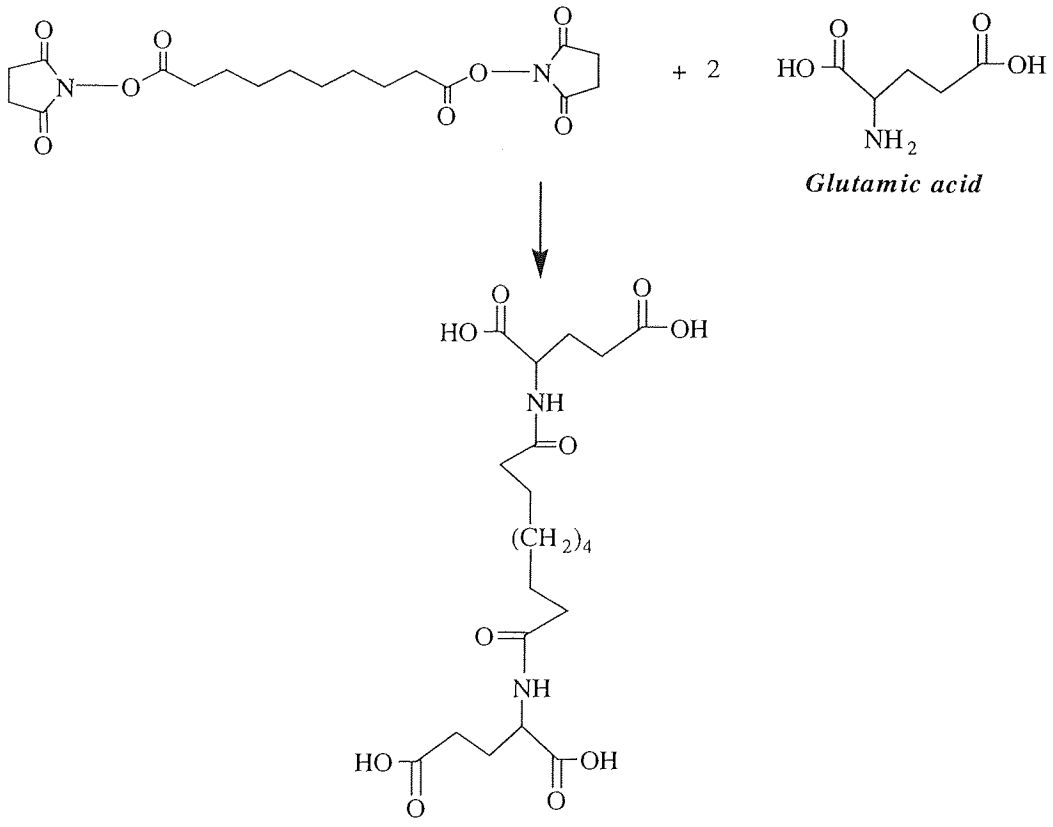


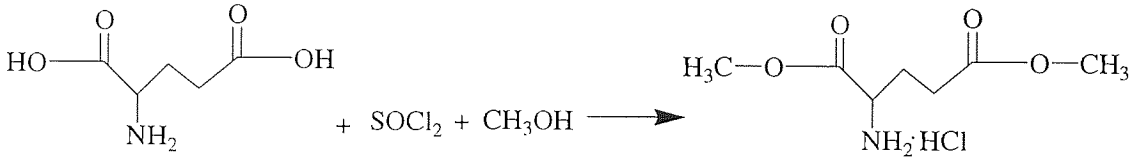
Figure 3.4 Synthesis of glutamic acid-SA monomer (GluSA)

2.9426 g (20 mmol) of L-Glutamic acid and 3.36 g (40 mmol) of $NaHCO_3$ were dissolved in distilled water at room temperature. N-hydroxysuccinimide active ester (3.96 g, 10 mmol) was added into the mixture with stirring and acetonitrile was added into the suspension until the active ester was dissolved. Stirring was continued for 72 hours, and then the acetonitrile was evaporated under vacuum to afford the aqueous solution. The solution was washed with $CHCl_3$ three times, then the water was evaporated, and the residue was dissolved in methanol, acidified with HCl. After filtration to remove the sodium salt, the methanol was evaporated to afford the yellow crude product. Purification by column chromatography using EtOAc: Hexane=1:1 yielded the product that was dried under vacuum.

3.2.2.2 Multi-step Method

In addition to the active method, the traditional method was also applied for preparation of the GluSA monomer. Three steps were described as following:

Step 1:

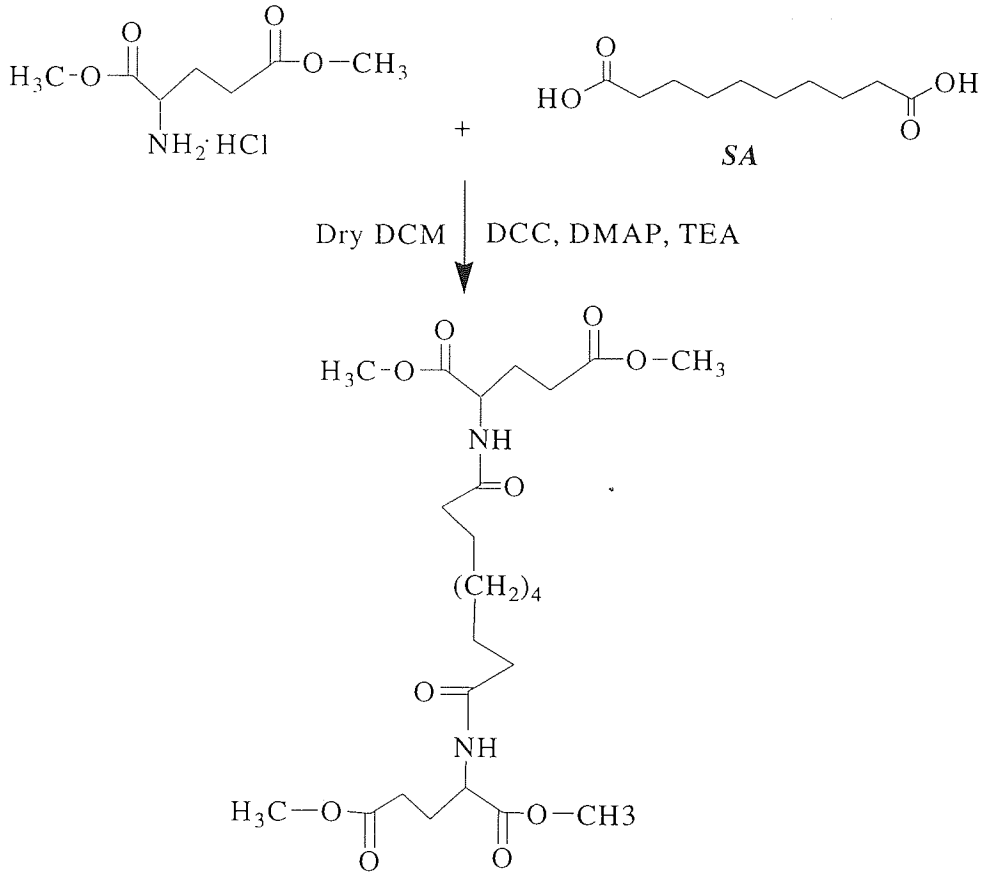


Glutamic acid ***Glutamic acid dimethyl ester HCl salt***

Figure 3.5 Synthesis of Glutamic acid dimethyl ester HCl salt

90 ml of methanol (MeOH) was added into a round bottom flask, and then the flask was immersed into an ice-water bath to cool down to 0°C. 17.93g (11 ml, 150 mmol) of thionyl chloride ($SOCl_2$) was added into methanol slowly and carefully along the side of flask, avoiding any contact with water at all time. Adding $SOCl_2$ gave off heat and the flask with mixture was cooled down in ice-water bath. Glutamic acid (10 g, 69 mmol) was added, and the mixture was stirred at room temperature overnight. The excess $SOCl_2$ and MeOH were removed under vacuum to afford a yellow viscous liquid purified by recrystallised from tetrahydrofuran (THF) and dried under vacuum.

Step 2:



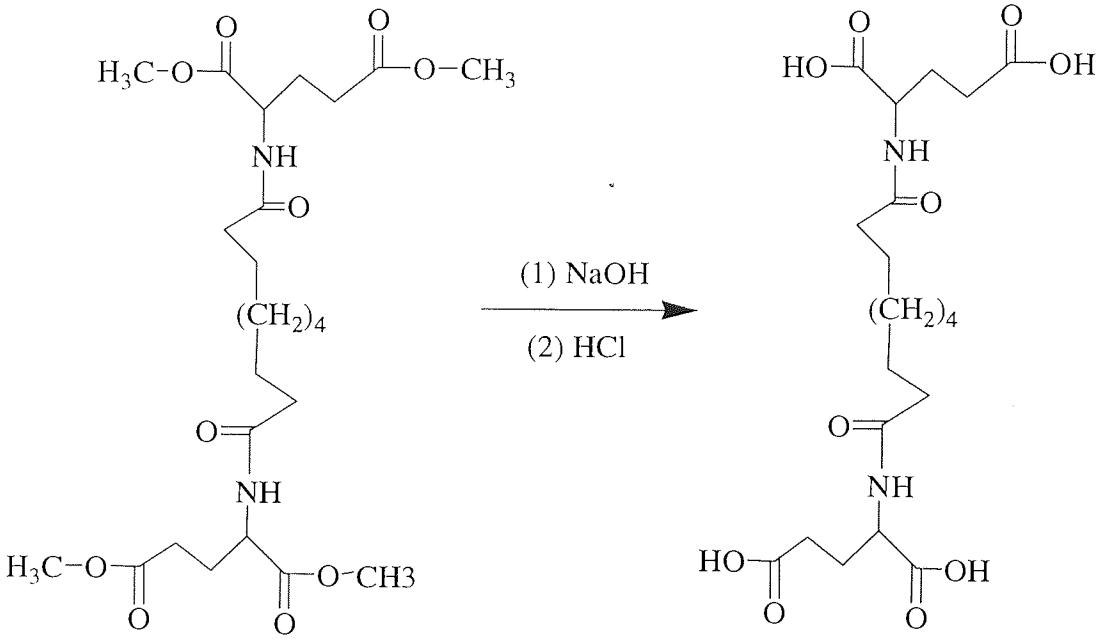
Glutamic acid -SA dimethyl ester

Figure 3.6 Synthesis of Glutamic acid-SA dimethyl ester

The glutamic acid-SA dimethyl ester (5 g, 24 mmol) was suspended in dry dichloromethane (DCM) and cooled down in ice-water bath. Addition of trietheylamine (TEA) (3.29 ml, 24 mmol) caused an increase in viscosity and additional DCM was used. After stirring for 10 min, SA (1.5918 g, 8 mmol), DCC (3.4090 g, 16.5 mmol) and DMAP (0.0602 g, 0.49 mmol) were added. This mixture was stirred at room temperature overnight. After filtration, the filtrate was collected and washed with 2 N HCl three times, saturated $NaHCO_3$ three times, and then

distilled water three times. The DCM solution was dried over $MgSO_4$, and filtratered. The DCM was evaporated under vacuum to obtain the product.

Step 3:



Glutamic acid -SA (GluSA)

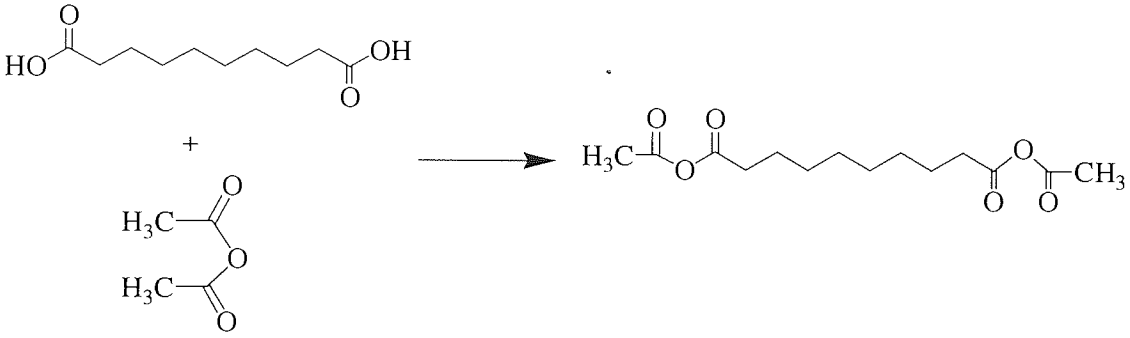
Figure 3.7 Synthesis of GluSA

Glutamic acid-SA dimethyl ester (5 g, 9.96 mmol) was dissolved in 20 ml of MeOH: THF (1:1, v:v). To the solution 44.304 ml of 1 M NaOH was added, and the mixture was kept stirring for 4-5 hours at room temperature. 20 ml of distilled water was added into the mixture. Then the mixture was washed with DCM three times. HCl was used to adjust the pH to 1. After evaporation of MeOH-THF and water, acetone was added to dissolve the product, and $MgSO_4$ was used to dry the acetone solution.

NaCl salt was removed by filtration, and the acetone was evaporated to obtain a yellow product that was dried under vacuum.

3.2.3 Preparation of Prepolymer

3.2.3.1 Sebacic Acid Anhydride (SAA)



Synthesis of SAA is described in full in ***Chapter 2.***

3.2.3.2 GluSA Anhydride (GluSAA)

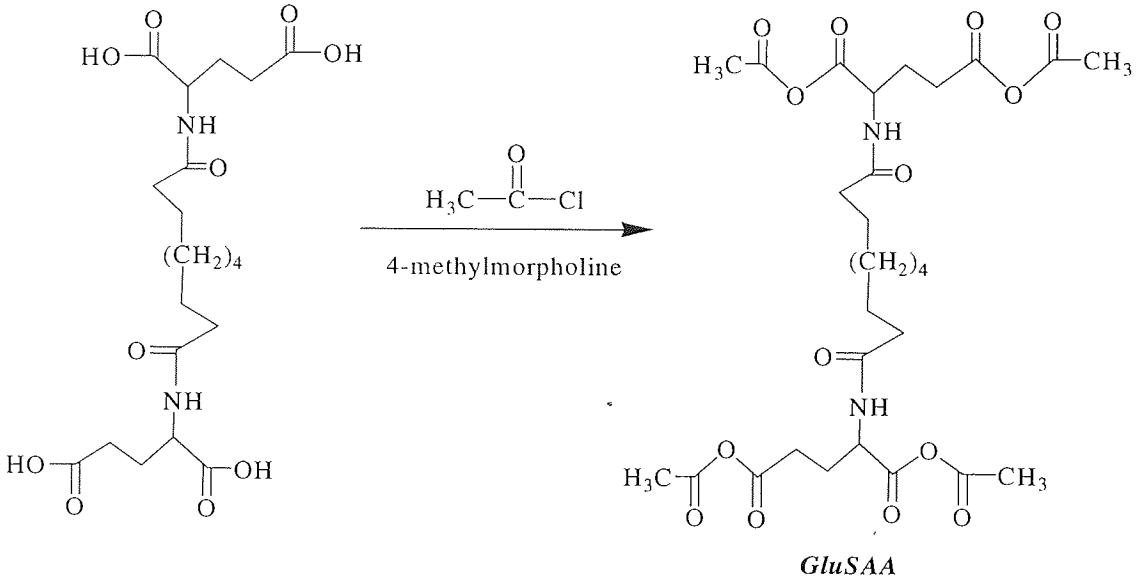


Figure 3.8 Synthesis of GluSAA

3 g (6.5 mmol) of GluSA was suspended in dry DCM with stirring in ice-water bath. To this suspension, 4.3 ml (39 mmol) of 4-methylmorpholine was added, and the mixture kept stirring for 10 minutes. 1.855 ml (26 mmol) of acetyl chloride was dissolved in 5 ml of dry DCM, and then dropped into the reaction mixture over 10 minutes. After addition of acetyl chloride, the mixture was stirred in an ice-water bath for 30 minutes. After filtration, the filtrate was collected and washed with saturated $NaHCO_3$ aqueous solution, followed by 0.5 N HCl and water, and dried over $MgSO_4$ (Bodanszky, 1994). DCM was evaporated under vacuum to obtain the thick liquid product.

3.2.3.3 1,3-Bis (p-carboxyphenoxy) propane Anhydride (CPPA)

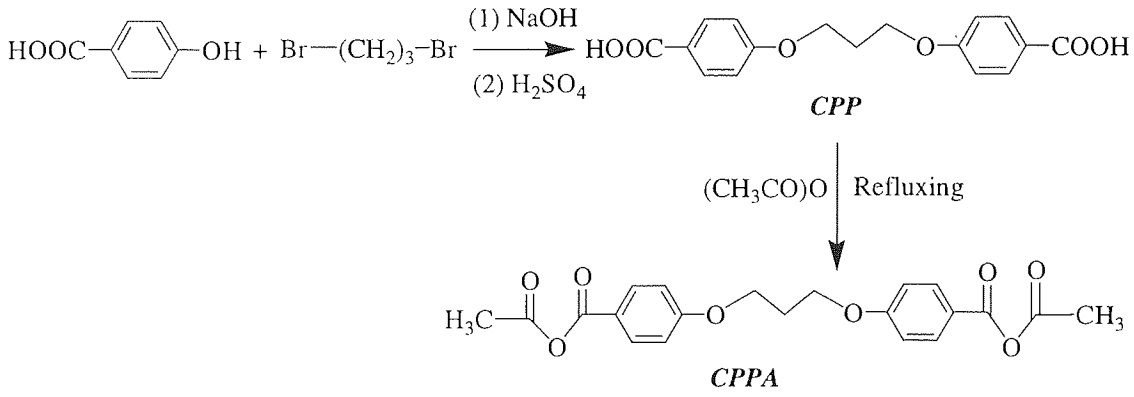


Figure 3.9 Synthesis of CPPA

A solution of 6.9 g (50 mmol) of 1,3-bis (p-carboxyphenoxy) propane and 4 g (100 mmol) of sodium hydroxide in 20 ml of water was refluxed and stirred in a flask equipped with a condenser and a dropping funnel. While the mixture was refluxing, 5.1 g (25 mmol) of 1,3-bis (p-carboxyphenoxy) propane was added through the funnel over a period of 1 hour. After the addition of 1,3-bis (p-carboxyphenoxy) propane, the reaction mixture was refluxed for 3 hours, and 1 g (25 mmol) of NaOH was added to the mixture, which was refluxed for a further 2 hours. Heating was stopped, and the reaction mixture was left standing overnight. The white precipitate was isolated by filtration and washed with 20 ml of methanol. The still wet precipitate was dissolved in 50 ml of distilled water, and then the solution was warmed to 60-70°C and acidified with 6 N sulfuric acid. While the mixture was still warm, the resulting product (CPP) was isolated by filtration and dried in a vacuum oven at 80°C. 6 g of 1,3-bis-(p-carboxyphenoxy) propane (CPP) and 65 ml of dry acetic anhydride were placed in a flask fitted with a condenser and a gas inlet tube. A slow stream of dry nitrogen (dried

through $CaCl_2$) was bubbled through the refluxing mixture. After 30 minutes, the hot mixture was filtered, and the slightly yellow filtrate was concentrated to a volume of about 15 ml by evaporating acetic anhydride under vacuum at a temperature not higher than 65°C. The remaining mixture was stored in a freezer overnight. The white crystals (CPPA) were isolated by filtration, washed with dry ether, and dried in a vacuum oven at 70°C.

3.2.4 Synthesis of Crosslinked Polymers

3.2.4.1 Crosslinked Copolymer (GluSA: SA)

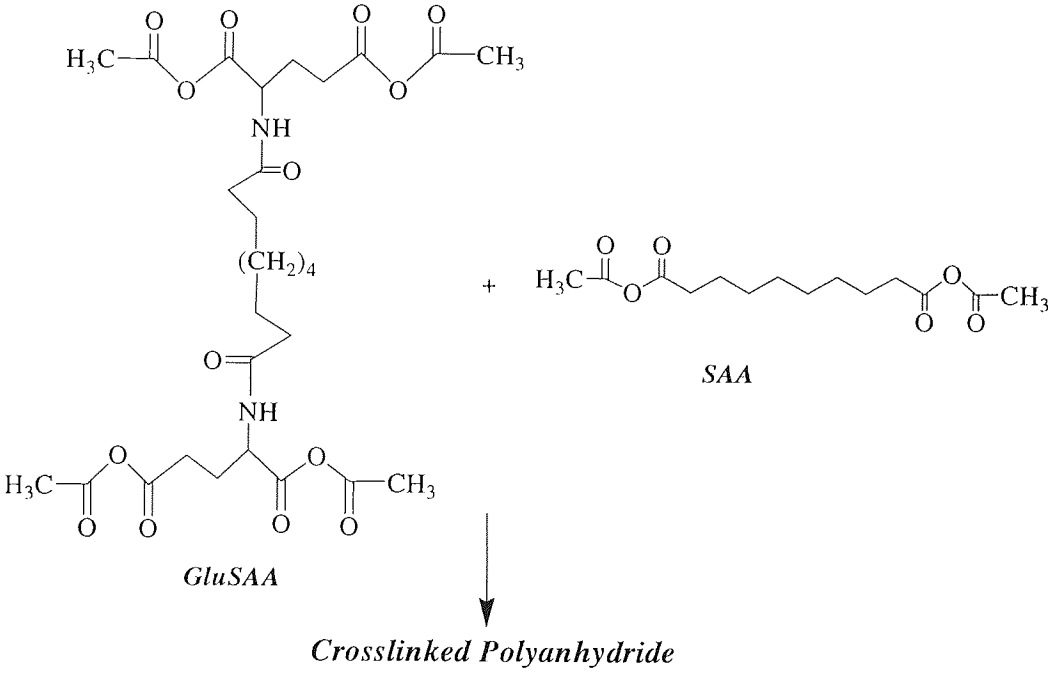


Figure 3.10 Synthesis of crosslinked copolymer (GluSA: SA)

SAA prepolymers and 2 molar percent catalyst, cadmium acetate, was ground into fine powder, and mixed with GluSAA prepolymer in a glass tube with a stirrer and a side arm equipped with a capillary nitrogen inlet, according to the mole ratios 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100. The tube was immersed in an oil bath at 180 °C. After the prepolymers were melted (within 1 minute), a high vacuum (2-3 mm Hg) was applied through the side arm. The condensation product, acetic acid, was collected in an acetone/dry ice trap. During the polymerisation, a strong nitrogen sweep with vigorous agitation of the melt was performed for 30 seconds every 15 minutes. After 30 minutes, the heating was stopped, and cooled to room temperature. The crude polymer was dissolved in dry dichloromethane, and the solution was filtered and dropped into dry petroleum ether (six times the volume of the solution). After several hours stirring at room temperature, the polymer was precipitated. After filtration, the precipitate was extracted with dry diethyl ether for several hours at room temperature, the ether was decanted and the residue was removed by evaporation under a high vacuum (Domb *et al.*, 1988).

3.2.4.2 Crosslinked Copolymer (CPP: SA: GluSA, 20:70:10)

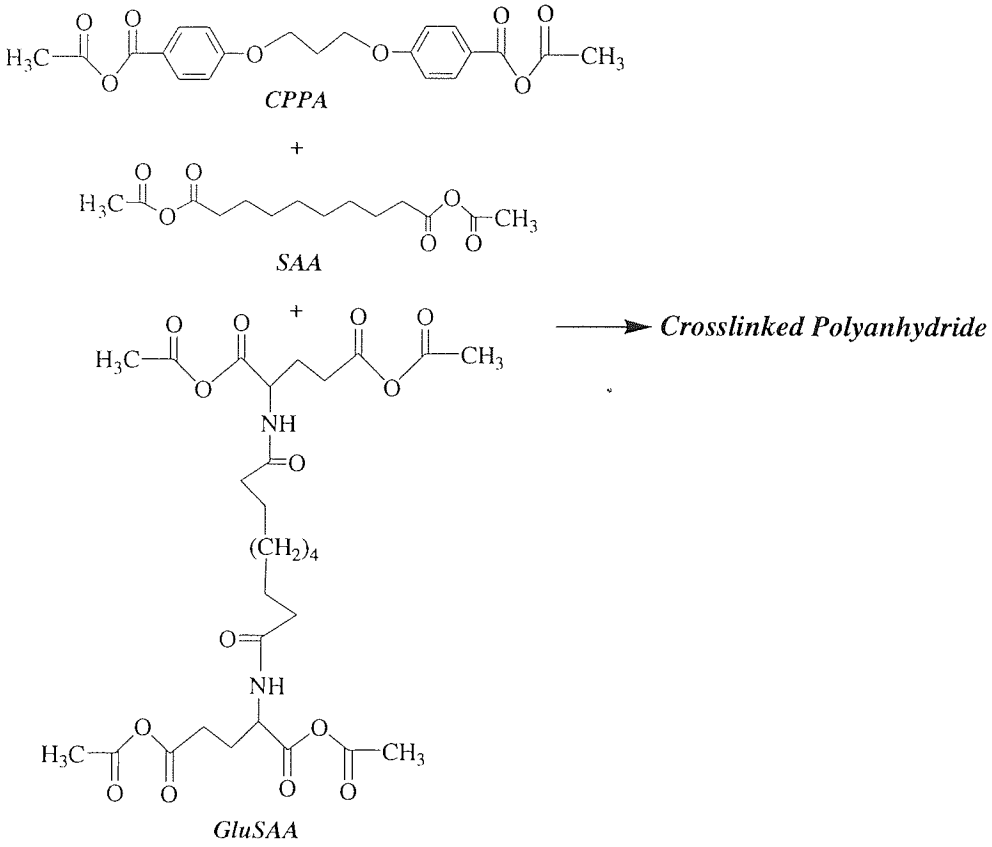


Figure 3.11 Synthesis of crosslinked copolymer (CPP: SA: GluSA, 20: 70: 10)

CPP, SAA prepolymers and 2 molar percent catalyst, cadmium acetate, was ground into fine powder, and mixed with GluSAA prepolymer in a glass tube with a stirrer and a sidearm equipped with a capillary nitrogen inlet, according to the mole ratio 20: 70: 10. The following procedure is same as the section ***3.2.4.1***.

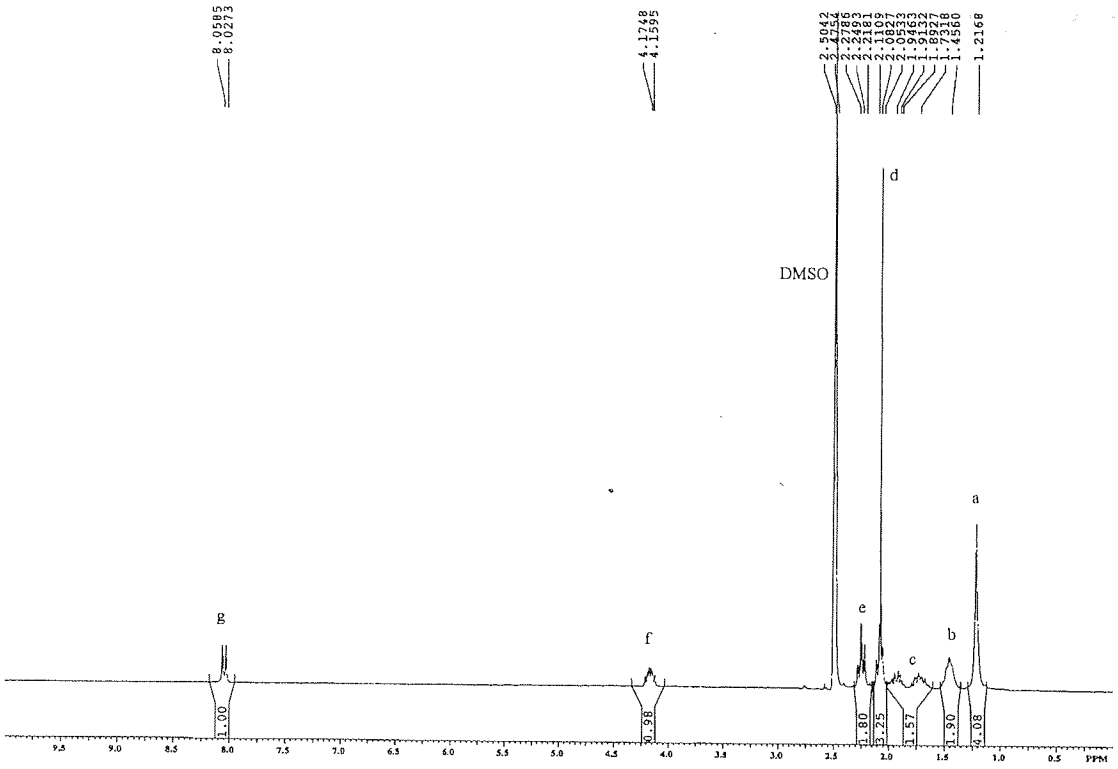
3.3 Results and Discussions

There are two methods to achieve the glutamic acid-SA monomer, which provided different yields (as shown in ***table 3.1***)

Method	Yield
Active Ester method	6-10%
Multi-step method	80-87%

Table 3.1 Yields of two methods used to prepare GluSA monomer

On comparing the preparation methods, although active ester methods had successfully synthesised GluSA, and it was easier to operate than multi-step method, the low yield of this method reduces its suitability for preparing polymers for pharmaceutical use. The multi-step method produced higher yield, and the starting materials and by-products can be removed (as evidenced by a clear ^{1}H NMR spectrum ***Figure 3.12***) by a simple operation (filtration and wash), avoiding column chromatography purification.



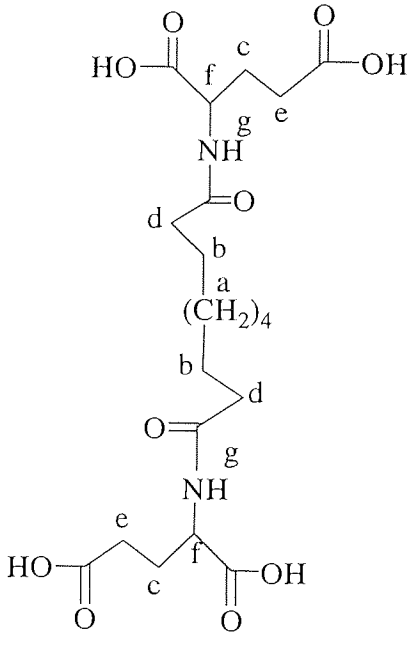


Figure 3.12 ^{1}H NMR spectrum of GluSA

Generally, polyanhydrides are synthesised by melt-polycondensation of mixed anhydrides of aliphatic acid and aromatic acid. Aliphatic mixed anhydride prepolymers are prepared by refluxing the carboxylic acid monomers in acetic anhydride. However, monomer GluSA refluxed in acetic anhydride produced a mixture, which ^{1}H-NMR spectrum showed was a mess. The mess result caused by heating GluSA in acetic anhydride was probably due to interfering side reactions. The amide-containing polymerisation was limited by possible side reactions of the free remaining electron pair on the secondary amide group, *e.g.* intermolecular cyclisation to form N-carboxyanhydrides (Staubi *et al.*, 1990). In this study, GluSA mixed anhydride prepolymer was prepared by reacting the GluSA monomers with CH_3COCl. The resulting product has the characteristic bonds (1820 and 1770 cm^{-1}) in its IR spectrum (***Figure 3.13***).

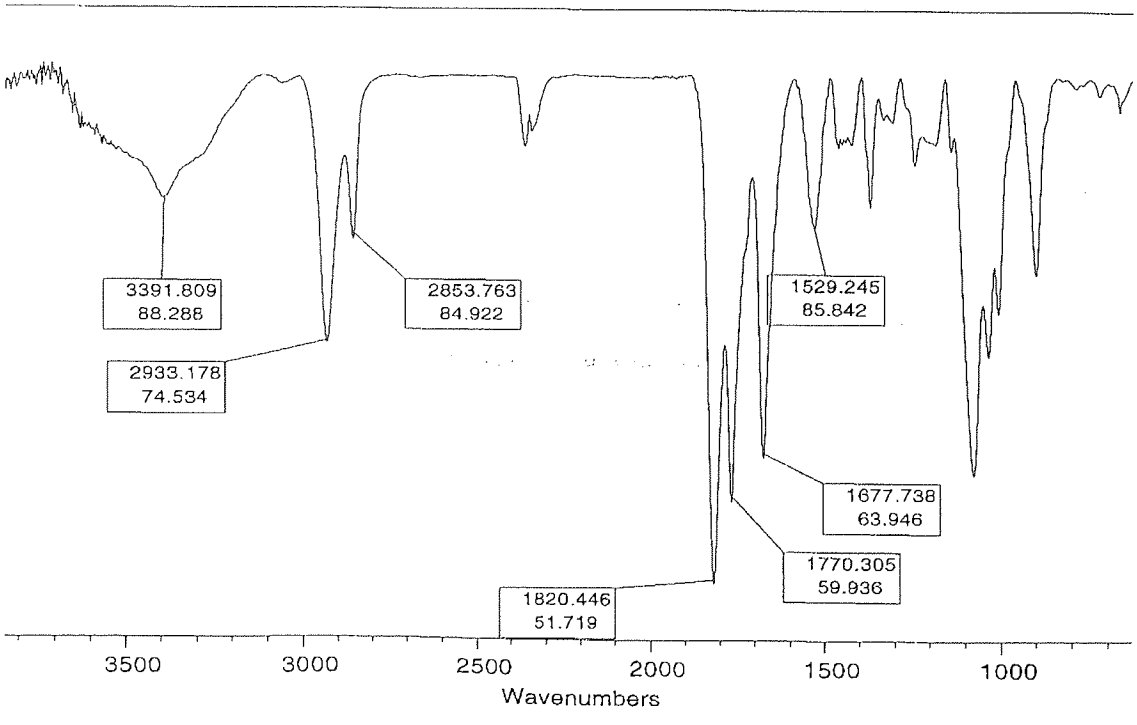


Figure 3.13 IR spectrum of GluSA mixed anhydride prepolymer

It was difficult to isolate and purify the prepolymer (GluSAA) without evoking decomposition. Washing the reaction mixture with $NaHCO_3$, HCl and water would effectively clean the prepolymer, but at the same time would induce decomposition. In addition, GluSAA prepolymer is not stable at room temperature within 2 hours as the peak at 1820 cm^{-1} became weaker and the peak at 1770 cm^{-1} became stronger in IR spectrum of GluSAA, which could mean that a cyclic anhydride was forming or the prepolymer was decomposing. It is possible that the cyclic formation of GluSA is a preferred stable product, and therefore, the melt-polycondensation must be carried out as soon as GluSAA was prepared.

Crosslinked copolymers were synthesised by melt-condensation. The solubility of the polymers in common organic solvents were determined at room temperature by dissolving 1 g of polymer in 2 ml of solvent, after 30 minutes stirring, the mixture was filtered and the solubility was determined gravimetrically (% w/v) (Domb and Maniar, 1993). The results are shown in ***Table 3.2***, and the physical properties of the copolymers of GluSA and SA are summarised in ***Table 3.3***.

Solvent	Polymer (GluSA:SA)										
	5:95	10:90	20:80	30:70	40:60	50:50	60:40	70:30	80:20	90:10	100:0
DCM	>30	>10	>10	100[a]	100[b]	100	100	100	100	100	100
$CHCl_3$	>30	>10	>10	100[a]	100[b]	100	100	100	100	100	100
THF	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Acetonitrile	0.04	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3.2 Solubility of Copolymer (GluSA:SA) (% w/v)

[a] After 30 min stirring, the polymer swelled in the solvent. Kept stirring for another 2 hours, the polymer dissolved in solvent.

[b] After 30 min stirring, the polymer swelled in the solvent. Kept stirring for another half hour, the polymer dissolved in solvent.

Polymer (GluSA:SA)	Molecular Weight [a] (Mw)	Melting Point [b] (°C)	Heat of Fusion [b] (J/g)	Physical Appearance
5:95	29.6 kDa	69-70	56.5±2.63	Slightly yellow solid
10:90	20.5 kDa	66-70	54.7±2.94	Yellow solid
20:80	16.8 kDa	61-64	45.5±1.87	Yellow flexible solid
30:70	10.2 kDa	45-60	9.9±1.15	Brown rubber
40:60	5.0 kDa	25-35	5.4± 1.04	Sticky rubber
50:50	8.5 kDa	*	*	Sticky syrup
60:40	6.5 kDa	*	*	Sticky syrup
70:30	6.4 kDa	*	*	Sticky syrup
80:20	2.7 kDa	*	*	Very sticky syrup
90:10	1.3 kDa	*	*	Very sticky syrup
100:0	1.0 kDa	*	*	Very sticky syrup

Table 3.3 Properties of Copolymer (GluSA:SA)

[a] Determined by GPC using polystyrene standard (n=3)

[b] Determined by DSC (n=3, mean ± s.d.)

* Not detectable

As shown in ***Table 3.2***, the series of polymers posses good solubility in DCM and $CHCl_3$ compared to poly (CPP: SA, 20: 80) with a solubility of >30 (%w/v) (Domb and Maniar, 1993). These polymers have low melting point (<100 °C). Additionally, in physical appearance, the poly (GluSA: SA, 20: 80) was flexible, and poly (GluSA: SA, 40:60) was a sticky rubber at room temperature.

The homopolymer (GluSA) is very sticky syrup with a low molecular weight. Copolymerisation with sebacic acid resulted in an increase in molecular weight and melting point as the sebacic acid content in the copolymer was increased. However, only a relatively low molecular weight of 29.6 kDa could be obtained at a ratio of GluSA: SA, 5: 95. This may be due to the linear prepolymer (GluSAA) reacting with itself or SA in various ways to yield longer and shorter chains, and adjacent chains could react with themselves to produce larger and smaller rings. This can be seen in IR spectra of the copolymers as well. The peak at 1810 cm^{-1} was strong, and the peak at 1740 cm^{-1} was reduced in IR spectra for copolymer (GluSA: SA) 5:95, 10:90, 20:80; however, the peak of 1810 cm^{-1} was reduced and the peak of 1740 cm^{-1} became stronger in the IR spectra for copolymer (GluSA: SA) 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. Moreover, the purity of prepolymer is a crucial factor for preparation of high molecular weight polymer. During the prepolymer manufactory, purification may not have been effective.

A small amount of GluSA was copolymerised with CPP and SA to synthesise copolymer (GluSA: CPP: SA, 10:20: 70). The properties of poly (GluSA: CPP: SA, 10:20:70) and poly (CPP: SA, 20: 80) are summarised in ***Table 3.4***.

Polymer	Physical Appearance	Solubility In DCM and $CHCl_3$ (% w/v)	IR [a]	Melting Point [b] (°C)	Heat of Fusion [b] (J/g)	Molecular Weight
P (GluSA: CPP: SA, 10:20:70)	Slightly yellow rubber	>10	1810cm^{-1} 1790cm^{-1} 1740 cm^{-1}	64±3	36.7± 3.17	53.7 kDa [c]
P (CPP: SA, 20:80) [d]	White solid	>30	1810cm^{-1} 1790cm^{-1} 1740 cm^{-1}	68 ± 4	64.0	50,000 ± 20,000

Table 3.4 Data Analysis for p (GluSA: CPP: SA, 10:20:70) and p (CPP: SA, 20:80)

[a] KBr disk

[b] Determined by DSC (n=3, mean ± s.d.)

[c] Soluble part determined by GPC using polystyrene standard (n=3)

[d] Data from Domb et al, 1999 and Kumar et al, 2002

Compared with the typical white solid polymer, poly (CPP: SA, 20:80), poly (GluSA: CPP: SA, 10:20: 70) was a slightly yellow rubber. Examination of the IR spectra of poly (GluSA: CPP: SA, 10:20: 70) revealed bonds at 1810 cm^{-1} (strong), 1790 cm^{-1} (most covered by 1810 cm^{-1}) and 1740 cm^{-1} (medium). However, due to its poor solubility in DCM and $CHCl_3$, the composition could not be studied using ^{1}H-NMR. The soluble fractions of poly (GluSA: CPP: SA, 10:20:70) in $CHCl_3$ were determined by ^{1}H-NMR, where only the peaks of CPP (7.99 ppm, 6.9 ppm and 4.2 ppm) and the peaks of SA (1.29 ppm, 1.63 ppm and 2.4 ppm) were seen. The practical mole ratio of CPP: SA was 17:73 in poly (GluSA: CPP: SA, 10:20:70). In the thermal study, the melting point of poly (GluSA: CPP: SA, 10:20:70) is close to that of p (CPP: SA, 20:80). However, compared with p (CPP: SA, 20:80), the heat of fusion of poly

(GluSA: CPP: SA, 10:20:70) decreased sharply because 10% of GluSA replaced 10% of CPP. This suggests that the degree of crystallinity is decreased due to a relatively homogeneous monomer distribution or crosslinking net building, hence less thermal energy was needed to overcome the intermolecular forces.

From these preliminary studies, it was seen that the physical appearance of polymers was sticky and rubbery at room temperature, and melting point and heat of fusion decreased with the addition of GluSA. These studies demonstrated that it might be possible to develop crosslinked polymers using GluSA as a crosslinking fragment.

Chapter 4 Synthesis and Characterisation of Aspartic Acid Based Polymers

4.1 Introduction

Products of polymer degradation need to be considered when designing biomedical implants. Because poly (amino acids) are naturally occurring, the possibility of associated toxicity may be reduced. Aspartic acid (***Figure 4.1***) is a unique amino acid that has a range of valuable current and potential applications, both as a homopolymer and as a component in copolymers. Poly (aspartic acid) is a degradable, water-soluble polymer. This polymer has proven to adsorb on sewage sludge, which is a key factor in determining its usefulness as a polymeric detergent component (Roweton *et al.*, 1997). In medical and pharmaceutical applications, poly (aspartic acid) and its related polymers also have been fostered for use as scaffolding for tissue growth, drug delivery, and other biomedical applications (Hayashi and Iwatsuki, 1990). One example is the use of poly (aspartic acid) as an inhibiting agent of amino-glycoside-induced nephrotoxicity (Kishore *et al.*, 1992). Amino-glycoside antibiotics are widely used to treat serious infections. Though these compounds can be damaging to the kidneys, poly (aspartic acid) given in concert with amino-glycoside antibiotics, inhibits the potentially toxic effects of these drugs. In addition, copolymers of poly (ethylene glycol) and poly (asparic acid) have been bound to a conjugate of adriamycin, an anticancer drug, at the poly (aspartic acid) chain to produce a novel targeted drug (Yokoyama *et al.*, 1990). In this study, aspartic acid was protected by reaction with sebacic acid to give monomer AspSA (***Figure 4.2***). Attempts were carried out to prepare mixed anhydride monomer, however, a cyclic anhydride (AspSAA, ***Figure 4.3***) with high yield was produced. This cyclic anhydride can be

reacted with poly (ethylene glycol) (PEG) to synthesis crosslinked polymers. However, the synthesised crosslinked polymers have a polyester structure.

Poly (ethylene glycol) (PEG) (***Figure 4.4***) has been frequently chosen as a drug delivery carrier due to its biocompatibility, minimal toxicity and antigenicity, and good solubility in water or common solvents (Dreborg and Akerblom, 1990). PEG has been copolymerised with linear aliphatic polyesters like poly (lactic acid) (PLA) for possible use in drug delivery and tissue engineering (Shah *et al.*, 1994; Deng *et al.*, 1999).

In this study, crosslinked poly (aspartic acid: PEG) was synthesised by the polymerisation reaction of prepolymer (AspSAA) and low molecular weight PEG using an acid catalyst.

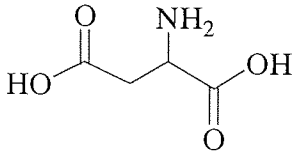


Figure 4.1 The structure of Aspartic acid

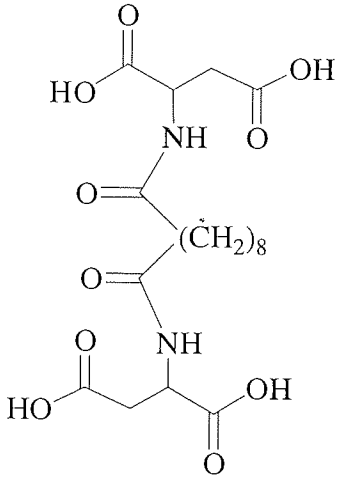


Figure 4.2 Aspartic acid-SA (AspSA)

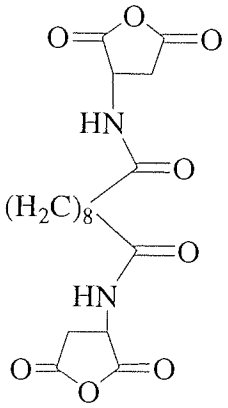


Figure 4.3 Aspartic acid-SA anhydride (AspSAA)

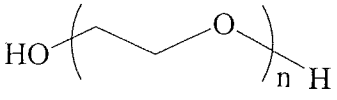


Figure 4.4 Poly (ethylene glycol) (PEG)

4.2 Experimental

4.2.1 Materials

Aspartic acid (Acros Organics)

Thionyl chloride (Sigma-Aldrich)

Sebacic acid (SA) (Sigma-Aldrich)

Dry acetic anhydride (Acros Organics)

1,3- dicyclohexylcarbodiimide (DCC) (Avocado Research Chemicals Ltd)

Trietheylamine (TEA) (Avocado Research Chemicals Ltd)

4-dimethylaminopyride (DMAP) (Avocado Research Chemicals Ltd)

Poly (ethylene glycol) (Mw 200 and 400) (Sigma-Aldrich)

p-toluenesulfonic acid monohydrate (Avocado Research Chemicals Ltd)

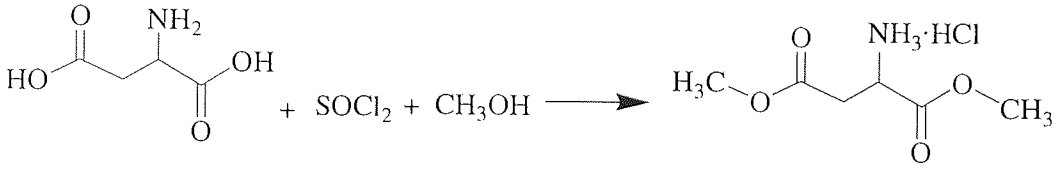
Dry Dichlorometnane (DCM) (Fisher scientific)

Dry Toluene (Merck)

All solvents of analytical grade (Fisher scientific)

4.2.2 Preparation of Aspartic acid –SA monomer (AspSA)

Step 1:

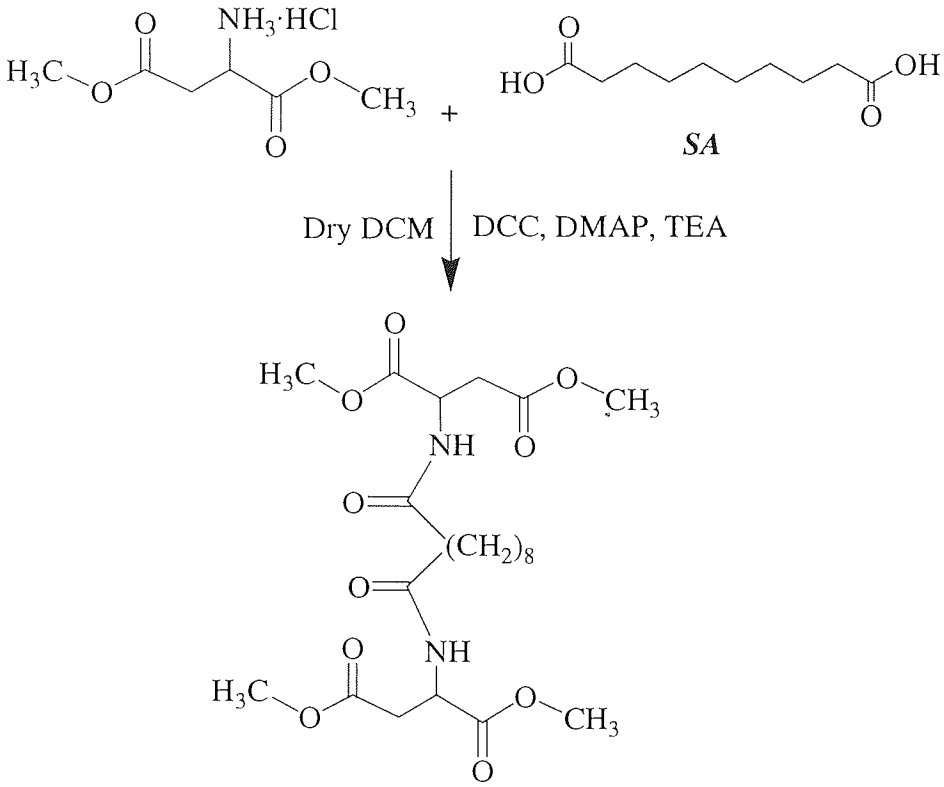


Aspartic acid ***Aspartic acid dimethyl ester HCl salt***

Figure 4.5 Synthesis of Aspartic acid dimethyl ester HCl salt

180 ml of methanol (MeOH) was added into a round bottom flask, and then the flask was immersed into an ice-water bath to cool down to 0 °C. 24.13 ml (330.60 mmol) of thionyl chloride ($SOCl_2$) was added into methanol slowly and carefully along the side of flask, avoiding any contact with water at all time as it could cause an explosion. When the mixture was cooled, 20 g (150.26 mmol) aspartic acid was added. The mixture was stirred at room temperature overnight, and the excess $SOCl_2$ and MeOH were removed under vacuum at 50°C to afford a yellow viscous liquid, purified by recrystallisation from tetrahydrofuran (THF) and dried under vacuum.

Step 2:



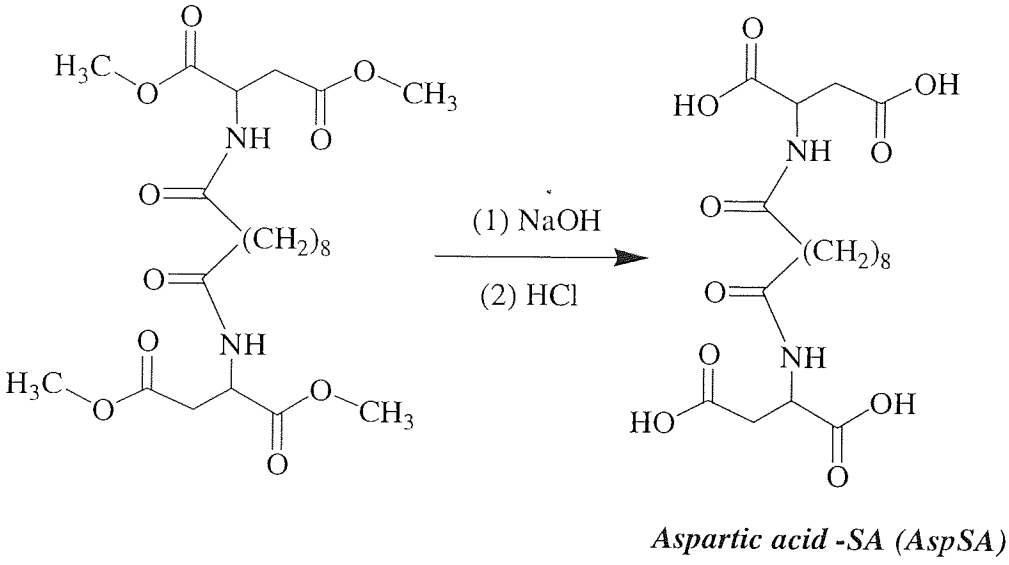
Aspartic acid -SA dimethyl ester

Figure 4.6 Synthesis of Aspartic acid-SA dimethyl ester

The aspartic acid-SA dimethyl ester (10 g, 50.63 mmol) was suspended in dry dichloromethane (DCM) and cooled down in an ice-water bath. With addition of trietheylamine (TEA) (7.04 ml, 50.60 mmol) the mixture became very viscous, and so more DCM was added. After stirring for 10 min, SA (3.4076 g, 16.87 mmol), DCC (7.2976 g, 35.43 mmol) and DMAP (0.1031 g, 0.84 mmol) were added. This mixture was stirred at room temperature overnight. After filtration, the filtrate was collected and washed with 2 N HCl three times, saturated $NaHCO_3$ three times, and then

distilled water three times, adding $MgSO_4$, which was removed by filtration, dried the DCM solution. The DCM was evaporated under vacuum to obtain a white product.

Step 3:



Aspartic acid -SA (AspSA)

Figure 4.7 Synthesis of AspSA

Aspartic acid-SA dimethyl ester (7.16 g, 14.67 mmol) was dissolved in 20 ml of MeOH: THF (1:1, v: v). To the solution, 65.374 ml of 1M NaOH was added, and the mixture was kept stirring for 4-5 hours at room temperature. 50 ml of distilled water was added into the mixture, which was then washed with DCM three times. HCl was used to adjust the pH to 1. After evaporation of MeOH-THF and water, acetone was added to dissolve the product, and $MgSO_4$ was used to dry the acetone solution. NaCl was removed by filtration, and the acetone evaporated to obtain a white product that was dried in vacuum for at least 24 hours.

4.2.3 Preparation of Prepolymer (AspSAA)

The prepolymer (AspSAA) cyclic anhydride can be synthesised by reaction of aspartic acid with thionyl chloride in chloroform or in excess acetic anhydride.

4.2.3.1 Reaction with Thionyl Chloride

In 1998, Won *et al.* reported a method reacting protected aspartic acid with thionyl chloride to obtain the cyclic anhydride.

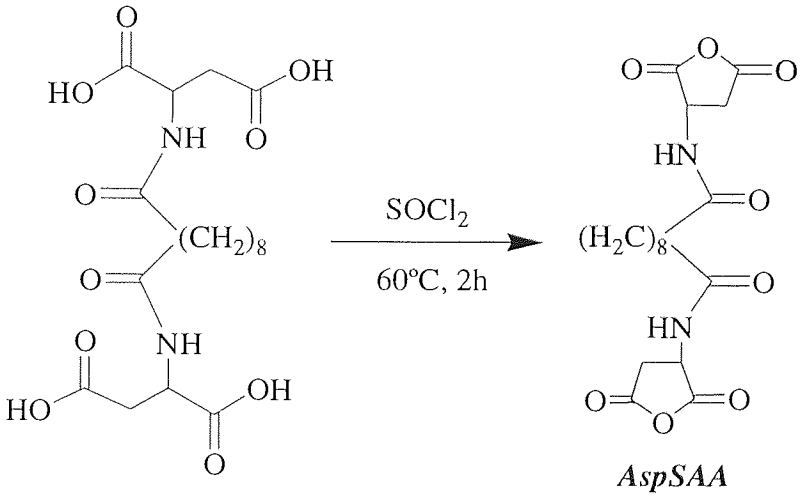


Figure 4.8 Synthesis of AspSAA by reaction with thionyl chloride

Thionyl chloride (10.86 ml, 148.92 mmol) was slowly added into a suspension of AspSA (2 g, 4.63 mmol) in 15 ml of chloroform, and the reaction mixture was refluxed at 65°C for 2 hours. The solvent and the excess thionyl chloride were

removed under reduced pressure. The residue was washed with a mixture of dry diethyl ether and dry petroleum ether (1:1 v:v) to produce AspSAA as fine crystals.

4.2.3.2 Reaction with Acetic Anhydride

Anhydride formation was carried out by cyclisation of AspSA acid in the presence of excess acetic anhydride (Won *et al.*, 1996).

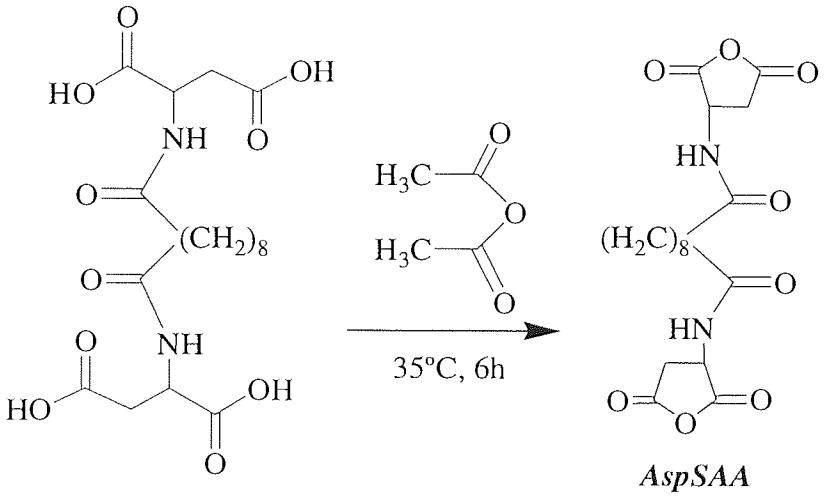


Figure 4.9 Synthesis of AspSAA by reaction with acetic anhydride

2 g of AspSA acid was added into 20 ml of dry acetic anhydride, and the mixture was stirred for 6 hours at 35°C. The AspSA acid was dissolved in acetic anhydride gradually over 1 hour. After reaction, the excess acetic anhydride was removed under vacuum, and 10 ml of dry diethyl ether was used to wash out any remaining acetic acid and anhydride. The resulting product was dried and stored under vacuum over P_2O_5.

4.2.4 Synthesis of Crosslinked Polymers

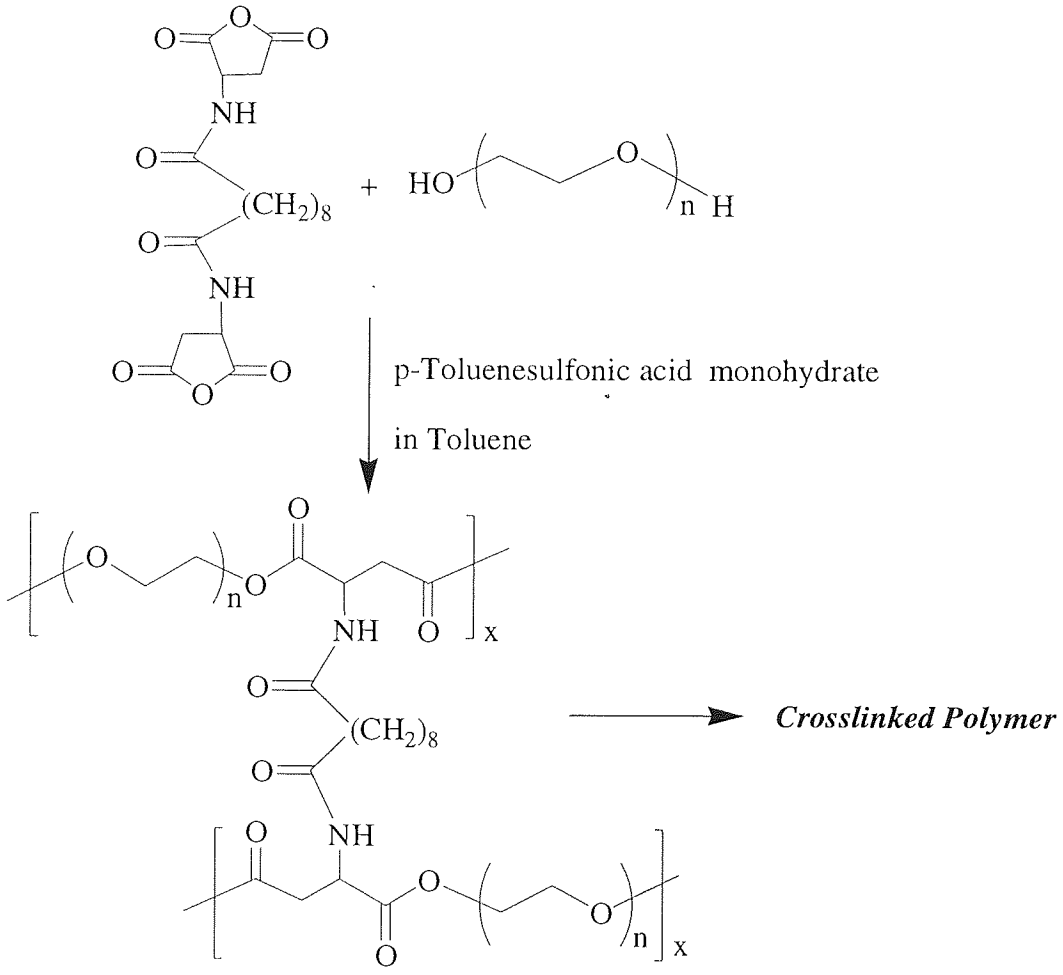


Figure 4.10 Synthesis of crosslinked copolymer (AspSA: PEG)

1g (2.5 mmol) of AspSA anhydride, poly (ethylene glycol) (5 mmol), p-toluenesulfonic acid monohydrate catalyst (0.008 mmol), and toluene (25 ml) were placed into a 50 ml round-bottomed flask equipped with a reflux condenser, and a nitrogen gas inlet. The reaction mixture was refluxed for a predetermined time under a nitrogen atmosphere. After evaporation of the solvent, a sticky, brown, rubbery product was obtained.

4. 3 Results and Discussions

The multi-step method was easily carried out to prepare the AspSA monomer. The yields for the three steps and preparation of prepolymer were high:

Step 1: 104% before the recrystallisation from tetrahydrofuran (THF) and 87% after.

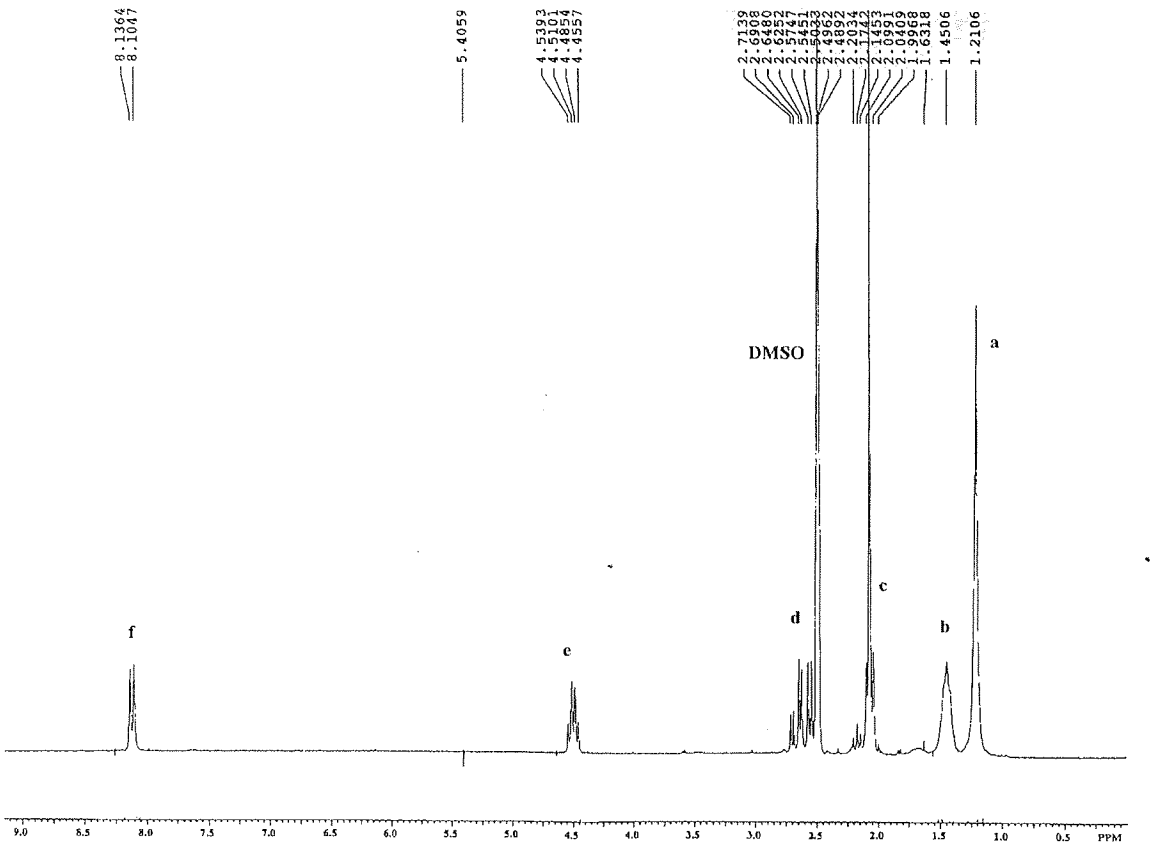
Step 2: 92.3%

Step 3: 89.2%

Preparation of prepolymer: 87% after reaction with thionyl chloride; 85% after reaction with acetic anhydride.

Attempts were made to prepare the AspSA mixed anhydride by refluxing AspSA in acetic anhydride and by reacting AspSA with CH_3COCl. Following the refluxing of AspSA in acetic anhydride, the NMR spectra showed that a mixture of cyclic and unknown components was obtained. NMR spectra showed that starting materials remained following the reaction of AspSA with CH_3COCl. This result may be attributed to intramolecular cyclisation to form a 5-membered cyclic anhydride.

^{1}H-NMR spectrum was used to determine the structure of the monomer AspSA and its cyclic anhydride (***Figures 4.11 and 4.12***).



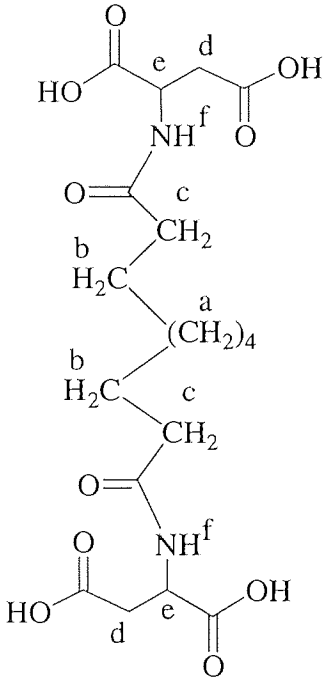
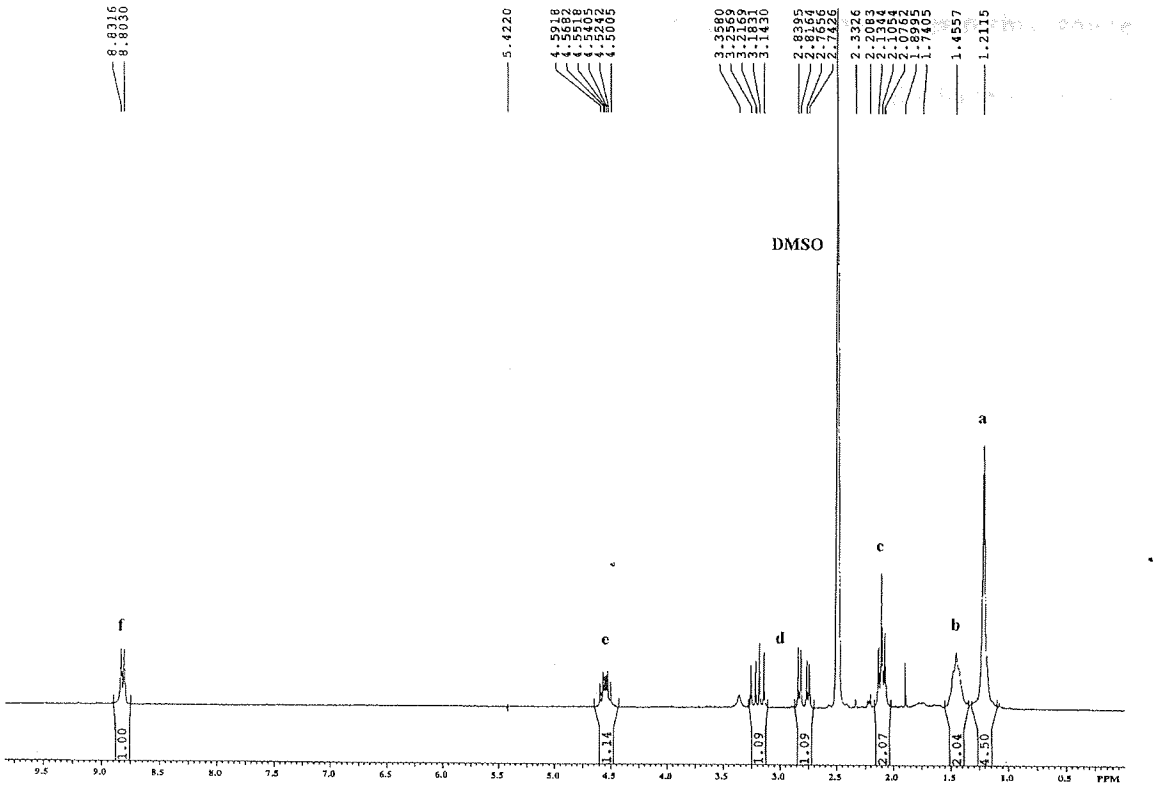


Figure 4.11 ^{1}H-NMR spectrum of AspSA monomer



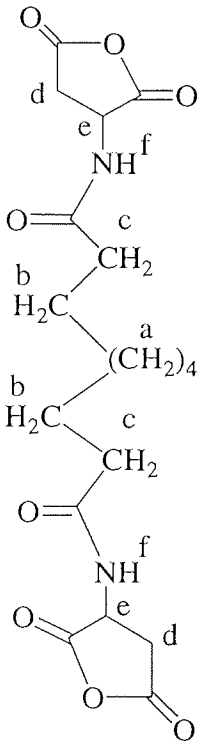


Figure 4.12 ^{1}H-NMR spectrum of prepolymer (AspSAA) cyclic anhydride

Anhydrides have been used as monomers in the preparation of polyester resins, where a high degree of polymerisation is not critical. The use of AspSA anhydride instead of AspSA acid for the preparation of polymers is desirable because the anhydride monomer can dissolve in toluene (whereas the acid is not soluble) for the removal of water (a by-product from the polycondensation reaction mixture) to minimise hydrolysis of the polymer and to shift the equilibrium of the reaction towards polycondensation (Won *et al.*, 1998).

Employing the acid catalyst, such as p-toluenesulfonic acid, sulphuric acid and phosphoric acid, in polycondensation of protected aspartic acid anhydride with PEG was effective in producing polymers with high molecular weight. Among the catalysts, p-toluenesulfonic acid monohydrate was the most effective (Won *et al.*, 1998). Furthermore, another advantage of using the catalyst is the acid catalyst is less toxic than the metallic-based catalysts.

In 1996 and 1998, Won *et al.* succeeded in synthesising a new biodegradable polymer (L-aspartic acid-PEG) from N-benzyloxycarbonyl protected L-aspartic acid anhydride (N-CBz-L-aspartic acid anhydride) and low molecular weight PEG. The optimal conditions for the polymerisation were obtained by reacting 0.12 mol % of p-toluenesulfonic acid with PEG 200 for 48 hours. This may be the result of the availability of the reactivity of the terminal hydroxyl groups of PEG, as the –OH groups in PEG are more hindered in higher molecular weight PEG because of long chain entanglement (Won *et al.*, 1998). According to their conditions, PEG 200 and PEG 400 were used in this study.

The effect of polymerisation time on polymer molecular weight was studied (***Table 4.1***). After 12 hours, the polymers can be dissolved in $CHCl_3$. After 24 hours, the solubility of polymers in $CHCl_3$ decreased sharply, although the Mw of the soluble part increased. When the reaction was carried out for 48 hours, a sticky, brown, rubbery product was obtained.

PEG / *Time (hours)*	*PEG 200*	*PEG 400*
12	2.3kDa [a]	2.1kDa [a]
24	6.9kDa [b]	7.3kDa [b]

Table 4.1 The molecular weight of polymers by polymerisation of AspSAA with PEG200 and PEG 400 for 12 hours and 24 hours

[a] Determined by GPC

[b] Soluble component determined by GPC

The solubilities of the polymer products in common solvents were determined at room temperature by dissolving 1 g of polymer in 2 ml of solvent, after 30 minutes stirring, the mixture was filtered and the solubility was determined gravimetrically (% w/v) (Domb and Maniar, 1993). Neither polymer products was soluble in the following solvents: acetone, acetonitrile, chloroform, dichloromethane (DCM), tetrahydrofuran (THF), dimethylformamide (DMF) and water.

The thermal properties of poly (AspSA: PEG200) and poly (AspSA: PEG400) were determined by DSC. No melting points were observed between 0 and 400 °C, which can be evidence of crosslinking of the polymer (Tamada and Langer, 1992). The solubility study and DSC analysis exhibited crosslinked polymers could be produced. The resulting polymers appear sticky and rubbery at room temperature.

As the resulting polymers cannot be dissolved in common organic solvents or water, it is very difficult to measure the molecular weight using GPC and to determine the structure of the polymers using ^{1}H-NMR. However, the infrared (IR) spectrum shows the characteristic absorption peaks of the polymers. Although the IR spectrum alone cannot completely define the structure of the polymers, it can be stated that the synthesis of the polymers was successful. The IR spectra for both poly (AspSA: PEG200) and poly (AspSA: PEG400) are in ***Figure 4.13*** and ***Figure 4.14*** respectively. All spectra showed the characteristic of the polymer products absorption peaks at 3420 and 3436 cm^{-1} (NH), and strong ester carbonyl bands at 1733 and 1739 cm^{-1} respectively. No characteristic absorption peaks at around 1860 and 1790 cm^{-1} for the cyclic anhydride were found.

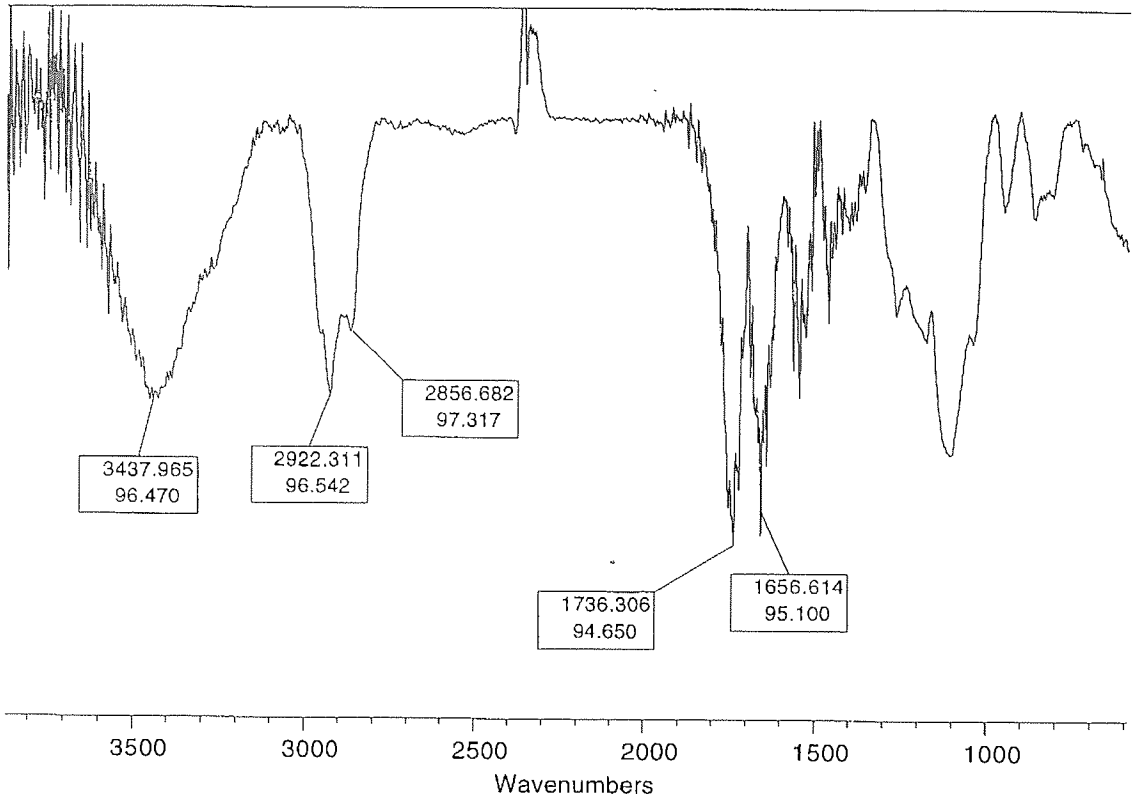


Figure 4.13 IR spectrum of poly (AspSA: PEG200)

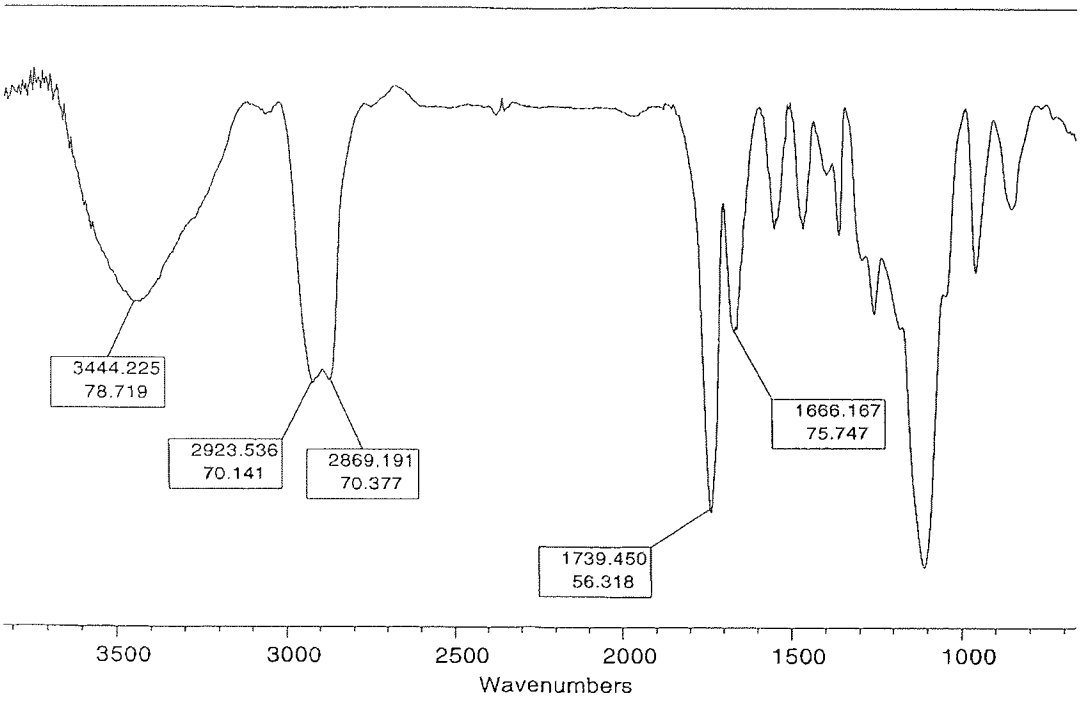


Figure 4.14 IR spectrum of poly (AspSA: PEG400)

Chapter 5 Polymer Degradation Studies

5.1 Introduction

Polyanhydrides are composed of a hydrophobic polymer backbone joined by anhydride linkages that readily split in the presence of water to free carboxylic acid groups. Generally the hydrophobicity of the monomer backbone is sufficient to prevent penetration of water into the core of the final polymer network. Therefore, the rate of hydrolysis of anhydride linkages is much greater at the polymer surface than in the bulk, resulting in a surface erosion mechanism (Burkoth *et al.*, 2000).

In general, chemical and physical changes accompany the degradation of biodegradable polyanhydrides, like the crystallisation of oligomers and monomers or the pH of the medium solution changes (Göpferich, 1996a). Some of these factors can have a substantial effect on the degradation rate. For example, the change in molecular weight is an important indicator for polymer degradation. Besides loss of molecular weight, other parameters have been proposed as measures for degradation, like changes in melting point. All of these are related but need not necessarily obey the same kinetics (Göpferich, 1996a).

A number of studies have focused on the degradation of the typical clinical polymers, poly (CPP: SA, 20:80) and poly (FAD: SA, 1:1) (Leong *et al.*, 1986; Shieh *et al.*, 1994; Domb and Nudelman, 1995; Göpferich, 1997). When poly (CPP: SA, 20:80) matrix discs were incubated in phosphate buffer (pH 7.4) at 37 °C, it was found that the molecular weight dropped exponentially during the first 24 hours. It is important that such investigations reveal the time scale over which degradation occurs, as the chemical degradation of bonds in the polymer chains is important among the variety

of parameters affecting the erosion of polymer bulk, therefore, yielding precious information on the expected time over which drugs may be released. However, the results of studing large matrix discs does not allow the assessment of the degradation properties unequivocally. With increasing dimensions, the result depends on other processes in addition to degradation, such as the diffusion of water into the polymer bulk. If the water diffusion is slow, the degradation of the polymer matrix disc is reduced due to the lack of water preventing the degradation inside the polymer bulk (Göpferich, 1996b).

As discussed in ***Chapter 1***, the degradation rate of polymers will depend on the pH of buffer used. Through chain scission, polymers are transformed into oligomers and monomers, which have different functional groups than the starting polymers. Thus, anhydrides are cleaved into carboxylic acids, esters and orthoesters into alcohols and carboxylic acids. The degradation products, therefore, influence the pH in the degradation medium (Göpferich, 1996b). During degradation studing for poly (CPP: SA, 20: 80), the pH of the buffer medium was measured whenever it was changed. The pH of buffer dropped to 5.7-6.0 from 7.4 after the first 24 hours degradation (Göpferich and Langer, 1993).

Due to degradation of polymer chains, large amounts of monomers are released into the pores created during erosion and finally diffuse into the buffer outside the discs. The solubility of SA and CPP was determined to make sure that they were sufficiently soluble in the degradation medium outside the polymer matrix. It was found that the solubility of both compounds, as they are acids, can be increased by increasing pH. Additionally, SA is at least five times more soluble than CPP at pH values even below

7.4. It was reported that the SA content reached a maximum, which coincided with the pH minimum in the buffer medium (Göpferich and Langer, 1993).

With the release of degradation products into the medium, the matrix weight changes. In 1993, Göpferich and Langer studied the weight change of three polyanhydrides discs, poly (SA), poly (CPP: SA, 20: 80) and poly (CPP: SA, 50: 50) during degradation. Their study indicated there were some common features for all three polymers. During the initial stage of degradation, the velocity of weight loss was relatively slow. After 24 hours all three polymers entered a phase of nearly constant state. This lasted for couple of days, after which the mass loss declined which indicated that processes other than only chain scission of the polymer became important. The results are good agreement with results for other polyanhydrides, such as poly (CPH: SA) and poly (FA: SA) (Göpferich, 1997).

For the investigation of changes in crystallinity during degradation, DSC was used. The monomers were examined to determine their melting point as well as their melting enthalpy (obtained by integration of the melting peaks). It was reported that SA has a melting point of $T_{SA} = 135.6 \pm 0.4$ °C and CPP of $T_{CPP} = 323.8 \pm 1.4$ °C. The melting enthalpy was determined to be $\Delta H_{SA} = 218 \pm 5$ J/g for SA and $\Delta H_{CPP} = 181 \pm 7$ J/g for CPP. The non-degraded poly (SA) has a melting peak at 81°C. With increasing time of degradation, a slight shoulder appeared in the range Tm= 100-110 °C, which was identified as crystallised SA. The non-degraded polymers had a Tm at 77 °C for poly (CPP: SA, 20:80) and at 56 °C for poly (CPP: SA, 50:50). Two additional peaks at 100-150 °C and at 250 –325 °C appeared after 2-3 days degradation. The former was again caused by crystallised SA; the latter corresponded

to CPP (Göpferich, 1997). In the poly (SA), the crystallinity increased with time whereas it decreased in the case of the two copolymers (CPP: SA), 20:80 and 50:50. The increasing crystallinity in poly (SA) during degradation indicates that the crystalline regions in this polymer are more resistant to degradation than the amorphous parts. The decreasing crystallinity in the copolymers indicates that the crystalline parts of the copolymers degrade substantially faster than those in the homopolymer, which is probably due to the disturbance of the crystallinity by the increasing presence of CPP monomer (Göpferich and Langer, 1993).

5.2 Experimental

Polymer samples (2-3 mg) were incubated in 1 ml solutions of pH 4, pH 7.4 and pH 10 in micro-vials. The micro-vials were stoppered and placed on a shaker. The samples were kept shaking at 100 shakes per minute at 37 °C, throughout the experiment duration. The degradation media were prepared as followings:

- ***Buffer solution pH 4*:** 55.1 parts of di-sodium citrate (0.1 mol/L) + 44.9 parts of HCl (0.1 mol/L). di-sodium citrate (0.1 mol/L) was prepared using Citric acid monohydrate (21.014 g/L) + 200 ml NaOH (1 mol/L) (Merck Index, 1989).
- ***Buffer solution pH 7.4:*** 1 tablet of phosphate-buffer saline (Sigma-Aldrich) was dissolved in 200 ml of double distilled water to obtain 0.1 M phosphate buffer saline (PBS) at 25 °C.
- ***Buffer solution pH 10:*** 53.4 parts of sodium carbonate (0.1 mol/L) + 46.6 parts of sodium hydrogen carbonate (0.1 mol/ L) (Merck Index, 1989).

At predetermined times, the samples were collected, and then centrifuged for 15 minutes at 21000 rpm (Micro Centaur Bench Top Centrifuge). The pH of the buffer solutions was measured using a glass rod pH detector at room temperature. The remaining pellet was washed with double distilled buffer three times, frozen and freeze-dried (Edwards Modylo Freeze-drier) before investigation. All procedures were carried out in triplicate by analysing three sets of 30-40 mg in separate vials.

The dried samples were stored in a desiccator at 0 °C for IR, DSC, GPC, and weight loss analysis. The degraded samples were pressed into KBr (Aldrich) pellets and analysed using infrared data manager software (Perkin Elmer). The thermal properties of the degraded samples were determined on a DSC-4 (Perkin Elmer) equipped with a microprocessor controller. The molecular weights of degraded polymers were determined using a GPC system relative to polystyrene standards. After freeze-drying, the samples were reweighed to obtain polymer weight loss during degradation.

5.3 Results and Discussion

In this study, the degradation of four classes of polymer was studied: poly (CP2B: SA), poly (GluSA: SA), poly (CPP: SA: GluSA, 20: 70: 10) and poly (AspSA: PEG).

5.3.1 Poly (CP2B: SA)

The peaks of interest in the IR spectra of polyanhydrides were the typical anhydride carbonyl peaks at 1810 cm^{-1} and 1740 cm^{-1}, and the carboxylic acid peak at 1700 cm^{-1}. The IR data for poly (CP2B: SA), 20: 80 and 50: 50 degradation for up to 5 days in pH 7.4 buffer medium are presented in ***Figure 5.1*** and ***Figure 5.2***.

One can readily see the emergence of the carboxylic acid peak at 1700 cm^{-1} from its initial shoulder on the 1740 cm^{-1} anhydride peak with increasing degradation time. At the same time, the anhydride carbonyl peaks at 1810 cm^{-1} decrease. However, for the poly (CP2B: SA, 50: 50), the aromatic anhydride bonds at 1740 cm^{-1} can still be detected after 5 days degradation. It is possible that the hydrophobic part (CP2B) inhibits initial water entrance and therefore slows the degradation.

Information about the free acid content of the degrading polymers can be obtained from the IR spectra as well. It can be seen in both figures that the broad –OH band at 3335 -2500 cm^{-1} increased with degradation. As the polymer degraded and formed soluble degradation products, there was a loss of –CH– from the polymer backbone as well as the formation of acid, resulting in the broad –OH band. IR spectra suggest that

the degrading system was a mixture that was composed of un-degraded polymer and stable oligomeric chains with high –OH content yet it retained the bulk of the polyanhydride backbone.

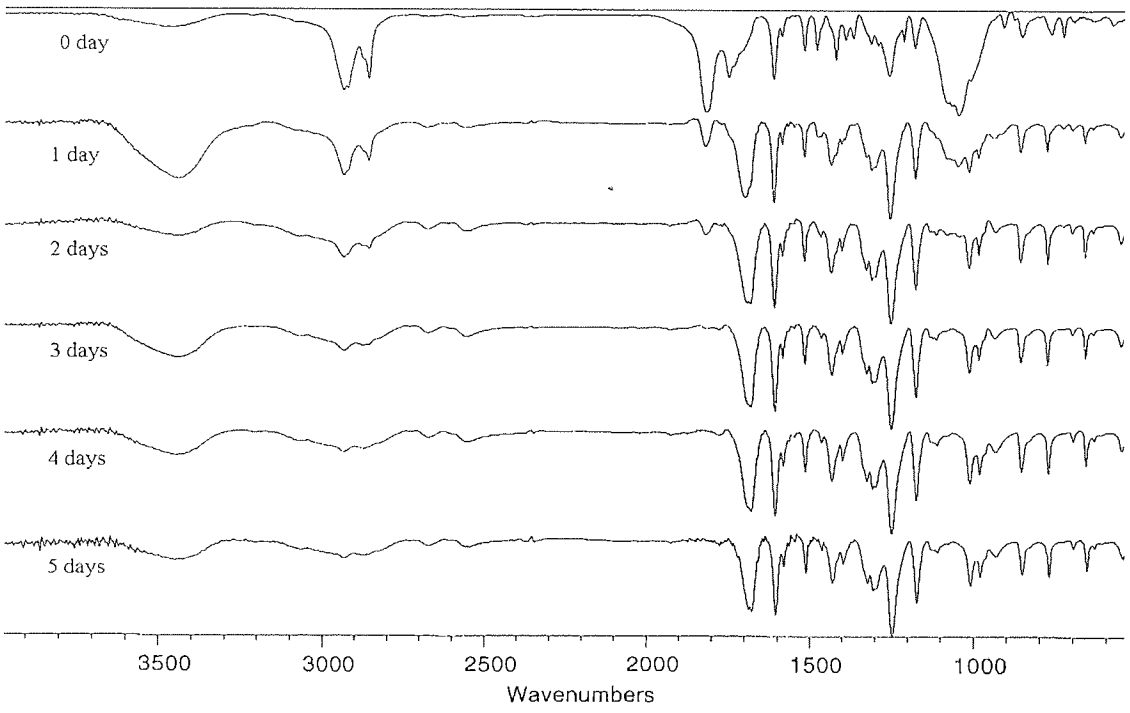


Figure 5.1 IR data for poly (CP2B: SA) 20:80 (Mw=35.1 kDa) during degradation in PBS

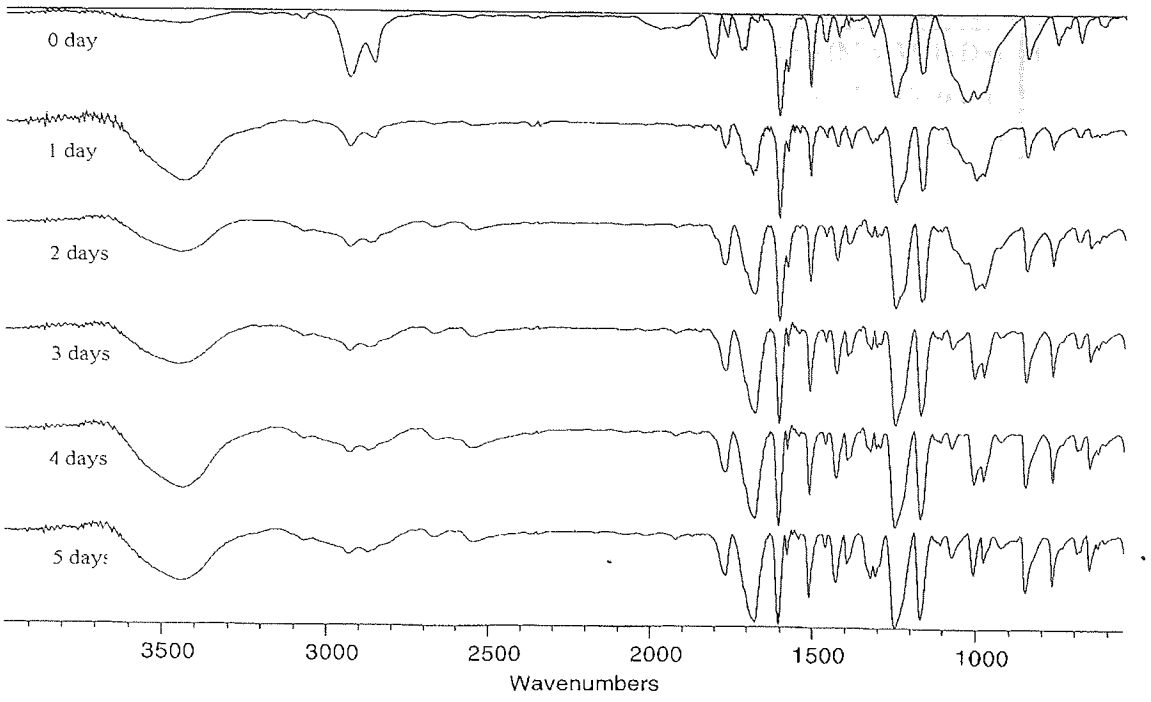


Figure 5.2 IR data for poly (CP2B: SA) 50:50 (Mw=22.4 kDa) during degradation in PBS

The influence of polymer molecular weight on the rate of polymer degradation was studied (***Figure 5.3***) using GPC. Results showed that there was no difference in degradation rates between different molecular weight polymers during their degradation in buffer medium pH 7.4. However, it has been reported that the initial molecular weight of polanhydrides is important for polyanhydride erosion (Göpferich and Langer, 1993). In the reported study, the erosion of polyanhydride was characterised by an induction period during which the rate of erosion was relatively slow. It was found that, during this period, significant molecular weight losses occurred within polymer, without significant device erosion. The length of this induction period was found to depend on the initial polyanhydride molecular weight, with increasing molecular weight leading to a longer induction period (D'Emanuele *et al.*, 1992).

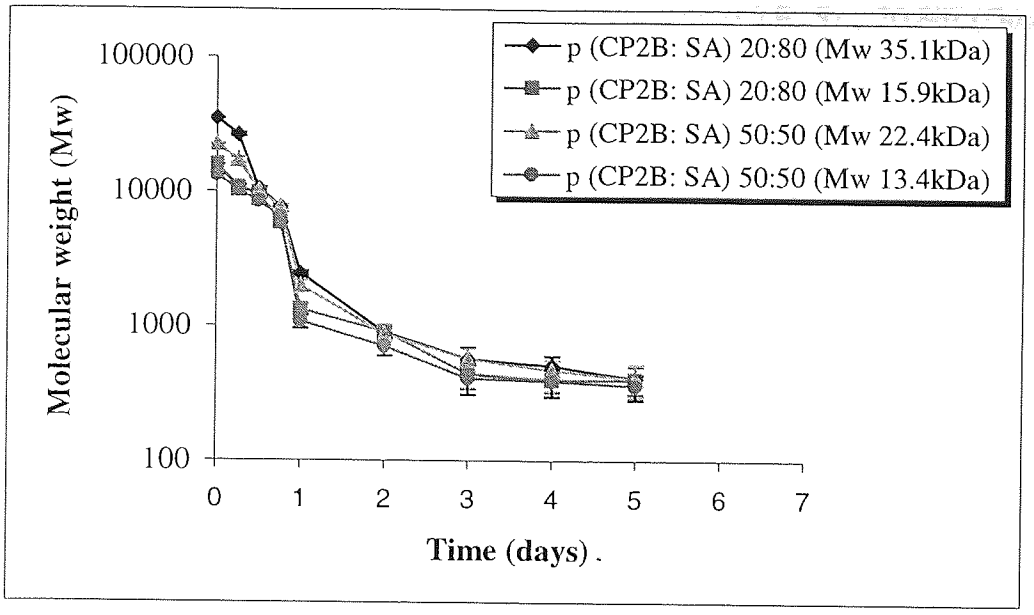


Figure 5.3 Molecular weight of poly (CP2B: SA) 20: 80 and 50: 50 during degradation in buffer pH 7.4 (n=3, mean ± s.d.)

It was expected that the microstructure of polyanhydrides would break down whereby the crystalline regions exhibit a higher resistance to degradation than amorphous regions, causing changes in polymer matrix crystallinity. When degradation of the polymer chains is faster than the diffusion of monomers to the matrix surface, the monomers can crystallise during erosion inside the porous network of the eroded polymer (Göpferich and Langer, 1993). DSC was used to examing the crystallinity of degrading polymers. The melting points of the monomers were determined to be 135.6 ± 4°C for SA and 307 ± 4 °C for CP2B (n=3, mean ± s.d.). ***Figure 5.4*** shows the thermograms of the poly (CP2B: SA, 20: 80) during the first period of degradation up to 7 days. The non-degraded materials showed one peak at T_m = 70 °C. The additional peaks at T_m=110-150 °C and at T_m=280-310 °C were visible once the degradation progressed. The former stemmed from crystallised SA; the latter caused

by CP2B. Similar results have been reported for poly (CPP: SA, 20:80) (Göpferich and Langer, 1993).

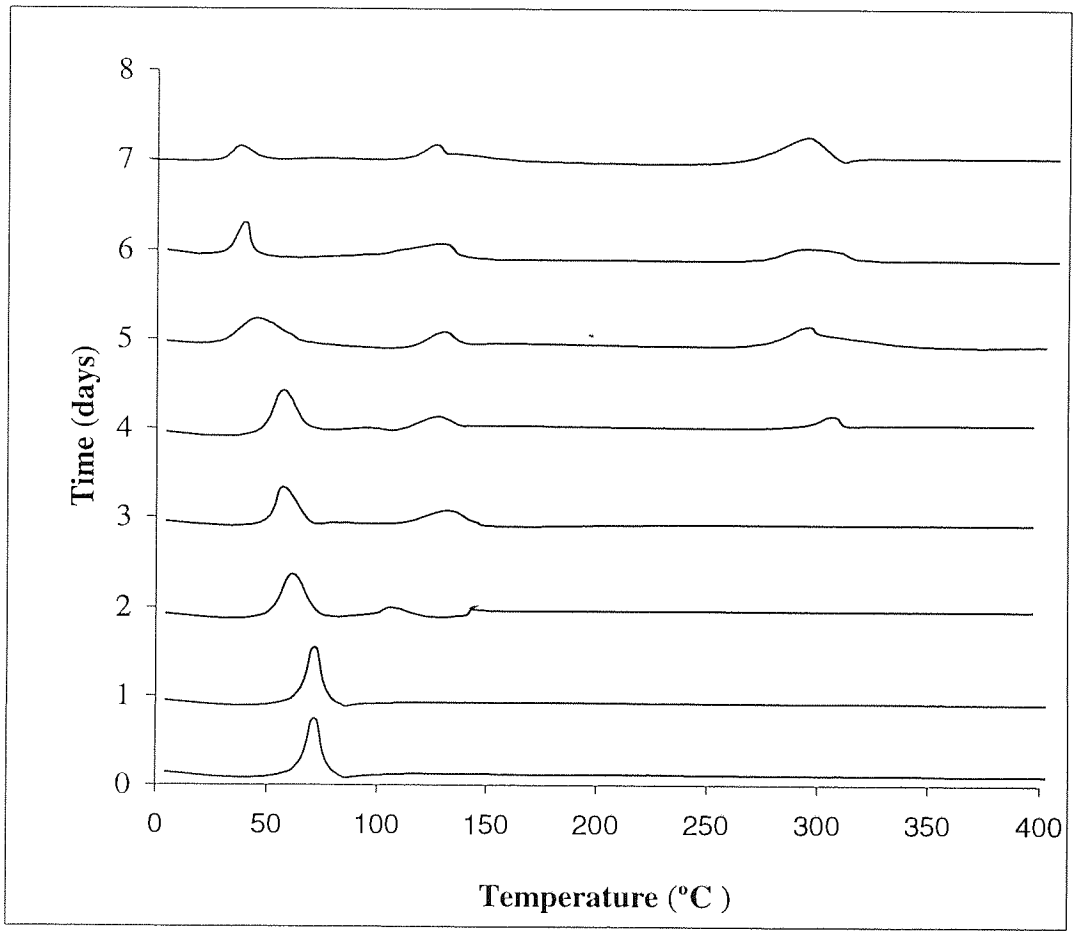


Figure 5.4 Changes of DSC thermograms during poly (CP2B: SA) 20: 80 degradation in PBS

Figure 5.5 shows that the melting enthalpies of poly (CP2B: SA, 20: 80) and poly (CP2B: SA, 50: 50) were subject to continuous changes during their degradation. The values reflected the changes within the polymers during degradation and revealed the polymer degradation and erosion mechanisms.

In the above two cases, at the beginning of degradation, the melting enthalpies decreased till day 7 because the polyanhydrides eroded and the crystalline parts were broken down. The polymers melting enthalpies increased again from day 7 to day 10 in case of p (CP2B: SA, 20: 80), from day 7 to day 16 in case of p (CP2B: SA, 50: 50). The thermogram indicated that the degraded crystalline parts had not left the polymer surface at that time. This may be due to the release of monomers created during degradation and erosion came to completeness between day 7 to day 10 and day 7 to day 16. The subsequently decreasing enthalpies reflected the further erosion occuring. The result suggests that the degradation and erosion rate of p (CP2B: SA, 50: 50) are slower that p (CP2B: SA, 20: 80) due to the higher ratio of hydrophobic part (CP2B) in the copolymer.

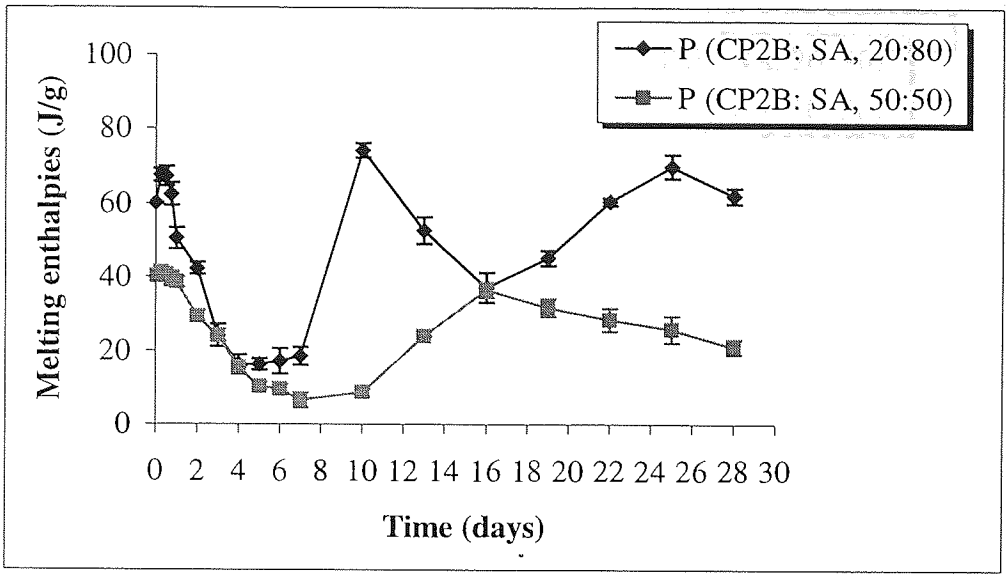


Figure 5.5 Melting enthalpies of p (CP2B: SA) 20: 80 and 50: 50 during their degradation in buffer pH 7.4 (n =3; mean ± s.d.)

The change in pH of PBS is shown in ***Figure 5.6***. The release of monomers was shown to decrease the pH at early time points. This suggests that the pH of buffer medium should be always kept high enough to allow the dissolution of all monomers in the vial to maintain sink conditions. The buffer medium pH of p (CP2B: SA, 20: 80) degradation became lower than that of p (CP2B: SA, 50: 50). This could be because the erosion rate of p (CP2B: SA, 20: 80) is faster than that of p (CP2B: SA, 50: 50), with an increased percentage of the hydrolytic component SA.

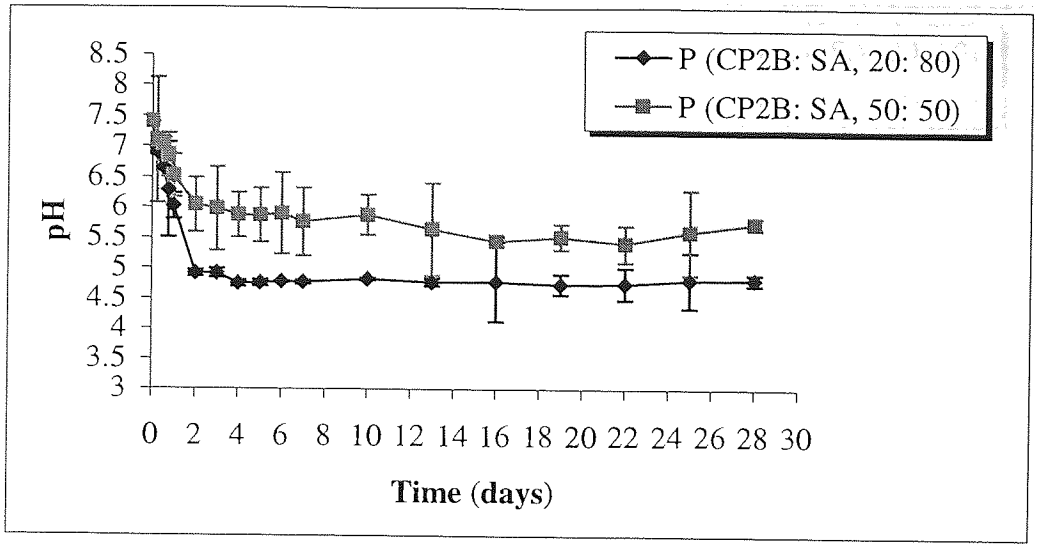


Figure 5.6 pH changes in PBS during p (CP2B: SA) 20: 80 and 50: 50 degradation (n=3; mean ± s.d.)

Results from polymer weight loss studies are shown in ***Figure 5.7***. It can be seen the rate of p (CP2B: SA) 20: 80 weight loss is faster than p (CP2B: SA) 50:50. The result could be explained in terms of the higher hydrolyticity of SA than CP2B. Additionally, it suggests that the release of SA into buffer determined the medium pH.

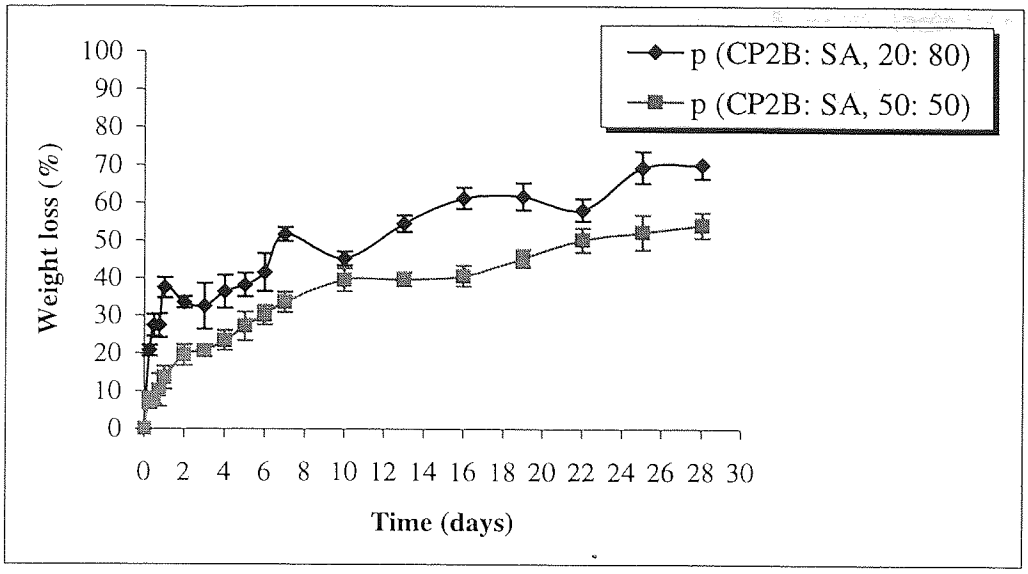


Figure 5.7 Weight loss during p (CP2B: SA) 20: 80 and 50: 50 degradation in PBS (n=3; mean ± s.d.)

5.3.2 Poly (GluSA: SA)

IR spectra for poly (GluSA: SA) 20: 80 incubated in buffer at pH 4.4, pH 7.4 and pH 10 (***Figures 5.8a, 5.8b and 5.8c***), show the emergence of the carboxylic acid peak at 1700 cm^{-1} with increasing degradation. At the same time, the anhydride carbonyl peaks at 1808 cm^{-1} decrease. After 7 days degradation in buffer medium pH 4.4, the anhydride carbonyl peaks at 1808 cm^{-1} were still visible. The polymers degraded fastest in buffer pH 10. It can be concluded that the anhydride bonds are more labile at high (alkaline) pH. This is expected since an acidic environment tends to hinder dissolution of the acid, preventing hydrolytic degradation of remaining polymer (Santos *et al.*, 1999). Compared to the linear poly (CP2B: SA) 20: 80 (Mw=35.1 kDa) and 50: 50 (Mw=22.4 kDa), the anhydride carbonyl peaks at 1808 cm^{-1} still can be

seen even after 5 days degradation in buffer medium pH 7.4. At the same time, it also can be seen in ***Figure 5.9,*** DSC revealed that no a visible signal for crystallised SA around T_m=110-150 °C within first 7 days degradation. These findings suggest that the poly (GluSA: SA) has a crosslinking network which can resist a longer time in buffer even though the polymers have a relative low molecular weight (Mw=16.8 kDa).

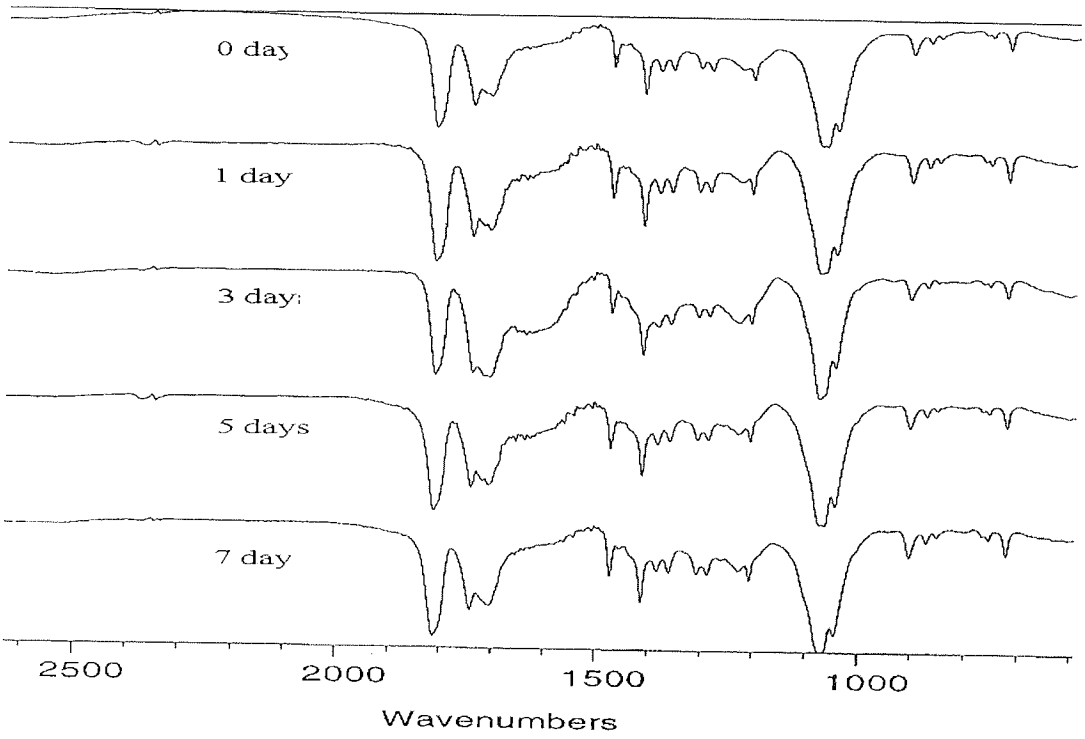


Figure 5.8a IR spectra for poly (GluSA: SA) 20: 80 in buffer pH 4.4

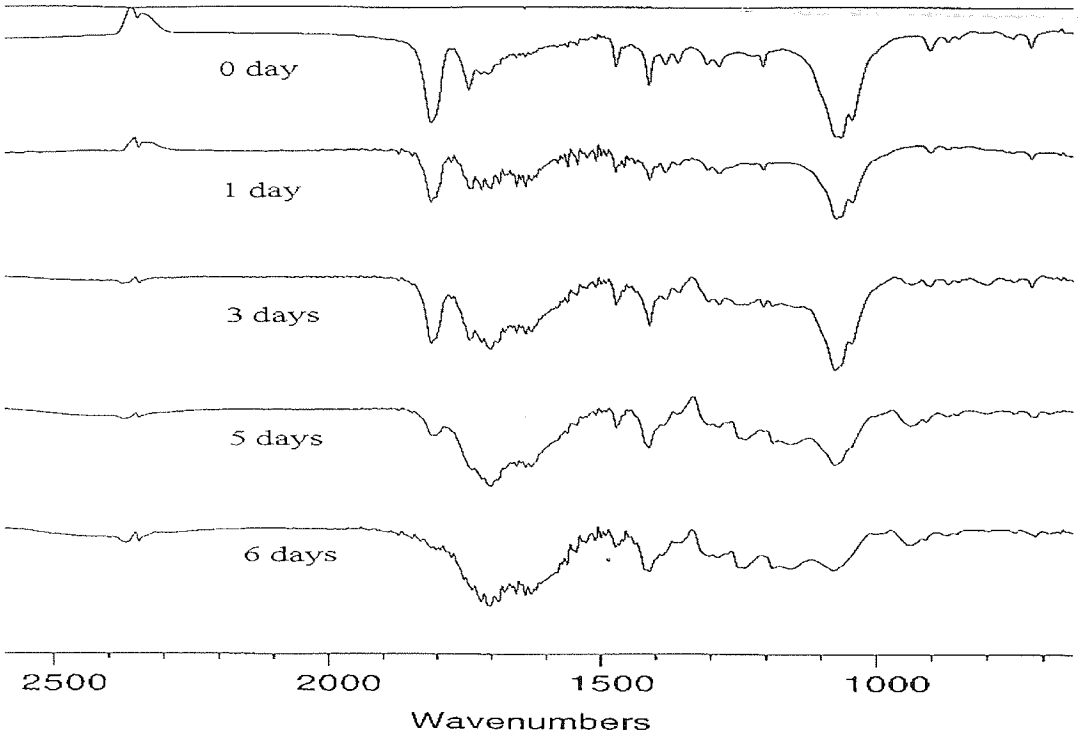


Figure 5.8b IR spectra for poly (GluSA: SA) 20: 80 in buffer pH 7.4

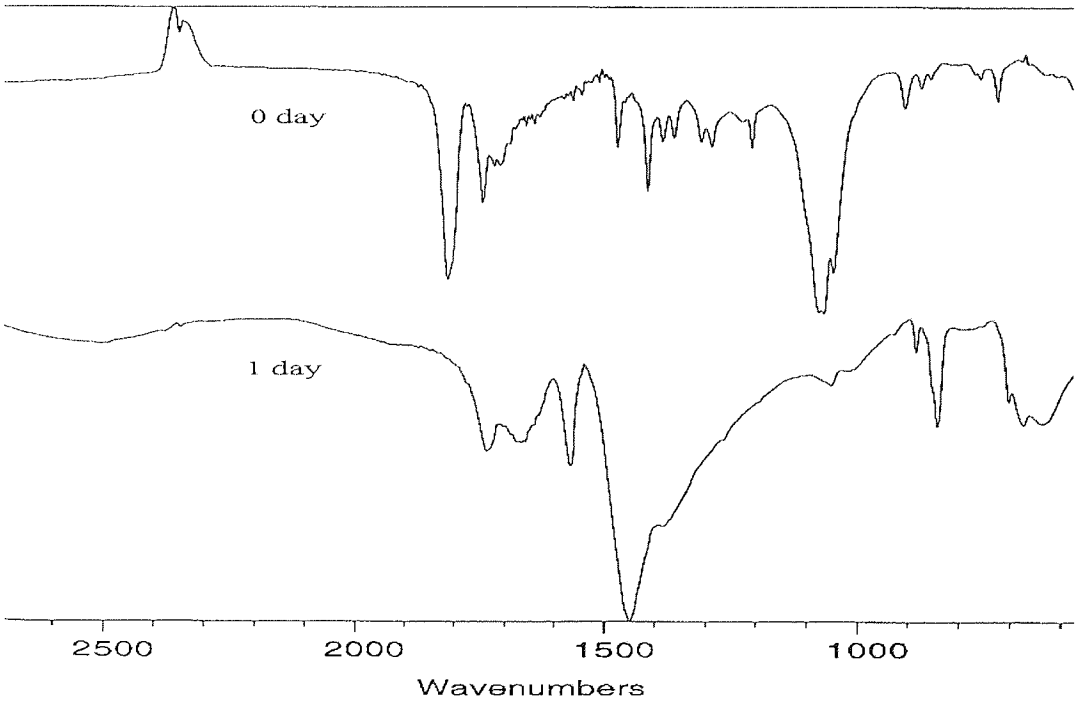


Figure 5.8c IR spectra for poly (GluSA: SA) 20: 80 in buffer pH 10

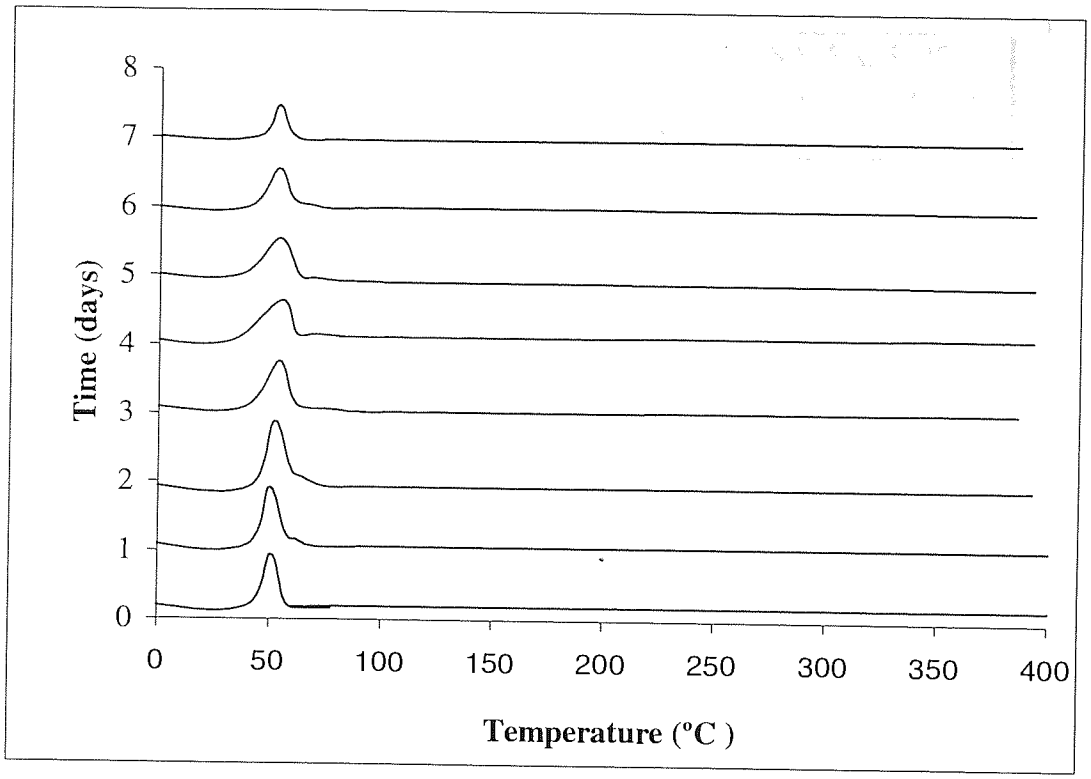


Figure 5.9 Changes of DSC thermograms during p (GluSA: SA) 20: 80 degradation in PBS

A sharp decrease in molecular weight was observed during the first 24 hours in PBS, followed by a very small change over another 4 days (***Figure 5.10***). ***Figure 5.11*** shows that increasing SA led to a slower weight loss. This could be because the crystalline region SA exhibited a high resistance to degradation. Additionally, with the polymer weight loss increasing, the pH in buffer medium decreased from 7.4 to around 4 after 24 hours degradation (***Figure 5.12***). Poly (GluSA: SA) 20:80 showed a faster rate of weight loss, which resulted in a lower pH value than p (GluSA: SA), 5:95. This may be because more degradation products were released in the case of p (GluSA: SA) 20:80 than in the case of p (GluSA: SA), 5:95.

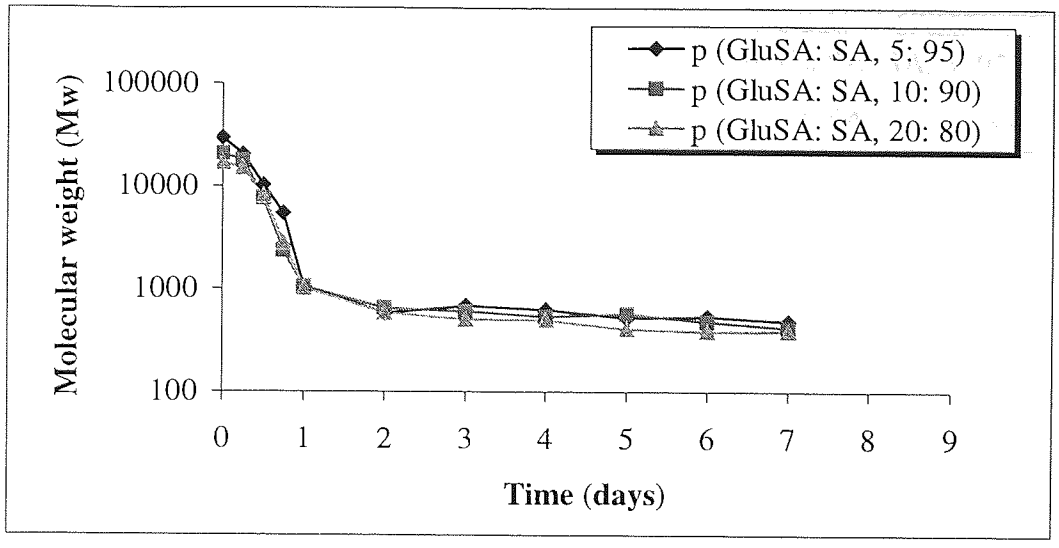


Figure 5.10 Polymers molecular weight changes during degradation

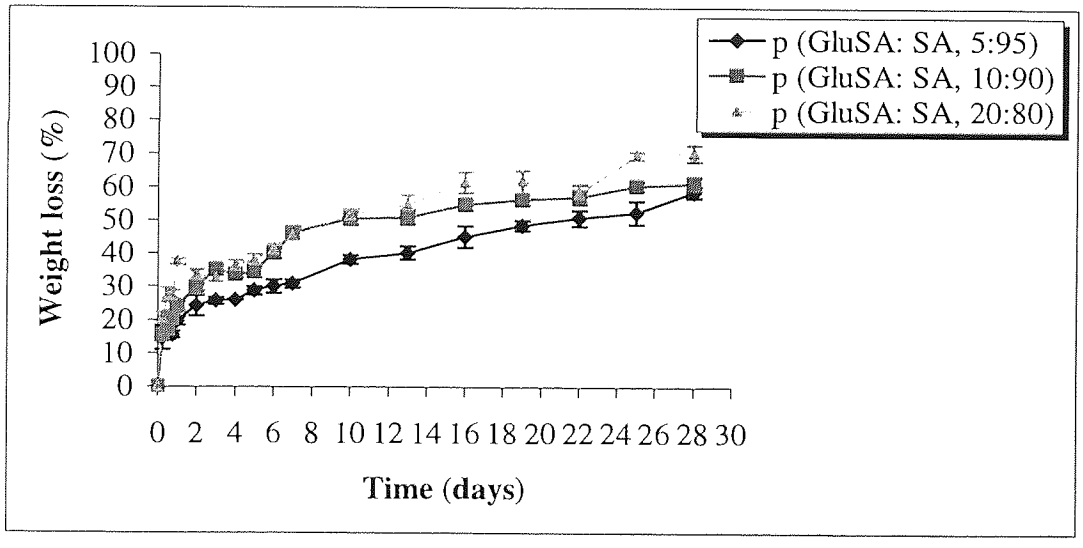


Figure 5.11 Weight loss during p (GluSA: SA), 5:95, 10:90 and 20:80 degradation (n=3; mean ± s.d.)

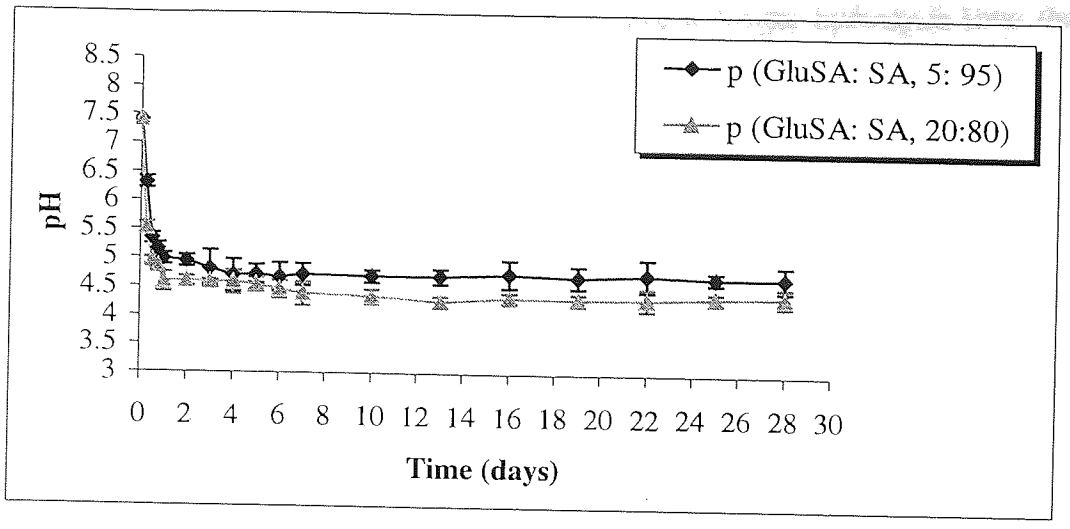


Figure 5.12 pH changes in PBS during p (GluSA: SA), 5:95, 20:80 degradation (n=3, mean ± s.d.)

5.3.3 Poly (CPP: SA: GluSA) 20: 70: 10

Bond cleavage is the most important factor in polymer degradation (Brunner and Göpferich, 1996). IR spectra for poly (CPP: SA) 20: 80 (Mw=50.5 kDa) and poly (CPP: SA: GluSA) 20:70:10 (Mw=53.7 kDa) degradation in PBS are presented respectively in Figure 5.13 and Figure 5.14. From these spectra, it can be seen the typical anhydride carbonyl peaks at 1810 cm^{-1} and 1740 cm^{-1} still can be detected for poly (CPP: SA: GluSA) 20:70:10 (Mw=53.7 kDa) even after 8 days degradation. On the other hand, after 5 days degradation, the anhydride carbonyl peaks at 1810 cm^{-1} and 1740 cm^{-1} disappeared for the poly (CPP: SA) 20: 80 (Mw=50.5 kDa) with similar molecular weight. Thus it suggests that the monomer, GluSA, acts as a crosslinking bridge during polymerisation to give a crosslinking network in the

polymer. Such a crosslinking network should have a longer hydrolysis time than linear polyanhydrides, but will eventually degrade.

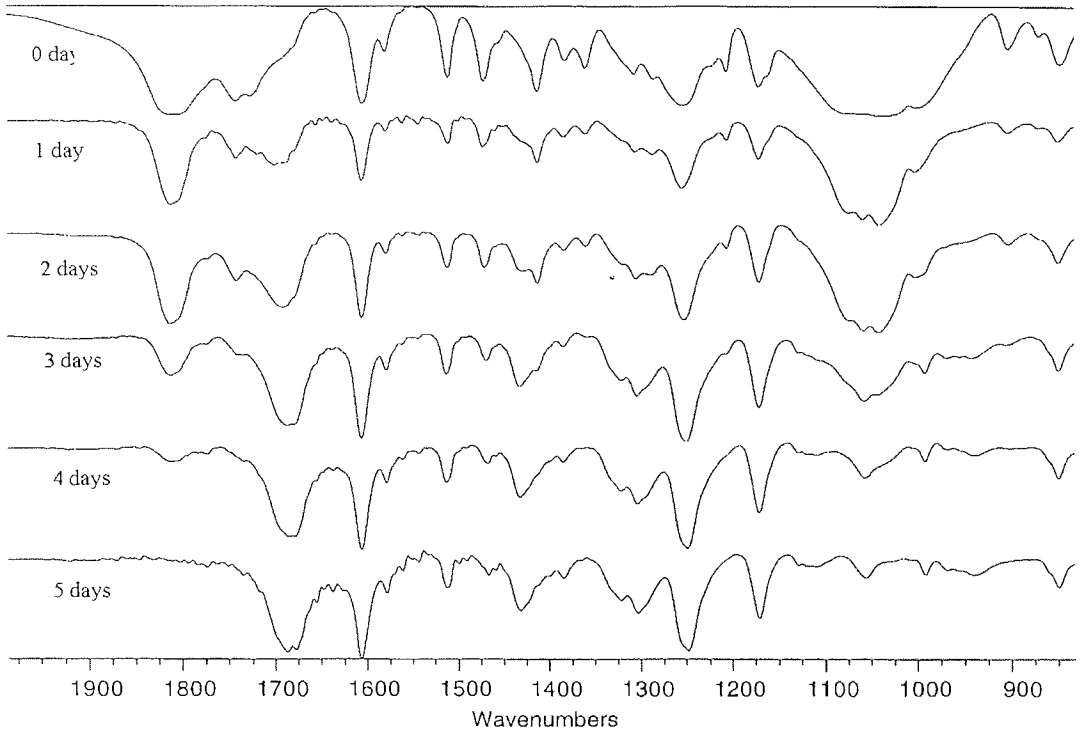


Figure 5.13 IR spectra for poly (CPP: SA) 20: 80 (Mw=50.5 kDa) degradation

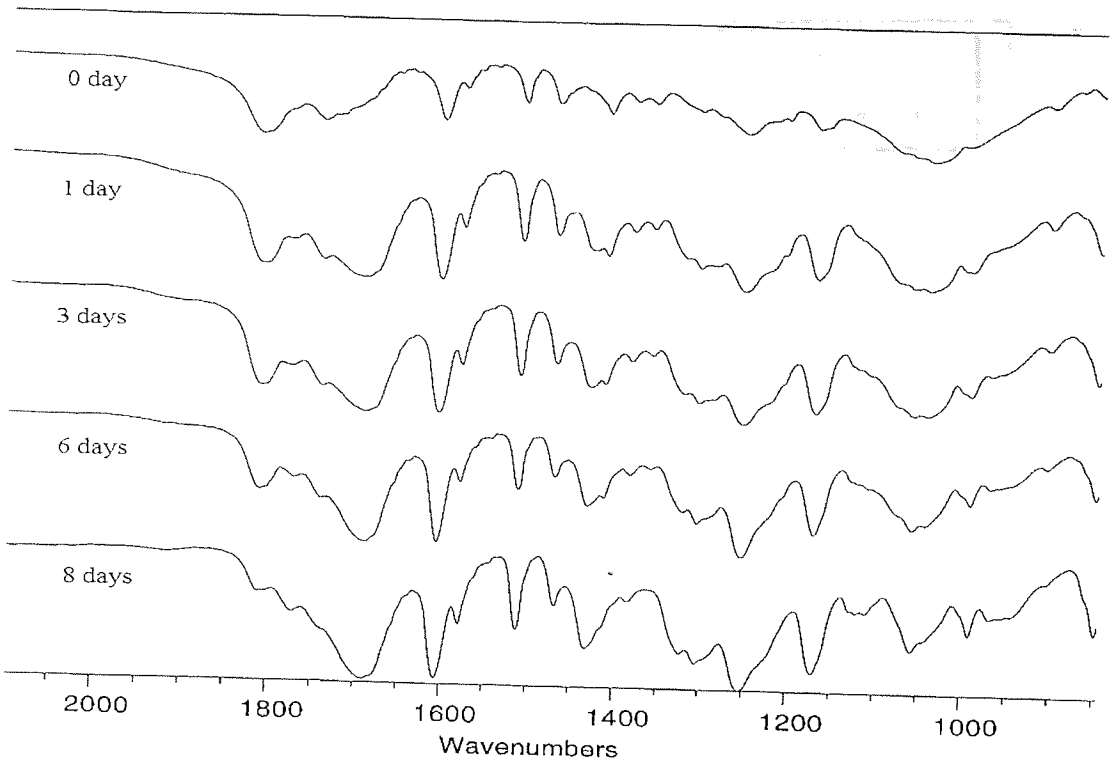


Figure 5.14 IR spectra for poly (CPP:SA:GluSA) 20:70:10 (Mw=53.7 kDa) degradation

The change in polymer molecular weight is shown in ***Figure 5.15***. The data from GPC showed there was no dramatic difference in Mw change between the two polymers during degradation. The molecular weight decreased very rapidly in first 24 hours from 50 kDa to around 5 kDa. It has been reported that polyanhydrides can lose 15 % of their molecular weight within about 1.5 hours even in anhydrous chloroform (Domb and Langer, 1989).

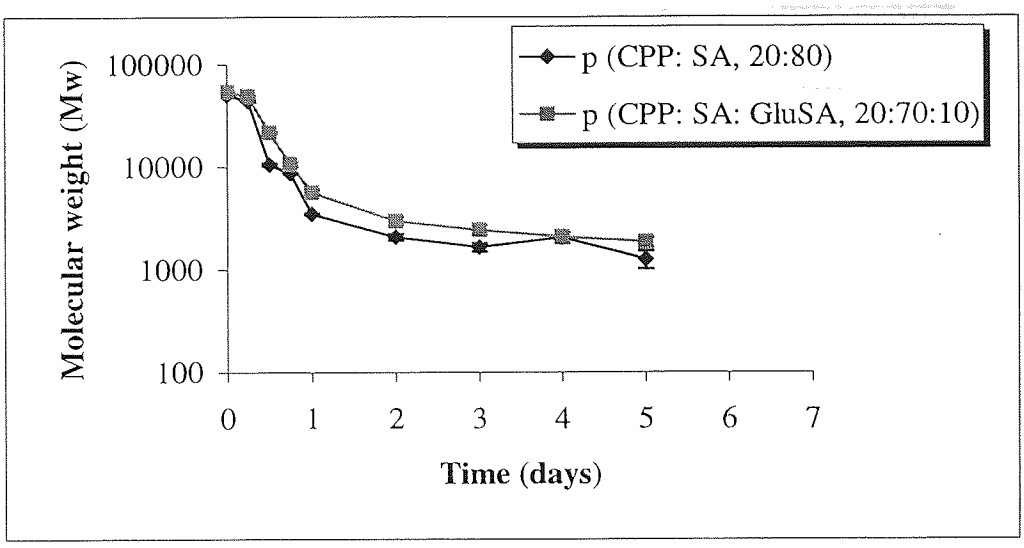


Figure 5.15 Molecular weight of p (CPP: SA) 20: 80 and p (CPP: SA: GluSA) 20:70:10 during their degradation in PBS (n=3, mean ± s.d.)

Figure 5.16 and *Figure 5.17* show the thermograms for the poly (CPP: SA) 20: 80 and poly (CPP: SA: GluSA) 20:70:10 during the degradation period. The two polymers showed a similar result. With time, a slight shoulder appeared around 100 °C, which can be identified as crystallised SA. For poly (CPP: SA) 20: 80, this occurred after one day, but after three days for poly (CPP: SA: GluSA) 20:70:10. It is possible that in poly (CPP: SA) 20: 80, the degree of copolymer crystallinity is higher as there is 10% more SA. The crystalline regions in polymers are more resistant to erosion than the amorphous parts (Göpferich and Langer, 1993).

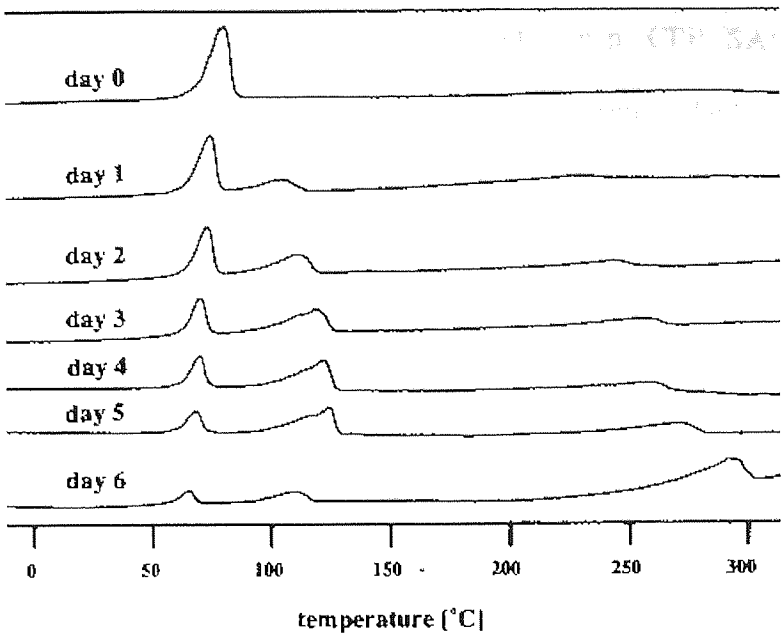


Figure 5.16 Changes in DSC thermograms during poly (CPP: SA) 20: 80 degradation

Data from Göpferich and Langer, 1993

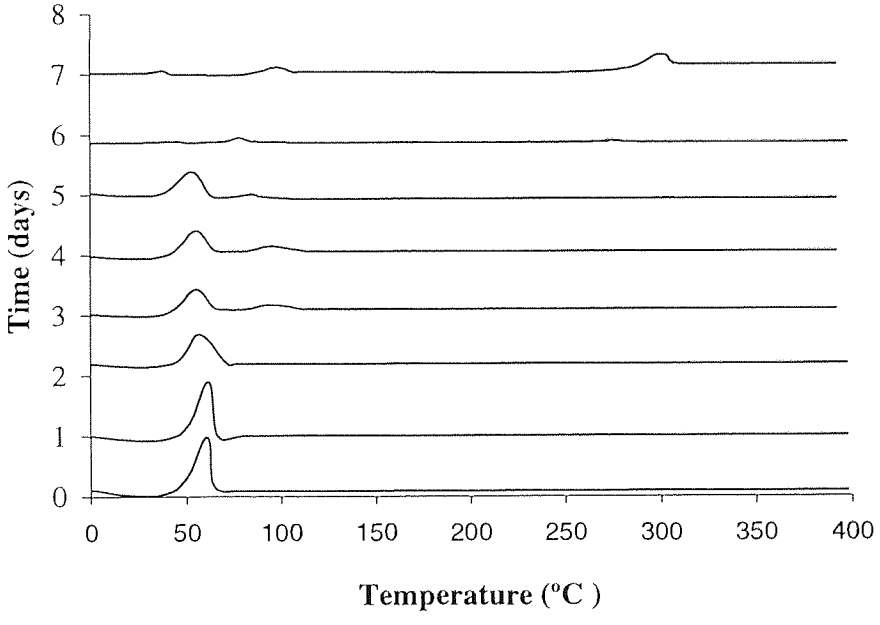


Figure 5.17 Changes in DSC thermograms during p (CPP: SA: GluSA) 20:70:10 degradation

Melting enthalpies are shown in ***Figure 5.18***. Between p (CPP: SA) 20: 80 and p (CPP: SA: GluSA) 20:70:10, there is no significant difference. This could mean that after adding the monomer GluSA, the copolymer undergoes surface erosion. At the beginning of the degradation, the melting enthalpies decreased due to the erosion of of polyanhydride and the crystalline parts were broken down by degradation. The melting enthalpies increased again, which could indicate the degraded crystalline part had not left the polymer.

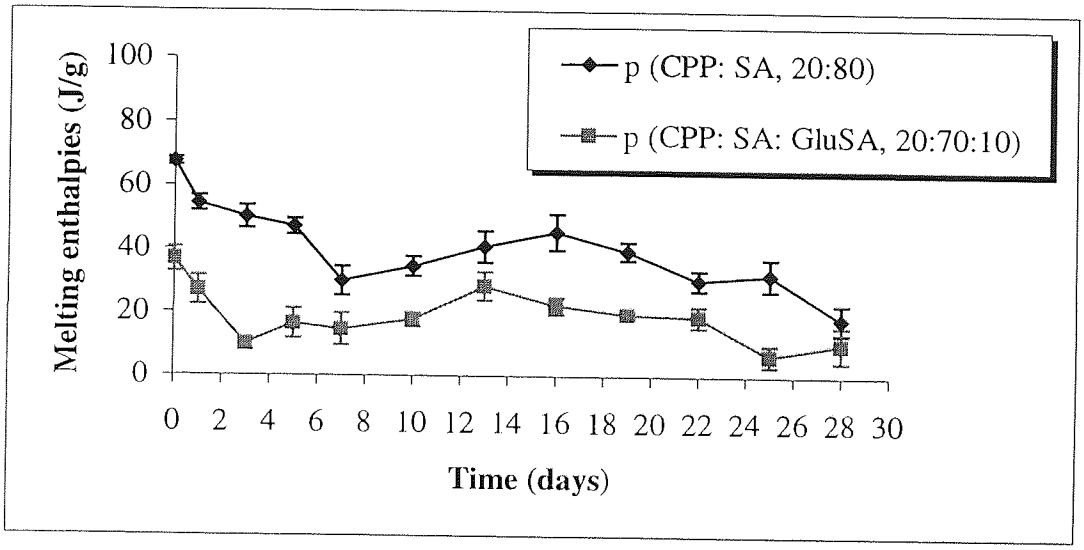


Figure 5.18 Melting enthalpies of p (CPP: SA) 20: 80 and p (CPP: SA: GluSA) 20:70:10 during their degradation in PBS (n =3; mean ± s.d.)

The pH in buffer medium was mainly determined by the polymer degradation and the solubility of degraded products (Göpferich and Langer, 1993). Although the IR data suggested that the anhydride carbonyl bond cleavage was faster in poly (CPP: SA: GluSA) 20:70:10 than that in poly (CPP: SA) 20:80, the pH decreased faster for poly (CPP: SA: GluSA) 20:70:10 than for poly (CPP: SA) 20:80 after one day degradation.

This may be due to the degradation product GluSA in p (CPP: SA: GluSA) 20:70:10. Then the pH became stable around 4 for both two polymers (***Figure 5.19***).

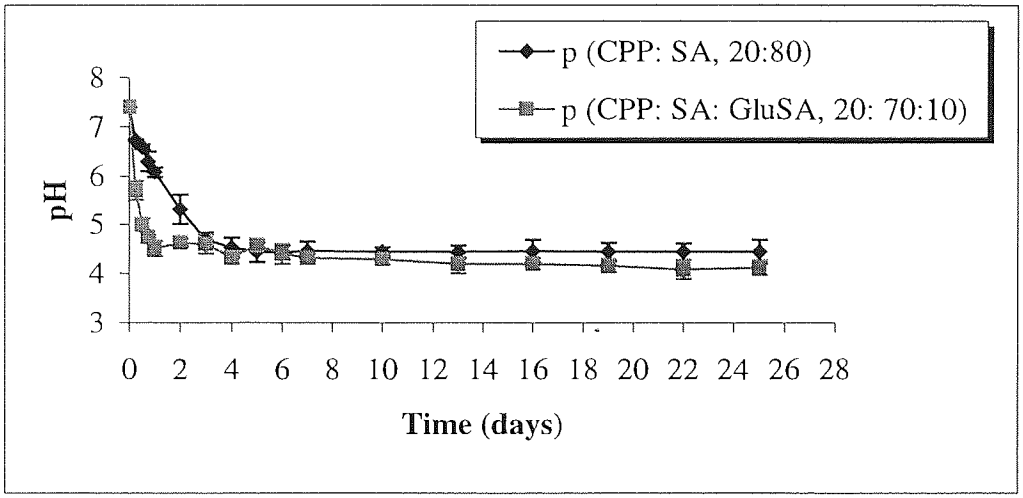


Figure 5.19 pH in PBS during p (CPP: SA) 20: 80 and p (CPP: SA: GluSA) 20:70:10 degradation (n=3, mean ± s.d.)

When the degraded products were released into the buffer medium, they can decrease the pH. The weight loss for poly (CPP: SA: GluSA) 20:70:10 is slightly faster than poly (CPP: SA) 20:80 (***Figure 5.20***), which means that more degradation products release to the PBS in unit time. Therefore, the results for polymers weight loss coincided with the pH changes.

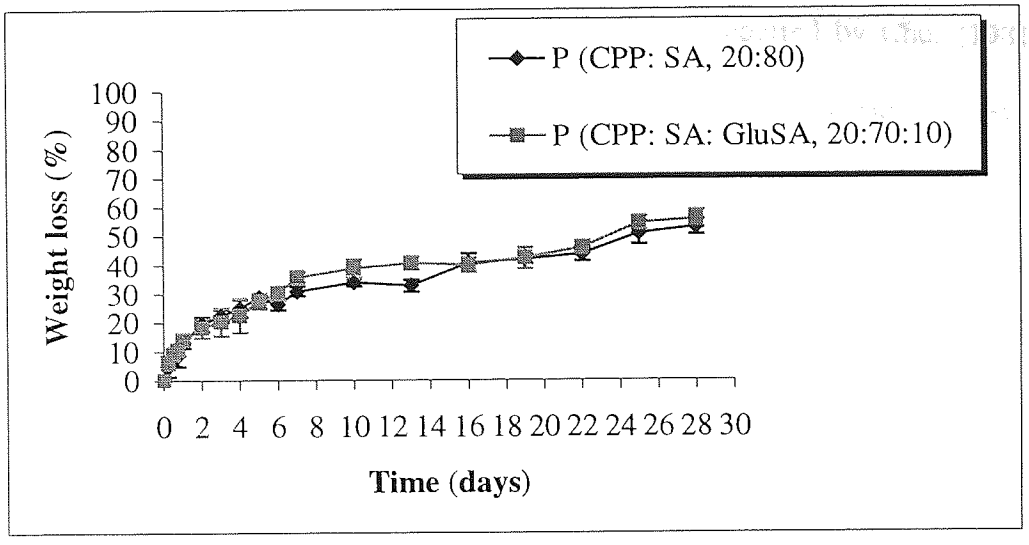


Figure 5.20 Weight loss during p (CPP: SA) 20: 80 and p (CPP: SA: GluSA) 20:70:10 degradation (n =3, mean ± s.d.)

5.3.4 Poly (AspSA: PEG)

The weight loss for the two copolymers, poly (AspSA: PEG 200) and poly (AspSA: PEG 400), at 37 °C in acidic (pH 4), physiological (pH 7.4) and alkaline (pH 10) buffers are presented in ***Figures 5.21*** and ***5.22***. In both cases, the mass of the copolymers decreased continuously with time after the onset of the degradation. As the pH of the buffer increased, the degradation rate of increased. At pH 4 and 7.4, there was an initial rapid decrease in weight for 7 days, followed by a slow and gradual degradation. In the alkaline buffer (pH 10), the copolymers had dramatically decreased in weight by over 80% after only 24 hours degradation. The two copolymers had the lowest degradation rate at pH 4.

This pH-dependent polyester hydrolysis was also reported by Chu. (1981). It was found that aliphatic polyesters, such as polyglycolide and poly (glycolide-co-lactide), hydrolytically degraded the most in a highly alkaline (pH 10.09) buffer followed the slightly alkaline (pH 7.4) and acidic buffer. The reasons for pH-dependent ester hydrolytic degradation were suggested to be due to the irreversible nature of the ester hydrolysis and the destruction of intermolecular hydrogen bonds. The study by Won *et al.* (1998) showed that increasing the molecular weight of the PEG in the copolymers decreased the overall molecular weight of the linear copolymers. Their reasons for this phenomenon was the availability of the reactivity of the terminal –OH groups in the high molecular weight PEG became hindered, due to long chain entanglement.

Within the seven days degradation, poly (AspSA: PEG 200) lost about 35% in weight, at the same time, poly (AspSA: PEG 400) lost about 60% in weight in buffer pH 7.4. After 20 days, retained 20% of its starting weight compared to 55% for poly (AspSA: PEG 200).

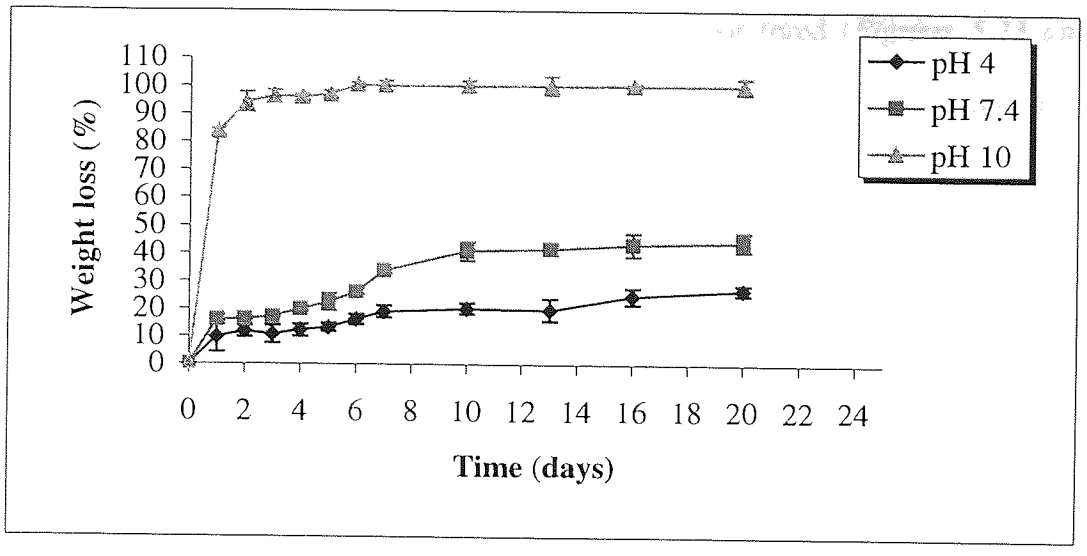


Figure 5.21 Weight loss during poly (AspSA: PEG 200) degradation in buffer with different pH (n=3, mean ± s.d.)

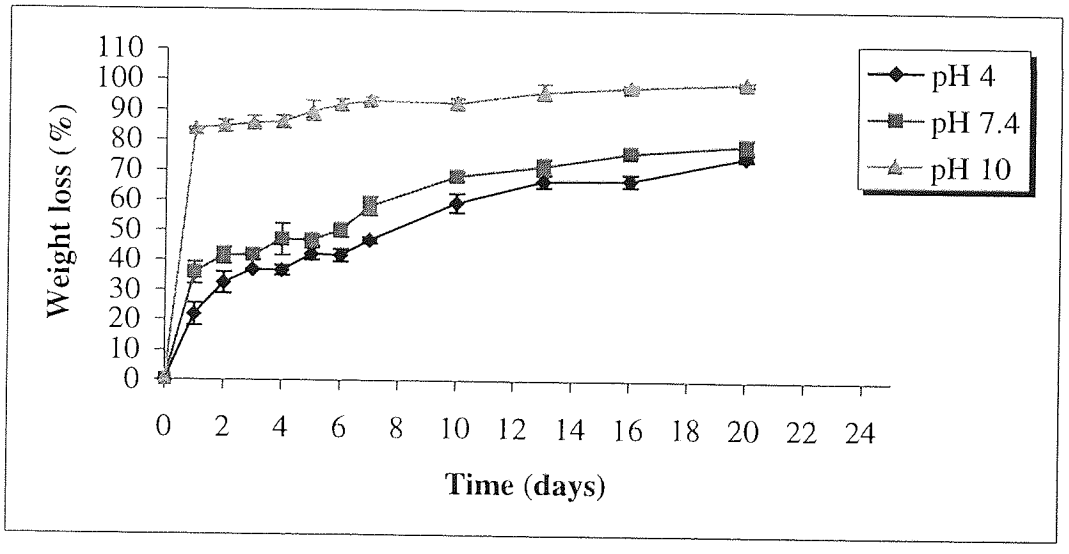


Figure 5.22 Weight loss during poly (AspSA: PEG 400) degradation in buffer with different pH (n=3, mean ± s.d.)

The variation in pH of the buffer solution was also monitored (***Figures 5.23 and 5.24***). Compared with polyanhydrides, the two polymers with polyester structure were not able to contribute significantly to the pH change at pH7.4. The explanation could be that the degraded products of poly (AspSA: PEG) were released more slowly than poly (CP2B: SA), poly (GluSA: SA) and poly (CPP: SA: GluSA). The poly (AspSA: PEG) were found to lose more weight than these polyanhydrides. This suggests that the solubility of degradation products increases in higher pH medium.

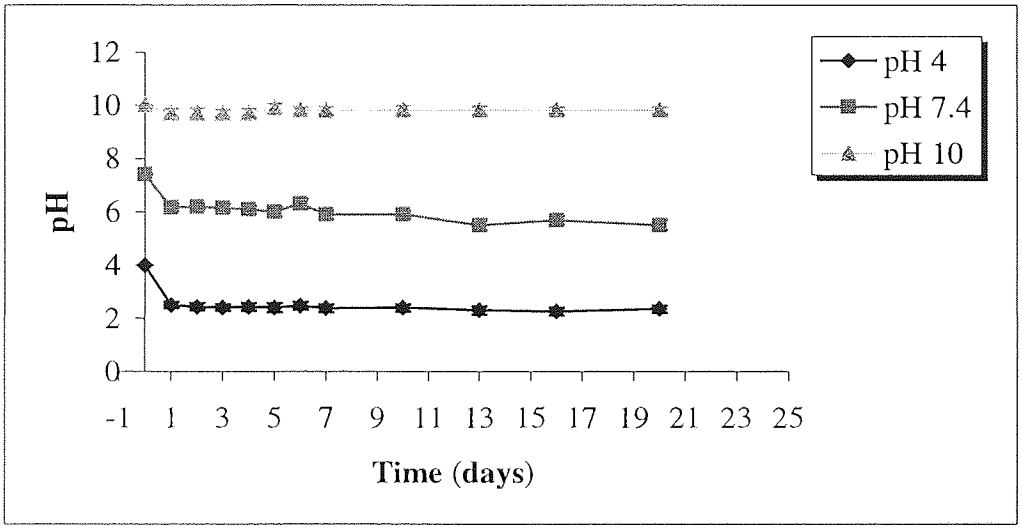


Figure 5.23 Changes in pH of the buffer medium during poly (AspSA: PEG 200) degradation (n=3, mean ± s.d.)

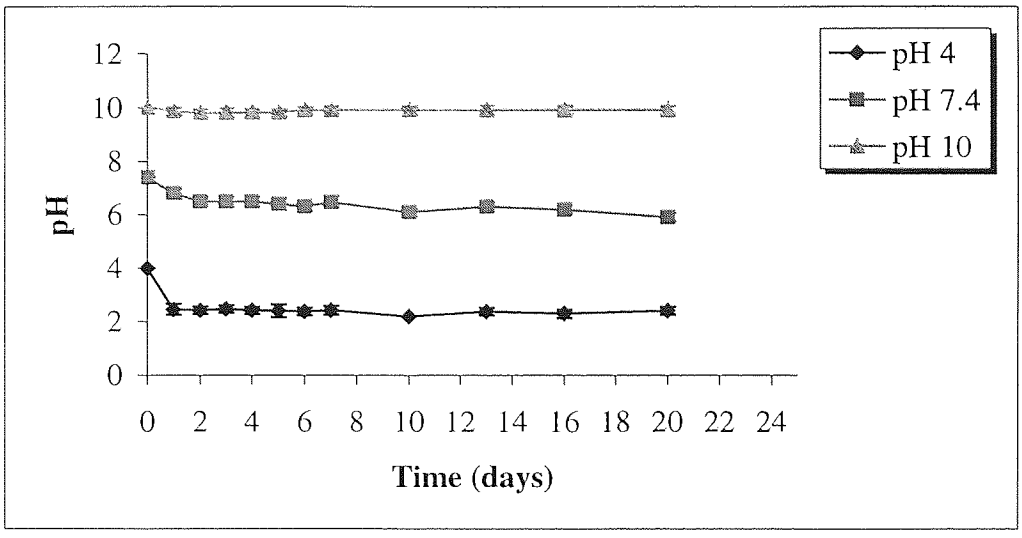


Figure 5.24 Changes of pH in the buffer medium during poly (AspSA: PEG 400) degradation (n=3, mean ± s.d.)

Chapter 6 Preparation and Characteristics of Microspheres

6.1 Introduction

In general, polyanhydrides microspheres can be prepared by four methods as detailed in ***Chapter 1***. Methods based on hot melt microencapulation (Mathiowitz and langer, 1987) and solvent removal (Mathiowitz *et al.*, 1988 and 1990), offer some advantages, but they also have several limitations. In the hot melt method, the microspheres are fabricated at the melting point of the polymer; this method is not suitable for the heat-sensitive drugs, such as proteins. In the latter method, solvent removal is conducted at room temperature and organic solvents only are used, which is advantageous for hydrolytically labile polyanhydrides. However, the danger of organic solvents residues in the microspheres limits the applicability of this method (Tamada and Langer, 1992). Furthermore, in both methods, drug is incorporated into a polymer by mixing particles of drug with particles of polymer, where the small drug particles can be heterogeneously distributed throughout the polymer. Therefore, a burst release may result when the drug islands located close to the matrix surface quickly dissolve after being immersed in buffer medium. If the drug can be homogeneously dispersed in the polymer matrix, this initial burst effect should be reduced. The development of solvent evaporation method and spray drying methods permit the preparation of microspheres of an injectable size that could release drugs at a controlled rate without any large initial burst (Tabata *et al.*, 1993). Microspheres have a distinct microstructure that depends on strongly on the preparation method applied and polymer used (Schugens *et al.*, 1994).

Scanning electron microscopy (SEM) is employed to view the surface morphology of microspheres and, if particles can be cut, their cross-sections. The sample was

mounted on an aluminium stub using carbon tape and coated with gold for 90 seconds in an argon atmosphere. The surface of microspheres was viewed under magnification using a Cambridge Instruments Stereoscan 90B. Images are taken by PIXIE thermal imaging system.

Other factors that affect the drug release behaviour include drug loading and pH of the release medium. In 1993, Tabata *et al.* reported that the release of bovine serum albumin (BSA) from poly (FAD: SA) microspheres prepared by double emulsion method displayed a near-constant rate without any large initial burst. In their study, almost 100% of the loaded BSA was released from microspheres within 30 days in 0.1 M phosphate buffer at 37 °C. They found that polymer molecular (Mw12.2 kDa, 29 kDa and 42.9 kDa used) had no effect on BSA release. However, the BSA release rate from p (FAD: SA) microspheres decreased with increasing amounts of FAD in the copolymer. This may be due to the more hydrophobic nature of the FAD monomer (Tabata *et. al*, 1993).

Microspheres made from tyrosine-containing poly (anhydride-co-imide), poly (TMA-Tyr: SA: CPP) using a double emulsion were studied by Hanes *et al.* (1998). The total BSA dose delivered from p (TMA-Tyr: SA: CPP, 20:50:30) microspheres over a period of 40 days increased from 0.35 to 131 μg BSA per mg microspheres for microspheres with initial BSA loading of 0.08 and 14.94 w/w, respectively. The percentage of BSA released during the first two days increased with protein loading. The results could indicate that BSA is released during the initial phase by a combination of microsphere erosion and protein desorption and diffusion from microsphere surfaces, or from small pores near the surface of microspheres. An

increase in the percentage protein loaded into the microspheres is likely to be accompanied by a proportionate increase in protein on or near the microspheres surface, accounting for the initially high BSA release rates (Hanes *et al.*, 1998). In the study, it was found that the overall BSA release rate increased with increasing ratio of TMA-Tyr or SA monomer in the polymer backbone. This is due to the less hydrophobic nature and higher water solubility of TMA-Tyr and SA compared with CPP. It should be noted that, in theory, it is possible to delivery drugs for periods ranging from hours to years just by changing the ratio of SA to CPP in the polymer (Leong *et al.*, 1985). However, in reality, it is limited by the stability of the protein at 37 °C in a hydrophobic and, depending on the monomers used, acidic environment.

In a study of drug releasefor polyanhydride microspheres prepared by spray drying, model drugs, including acid orange and bovine somatotropin (STH), were incorporated into microspheres prepared from p (CPP: SA, 20: 80), P (SA) and p (FA: SA, 20: 80) (Mathiowitz *et al.*, 1992). The fast release rates resulting in release over 24 hours were obtained in these cases. This could be due to the fast polymer degradation and the small size of microspheres (1-10 micron). It is important that during spray drying, the polymers tend to lose their degree of crystallinity. The phenomenon is known in spray drying of both polymers as well as small molecules (Mathiowitz *et al.*, 1990a and Ron *et al.*, 1991). The fast drying process provides very short time for the polymer to precipitate, resulting in an amorphous structure. Addition of drugs, especially those soluble in the organic solvents, further decreases the degree of crystallinity (Mathiowitz *et al.*, 1992). It has been reported that the main problem encountered in the spray drying of a polymer solution is the formation of fibres as a result of insufficient forces present to break up the liquid filament into

droplets. Therefore, the successful dispersion of the filament into polymer droplets depends greatly on the type of polymer, and to a less degree on the viscosity of the spray solution.

In this study, microspheres were prepared by the modified solvent evaporation method (water in oil in water, w/o/w double emulsion) and the spray drying method. The following polymers were used: p (CP2B: SA, 20: 80), p (CP2B: SA, 50:50), p (GluSA: SA, 5: 95), p (GluSA: SA, 10: 90) and p (GluSA: SA, 20:80). The characterisation of blank microspheres and BSA-loaded microspheres (10% w/w, theoretical loading) and the release of BSA were studied.

6.2 Experimental

6.2.1 Materials

Poly (vinyl alcohol) (PVA) with 13,000-23,000 average molecular weight and 87-89% hydrolysed (Sigma-Aldrich)

Bovine serum albumin (BSA) (Sigma-Aldrich)

Bicinchoninic acid (BCA, 4,4'-dicarboxy-2, 2'-biquinoline, sodium salt) protein assay reagent (Sigma-Aldrich)

Sodium dodecyl sulphate (SDS) (Avocado)

Sodium azide (NaN_3) (Sigma-Aldrich)

Phosphate buffer saline tablets (Sigma-Aldrich)

Dichloromethane (DCM) (Fisher scientific)

Reagents of analytical grade and double distilled water were used throughout this study.

6.2.2 Preparation of Microspheres by Double Emulsion (W/O/W) Method

2% w/v and 0.1% w/v polyvinyl alcohol (PVA) solution were prepared by dissolution of 0.2 g and 0.1 g PVA in 10 ml and 100 ml double distilled water. 500 µl of aqueous solution containing 2% (w/w) BSA was emulsified into 5 ml DCM containing 0.1 g of polymer by probe sonication (Soniprep 150), output 50 w for 3 minutes on ice bath, to form the primary emulsion. The organic solution of polymer in DCM, 2% and 0.1% PVA solution were cooled in an ice bath for one hour before use. The primary emulsion was poured into 10 ml of 2% (w/v) PVA aqueous solution and mixed vigorously on a vortex mixer for 1 minute to form the double emulsion. The resulting double emulsion was added into 100ml 0.1% (w/w) PVA solution and stirred at room temperature for 4 hours on a magnetic stirring plate, to allow the DCM to evaporate completely and to harden the microspheres. The hardened microspheres were collected by centrifugation at 10000 rpm for 35 min (JA-14 rotor, Beckman Centrifuge U.K.). The microspheres was washed twice with double-distilled water after each centrifugation and then freeze-dried (as shown in ***Figure 6.1***). The free-flowing powder was stored in a desiccator in refrigerator at 4°C.

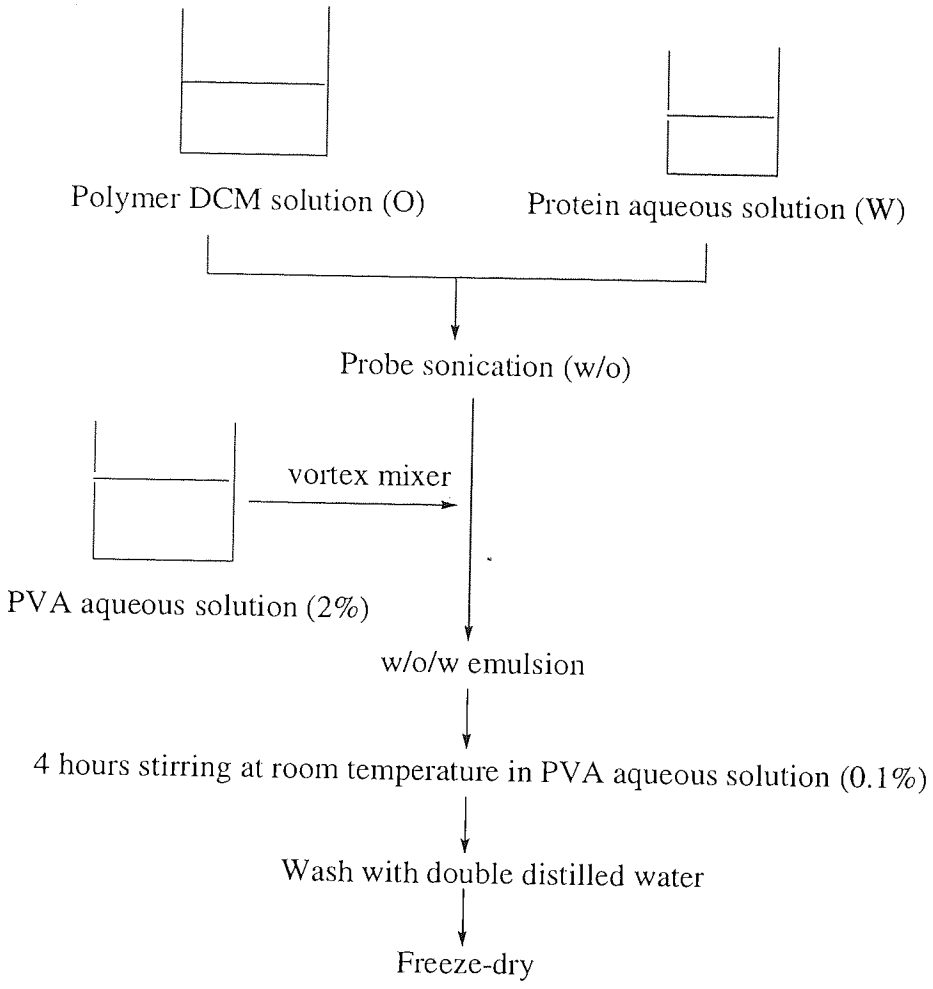


Figure 6.1 Preparation of polyanhydride microspheres by double emulsion-solvent evaporation method

6.2.3 Preparation of Microspheres by Spray Drying (SD) Method

Microspheres were prepared using a Büchi 190 mini spray dryer (***Figure 6.2***). 0.4 ml 10% (w/v) BSA aqueous solution was emulsified into 20 ml 2% (w/v) polymer in DCM solution, using probe sonication at output 70 to 80 W for 3 minutes on ice, until an emulsion was formed. Microspheres were then obtained by spray drying the polymer-drug emulsion through a 0.7 mm nozzle. The emulsion was stirred on ice before feeding to prevent droplet coalescence. Process parameters were as follows:

Inlet air temperature: 45-47 °C

Outlet temperature: 39-40 °C

Aspirator setting: 100%

Pump setting: 5-6 ml/min

Spray flow (normliter/h): 500

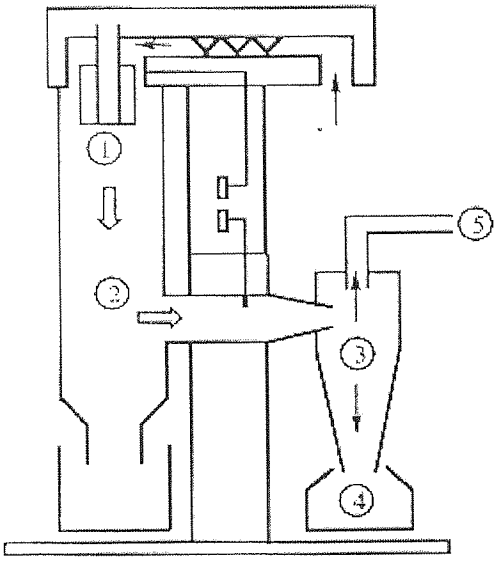


Figure 6.2 Mini Büchi Spray dryer apparatus: (1) 0.7mm nozzle; (2) spray chamber; (3) cyclone; (4) collector; (5) aspirator

Adapted from Conte *et al.*, 1994

6.2.4 Determination of Yield

The microspheres were weighed after drying, and the percentage yield was calculated by the following formula:

$$\text{Yield} = \frac{\text{Weight of dried microspheres}}{\text{Weight of polymer + BSA}} \times 100\ \%$$

6.2.5 Microsphere Degradation and Weight Loss Studies

2-3 mg of microspheres (n=3) were suspended in 1 ml of media buffer, and placed on an orbital shaker at 37 °C. At predetermined times, microspheres were collected by centrifugation, washed three times with double distilled water, freeze-dried and 21000 rpm for 10 minutes, freeze-dried (Edwards Modylo freeze drier) and stored in a desiccator in refrigerator at 4°C for SEM, GPC, and IR analyses, or weighed for the weight loss study. The degradation media buffers were prepared as detailed in ***Chapter 5***.

6.2.6 BCA Assay

A bicinchoninic acid (BCA) assay method of protein determination (Smith *et al.*, 1985) was used to determine the concentration of BSA in the release samples. The water-soluble sodium salt, BCA is sensitive, stable and highly specific for the Cu (I) ion forming an intense purple complex at 60°C in an alkaline environment (***Figure***

6.3). This colour generation forms the basis of the analytical method, capable of monitoring the amount of Cu (I) ion produced when the peptide bonds of a protein, complex with the alkaline Cu (II) ion (Biuret reaction). The absorbance of the purple complex at room temperature at 572 nm increases proportionally over a broad range of protein concentrations (0.5-1200 μg / ml). 200µl of the working reagent, consisting of 50 parts of BCA and 1 part 4 % $CuSO_4$, was added to 10µl of the protein sample on a 96 well microtitre plate (Fisher, Loughborough, U.K.). The solution was incubated at 60°C for one hour, cooled to room temperature and the absorbance was read using an MRX microplate reader (Dynex Technologies) at 570nm. Each absorbance is the average of at least 4 readings. A standard calibration was carried out each time from 10 µg/ml to 250 μg /ml (***Figure 6.4***). The calibration curve was constructed by plotting the absorbance of a series of protein standards subjected to the same conditions as the samples.

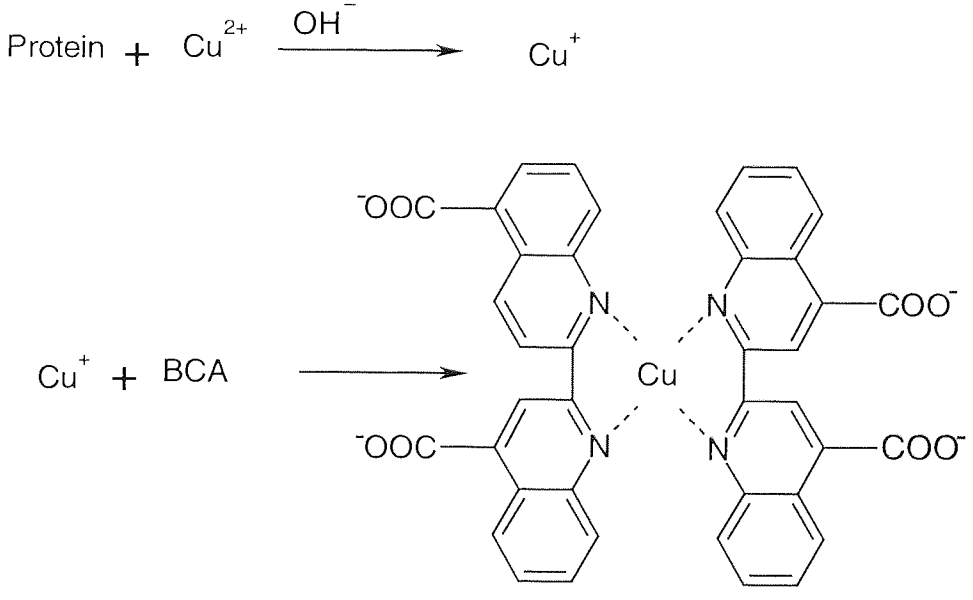


Figure. 6.3 Formation of purple complex with BCA and cuprous ion

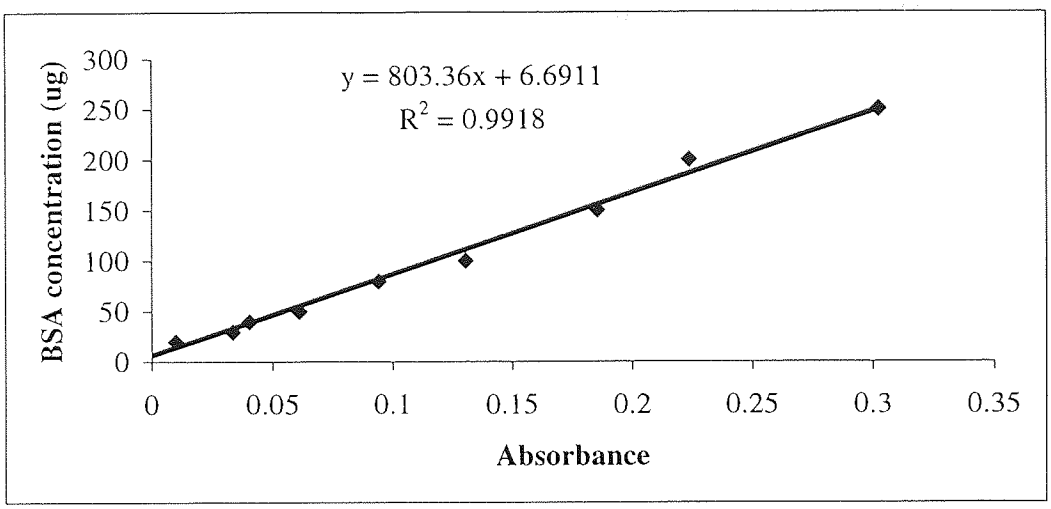


Figure. 6.4 Calibration curve of the series of BSA in 0.1 M pH7.4 PBS

6.2.6.1 Determination of Entrapment Efficiency

The method used to determine protein entrapment was adapted from Hora *et al.* (1990). 2-3 mg of the freeze-dried or spray-dried microspheres, accurately weighed, were incubated in 1ml of 1 M NaOH. Sodium hydroxide catalyses the hydrolysis of the polymer. Extraction of the protein occurred after degradation of the polymer for 4 hours shaking in a 37°C water bath until the solution became clear. The resulting solution was neutralised using 1 M HCl and the BSA concentration was determined using the bicinchoninic acid (BCA) assay. From this result, the percentage (w/w) of BSA entrapped *per* dry weight of microspheres was calculated. Each sample was assayed in triplicate. The entrapment efficiency was caculated using the following formula:

$$\text{Entrapment efficiency} = \frac{\text{Actual BSA loading}}{\text{Theoretical BSA loading}} \times 100\ \%$$

6.2.6.2 Release of BSA from Microspheres

2-3 mg of microspheres (n=3) were incubated at 37°C in 1 ml of media buffer pH 4, pH 7.4 and pH 10 (see ***Chapter 5***). The microsphere suspensions were kept shaking thoroughout the duration of the experiment. The release buffer media solution pH 7.4 contained 2% w/v SDS. NaN_3 was added at a concentration of 0.02 mg / ml as an antibacterial agent. 100 µl of supernatant solution was removed at predetermined times following centrifugation at 21000rpm for 10 min (Micro Centaur Bench top centrifuge), 100 µl of the fresh buffer was added to the samples. The supernatant solutions was kept frozen till a BCA assay was carried out. Each experiment was performed in triplicate and results were the mean of three samples.

6.3 Results and Discussions

6.3.1 Microsphere Preparation

Table 6.1 summarises the yield, actual loading, encapsulation efficiency and size of microspheres prepared by double emulsion (W/O/W) and spray drying (SD) methods.

Polymer	Mw[a] (kDa)	Preparation method	Yield (%)	Actual loading[b] (%)	Encapsulation efficiency (%)	Size[c] µm
CP2B: SA 20:80	35.1	W/O/W	60-78	9.1 ± 0.51	91	10-50
		SD	30-35	7.3 ± 0.38	72.1	2-10
CP2B: SA 50:50	22.4	W/O/W	60-75	8.4 ± 0.67	91.7	10-50
		SD	30-35	7.1 ± 0.43	67.0	2-10
GluSA:SA 5:95	29.7	W/O/W	60-70	7.5 ± 0.28	74.6	10-50
GluSA:SA 10:90	20.5	W/O/W	65-75	7.8 ± 0.36	75.4	10-50
GluSA:SA 20:80	16.8	W/O/W	65-70	8.6 ± 0.44	86.6	10-50

Table 6.1 Characteristics of different microspheres prepared by double emulsion (W/O/W) and spray drying (SD) methods

[a] Polymer molecular weight determined by GPC before microencapsulation

[b] n=3, mean ± s.d.

[c] Size determined by SEM

A higher yield was achieved using the double emulsion method than spray drying method. It is possible that some microspheres stick on the cylinder during the spray-drying preparation process. However, spray drying method is a reproducible, rapid, and easy-to-scale-up method, which should offer some advantages for preparing polyanhydride microspheres. Employing a spray drying method, the microsphere preparation process is completed in a shorter period of time than double emulsion method. It is anticipated that this should lead to less polymer degradation. Polymers with higher molecular weights have improved mechanical and film-forming properties, which can improve the stability of microspheres forming, and more protein can be encapsulated (Youan *et al.*, 1999). So it is important to decrease polymer degradation and remain its high molecular weight during microspheres preparation. Hydrolysis of the anhydride bonds will cause degradation of the polyanhydrides during microsphere preparation, and degradation occuring during double emulsion method is attributed to a long hardening time (4 hours in aqueous PVA solution).

IR spectra for p (CP2B:SA) 20:80 before and after microsphere preparation suggested that polymer degradation took place during micrsphere preparation by both methods (***Figure 6.5***). The carboxylic acid peak at 1700 cm^{-1} was stronger for microspheres prepared by a double emulsion method than by a spray drying method. However, results obtained by GPC (***Table 6.2***) indicated that both methods resulted in a rapidly reduced Mw, and there was little differences between the microspheres prepared by different methods.

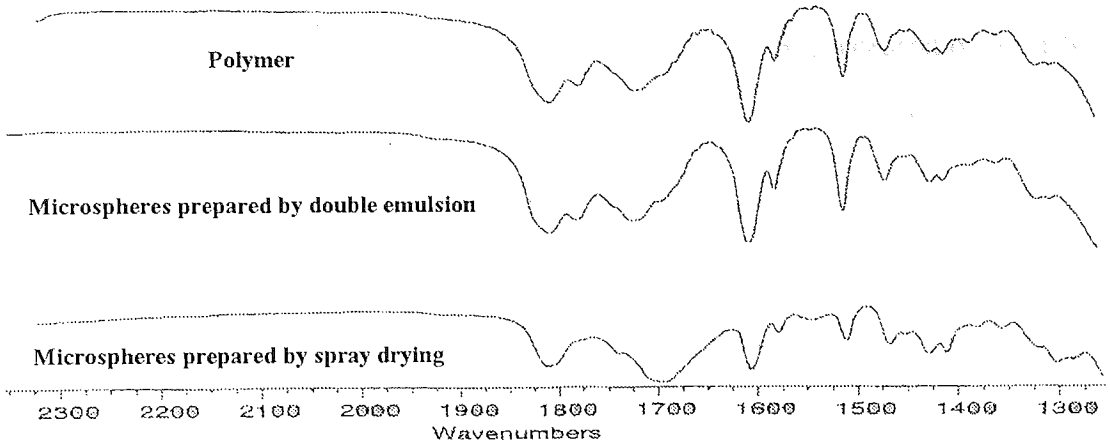


Figure 6.5 IR spectra for p (CP2B:SA) 20:80 and its microspheres prepared by spray drying and double emulsion methods

P (CP2B:SA) 20:80	35.1 kDa
Spray drying (SD)	10.2 kDa
Double emulsion (w/o/w)	9.8 kDa

Table 6.2 Changes in Mw for p (CP2B:SA) 20:80 microspheres prepared by spray drying and double emulsion methods

The microspheres obtained using the double emulsion method had higher encapsulation efficiencies than those prepared by spray drying method both in the case of microspheres of p (CP2B: SA) 20:80 and microspheres of p (CP2B: SA) 50:50 (see ***Table 6.1***). Similar results were also obtained by Chiba *et al.* (1997). In their study, BSA was efficiently encapsulated into poly (anhydride-co-imide) microspheres using double emulsion method. High protein encapsulation efficiency (>70%) is a distinct advantage of the double emulsion method. Furthermore, the encapsulation process may be performed at a low temperature (in an ice bath) to minimise thermal

drug inactivition (Chiba *et al.*, 1997). Low BSA incorporation levels achieved using the spray drying method were probably because of a high concentration of BSA aqueous solution (10%) used and a large volumme (20 ml) of water-oil mixture emulsified. It is hard to get a homogenous emulsion for a big volumme using lab sonication. On the other hand, if large droplets formed before feeding, the spray nozzle could split the emulsion into its individual components, which may stick on the cylinder due to the higher boiling point of water. Thus, it is possible that some microspheres harvested in the product collector could have little or no drug content due to a loss of products in the drying chamber. These problems may be avoided by decreasing the spray flow to decrease the microsphere size or decreasing the concentration of BSA solution as it has been reported that the best encapsulation efficiencies for spray drying are always obtained with the lowest amount of drug added to the polymer (Pavanetto *et al.*, 1993).

A high encapsulation efficiency (>70%) was achieved in the case of poly (GluSA: SA) microspheres prepared using w/o/w method. This may indicate that poly (GluSA: SA), a fast degrading crosslinking polymer, could act as a drug delivery carrier as good as linear polyanhydrides.

It was found that, in the double emulsion process, the size distribution of microspheres was greatly dependent on the sonication conditions for preparation of the inner emulsion (Tabata *et al.*, 1993). No change in the size distribution was observed by changing the exposure time of sonication in the range from 10 to 30 seconds. And microspheres did not have an injectable size (<150 mm) if exposure time was less than 10 seconds. However, sonication could enhance polymer

degradation and protein activity loss (Tabata *et al.*, 1993). In order to allow a comparison for the microspheres prepared from different polymers, the same sonication conditions were maintained through this study.

6.3.2 Microsphere Surface Morphology

6.3.2.1 Microspheres Prepared by the Double Emulsion Method

Figure 6.6 shows the morphological characterisation of p (CP2B: SA) microspheres prepared by double emulsion method. All microspheres were spherical in shape. The surface of p (CP2B:SA), 20:80 microspheres possessed more small pores compared with p (CP2B:SA), 50:50 microspheres. In other cases in the literature, it was also found that the more crystalline content (*ie.* increasing SA) microspheres appeared more porous external surface (Mathiowitz *et al.*, 1990b and c). This could be a result of fast precipitation that is typical of the crystalline polymers. The amorphous p (CP2B: SA) 50:50 microspheres were relatively smooth and dense for both blank and BSA-loaded samples.

It was seen that when BSA was encapsulated (***Figures 6.6 C and D***), no BSA traces were found on the external surface of polymers, which suggests that the BSA was successfully encapsulated into the polymer matrix.

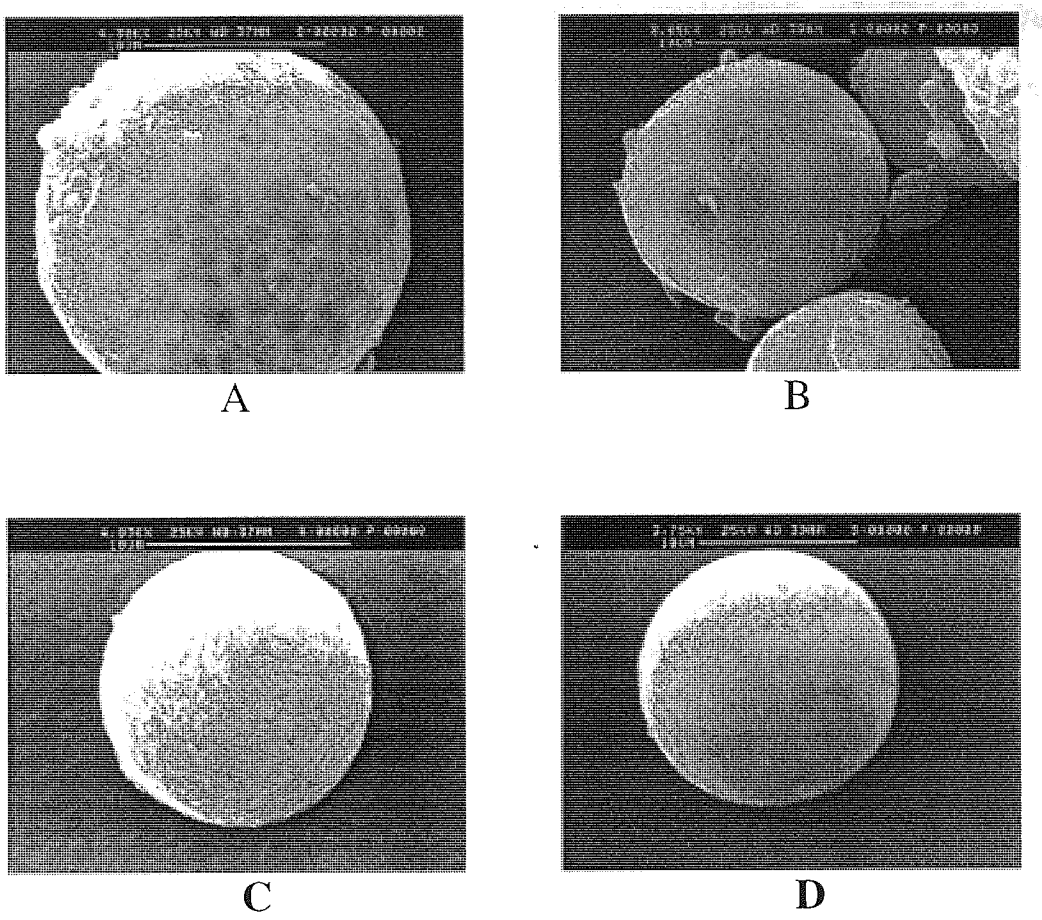


Figure 6.6 SEM of P (CP2B: SA) microspheres prepared by double emulsion: **(A)** blank P (CP2B: SA, 20:80) microspheres; **(B)** blank P (CP2B: SA, 50:50) microspheres; (**C**) BSA loaded P (CP2B: SA, 20:80) microspheres; **(D)** BSA loaded P (CP2B: SA, 50:50) microspheres.

The morphological changes in blank p (CP2B: SA) microspheres during degradation and BSA loaded p (CP2B: SA) microspheres during degradation *in vitro* (0.1 M pH 7.4 PBS, 37°C) are shown in ***Figures 6.7*** and ***6.8***, respectively. It can be seen in *Figure 6.7* that immediately after preparation, polymer fragments were visible on the microspheres surface for both polymer microspheres, which could be related to a high viscosity of polyanhydride DCM solution for microsphere preparation. Polymers with

higher molecular weight would have improved mechanical and film-forming properties (Domb *et al.*, 1988). However, with the molecular weight increasing, an aggregation could occur as a result of viscosity of polymer DCM solution increasing (Youan *et al.*, 1999). After 7 days, blank p (CP2B: SA, 20:80) microspheres had degraded more than blank p (CP2B: SA, 50:50) microspheres. This may be because the increased hydrophobic monomer (CP2B) in p (CP2B: SA, 50:50) inhibited the initial water entrance and therefore resulted in a slow degradation. Although after 7 days degradation, anhydride bonds on microsphere surface had been hydrolysed and some particles appeared to have collapsed, the blank microspheres did not lose their bulk structure. This could be a result of a large proportion of the poorly water-soluble monomers remaining.

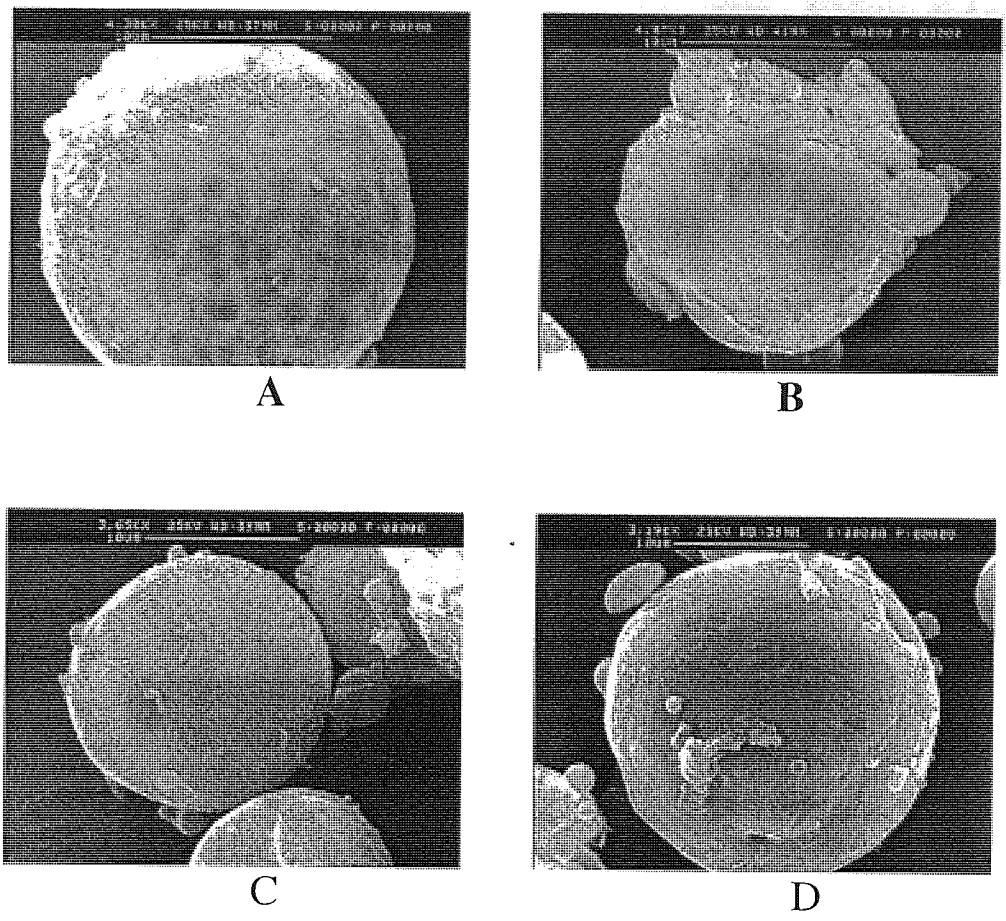


Figure 6.7 SEM of blank P (CP2B: SA) microspheres prepared by double emulsion during degradation *in vitro*: (**A**) blank P (CP2B: SA, 20:80) microspheres (day 0); (**B**) blank P (CP2B: SA, 20:80) microspheres (day 7); (**C**) blank P (CP2B: SA, 50:50) microspheres (day 0); (**D**) blank P (CP2B: SA, 50:50) microspheres (day 7).

In ***Figure 6.8***, for BSA-loaded microspheres made from p (CP2B: SA, 20:80), the microsphere surface appeared highly porous after 7 days degradation. However, the spherical shape of microspheres was still observed. This is the same for the blank microspheres. This result indicated that the microspheres was attacked from surface to core. After 14 days, the p (CP2B: SA, 20:80) microspheres lost their spherical

structure. A similar profile for degradation was observed for microspheres made from p (CP2B: SA, 50:50).

Blank microspheres and BSA-loaded microspheres appear to degrade differently. Presumably, BSA-loaded microspheres can degrade faster than blank microspheres prepared from the same polymer, because once BSA release is initiated, channels may form throughout the polymer matrix. As a result, microsphere degradation is controlled by a combination of diffusion and hydrolysis.

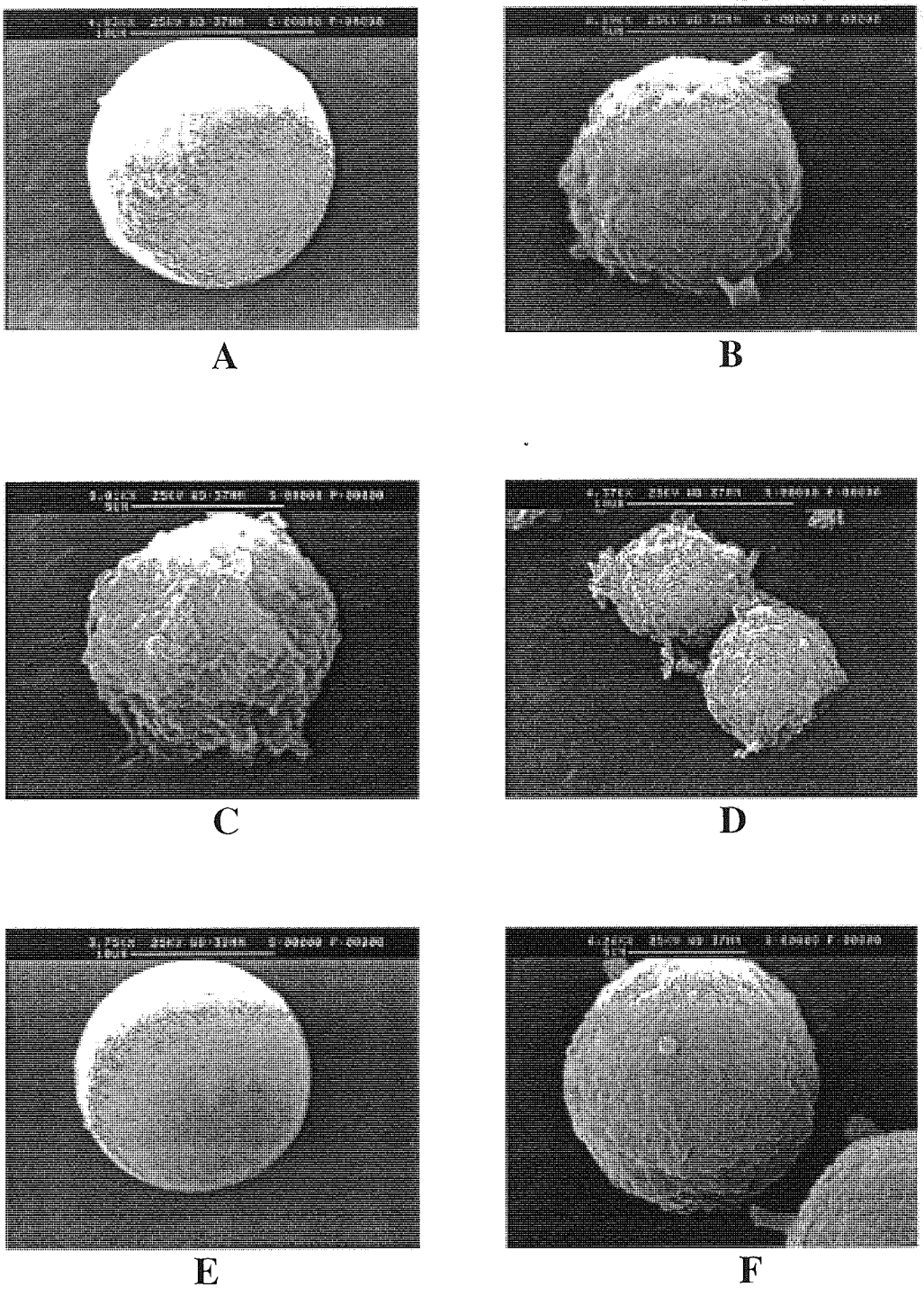


Figure 6.8 SEM of BSA-loaded P (CP2B: SA) microspheres prepared by double emulsion during degradation *in vitro*: (**A**) P (CP2B: SA, 20:80) microspheres (day 0); (**B**) P (CP2B: SA, 20:80) microspheres (day 7); (**C**) and (**D**) P (CP2B: SA, 20:80)

microspheres (day 14); (E) P (CP2B: SA, 50:50) microspheres (day 0); (F) P (CP2B: SA, 50:50) microspheres (day 7).

Scanning electron micrographs of BSA-loaded microspheres made from poly (GluSA: SA) are shown in ***Figures 6.9 A, B*** and ***C***. SEM of BSA-loaded microspheres prepared from poly (GluSA: SA), 20:80 after degradation (day 7) is shown in ***Figure 6.9 D***. With an increasing percentage of monomer GluSA in copolymer, the surface of microspheres becomes less porous and denser. This may be due to the decreasing degree of crystallinity of polymer as a result of the crosslinked structure. It was seen that after 7 days in buffer, poly (GluSA: SA), 20:80 microspheres had degraded significantly, and the microspheres appeared to have lost all structural integrity. This indicated that the polymer had degraded completely at the point. From the SEM study, it seemed the poly (GluSA: SA), 20:80 microspheres degraded faster than poly (CP2B: SA, 20:80) microspheres. It could be related to the lower hydrophobicity of poly (GluSA: SA, 20:80) than that of poly (CP2B: SA, 20:80).

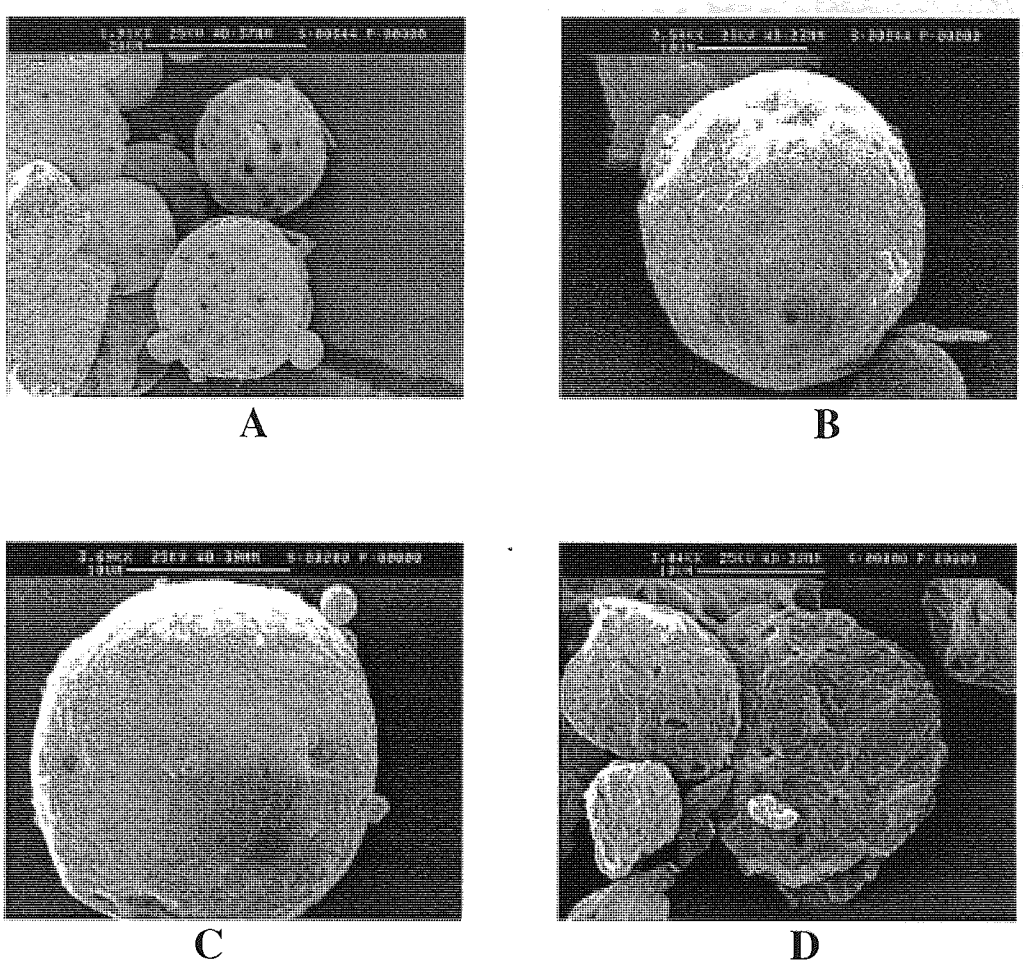


Figure 6.9 SEM of BSA loaded P (GluSA: SA) microspheres prepared by double emulsion: (**A**) P (GluSA: SA, 5:95) microspheres; (**B**) P (GluSA: SA, 10:90) microspheres; (**C**) P (GluSA: SA, 20:80) microspheres; (**D**) P (GluSA: SA, 20:80) microspheres during degradation *in vitro* (day 7).

6.3.2.2 Microspheres Prepared by Spray Drying Method

Scanning electron micrographs of microspheres made from p (CP2B: SA, 20:80) and p (CP2B: SA, 50:50) are shown in ***Figure 6.10***. The surfaces of blank and BSA-loaded p (CP2B: SA) microspheres were smooth and dense. However, the microspheres tended to fuse with each other. The same phenomenon was observed with p (CPP: SA) microspheres (Mathiowitz *et al.*, 1992). Lowering the concentration of the polymer solution may prevent the aggregation, which could be a result of low viscosity of polymer DCM solution. It is possible that the low melting point of polymer is the main reason for the high degree of fusion during spray drying is (Mathiowitz *et al.*, 1992). The aggregation was reduced for p (CP2B: SA) 20:80 (Tm 70°C) microspheres compared to p (CP2B: SA) 50:50 (Tm 62 °C) microspheres, owing to the different melting points of the two polymers. This is because the polymer with low melting point should be sufficiently hard in shorter time than one with high melting point.

During microsphere preparation, some of the spheres accumulated in the spray drier trap, and this could be related to the aggregation of the microspheres. When the surface concentration of the sprayed droplets reaches saturatation, crusts will form. If the crusts are sufficiently dry or hard, there is no change in appearance and the dried particles are spherical. If the crusts are not dry or hard enough, the microspheres would appear deformed or adhere to each other in the spraying chamber and result in aggregation. The aggregation during the evaporation process prevented some of the microspheres from reaching the final collecting tube and accumulated in the trap of the spray drier. This may be responsible for the lower yields in spray drying.

Degradation of BSA-loaded microspheres made from p (CP2B: SA) are shown in *Figures 6.10 E* and *F*. No significant changes were visible during the degradation. The microspheres remained spherical, with no visible pores, softening or fusion. This could be due to slow penetration of water into the polymer matrix owing to the high hydrophobicity of p (CP2B: SA). Furthermore, due to the high aggregation and therefore low permeation of hydrophilic, low molecular weight ions of the phosphate buffer through the polymer matrix, degradation products may not be released into the buffer. This matches with pH change in buffer medium (see ***Section 5.3***) Therefore, there were no clear erosion signs observed using SEM after 7 days degradation.

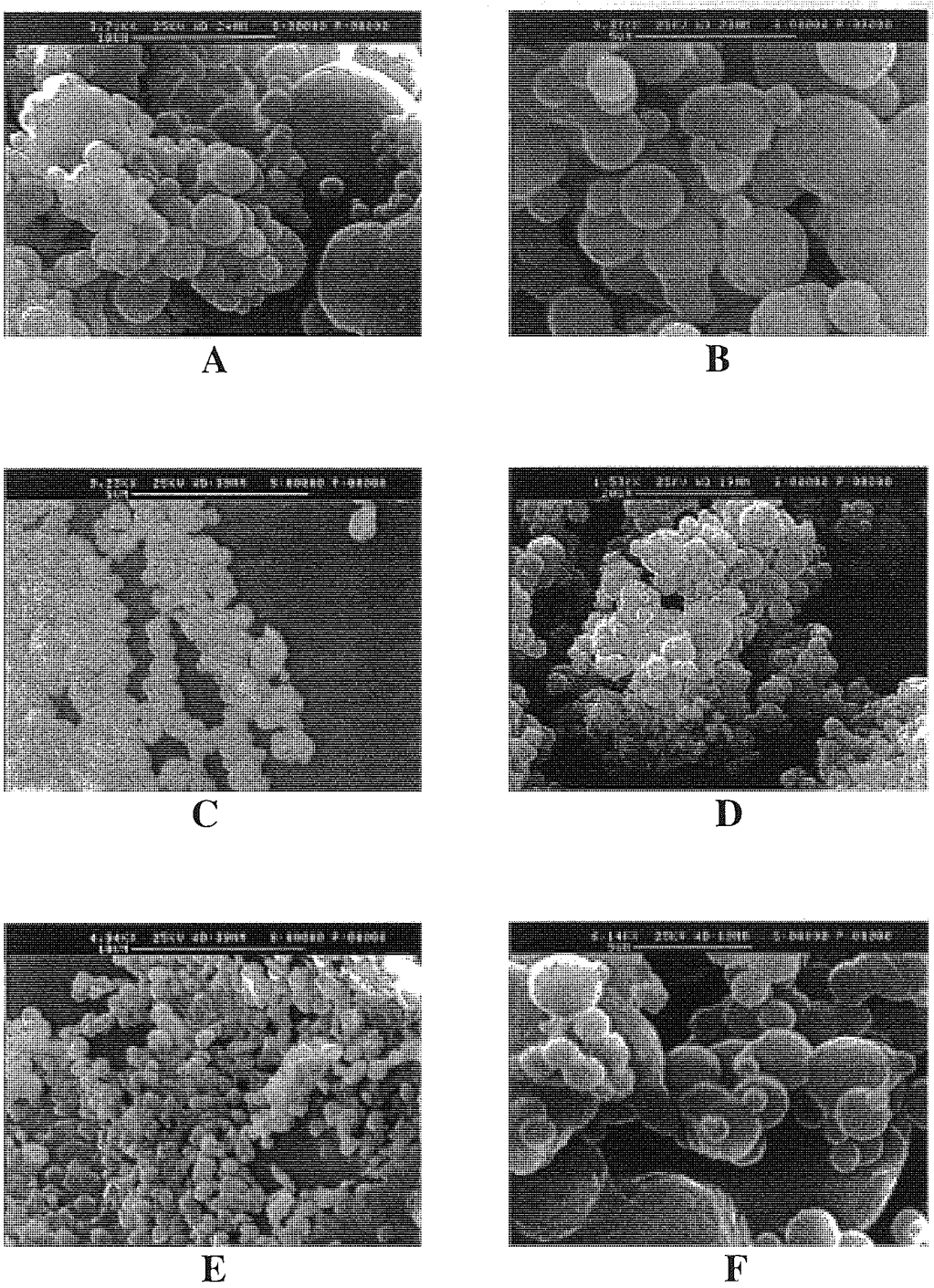


Figure 6.10 SEM of P (CP2B: SA) microspheres prepared by spray drying method: (**A**) blank p (CP2B: SA, 20:80) microspheres; (**B**) blank p (CP2B: SA, 50:50) microspheres; (**C**) BSA loaded p (CP2B: SA, 20:80) microspheres; (**D**) BSA-loaded p (CP2B: SA, 50:50) microspheres; (**E**) BSA loaded p (CP2B: SA, 20:80) microspheres

during degradation *in vitro* (day 7); (F) BSA loaded p (CP2B: SA, 50:50) microspheres during degradation *in vitro* (day 7).

Blank micropheres prepared from p (GluSA: SA, 20: 80) using spray drying are shown in ***Figure 6.11***. It can be seen that a high degree of aggregation was observed. This could be due to the low melting point of polymer (Tm 61-64 °C). It also could be explained by the high viscosity of polymer DCM solution as a result of crosslinking network in polymer. The yield decreased to 10-15%. Therefore, crosslinked poly (GluSA: SA) was not suitable for preparation of microspheres using spray drying method.

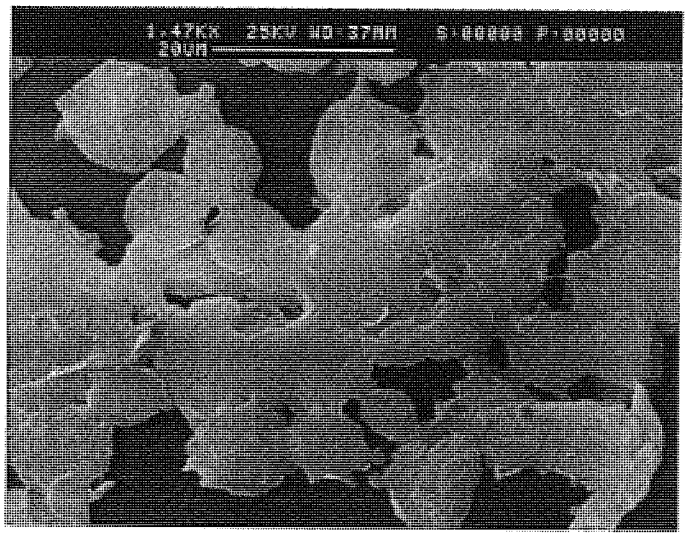


Figure 6.11 SEM of blank P (GluSA: SA), 20:80 microspheres prepared by spray drying method

6.3.3 Microsphere Degradation and BSA Release Studies

Microsphere degradation also can be monitored using GPC and IR. The results of IR analysis for p (CP2B: SA) microspheres during degradation were similar to those of the unprocessed polymers (as shown in ***Figure 6.12***). The anhydride carbonyl peaks at 1810 cm^{-1} decreased with time, while at the same time, the carboxylic acid peak at 1700 cm^{-1} became stronger. However, for the poly (CP2B: SA, 50: 50) microspheres, the aromatic anhydride bonds at 1740 cm^{-1} were still detected after 5 days degradation, which could be due to the high hydrophobicity of CP2B. The Mw changes for p (CP2B: SA) microspheres are shown in ***Figure 6.13***. It was found that the Mw of microspheres decreased very rapidly in buffer. It was reported that there was no correlation between the rate of drug release and polymer degradation expressed as % decrease in the molecular weight, which at first glance might appear to be contradictory (D'Emanuele *et al.*, 1992). The drug release rate would depend on the rate of erosion expressed as volume of the matrix dissolved *per* unit time, times the drug load rather than the rate of polymer degradation (Domb *et al.*, 1997). However, the erosion of polyanhydride was characterised by an induction period during which the rate of erosion was relative slow (see ***Chapter 5***). It was found that during this period significant molecular weight losses occurred within the polymer, without significant device erosion. The length of the induction period depends on the initial polyanhydride molecular weight increasing molecular weight leads to a longer induction period (D'Emanuele *et al.*, 1992).

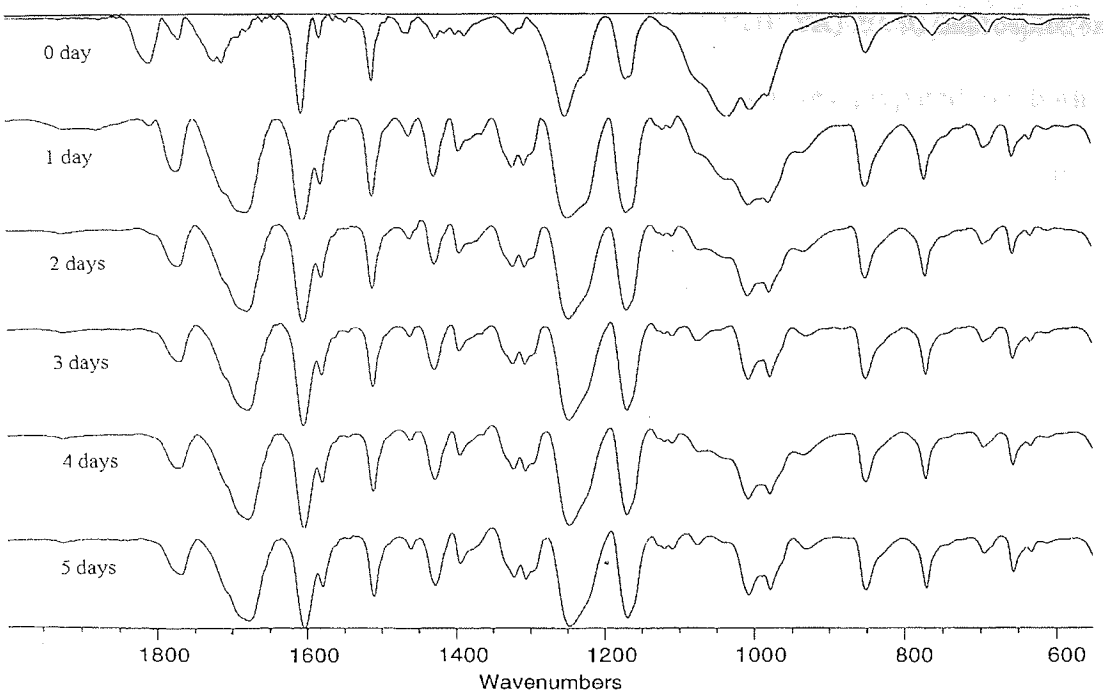


Figure 6.12 IR data for BSA-loaded poly (CP2B: SA) 50:50 microspheres prepared by double emulsion during degradation in PBS pH 7.4

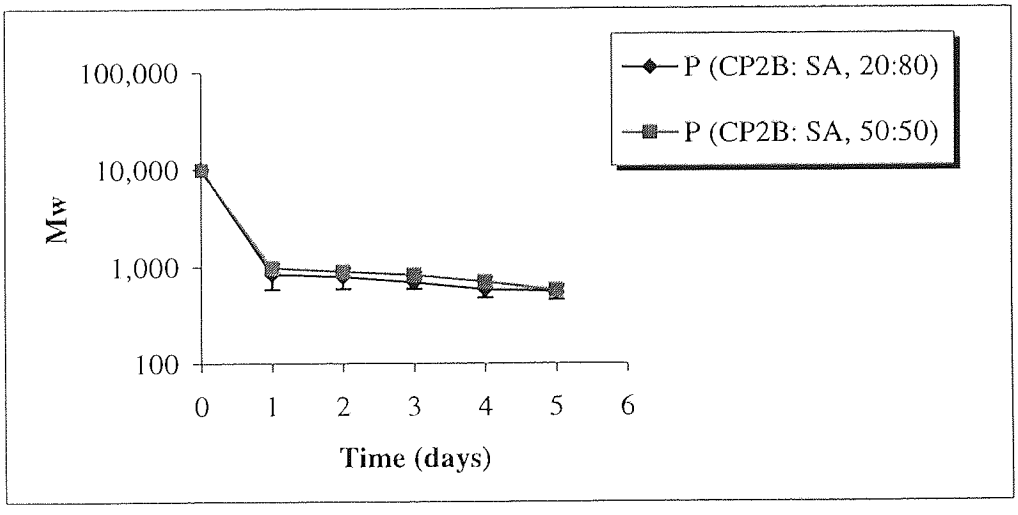


Figure 6.13 Molecular weight of poly (CP2B: SA) 20: 80 and 50: 50 during degradation in PBS pH 7.4 (n=3, mean ± s.d.)

It can be seen from ***Figure 6.14*** that release from p (CP2B: SA) 20:80 microspheres is slightly faster than from p (CP2B: SA) 50:50 microspheres prepared by both by double emulsion and spray drying methods. This could be due to the higher hydrophobicity of p (CPB: SA) 50:50 preventing water uptake. About 5-13 % BSA was leased over 24 hours for those microspheres. It was reported that the release of the incorporated material could occur *via* two independent processes. The first is diffusion of the drug through fluid-filled pores, formed by the dissolution of the incorporated drug particles; the second is *via* erosion of the polymer matrix as the anhydride bonds are hydrolysed. The total release of drug will be the sum of these two release rates (Mathiowitz *et al.*, 1992). In this case, BSA release could be controlled by a combination of diffusion and polymer erosion initially, flowed by erosion-controlled release at later time. Additionally, if the BSA islands located close to the matrix surface, it is possible that BSA will quickly dissolve once being immersed in solution.

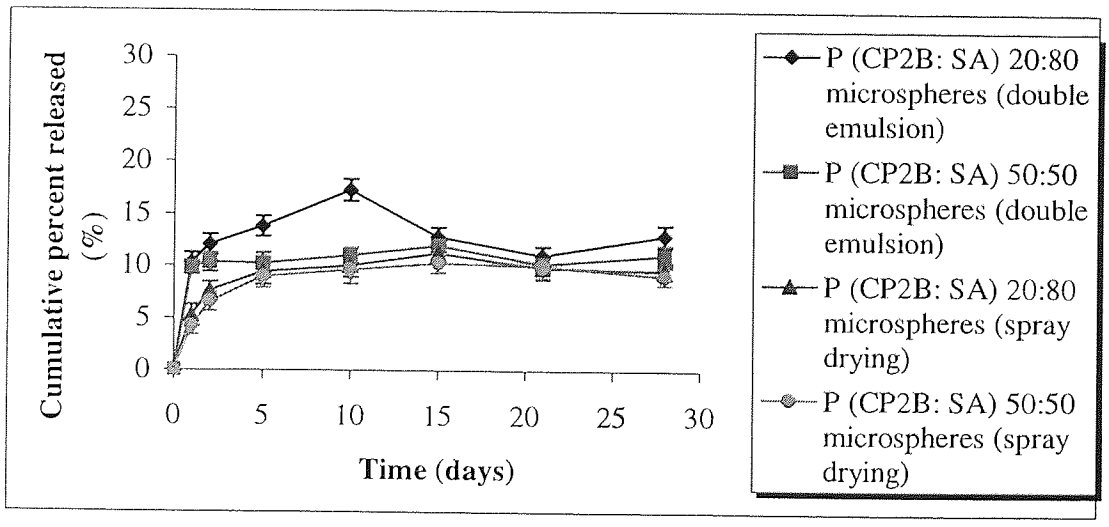


Figure 6.14 Release of BSA from p (CPB: SA) microspheres prepared by double emulsion and spray drying in PBS containing 2% SDS (n=3; mean ± s.d.)

In release studies *in vitro*, SDS was used to reduce BSA re-adsorption onto the polymer surface. It was seen that the cumulative percent release of BSA increased from 32 % to 37 % in the case of p (GluSA: SA, 20:80) microspheres (***Figure 6.15***). However, the cumulative release value for microspheres fell after about one week. This may be because the released BSA could come back to adsorb on the polymer surface again.

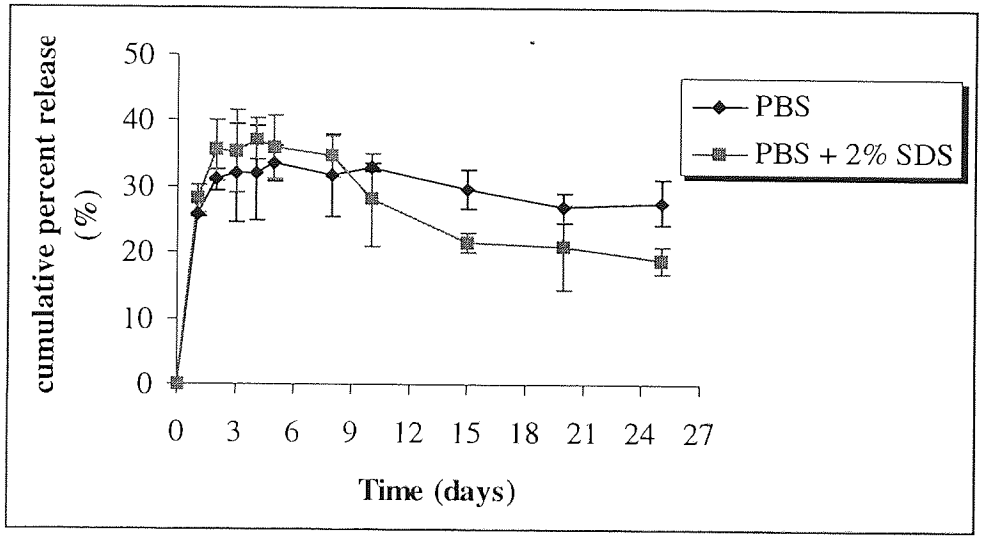


Figure 6.15 Release profiles for p (GluSA: SA, 20:80) microspheres prepared by double emulsion method in PBS and PBS containing 2% w/v SDS (n=3; mean ± s.d.)

It is expected that drug release should correlate with weight loss, a more appropriate indicator of erosion rate than the decrease in polymer molecular weight (Domb *et al.*, 1997). ***Figure 6.16*** shows the microsphere weight loss and the cumulative percent release of BSA. It was seen that the microspheres weight loss was higher than cumulative percent release of BSA after one week. Combined with the fact that pH in buffer decreased from 7.4 to 4 in PBS during polymer degradation period (see

Chapter 5), it was found, in the first few days, the polymer degradation products could leave the polymer matrix and dissolve into buffer. Their fast erosion may correspond to, at least partially, the relatively fast BSA release. With the degradation products increasing, the pH in buffer is decreased, which could result in slower polymer erosion and poor dissolution of degradation products. Thus the subsequent dissolution of degradation products becomes the rate-limiting step in protein release (Hanes *et al.*, 1998).

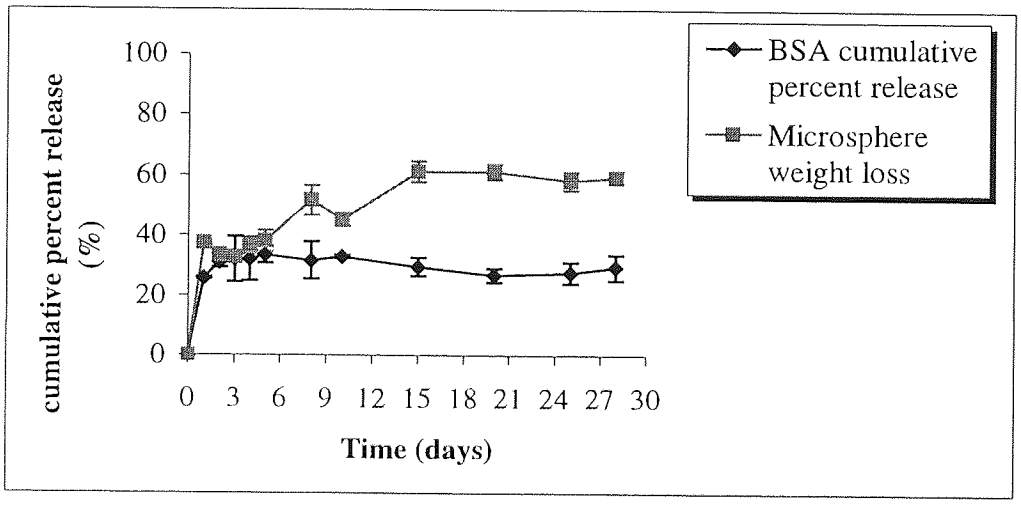


Figure 6.16 Weight loss and the cumulative percent release of BSA for p (GluSA: SA, 20:80) microspheres prepared by double emulsion in PBS (n=3; mean ± s.d.)

The effect of buffer pH on BSA release profiles from p (GluSA: SA, 20:80) microspheres prepared by double emulsion is shown in ***Figure 6.17***. BSA release rate was significantly reduced at low pH and enhanced under basic conditions. The reduced BSA release rate at low pH may be likely due to the decreased solubility of the monomers under acidic conditions. At higher pH conditions, the monomers have a relatively high solubility and microsphere erosion can occur more quickly, leading to

a faster BSA release. The "stability" of polyanhydride microspheres at low pH could be an advantage for oral drug delivery of vaccines when mucosal immunity is desired, since microspheres less than 10 micron in diameter are known to be taken up from the intestine into Peyer's patches (Eldridge *et al.*, 1990; Chiba *et al.*, 1997).

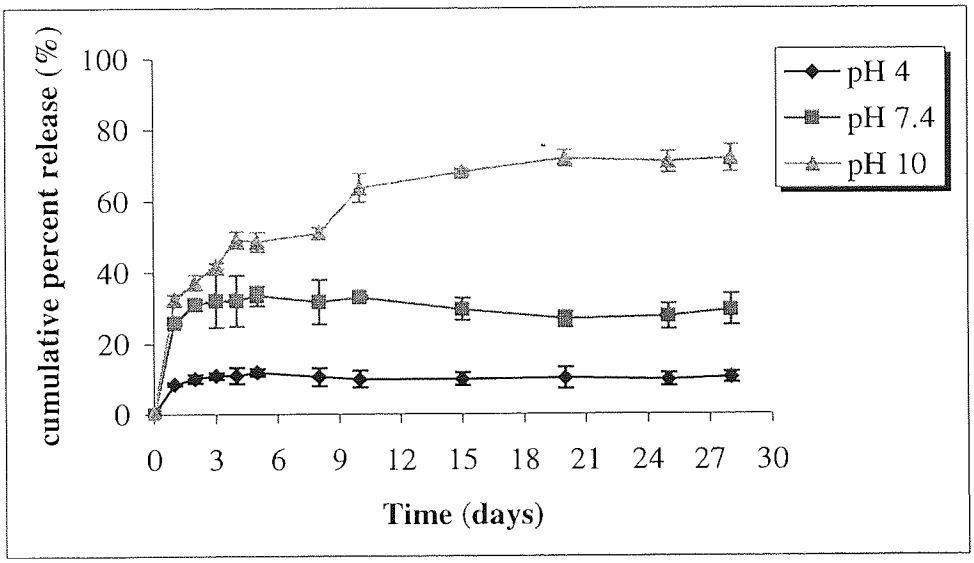


Figure 6.17 The effect of pH of buffer on BSA release profiles from p (GluSA: SA, 20:80) microspheres prepared by double emulsion (n=3; mean ± s.d.)

Chapter 7 Conclusion

Two biodegradable polyanhydrides have been synthesised using the melt-polycondensation method in the presence of cadmium acetate as a catalyst that resulted in a polyanhydride with a higher molecular weight. One is an unsaturated polyanhydride, poly (CP2B: SA), which has double bonds along the polymer backbone. The other is an amino acid (glutamic acid) based polyanhydride, poly (GluSA: SA), where, the monomer GluSA that made from glutamic acid and sebacic acid, acts as a crosslinking bridge to produce a crosslinked polyanhydride. A new biodegradable polyester, p (AspSA: PEG), has also been prepared from low molecular weight PEG and AspSA anhydride, made from aspartic acid and sebacic acid. The resulting copolymers have a crosslinked structure.

The two major factors that determine the degradation of the investigated polyanhydrides are the properties and portions of the monomers in polymers and the pH of the buffer medium. Degradation occurred more rapidly in poly (CP2B: SA) with low percentages of hydrophobic monomer (CP2B). In the case of poly (GluSA: SA), the polymers with high percentages of GluSA degraded faster. The hydrolytic degradation of these polymers was pH-dependent and characterised by a rapid decrease in molecular weight, particularly at an alkaline pH. A fast hydrolysis of the anhydride bonds, the backbone of the polymers, was believed to be the mode of degradation.

Poly (CP2B: SA) 20: 80 and 50: 50 were used to prepare microspheres using solvent evaporation process and spray drying method. Poly (GluSA: SA) was found not to be suitable to prepare microspheres using spray drying method. This is probably due to their low melting point, but the double emulsion method produced good quality

microspheres for poly (GluSA: SA). Microspheres made from both types of polymers appeared spherical and encapsulated about 70% of BSA.

Increased BSA release was achieved in an alkaline medium (pH 7-10). This is due to an increase in the polymer erosion and dissolution of the degraded acid monomers from the microsphere surface under alkaline conditions. Polymer composition also played an important role in the release of BSA. The BSA release rate increased with increasing hydrophilic momnomer SA percentage in poly (CP2B: SA). The crosslinked poly (GluSA: SA, 20:80) microspheres have a higher release rate than poly (CP2B: SA, 20:80) within 30 days. This suggested the amino acid component in the polymer has played a big role in improving the microenvironment of the microspheres, such as reducing acidity through neutralising released the acid monomers. Therefore this crosslinked polymer may be a potential candidate for protein delivery in the form of microspheres.

Chapter 8 References

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