INCORPORATION AND RELEASE OF MACROMOLECULES FROM BIODEGRADABLE POLYMER VEHICLES

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Doctor of Philosophy

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The initial objective of this work was to evaluate and introduce fabrication techniques based on W/O/W double emulsion and O/W single emulsion systems with solvent evaporation for the incorporation of a surrogate macromolecule (BSA) into microspheres and microcapsules fabricated using P(HB-HV), PEA and their blends. Biodegradation, expressed as changes in the gross and ultrastructural morphology of BSA loaded microparticulates with time was monitored using SEM concomitant with BSA release. Spherical microparticulates were successfully fabricated using both the W/O/W and O/W emulsion systems. Both microspheres and microcapsules released BSA over a period of 24 to 26 days. BSA release from P(HB-HV)20% PCL 11 microcapsules increased steadily with time, while BSA release from all other microparticulates was characterised by an initial lag phase followed by exponential release lasting 6-11 days. Microcapsules were found to biodegrade more rapidly than microspheres fabricated from the same polymer. The incubation of microparticulates in newborn calf serum; synthetic gastric juice and pancreatin solution showed that microspheres and microcapsules were susceptible to enzymatic biodegradation. The *in vitro* incubation of microparticulates in Hank's buffer demonstrated limited biodegradation of microspheres and microcapsules by simple chemical hydrolysis. BSA release was thought to ocurr as a result of the macromolecule diffusing through either inherent micropores or via pores and channels generated *in situ* by previously dissolved BSA. However, in all cases, irrespective of percentage loading or fabrication polymer, low encapsulation efficiencies were obtained with W/O/W and O/W techniques (4.2±0.9%-15.5±0.5%,n=3), thus restricting the use of these techniques for the generation of microparticulate sustained drug delivery devices.

In order to overcome this low encapsulation efficiency, a W/O single emulsion technique was developed and evaluated in an attempt to minimise the loss of the macromolecule into the continuous aqueous phase and increase encapsulation efficiency. Poly(lactide-co-glycolide) [PLCG] 75:25 and 50:50, PEA alone and PEA blended with PLCG 50:50 to accelerate biodegradation, were used to microencapsulate the water soluble antibiotic vancomycin, a putative replacement for gentamicin in the control of bacterial infection in orthopaedic surgery especially during total hip replacement. Spherical microspheres (17.39±6.89μm,n=74-56.5±13.8μm,n=70) were successfully fabricated with vancomycin loadings of 10, 25 and 50%, regardless of the polymer blend used. All microspheres remained structurally intact over the period of vancomycin release and exhibited high percentage yields(40.75±2.86%-97.16±4.3%,n=3)and encapsulation efficiencies (47.75±9.0%-96.74±13.2%,n=12). PLCG 75:25 microspheres with a vancomycin loading of 50% were judged to be the most useful since they had an encapsulation efficiency of 96.74+13.2%, n=12 and sustained therapeutically significant vancomycin release (15-25μg/ml) for up to 26 days.

This work has provided the means for the fabrication of a spectrum of prototype biodegradable microparticulates, whose biodegradation has been characterised in physiological media and which have the potential for the sustained delivery of therapeutically useful macromolecules including water soluble antibiotics for orthopaedic applications.

KEYWORDS: Microspheres and Microcapsules; Biodegradation of Poly(α,β esters); O/W, W/O/W and W/O emulsion with solvent evaporation; Poly(lactide-co-glycolide); Poly(hydroxybutyrate/hydroxyvalerate).

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List of Abbreviations

PHB	-	Poly(hydroxybutyrate)
PHV	-	Poly(hydroxyvalerate)
P(HB-HV)	-	Poly(hydroxybutyrate/hydroxyvalerate)
PGA	-	Poly(glycolic acid)
PLA	-	Poly(lactic acid)
PLCG	-	Poly(lactide-co-glycolide)
PCL	-	Poly(caprolactone)
PEA	-	Poly(ethylene adipate)
PVA	-	Poly(vinyl alcohol)
O/W	-	Oil in water
W/O/W	-	Water in oil in water
W/O	-	Water in oil
BSA	-	Bovine serum albumin
BCA	-	Bicinchoninic acid assay
SEM	-	Scanning Electron Microscopy
PWL	-	Percentage weight loss

CHAPTER ONE

INTRODUCTION.

1.0 Controlled release of macromolecules.

Significant advances have been made over the last two decades in the area of controlled release for the delivery of a variety of biologically active molecules used in agricultural, veterinary and pharmaceutical spheres. The literature refers to extended, sustained and controlled release and no distinction is made between them for the purpose of this thesis[1].

The present interest shown by the pharmaceutical industry in the area of controlled release is partly a reflection of the high costs associated with establishing a new drug in the market place. It can take up to 10 years and £32m pounds to get a new drug on to the market[2]. Consequently the number of new drugs reaching the market has plummeted in recent years. Pharmaceutical drug companies now focus on the development of new drug formulations which achieve optimal therapeutic effect not solely on the basis of the drug itself but also on the basis of the principles of release, absorption, distribution, metabolism and elimination. These new formulations allow extensions to be sought on lucrative patents[1,3].

The advantages of having a drug released for an extended period, in a controlled manner, are easily appreciated by comparing the delivery profiles from controlled release and conventional dosage forms. Conventional methods of drug dosing such as tablets and intravenous injection result in an initial burst in the systemic level of bioactive agent which often exceeds the therapeutic level. Once bodily elimination has countered this threat, drug activity is steadily reduced to a level which is ineffective in treating the

patient[4]. Controlled delivery eliminates this cyclical aspect of drug administration and facilitates the maintenance of a constant plasma level of the drug. This is important for the administration of drugs such as leuprolide acetate which has a narrow therapeutic range. It also reduces the need for constant dosing, therefore patient compliance is less of a problem.

Initial investigations into controlled polymer based release devices focused on the use of biocompatible non-biodegradable materials such as silicone rubber to release low molecular weight hydrophobic molecules[5]. Generally they were implantable systems and consequently demanded surgical retrieval once the drug had been exhausted. As a consequence of this, attention was drawn to the use of biodegradable polymers. There are various definitions of "biodegradable" and "bioerodible" to be found in the literature[6]. In this thesis biodegradation refers to hydrolytic, enzymatic or bacteriological degradation processes which do not change the physical form of the polymer[7]. In comparison bioerosion involves a change in the physical form of the polymer, i.e. weight loss, from the polymer matrix[7].

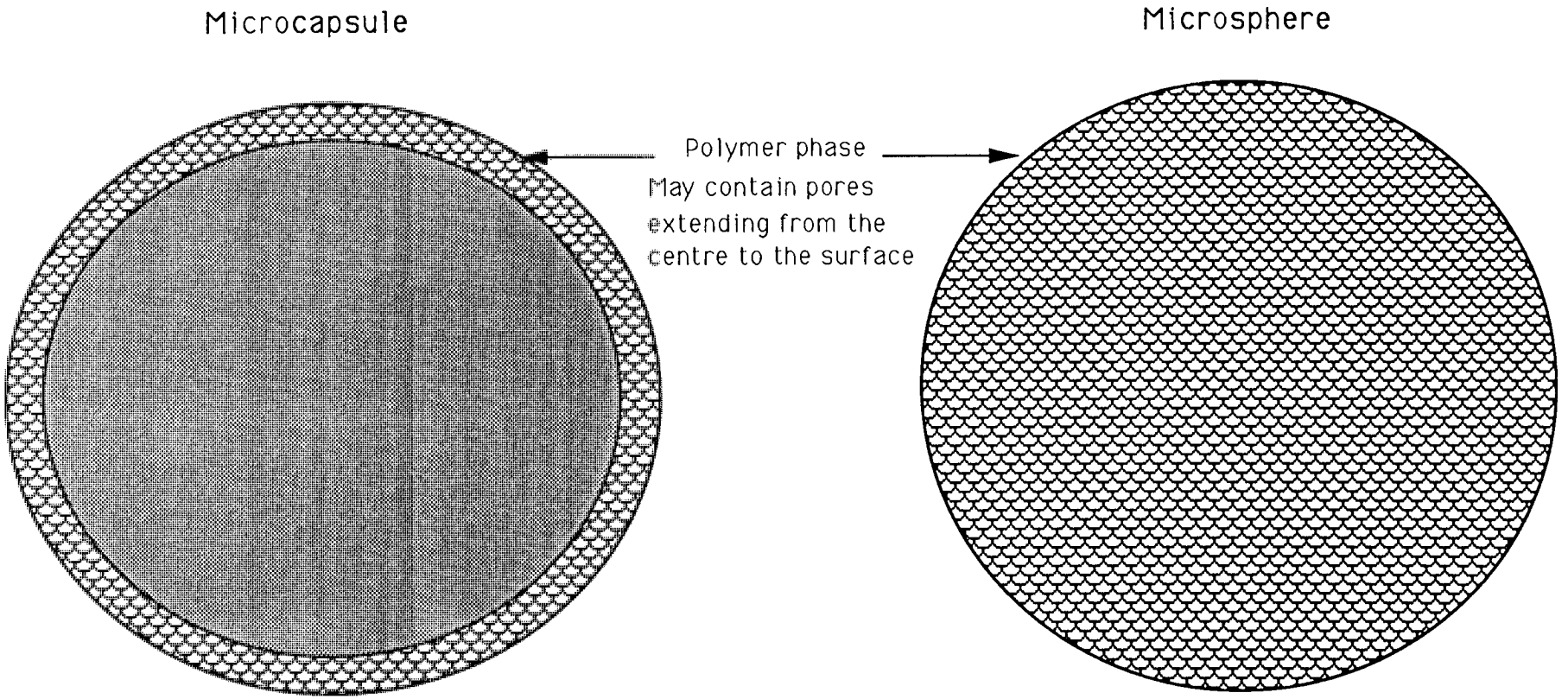
Naturally occuring biodegradable polymers such as collagen[8], fibrinogen[9], albumin[10], gelatin[11] and polysaccharides ie acacia (gum arabic)[12] have been used to produce microparticles. However, there are several problems, such as uncertain purity, associated with the use of these polymers as medical devices[13]. Attempts to use reconstituted collagen as a vehicle for the delivery of pilocarpine or medroxyprogesterone resulted in non reproducible and inconsistent release rates[14]. Therefore attention has been focused on the utilisation of synthetic biodegradable polymers, where the

processing conditions, availability and cost can be more efficiently controlled. There is an enormous number of potentially biodegradable materials available under the broad heading of synthetic polymers. These include poly(amines) eg polycapsolactam, poly(orthoesters), eg polyorthoformate, poly(alkyl-α-cyanoacrylates), poly(α β esters), eg.poly(lactic acid) and poly(ethylene adipate). Poly(α, β esters) have the advantage of being both biodegradable and biocompatible in so far as they biodegrade to toxicologically acceptable products which can easily be metabolized or eliminated from the body[15]. Poly(α, β esters) are the most commonly used biodegradable polymers for the manufacture of microparticle controlled release devices. Poly(α esters) include Poly(glycolic acid) [PGA][16], poly(lactic acid) [PLA][17,18,19,20,21], poly(glycolic-co-lactic acid) [PLCG][18,20,22,23]. However, due to the presence of a chiral centre, there are three possible forms of PLA, which are those deriving from the optically active D(-) and L(+) forms of the parent acid, together with the racemic D,L form. These are respectively abbreviated to P(D-LA), P(L-LA) and P(D,L-LA) in this thesis. Poly (β esters) include poly-ε-caprolactone (PCL)[24,25], poly-β-hydroxybutyrate[26], and copolymers of the latter with 3-hydroxyvalerate P(HB-HV)[26,27].

Under the broad heading of microparticulate drug delivery vehicles may be included nanoparticles (microemulsions, nanospheres and nanocapsules)[28], liposomes[29], and microparticles (microspheres and microcapsules)[30]. Liposomes have and continue to attract enormous attention commercially. They are spherical vehicles which are prepared with diameters ranging between 300 Angstroms to 1000 Angstroms[31]. They are composed of phospholipid bilayers and have the ability to carry both hydrophilic and

hydrophobic species[32]. Being composed of biocompatible materials, they initially appeared to be promising polymeric delivery devices. Unfortunately, they present significant problems, including low encapsulation efficiency and structural instability and weakness. Furthermore they are difficult to stabilize during storage and cannot be sterilized. Nanoparticles are far more stable than liposomes and generally consist of polymeric material into which a drug is either dissolved, entrapped or bound to the surface[28]. Microparticles [microspheres and microcapsules] are usually 1 to 1000μm in diameter and appear to have no clear definition in the literature. Jalil and Nixon describe monolithic particles (microspheres) as microcapsules[17], while Ogawa, Yamamoto, Okada, Yashiki and Shamamoto describe monolithic particles as microcapsules, but closer examination of their work reveals that they are actually referring to reservoir type devices with aqueous based centres surrounded by a polymer membrane[33]. More-over, Bodmeier, Chen and Tyle describe the preparation of both monolithic polymer particles and reservoir type devices, but apply the term microsphere to each type of particle[34]. In contrast, Tsai, Havard, Hogan, Malanga, Kandzari and Ma refer to monolithic polymer particles containing randomly distributed hydrocortisone as microcapsules[35], whereas Maulding uses the two terms synonymously[36]. For the purposes of this thesis microspheres are referred to as solid uniform (monolithic), generally spherical, polymer particles containing a randomly dispersed drug, figure 1.0[37]. Although this implies a solid polymer sphere, polyester microspheres prepared using single emulsion solvent extraction methods are sometimes macro-porous with honey-combed interiors. Microcapsules, on the other hand, are devices which consist of a non-polymer or aqueous core surrounded by a polymer membrane.

Figure 1.0. General structure of a microcapsule and microsphere.



This terminology is also adopted by Cha and Pitt[38], Bodmeier and McGinty[19], Dubertnet, Benoit and Couraze and Duchene[25].

In relation to other forms of drug delivery, microparticles have gained in popularity over the last decade for various reasons. In the case of parenterally administered systems, these reasons include ease of administration, where injection can replace surgical implantation. With regard to oral dosage forms, microparticulate systems distribute more uniformly in the gastrointestinal tract, thereby resulting in a more uniform drug absorption. They also present fewer parochial adverse reaction, and ensure the avoidance of inadvertent intestinal retention on chronic dosing, when compared to single unit dosage forms[34,39]. Additionally, the products of microencapsulation offer opportunities for targeting to specific body sites. One of the simplest methods of targeting microparticle drug delivery devices to their intended sites of action is by direct administration. One illustration of this is the process of chemoembolization whereby microparticles are injected into, and occlude, small blood vessels close to the target area. Direct administration has also been used to localize drug-loaded microparticles within the ocular environment[40] and the periodontal pocket[41]. However, a considerable quantity of drug delivery research currently centres on the concept of 'site-specific drug delivery' which refers for example to the development of microparticle systems that are able to direct themselves to specific targets within the body[42]. A critical factor in determining the effectiveness of microparticles that are intended for site-specific drug delivery relates to their interaction with the phagocytic cells of the reticuloendothelial system [RES]. This point is relevant to both oral systems and those which are injected directly into the bloodstream.

For example, orally administered microparticles can be taken up from the gastrointestinal tract as a result of phagocytosis by mucosal cells which line the lumen. It is therefore favourable that ingested microparticles are readily phagocytosed by these cells if they are to reach the target tissues in the gut area such as the Peyer's patches[43]. In contrast, if microparticles that have been injected directly into the bloodstream are recognized as foreign by the phagocytic cells of the reticuloendothelial system they are likely to be totally removed from the circulation within a few minutes[44]. Clearly therefore, unless the reticuloendothelial system is actually the target, it is essential that these microparticles are not phagocytosed before they reach their site of action. In addition microparticles injected into the circulation cannot be greater than 2-5μm in size if they are not to occlude in the small arterioles of the lungs. Eldridge and co-workers administered microspheres prepared from various polymers to mice, and found that the uptake of polymer particles by the mucosal cells lining the gut was dependant on particle size and hydrophobicity[43]. Microspheres in the 1-10μm size range, made from hydrophobic polymers such as poly-β-hydroxybutyrate, were the most readily phagocytosed. Similarly, Davis and Illum have emphasised the importance of surface charge and hydrophilicity in relation to the removal of microparticles from the circulation by phagocytic cells of the reticuloendothelial system[45]. Moreover, these workers have actually demonstrated that microparticle sequestration by the RES can be prevented via the application of hydrophilic coatings based on the poloxamer and poloxamine series. This procedure also offers some degree of target selectivity, for example microparticles coated with poloxamine 908 remain for extended periods in the circulation whilst those coated with poloxamer 407 are directed to bone[45]. It was suggested that

the attachment of a homing ligands to the surface of microparticles provided opportunities for more sophisticated site specific targeting[45]. However, the development of microparticulate delivery devices, specially coated, for *in vivo* targeting purposes and which also provide therapeutically useful release kinetics, is likely to be a complex exercise. The work in this thesis has concentrated on the use of poly(α,β esters) for the fabrication of microspheres and microcapsules and their use in the controlled release of proteins and antibiotics.

1.1 The incorporation and release of macromolecules from a polymeric matrices.

In recent years there has been an exponential increase in the production of genetically engineered biologically active proteins with high specific activity potentially available for therapeutic applications. Included in these are peptide hormones, releasing hormones, antibodies, cytokines, tissue growth factors and antibiotics[46]. The production of these materials has only served to highlight the immense difficulties associated with the delivery of such macromolecules to target tissues. Thus oral administration is ineffective either as a result of proteolytic activity in the gastrointestinal tract, or by poor absorption through the intestinal wall. In addition, vaginal[46–51], rectal, intranasal[52] and buccal[53] administration are all associated with low and variable bioavailability[47]. Therefore intravenous administration is the usual method employed, but this too has drawbacks including the need for frequent injections, together with constant under and overdosing caused by the inability to maintain constant idealized plasma concentrations. In addition, these macromolecules generally have

very short elimination half lives[47]. To exploit the full potential of these therapeutic macromolecules novel delivery devices must be generated which will protect them from degradation in the *in vivo* environment of the body whilst allowing release at a controlled therapeutically significant rate close to the site of action.

Many problems are encountered in incorporating large macromolecules into polymeric devices. Many of these are a reflection of polymer-polymer incompatibility phenomena, ie interactions between the polymer matrix and the incorporated macromolecule. The mutual incompatibility which is normally exhibited by two polymers means that homogenous distribution is difficult to achieve and solubility problems arise with higher molecular weight macromolecules (>1500)[6]. Thus the incorporation of macromolecular drugs into polymer matrices frequently requires the difficult combination of an aqueous solvent system and an organic solvent system, with further complications arising from drug hydrolytic and thermal instability as a result of raised temperature and an acid or alkaline environment. Furthermore chemical reactions may occur between the bioactive agent and the fabrication polymer. It has been reported that amines such as meperidene, methodone and promethazine accelerate the hydrolysis of poly(L-lactic acid) microspheres[15]. The enhanced decomposition of peptides resulting in a loss of biological activity has been reported to be caused by carboxylate groups formed by partial hydrolysis of poly (α ester) matrices[32].

Controlled sustained release from a polymeric matrix is far more difficult to achieve with macromolecules such as proteins and high molecular weight molecules than it is with conventional low

molecular weight drugs. A typical poly (α,β ester) matrix, above the glass transition temperature, shows both ordered (crystalline) regions and discontinuous (amorphous) regions. Most low molecular weight drugs are poorly soluble in the polymer phase and are unable to penetrate the crystalline regions. Therefore permeation of the drug through the polymer occurs via gaps created in the discontinuous amorphous region of the matrix. Diffusivity is very sensitive to the size of the penetrating molecule. Thus, whereas conventional drugs, when entrapped within the amorphous regions of the polymer above the glass transition temperature may escape by partition dependant [or Fickian] diffusion through the polymer matrix, this is not an option usually open to macromolecule species[47]. Holland *et al* have described four possible mechanisms for the release of macromolecules from polymeric matrices : Diffusion controlled release through swollen colloids; macromolecule discharge by aqueous diffusion, with the consequential emergence of matrix porosity; release by diffusion following the erosion of cross linked polyester hydrogels and release as a consequence of the erosion of α.β esters[6].

In general the release of a macromolecule from polyester matrices can only be effected by the leaching of the entrapped species into the dissolution medium. This leaching process is dependant on the temperature, pH, type of dissolution media and solubility of the drug in the penetrating medium[54]. For macromolecules not at the surface of the polymeric device, release may occur as a result of the dissolution media gradually penetrating through the aqueous pores or channels which are typically generated *in situ* as mentioned above, either by macromolecule leaching, followed by bioerosion of the polymer matrix or by a combination of

the two. This basically describes the mechanism for macromolecule release from monolithic microspheres although biodegradation can also play a part depending upon the rate of biodegradation of the fabricating polymer. Holland and colleagues found that polymer hydrolysis facilitated the release of an entrapped drug from a poly(α,β ester) matrix by a mechanism related to biodegradation [ie a molecular weight reduction and a diminution in the level of crystallinity] and/or bioerosion [ie physical breakdown of the polymer matrix and the generation of porosity]. In the former case, partition dependant diffusion led to macromolecule release, since diffusion coefficients were expected to increase as the molecular weight and chain entanglements of the polymer decreased. Alternatively, bioerosion related drug release typically involved either the diffusion of the active species through water filled pores which arose from the elution of solubilised low molecular weight polymer fractions into the dissolution media, or by a total collapse of the polymer matrix[38].

In terms of the controlled release of macromolecules zero order kinetics (a constant non time dependant rate) for the release of macromolecules or conventional drugs from polymeric matrices is rarely achieved. Pseudo zero-order release has been achieved for both monolithic and swellable hydrogels by alteration of the matrix geometry[38]. Cha and Pitt obtained near zero order kinetics for the release of L-methodone from poly(α,β ester) microspheres by combining different microsphere formulations[38]. However, the release kinetics for both macromolecules and conventional drugs from monolithic microspheres usually follows a square root of time dependence as described by the Higuchi models of release[56,57]. Reservoir type microcapsules on the other hand offer the possibility

of zero order release characteristics, provided, the membrane of the microcapsule remains intact and unchanged during release, the movement of core molecules through the polymer shell is the rate limiting step in the dissolution process. The concentration of core macromolecules inside the limiting shell surface remains constant (this condition is fulfilled when there is a saturated solution of the core material inside the shell and an excess of solid core material is available to dissolve) and all of the microcapsules in a dose have similar release rates and macromolecular drug loads[54].

1.2 Techniques for the microencapsulation of macromolecules using poly(α,β esters).

There are numerous techniques for microencapsulation described in the literature. The choice of technique often depends on the type of polymer being used and the core material to be microencapsulated. The preparative technique will have a profound effect on the morphology, particle size and release profile of the microencapsulated material. Therefore when choosing a preparative technique it is important to take into consideration the intended end-use of the microparticle whilst keeping in mind factors such as the batch reproducibility; scale up efficiency; sterilisation; *in vitro* and *in vivo* toxicology and pharmaceutical efficiency.

Li, Kowarski, Feld and Grim have divided microencapsulation techniques into four basic groups:- mechanical methods; electrostatic methods; phase separation or coacervation (including emulsification techniques) and interfacial polymerization methods[58]. However, the work in this thesis has involved exclusively microencapsulation with poly(α,β esters), therefore

detailed attention has only been directed towards those techniques which are suitable for use with these materials.

Electrostatic methods of microencapsulation are intrinsically rare, and involve mixing the microencapsulating polymer and the core material when both are aerosolized. Each phase must be liquid during the encapsulation stage and the fabricating polymer must be capable of surrounding the oppositely charged core material[58].

In contrast to the electrostastic methods, interfacial polymerisation has been widely applied as a microencapsulation technique. Here, microencapsulation is achieved via the polymerization and precipitation of a monomer at the interface of two immiscible liquids. Thus, a monomer-containing dispersed phase is emulsified into a continuous phase until the desired particle size is reached, at which point a cross linking agent is added[58]. Arshady has listed several different types of interfacial polymerisation : suspension; emulsion; dispersion; precipitation and bimodal [referring to combinations of the previous five][59]. Nevertheless, microencapsulation with poly(α,β esters) is more typically carried out using mechanical or phase separation processes.

1.2.1 Mechanical methods.

A considerable number of mechanical methods used for the process of microencapsulation have been described in the literature and most of these utilize special equipment. Li *et al* have produced a comprehensive review of microencapsulation technologies and

equipment including spray drying, spray congealing, pan coating, spheronisation, fluidised bed coating and multiforce-centrifugal processing[58]. Of these the most widely reported mechanical method is spray-drying which has been used to form monolithic microspheres from a variety of poly(α,β esters), including poly(D L, lactic acid) and poly(β-hydroxybutyrate)[60,61]. The process involves the dispersion of a core material in a coating solution which contains the dissolved coating substance. This dispersion is atomised by forcing it through a fine nozzle into a stream of heated air. The heated air provides the latent heat required to remove the solvent from the coating material and so form hardened microparticles. The microparticles are generally spherical and range from 5-600um in size[58]. Bodmeier and Chen encountered problems with sphericity[42]. They used spray drying to encapsulate the relatively small molecules progesterone, theophylline and caffeine with poly(lactic acid). Although an increased encapsulation efficiency was obtained for progesterone using spray drying, compared with solvent evaporation methods, fibrous strands were produced rather than microdroplets before the casting solution was eluted from the nozzle of the equipment. The production of fibrous strands was thought to be dependant on the nature of the polymer. These findings were consistent with work carried out by Aktar[61]. He attempted to produce microspheres with poly(β–hydroxybutyrate) using the spray drying method and found that the use of high molecular weight P(HB-HV) polymers gave rise to a poor quality product. The use of low molecular weight polymer resulted in the production of a fine, free flowing powder. Aktar concluded that non-film forming polymers were the most suitable for spray drying, since a high degree of chain entanglement made the formation of microdroplets practically impossible. Although spray drying has the

advantage of being simple and reproducible, the regulation of important functional parameters such as the rate of release of encapsulated material via intentional changes in particle morphology are very limited. Spray drying also involves the use of temperatures well in excess of 37°C, which limits its usefulness for encapsulating heat sensitive macromolecules such as polypeptides.

1.2.2 Phase Separation Techniques.

In general, microencapsulation by phase separation is achieved by polymer precipitation, as a consequence of either non-solvent addition or solvent evaporation. Although in some instances both mechanisms may play a role[62]. Vidmar, Smoloic, Bubalo and Jalsenjak have used phase separation by non-solvent addition to microencapsulate water soluble oxytetracycline HCL[63]. They suspended the drug in a solution of poly(lactic acid) in dichloromethane and induced phase separation by the addition of n-heptane under constant stirring. However, the product consisted of highly aggregated microcapsules.

A similar technique has been employed by Sanders, Kent, MacRae, Vickery, Tice and Lewis to encapsulate nefarelin acetate, a water soluble LHRH analogue[64]. Poly(D, L lactic co glycolic acid) microcapsules were generated using a non-solvent addition microencapsulation technique in which a water in oil [W/O] emulsion was formed by combining an aqueous solution of the drug and a solution of the co-polymer in methylene chloride. An unspecified non-solvent was added to the initial W/O emulsion to initiate

polymer precipitation. In order to extract residual methylene chloride and thus microcapsules the non-solvent plus emulsion was added to a larger volume of non-solvent. In theory, microparticles generated by this process would be reservoir devices or microcapsules consisting of an aqueous solution of drug encapsulated within a polymer membrane. However, no information was provided on the ultrastructure or surface morphology of the microparticles[64]. In 1987, Gardner filed a U.S. patent for a phase separation microencapsulation process that was claimed to result in the production of reservoir type microcapsules from homo and copolymers of glycolic and lactic acid[62]. This process was partially based upon the concept of non solvent addition, but it also utilized solvent evaporation as a means of inducing polymer precipitation. This hybrid process involved dissolving the encapsulating polymer in a mixture of two miscible organic liquids, one of which was a solvent for the polymer, whilst the other was a non solvent, the solvent has the higher vapour pressure. The two liquids were employed in a ratio such that the resulting polymer solution was very close to its phase separation or cloud point. An aqueous solution or suspension of the material for encapsulation was then emulsified into the organic phase. Vigorous agitation was required to create the emulsion and thereafter to maintain it whilst solvent/non solvent evaporation was allowed to proceed. The lower vapour pressure of the solvent ensured that the proportion of non solvent in the solvent/non-solvent mixture increased as a result of solvent/non-solvent evaporation. This, coupled with the fact that the polymer solution was initially close to its cloud point, ensured that phase separation, and thus polymer precipitation, occurred rapidly. The phase separated polymer then migrated to the surface of the aqueous microdroplets and began to microencapsulate them.

When encapsulation had been completed and the solvent evaporation phase was over, non solvent addition was utilized to effect the hardening of the microparticles.

The technique of emulsification with solvent evaporation is one of the most extensively used methods of microencapsulation and the most commonly employed with poly(α,β-esters). poly(D, L-lactic acid)[19,65,66,67], poly(L-lactic acid)[17,18,38,68], poly(β–hydroxybutyrate)[69,70], poly(glycolic acid)[16] and poly-ε–caprolactone[24,25] have all been employed as fabricating materials. Depending on the fabrication technique chosen a reservoir type microcapsule or a monolithic microsphere can be generated. A double emulsion technique (W/O/W) with solvent evaporation can be used to generate microcapsules, while a single (O/W or W/O) emulsion technique with solvent evaporation can be used to generate microspheres. The choice of whether a O/W or W/O single emulsion technique is used to form microspheres ultimately depends upon the aqueous solubility of the macromolecule to be microencapsulated.

The most commonly reported technique for the microencapsulation of drugs with little aqueous solubility is the single emulsion solvent evaporation process, O/W system[71]. This technique has been used to encapsulate norethisterone, local anaesthetics[72], bleomycin sulphate[73], lomustine and progesterone[66], quinidine sulphate [19] and many others. The technique involves dissolving the biodegradable polymer and the material to be entrapped in a chlorinated solvent, usually dichloromethane. This organic phase is then emulsified (using a high shear speed) with an aqueous phase to form a stable emulsion. The solvent is removed by evaporation at an elevated temperature, thus leaving microspheres.

A wide range of preparative variables can affect the nature of the final product. These include factors related to the nature of the polymer [such as polymer type, it's molecular weight and the polydispersity], the organic solvent system [such as the heat of evaporation, the level of water miscibility] and the drug [such as its solubility in each phase]. The polymer concentration; the drug loading; polymer organic solvent-drug-aqueous phase interactions; the dispersed to continuous phase ratio; the type and concentration of the aqueous phase emulsifier; the physical emulsification process; the temperature and pressure and the solvent evaporation period may also play a role in determining the nature of the final product. Several workers have reported a relationship between the viscosity of the organic dispersed phase and the size of the resulting microparticles. Thus, Spenlehauer, Vert, Benoit, Chabot and Veilland noted that an increase in either the drug or the polymer concentration enhanced the mean diameter of cisplatin loaded poly(D,L-lactic acid) microspheres[65]. Wakiyama and colleagues reported that increasing the molecular weight of poly(D,L lactic acid) increased microsphere diameter[74]. The polymer molecular weights used in their investigations ranged from 9,100 to 25,000, which implied intrinsic viscosity values in benzene at 30°C of between 0.32 and 0.7 $Kgm^{-1}s^{-1}$. Methylene chloride was utilized as the dispersed phase solvent, and three different drugs were separately encapsulated. Butamben-loaded microspheres prepared using the lowest molecular weight polymer had a mean diameter of 50.6μm, but this increased to 60.2μm when the molecular weight of the fabricating polymer was raised to 25,000. In contrast, when either of the local anaesthetics dibucaine or tetracaine was used as the encapsulated species, microspheres prepared from the lowest

and highest molecular weight polymers had diameters that were not significantly different[74].

In the O/W process for the fabrication of microspheres, the presence of a surfactant in the continuous phase is required to stabilize the initial O/W emulsion and to subsequently prevent the microspheres from agglomerating. The hydrophilic colloid most commonly employed as a continuous phase surfactant in microencapsulation work with poly(α,β esters) is poly (vinyl alcohol) (PVA) which may be used either alone or in combination with other colloids such as methylcellulose[65,67]. The work of Cavalier, Benoit and Thies with hydrocortisone loaded poly(lactic acid) microspheres clearly demonstrates the important influence that the continuous phase surfactant system can have on microsphere formation and particle shape[67]. Thus, when 0.27% PVA was exclusively used as the aqueous phase emulsifier, spherical microspheres were produced at drug : polymer ratios of below 0.4. However, increasing the drug : polymer ratio to 0.56 resulted in the production of misshapen particles, whilst at drug : polymer ratios exceeding 0.6%, microsphere formation was not possible due to the instability of the emulsion and the consequent production of large aggregates. The use of 400cps grade methycellulose, at concentrations ranging from 0.05% to 0.3%, instead of PVA, as the aqueous emulsifier facilitated the production of what appeared to be largely oval shaped microspheres at drug : polymer ratios ranging from 0.6 to 1.0. It was concluded that methylcellulose-400 generated a stable emulsion, but that the high solution viscosity prevented the formation of spherical particles. However, a combination of 0.05% methylcellulose-400 and 0.27% PVA in the continuous phase led to the production of spherical particles at

these drug : polymer ratios. The rheological properties of this mixture were therefore judged to be superior to those of the individual colloids. Moreover, a less viscous form of methylcellulose [10cps grade] resulted in the production of spherical particles at drug : polymer ratios exceeding 0.6 with or without the presence of 0.27% PVA[67].

In order to improve the encapsulation efficiency of water soluble compounds into biodegradable polymer systems Tsai, Howard, Hogan, Malanga, Kandzari and Ma used a W/O emulsification technique with solvent evaporation[35]. This technique generated microspheres containing up to 13.8% by weight of mitomycin c. The W/O emulsion system employed consisted of light mineral oil plus Span 65 as the continuous phase and mitomycin c in PLA/acetonitrile as the dispersed phase. The emulsion was stirred for 25 minutes at 55°C under vacuum to remove solvent. Jalil and Nixon used a modification of this technique to compare the core loading, particle size, ultrastructural and gross morphology of microspheres prepared by both the O/W and W/O emulsion systems[17]. In the W/O system the dispersed phase consisted of acetonitrile, mitomycin c and poly(L-lactic acid) while light mineral oil and Span 40 rather than Span 60 was used as the continuous phase. The use of span 40 resulted in the fabrication of smaller microspheres than those fabricated with span 60. Unlike Tsai et al[35], Jalil and Nixon washed the microspheres in petroleum ether (40-60°C) rather than mineral oil. The O/W emulsion system used by Jalil and Nixon was a modification of a previous method used by Beck and colleagues to encapsulate progesterone[71]. Jalil and Nixon obtained an encapsulation efficiency of 17.78% for phenobarbitone using their O/W emulsion system. As expected saturating the

continuous phase with phenobarbitone, improved the encapsulation efficiency based on the theoretical drug loading to 37%. By contrast the W/O system employed was characterised by a high encapsulation efficiency. 92.24% of the theoretical phenobarbitone loading was encapsulated leaving a few crystals of phenobarbitone in the continuous phase. However, whereas microspheres generated by the O/W system possessed "good surface characteristics", the W/O microspheres had phenobarbitone crystals deposited on their surface and were slightly irregular in shape[17]. Bodmeier and co-workers have acknowledged that water soluble drugs are most efficiently microencapsulated within polymers such as poly(α,β esters) using non-aqueous (W/O) emulsion solvent evaporation[75]. However, these workers also suggested that an aqueous continuous phase was beneficial, both financially in the product clean up process and for the recovery of the continuous phase, during fabrication. Bodmeier and colleagues have also evaluated the double emulsion with solvent evaporation technique and have suggested that the partitioning of water soluble drugs into the aqueous phase may be reduced by the presence of an organic polymer solution which would form a physical barrier between the inner aqueous phase and the aqueous continuous phase, thus increasing the encapsulation efficiency of the microcapsule[75]. Using poly(D, L-LA) as the microencapsulating polymer, Bodmeier and colleagues demonstrated that the W/O/W emulsion system generated microcapsules containing more than twice as much of the mixed-action adrenergic agonist pseudoephedrine HCL as microspheres prepared using an O/W emulsion[75]. The seminal work on W/O/W emulsification with solvent evaporation was carried out by Vranken and Claeys and their technique is documented in U.S. patent 3523 906[62]. Ogawa and coworkers described the microencapsulation of

the hydrophilic peptide leuprolide acetate within poly(D, L-lactic acid) and poly(glycolic-D,L-lactic acid) microcapsules using a similar technique, based on U.S. patent 4652 441[30].

1.3 The application of microencapsulation for the delivery of macromolecules.

Whilst microencapsulation has found applications in a number of industries including computing[76]; horticulture; agriculture; fish farming[77]; printing[78]; aerospace[32] and the pharmaceutical industry[21], undoubtedly the most important role for microencapsulation, in the pharmaceutical industry, is in the field of the controlled and sustained delivery of macromolecules. The potential applications for biocompatible controlled and sustained delivery devices for therapeutically useful macromolecules are endless. One such application and one which has formed part of the work in this thesis is the developement of a device for the delivery of specific antibiotics for use in orthopaedic surgery. Total hip replacement (arthroplasty) was first performed in 1938 by Dr Wiles, who used a stainless steel prosthesis. The use of a high molecular weight polyethene acetabular cup and a stainless steel femoral component was initially accredited to Charnley[79]. The whole area of total hip replacement is a dynamic one. New designs, fabrication materials, adhesives and improved surgical procedures are constantly being sought as the number of hip replacement operations carried out each year continues to increase. As with all invasive surgery there are various complications associated with total hip arthroplasty. These include thromboembolism, dislocation, loosening, fracture or perforation of the femur during the operation, ectopic bone formation, stress induced osteoporosis, cancer and

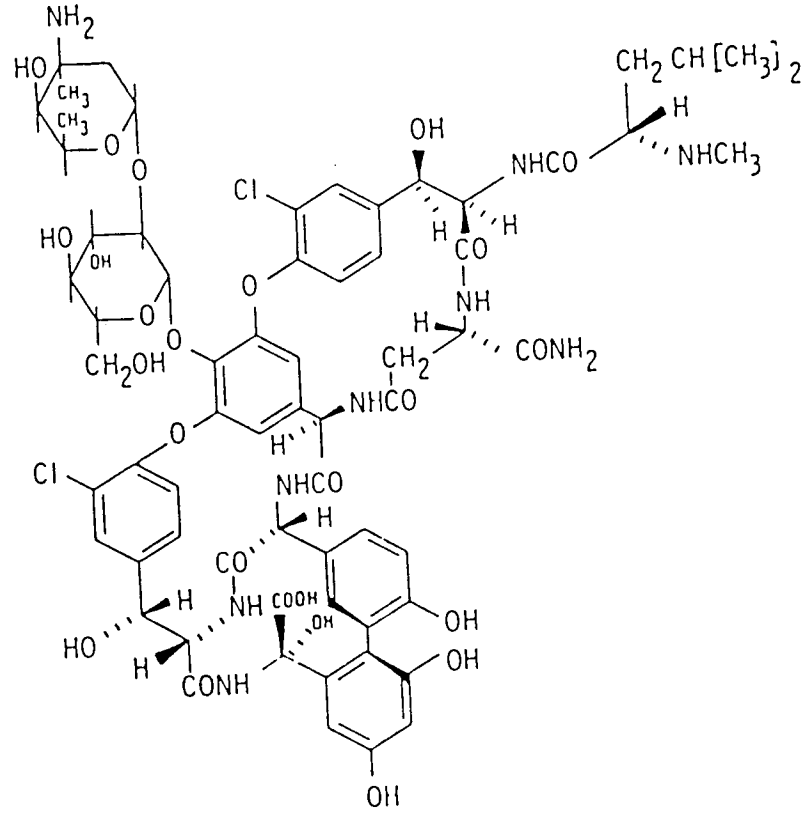
probably the most common malady microbial infection. Established infection continues to represent a significant morbidity with respect to hip joint replacement and the management of open fractures. The localised delivery of certain antibiotics perhaps biofilm specific, from an injectable/implantable biodegradable microparticulate vehicle offers greater advantage over currently available forms of systemic and topical delivery and has particular application in the management of infection in endoprosthesis and osteomyelitis. In addition biodegradability of the microparticulate will render retrieval surgery unnecessary and make the procedure economically attractive. Levels of infection vary depending on the precautions taken during the surgical procedure e.g the use of total body exhaust systems, exposure to ultra violet light, double gloving, use of clean air. *Staphylococcus aureus, staphylococcus albus; streptococci spp; Escherichia coli* and *Pseudomonas* [80,81] are the most common infecting organisms. Although generally more than one organism is responsible[82]. Most infections are the direct result of the invasion of infecting organisms at the site of the operation, i.e. from patients skin, surgical staff and wound bandages. If a long period elapses between the operation and the onset of the infection the source may be a bacteriaemic one. Post-operative infection after total hip arthroplasty or prior to replacement, especially in aged patients is usually life threatening. Infection is usually painful, disabling and extremely costly. Mortality due to infection is reported to be between 7 and 62%[82]. Three remedial treatments for infection are usually followed. Firstly, antibiotic treatment, secondly, incision and drainage where the infected tissue and scar tissue is debrided and the cavity washed with an antibiotic. The third, final and most drastic solution is revision surgery. This involves the partial or complete removal of the prosthesis, possibly

followed by replacement with a new prosthesis. However, replacement cannot be effected until residual infection is removed and this involves the extended hospitalisation of the recipient and great cost to the health service. One approach is to use antibiotic impregnated bone cement in both primary and revision surgery to lower the incidence of infection[83]. Antibiotics that have been used include penicillin, gentamicin, erythromycin, tetracycline, tobramicin, methcillin, clindamycin, cephalothin, lincomycin, bacitracin, dicloxacillin and oxacillin. The antibiotic can also be administered locally to the joint space in the postoperative period when infection is most likely. When added to the bone cement the antibiotic diffuses out over a period of days and lies in close contact to the important bone/cement interface. Buchholtz and colleagues have reported a significant fall in the incidence of infection from 5% to 0.8% when using gentamicin impregnated bone cement[83]. Lynch *et al* have compared plain and gentamicin impregnated cement in both primary hip replacement and revision surgery and found that in primary surgery the infection rate did not significantly change while in revision surgery the infection rate fell from 3.46% to 0.8%[84]. There is, however, a ground swell of opinion that gentamicin impregnated bone cement is probably not the best method for the prevention of infection in hip replacement. Hope and co-workers noted that significant numbers of patients undergoing revision surgery, as a result of coagulase negative staphylococcal infections, had been given gentamicin in the bone cement at the time of their primary surgery[85]. It appeared that the concentration of gentamicin released from the bone cement was not sufficient to destroy the bacteria at the bone cement interface, and once all the gentamicin had been released the bacteria were able to flourish. The use of gentamicin impregnated bone cement is therefore a very

crude approach to the control of bacterial infection in orthopaedic surgery. A more attractive strategy is to use gentamicin impregnated polymethylmethacrylate (PMMA) beads[85]. These beads are still routinely used during incision and drainage and during delayed prosthesis replacement. The infected prosthesis is removed and gentamicin-PMMA beads are packed into the bone space to eradicate infection before a new prosthesis is fitted. As with impregnated bone cement it is now generally recognised that gentamicin-PMMA beads do not release sufficient gentamicin over time to eradicate all potential infections. Furthermore, in recent years gentamicin resistant micro-organisms such as *Psuedomonas aeruginosa* have become more prevalent, especially in hospital environments[86]. Gentamicin is a broad spectrum antibiotic that has the ability to inhibit protein synthesis in susceptible, principally gram negative, bacteria. It does this by diffusing through aqueous channels formed by proteins in the outer membrane of bacteria and thereby enters the periplasmic space. By a mechanism of permeation, driven by a membrane potential, gentamicin passes across the cytoplasmic membrane and binds to ribosomes resulting in the inhibition of protein synthesis. Gentamicin disrupts the normal cycle of ribosomal function by interfering, at least in part, with the initiation of protein synthesis and also by inducing misreading of the mRNA template. Bacteria may be resistant to the antimicrobial activity of gentamicin as a result of either the failure of the antibiotic to permeate the bacterial membrane, low affinity of the drug for the bacterial ribosome or inactivation of the drug by microbial enzymes. The last mechanism is by far the most important explanation for the acquired microbial resistance to gentamicin. Once the gentamicin has reached the periplasmic space it may be

altered by microbial enzymes that phosphorylate, adenylate,or acetylate specific hydroxyl or amino groups on the antibiotic. The genetic information for these enzymes is acquired primarily by conjugation and transfer of DNA as plasmids and resistance transfer factors. These plasmids have become widespread in nature and especially in hospital environments and code for a large number of enzymes that have markedly reduced the clinical usefulness of gentamicin. Indeed gentamicin resistant strains of *Staphylococci* emerge rapidly during exposure to the drug[86]. In some hospitals, the ward flora have undergone considerable alterations in susceptibility to antibiotics during the last 20 years, with a gradual increase in resistance to gentamicin. The relative frequency of these changes varies dramatically even in different units within a single hospital[86]. For these reasons attention is turning away from the use of gentamicin to combat infections associated with total hip arthroplasty and turning towards the use of other antibiotics such as vancomycin. Vancomycin hydrochloride (figure 1.1) is a complex and unusual tricyclic glycopeptide with a molecular weight of about 1500[87]. It is produced by certain strains of *Streptomyces orientalis* and although naturally a light brown powder it is marketed for intravenous use as a sterile white powder, of which 0.1g completely dissolves in 1ml of water[88]. A 5% solution has a pH of between 2.8 and 4.5 and the antibiotic can be identified and assayed by radial diffusion using *Bacillus subtilus* as the susceptible organism[87].

Figure 1.1 The structure of Vancomycin Hydrochloride.[87]



Vancomycin hydrochloride is poorly absorbed after oral administration and large quantities are excreted in the faeces. For parental therapy the drug is administered intravenously and a single intravenous dose of 1g in adults produces plasma concentrations of 15-30μg/ml 1 hour after a 1-2 hour infusion[88]. The drug has a half life in the circulation of about 6 hours with approximately 30% binding to plasma proteins. Vancomycin is primarily active against gram positive bacteria and it's antimicrobial action is a result of its ability to bind with high affinity to the precursors of the bacteria cell wall. The D-alanyl-D-alanine portion of cell wall precursor units appears to be a crucial site of attachment[89]. Furthermore Williams *et al* have shown that two vancomycin

molecules can bind together because their backs (pointing away from the cell wall binding pocket) are an exact match for, and can therefore recognise each other[90]. This dimerisation increases the affinity of vancomycin for the bacterial cell wall and results in the destabilisation of and eventual destruction of the outer fortification of the bacterium. The potential importance of vancomycin for the treatment of infection is reflected by its sales figures, approximately one billion American dollars per annum in the United States alone.

1.4. The use of biodegradable polymers in the fabrication of microparticulate delivery devices.

1.4.1 Biodegradation

The various definitions of biodegradation have been comprehensively reviewed by Holland and colleagues[6]. These authors, indicate that there is considerable confusion in the literature as to the meaning of the term 'biodegradation'. Taylor has defined "biodegradation" as the breakdown of the polymeric material of living organisms or their secretions[91], a definition which is similar to those held by Williams[92], Potts[93], Griffin[94] and Kopek and Ulbrich[95]. Gilbert, Stannet, Pitt and Schindler define biodegradation as the simple hydrolytic breakdown of polymeric materials[96], whereas Gilding, on the other hand, reports that the term biodegradable polymers is widely used to convey the meaning of any polymer that degrades in the human body[97]. Zaikov has considered the *in vivo* degradation of polymers in the terms of three degradation components, without giving a definition for

biodegradation *per se*[98]. The degradative components were water (diffusivity), salt content, together with pH of the physiological environment and enzymatic attack. In the present work the term 'biodegradation' will refer to hydrolytic, enzymatic or bacteriological degradation processes occuring in a polymer in a physiological environment. These processes do not necessarily proceed to a stage where the physical form of the polymer is altered[6].

The initial molecular weight of a polymer is undoubtedly the single most important parameter controlling biodegradation rate, with the rate of biodegradation increasing with decreasing molecular weight[6]. The crystallinity of the sample also exerts an influence over biodegradation[6]. High levels of crystallinity result in reduced rates of biodegradation and this is clearly demonstrated by the copolymer poly(D-(-)-3-hydroxybutyrate/hydroxyvalerate), Biopol[6]. As the proportion of Poly(hydroxyvalerate) increases in the copolymer the crystallinity decreases thereby increasing the rate of biodegradation[6].

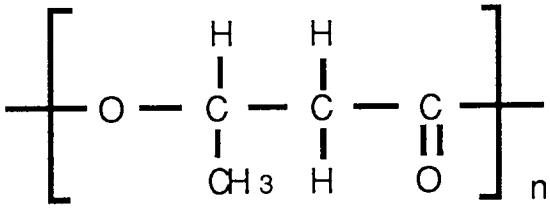
Physico-chemical factors also play a role in the rate of biodegradation of poly(α,β esters). For example, increased temperature and alkalinity speed up the rate of hydrolysis of P(HB-HV)[99]. Polymer processing can also play a part in determining the rate of polymer biodegradation. Yasin, Holland and Tighe found that melt processing reduced the molecular weight of P(HB-HV) 12% PHV from 390 $x10^3$ to 360 x 10^3 and injection moulding reduced this further to 190 x 10^3.[102]. Various fabrication forms of Biopol have differing stabilities to hydrolytic attack. Cold compressed tablets of P(HB-HV) have been shown to be less stable than solvent cast

films which in turn were less stable than injection moulded pieces. This is solely because the processing conditions used in the fabrication of the polymer have a marked effect on such properties as molecular weight and crystallinity of the material[100].

1.4.2 Poly(D-(-)-3-hydroxybutyric acid) [PHB] and copolymers with 3-hydroxyvalerate [P(HB-HV)]

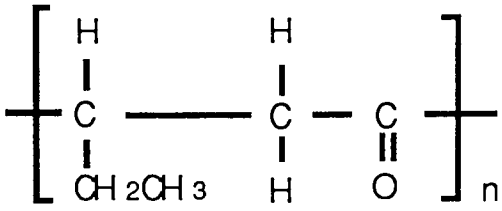
Poly-B-hydroxybutyric acid is an optically active alphatic polyester produced by different strains of the bacterium Bacillus megaterium[101], figure 1.2 below.

Figure 1.2 Poly(hydroxybutyrate) [PHB]



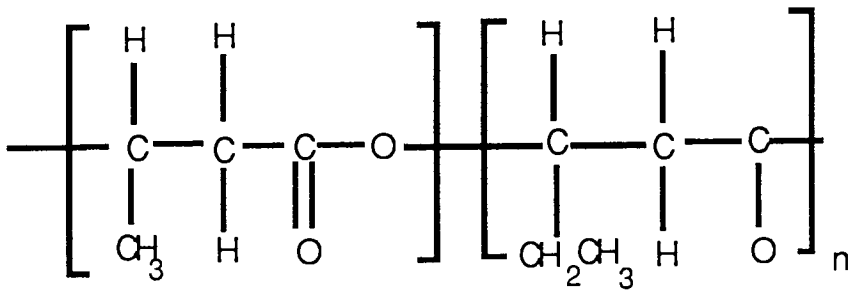
The polymer serves as an energy and carbon source and is stored in the bacterium as highly crystalline hydrophobic granules 0.2 to 0.7 μm in diameter. Although Lemoigne was the first to isolate PHB from the cells of Bacillus megaterium in 1925[101], only recently has it been possible to obtain this polymer in commercially significant amounts. I.C.I. manufacture PHB from *Azobacter beijeinckii* cultures[61,102,103] and these bacteria can also be manipulated to synthesize a range of PHB copolymers with polyhydroxypentanoic acid (which is more commonly referred to as polyhydroxyvaleric acid PHV), figure 1.3.

Figure 1.3 Poly(hydroxyvalerate)[PHV]



Copolymers of P(HB-HV) containing between 0 and 30% PHV are available under the trade name Biopol®[6]. The PHB/PHV copolymer (Figure 1.4) is less crystalline, more flexible and more readily processable than PHB alone. The addition of PHV has also been shown to increase the thermal stability of PHB[101].

Figure 1.4 Poly(hydroxybutyrate/hydroxyvalerate) P[HB-HV]



There is substantial evidence in the literature to suggest that the PHB homopolymer and its principle degradation products, D(-)-3-hydroxybutyrate, are well tolerated *in vivo*[104,105]. Work carried out by Korsatko and co-workers involving the subcutaneous implantation of PHB tablets into mice showed that the PHB homopolymer was biocompatible and toxicologically acceptable[104] . Similarly, Prouton, Kennedy, Notarlinni and Gould showed that the inflammatory response induced when PHB microspheres were injected intramuscularly into rats was due to the trauma of the injection

rather than PHB *per se*[105]. Indeed, little is known about the biocompatibility of P(HB-HV) copolymers in man. Although both monomers are normal physiological metabolites, there is some evidence to suggest that they inhibit cellular growth at high concentrations[105]. Prouton *et al* reported that the population doubling time of cultured mouse fibroblast cells was extended when exposed to D(-)-ß-hydroxybutyrate concentrations exceeding 10mg/ml [relative to control samples grown in a medium free of this monomer] and that growth was totally inhibited at levels above 25mg/ml[105]. However, this should not be a serious concern in relation to PHB based implantable delivery devices, as slow biodegradation and rapid metabolism of D(-)-ß-hydroxybutyrate should ensure that *in vivo* concentrations of D(-)-ß-hydroxybutyrate remain at tolerable levels. In addition, PHB is not yet licensed with either the U.K. or U.S.A. regulatory authorities for use as an internal medical device.

P(HB-HV) is chemically biodegraded by an ester hydrolysis mechanism[99,100,106,107]. After 500 days incubation at 37°C in an aqueous environment (pH 7.4), P(HB-HV) plaques have been reported to show losses of approximately 5% by weight of the polymer mass[55]. The rate of hydrolytic degradation of P(HB-HV) is reported to depend on copolymer ratio and composition, the degree of polymer crystallinity, polymer molecular weight and polymer processing conditions[99,100,107,108]. An increase in the percentage PHV content of P(HB-HV) has been shown to decrease the molecular weight of the polymer and to increase the rate of degradation. This is thought to be due to the decrease in polymer crystallinity that occurs with the addition of PHV[99]. The rate of chemical degradation has also been shown to be effected by temperature and pH[6,99,109,110]. Accelerated degradation has been demonstrated at 70°C compared with 37°C, and

an increase in the degradation rate has been seen with increased alkalinity. Degradation at elevated temperature and alkalinity shows the same characteristics as can be seen under conditions of physiological temperature (37°C) and pH (7.4) but within a condensed time scale which is useful experimentally[99]. The hydrolytic biodegradation of P(HB-HV) occurs in two distinct phases. The first phase is surface erosion of the polymer due to random chain scission. An increase occurs in the concentration of hydroxyl and carboxyl groups at the polymer surface as a consequence of ester hydrolysis at the polymer-water interface. These changes have been measured as changes in the surface energy and are represented as a pitting of the polymer surface[99,107,110]. During surface erosion an increase can be seen in polymer surface rugosity and pores or micropores can be seen to develop. These characteristics have been quantified using goniophotometric scattering and microscopy techniques. A small amount of dry polymer weight loss can also be detected as a result of the surface erosion[99,110].

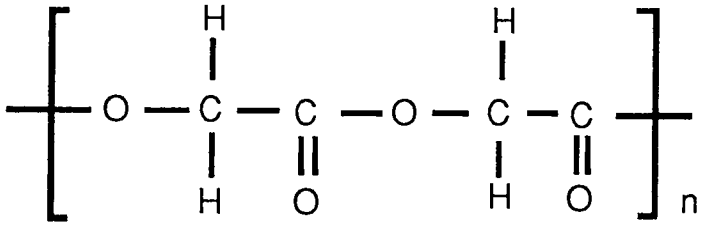
The second phase in the chemical hydrolytic degradation of P(HB-HV) is bulk polymer erosion. This occurs as the products of chain scission diffuse out from the amorphous regions of the polymer matrix through the pores created by surface erosion. Initially, only low molecular weight products are able to diffuse out. However, as the matrix becomes progressively more porous, loss of high molecular weight products occurs. This is seen as a dramatic loss in polymer dry weight as the amorphous region is eroded[3,8,107,110]. From these observations it is clear that the *in vivo* degradation of unfilled microparticulates composed of P(HB-HV) will be predominantly by ester hydrolysis. It is also possible that contributions to degradation may be made by low level esterase

activity[110]. The majority of macromolecule release, (bovine serum albumin) from loaded PHB/PHV microspheres has been reported to occur during random chain scission of the polymer matrix, prior to any significant polymer weight loss due to bulk erosion[107].

1.4.3 Poly(glycolic acid) [PGA]

The first synthetic absorbable sutures were made from poly(glycolic acid) and have been available commercially for many years under the trade name of Dexon®. PGA (see Figure 1.5) is the most hydrophilic of the polyesters, with a high molecular weight (>20,000) and high crystallinity which gives Dexon sutures greater tensile strength than either cat-gut or silk[6]. The high crystallinity of PGA not only controls the mechanical properties of the polymer but also its biodegradation characteristics.

Figure 1.5 Poly(glycolic acid) [PGA]



A series of biodegradation studies has been conducted on PGA (mostly in the form of Dexon sutures) by Chu[111–116]. Chu described *in vitro* degradation as a two stage process in which, initially, from day 0 to 21, water moves into the amorphous regions of the polymer causing random hydrolytic chain scission of the ester groups. As there is a degrading of the amorphous regions, crystallinity increases. The second stage takes place from day 21 to 49 and

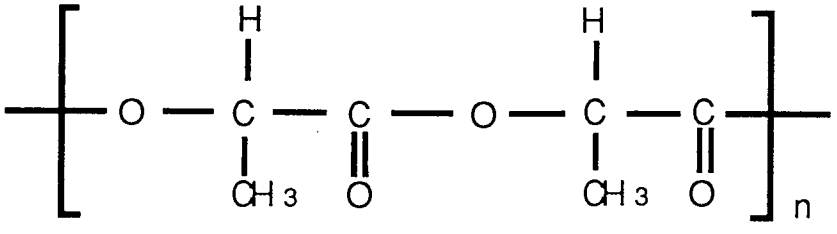
maximum crystallinity occurs around the transition between stage 1 and stage 2. As all the amorphous regions are degraded, hydrolytic attack focuses on the crystalline regions and by day 49 tensile strength is completely lost, although 58% of the suture remains.

Strongly alkaline environments have been shown to increase the rate of hydrolysis of PGA with respect to normal physiological (pH7.4) or acidic conditions[111,113,116]. In addition, Williams and colleagues have shown that certain enzymes, namely ficin, carboxypeptidase A, α-chymotrypsin and clostidiopeptidase A, affect the rate of *in vitro* biodegradation of PGA[117] and Herman has suggested that tissue esterases play an important part in PGA degradation[118].

1.4.4 Poly(lactic acid) [PLA]

Lactic acid is present in nature both as an immediate and as an end product in carbohydrate metabolism. Lactic acid (Figure 1.6) is optically active and so exists as both the D(-) and L(+) isomers and the racemic D,L form. The L(+) isomer is metabolised in the body.

Figure 1.6 Poly(lactic acid) [PLA]



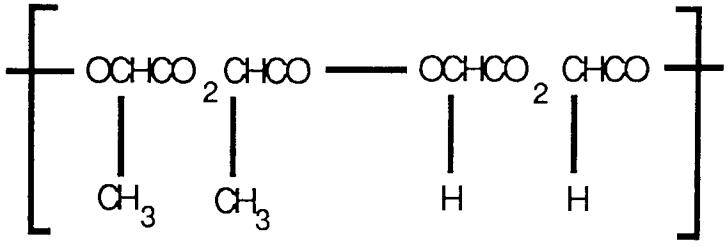
Poly(L-LA) is a tough, semi-crystalline polymer having a crystallinity of about 37% and a Tg and TM of 36°C and 180°C respectively. Combined with the effect of crystallinity and

increasing hydrophobicity due to the presence of extra methyl groups compared with PGA, water uptake is restricted to about 2% and biodegradation of P(L-LA)is slower than that of PGA. Reed and Gilding showed that the *in vitro* degradation of P(L-LA) resulted in a 10-15% loss in weight within 16 weeks[119]. Conversely, P(D,L-LA) is amorphous and has only one morphological phase. This allows an even distribution of active component throughout the polymer matrix of microspheres. Therefore, although P(D,L-LA) degrades more rapidly than P(L-LA) it is the preferred choice for microsphere preparation[6]. The fact that PGA biodegrades more rapidly than P(D,L-LA) has been exploited in the synthesis of poly(lactide-co-glycolide) [PLCG] copolymers, which have been used extensively for both controlled and sustained drug delivery systems and surgical fixation devices[120,121].

1.4.5 Copolymers of poly(lactic acid with glycolic acid) [PLCG].

Copolymers of PLA and PGA were initially synthesized to generate a polymer which combined the high tensile strength of PGA with the slower degradation of PLA. The physico-chemical and degradation properties of PLCG, Figure 1.7, depend on the molar ratio of the two monomers within the polymer chain, and on the polymer molecular weights[13].

Figure 1.7 Poly(D,L-Lactide-co-glycolide) [PLCG]



The copolymer compositions of the poly(D,L-lactide-co-glycolide) system have been studied by Pitt and colleagues[120,121]. They found that the release of progesterone from poly(D,L-lactide-co-glycolide) copolymer films, with 19 and 21% W/W glycolide content, occurred biphasically. The first release phase took place over a period of 28 to 31 days while the second release phase, which was very much greater than the first, was then observed. Work by Beck and Tice and co-workers has shown that the release of steroids such as norethisterone and testosterone from poly(D,L-lactide-co-glycolide) occurs in two stages. This biphasic release depends upon diffusion followed by biodegradation[64,122–126].

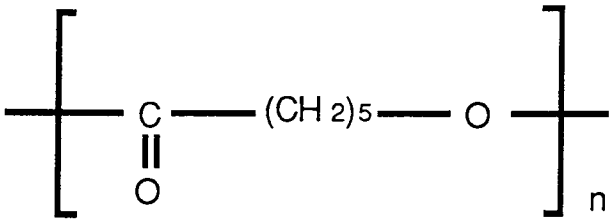
Hutchinson and colleagues have investigated the *in vitro* and *in vivo* release of polypeptides (luteinizing hormone-releasing hormone, LHRH)) from poly(D,L-lactide-co-glycolide) copolymers containing 25 to 100% P(D,L-LA)[47,127]. Hutchinson found that the release of LHRH was biphasic and the result of an initial surface release, followed by a lag period and then a continuous low level release until exhaustion. He explained the bisphasic release profile on the basis of an initial diffusion of the polypeptide through aqueous pores generated in the dosage form. Hutchinson stated that

these aqueous channels were generated by two distinct and separate mechanisms. The first involved the leaching of drug from polypeptide domains at or near the surface. The second mechanism involved biodegradtion of the polymer and was associated with the generation of microporosity

1.4.6 Poly(caprolactone) [PCL]

Poly(caprolactone) [PCL] is a crystalline aliphatic polyester, (Figure 1.8), which biodegrades in a similar way to P(D, L)LA, i.e. random chain scission as a result of ester hydrolysis[127]. The low molecular weight fragments are taken up by macrophages and degraded intracellularly[106].

Figure 1.8 Poly(caprolactone) [PCL]

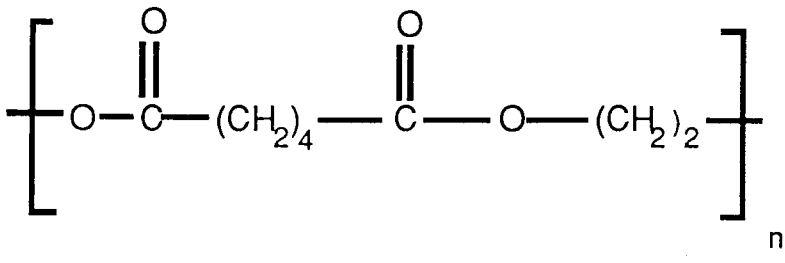


PCL is degraded by micro-organisms such as P.*pullans* and has been evaluated for use as a biodegradable packaging material[129]. PCL forms compatible blends with a wide range of other polymers including poly(lactic acid)[128] and poly(glycolic acid)[130] and has been shown to increase the porosity of PHB/HV microcapsules when added during fabrication[106,131].

1.4.7 Polyethylene Adipate [PEA]

Although polyethylene adipate is a widely available aliphatic polyester[132] very little previous work on the chemical degradation or biodegradation of this polymer has been reported. Luderwald pyrolysed PEA and using mass spectrometer analysis claimed that the results indicated cleavage at the ester groups to give ketenic and hydroxyl ends[133]. Hashimoto and Yamashita, however, degraded the polymer and reported a cyclic dimer to be the main product accompanied by a cyclic monomer and a cyclic 3-oxopentamethylene ester[134]. McNeill and Basan found that when PEA was degraded, the main products were cyclic oligomers[132]. These workers concluded that in this respect PEA behaved similarly to other aliphatic polyesters such as poly(lactic acid) and poly(glycolic acid) and carbon dioxide, acetaldehyde and 2-ethylacrolein were also formed. However, these workers were unable to confirm Luderwald's claim that ketenic and hydroxyl ends resulted from cleavage at the ester group. The mechanism of biodegradation of poly(ethylene adipate) in physiological systems remains to be investigated.

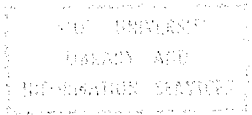
Figure 1.9 Polyethylene Adipate



1.5 *Aims and Objectives of Work*

The controlled release of low molecular weight species by diffusion from polymers is well understood and an increasingly exploited process. The growing interest in the controlled release of clinically useful macromolecules such as antibiotics has highlighted the relative difficulties associated with the delivery of high molecular weight compounds by this process. In addition, the impact of polymer biodegradability and the mechanisms involved on the controlled release of bioactive macromolecules remains to be evaluated. Many problems are encountered when attempts are made to incorporate macromolecules into polymeric matrices. Most of these derive from either interactions between the polymer matrix and the incorporated macromolecule, i.e. polymer-polymer compatibility phenomenon or the inability of the biological macromolecule to withstand solvents and the temperature involved in the fabrication of synthetic polymers. The mutual incompatibility normally encountered between natural and synthetic systems means that homogeneous distribution is difficult to achieve.

This study sets out to address these problems, with the central objective of the work being to evaluate and introduce techniques for the microencapsulation of selected biologically active macromolecules in microparticulates fabricated using synthetic biodegradable polyesters and their blends. Judicious choice of blend should provide an opportunity for controlling the rate of polymer (blend) biodegradation. Microspheres and microcapsules have advantages over other types of delivery device in that they can be fabricated in different sizes and this will allow



easy administration to specific body sites, such as joint spaces. They may be used parentally and their biodegradability removes the need for further remedial surgery.

Subsequent work will be carried out to monitor changes in the gross and ultrastructural morphology of microcapsules and microspheres prepared using both the W/O/W double emulsion and O/W single emulsion fabrication techniques prior to and during biodegradation. Poly(hydroxybutyrate/hydroxyvalerate) [P(HB-HV)] and poly(ethylene adipate) [PEA] will be used as fabrication materials in this study. Both polymers will be blended with 20% polycaprolactone type II [PCL 11] in an attempt to induce inherent microporosity within the microparticulate and thus afford a mechanism for the release of the encapsulated macromolecule. In this regard, initial assessments will be made of microparticulate yield, overall shape, surface topography, ultrastructural morphology and size distribution. The effect of percentage loading with BSA, a surrogate macromolecule on these parameters together with the encapsulation efficiency will be investigated. The release of BSA will be monitored over an extended period of time, but not exceeding 30 days, using the flexible and sensitive bicinochoninic acid assay. The potential of these microparticulates as suitable controlled and sustained delivery vehicles for macromolecules will be evaluated.

In order to gain some understanding of the mechanism by which P(HB-HV) and PEA microcapsules and microspheres might biodegrade *in vivo* when utilised in parental delivery, oral or gastrointestinal delivery the microparticulates will be incubated in new-born calf serum, synthetic gastric juice and pancreatin respectively. The *in vitro* incubation of microparticulates in Hank's

buffer, pH 7.4 will provide information on the effect of simple chemical hydrolysis on microparticulate integrity and provide some insight into the mechanisms involved in the release of macromolecules.

Preliminary indications have suggested that microparticulates fabricated using both the W/O/W and O/W emulsion systems have very low encapsulation efficiencies. In order to overcome this drawback a W/O single emulsion system with solvent evaporation will be developed and evaluated in an attempt to minimise the loss of macromolecule into the continuous aqueous phase. In this study the fabrication polymers used will be 75:25 and 50:50 poly(lactide - co- glycolide) [PLCG]; poly(ethylene adipate) alone; 50:50 blends of poly(ethylene adipate) with poly(lactide-co-glycolide) 50:50, and blends of 75% poly(hydroxybutyrate/hydroxyvalerate) with 25% poly(lactide-co-glycolide) 50:50. Poly(lactide-co-glycolide) will be used since it has established biocompatibility.

Microparticulate vehicles (microspheres) based on poly(lactide-co-glycolide) will be fabricated with a view to their possible use in combating infection during total hip replacement. These microspheres will be loaded with the antibiotic vancomycin at various percentage loadings (based on the dry weight of polymer used). Vancomycin is an antibiotic primarily active against gram negative bacteria which are thought to be principally responsible for infections during and subsequent to total hip replacement. The effect of fabrication technique on microsphere shape, surface morphology, percentage yield, size distribution and encapsulation efficiency will be assessed. The release of vancomycin will be monitored for an extended period of time in Hanks buffer, pH 7.4 and

new-born calf serum and the concentration monitored using a microbiological radial diffusion assay. The biodegradation of loaded and unloaded microspheres will be monitored in Hanks buffer and new-born calf serum to provide some insight into the basic mechanisms involved and the roles these play in vancomycin release relevant to the use of the vehicles in orthopaedic applications.

CHAPTER TWO

MATERIALS AND METHODS.

2.0 *Materials.*

Poly[vinyl-alcohol] (PVA) [88% hydrolysed, average Mw 77000-79000]; light mineral oil [density 0.838 g/cm^3, HPLC grade]; dichloromethane [HPLC grade]; acetonitrile [99.9% HPLC grade] and petroleum ether (boiling point 40-60°C) were obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K. and used without further purification.

The emulsifying agent Sorbitan Monopalmitate (Span 40); Agarose type VII (low temperature gelling >15°C); Bicinchoninic Protein Assay reagent [4,4 Dicarboxy-2,2,Biquinoline], Bovine Serum Albumin (fraction V RIA grade) and the antibiotic Vancomycin Hydrochloride (11030ug vancomycin base/mg, 2.5 mol/mol H_2O); pepsin from porcine stomach mucosa and porcine pancreatin (containing many enzymes including, trypsin, pepsin, lipase, ribonuclease and protease) were all supplied by the Sigma Chemical Company, Poole U.K

Sterile Hanks balanced salt solution (HBSS) (appendix 1), non heat treated newborn calf serum (batch number 30G9510D from New Zealand, mycoplasma and virus screened, containing protease and low levels of esterase) and penicillin (10,000ul/ml)/streptomycin (10,000 ug/ml) solution were supplied by Gibco, Life technologies Ltd, Paisley, Scotland. All other basic chemicals were of analytical grade and double distilled water was used throughout.

2.1. Polymers.

POLY-β-HYDROXYBUTYRATE/ 3-HYDROXYVALERATE [P(HB-HV)] containing 10.8 mole % hydroxyvalerate and with a M_W of 330,000

was obtained from ICI Biopolymers Ltd., Stockton-on-Tees, Cleveland, U.K., under the trade name Biopol®.

POLY-$\mathcal{E}$- CAPROLACTONE TYPE 11 [PCL] was obtained in pellet form from the Aldrich Chemical Company Ltd, Gillingham, Dorset, UK. The weight average and number average molecular weights (M_w and M_n) were M_w= 64000 and M_n= 34,200 respectively and the polydispersity was 2.0015. The M_w, M_n and polydispersity determinations were made by gel permeation chromatography (GPC) at the polymer supply and characterization centre, RAPRA Technology Ltd., Shawbury, Shrewsbury, Shropshire, U.K.

POLYETHYLENE ADIPATE [PEA] was supplied by the Aldrich Chemical Company Ltd, Gillingham, Dorset UK. M_w, M_n and polydispersity were determined by gel permeation chromatography (GPC) at the Polymer Supply and Characterisation Centre, RAPRA Technology Ltd., Shawbury, Shrewsbury, Shropshire, U.K. Polyethylene adipate M_w= 1.79×10^4, M_n= 9.09×10^3, polydispersity= 1.779.

POLY[D,L-LACTIDE-CO-GLYCOLIDE] [PLCG], 75:25 and 50:50 were synthesized by Boehringer Ingelheim Germany and supplied by Alpha Chemicals Ltd, Bracknell, UK. PLCG 75:25 (Resomer 752) had a Mw of approximately 18,640 and M_n of 6,511 with a polydispersity of 2.8815 and inherent viscosity 0.27 DL/G. The molar ratio of lactide to glycolide was 75:25 with residual monomer contents of 0.5% and 0.5% respectively. PLCG 50:50 had an actual molar ratio of 48 lactide:52 glycolide with residual monomer contents of 0.5% and 0.5% respectively and a M_w of 14,810 and a M_n of 5,716. The polydispersity was 2.8965 and the inherent viscosity 0.21 DL/G.

Both PLCG 75:25 and 50:50 were stored at 4°C to prevent microbial deterioration.

2.2 *Fabrication of P(HB-HV)20%PCL 11, PEA20% PCL 11 and 50%P(HB-HV)50% PEA blend microspheres using oil in water emulsification with solventevaporation*[71].

Microspheres were prepared essentially using a single emulsification solvent extraction technique as originally described by Beck and colleagues[71]. The basis of the method is described in the protocol, figure 2.1 (page 75) and consists of the formulation of a stable oil in water emulsion by the emulsification of an organic phase (polymer + solvent) in an aqueous phase (PVA in double distilled water) followed by evaporation of the solvent from the droplets in the emulsion leading to the precipitation of microspheres.The organic phase was prepared by dissolving the polymer (refer to table 2.1 on pg 76) in dichloromethane to produce a 5% w/v solution. Polymer solvation was effected by ultrasonication at 40°C for 30 minutes. The aqueous phase consisted of 1.5% PVA in double distilled water. Both the organic and aqueous phases were cooled in an ice bath for one hour to reduce their viscosity. The oil in water emulsion was prepared by gradually adding the organic phase drop by drop into 150mls of aqueous phase emulsified at a high speed of 10,000 rpm for 5 minutes using a Silverson stirrer fitted with a disintegrating head. The solvent was encouraged to evaporate from the emulsion by agitating with a magnetic stirrer for 12 hours at room temperature under a fume hood. The microspheres were sedimented by centrifugation at 15000 rpm for 90 seconds, resuspended in double distilled water and subsequently recentrifuged. This washing procedure was repeated three times.

Figure 2.1 PREPARATION OF MICROSPHERES USING AN OIL IN WATER EMULSIFICATION TECHNIQUE.[71]

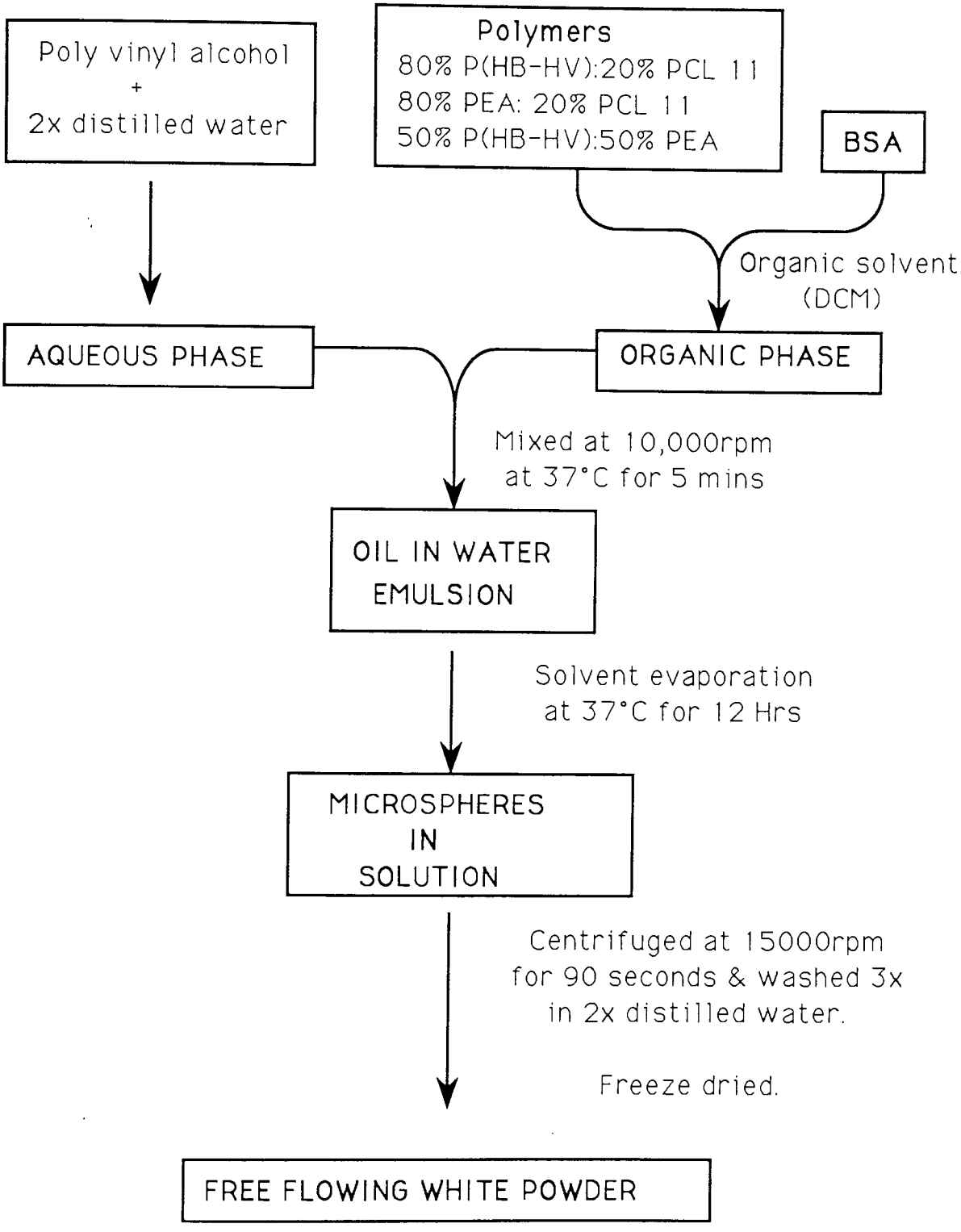


Table 2.1. **Polymers used in the fabrication of microspheres and microcapsules.**

Polymer	Theoretical BSA loading (W/W) of microspheres and microcapsules fabricated for release studies.	Theoretical BSA loading (W/W) of microspheres and microcapsules fabricated for biodegradation studies.
80% P(HB-HHV):20% PCL TYPE 11	10% 20% 30% 40% 50%	5% 10% 15% 20% 25%
80% PEA:20% PCL TYPE 11	10% 20% 30% 40% 50%	5% 10% 15% 20% 25%
50% P(HB-HV):50% PCL TYPE 11	10% 20% 30% 40% 50%	– – – – –

The microspheres were then pre-frozen in a little added 2x distilled water at -20°C, and freeze dried in an Edwards Modulyo 4L freezedrier to give a white powder. The microspheres were stored desiccated at -20°C until required for biodegradation studies.

Incorporation of bovine serum albumin into microspheres.

Bovine serum albumin powder (fraction v, M_W 65,000) was suspended in the polymer solution as part of the organic phase. In release studies the bovine serum albumin was loaded into microspheres as a representative surrogate peptide at 10%, 20%, 30%, 40% and 50% of the dry weight of polymer used. For biodegradation studies microspheres were loaded with BSA at 5, 10, 15, 20 and 25% of the dry weight of polymer used.

2.3 Preparation of microcapsules using a double emulsion solvent extraction technique[33]

The double emulsion solvent extraction technique as described by Okada and colleagues utilized a water in oil in water emulsion system to encapsulate leuprolide acetate[33]. The technique is summarised in figure 2.2, page79.

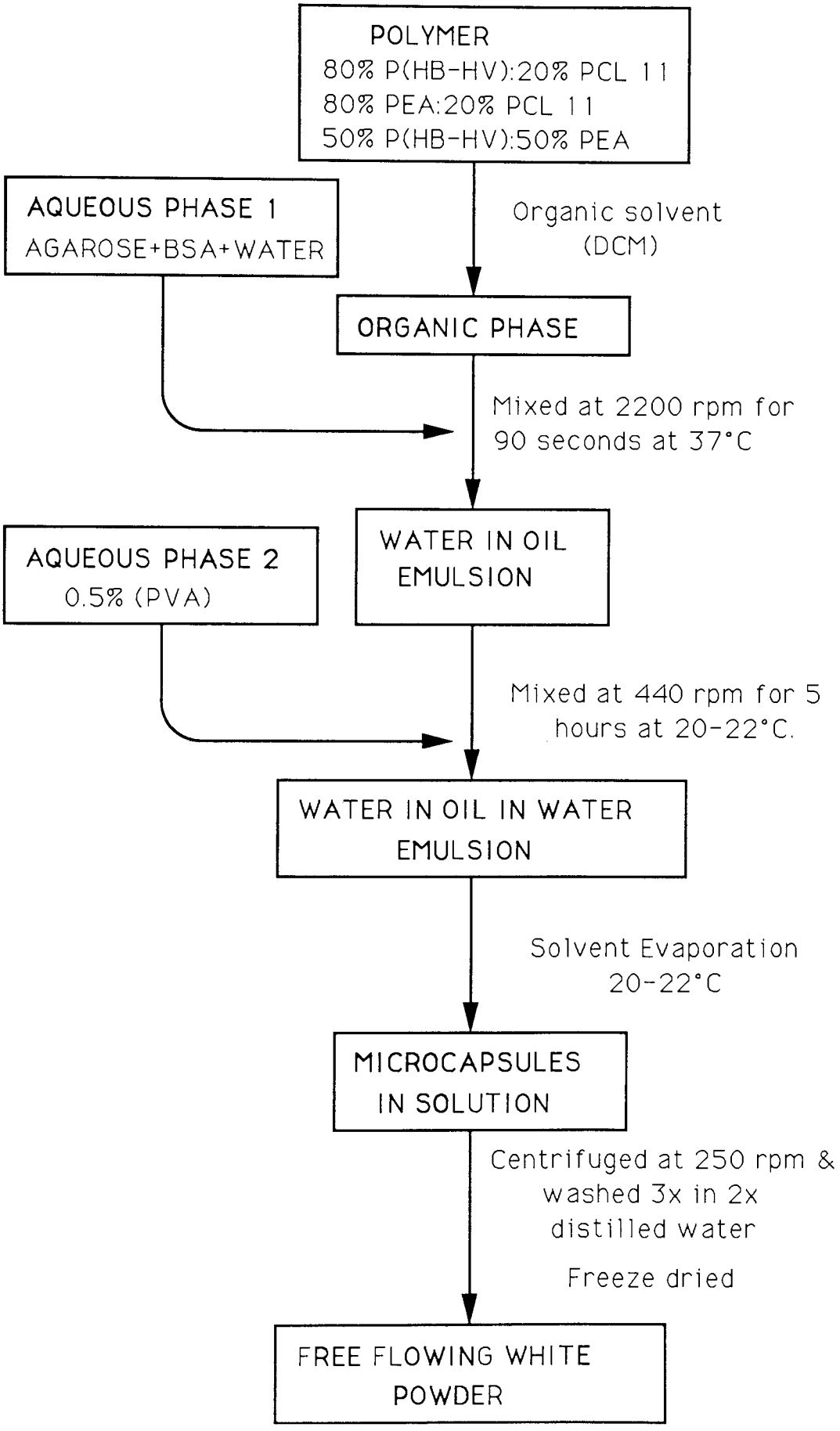
The organic phase consisted of a 6% w/v solution of polymer (80%P(HB-HV)20% PCL 11, 80% PEA20% PCL 11 and a 50:50 blend of P(HB-HV):PEA) in dichloromethane ultrasonicated at 40°C for 30 minutes. The inner aqueous phase 1 was prepared by dissolving 0.056g of type V11 low temperature gelling agarose (2.25% W/V) in 2.5ml of warm distilled water by ultrasonication at 37°C for 15 minutes. 1ml of this inner aqueous phase 1 was combined with 15mls of the organic polymer solution in a specially designed sealed mixing unit attached to a Heidolph stirrer which allowed the slow addition of the inner aqueous phase 1 after polymer mixing had

commenced. The two components were emulsified at 37°C in a water bath using a high shear head at 2200rpm for 90 seconds. All the apparatus was pre-warmed to 37°C to prevent the inner aqueous phase 1 from cooling and thereby resulting in the generation of an agarose gel prior to emulsification with the organic phase. Keeping the aqueous phase 1 at 37°C also made it easier to work with. After emulsification the sealed unit and it's contents were removed from the bath and rapidly cooled to approximately 20°C to increase the viscosities of the inner water phase and O/W emulsion itself. Phase combination and the generation of a water in oil in water emulsion was effected by adding 15ml of the water in oil emulsion to 150ml (0.5% W/V) of PVA (aqueous phase 2) at room temperature (20-22°C). The PVA solution was continuously stirred, at 440rpm using a Heidolph mixer fitted with a three blade propeller in an off centre position and solvent evaporation was allowed to proceed for 5 hours under an extraction hood. After this time the remaining solution was gently centrifuged (250rpm) at room temperature until sedimentation of the microcapsules had occurred. The supernatant was removed by aspiration and the microcapsules resuspended in double distilled water. The washing procedure was repeated 3 times and the final suspension of microcapsules sized in a series of wet state sieves (Endicott 153-300um) prior to prefreezing at -20°C and freeze drying in an Edwards Modulyo 4L freeze drier overnight to produce a free flowing preparation of white microcapsules. The microcapsules were stored desiccated at -20°C until required.

The incorporation of bovine serum albumin into microcapsules.

BSA was incorporated via the inner aqueous agarose hydrocolloid phase 1 at theoretical loadings of 10%,20%,30%,40%

Figure 2.2 PREPARATION OF MICROCAPSULES USING A DOUBLE EMULSION SOLVENT EXTRACTION TECHNIQUE [33]



and 50% by dry weight of polymer for use in release experiments and 5,10,15,20 and 25% by dry weight of polymer for use in biodegradation experiments (Table 2.1, page 76).

The double emulsion solvent extraction method was then carried out as described in the protocol, fig 2.2, page 79.

2.4 Preparation of microspheres using a water in oil single emulsion solvent extraction method[35].

The W/O single emulsion solvent extraction method was originally developed by Tsai et al in an attempt to increase the encapsulation efficiency of water soluble macromolecules such as mitomycin c[35]. The method was further developed by Jalil and Nixon and their modification is the basis of the technique used here and summarised in the protocol, fig 2.3[17], page 82. A range of polymers and polymer blends have been utilized in the technique, details of which are given in table 2.2, page 81. PLCG 50:50 was blended with P(HB-HV) and PEA, both slowly biodegrading polymers, in an attempt to increase their rate of biodegradation.

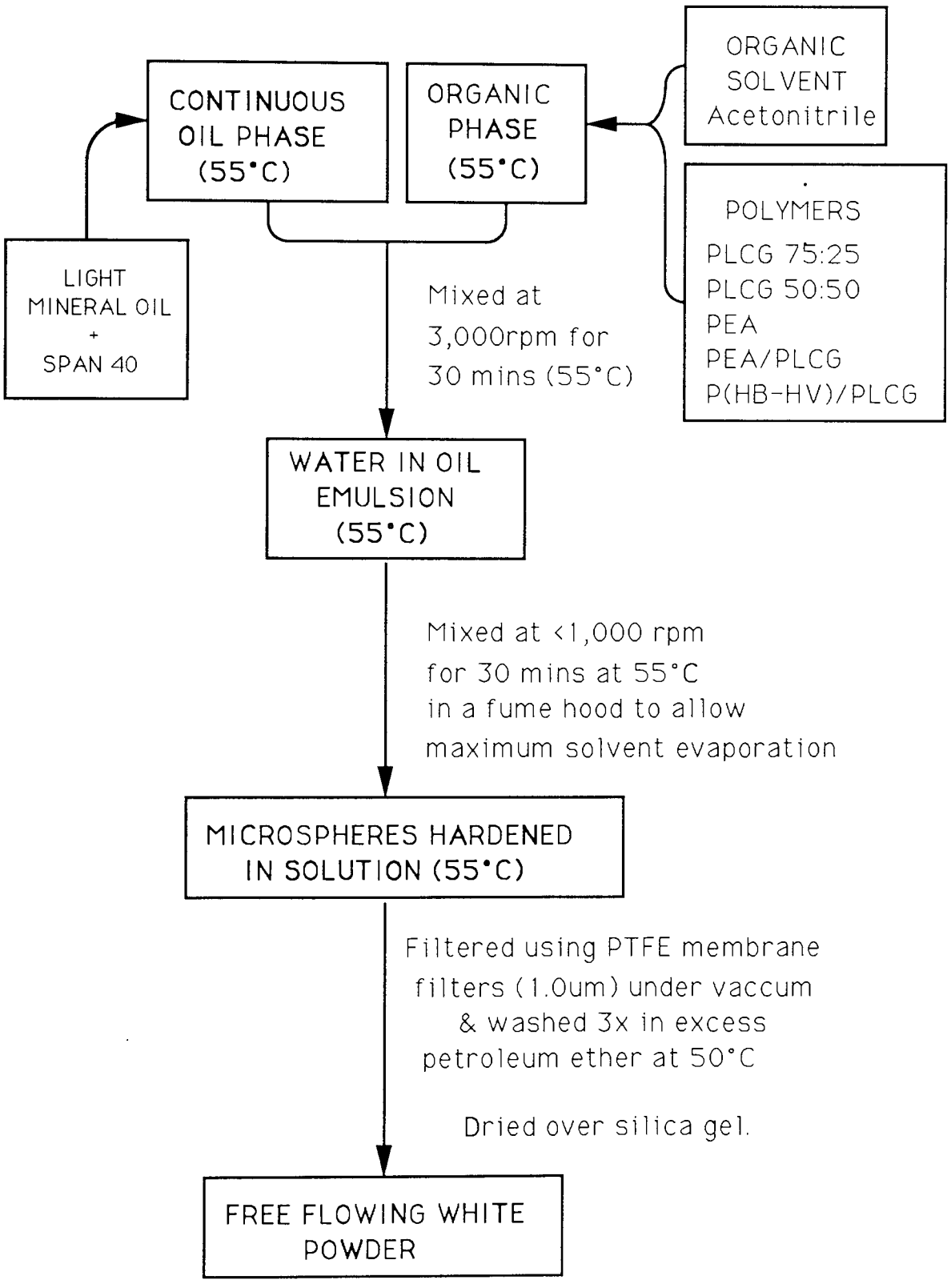
In the W/O single emulsion solvent extraction method the organic phase was prepared by dissolving 1g of polymer, using ultrasonication for 15 minutes at 35°C, in 30ml of acetonitrile This organic phase was then maintained at 55°C in a water bath.

The continuous phase consisted of 125g of light mineral oil containing 2% w/w (2.5g) span 40 maintained at 55°C in a water bath. The organic phase was gradually added to the continuous phase and the whole emulsion stirred at 3000rpm for 30 minutes at 55°C using a silverson emulsifier. The emulsion was lightly mixed at 55°C with a Heidolph stirrer at <1000rpm for a further 30 minutes to ensure solvent evaporation. The temperature was maintained at 55°C throughout the process to remove solvent and induce initial

Table 2.2 Formulation details for vancomycin loaded microspheres.

Formulation code	Polymers used	Vancomycin loading [% w/w]
PLCG (75:25)10% VANCO	100% PLCG 75:25	10%
PLCG (75:25)25% VANCO	100% PLCG 75:25	25%
PLCG (75:25)50% VANCO	100% PLCG 75:25	50%
PLCG (50:50)10% VANCO	100% PLCG 50:50	10%
PLCG (50:50)25% VANCO	100% PLCG 50:50	25%
PLCG (50:50)50% VANCO	100% PLCG 50:50	50%
PEA25% VANCO	100% PEA	25%
PEA/PLCG25% VANCO	50% PEA/50% PLCG(50:50)	25%
P(HB-HV)/PLCG10% VANCO	75% P(HB-HV/25% PLCG(50:50)	10%
P(HB-HV)/PLCG25% VANCO	75% P(HB-HV)/25% PLCG(50:50)	25%
P(HB-HV)/PLCG50% VANCO	75% P(HB-HV)/25% PLCG (50:50)	50%

Figure 2.3 PREPARATION OF MICROSPHERES USING A WATER IN OIL SOLVENT EXTRACTION TECHNIQUE.[17]



microsphere hardening. The emulsion was then cooled to 35°C and left for 1 hour to allow the microspheres to harden fully whilst preventing the precipitation of span 40. All of these steps were carried out in a fume cupboard.

The microspheres were separated by filtration through specially supported solvent resistant filters (Whatman polypropylene backed PTFE membrane filters) with a pore size of 1.0um under vacuum. Span 40 was removed by washing with excess petroleum ether at 50°C. The microspheres were weighed dry and sized using SEM.

Incorporation of water soluble antibiotic vancomycin.

Vancomycin was incorporated into PLCG (75:25), PLCG (50:50) and P(HB-HV)/PLCG (50:50) microspheres at 10% 25% and 50% loadings and in PEA and the blend 50%PEA/50% PLCG (50:50) microspheres at 25% loading by dry weight of polymer. The formulation details for vancomycin loaded microspheres prepared using the W/O emulsification technique are summarised in table 2.2, page 81. Vancomycin was incorporated into the organic phase together with the appropriate polymer. However, P(HB-HV) is insoluble in acetonitrile and to overcome this the polymer was initially dissolved in a minimum volume of DCM (10ml) and then added to the acetonitrile. Dissolution of the polymer was effected by ultrasonication.

2.5 Microparticle ultrastructural morphology and surface topography.

Microspheres and microcapsules were thoroughly dried, mounted on stubs and sputter coated with gold in an Emscope SC220 sputter coater before being examined on a Cambridge Instruments

Stereoscan 90 Scanning Electron Microscope equipped with polaroid photographic facility. The size distribution of microspheres and microcapsules was assessed from the sizing bars present on the micrographs. The biodegradation of both microspheres and microcapsules has been monitored during incubation in various media over a period of 28-35 days. SEM micrographs are representative of at least 25 randomly selected microparticles in terms of their initial appearance and change in morphology due to biodegradation with time.

2.6 Measurement of encapsulation efficiency

0.2g of previously prepared microspheres or microcapsules were combined with 2ml of DCM in a glass vial and shaken thoroughly for 30 minutes. To this was added 2ml of double distilled water and the vial was shaken for a further 30 minutes and allowed to stand overnight to ensure complete partitioning of the water soluble macromolecule into the aqueous phase. The upper aqueous layer was carefully drawn off using a sterile pipette. A sample of the aspirate was then assayed for either BSA or Vancomycin using the Bicinochoninic Acid Assay[135] or Radial Diffusion Assay[136] respectively. Encapsulation efficiency was defined as the ratio of actual macromolecule content (ug/100ml) to the theoretical macromolecule loading (ug/100ml), expressed as a percentage.

2.7 The release of macromolecules from microspheres and microcapsules.

2ml of Hank's buffer or newborn calf serum was added to O.2g of microspheres or microcapsules in 10ml sterilin tubes and incubated at 37°C with daily agitation. The daily release of macromolecule was monitored for upto 30 days. Each day the tubes

were lightly centrifuged to sediment the particles and the supernatant removed for assay. The incubation medium was replaced with 2ml of fresh medium to maintain "sink conditions" and discourage microbial growth.

2.7.1 Bicinochoninic acid (BCA) assay for protein[135].

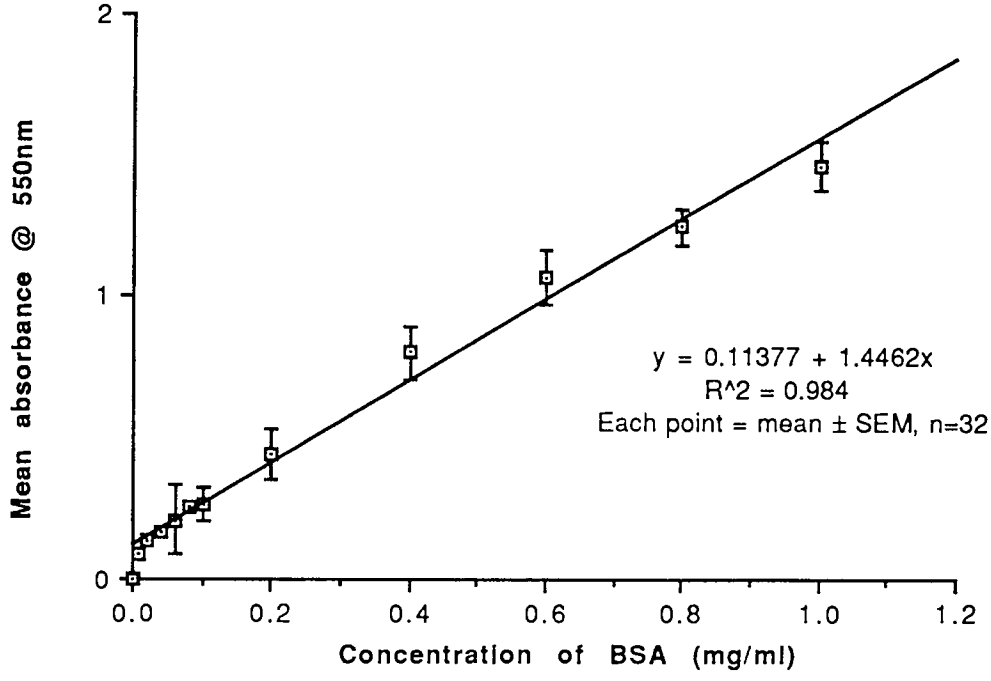
Bicinochoninic acid, sodium salt is a sensitive stable, water soluble compound capable of forming an intense purple complex with the cuprous ion Cu(1) at 60°C in an alkaline environment. This colour generation forms the basis of the analytical method capable of monitoring the amount of Cu(1) ion production when the peptide bonds of a protein complex react with alkaline Cu(11) ion (Biuret reaction). The colour produced in this reaction is stable and found to intensify over a range of increasing protein concentrations and increased sensitivity can be achieved by increasing the incubation time, the incubation temperature or both. The absorbance of the purple colour at room temperature and 562nm increases proportionally over a broad range of protein concentrations and the use of a standard protocol allowed the determination of protein in the range of 100µg/ml to 1200µg/ml. A micro protocol was used to determine protein concentrations in the range of 5µg/ml to 250µg/ml when protein concentrations were expected to be low, or if only a small amount of sample was available for assay.

The BCA protein assay working reagent consisted of 50 parts reagent A (appendix 2) mixed with 1 part reagent B (appendix 3).

10µl of the release sample or standards was mixed with 200ul of working reagent in each well of a 96 well Elisa plate and left for 30 minutes at 60°C. The absorbance of each well was read at 562nm. The BSA concentration in the sample was computed by reference to a standard curve of a range of BSA concentrations (100ug/ml to

1200ug/ml) in Hanks buffer, pH 7.4, versus absorbance at 562nm and expressed as BSA mg/ml. A typical BSA standard curve is shown in fig 2.4 below.

Fig 2.4 BSA standard curve in Hank's buffer pH7.4.



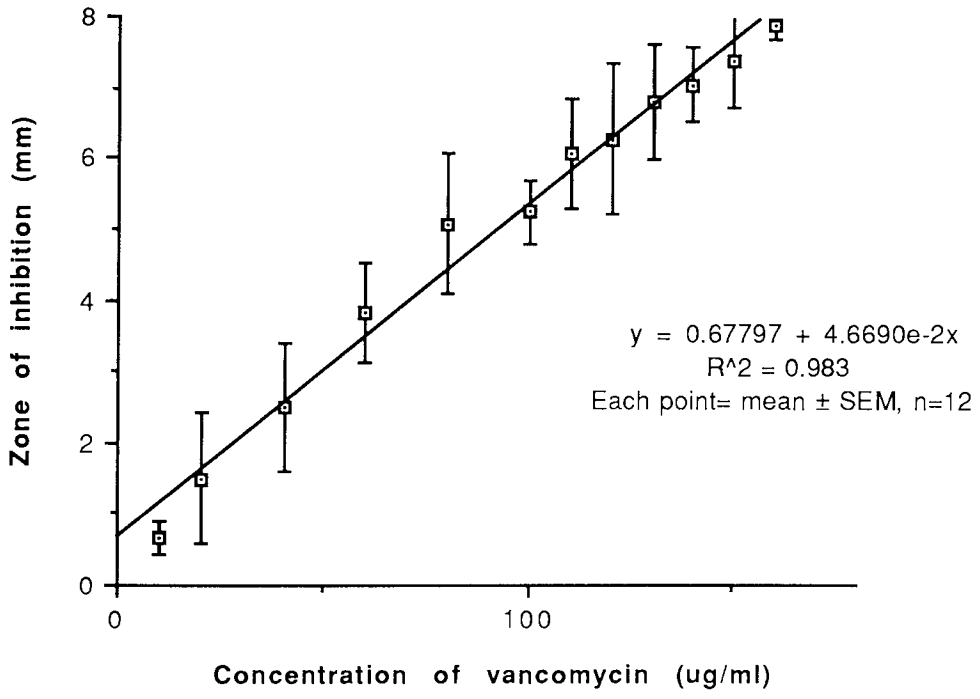
2.7.2 Vancomycin Radial Diffusion Assay[136].

Cultures of the organism *Sarcina Lutea*, which is susceptible to the antibiotic vancomycin, were plated out and grown at 37°C for 3 days. A quarter of a plate of S.Lutea was then dispersed into 3ml of nutrient broth and left rotating at 37°C overnight. 300ml of oxoid D.S.T Agar (12g agar in 300ml x2 distilled water) was autoclaved and left to cool in a water bath at 50°C for 30-60 minutes.
To this was added the *S.Lutea* in 3mls of nutrient broth and after gentle mixing the agar was poured into a 243 x 243 x 18mm bioassay dish (supplied by Gibco, Life technologies Ltd, Paisley, Scotland). Once set the lid was removed and the plate inverted in a warm room at 37°C. After 15-30 minutes the lid was replaced and the plate transferred to a cold room at 4°C for a further 15-30 minutes to ensure the agar was firm enough for 36, 5mm diameter

wells to be cut into the plate using the end of a Pasteur pipette. A 100µl aliquot of either sample or standard was added to each well in a recorded but random sequence and the plate left overnight at 37°C. The following day the diameter of the zone of inhibition around each well was measured using a ruler. The agar was then removed from the plate and the latter disinfected and washed in 70% alcohol before being reused.

A standard curve was constructed using a range of known vancomycin concentrations in either Hank's buffer pH 7.4 or newborn calf serum versus diameter of the zone of inhibition in mm.
A typical vancomycin standard curve is shown in fig 2.5 below.

Fig 2.5 Vancomycin standard curve in newborn calf serum.



2.8 Biodegradation of microspheres and microcapsules.

The extent of biodegradation of microspheres and microcapsules in various media was monitored both gravimetrically as a change in dry weight with time and using Scanning Electron

Microscopy (S.E.M). 0.2g of microspheres or microcapsules were added to sterilin tubes containing 2mls of either Hank's buffer, newborn calf serum, synthetic gastric juice or pancreatin solution incubated at 37°C with daily agitation.The incubation medium was changed by aspiration every 3 days to prevent microbial growth. At seven day intervals a small sample of microspheres or microcapsules was removed from each tube using a Pasteur pipette and dried in a desiccator for 24 hours prior to examination of ultrastructural morphology using S.E.M. After 30 to 35 days incubation the tubes were centrifuged as described above and the microspheres or microcapsules resuspended in double distilled water to wash off all the incubation media. This procedure was repeated three times. The microspheres and microcapsules were then dried in a desiccator for 7 days or to a constant weight and the percentage weight loss (PWL) calculated by difference as indicated below.

$$\frac{\text{Dry weight loss after 30 days (g)}}{\text{Original dry weight of sample (0.02g)}} \times 100 = \% \text{ weight loss}$$

2.9 Statistical analysis

Results were expressed as mean values ± SEM and statistical significance of the difference between points was confirmed by Student's t test (non-paired). Regression coefficents were calculated for the standard curves.

CHAPTER THREE

THE INCORPORATION AND RELEASE OF BSA FROM MICROSPHERES AND MICROCAPSULES COMPOSED OF P(HB-HV), PEA AND A 50:50 BLEND OF P(HB-HV)/PEA.

3.0 *Introduction.*

The encapsulation of water soluble molecules by both the double emulsion and O/W single emulsion methods with solvent extraction have been comprehensively described in the literature[30,66,72,73,75]. Bodmeier and colleagues have demonstrated that it is possible to incorporate low molecular weight water soluble pseudoephedrine HCL into microcapsules and microspheres using these techniques[75]. They found that the double emulsion method gave microcapsules with a 30.1% pseudoephedrine content compared to a pseudoephedrine content of 14.3% for microspheres fabricated using the single emulsion (O/W) solvent extraction technique[75]. This was attributed to the organic solvent/polymer solution acting as a barrier between the drug containing internal and external aqueous phases, therefore drug loss from the microcapsule as a result of drug partitioning in to the aqueous phase was minimalised. The O/W single emulsion solvent extraction system has been used to encapsulate progesterone into P(HB-HV) microspheres[27] and various steroids (hydrocortisone, cisplatin) with varying water solubilities into poly(D,L-lactide) microspheres[67,74]. Whilst many workers claim to have successfully encapsulated water soluble macromolecules using both W/O/W and O/W techniques many of the fabrication variables/conditions were different to the ones used in this study. Work carried out by Fong, Josephine, Pearson and Maulding showed that thioridazine, ketotifen and hydrocortisone release was affected by one or more of the following factors, percentage drug loading; polymer molecular weight; polymer composition or polymer concentration in the organic phase of the emulsion[137]. Probably one of the most prominent factors in this regard is the nature of the encapsulating polymer. Ogawa *et al* emphasised this by showing that the

efficiency of both PLA and PLCG microcapsules to encapsulate leuprolide acetate fell from 70% to 44% by only slightly changing the composition of the encapsulating polymer[30].

Once encapsulated the release of entrapped macromolecules, such as proteins, from polyester microparticulates is unlikely to be as a result of partition dependant diffusion because proteins do not show appreciable solubility in the polymer phase due to structural dissimilarities and mutual incompatibility[47]. The free volume within the polymer regions arising from segmental motion is invariably too small to accommodate macromolecules[47]. However, the release of macromolecules such as bovine serum albumin from microcapsules may be effected by increasing the porosity of the capsule membrane. Porosity may be an inherent feature, but can also be generated within the microparticle by polymer degradation[38] or the modification of the fabrication process[131]. Embleton has described a manoeuvre designed to generate a microporous network within the polymer membranes of microcapsules fabricated using the double emulsion technique[131]. The process involves the addition of polycaprolactone type 11 (PCL 11) to the bulk polymer, in this case P(HB-HV). The PCL is inherently more soluble than P(HB-HV) in the organic phase and during solvent evaporation is eluted through the hardened P(HB-HV) capsule wall generating micropores of average size, 2um. For this reason 20% PCL11 was blended with 80% P(HB-HV) and 80% PEA in the fabrication of both microspheres and microcapsules in the present study.

3.1 *Methodology - fabrication of microcapsules and microspheres.*

The polymer blends P(HB-HV)20% PCL 11; PEA20% PCL 11 and P(HB-HV)50% PEA (table 2.2, page 81) were used to fabricate microspheres via the O/W single emulsion solvent extraction technique as detailed in section 2.2, page 74, and microcapsules were fabricated using the double emulsion solvent extraction technique as described in section 2.3, page 77. Microparticle topography and ultrastructural morphology, size distribution and encapsulation efficiency were determined as described in general methods sections 2.5, and 2.6, pages 83 and 84 respectively.

Release experiments were monitored in Hank's buffer pH 7.4 at 37°C. BSA release was measured using the Bicinochoninic acid assay, section 2.7.1, page 85.

3.2 *Results.*

3.2.1 *Microparticle topography and ultrastructural morphology*

All products from both O/W and W/O/W fabrication techniques produced white free flowing powders when freeze dried. SEM demonstrated that both the microspheres and microcapsules generated were largely spherical with microspheres ranging from 0.6μm to 50μm in diameter and microcapsules ranging from 10-200μm in diameter.

Microspheres produced from P(HB-HV)20% PCL 11, Plate 3a, showed inherent microporosity and generally had a more rugged surface topography than PEA 20% PCL 11 microspheres, plate 3b, which showed no sign of microporosity. A mixture of two distinct types of microspheres were fabricated from the polymer blend

P(HB-HV)20% PCL 11, plate 3c. One type showed microporosity and were larger than the second type which resembled PEA20% PCL 11 microspheres.

Morphological examination of P(HB-HV)20% PCL 11 microcapsules generated by W/O/W emulsification revealed a smooth surface with surface micropores suggesting inherent porosity, plate 3d. These pores ranged from 2.5 to 6.5 μm in diameter. PEA20% PCL 11 microcapsules were spherical with surface ridges and cracks. There did not appear to be any surface micropores, plate 3e. As with microspheres fabricated from P(HB-HV)20% PEA, a mixture of two distinct types of microcapsules were generated. Some had surface characteristics of P(HB-HV)20% PCL 11 microcapsules while others had surface characteristics of PEA20% PCL 11 microcapsules, plate 3f.

There appeared to be little surface deposition of BSA on either microspheres or microcapsules prior to incubation in Hank's phosphate buffer. This was undoubtedly the result of the washing procedure carried out during both fabrication processes.

3.2.2 *The size distribution of microspheres and microcapsules.*

All microparticles were sized at 20% loading with microspheres being generally smaller than microcapsules. This is a result of the higher rate of stirring (10,000 rpm) during microsphere preparation compared with microcapsule fabrication (2200 rpm). P(HB-HV)20% PCL 11 microspheres range from 6 to 50μm in diameter, with a mean diameter of 26.28μm ± 2.34, n=25, (figure 3.1.1). The greatest proportion of P(HB-HV)20% PCL 11 microspheres (24%) had a diameter of between 21 and 25μm. The size of PEA20% PCL 11 microspheres ranged from 0.6 to 5μm in

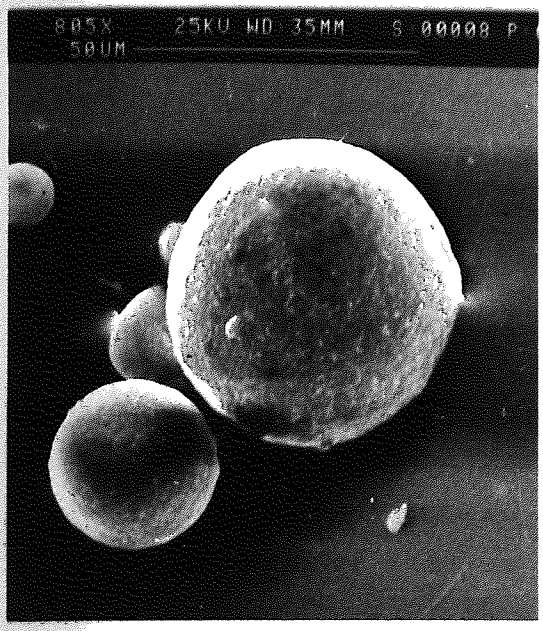


Plate 3a

P(HB-HV)20% PCL11 microspheres prior to incubation (X805)

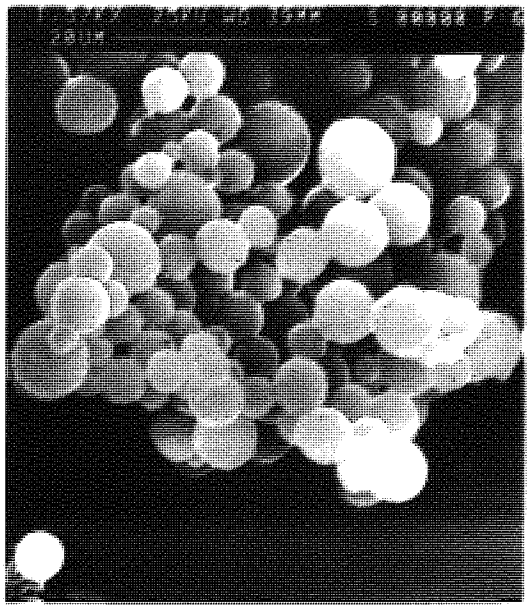


Plate 3b

PEA20% PCL11 microspheres prior to incubation (KX1,57)

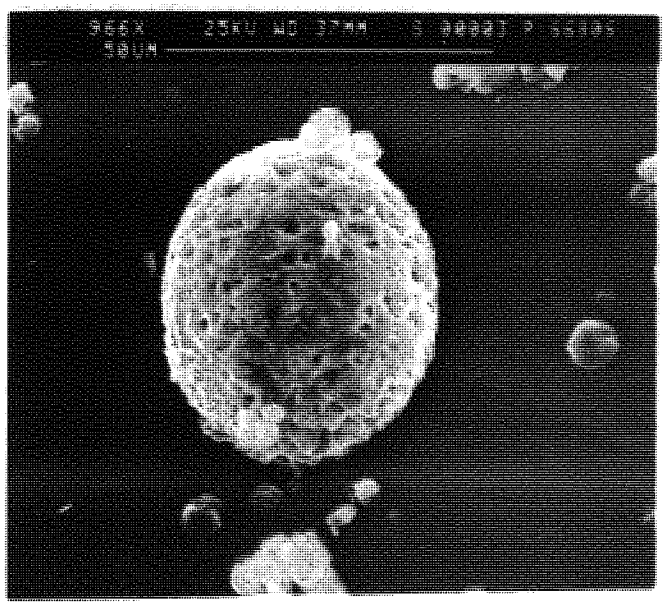


Plate 3c

50%P(HB-HV)50% PEA microspheres prior to incubation (X966).

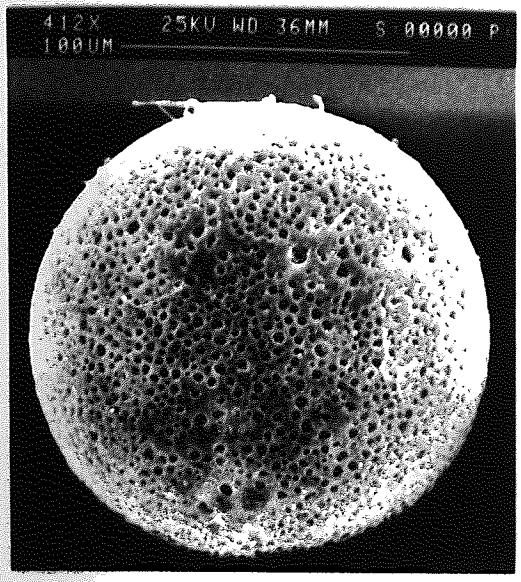


Plate 3d

P(HB-HV)20% PCL11 microcapsule prior to incubation (X412)

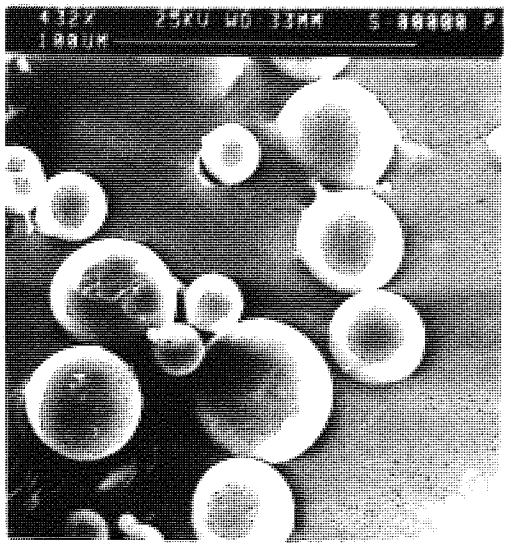


Plate 3e

PEA20% PCL11 microcapsules prior to incubation (X432)

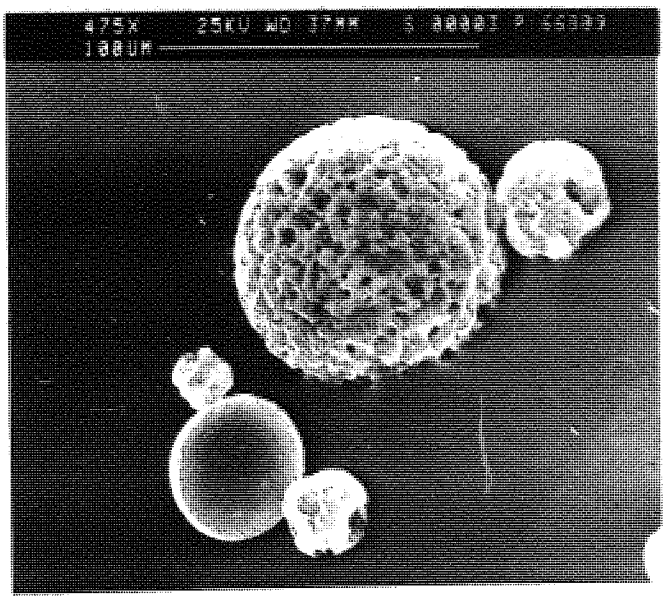


Plate 3f

50%P(HB-HV)50%PEA microcapsules prior to incubation (X475)

Figure 3.1.1

Size distribution of P(HB-HV)/20% PCL11 microspheres. (26.28 ±10.24μm, n=25)

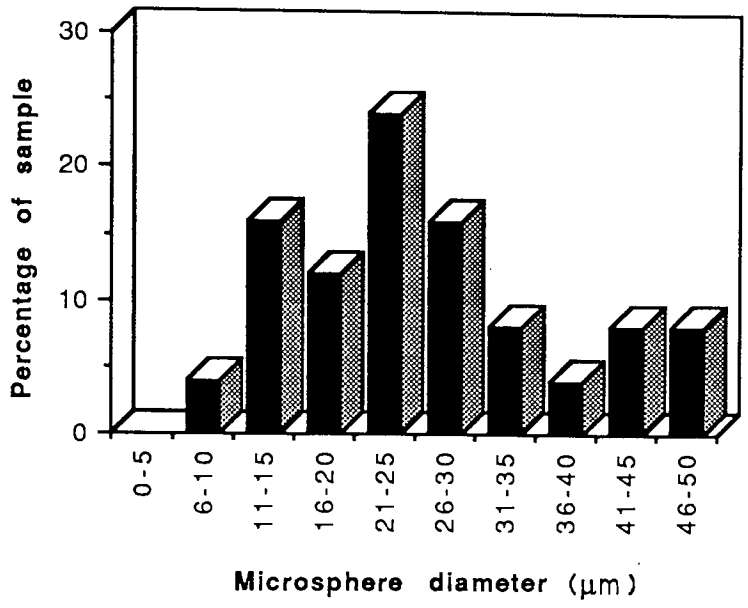


Figure 3.1.2

Size distribution of PEA/20% PCL 11 microspheres. (2.88 ± 11.0μm, n=25)

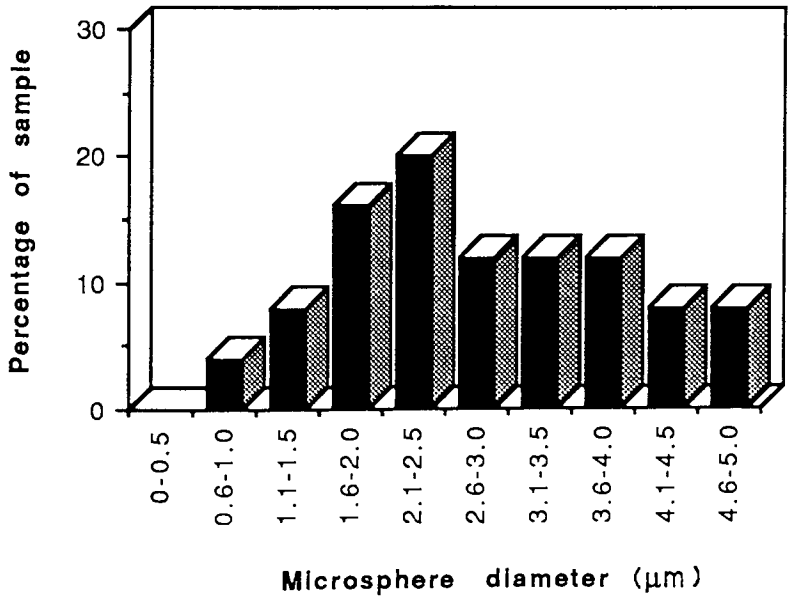


Figure 3.1.3

Size distribution of P(HB-HV)/50% PEA microspheres. (8.86 ± 11.0μm, n=25)

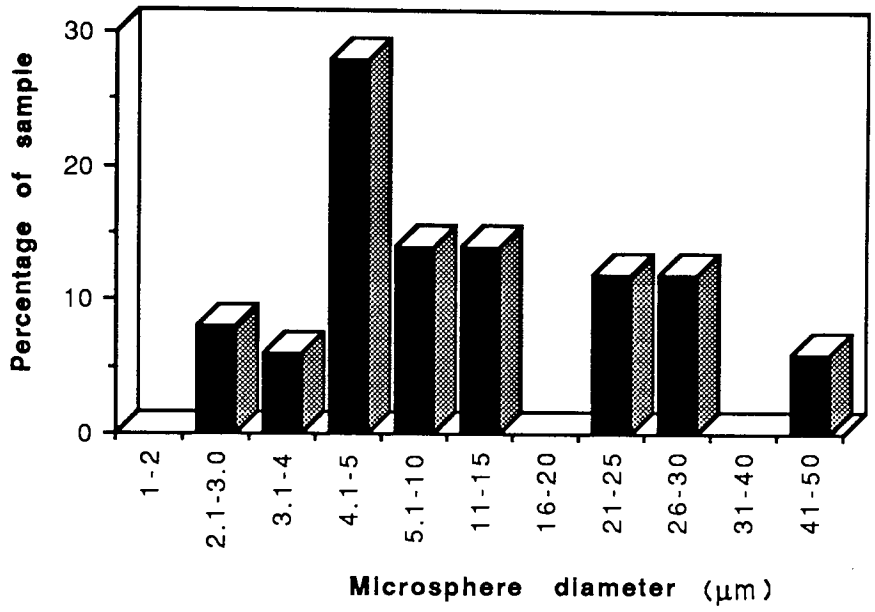


Figure 3.1.4

Size distribution of P(HB-HV)/20% PCL11 microcapsules. (100.24 ± 10.11μm, n=25)

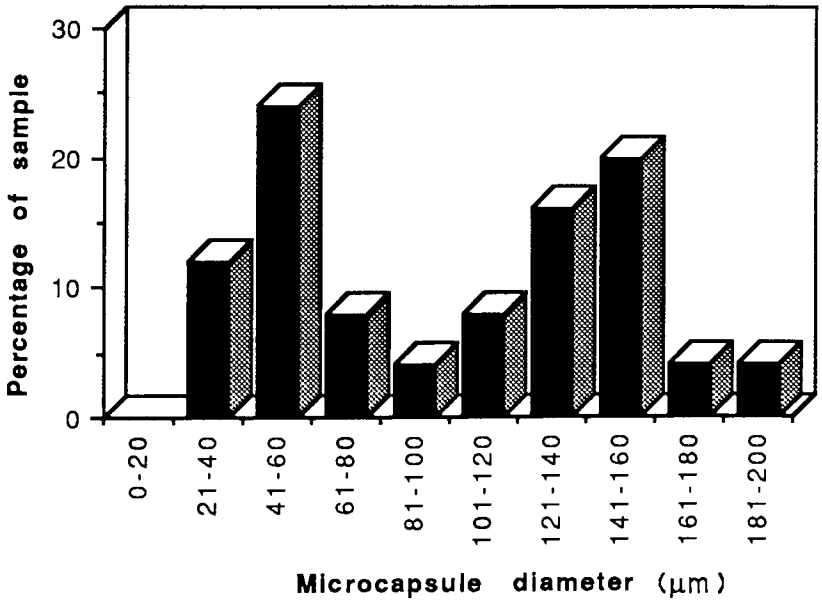


Figure 3.1.5

Size distribution of PEA/20% PCL 11 microcapsules. (37.04 ± 5.47μm, n=25)

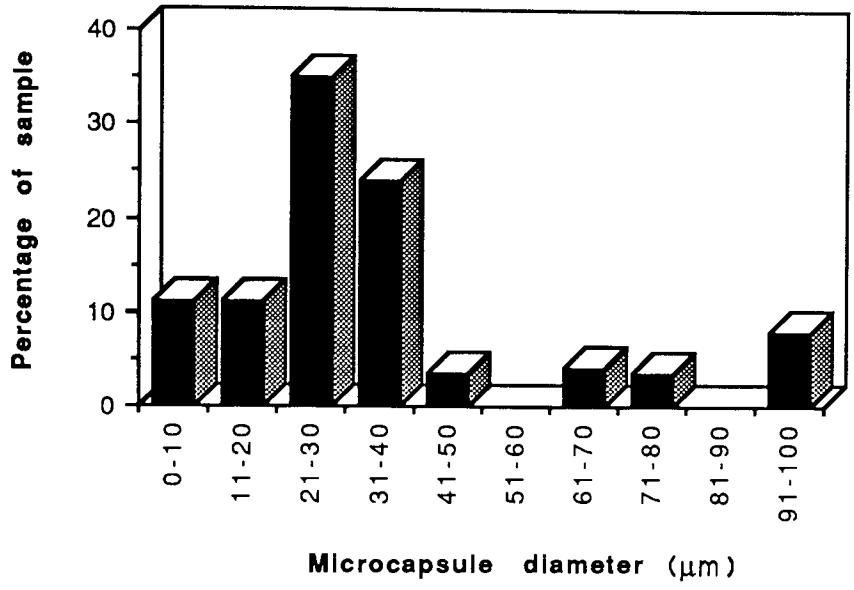


Figure 3.1.6

Size distribution of P(HB-HV)/50% PEA microcapsules. (48.57 ± 26.17μm, n=25)

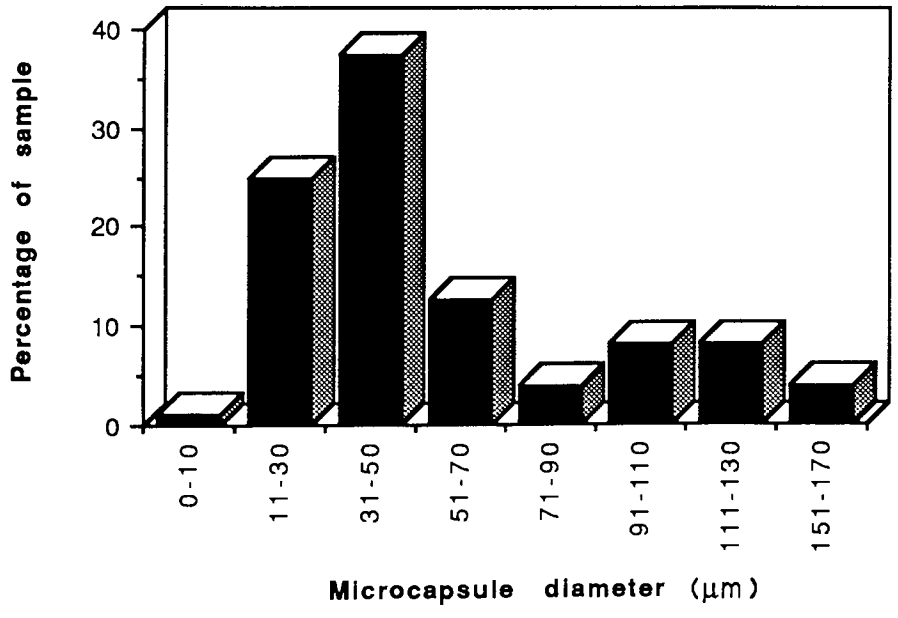


Table 3.1

The theoretical % BSA loading and mean encapsulation efficiency of microspheres and microcapsules. (mean values ± SEM, n=4)

Polymer Formulation	Theoretical Loading [%]	Microsphere Encapsulation Efficiency [%]	Microcapsule Encapsulation Efficiency [%]
P(HB-HV)20% PCL 11	10	5 ± 0.01	5.5 ± 0.06
	20	6.5 ± 0.005	4.3 ± 0.4
	30	9.2 ± 0.07	7.7 ± 0.07
	40	12 ± 0.016	12 ± 0.02
	50	15 ± 0.5[a]	11.5 ± 0.05[a]
PEA20% PCL 11	10	4.2 ± 0.09	6.6 ± 0.2
	20	4.6 ± 0.04	8.1 ± 0.02
	30	7.5 ± 0.06	12.5 ± 0.06
	40	8.3 ± 0.03	15 ± 0.05
	50	11 ± 0.1[a]	12 ± 0.04[a]
P(HB-HV)50% PEA	10	6 ± 0.1	5 ± 0.03
	20	8.5 ± 0.03	5.5 ± 0.04
	30	11.5 ± 0.14	7.5 ± 0.025
	40	13.5 ± 0.09	10 ± 0.29
	50	14.5 ± 0.06[a]	12 ± 0.03[a]

a, significantly elevated, $p<0.05$ when compared with all other % loadings for that polymer blend.

diameter with a mean diameter of 2.88 ±0.23μm, n=25, (figure 3.1.2). The greatest proportion of PEA microspheres (20%) had a diameter of between 2.1 and 2.5μm. P(HB-HV)/20% PCL 11 microcapsules (figure 3.1.4) ranged from 21 to 200um in diameter, with a mean diameter of 100.24 ±10.11μm, n=25. The greatest proportion of P(HB-HV)20% PCL 11 microcapsules had a diameter of between 41 and 60μm (24%). PEA/20% PCL 11 microcapsules (figure 3.1.5) ranged from between 8 to 100μm in size, with a mean diameter of 37.04 ±5.47μm, n=25 and the greatest proportion were between 21 and 30μm in size.

3.2.3 *Effects of BSA loading on encapsulation efficiency and release kinetics.*

The effect of percentage BSA loading on the encapsulation efficiency and dissolution kinetics was investigated by fabricating microspheres and microcapsules with BSA loadings of 10,20,30,40 and 50% by dry weight of polymer. The encapsulation efficiencies achieved were low in all cases and appeared to be directly related to initial theoretical percentage loading. Microspheres fabricated using the O/W single emulsion system attained the highest encapsulation efficiency with an initial theoretical loading of 50% regardless of polymer formulation (table 3.1). P(HB-HV)/50% PEA microcapsules fabricated using the W/O/W double emulsion system also achieved their highest encapsulation efficiency at a theoretical loading of 50%. In the case of microcapsules fabricated from the polymer blends P(HB-HV)20% PCL 11 and PEA20% PCL 11, the optimum theoretical loading was 40% with encapsulation efficiencies of 12% and 15% respectively. P(HB-HV)20% PCL 11 and PEA/20% PCL11 microcapsules showed the greatest encapsulation efficiency, at 15% and 17% respectively.

3.2.4 *In vitro BSA release from microspheres and microcapsules*

BSA release profiles obtained over a 30 day period at 37°C indicated that the total cumulative release of BSA from both microcapsules and microspheres fabricated from P(HB-HV)20% PCL 11, PEA20% PCL 11 and P(HB-HV)50% PEA increased with % loading. The release of BSA from microspheres was characterised by an initial lag period lasting from 6-9 days followed by an exponential release, which lasted from 6-11 days. This was followed by a period of low level sustained release which gradually decreased until the vehicle was either exhausted ie BSA could not be detected, or sampling was discontinued. Microspheres prepared from P(HB-HV)/20% PCL 11 exhibited sigmoidal release curves at all percentage loadings (figure 3.1.7, page 103). With an initial lag phase followed by exponential release starting at approximately day 6. At this point there was no significant difference in BSA release from 40% and 50% BSA loaded microspheres. With 10%, 20% and 30% loaded microspheres BSA release began to decline after day 13 but 40% and 50% loaded microspheres continued to release BSA up to day 17, although there was still no significant difference in BSA release between 40% and 50% loaded microspheres at day 15, figure 3.1.7 page 103. P(HB-HV)20% PCL 11 microspheres, at all percentage loadings had stopped releasing detectable BSA by day 24. Microspheres prepared from PEA20% PCL 11 effectively stopped releasing BSA by day 22, irrespective of the percentage loading, figure 3.1.8, page104. In this case the release profile was characterised by an extended lag phase lasting

some 10 days followed by exponential release phase whose magnitude increased with increasing BSA loading up to a loading of 40%. There was no significant difference in BSA release from 40% and 50% loaded microspheres at day 6 or day 15. BSA release from 40% and 50% loaded microspheres was maintained up to day 15 after which release gradually decreased to undetectable levels by day 27-28, figure 3.1.8. BSA release profiles obtained from P(HB-HV)50% PEA microspheres were again characterised by an initial lag phase followed by an exponential release of BSA from day 6 which continued upto day 16. Low level sustained release could be detected up to day 23, figure 3.1.9. By day 15, 50% BSA loaded PEA20% PCL 11 microspheres released significantly more BSA than similarly loaded P(HB-HV)20% PCL 11 and P(HB-HV)50% PEA microspheres, $p<0.025$ for both.

Figures 3.2.0, 3.2.1 and 3.2.2 respectively summarise the total cumulative release of BSA from P(HB-HV)20% PCL 11, PEA20% PCL 11 and P(HB-HV)50% PEA microcapsules over a 30 day period. BSA release increased with theoretical percentage loading regardless of the polymer blend used. Clearly, although the general profile of release was very similar for PEA20% PCL 11 and P(HB-HV)50% PEA microcapsules, the former showed an extended lag phase which might of been the product of less BSA deposited peripherally within the microcapsule shell. With the two polymer blends, PEA20% PCL 11 and P(HB-HV)50% PEA, the majority of BSA load was released over a 6 to 8 day period, eg. 50% BSA loaded PEA20% PCL 11 and P(HB-HV)50% PEA microcapsules released 94% and 98% of total BSA load respectively during the exponential phase. By day 15, 50% BSA loaded P(HB-HV)50% PEA microcapsules showed a significantly greater BSA release than 50% loaded PEA20% PCL 11 microcapsules, $p < 0.05$. BSA release from P(HB-HV)20% PCL 11 microcapsules was

characterised by steady increase with time, figure 3.2.0. The rate of BSA release, increased with increasing in percentage loading and significantly more BSA was released from 50% loaded microcapsules than from 40% loaded microcapsules by day 15, $p < 0.05$. However, significantly more BSA was released from 50% loaded P(HB-HV)20% PCL 11 microcapsules at day 15 than from similarly loaded P(HB-HV)20% PCL 11 microcapsules, $p < 0.05$. No BSA release could be detected from P(HB-HV)20% PCL 11 microcapsules after 24 days incubation.

Figure 3.1.7 The total cumulative release of BSA from P(HB-HV)PCL 11 microspheres in Hank's buffer,pH 7.4 (mean values ± SEM, each point n=12) .

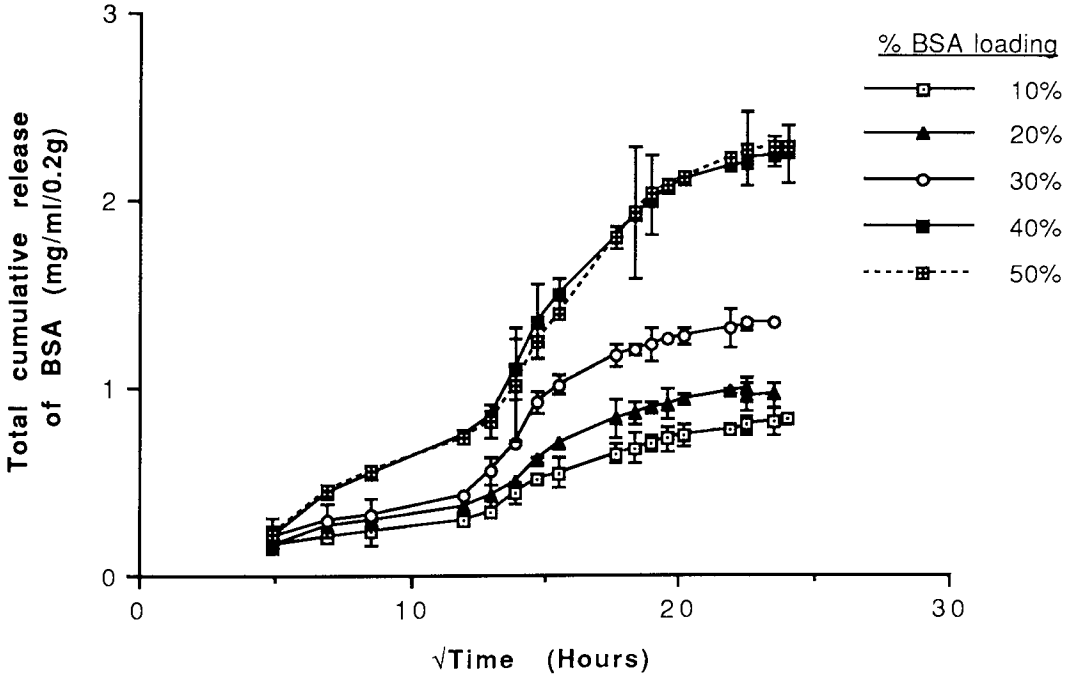


Figure 3.1.8 The total cumulative release of BSA from PEA/20% PCL 11 microspheres in Hank's buffer, pH 7.4, (mean values ± SEM, each point n=12)

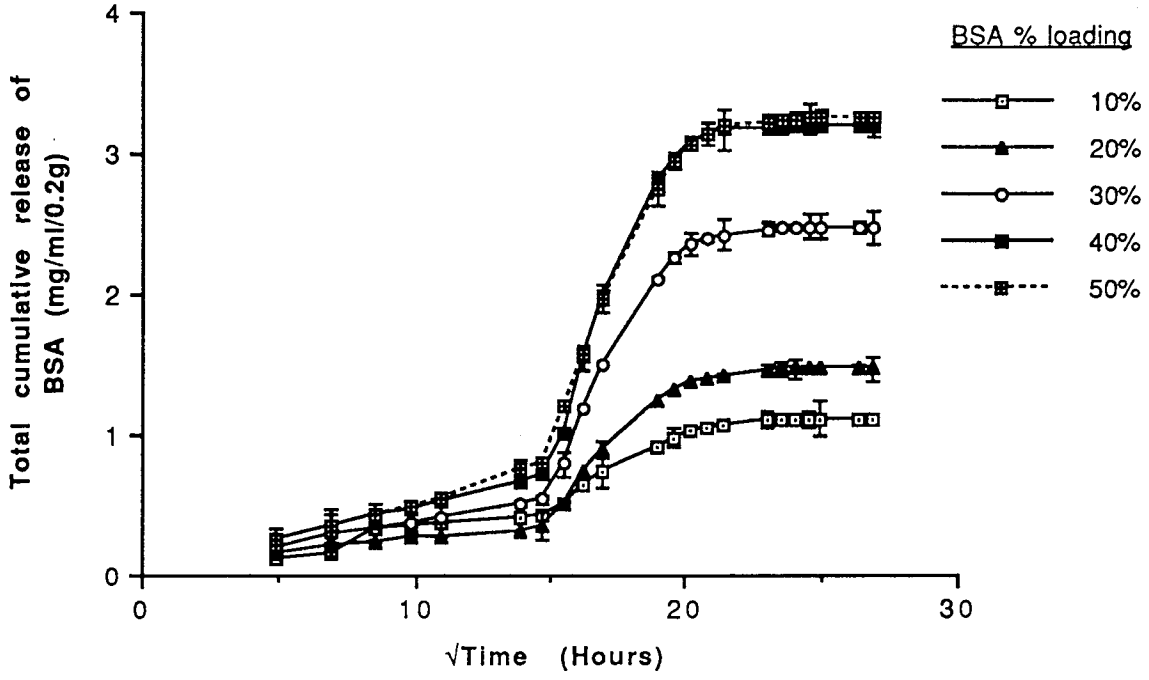


Figure 3.1.9 The total cumulative release of BSA from P(HB-HV)/50% PEA microspheres in Hank's buffer, pH 7.4, (mean values ± SEM, each point n=12).

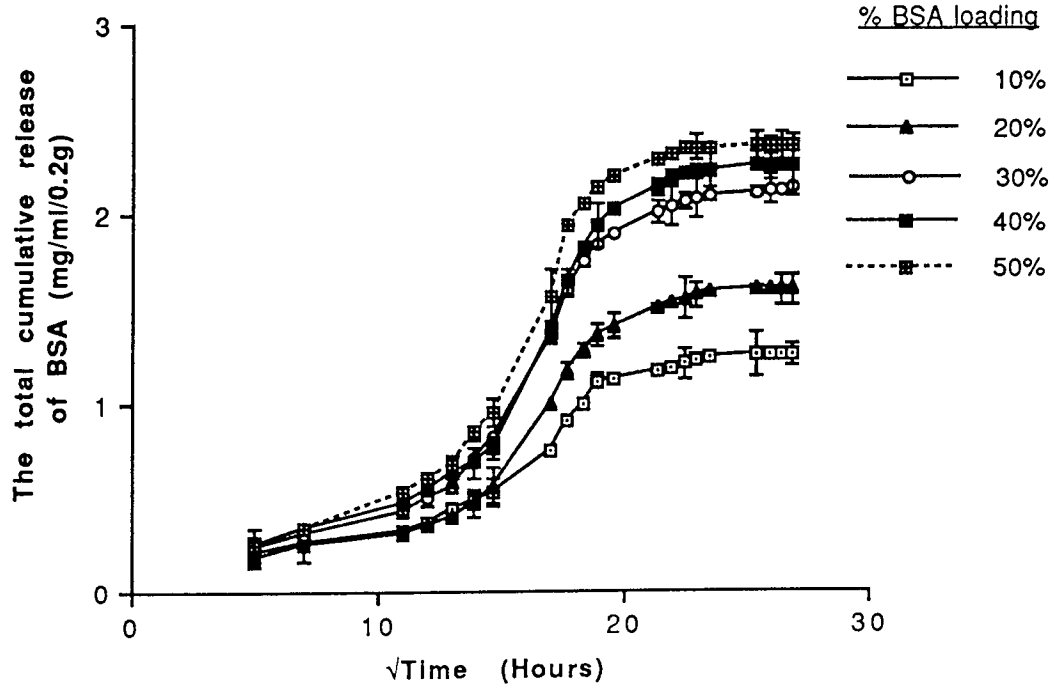


Figure 3.2.0 The total cumulative release of BSA from P(HB-HV)/20% PCL 11 microcapsules in Hank's buffer, pH 7.4 (mean values ± SEM, each point n=12.

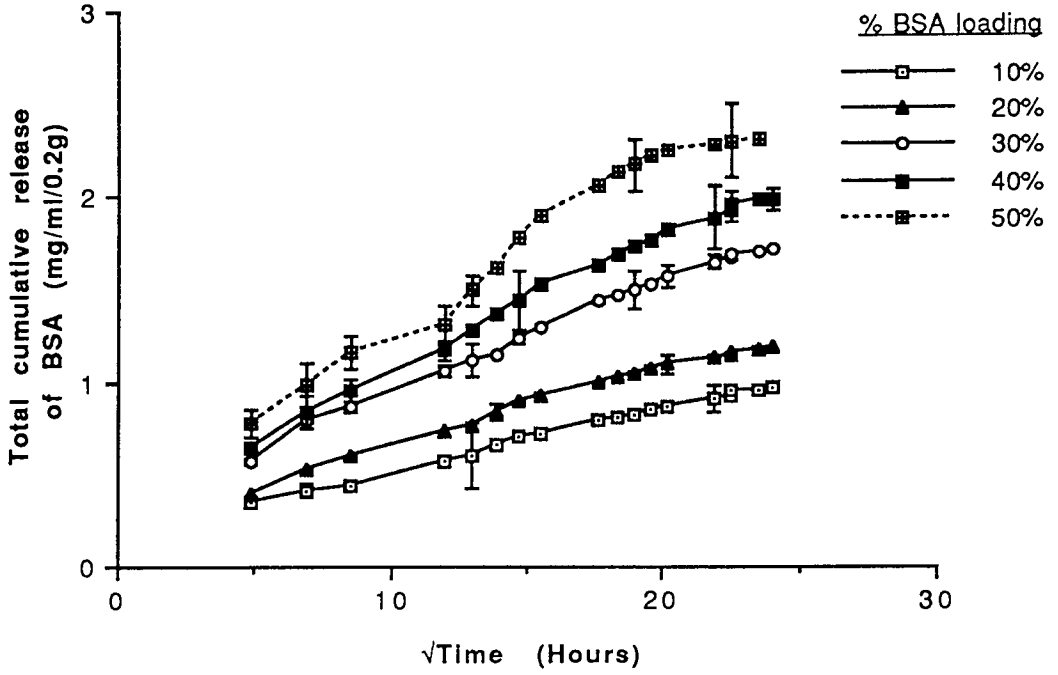


Figure 3.2.1 The total cumulative release of BSA from PEA/20% PCL 11 microcapsules in Hank's buffer, pH 7.4 (mean values ± SEM, each point n=12)

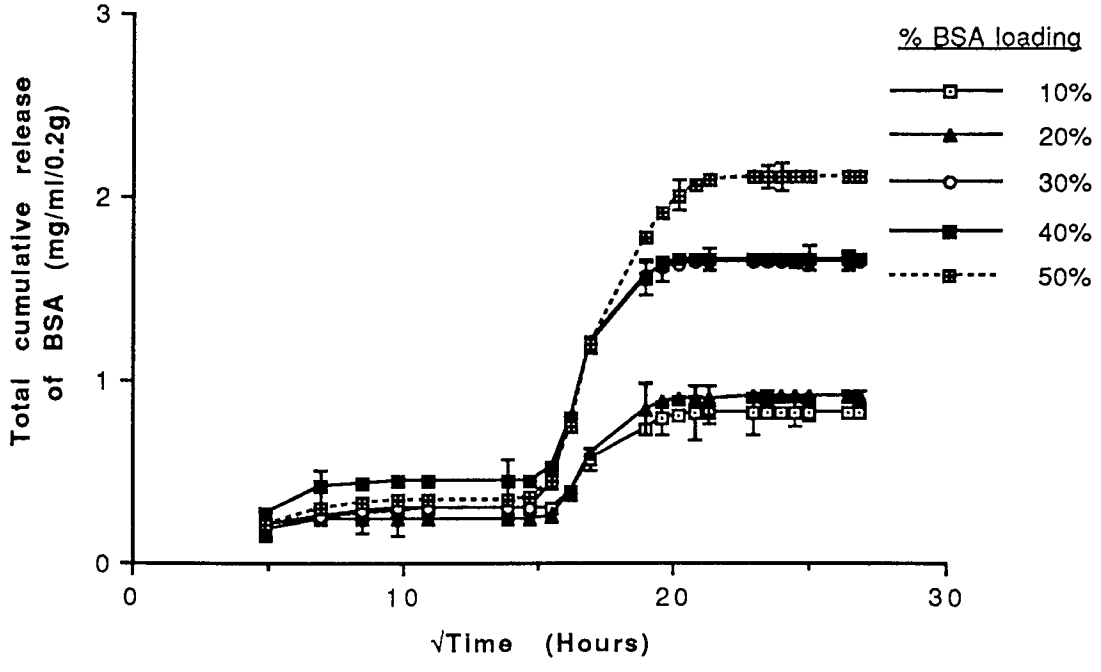
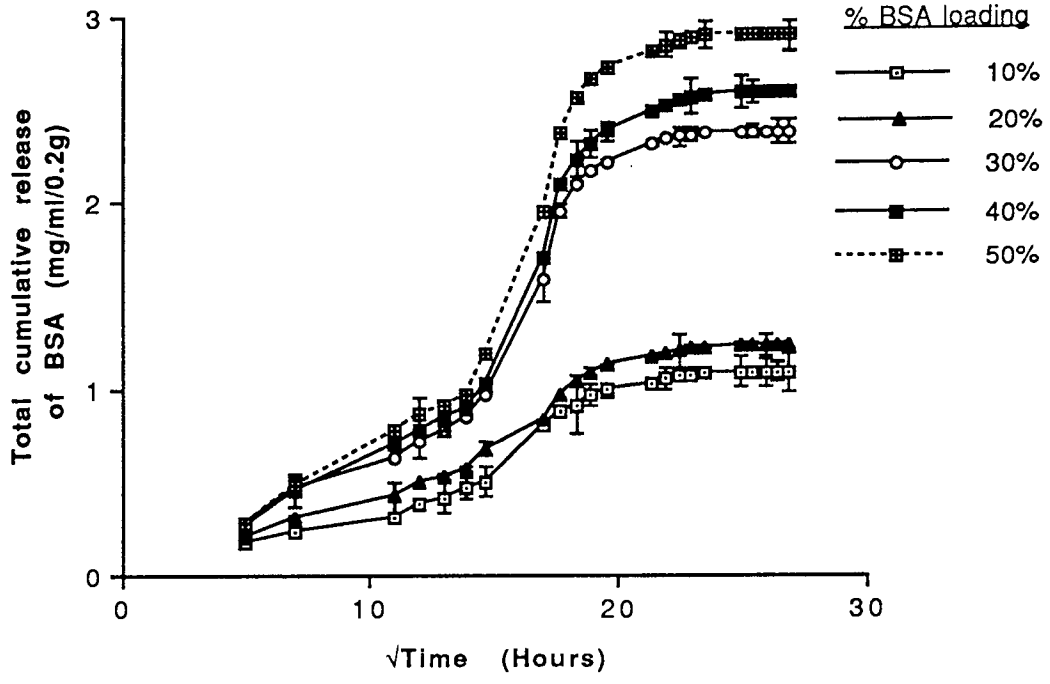


Figure 3.2.2 The total release of BSA from P(HB-HV)/50% PEA microcapsules in Hank's buffer, pH7.4 (mean values ± SEM, each point n=12).



3.3 Discussion

Both microspheres and microcapsules released BSA over a period of between 24 and 26 days. BSA release from P(HB-HV)20% PCL 11 microcapsules increased steadily with time. This suggested that the release of the surrogate macromolecule BSA,from P(HB-HV)20% PCL 11 microcapsules was via diffusion through inherent micropores. The latter run through the polymer capsule from the hollow central reservoir to the external environment. Such micropores could be identified morphologically down the electron microscope and were between 2μm and 6μm in diameter. Very small micropores (0.2 to 0.8μm) were also present on the surface of P(HB-HV)20% PCL 11 microspheres. However, the release of BSA from P(HB-HV)20% PCL 11 microspheres was not zero order but just like release from microspheres and microcapsules fabricated from

PEA20% PCL 11 and P(HB-HV)50% PEA was characterised by an initial lag phase followed by exponential release lasting 6-11 days. The micropores present at the surface of P(HB-HV)20% PCL 11 microspheres were much smaller in diameter than those associated with microcapsules fabricated from the same polymer blend. It is possible that the formation of a finer microemulsion during the microsphere fabrication process might have been instrumental in the generation of these micropores and their small size might of retarded the flux of BSA through the microsphere matrix. Although PCL 11 was blended with PEA during the fabrication of PEA microspheres and microcapsules, there was no evidence of micropore formation. The presence of precipitated polymer during PEA microparticle fabrication indicated that a stable emulsion was not formed possibly due to a lack of miscibility. It is possible that the precipitation rates of PEA and PCL 11 are so different that the PCL 11 remains seperated from PEA during microparticle preparation and is therefore not forced out of the polymer matrix forming micropores when the matrix contracts as is the case when blended with P(HB-HV).

It is likely that BSA was released from P(HB-HV)20% PCL 11 microspheres and P(HB-HV)50%PEA and PEA20% PCL 11 microspheres and microcapsules via channels created in the microcapsule membrane or the microsphere matrix by the dissolution in penetrating incubation medium. The BSA would diffuse down a concentration gradient to the exterior environment by Fickian diffusion creating pores and channels, which would subsequently facilitate the further efflux of BSA through the microcapsule or from deeper in the microsphere matrix. Clearly the distribution of BSA within the microsphere matrix will significantly influence release kinetics. BSA crystals were not

observed on the surface of either microspheres produced from P(HB-HV)20% PCL 11 microspheres or microcapsules fabricated from P(HB-HV)20% PCL 11 and PEA 20% PCL 11 consequently no evidence of a burst release was observed in the release profiles. On the contrary, an initial lag phase occurred which lasted 6-8 days followed by a period of exponential BSA release. This could be explained by the sparse distribution of BSA at the compressed periphery of the microsphere and a greater concentration of BSA at the central region. As the incubation media gradually penetrates into the matrix of the microsphere it dissolves small quantities of peripherally located BSA which subsequently leaches out of the polymer matrix leaving pores and channels. At a critical point around 6-8 days the penetrating incubation media begins to dissolve the centrally located BSA which diffuses out of the matrix through the many pores and channels. This results in a substantial release of BSA over the subsequent 6-11 day period and the eventual exhaustion of the encapsulated macromolecule.

Although BSA release could be maintained for up to 24 days the encapsulation efficiencies of both microcapsules and microspheres were very low. Various explanations have been put forward to account for this. Jalil and Nixon have suggested that water soluble macromolecules partition down a concentration gradient into the continuous aqueous phase during the O/W or W/O/W fabrication process, resulting in a low encapsulation efficiency[13]. In the case of microcapsules prepared using the W/O/W solvent extraction technique the purpose of adding agarose to the inner aqueous phase, was to maintain the integrity and seperate the inner aqueous phase from the continuous phase thus preventing the diffusion of BSA into the outer continuous phase. It is possible that direct contact between the inner aqueous phase and the continuous

phase may have occurred as a result of a local demulsification caused by vigorous stirring. This removed the barrier between the two phases and allowed the partitioning of BSA to take place.

A second possible reason for the low encapsulation efficiencies seen with microcapsules produced using the W/O/W technique is the presence of contractile forces operating at the time of fabrication. During the course of dichloromethane extraction, and especially as the polymer precipitates from solution, the organic phase experiences contraction forces. These are caused by the substantial volume decrease which accompanies the precipitation of the polymer out of solution. The intense contraction in the polymer phase must be accompanied by a substantial reduction in the volume of inner aqueous phase, which it encapsulates. The inner aqueous phase is lost through the micropores formed by the precipitation of PCL 11 to the continuous aqueous phase. The true effect of these contractile forces on encapsulation efficiency can not be judged without further investigations.

CHAPTER FOUR

IN VITRO BIODEGRADATION OF MICROSPHERES AND MICROCAPSULES FABRICATED FROM POLY[β–HYDROXYBUTYRATE/HYDROXYVALERATE] AND POLY[ETHYLENE ADIPATE].

4.0 *Introduction.*

Poly-β-Hydroxybutyrate/3-Hydroxyvalerate [P(HB-HV)] and Polyethylene adipate [PEA] are essentially biodegraded by a hydrolytic mechanism[6]. The rate of ester hydrolysis is affected by inherent factors such as polymer molecular weight, crystallinity, copolymer content and by external factors such as pH, temperature and polymer processing[6]. There is also some evidence to suggest that enzymatic action may play a part in the biodegradation process[6,138]. Therefore, although *in vitro* degradation of polyesters by simple chemical hydrolysis has been shown to give a reasonably direct correlation with degradation *in vivo,* biodegradtion in different body sites will be profoundly effected by local environmental pH and may also be the result of enzymatic attack.

In order to gain some understanding of how microspheres and microcapsules composed of P(HB-HV)20% PCL 11 and PEA20% PCL 11 biodegrade *in vivo* when administered via a parenteral route or by the gastrointestinal route the microparticulates were incubated in either newborn calf serum or pancreatin and synthetic gastric juice respectively. Incubation in Hank's buffer, pH 7.4, will provide information on polymer degradation resulting from simple chemical hydrolysis and provide some insight into the possible mechanism and morphological basis for the release of a surrogate protein such as BSA *in vitro.*

4.1 *Methodolgy.*

P(HB-HV) and PEA microspheres were fabricated using the O/W emulsion solvent extraction technique section 2.2, page 74, while microcapsules were prepared with the same polymers using the double emulsion solvent extraction technique, section 2.3

page 77. Both polymers were blended with 20% PCL 11, a compound which is hydrophobic and crystalline in nature, in an attempt to induce macroporosity into the microparticle matrix[131]. The microparticulates were loaded with 5,10,15,20 and 25% BSA by weight of polymer, table 2.1, page 76.

Four different types of incubation media were used for the *in vitro* biodegradation studies. Hank's buffer, pH 7.4, undiluted non heat-treated newborn calf serum, synthetic gastric juice pH 0.8 (which contained 10% pepsin) and 1.5% porcine pancreatin solution, pH 7.4 which was made up with Hank's buffer, pH 7.4 and contained 2% penicillin/streptomycin to suppress microbial activity. 0.2g of microspheres or microcapsules was added to screw capped sterile tubes containing 2ml of either Hank's buffer, pH 7.4; newborn calf serum; synthetic gastric juice or pancreatin solution each containing 0.1ml of amphotericin B (fungizone) to inhibit any subsequent fungal growth and incubated at 37°C with daily agitation. Biodegradation of the microparticulates loaded with 5,10,15,20 and 25% BSA was monitored gravimetrically after a 30 day period, section 2.8, page 87. Changes in gross and ultrastructural morphology were monitored using SEM as previously described, section 2.8, page 87.

4.2 *Results.*

4.2.1 *Percentage weight loss (PWL) from microspheres and microcapsules.*

The lowest PWL from both microspheres and microcapsules irrespective of % BSA loading was observed after incubation in Hanks buffer, figure 4.1. In this buffer microspheres and

microcapsules composed of P(HB-HV)20% PCL 11 showed a significantly higher PWL than PEA microparticulates, $p < 0.05$. However, P(HB-HV)20% PCL 11 microcapsules showed a significanctly higher mean PWL (28.11 ± 0.917%) than P(HB-HV)20% PCL 11 microspheres (25.78 ± 0.8%). P(HB-HV)20% PCL 11 microspheres and microcapsules demonstrated a gradual increase in PWL with increasing BSA loading up to 15% but decreased thereafter, figure 4.1. The PWL from PEA/20% PCL 11 microspheres and microcapsules was low in Hank's buffer (12.9 ± 0.79% and 12.5 ± 0.15% respectively) and not significantly influenced by BSA loading.

High PWL values were observed for both microspheres and microcapsules after 30 days incubation in newborn calf serum, figure 4.2. Microcapsules showed significantly higher PWL values than microspheres ($p < 0.05$) irrespective of the fabrication polymer used. Although, PEA20% PCL 11 microcapsules and microspheres demonstrated significantly higher PWL ($p < 0.05$) than corresponding P(HB-HV)20% PCL 11 microcapsules and microspheres, Figure 4.2.

As with newborn calf serum, microcapsules incubated in 1.5% pancreatin solution showed significantly higher PWL values than microspheres fabricated from the same polymer, irrespective of percentage loading, ($p < 0.05$), figure 4.3. Generally, PEA20% PCL 11 microspheres and microcapsules showed significantly higher ($p<0.05$) mean PWL values in pancreatin solution than P(HB-HV)20% PCL 11 microspheres and microcapsules except at the very highest BSA loading, Figure 4.3.

Similar trends in PWL were also observed for microspheres and microcapsules after 30 days incubation in synthetic gastric juice containing 10% pepsin, figure 4.4.

Figure 4.1 PWL from microspheres and microcapsules after 30 days incubation in Hank's buffer at pH 7.4 and 37°C (mean values ± SEM, n=4)

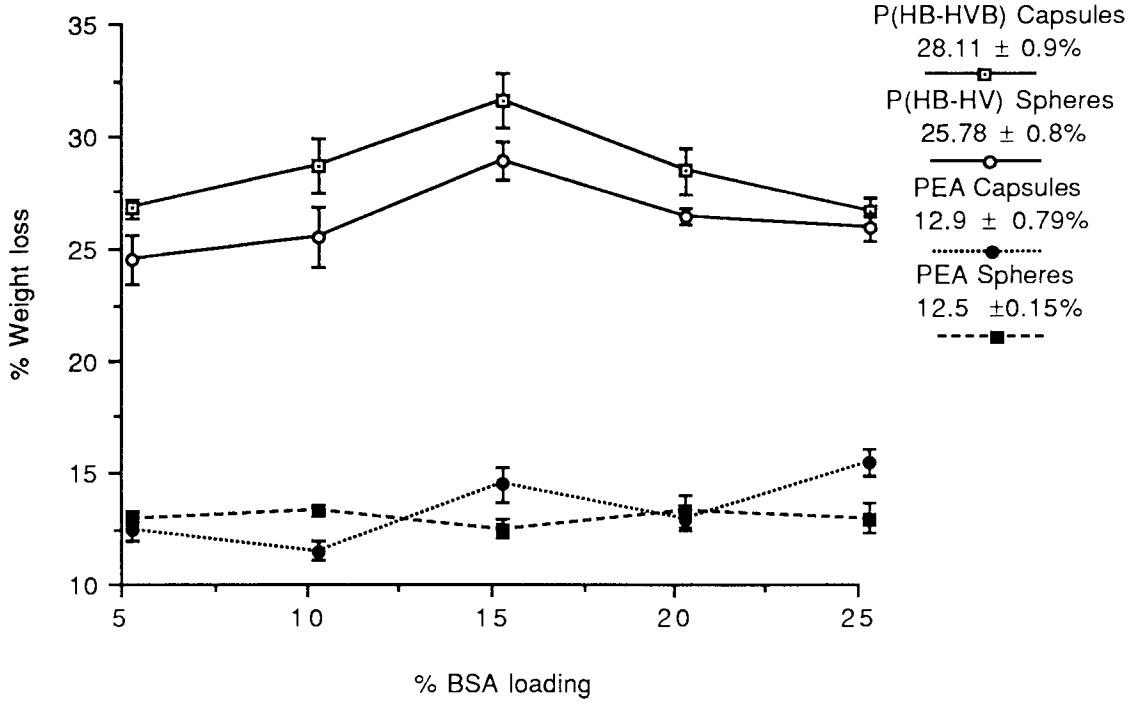


Figure 4.2 PWL from microspheres and microcapsules after 30 days incubation in newborn calf serum at 37°C. (mean values ± SEM, n=4)

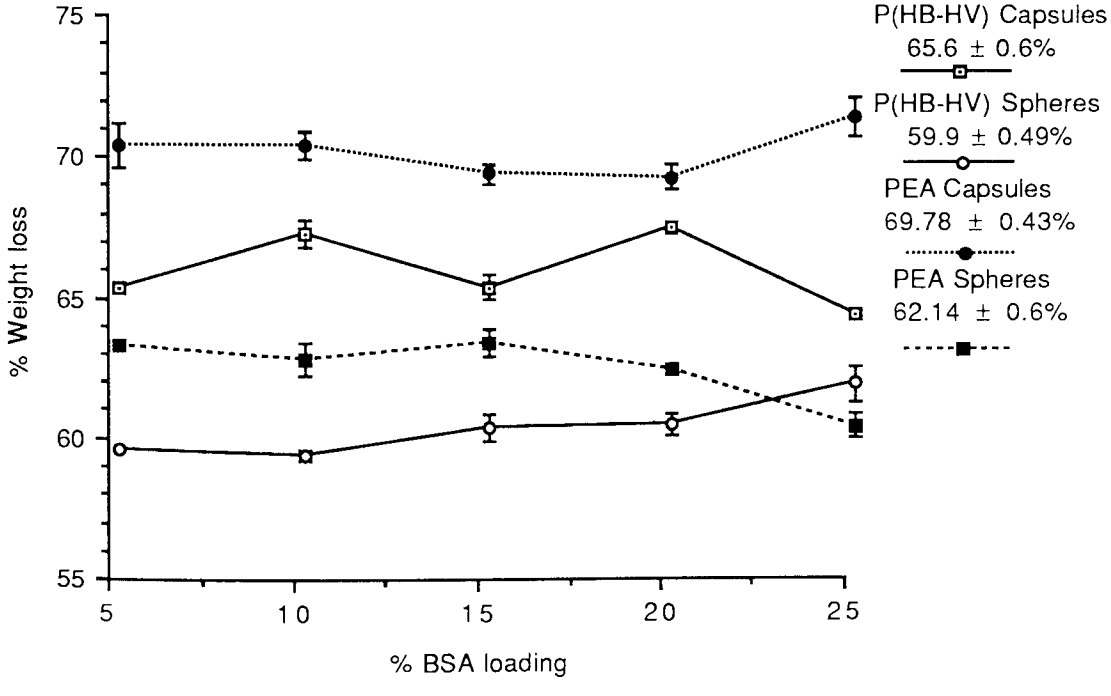


Figure 4.3 PWL from microspheres and microcapsules after 30 days incubation in 1.5% pancreatin at 37°C. (mean values ± SEM, n=4)

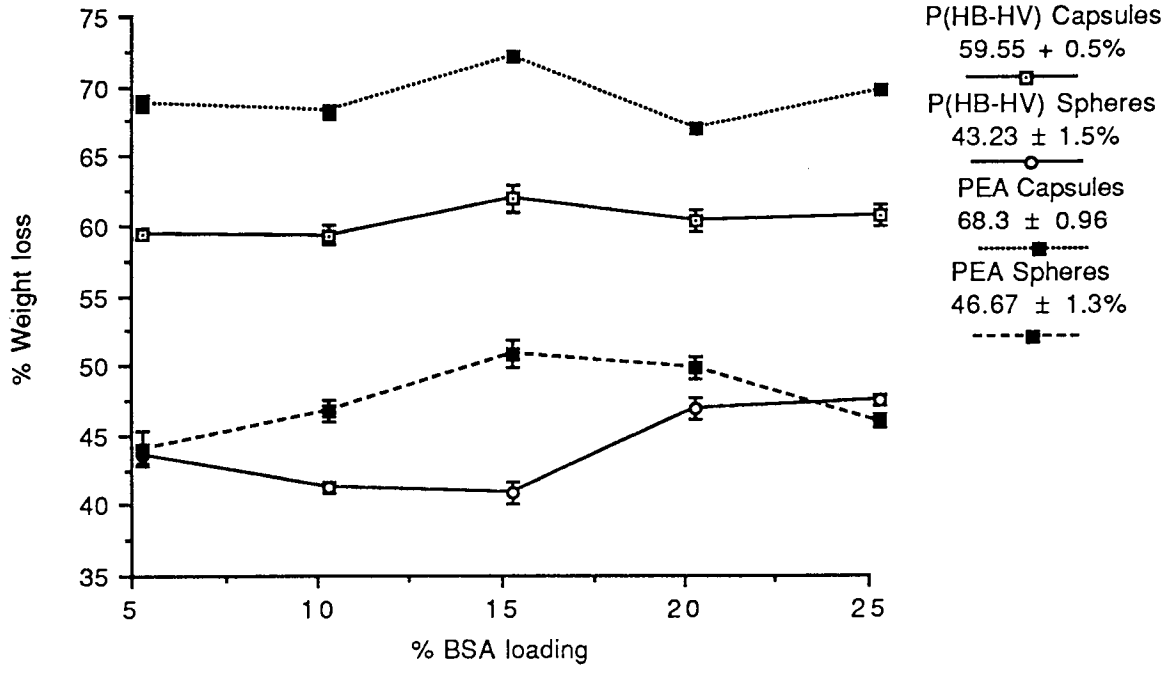
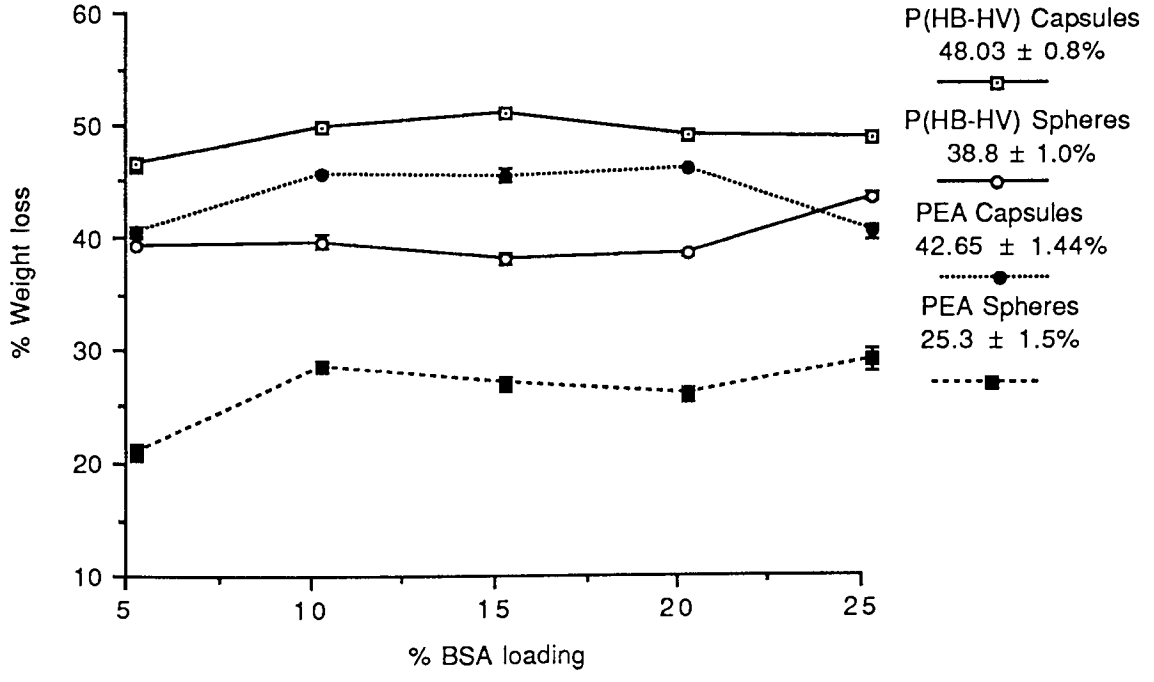


Figure 4.4 PWL from microspheres and microcapsules after 30 days incubation in synthetic gastric juice containing 10% pepsin at 37° C (mean values ± SEM , n=4).



Again, microcapsules showed significantly higher PWL values than microspheres, irrespective of the fabrication polymer. Furthermore, P(HB-HV)20% PCL 11 microcapsules and microspheres, showed significantly greater ($p < 0.05$) PWL in synthetic gastric juice than microcapsules and microspheres fabricated using PEA20% PCL 11 at all percentage BSA loadings, figure 4.4.

Overall these PWL data suggest that P(HB-HV)20% PCL 11 microcapsules and microspheres show a greater percentage weight loss than either PEA20% PCL 11 microcapsules or microspheres in Hank's buffer. This differentiation between polymers in terms of PWL could be the result of greater chemical hydrolysis of P(HB-HV)20% PCL 11 compared with PEA20% PCL 11. However, incubation in newborn calf serum, pancreatin and synthetic gastric juice resulted in the differentiation of microcapsules from microspheres in terms of PWL, with microcapsules consistently exhibiting greater PWL values than microspheres irrespective of fabrication polymer. It is possible that when incubated in these media, enzymatic biodegradation of either BSA or the polymer matrix may have occurred, which resulted in the generation of pores and channels and a subsequent PWL. In newborn calf serum such enzymatic biodegradation could have resulted from low level esterase activity or significant protease activity originating from alpha and beta globulin fractions of plasma proteins. In the case of synthetic gastric juice, the enzyme pepsin, a carboxy peptidase, may result in enzymatic degradation and pancreatin solution contains numerous enzymes such as trypsin, chymotrypsin, elastase, carboxy peptidase A, cholesterol ester hydrolase, phospholipase A_2, lipase and amylase which could potentially result in the enzymatic biodegradation of P(HB-HV) and PEA. Clearly, due to their inherent architecture,

microcapsules are more susceptible to a loss of structural integrity resulting from either enzymatic and/or hydrolytic attack than microspheres.

When incubated in newborn calf serum and pancreatin solution PEA20% PCL 11 microparticulates showed greater mean PWL values than P(HB-HV)20% PCL 11 microcapsules or microspheres. As both newborn calf serum and pancreatin have a high enzyme content it might this suggest that PEA is more susceptible to enzymatic biodegradation than P(HB-HV)20% PCL 11, or at least to biodegradation by the enzymes present in these two media. However, despite the presence of pepsin in gastric juice the PWL data for P(HB-HV)20% PCL 11 and PEA20% PCL 11 microparticulates incubated in this medium resembled PWL data obtained when microparticulates were incubated in Hank's buffer. Indeed P(HB-HV)20% PCL 11 microparticulates showed greater mean PWL values than microparticulates fabricated using PEA20% PCL 11, although the weight losses were higher in gastric juice than in Hank's buffer. This might suggest that a more chemically associated hydrolytic effect, ie pH influence, was responsible for the PWL in synthetic gastric juice, with the presence of pepsin resulting in slightly greater PWL value than was found in Hank's buffer. This would result in an overall weight loss picture whereby weight loss of microparticulates in gastric juice would fall half way between Hank's buffer on the one hand and newborn calf serum and pancreatin on the other.

Weight loss from PEA20% PCL 11 microcapsules and microspheres incubated in Hank's buffer did not appear to be influenced to any great extent by % BSA loading. However, the PWL of P(HB-HV)20% PCL 11 microparticulates increased significantly

between 5% to 15% BSA loading ($p < 0.05$) and then declined significantly when the BSA loading was increased from 15% to 25% ($p < 0.05$). In newborn calf serum the PWL shown by PEA20% PCL 11 and P(HB-HV)20% PCL 11 microcapsules appeared to fluctuate, showing no real trend, other than differing absolute PWL terms, ie the PWL from 25% BSA loaded PEA20% PCL 11 microcapsules was significantly greater than from 25% BSA loaded P(HB-HV)20% PCL 11 microcapsules, $p < 0.05$. However, the PWL of PEA20% PCL 11 microspheres appeared to decline with percentage loading, while the PWL of P(HB-HV)20% PCL 11 microspheres tended to gradually increase with loading up to 25%. In pancreatin, other than the obvious absolute differences in PWL at each percentage BSA loading, PWL loss from PEA20% PCL 11 and P(HB-HV)20% PCL 11 microcapsules did not appear to vary significantly with percentage loading. PEA20% PCL 11 microspheres, on the other hand, showed an initial increase in PWL with percentage BSA loading followed by a significant decline in PWL as the loading increased from 15% to 25%. P(HB-HV)20% PCL 11 microspheres tended to show an increase in PWL with increasing BSA loading. It is possible that the macroporosity of P(HB-HV)20% PCL 11 microspheres allowed enzymes present in newborn calf serum and pancreatin solution to quickly penetrate and breakdown BSA dispersed within the polymer matrix, resulting in increased PWL with increased BSA loading. The absence of macroporosity in microspheres fabricated using PEA20% PCL 11 might have bought about the reverse situation in these two media, thereby resulting in a general decline in PWL values as the BSA % loading increases.

4.2.2 *Morphological appearance of microspheres and microcapsules after incubation in Hank's buffer, newborn calf serum, pancreatin and synthetic gastric juice.*

Changes in the surface topography and ultrastructural morphology of 20% BSA loaded microparticulates incubated in Hank's buffer, newborn calf serum, pancreatin and synthetic gastric juice were monitored over a 30 day period using SEM as described previously, section 2.8, page 87.

4.2.3 *The biodegradation of P(HB-HV)20% PCL 11 microcapsules and microspheres in Hank's buffer, newborn calf serum, pancreatin and synthetic gastric juice.*

Plates 4.1.1a and 4.1.2a show the appearance of freshly prepared freeze dried 20% BSA loaded P(HB-HV)20% PCL 11 microcapsules and microspheres respectively prior to incubation. The microcapsules were generally larger in diameter (100.24 ± 10.11μm, n=25) and appeared to have a more well defined spherical structure than microspheres (26.28 ± 2.34μm, n=25). This observation confirmed the size distribution of microcapsules and microspheres previously presented. Plates 4.1.1b and 4.1.2b reveal the inherent macroporosity of P(HB-HV)20% PCL 11 microcapsules and the presence of surface micropores in microspheres fabricated from the same polymer. Microcapsule pore size ranged from 2.5um to 6.5um in diameter compared with 0.2um to 0.8um in diameter for microspheres and the pores appeared to be more uniformally distributed in microcapsules than in microspheres. BSA deposits were not evident on the surface or in the outermost pores of either microcapsules or microspheres. Presumably any BSA which might have accumulated on the surface of the

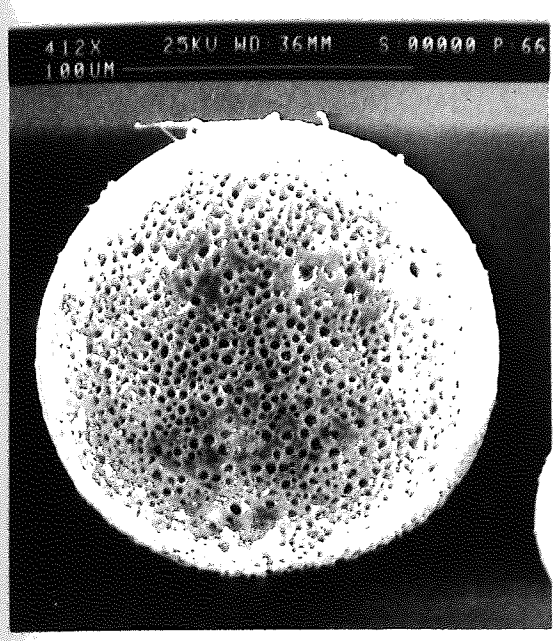


Plate 4.1.1a
P(HB-HV) Microcapsule (x412) prior to incubation.

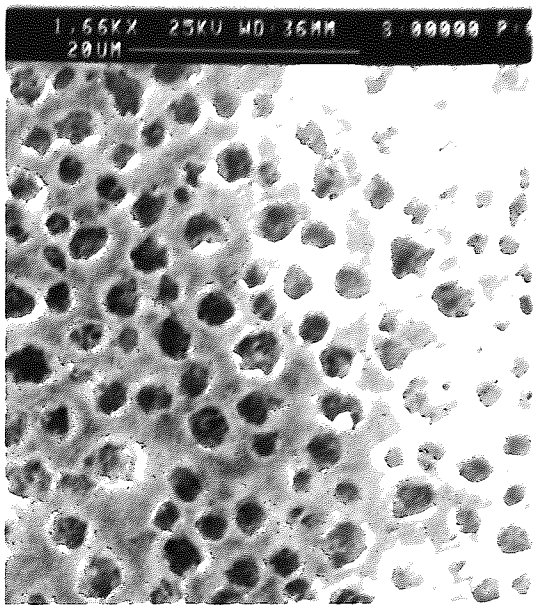


Plate 4.1.1b
P(HB-HV) Microapsule (KX1,66) prior to incubation showing surface macropores.

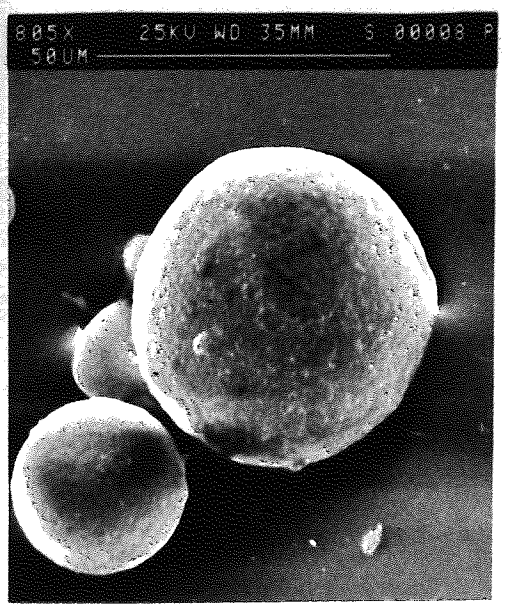


Plate 4.1.2a
P(HB-HV) Microsphere (x805) prior to incubation.

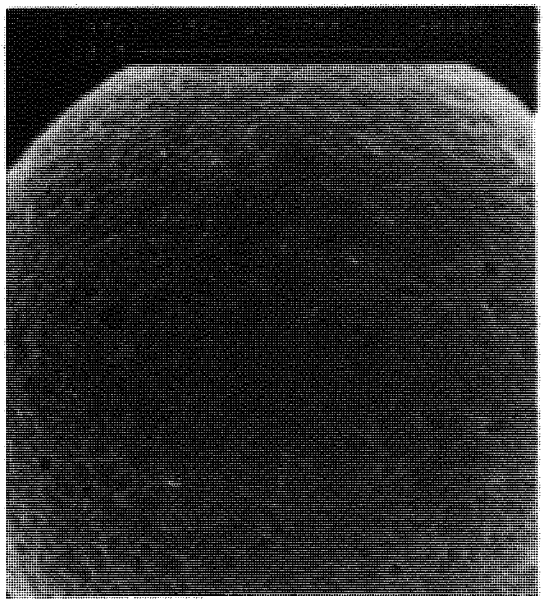


Plate 4.1.2b
P(HB-HV) Microsphere (KX1,95) prior to incubation showing surface micropores.

microparticulates would have been washed off during fabrication.

Biodegradation of microcapsules and microspheres in Hank's buffer, pH 7.4.

After 30 days incubation in Hank's buffer P(HB-HV)20% PCL 11 microcapsules (plate 4.1.3a) and microspheres (plate 4.1.3b) showed an increase in pore diameter suggesting increased porosity. The process was more marked in microcapsules, with the surface becoming pitted and rugose in places as pores coalesced. Microspheres, tended to maintain their relatively spherical shape but were characterised by some surface pitting, suggesting surface biodegradation as shown in the bottom right hand side of plate, 4.1.3b. These observations suggest that microcapsule biodegradation is more rapid and extensive than that of microspheres and this assumption is supported by the higher mean PWL values recorded for P(HB-HV)20% PCL 11 microcapsules compared with P(HB-HV)20% PCL 11 microspheres of 28.11 ± 0.917% and 25.78 ± 0.8% respectively.

Biodegradation of. microcapsules and microspheres in newborn calf serum.

Plates 4.1.4a, 4.1.4b, 4.1.4c and 4.1.4d illustrate the progressive biodegradation of P(HB-HV)20% PCL 11 microcapsules after 9,16,23 and 30 days incubation in newborn calf serum respectively. After 9 days, an extensive degree of macroporosity had developed with increased pore diameter, but no evidence of bulk erosion or disruption. After 16 days some bulk erosion had occurred with the disintergration of the spherical structure resulting in the breaking open of the microcapsules, plate 4.1.4b. After 23 days,

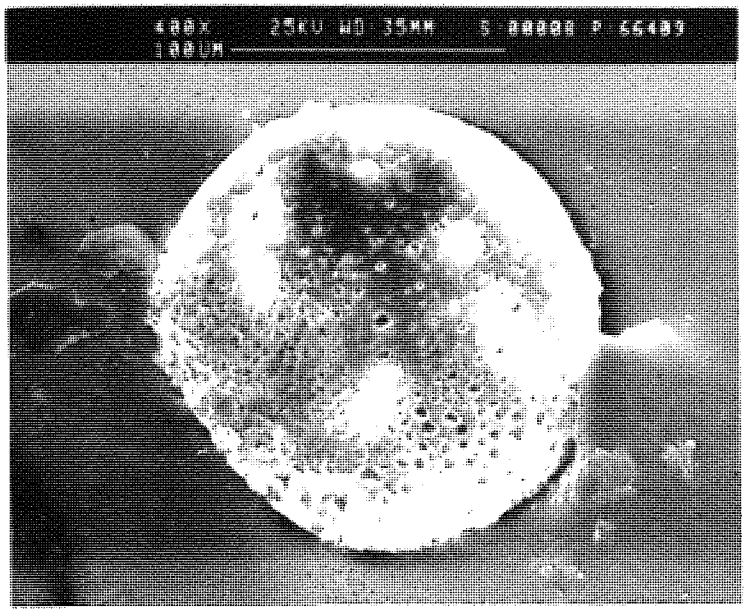


Plate 4.1.3a
P(HB-HV) Microcapsule (X400)
after 30 days incubation
in Hank's buffer, pH 7.4.

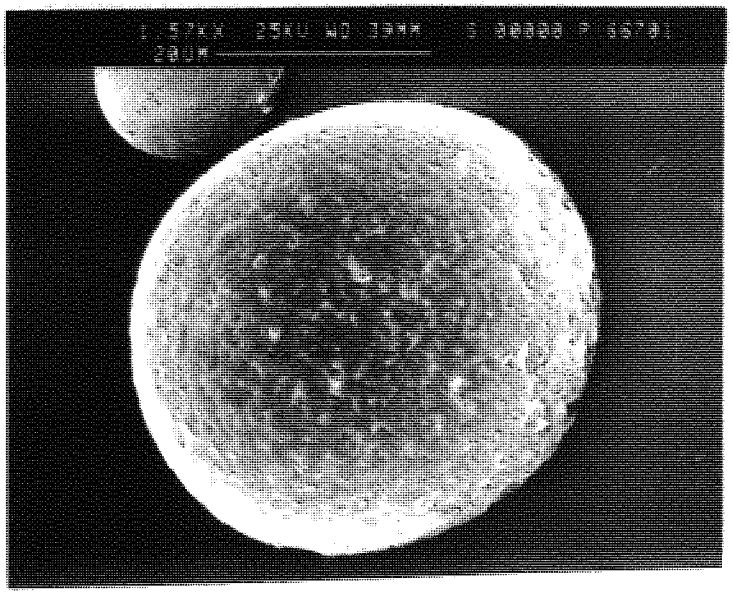


Plate 4.1.3b
P(HB-HV) Microsphere (KX1,57)
after 30 days incubation
in Hank's buffer, pH 7.4.

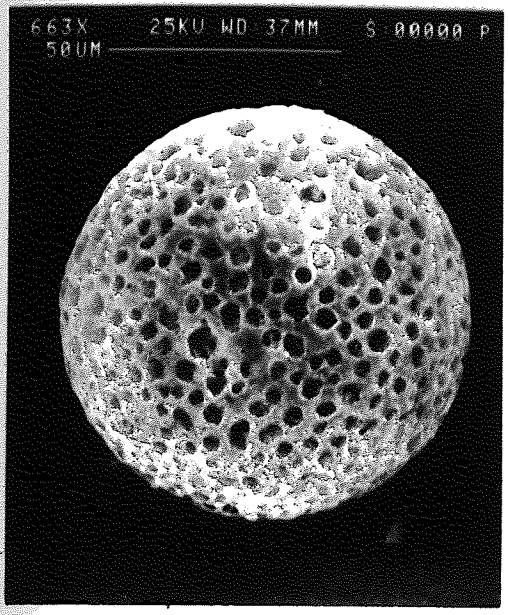


Plate 4.1.4a
P(HB-HV) Microcapsule (X663)
after 9 days incubation
in new-born calf serum.

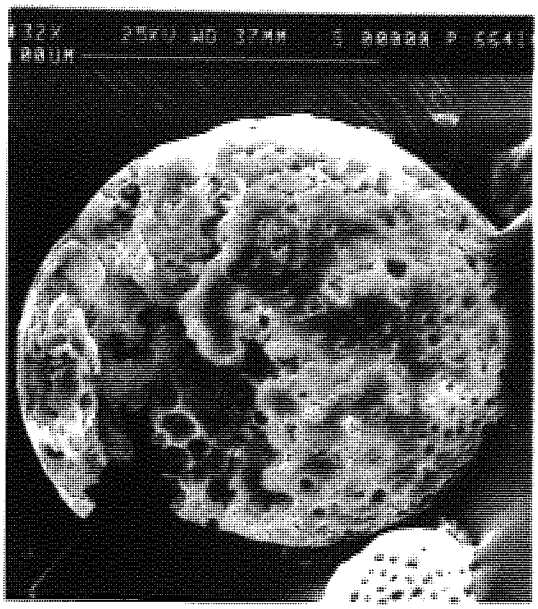


Plate 4.1.4b
P(HB-HV) Microcapsule (X432)
after 16 days incubation
in new-born calf serum.

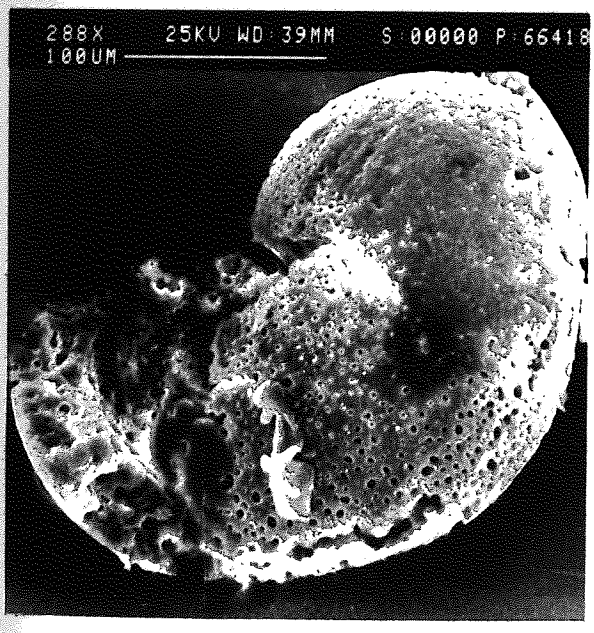


Plate 4.1.4c
P(HB-HV) Microcapsule (X288)
after 23 days incubation
in new-born calf serum.

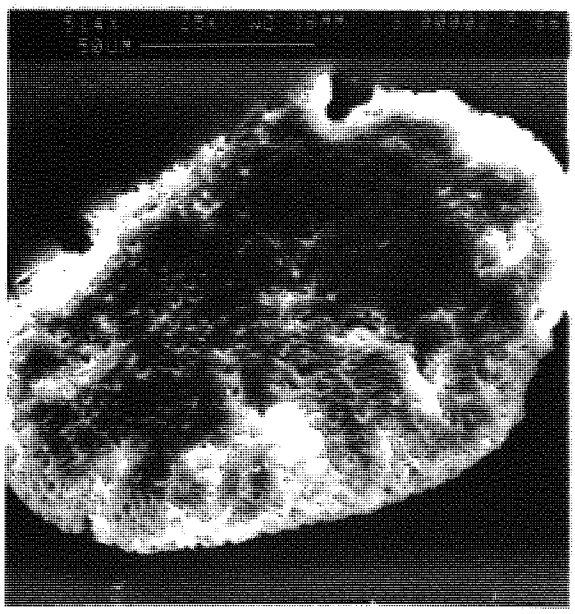


Plate 4.1.4d
P(HB-HV) Microcapsule (X432)
after 30 days incubation
in new-born calf serum.

further breakdown of the capsule wall was observed revealing the macroporous nature and thickness of the microcapsule wall, ie between 40 and 50μm. By day 30 the microcapsules had completely lost their spherical shape and started to disintegrate, plate 4.1.4d.

P(HB-HV)20% PCL 11 microspheres incubated in newborn calf serum showed heavy surface pitting and exaggerated micopores after 9 days, plate 4.1.5a. The rims of pores showed evidence of bioerosion and coalescence after 16 days incubation (plate 4.1.5b). The outer surface of the matrix became punctuated with a reduced number of large deep pits, whose continued coalescence led to the eventual bulk degradation of the microsphere matrix. The general spherical shape of the microspheres was maintained but their overall diameter reduced, suggesting extensive surface erosion. Overall, these observations support the substantial PWL values obtained for microcapsules and microspheres incubated in newborn calf serum, page114.

Biodegradation of microcapsules and microspheres in pancreatin solution.

Plates 4.1.6a, 4.1.6b, 4.1.6c and 4.1.6d illustrate the progressive biodegradation of P(HB-HV)20% PCL 11 microcapsules in 1.5% pancreatin solution (pH 7.4) after 9,16, 23 and 30 days incubation respectively. After 9 days the macroporosity of both the surface and matrix of the microcapsules had increased significantly (plate 4.1.6a) and there was some evidence of the coalescence of macropores (lower right hand corner). After 16 days some surface erosion of the microcapsule surface could be seen (plate 4.1.6b) which became more pronounced after 23 days (plate 4.1.6c). Large gaps in the polymer membrane had begun to appear and by day 30 had

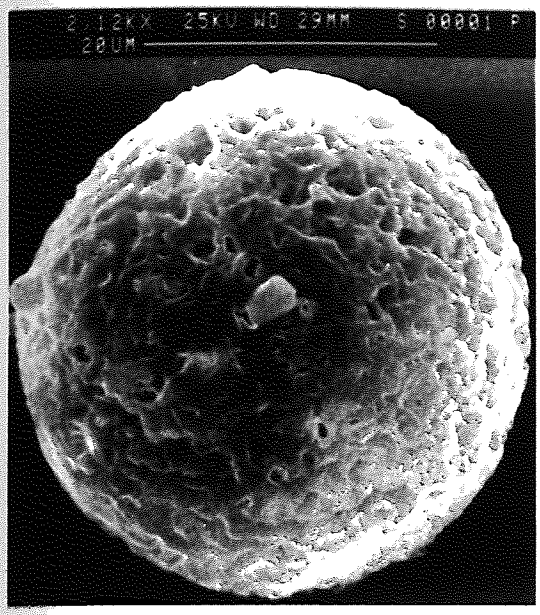


Plate 4.1.5a
P(HB-HV) Microsphere (KX2,12)
after 9 days incubation
in new-born calf serum.

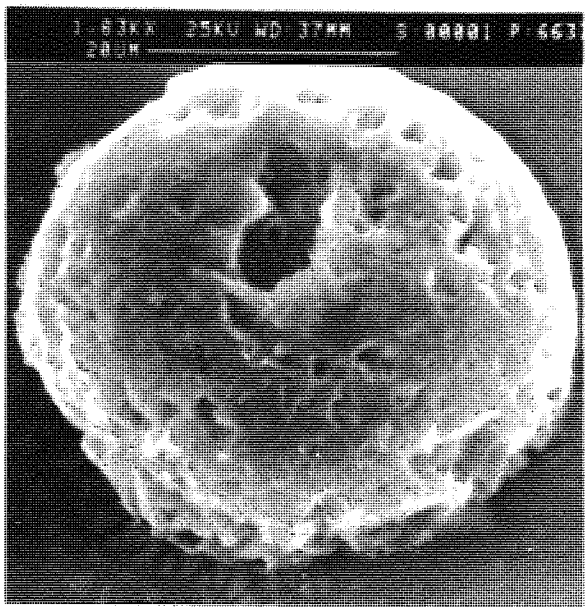


Plate 4.1.5b
P(HB-HV) Microsphere (KX1,83)
after 16 days incubation
in new-born calf serum.

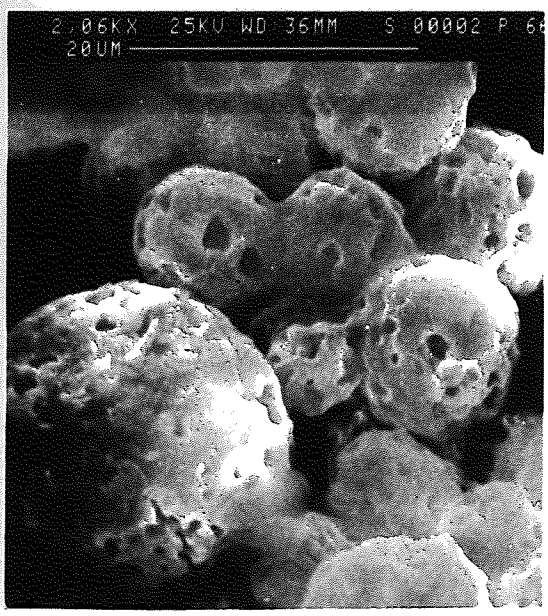


Plate 4.1.5c
P(HB-HV) Microsphere (KX2,06)
after 23 days incubation
in new-born calf serum.

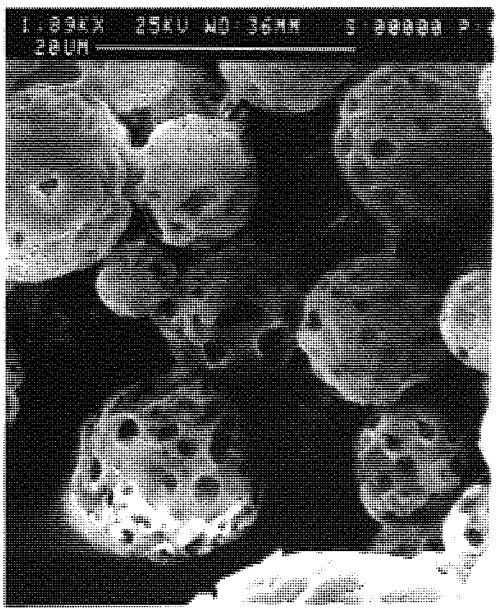


Plate 4.1.5d
P(HB-HV) Microsphere (KX1,89)
after 30 days incubation
in new-born calf serum.

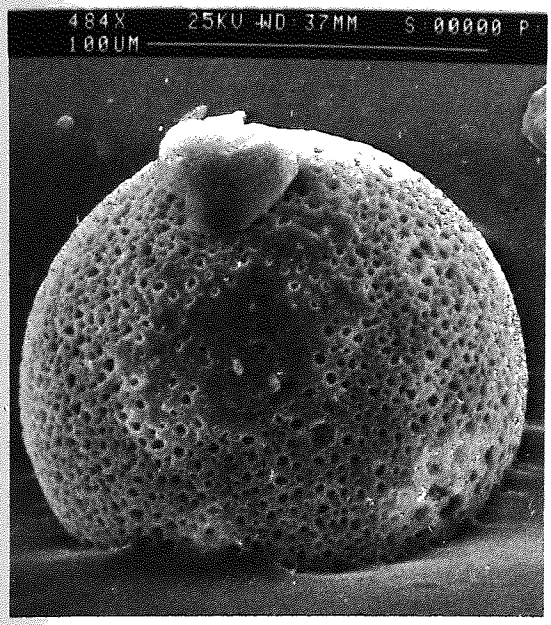


Plate 4.1.6a
P(HB-HV) Microcapsule (X484)
after 9 days incubation
in 1.5% pancreatin solution.

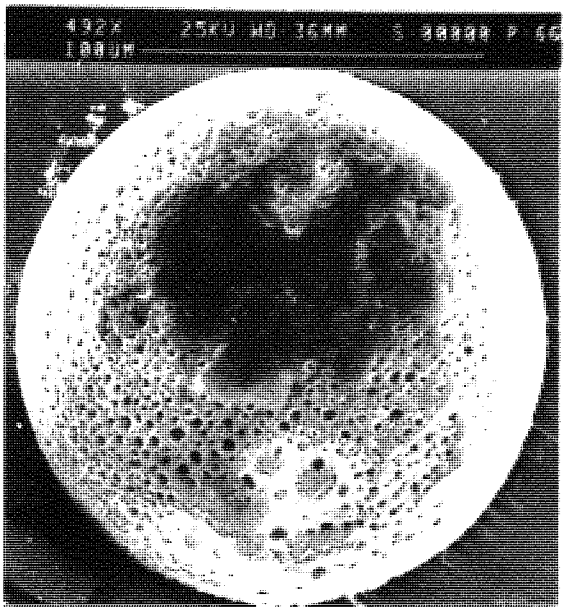


Plate 4.1.6b
P(HB-HV) Microcapsule (X492)
after 16 days incubation
in 1.5% pancreatin solution.

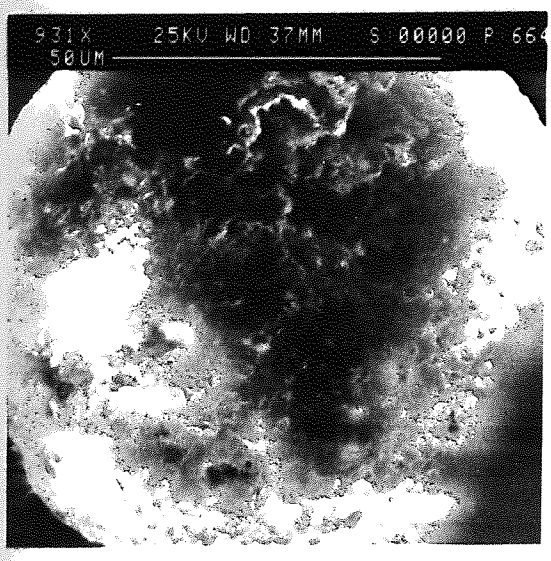


Plate 4.1.6c
P(HB-HV) Microcapsule (X931)
after 23 days incubation
in 1.5% pancreatin solution.

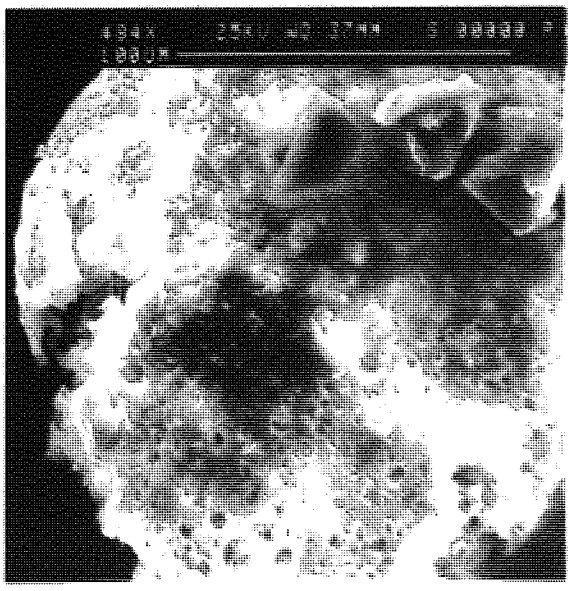


Plate 4.1.6d
P(HB-HV) Microcapsule (X484)
after 30 days incubation
in 1.5% pancreatin solution.

become so exaggerated as to lead to the gradual disintegration of the microcapsule, (plate 4.1.6d). The rate of disintegration of the microcapsule was judged not to be as rapid as that seen with newborn calf serum.

Significant micropores were not apparent on the relatively smooth surface of P(HB-HV)20% PCL 11 microspheres after 9 days incubation in pancreatin solution, plate 4.1.7a. However, surface pitting was evident by day 16 (plate 4.1.7b) and by day 23 the microspheres had fractured, forming discreet plates at various places on the surface and exposing interior parts of the microsphere matrix, plate 4.1.7c. By day 30 the microspheres had begun to disintergrate along the fracture lines resulting in the breakup of the microsphere matrix, plate 4.1.7d.

Biodegradation of microcapsules and microspheres in synthetic gastric juice containing 10% pepsin.

The structural deterioration of P(HB-HV)20% PCL 11 microcapsules, incubated in synthetic gastric juice containing 10% pepsin, after 9,16,23 and 30 days, is illustrated in plates 4.1.8a, 4.1.8b, 4.1.8c and 4.1.8d respectively. Significant macroporosity was evident by day 9 (plate 4.1.8a) which was exaggerated by pore coalescence at day 16 (plate 4.1.8b). By day 23 this increased macroporosity was accompanied by extensive bioerosion of the microcapsule membrane, although there was no significant decrease in size, plate 4.1.8c. By day 30 there was a further increase in pore diameter and the microcapsules had begun to lose their spherical shape, although they remained structurally intact, plate 4.1.8d.

P(HB-HV)20% PCL 11 microspheres incubated in gastric juice showed little evidence of bioerosion by day 9 (plate 4.1.9a) and surface macropores and micropores were absent. By day 16 there

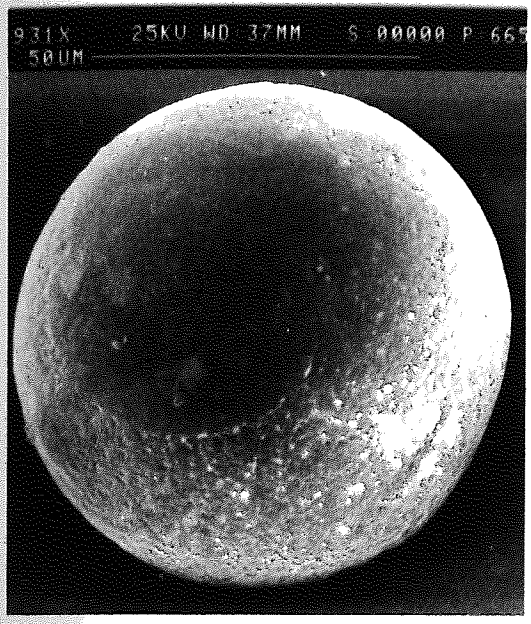


Plate 4.1.7a
P(HB-HV) Microsphere (X931)
after 9 days incubation
in 1.5% pancreatin solution.

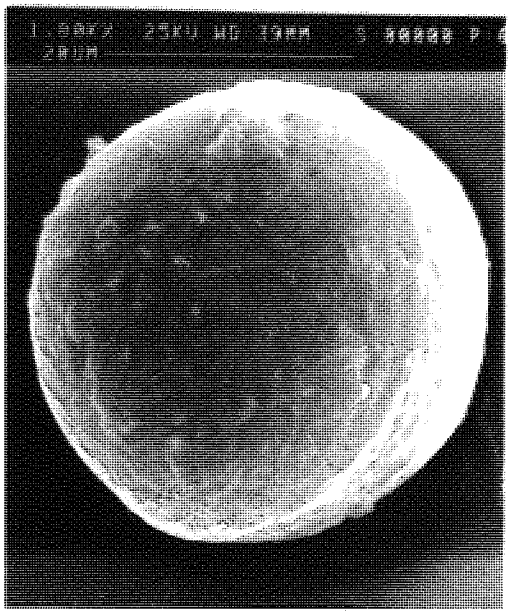


Plate 4.1.7b
P(HB-HV) Microsphere (KX1,80)
16 days after incubation
in 1.5% pancreatin solution.

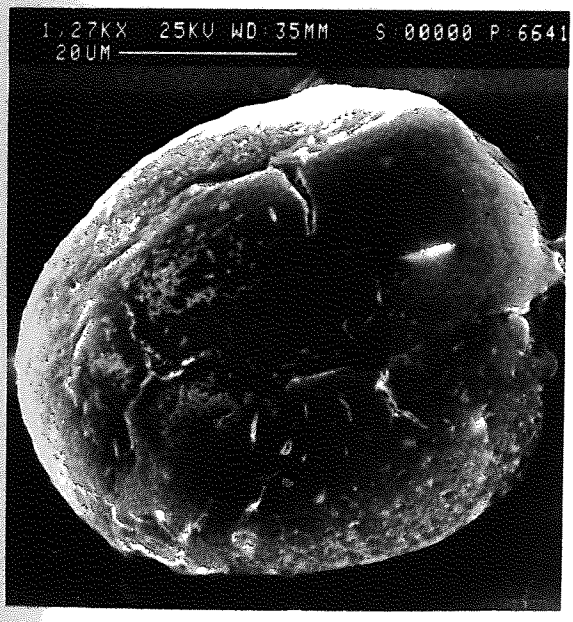


Plate 4.1.7c
P(HB-HV) Microsphere (KX1,27)
after 23 days incubation
in 1.5% pancreatin solution.

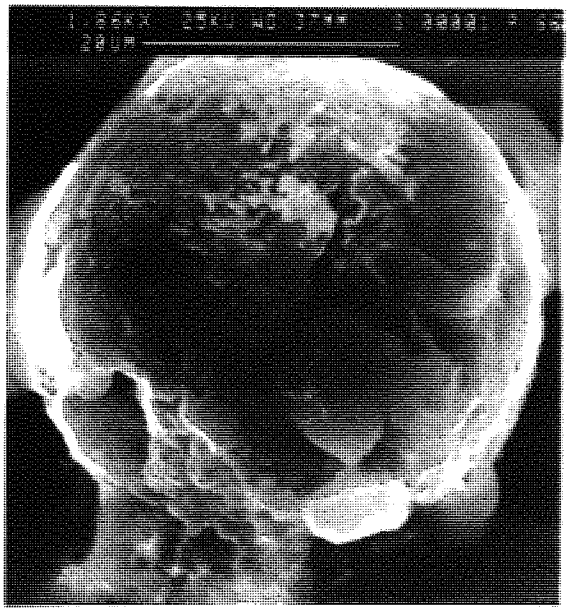


Plate 4.1.7d
P(HB-HV) Microsphere (KX1,86)
after 30 days incubation
in 1.5% pancreatin solution.

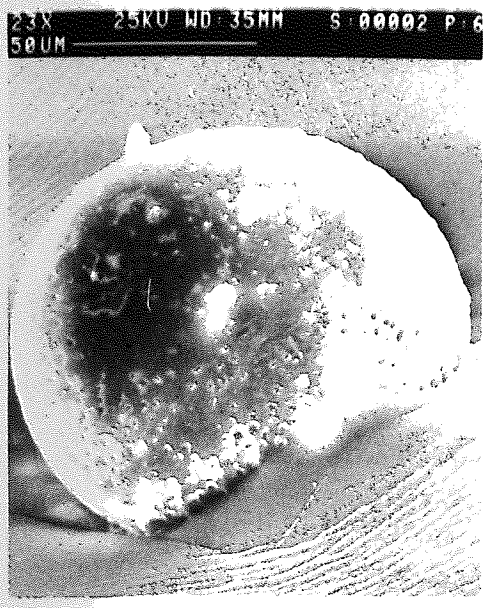


Plate 4.1.8a
P(HB-HV) Microcapsule (X523)
after 9 days incubation
in gastric juice.

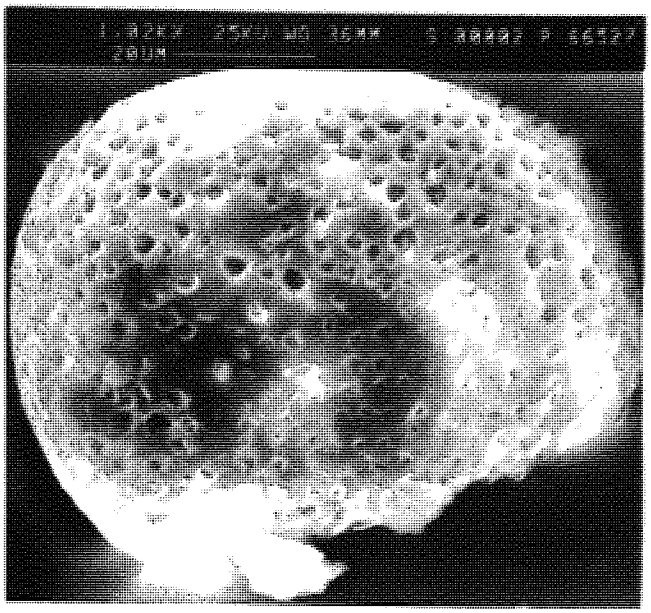


Plate 4.1.8b
P(HB-HV) Microcapsule (KX1,02)
after 16 days incubation
in gastric juice.

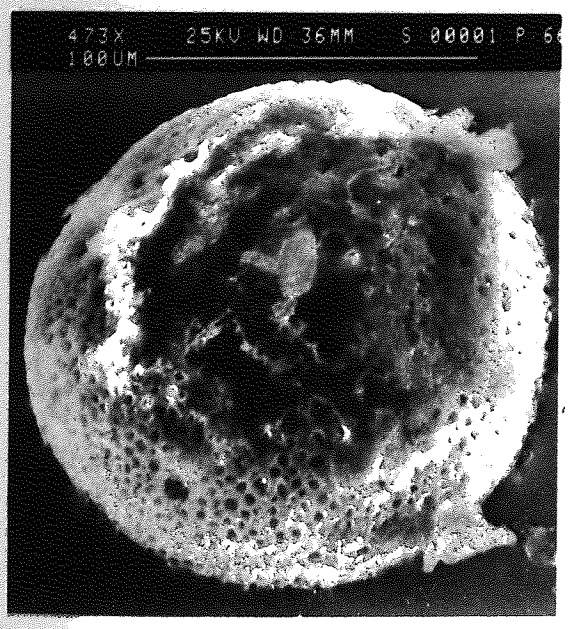


Plate 4.1.8c
P(HB-HV) Microcapsule (X473)
after 23 days incubation
in gastric juice.

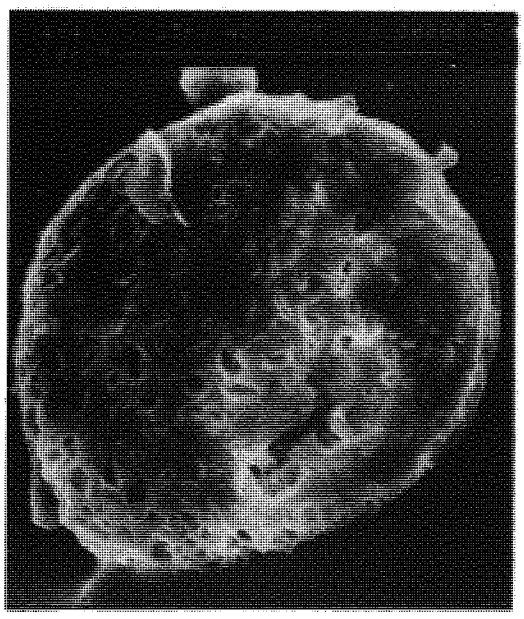


Plate 4.1.8d
P(HB-HV) Microcapsule (X484)
after 30 days incubation
in gastric juice.

Plate 4.1.9a
P(HB-HV) Microsphere (X913)
after 9 days incubation
in gastric juice.

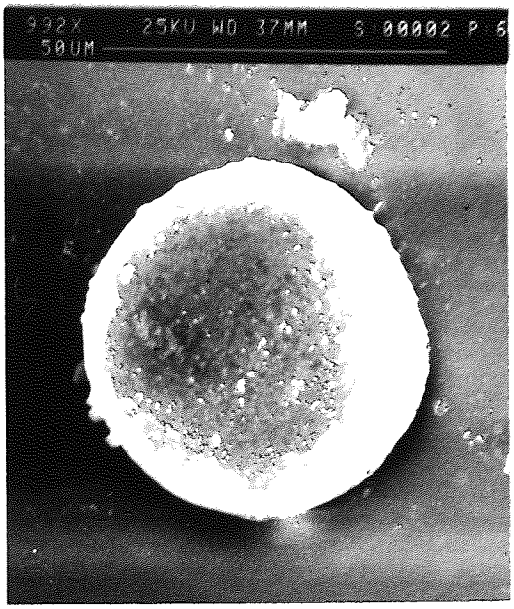


Plate 4.1.9b
P(HB-HV) Microsphere (x992)
after 16 days incubation
in gastric juice.

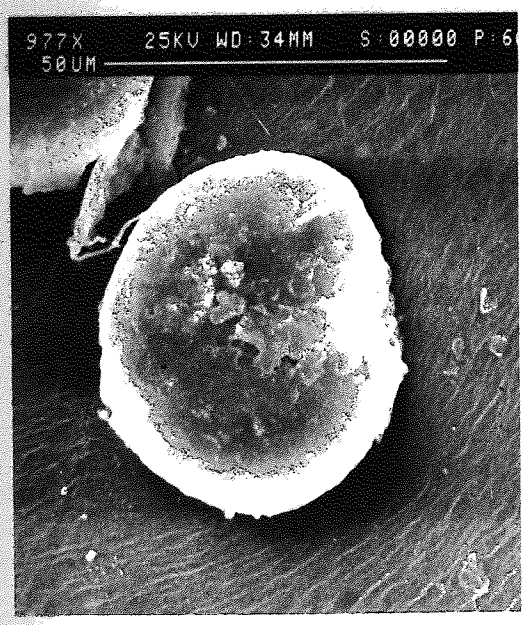


Plate 4.1.9c
P(HB-HV) Microsphere (X977)
after 23 days incubation
in gastric juice.

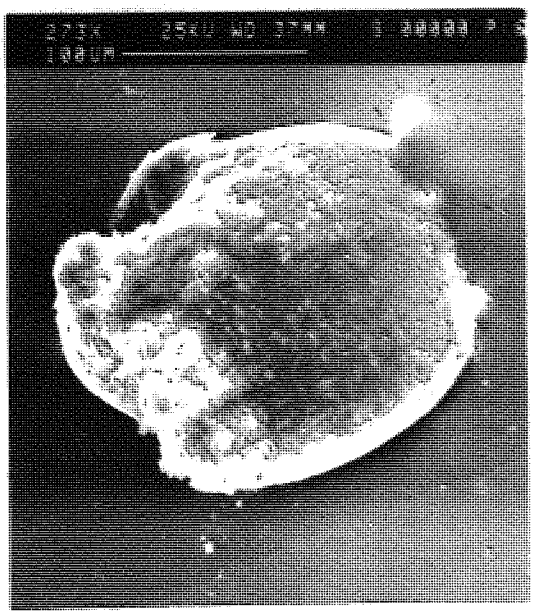


Plate 4.1.9d
P(HB-HV) Microsphere (x273)
after 30 days incubation
in gastric juice.

was some evidence of a loss of shape and surface bioerosion, plate 4.1.9b. Further surface bioerosion and flaking was evident by day 23, plate 4.1.9c and by day 30, bulk erosion of the matrix and disintegration had begun to occur, plate 4.1.9d. Gastric juice appeared to be the least active of the three physiological solutions in terms of particulate biodegradation and its effects appeared to be intermediate between Hank's buffer on the one hand and newborn calf serum and pancreatin on the other perhaps because of the low pH (0.8) and the presence of pepsin.

4.2.4. The biodegradation of PEA20% PCL 11 microcapsules and microspheres in Hank's buffer, newborn calf serum, pancreatin and synthetic gastric juice.

Plates 4.2.0a, 4.2.0b and 4.2.0c show the smooth surface of freshly fabricated freeze dried PEA20% PCL 11 microcapsules and microspheres prior to incubation in Hank's buffer, newborn calf serum, pancreatin and synthetic gastric juice. Microcapsules were significantly larger than microspheres and both were essentially spherical in shape. However, at high magnification, PEA microcapsules possessed small hairline ridges and cracks in their surface, plate 4.2.0b, which may have resulted from the solvent evaporation stage during fabrication. Neither PEA20% PCL 11 microcapsules, Plate 4.2.0c nor PEA20% PCL 11 microspheres had surface micropores, plate 4.2.0b.

No discernable biodegradation of either PEA20% PCL 11 microcapsules or microspheres was visible after 30 days incubation in Hank's buffer (micrographs not shown). However, PWL values indicated that 12.9 ± 0.79% and 12.5 ± 0.15% of the mass had been lost from microcapsules and microspheres respectively and that

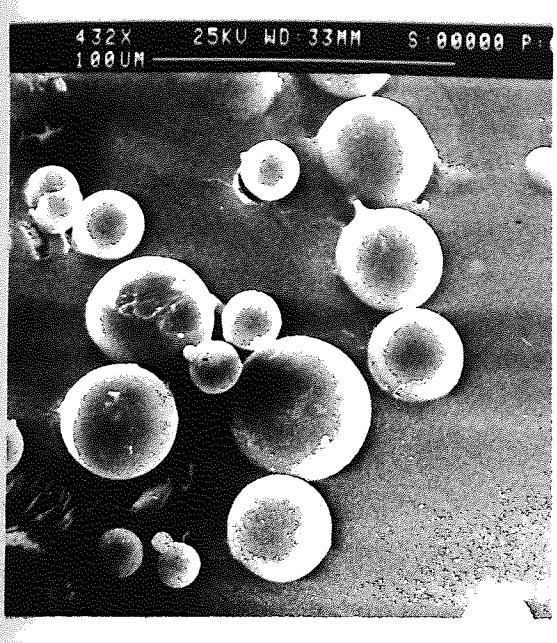


Plate 4.2.0a
PEA Microcapsule (X432)
prior to incubation.

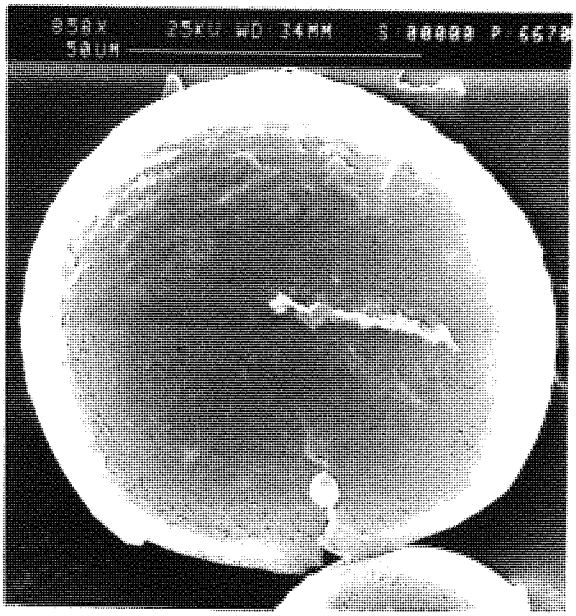


Plate 4.2.0b
PEA Microcapsule (X858)
prior to incubation.

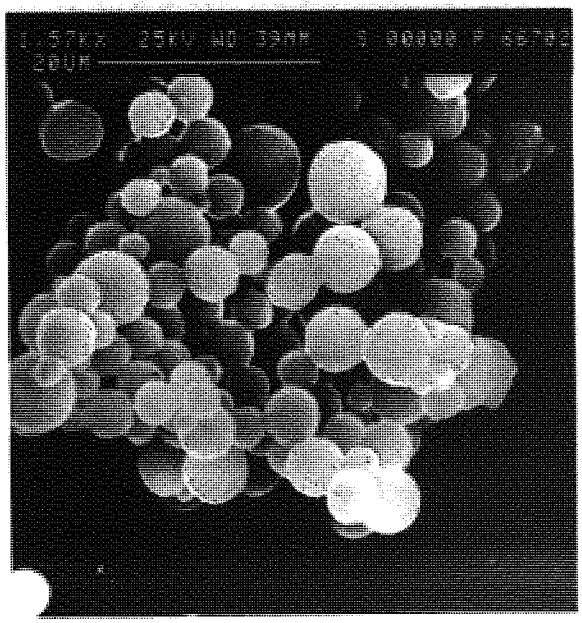


Plate 4.2.0c
PEA Microspheres (KX1,57)
prior to incubation.

weight loss from microcapsules was slightly higher than that from microspheres.

Biodegradation of PEA microcapsules and microspheres in newborn calf serum.

Plates 4.2.1a, 4.2.1b, 4.2.1c and 4.2.1d show the progressive biodegradation of PEA20% PCL 11 microcapsules after 9, 16, 23 and 30 days incubation respectively in new born calf serum. After 9 days extensive pitting of the microcapsule surface was observed although no pores were seen to develop. By day 16, extensive surface erosion and reduction in size could be seen as the microcapsules began to lose their spherical shape. After 23 days incubation, large holes appeared in the outer capsule coat, plate 4.2.1c and by day 30 the capsule membrane had fragmented and almost completely broken down, plate.4.2.1d.

After 9 days incubation in newborn calf serum, PEA/20% PCL 11 microspheres showed considerable bulk erosion and crumbling, plate 4.2.2a, although no discrete pores were present or seen to subsequently develop, the surface topography suggested that heavy surface pitting had occurred prior to bulk erosion. By day 16 the degree of bulk erosion had increased, leading to the loss of spherical shape and further crumbling, plate 4.2.2b. From day 23 to day 30 there was extensive disintegration of the matrix and reduction in size, plates 4.2.2c and d. This comprehensive biodegradation supports the high values of PWL obtained for PEA microspheres in newborn calf serum.

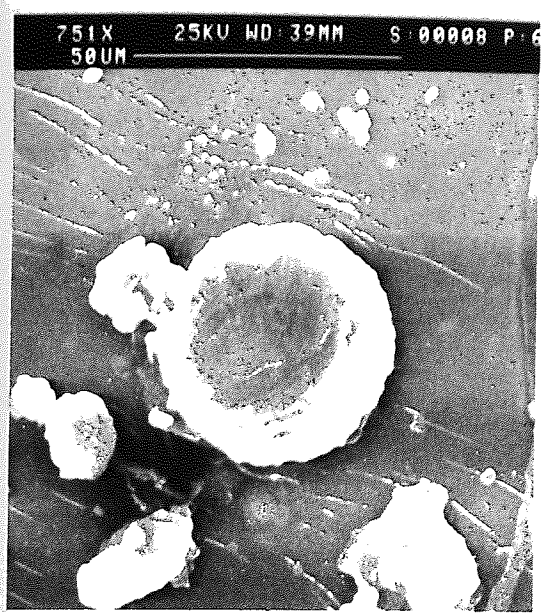


Plate 4.2.1a
PEA Microcapsule (X751)
after 9 days incubation
in new-born calf serum.

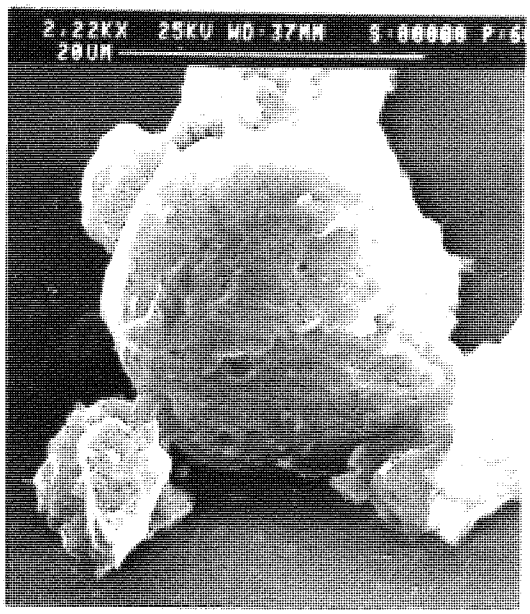


Plate 4.2.1b
PEA Microcapsule (KX2,22)
after 16 days incubation
in new-born calf serum.

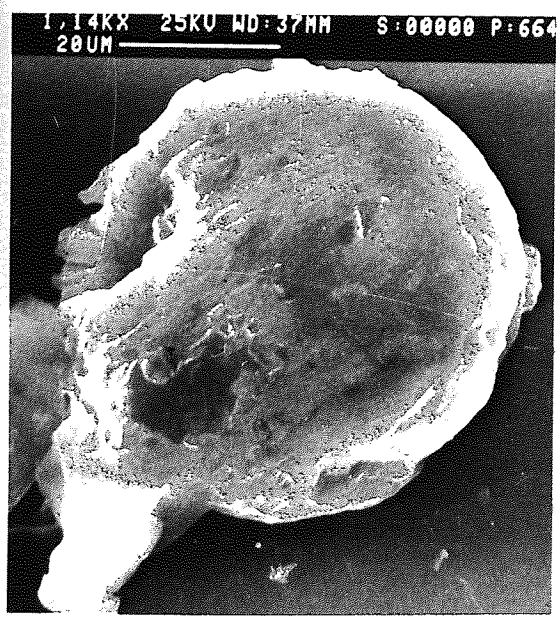


Plate 4.2.1c
PEA Microcapsule (KX1,14)
after 23 days incubation
in new-born calf serum.

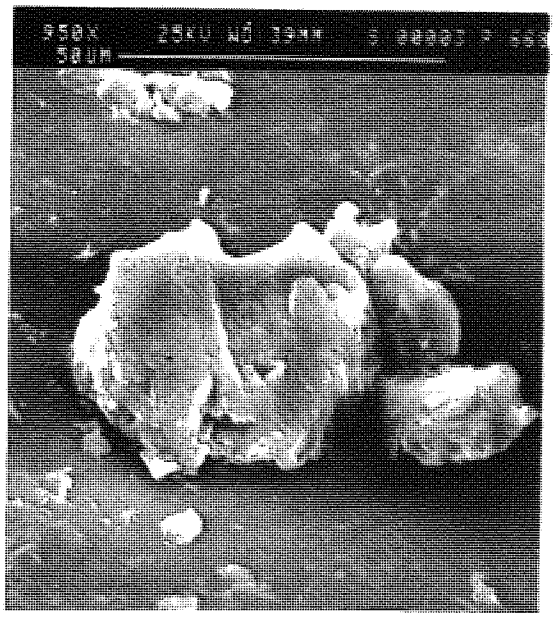


Plate 4.2.1d
PEA Microcapsule (X950)
after 30 days incubation
in new-born calf serum.

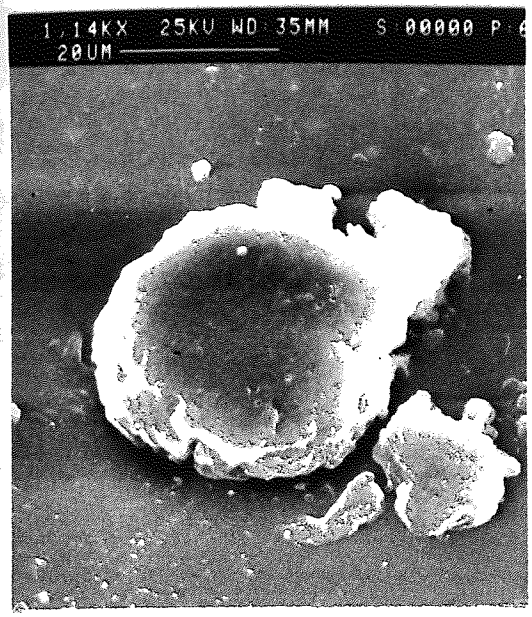


Plate 4.2.2a
PEA Microsphere (KX1,14)
after 9 days incubation
in new-born calf serum.

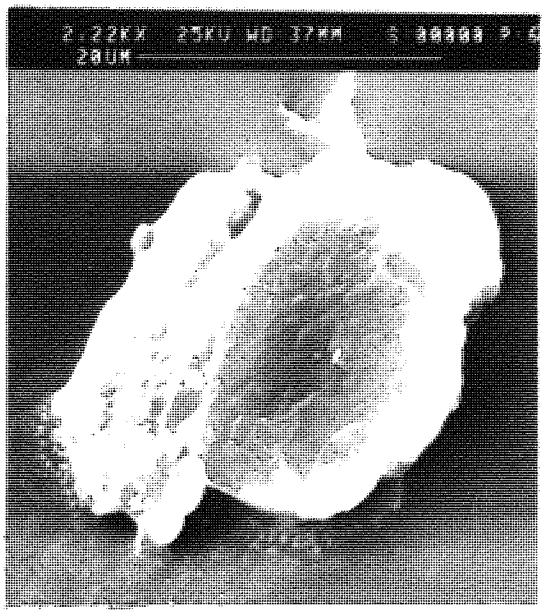


Plate 4.2.2b
PEA Microsphere (KX2,22)
after 16 days incubation
in new-born calf serum.

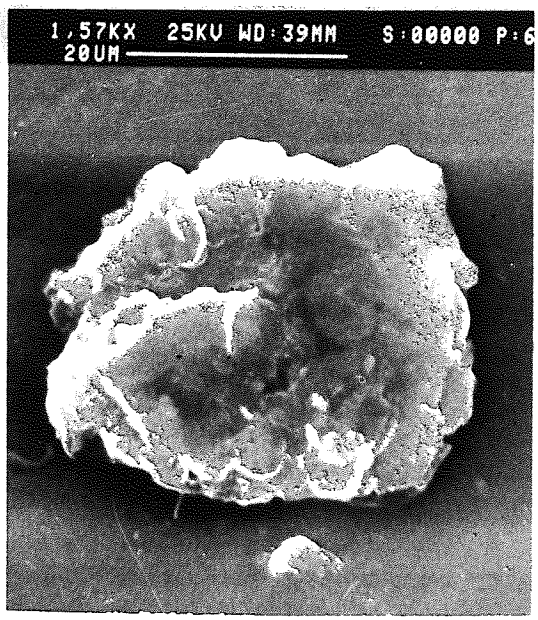
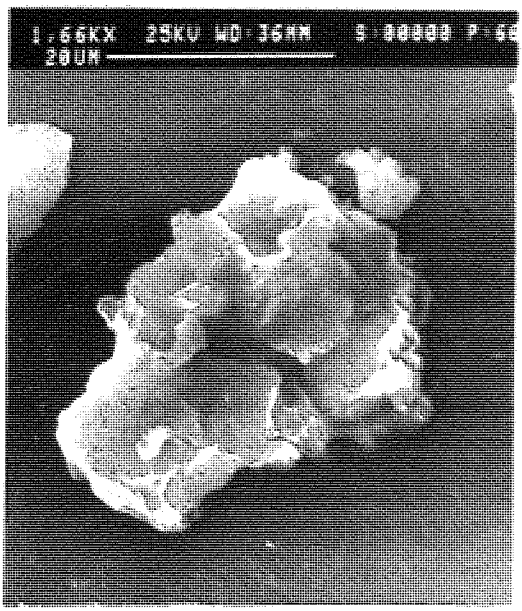


Plate 4.2.2c
PEA Microsphere (KX1,57)
after 23 days incubation
in new-born calf serum.



4.2.2d
PEA Microsphere(KX1,66)
after 30 days incubation
in new-born calf serum.

Biodegradation of PEA microcapsules and microspheres in pancreatin solution.

Plates 4.2.3a, 4.2.3b,4.2.3c and 4.2.3d illustrate the progressive biodegradation of PEA20% PCL 11 microcapsules after 9,16,23 and 30 days incubation respectively in pancreatin solution. After 9 days the microcapsule coat had been eroded away at a single location on the surface. No pores were present or seen to subsequently develop. By day 16, microcapsules began to fracture, with large fragments of the membrane beginning to peel off leading to total collapse and disintegration. By day 23 the microcapsules had begun to lose their spherical shape as the membrane fragmented, plate 4.2.3c and by day 30 no discrete microcapsules remained, only fragments of capsule wall, plate 4.2.3d.

Incubation of PEA20% PCL 11 microspheres in pancreatin solution produced significant pitting of the surface after 9 days, plate 4.2.4a. By day 16 (plate 4.2.4b) microspheres had lost their spherical shape and fractures had begun to appear in the surface, plate 4.2.4b. After 23 days incubation the microspheres started to fragment and pieces began to flake off the surface leading to complete disintegration by day 30, plate 4.2.4d. Pancreatin, therefore produced a significant and rapid biodegradation of PEA microparticles and this was reflected in the high PWL values observed for this polymer (page115). PWL data also confirmed that PEA microcapsules biodegraded more quickly and extensively than PEA microspheres.

Biodegradation of PEA microcapsules and microspheres in synthetic gastric juice.

Very little biodegradation of the PEA20% PCL 11

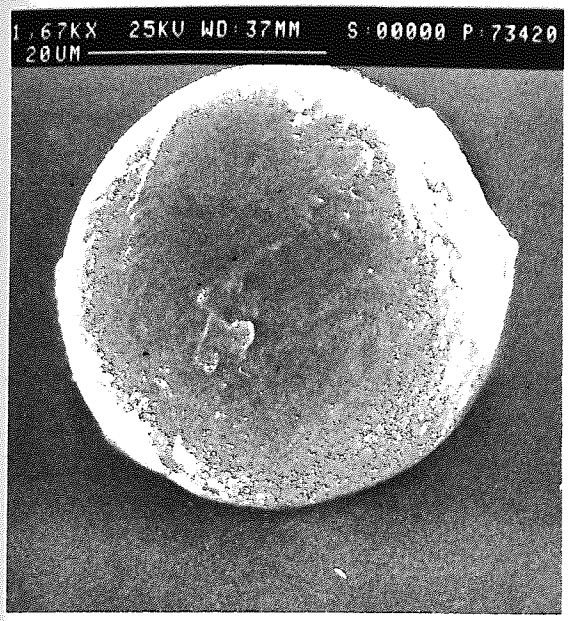


Plate 4.2.3a
PEA Microcapsule (KX1,67)
after 9 days incubation
in pancreatin solution.

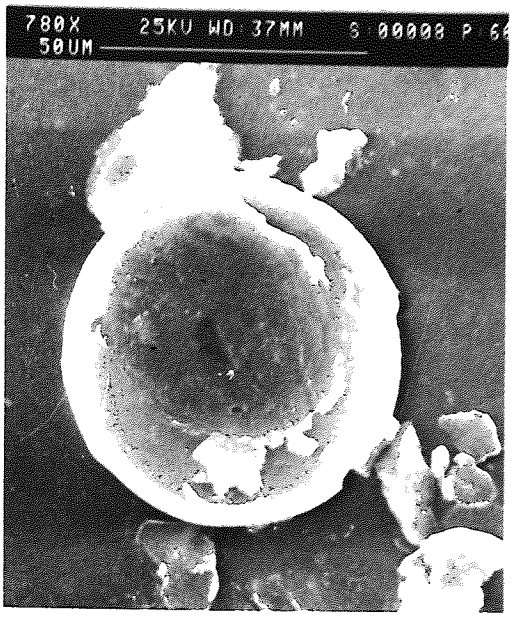


Plate 4.2.3b
PEA Microcapsule (x780)
after 16 days incubation
in pancreatin solution.

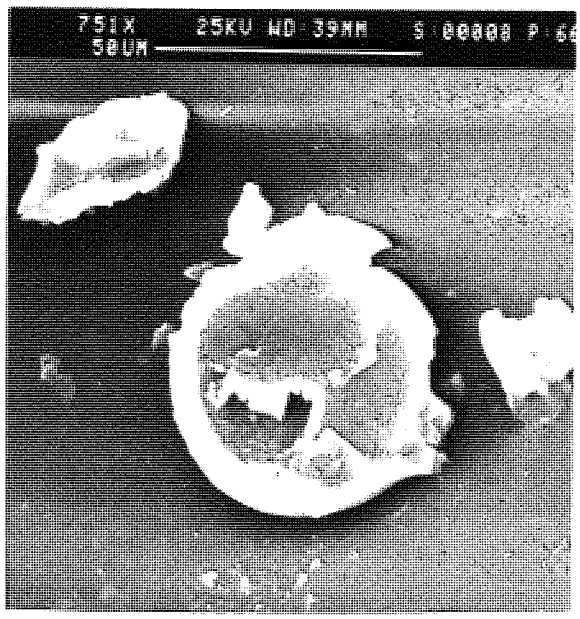


Plate 4.2.3c
PEA Microcapsule (x715)
after 23 days incubation
in pancreatin solution.

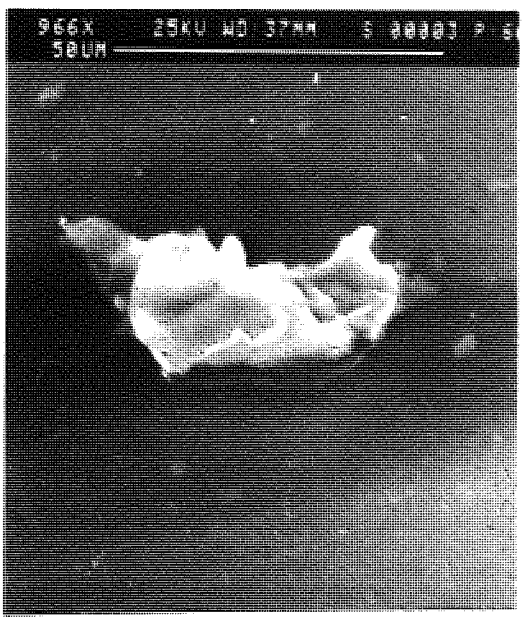


Plate 4.2.3d
PEA Microcapsule (x966)
after 30 days incubation
in pancreatin solution.

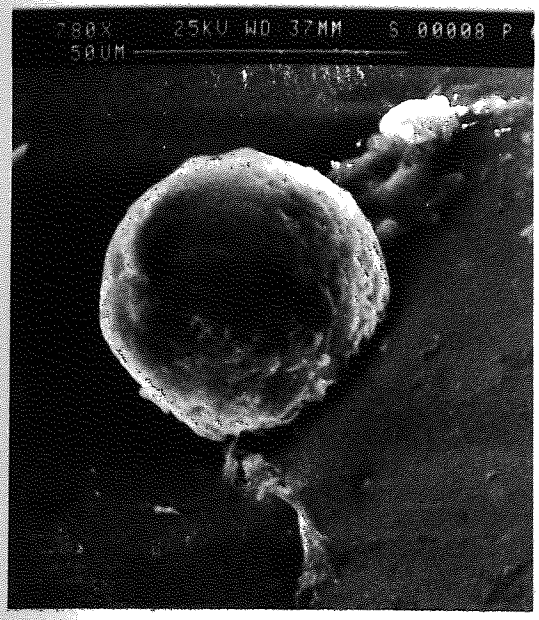


Plate 4.2.4a
PEA Microsphere (X708)
after 9 days incubation
in pancreatin solution.

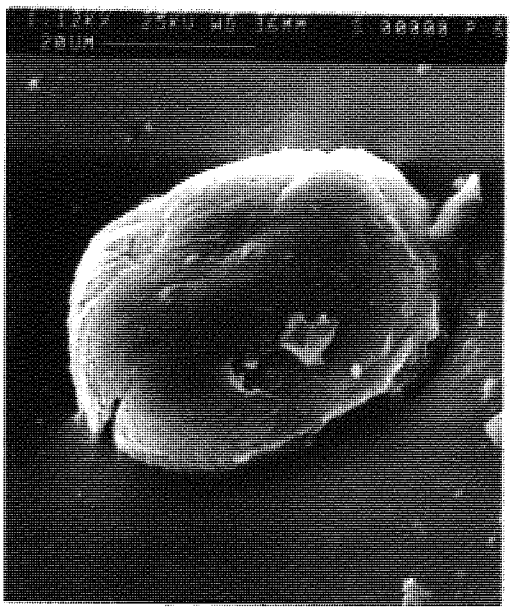


Plate 4.2.4b
PEA Microsphere (KX2,12)
after 16 days incubation
in pancreatin solution.

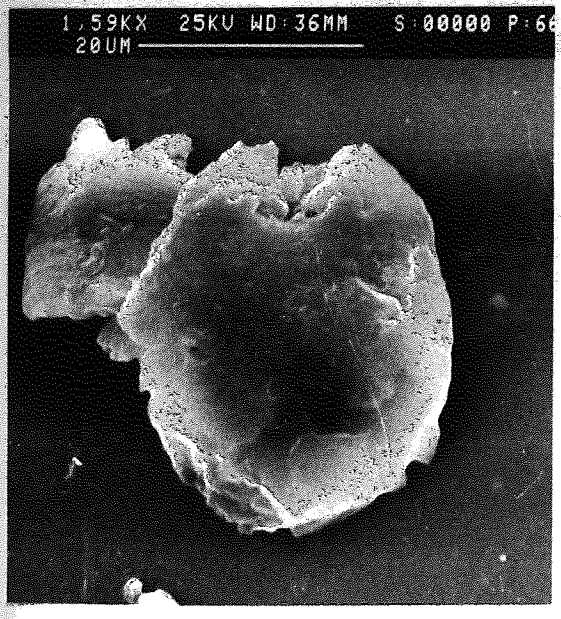


Plate 4.2.4c
PEA Microsphere (KX1,59)
after 9 days incubation
in pancreatin solution.

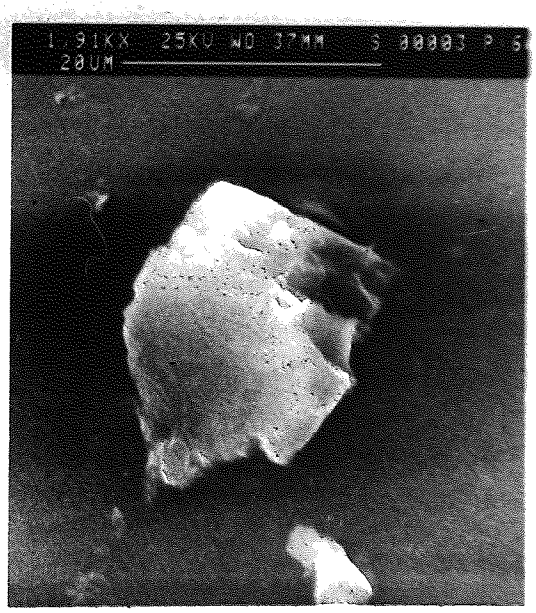


Plate 4.2.4d
PEA Microsphere (KX1,91)
after 30 days incubation
in pancreatin solution.

microcapsules was observed after 30 days incubation in synthetic gastric juice, (micrographs not shown). A small proportion of the microcapsules did show some shallow pitting on their surface, but this was by no means comprehensive. PEA20% PCL 11 microspheres showed no evidence of biodegradation after 30 days incubation in synthetic gastric juice.

4.3. Discussion.

Surface erosion (random chain scission at the polymer/medium interface) only gives rise to small changes in dry microparticulate mass. Bulk erosion occurs as the products of chain scission diffuse out from the amorphous region of the polymer/BSA matrix through surface pores previously occupied by solubilised BSA, causing significant weight loss. Mean weight loss values (PWL) can be used to indicate the degree of microparticulate bulk erosion.

In the four incubation solutions used microcapsules were found to biodegrade more rapidly than microspheres fabricated from the same polymer. Although, in the case of P(HB-HV)20% PCL 11 microcapsules and microspheres, the greater PWL values exhibited by microcapsules in all four incubation media could be the result of differences in microporosity, ie P(HB-HV)20% PCL 11 microcapsules possessed larger surface pores than microspheres fabricated from the same polymer. PEA20% PCL 11 microcapsules were also shown to breakdown more rapidly than PEA20% PCL 11 microspheres, although no distinct micropores are seen in either micrparticulate. Therefore, differences in internal structure, ie the presence of a microencapsulated internal reservoir rather than a solid matrix,

would appear to be responsible for the greater PWL values observed for microcapsules compared with microspheres, fabricated from the same polymers blends.

A small amount of microcapsule membrane surface bioerosion has the potential to expose the majority of the BSA present in the aqueous core of the capsule. The same small amount of surface bioerosion of a microsphere will only degrade a peripheral portion of the polymer/BSA matrix. The BSA will go into solution leaving channels or pores in the polymer through which BSA more deeply dispersed in the matrix will escape. Therefore microcapsules undergoing the same degree of surface bioerosion as microspheres are likely to show higher PWL values because microcapsules are inherently more fragile than microspheres and breakdown or fragment more easily. Although this explains in part the different PWL values obtained for microcapsules and microspheres, encapsulation efficiencies for both microcapsules and microspheres in this study were very low (table 3.1). It would seem likely that microcapsule structure *per se* results in a greater and possibly more rapid polymer/aqueous solution interaction thereby facilitating more extensive enzymatic or hydrolytic biodegradation and consequently greater resultant PWL values.

Ester hydrolysis is the main mechanism of biodegradation of P(HB-HV), PCL 11 and PEA in Hank's buffer, pH 7.4 and both SEM and PWL studies confirmed that both microcapsules and microspheres fabriacted from these polymers showed very limited biodegradation in this medium.

When incubated in an aqueous medium such as Hank's buffer, BSA present on the outside, at the periphery and in the core of P(HB-HV)20% PCL 11 microcapsules and in the matrix of P(HB-HV)20% PCL

11 microspheres, will be lost by dissolution into the surrounding medium. This results in an increase in pore size of P(HB-HV)20% PCL 11 microcapsules and induces channel and pore formation in microspheres. SEM studies revealed such an increase in the pore size of P(HB-HV)20% PCL 11 microcapsules and microspheres after 30 days incubation in Hank's buffer. Some surface pitting of microspheres was also evident. Both the increase in pore size (most dramatic in microcapsules) and surface pitting indicate the occurrence of surface erosion. This could account for the low PWL seen from P(HB-HV)20% PCL 11 microcapsules and microspheres in Hank's buffer. With microcapsules, BSA and agarose is lost from the core by exchange through aqueous pores. This is reflected in the higher PWL from microcapsules compared with microspheres.

PEA microparticulates showed little biodegradation after 30 days incubation in Hank's buffer and the low PWL values probably reflected the low level of BSA dissolution from the peripheral matrix of the microparticulates. There was some suggestion that because of the smaller size and therefore increased surface area of PEA20% PCL 11 microspheres and microcapsules compared with microspheres and microcapsules fabricated using P(HB-HV)20% PCL 11, PEA microparticles might biodegrade at a faster rate. However, this was not shown to be the case, PEA20% PCL 11 microspheres and microcapsules were less susceptible to hydrolytic biodegradation in Hank's buffer than P(HB-HV)20% PCL 11 microparticulates at pH 7.4. This may be due solely to the chemical structure of PEA, but is most likely to be due to the absence of micropores in the surface of the PEA microcapsules and the lack of macroporosity in the matrix of microspheres.

The most extensive biodegradation of microspheres and

microcapsules, irrespective of fabrication polymer, occurred in non-heat treated new-born calf serum (pH 7.1). Ester hydrolysis was expected to occur in this medium at a similar rate to that seen in Hank's buffer. Enzymatic degradation would be facilitated by the low level esterase activity present in the serum and significant protease activity might originate from alpha and beta globulin fractions of plasma proteins.

PWL data indicated that PEA20% PCL 11 microcapsules and microspheres were more susceptible to biodegradation in newborn calf serum than P(HB-HV)20% PCL 11 microparticulates. Increased porosity develops as P(HB-HV)20% PCL 11 microcapsules biodegrade and the structural integrity of these microcapsules appears to be lost as a consequence of polymer degradation, increasing pore size and coalesence of pores forming channels in addition to BSA dissolution. P(HB-HV)20% PCL 11 microspheres show heavy surface pitting in newborn calf, serum which progresses to a characteristic sponge like appearence and reduction in size. It was assumed that, because the synthetic gastric juice was very acidic (pH 0.8) and that hydrolytic polymer degradation and the solubility of the products of polymer hydrolysis increase with alkalinity, very low levels of degradation by ester hydrolysis would occur. However, SEM and PWL studies demonstrated a more extensive biodegradation of all microparticulates in gastric juice than in Hank's buffer which was essentially neutral, pH7.4. It may be that enzymatic degradation of BSA and possibly of the bulk polymer itself by pepsin, a carboxy peptidase, may occur in addition to low level hydrolytic degradation of the fabrication polymer. SEM studies showed that after incubation in synthetic gastric juice, P(HB-HV)20% PCL 11

microcapsules were degraded more extensively than PEA microcapsules and P(HB-HV)20% PCL 11 microspheres in turn were degraded more extensively than PEA microspheres after 30 days. The lack of effect of gastric juice on PEA microparticles may have been due to the absence of micropores and internal macroporosity. Once the BSA located in the periphery of the PEA20% PCL 11 microcapsules or microspheres was lost by dissolution and enzymatic attack no further breakdown of the polymer matrix could occur other than low level hydrolytic or enzymic erosion at the polymer medium interface.

1.5% pancreatin solution (pancreatic enzyme solution) produced an extensive biodegradation of both microcapsules and microspheres, significantly greater than that seen with either Hank's buffer or synthetic gastric juice. Pancreatin comprises a range of enzymes including trypsin, chymotrypsin and elastase (serine proteases), carboxy peptidase A (a zinc protease), cholesterol ester hydrolase, phospholipase A_2 lipase and amylase. An enzymatic degradation mechanism will therefore predominate in this solution, not least, directed towards the breakdown of BSA and ester bonds in the bulk polymer. Although both amphotericin B and penicillin/streptomycin were added to the pancreatin solution during incubation, some microbial growth persisted. It may be that the presence of these microorganisms enhanced the biodegradation of the microparticulates. Koosha, Muller and Davies have reported that P(HB-HV) is biodegraded by anaerobic, bacteria and, more slowly, by aerobic bacteria and fungi[139]. In addition, Yammada and colleagues have documented the degradation of P(HB) by *Pseudomonas pickettii,* a bacterium isolated from laboratory media[140].

PEA20% PCL 11 microparticulates were more susceptible to biodegradation in pancreatin solution than P(HB-HV)20% PCL 11 microparticulates. The exact reasons for this are not clear. It may be that the presence of trypsin, chymotrypsin and protease activity in the solution played a significant role in BSA degradation and encouraged the production of new channels and pores. However, while this would explain why PEA20% PCL 11 degrades more quickly than P(HB-HV)20% PCL 11 in this solution, it may be that PEA20% PCL 11 is in some way more susceptible to enzymatic hydrolysis than P(HB-HV)/20% PCL 11. Morphological examination revealed that PEA20% PCL 11 microcapsules and microspheres appeared to degrade in a similar manner, by surface erosion, neither particle developing channels or pores. The surface of PEA microparticulates fractured into segments which remained almost intact over the 30 day incubation period. It would, therefore, appear that the majority of the PWL shown by PEA20% PCL 11 microparticulates is a function of significant BSA dissolution at the microparticle surface; polymer hydrolysis only causing the polymer to fragment. P(HB-HV)20% PCL 11 biodegradation was more extensive in pancreatin solution than in synthetic gastric juice. This might be expected since hydrolytic degradation will proceed faster at pH (7.4) and P(HB-HV)20% PCL 11 would be more susceptible to the enzyme rich cocktail in the pancreatin solution. Pancreatin induced surface erosion was well demonstrated by P(HB-HV)20% PCL 11 microcapsules, although this did not lead to fragmentation or the loss of spherical integrity. With P(HB-HV)20% PCL 11 microspheres, fractures developed in the microsphere surface allowing pancreatin access to the internal matrix of the microspheres and the loss of pieces of polymer by flaking.

CHAPTER FIVE.

MICROENCAPSULATION AND RELEASE OF THE ANTIBIOTIC VANCOMYCIN FROM MICROSPHERES PREPARED FROM POLY (D,L-LACTIDE-CO-GLYCOLIDE), POLY (ETHYLENE ADIPATE) AND POLY(β-HYDROXYBUTYRATE/HYDROXYVALERATE).

5.0 Introduction.

Previous work described in this thesis, had established that the efficiency of the microencapsulation of the surrogate macromolecule, BSA into microspheres prepared using the O/W single emulsion solvent extraction technique was relatively low (4.2 ± 0.09 to 15 ± 0.5). Whilst vehicles could be readily produced by this method it became apparent that the vast majority of any water soluble macromolecule being encapsulated was rapidly lost to the aqueous continuum during fabrication. Bodemeier & McGinity found that the diffusion of material into the aqueous phase during the fabrication of microspheres composed of poly (DL-lactide) led to the loss of ionizable drugs such as quinidine and quinidine sulphate, this loss could be minimized and drug loading improved by adjusting the pH of the aqueous phase and suppressing ionization[141]. Quinidine base ionizes in acidic pH and could not be entrapped at pH 2, whilst it was successfully encapsulated at pH values above 9. However, these workers reported increasingly distorted microsphere surfaces with increased pH of the aqueous continuum. They also reported that microsphere structure became increasingly distorted and porous when the KCL concentration of the aqueous continuous phase was increased.

It is well established that drug loading of moderately water soluble drugs can be improved by the previous addition of core material to the aqueous continuous phase[142]. Bodemeier and McGinity[142] demonstrated an improved loading of quinidine sulphate by this manoeuvre and Wakiyama found improved loading of tetracaine, which is a local anaesthetic, when the drug was previously added to the aqueous continuum[72]. Jalil and Nixon however, found that the microencapsulation of phenobarbitone in poly(L-lactic acid) using the

O/W emulsion method, whilst providing an improved core loading when the aqueous continuum was presaturated, resulted in the formation of numerous free crystals on the surface of the microspheres[17,143].

With this in mind, in the present work, any marginal increase in encapsulation efficiency achieved by presaturating the aqueous phase would have to be offset against the use of excessive quantities of expensive vancomycin in the subsequently discarded aqueous phase. On this basis it was decided to use a technique pioneered by Tsai and coworkers in which a W/O emulsion system was employed. In their method poly(lactic acid) and mitomycin C were dissolved in acetonitrile and emulsified with light mineral oil containing span 65 as an emulsifier[35]. Subsequent removal of the acetonitrile by evaporation at 55°C resulted in the precipitation of polymer containing drug in the form of monolithic microspheres. Span 65 was removed by washing with 40-60°C petroleum ether at 50°C. This technique was later improved by Jalil & Nixon who compared the morphology, particle size and encapsulation efficiency of phenobarbitone loaded microspheres prepared by both the O/W and W/O emulsion systems[17]. The W/O system produced microspheres with a much greater percentage loading (45.12%) than those produced by the O/W technique (8.89%). The effects of various parameters, i.e. stirring rate, emulsifier type and concentration[144], temperature of the solvent[145] and polymer molecular weight[146] on microsphere fabrication from poly(L-lactide) [L-PLA], poly(D,L-lactide)[D,L-PLA] and poly(D,L-lactide-co-glycolide) [PLCG] using the W/O emulsion solvent extraction technique, have now been evaluated. Since the W/O system appeared to give improved microencapsulation efficiencies it was decided to use this technique for the encapsulation of vancomycin hydrochloride.

In this study poly(D,L-lactide-co-glycolide) [PLCG] 50:50 and 75:25, poly (ethylene adipate) [PEA] and blends of the same were chosen as candidate biodegradable polymers for the fabrication of microspheres containing vancomycin. Poly(D,L-lactide-co-glycolide) was chosen because it is one of a limited number of biodegradable polymers considered to be biocompatible and has been used extensively in the pharmaceutical industry. Poly[β-hydroxybutyrate/3 hydroxyvalerate], [P(HB-HV)] and PEA have been blended with PLCG 50:50 in an attempt to accelerate the biodegradation rate of these two polymers. The basis of the present studies was to fabricate biodegradable microspheres composed of PLCG 50:50 and 75:25; PEA; 50/50 PEA with PLCG 50:50 and 75%P(HB-HV) and 25%PLCG 50:50 blends using W/O emulsification with solvent extraction and to assess the percentage yield, size distribution, surface topography and ultrastructural morphology of the microspheres. The microspheres have been loaded with vancomycin at 10%, 25% and 50% by weight of polymer and the actual encapsulation efficiency determined. This has been followed by evaluation of the kinetics of static vancomycin release into Hank's buffer and newborn calf serum *in vitro* at 37°C over 30-35 days.

5.1 Methodology.

Microspheres were fabricated from the following polymers and blends (PLCG 50:50; PLCG 75:25; PEA; 50% PEA 50% PLCG 50:50 and 75%P(HB-HV) 25%PLCG 50:50) by the W/O single emulsion solvent extraction technique detailed in section 2.4, page 80, and summarized in figure 2.3, page 72. Microspheres were loaded with vancomycin HCL at 10%, 25% and 50% ie 1g of polymer was combined with 1g of

vancomycin for a core to polymer ratio of 1:1 (50% loading); with 0.333g of vancomycin for a 1:3 ratio (25% loading) and with 0.1g of vancomycin for a 1:10 ratio, (10% loading). Span 40 was chosen as the emulsifier in this system because work carried out by Jalil and Nixon[143] had shown that this surfactant resulted, on average, in the production of smaller microspheres than microspheres fabricated using either Span 65 or Span 80. The resultant microspheres were air dried and stored in a desiccator over silica gel to ensure the removal of residual moisture. The percentage yield was given by

$$\frac{\text{Actual Yield (g)}}{\text{Theoretical maximum Yield (g)}} \times 100\%$$

The size distribution of microspheres and vancomycin encapsulation efficiency were determined as described in section 2.5, page 83 and 2.6, page 84. In release studies 0.2g quantities of microspheres were incubated at 37°C in 2ml of either Hank's buffer, pH 7.4 or newborn calf serum for a period of up to 30-35 days (or until no further release of vancomycin could be detected). Vancomycin was assayed by radial diffusion, section 2.7.2, page 86. The concentration of vancomycin in samples was either extrapolated directly from standard curves of clearing zone diameter (mm) versus a range of vancomycin concentration, 10-200 μg/ml or determined from the computer generated regression equations of standard curves prepared using distilled water, Hank's buffer and new born calf serum (figures 5.1.1, 5.1.2, 5.1.3 and 5.1.4 respectively) The rate of vancomycin release was expressed either as the day to day release, the total

Figure 5.1.1 Vancomycin standard curve in distilled water (mean values ± SEM, n=12 for each point)

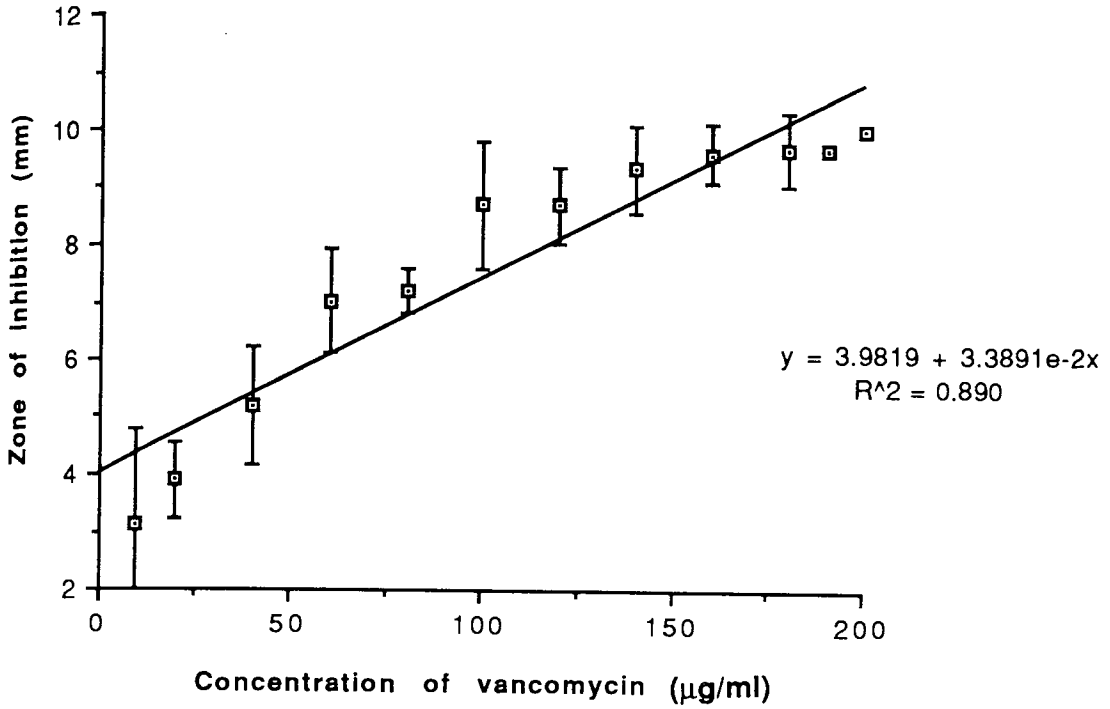


Figure 5.1.2 Vancomycin standard curve in Hank's buffer, pH 7.4 (mean values ± SEM, n=12 for each point)

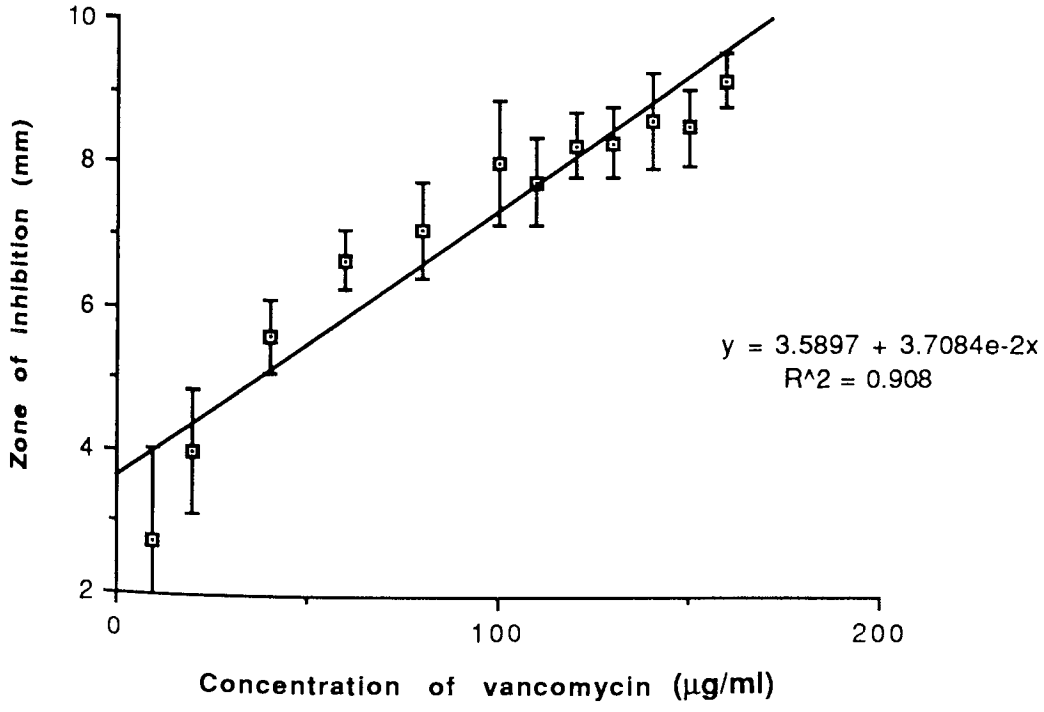


Fig 5.1.3. Standard curve using low concentrations of vancomycin in Hank's buffer, (mean values ± SEM, n=12 for each point).

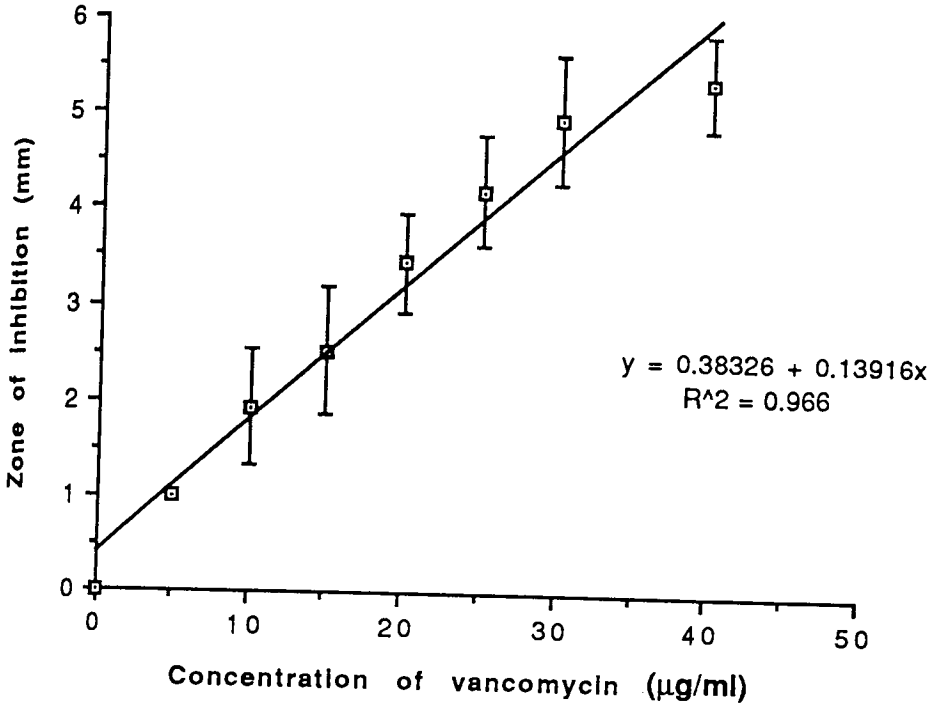
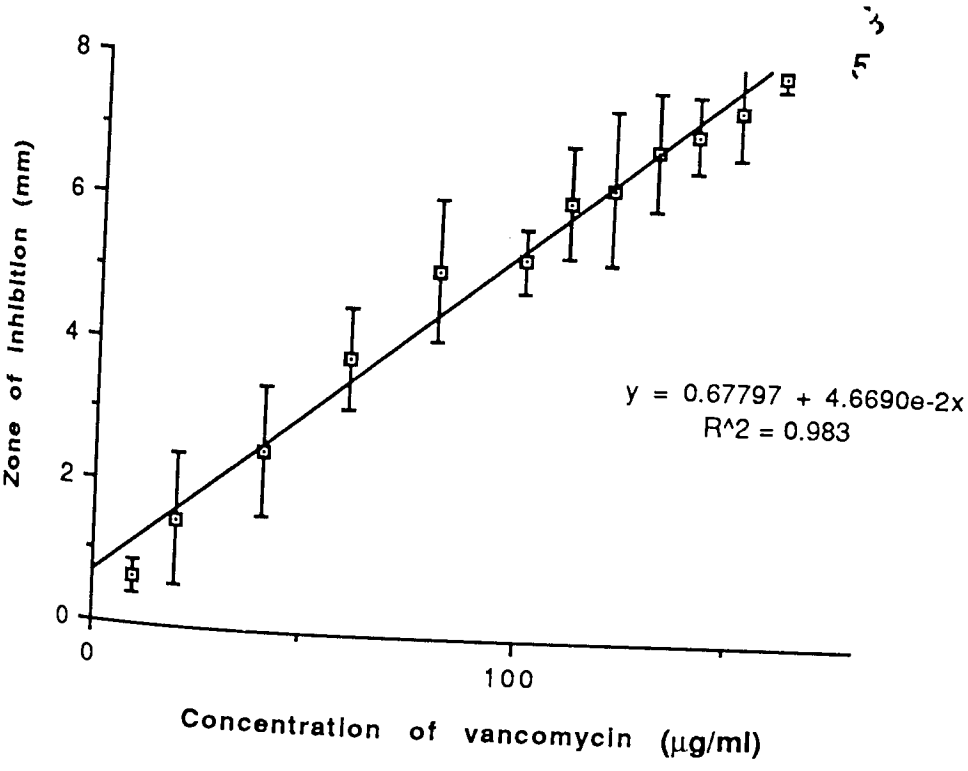


Fig 5.1.4 Vancomycin standard curve in new-born calf serum, (mean values ± SEM, n=12 for each point.)



cumulative release or the total cumulative percentage release versus the square root of t.

5.2 Results.

Spherical monolithic microspheres were successfully fabricated from PLCG 50:50, PLCG 75:25, PEA and the blend 50% PEA/50%PLCG 50:50 using the w/o single emulsion solvent extraction technique. The surface of unloaded PLCG 50:50 microspheres appeared smooth (plate 5.1a), page153 although when loaded with 25% and 50% vancomycin, crystals were deposited and embedded in the surface, plates 5.1b and 5.1c. Unloaded PLCG 75:25 microspheres (plate 5.2a and 5.2b, page154) also had a smooth surafce, but vancomycin crystals were associated with the surface of 25% and 50% loaded microspheres, plates 5.2c and 5.2d. Close examination of the internal structure of a fractured microsphere (plate 5.2b) revealed a macroporous honeycomb like matrix.

Unloaded PEA microspheres had a more rugose surface (plate 5.3a) than unloaded PEA/PLCG microspheres, which were spherical in shape with a smooth surface, plate 5.4a. 25% vancomycin loaded PEA microspheres, plate 5.3b showed limited evidence of crystal deposition on their surface but this was significantly increased on similarly loaded 50% PEA/50% PLCG 50:50 microspheres, plate 5.4b.

Unloaded 75% P(HB-HV)/25% PLCG 50:50 blend microspheres, plate 5.5a, page156 were irregularly shaped with a rough pitted surface. Vancomycin crystals were observed embedded in the surface of microspheres loaded with 25% vancomycin, plate 5.5b. Two reasons may account for vancomycin deposition on the surface of microspheres. Firstly vancomycin is poorly soluble in petroleum ether, therefore

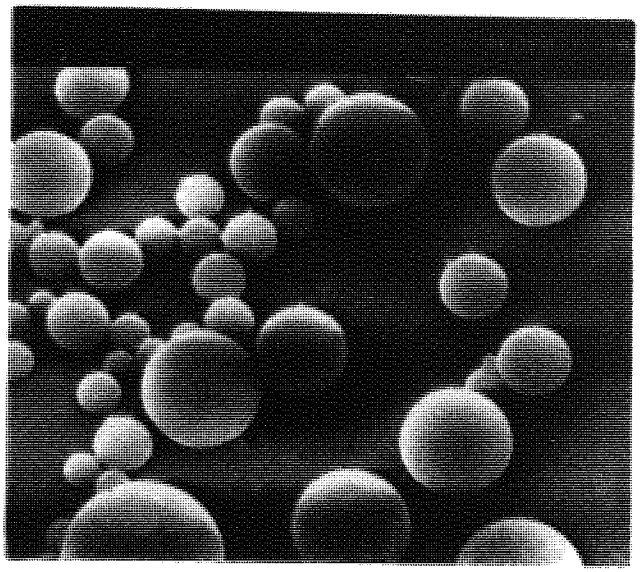


Plate 5.1a

Unloaded PLCG 50:50

microspheres (x948)

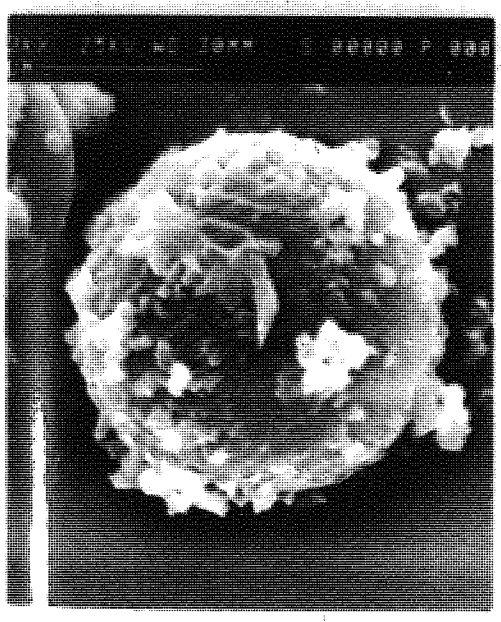


Plate 5.1b

25% vancomycin loaded PLCG

50:50 microspheres (KX1,13).

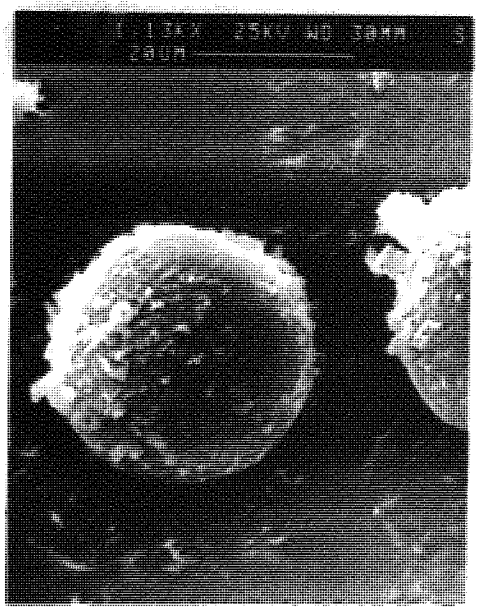


Plate 5.1c

50% vancomycin loaded PLCG

50:50 microspheres (KX1,13).

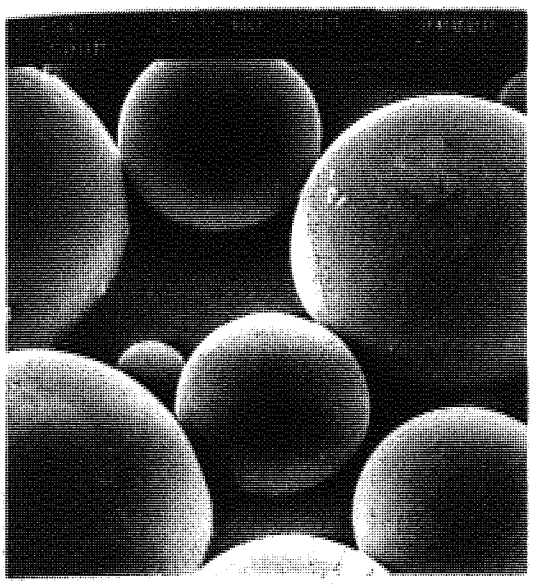


Plate 5.2a

Unloaded 75:25 PLCG microspheres (X984).

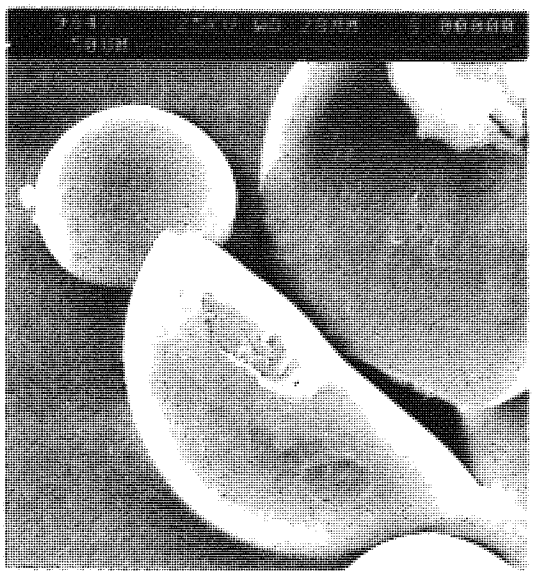


PLate 5.2b

Unloaded 75:25 PLCG microspheres(X984).

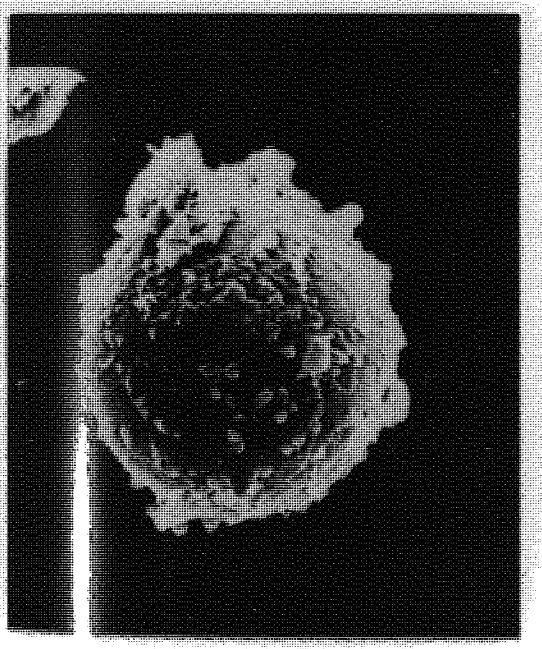


Plate 5.2c

25% vancomycin loaded 75:25 PLCG microsphere (X607).

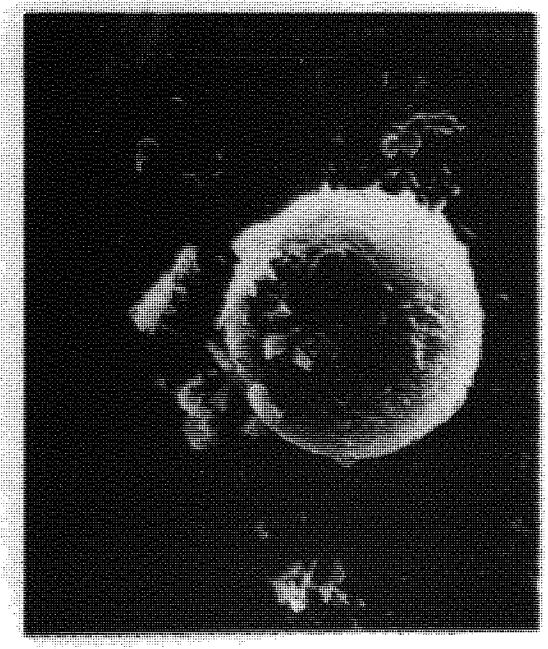


Plate 5.2d

50% vancomycin loaded PLCG 75:25 microsphere (KX1,18).

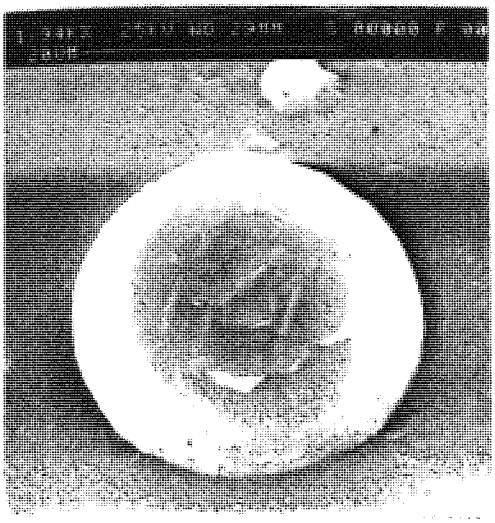


Plate 5.3a

Unloaded PEA

microsphere (KX1,94)

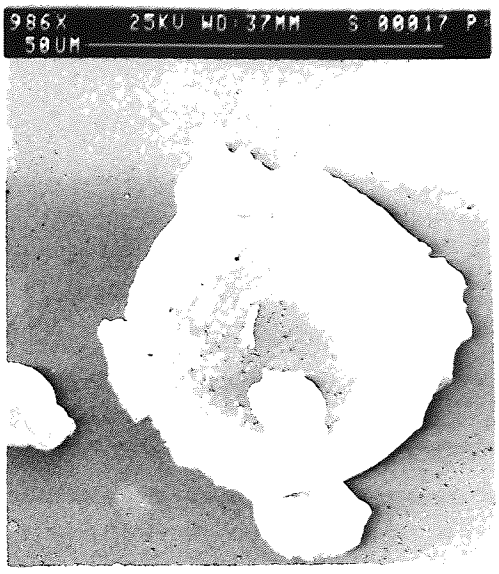


Plate 5.3b

25% vancomycin loaded PEA

microsphere (X986)

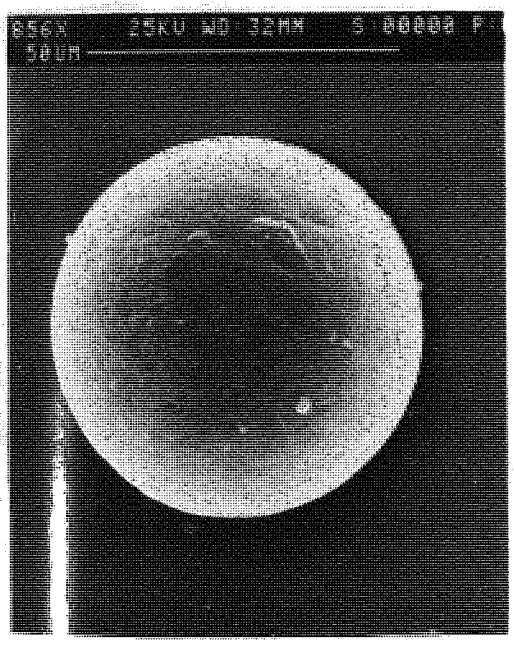


Plate 5.4a

Unloaded PEA/PLCG

microsphere (X856)

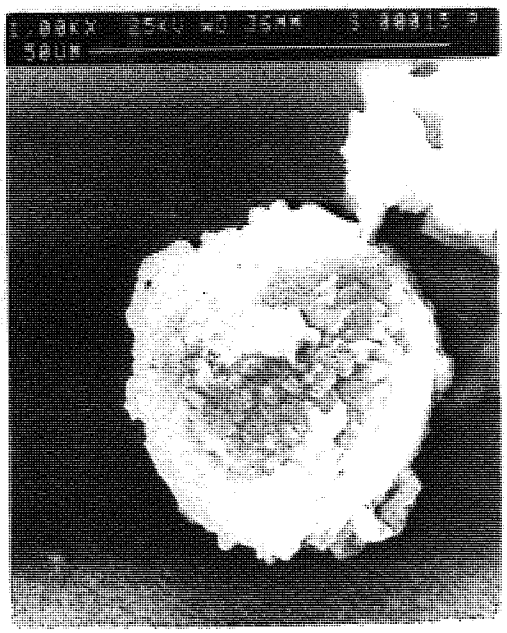


Plate 5.4b

25% vancomycin loaded PEA/PLCG

microsphere (KX1,00)

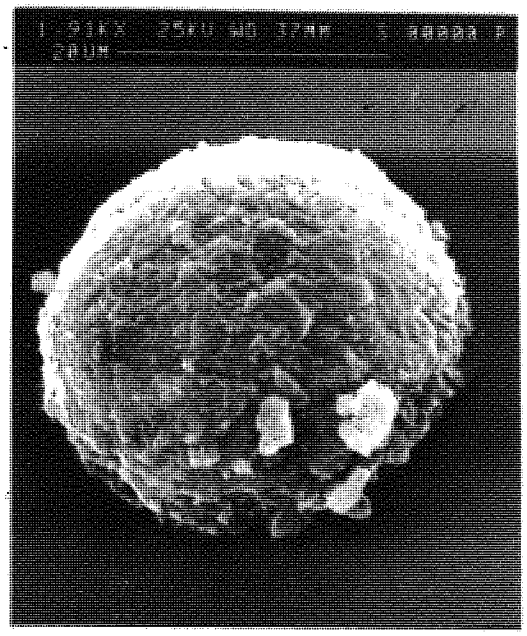


Plate 5.5a

Unloaded P(HB-HV)/PLCG

microsphere (KX1,91)

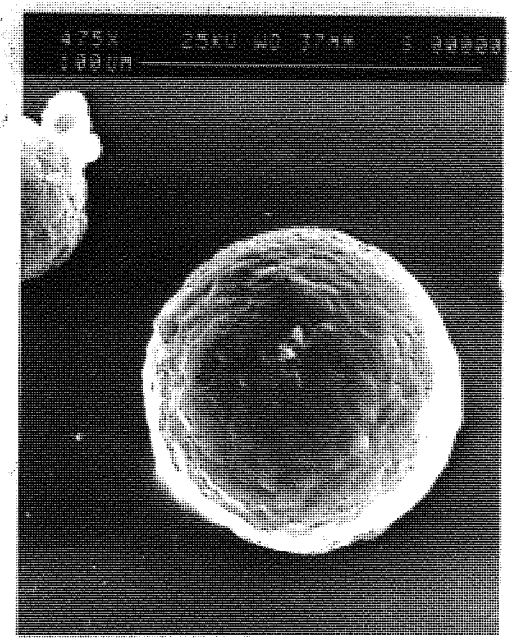


Plate 5.5b

25% vancomycin loaded

P(HB-HV)/PLCG microsphere (475X)

washing the microspheres with petroleum ether to remove span 40 and the light mineral oil would not result in the removal of surface deposites of vancomycin and secondly because of the high vancomycin loading used the amount of polymer was insufficient to accomodate all the vancomycin crystals.

5.2.1 Percentage yield of vancomycin loaded microspheres.

The average percentage yields obtained during the fabrication of vancomycin loaded microspheres were generally high Table 5.1. Increasing the percentage vancomycin load tended to reduce the percentage yield irrespective of the polymer used. This reduction was most marked with 75% P(HB-HV)/25% PLCG 50:50 microspheres. Here the % yield of 50% vancomycin loaded microspheres was reduced by some 28.5% compared with 10% vancomycin loaded microspheres, Table 5.1, over page.

5.2.2 Size distribution of unloaded and vancomycin loaded microspheres.

For each polymer and polymer blend, the size distribution of 65-75 microsphere diameters was evaluated from SEM thermal prints.

Unloaded PLCG 50:50 microspheres ranged between 5-50μm in diameter, with a mean of 17.39 ± 6.89μm (n=74). The greatest proportion had a size range of between 10-20μm, figure 5.1.5. Percentage loading had no statistically significant effect on the size distribution of PLCG 50:50 microspheres, with 25% vancomcyin loaded microspheres ranging from between 5-50μm in diameter, figure 5.1.6. However 25% vancomycin loaded microspheres had a mean diameter of

Table 5.1 **Average percentage yields obtained for vancomycin containing microspheres, (mean values ± SEM)**

Polymer	n	% vancomycin loading	Average % Yield
PLCG 50:50	3	10	95.96 ± 1.76
	3	25	84.76 ± 4.6
	3	50	79.24 ± 4.02[a]
PLCG 75:25	3	10	97.16 ± 4.3
	2	25	90.89 ± 3.2
	3	50	91.85 ± 2.8
PEA	3	25	54.43 ± 7.69
PEA/PLCG	3	25	40.75 ± 2.86
P(HB-HV)/PLCG	3	10	90.18 ± 0.92
	3	25	80.19 ± 3.34
	3	50	64.48 ± 2.9[a]

a, significantly reduced, $p<0.05$ compared with 10% vancomycin loaded microspheres fabricated using the same polymer.

19.87 ± 1.07µm compared with 17.39 ± 6.89µm for unloaded PLCG 50:50 microspheres. Furthermore microspheres loaded with 25% vancomycin had double the proportion of microspheres in the 20-30µm diameter size range compared to unloaded microspheres, figure 5.1.6.

PLCG 75:25 microspheres had a mean diameter of 26.34 ±6.4 µm

Figure 5.1.5 Size distribution of unloaded PLCG 50:50 microspheres, (mean values ± SEM, n=74).

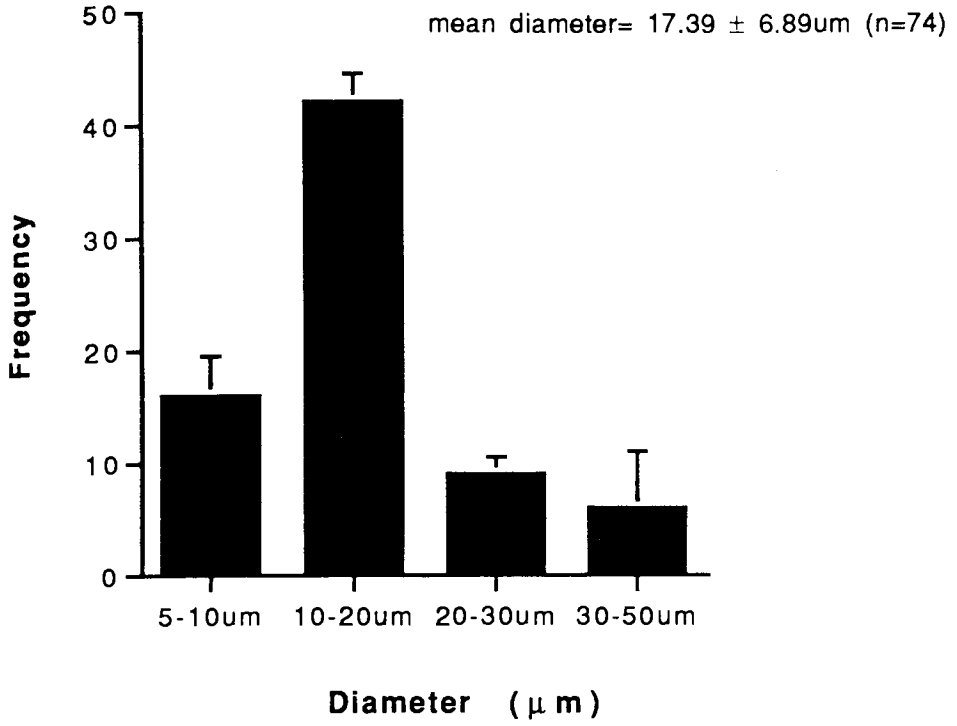


Figure 5.1.6 Size distribution of 25% vancomycin loaded PLCG 50:50 microspheres, (mean values ± SEM, n=72).

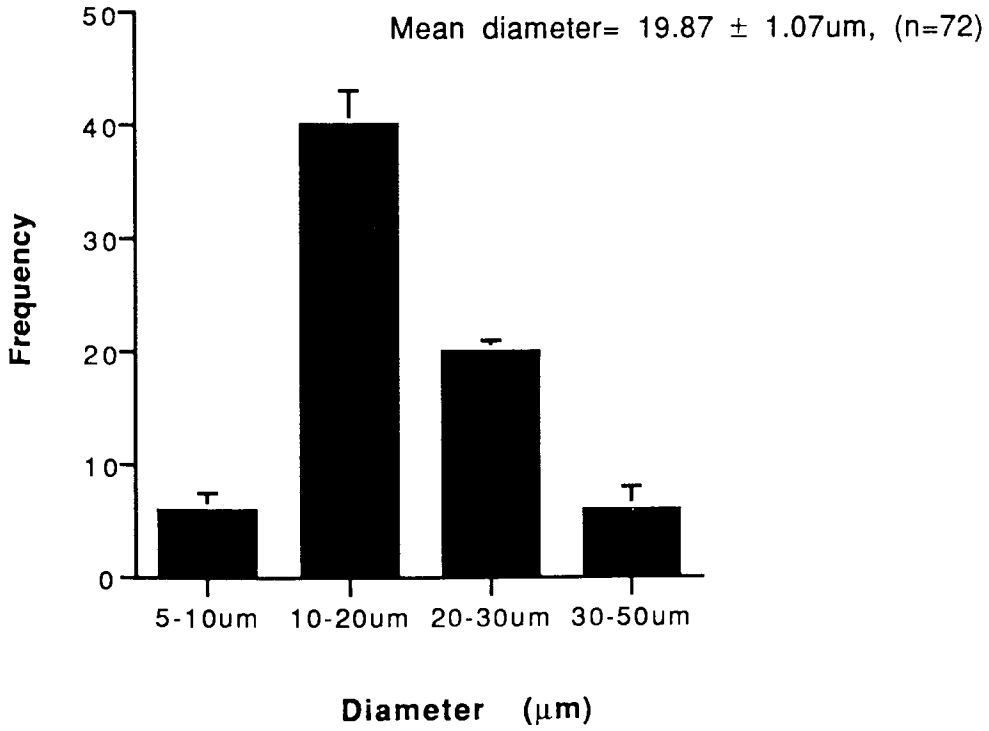


Figure 5.1.7 Size distribution of unloaded PLCG 75:25 microspheres. (mean values ± SEM, n=65)

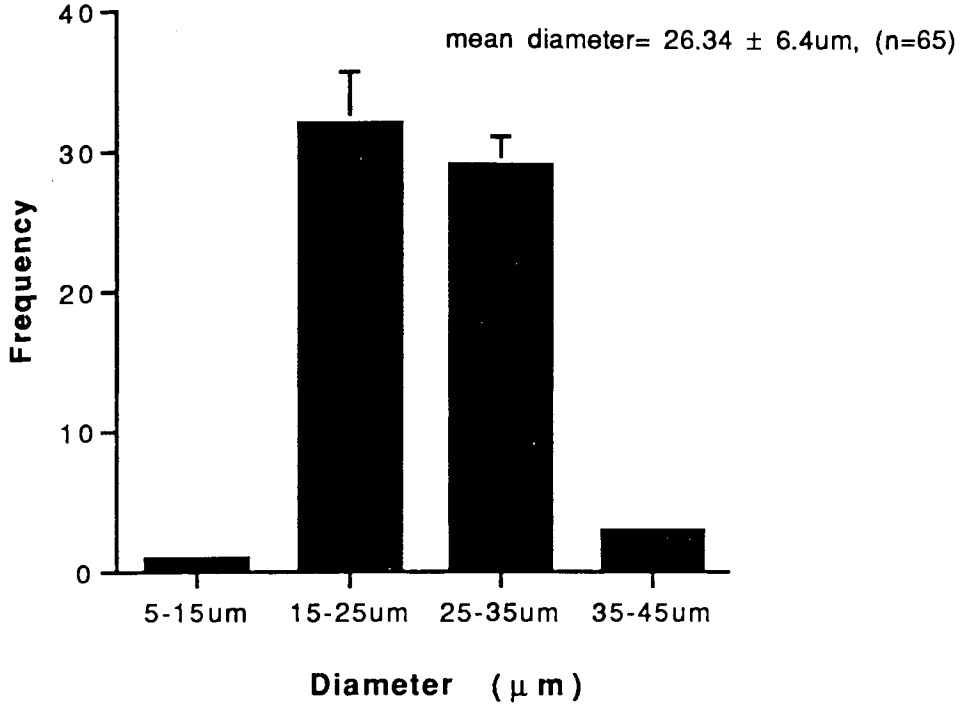


Figure 5.1.8 Size distribution of 25% vancomycin loaded PLCG 75:25 microspheres, (mean values ± SEM, n=65).

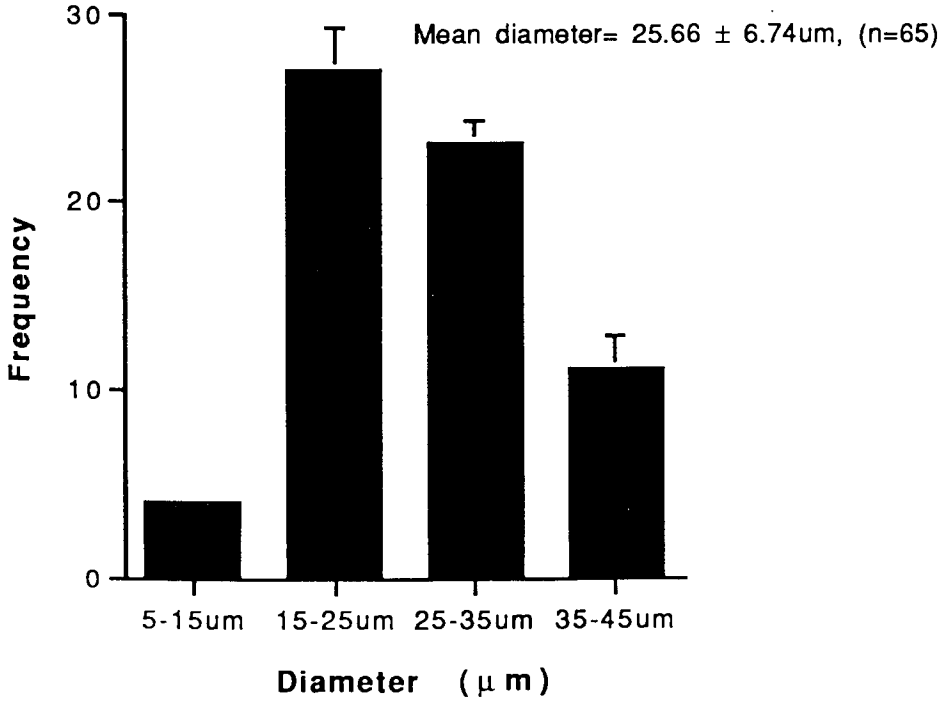


Figure 5.1.9 Size distribution of 50% vancomycin loaded PLCG 75:25 microspheres, (mean values ± SEM, n=65)

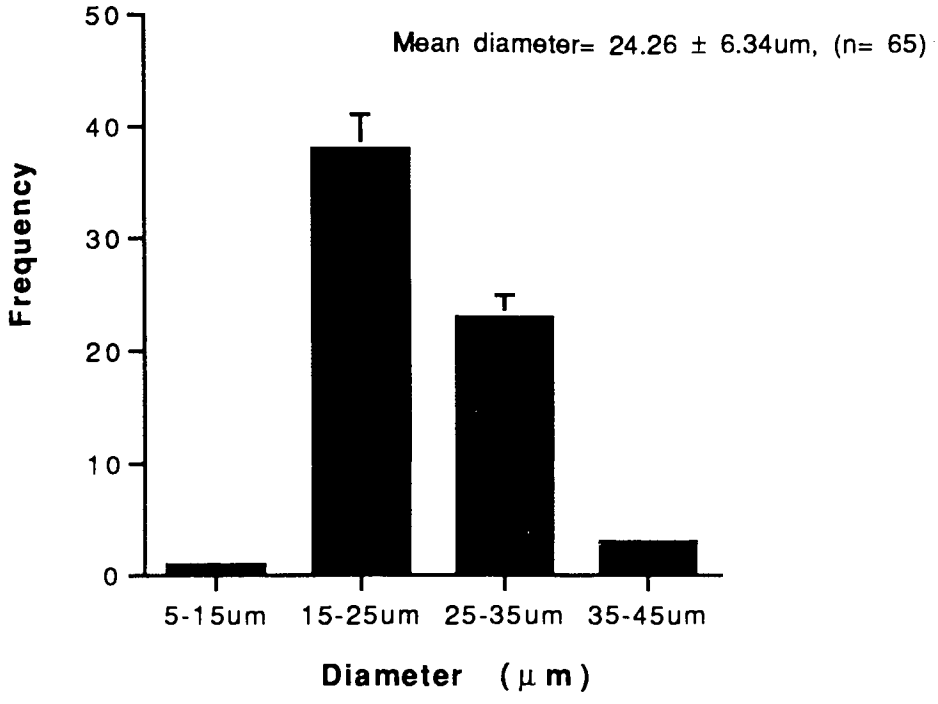


Figure 5.2.0 Size distribution of unloaded PEA microspheres, (mean values ± SEM, n=70).

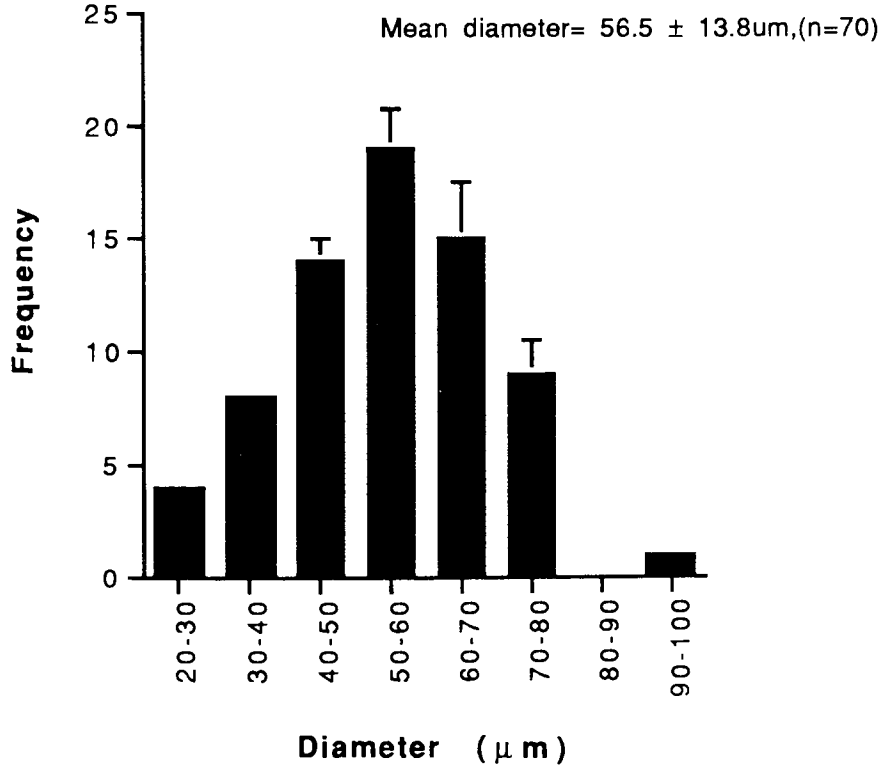


Figure 5.2.1 Size distribution of unloaded PEA/PLCG microspheres, (mean values ± SEM, n=70)

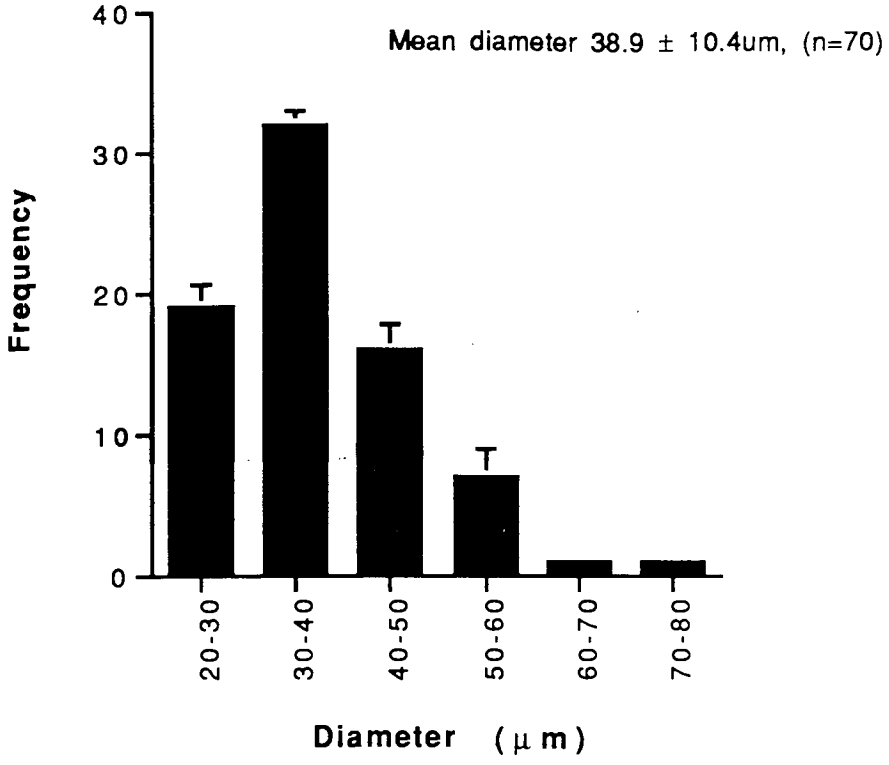
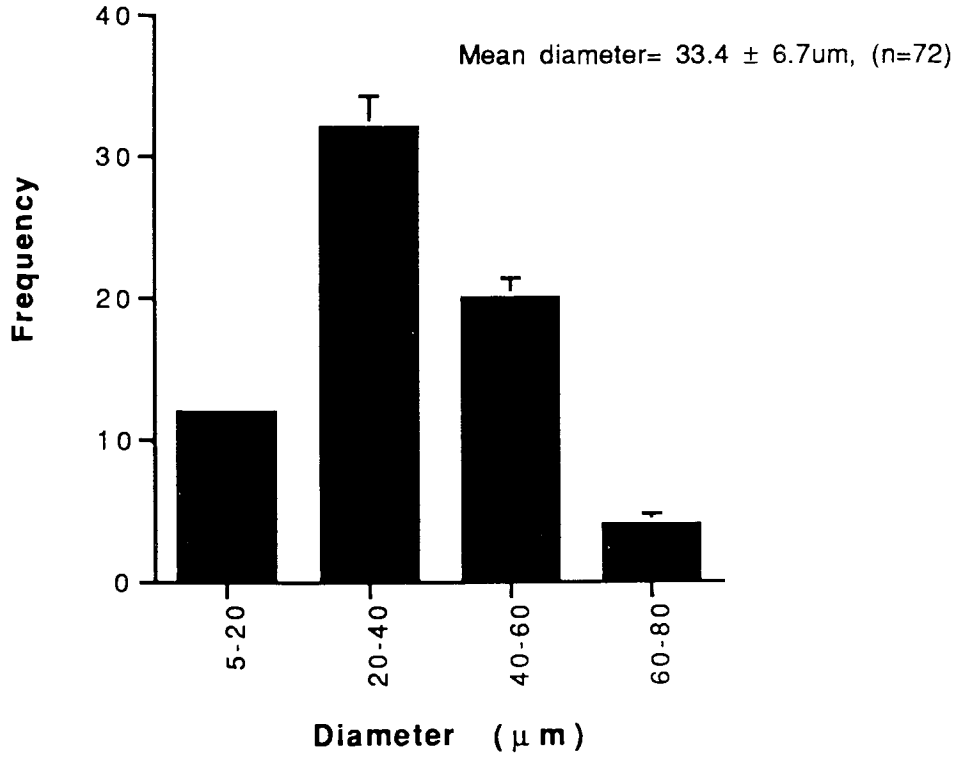


Figure 5.2.2 Size distribution of unloaded P(HB-HV)/PLCG microspheres, (mean values ± SEM, n=72)



with 94% falling in the in 15-35μm range (figure 5.1.7). The mean diameter decreased slightly but not significantly to 25.66 ± 6.77μm (n=65) when PLCG 75:25 microspheres were loaded with 25% vancomycin and to 24.26 ± 6.34μm (n=65) when loaded with 50% vancomycin, figures 5.1.8 and 5.1.9.

PEA microspheres tended to be larger than PLCG and ranged from 28-96μm in diameter with a mean of 56.5 ± 13.8μm (n=70), figure 5.2.0. Blending PEA with 50% PLCG 50:50 tended to increase the proportion of small microspheres with a mean diameter of 38.9 ± 10.4 over a range of 24-77μm, figure 5.2.1.

Unloaded 75% P(HB-HV) 25% PLCG 50:50 microspheres ranged from 5-78μm in diameter, with a mean diameter of 33.4μm ± 6.7μm (n=72), figure 5.2.2.

5.2.3 Encapsulation efficiency of vancomycin loaded microspheres

The mean encapsulation efficiencies of 10%,25% and 50% vancomycin loaded microspheres fabricated using the various polymers and their blends have been summarized in Table 5.2. Only in the case of PLCG 75:25 did the mean encapsulation efficiency increase significantly with percentage loading. In all other cases percentage vancomycin loading had no significant effect on the encapsulation efficiency, Table 5.2. Maximum encapsulation efficiencies for PLCG 50:50 and 75% P(HB-HV) 25%PLCG 50:50 microspheres were observed with 10% vancomycin loading ie 79.14 ± 8.4% and 59.49 ± 9.49% respectively. Mean encapsulation efficiency for PLCG 50:50 was always significantly higher than that of 75% P(HB-HV) 25% PLCG 50:50 microspheres with the same % vancomycin loading Table 5.2.

Table 5.2. Mean encapsulation efficiencies of vancomycin loaded microspheres (mean values ± SEM, n=12).

Polymer	n	% vancomycin loading	Mean encaps efficiency (%)
PLCG 50:50	12	10%	79.14 ± 8.4
	12	25%	64.19 ± 2.94
	12	50%	70.52 ±14.92
PLCG 75:25	12	10%	70.52 ± 3.41
	12	25%	89.83 ± 6.67
	12	50%	96.74 ± 13.2[a]
PEA	12	25%	66.42 ± 7.0
PEA/PLCG	12	25%	67.65 ± 3.6[c]
P(HB-HV)/PLCG	12	10%	59.49 ± 9.49[b]
	12	25%	47.75 ± 9.0[b]
	12	50%	50.23 ± 4.3[b]

a, significantly elevated, $p < 0.05$, compared with 10% vancomycin loaded microspheres fabricated using PLCG 75:25.

b, significantly reduced, $p < 0.05$, compared with the corresponding % vancomycin loading of PLCG 50:50 microspheres.

c, significantly elevated, $p < 0.05$, compared with 25% vancomycin loaded microspheres fabricated using 75% P(HB-HV)/25% PLCG.

5.2.4 The release of vancomycin from microspheres.

The concentration of vancomycin in release samples from incubated microspheres was determined by extrapolation from the radial diffusion standard curves for either Hank's buffer, figure 5.1.2 or newborn calf serum, figure 5.1.4.

Vancomycin release from PLCG 50:50 microspheres.

The daily vancomycin release profile from 25% and 50% loaded 50:50 PLCG microspheres incubated in Hank's buffer was characterized by an initial short burst release phase lasting 2 days, presumably a function of the high solubility of vancomycin associated with the surface and superficial layers of the microspheres, followed by a much reduced but physiologically meaningful level (mean levels of vancomycin release of 120 ± 0.07μg/ml and 17± 0.09μg/ml for 25% and 50% vancomycin loaded microspheres respectively) of sustained vancomycin release for up to at least 15 days, figure 5.2.3. The total cumulative release of vancomycin in Hank's buffer increased proportionally with percentage loading. Significantly elevated levels of vancomycin were released from 50% loaded microspheres compared with 10% and 25% loaded microspheres, $p<0.05$. Detectable release of vancomycin from 10% loaded microspheres ceased after 4 days, figure 5.2.4. A similar release profile was observed for PLCG 50:50 microspheres incubated in newborn calf serum at 37°C (Figure 5.2.5). Although the extent of the vancomycin burst release phase from 25% and 50% loaded microspheres was somewhat less pronounced in newborn calf serum than in Hank's, it was significantly more protracted and lasted for about 8 days, figure 5.2.5.

Figure 5.2.3 The effect of percentage loading on the daily release of vancomycin from PLCG 50:50 microspheres in Hank's buffer, pH 7.4. (mean values ± SEM, n=12)

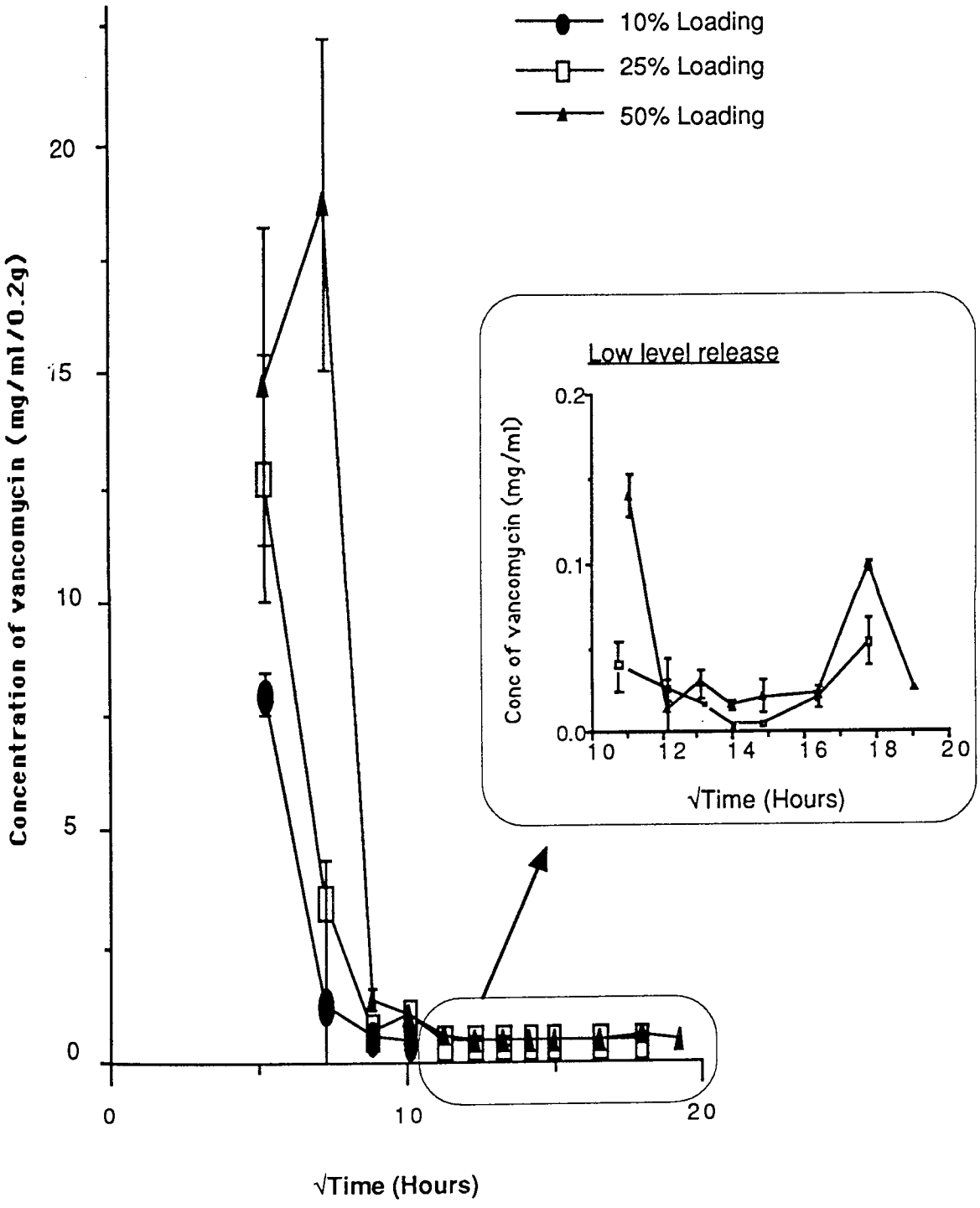
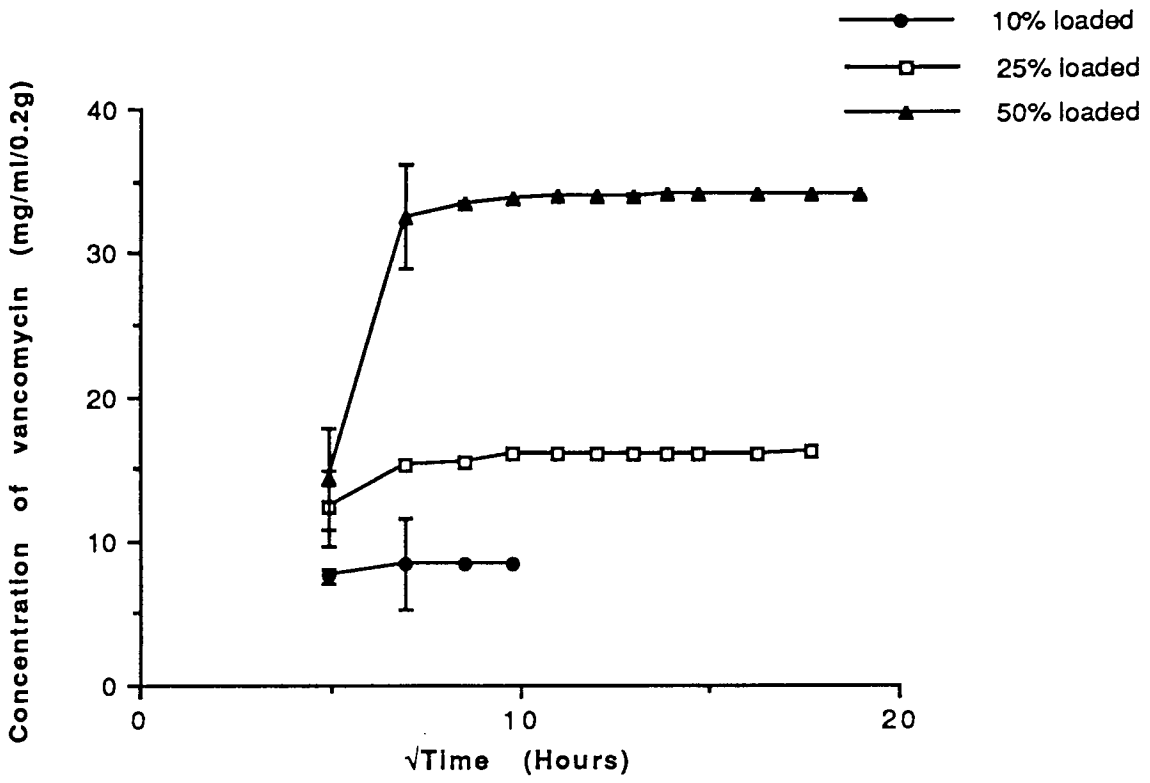


Figure 5.2.4

The effect of percentage loading on total cumulative release of vancomycin from PLCG 50:50 microspheres in Hank's buffer, pH 7.4 at 37°C. (mean values ± SEM, (n=12).



Subsequent low level vancomycin release lasted for a further 11 days, although the level of release was less physiologically significant. The total mean cumulative release of vancomycin increased with increasing percentage loading and 10% loaded microspheres ceased to release detectable amounts of vancomycin after 4 days, figure 5.2.6.

Figure 5.2.5

The effect of percentage loading on the daily release of vancomycin from PLCG 50:50 microspheres in new born calf serum, 37°C. (mean values ± SEM, n=12)

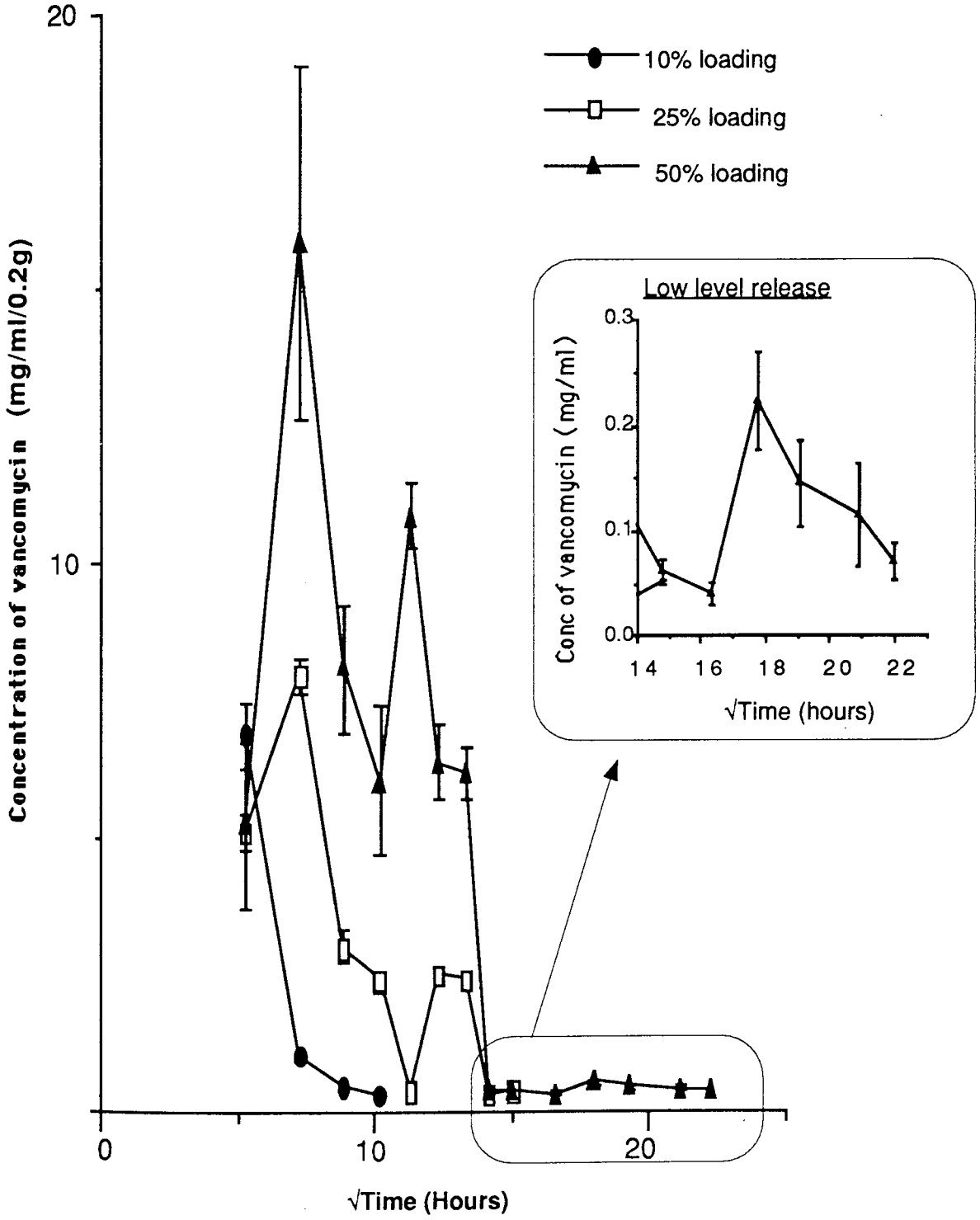
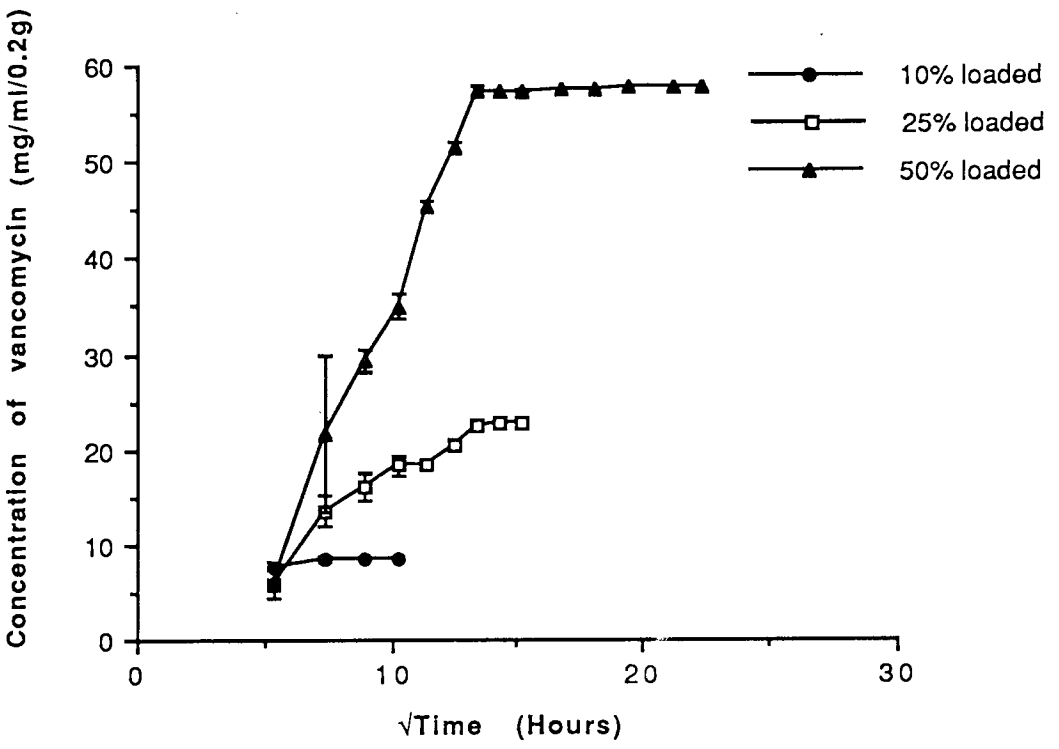


Figure 5.2.6

The effect of percentage loading on the cumulative release of vancomycin from PLCG 50:50 microspheres in newborn calf serum at 37°C. (mean values ± SEM, n=12)



Release from PLCG 75:25 microspheres.

The total mean cumulative release of vancomycin from 25% and 50% loaded PLCG 75:25 microspheres incubated in newborn calf serum at 37°C was characterized by an initial burst release followed by a low level sustained release extending to 26 and 27 days respectively. The concentration of vancomycin actually released from 50% loaded microspheres during the initial burst phase was significantly different ($p<0.05$) and approximately 3x that from 25% loaded microspheres, figure 5.2.7.

The total mean cumulative vancomycin release from 50% PLCG loaded microspheres, after 2 days incubation in newborn calf serum at 37°C appeared to exceed the actual concentration of vancomycin

incorporated into microspheres as determined by radial diffusion assay. This inaccuracy was thought to be a consequence of the very high dilutions required to reduce the sample concentration, so that the vancomycin concentration could be extrapolated from the bioassay standard curve. This factor also explains the large standard errors of the means associated with the first five points on the release profiles, where significant dilution was called for. In order to account for this anomaly a total cumulative percentage release profile was constructed in which the total mean cumulative release after 30 days was taken as 100%. Values for vancomycin release which fell below this value were expressed as a percentage of the maximum vancomycin release obtained after 30 days, figure 5.2.8. On this basis 97.91% of the total mean vancomycin release from 25% loaded microspheres occurred within the initial 4 day period of burst release. A period of sustained release extending to 13 days then followed during which the remaining 2.09% of the vancomycin load was released. 50% loaded microspheres released 98.43% of the mean total vancomycin content within the first 5 days of the burst release phase, with the remaining 1.57% being released at a low, sustained level over the subsequent 25 days, figure 5.2.8. The concentration of vancomycin released after the initial burst release and over the subsequent 26 days and 25 days from 25% and 50% loaded microspheres respectively, although significantly different ($p<0.05$), was low. However, on a day to day basis the vancomycin concentration remained therapeutically significant, figure 5.2.9. 10% loaded microspheres exhibited almost no burst release and the amount of vancomycin incorporated did not appear to be sufficient to maintain a significant detectable release of vancomycin from microspheres beyond day 5 when incubated in newborn calf serum, figure 5.2.9.

Figure 5.2.7 The effect of percentage loading on the total mean cumulative release of vancomycin from PLCG 75:25 microspheres in newborn calf serum at 37°C. (mean values ± SEM, n=12)

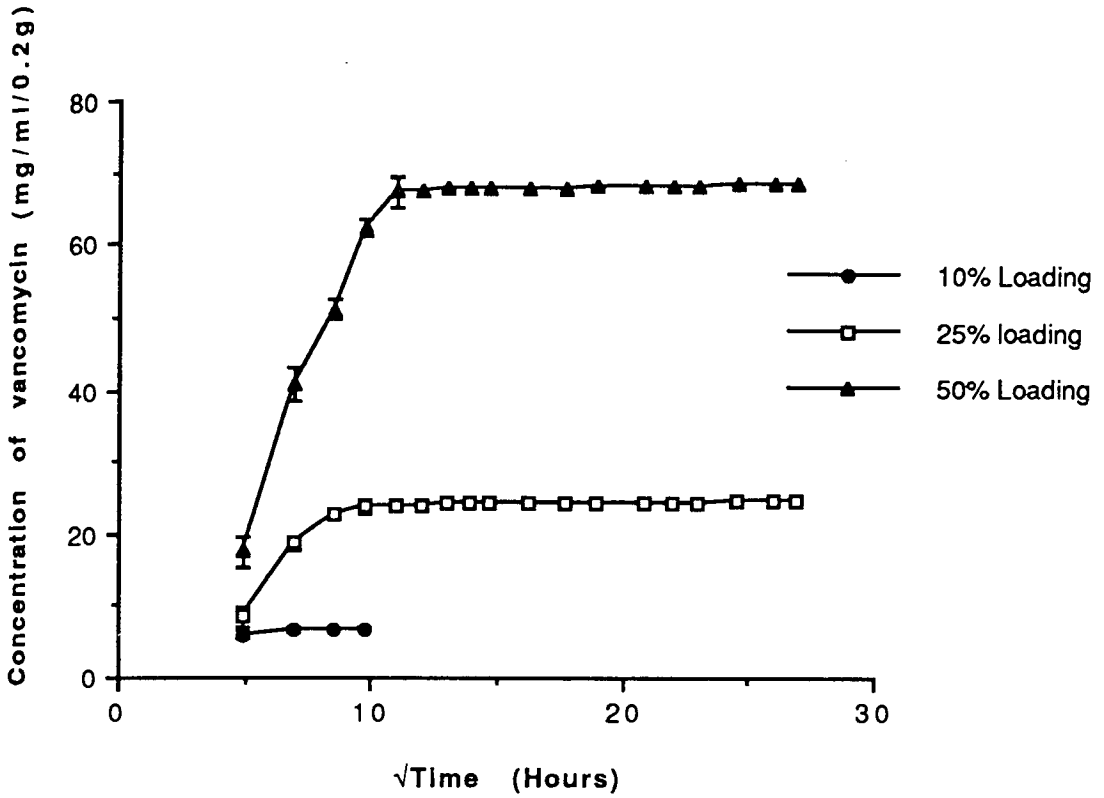


Figure 5.2.8 The total cumulative percentage release of vancomycin from PLCG 75:25 microspheres in newborn calf serum at 37°C. (mean values ± SEM, n=12)

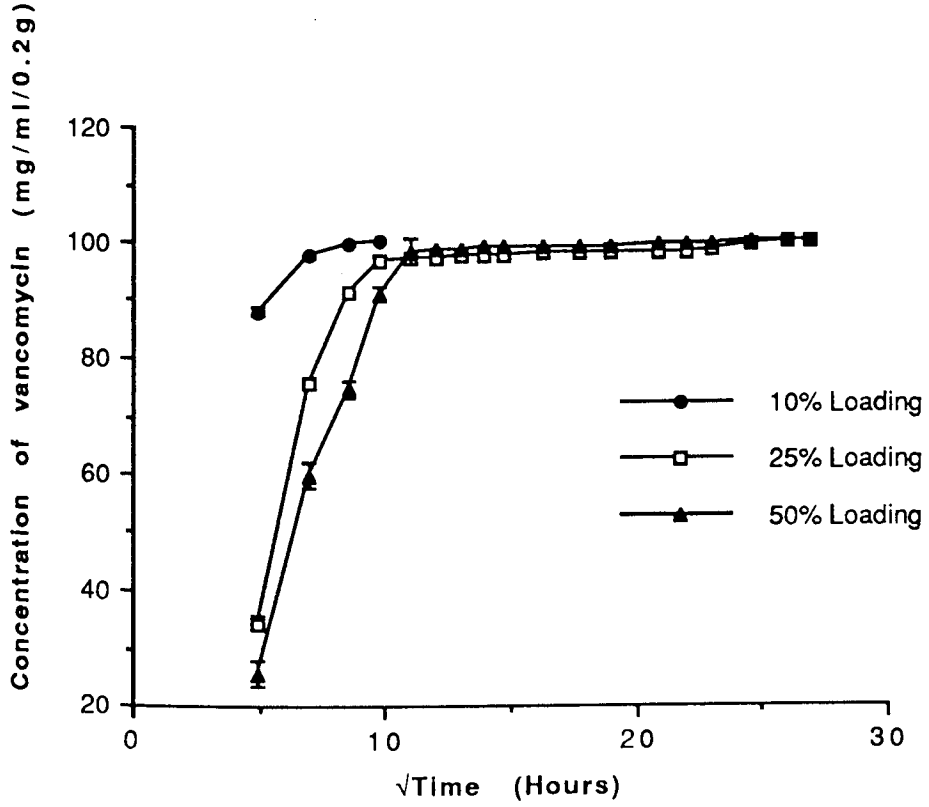


Figure 5.2.9 The effect of percentage loading on the daily release of vancomycin from PLCG 75:25 microspheres incubated in newborn calf serum at 37°C. (mean values ± SEM, n=12)

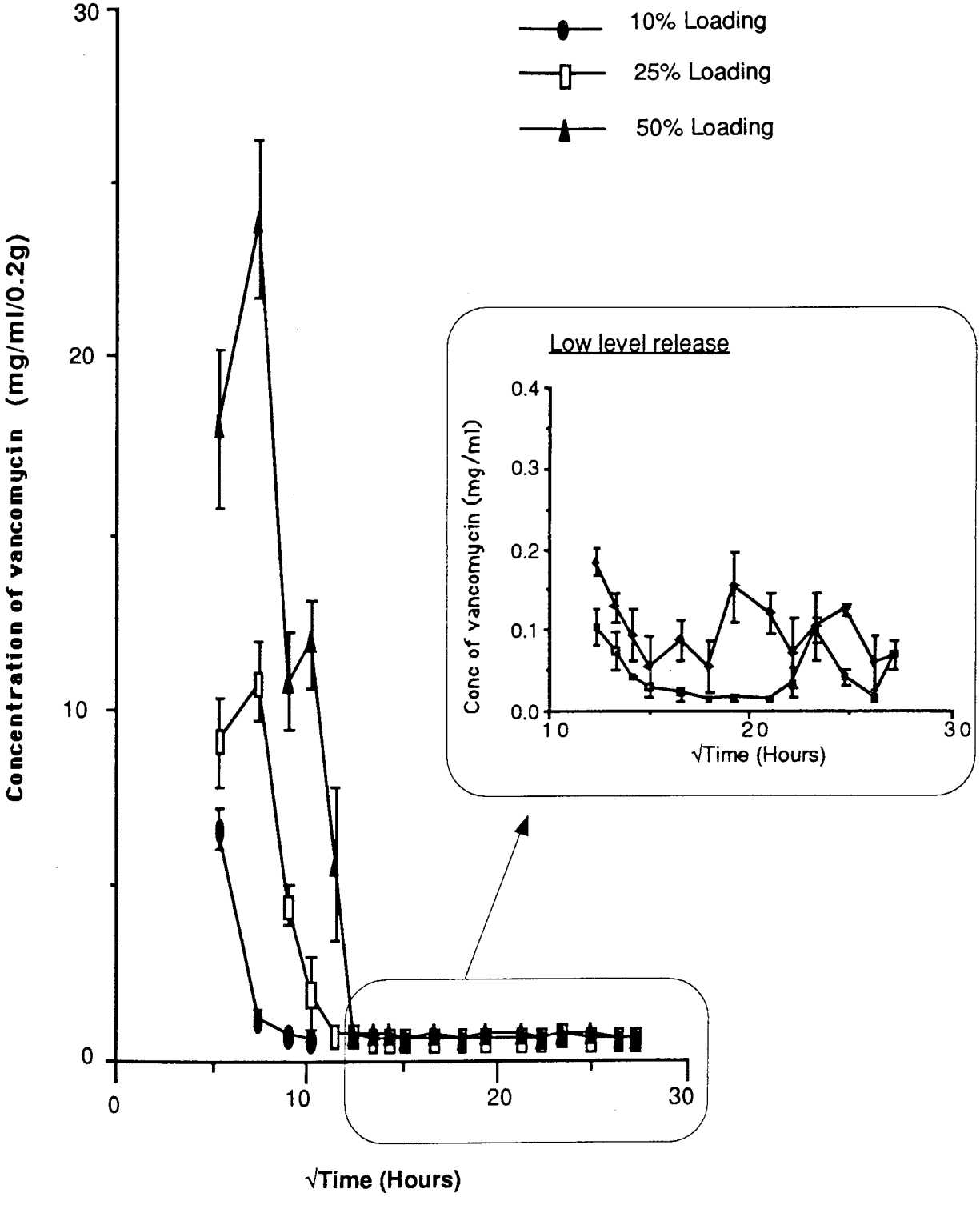
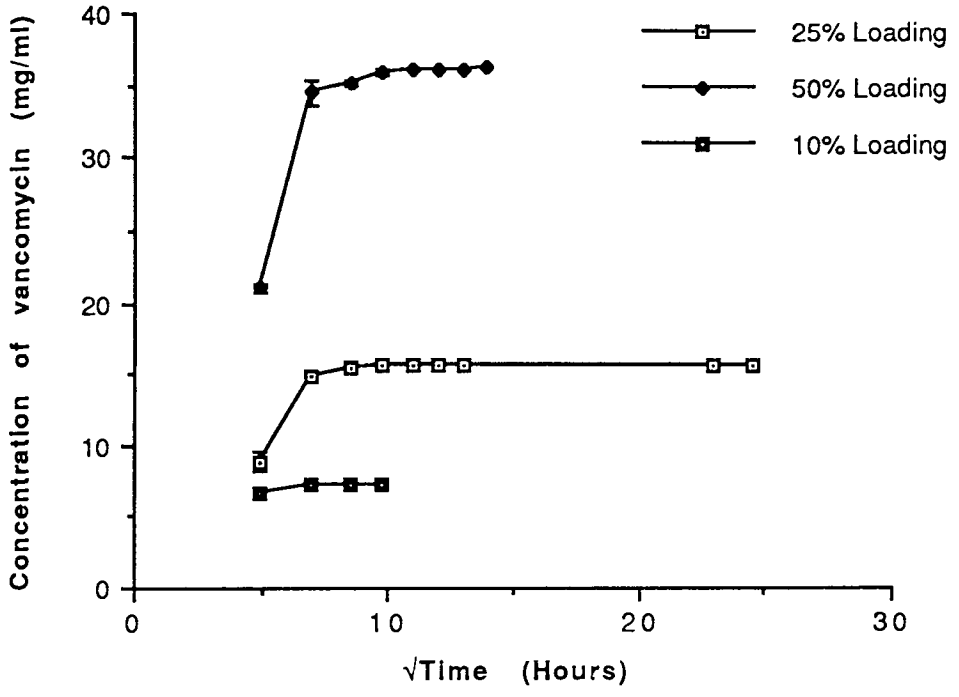
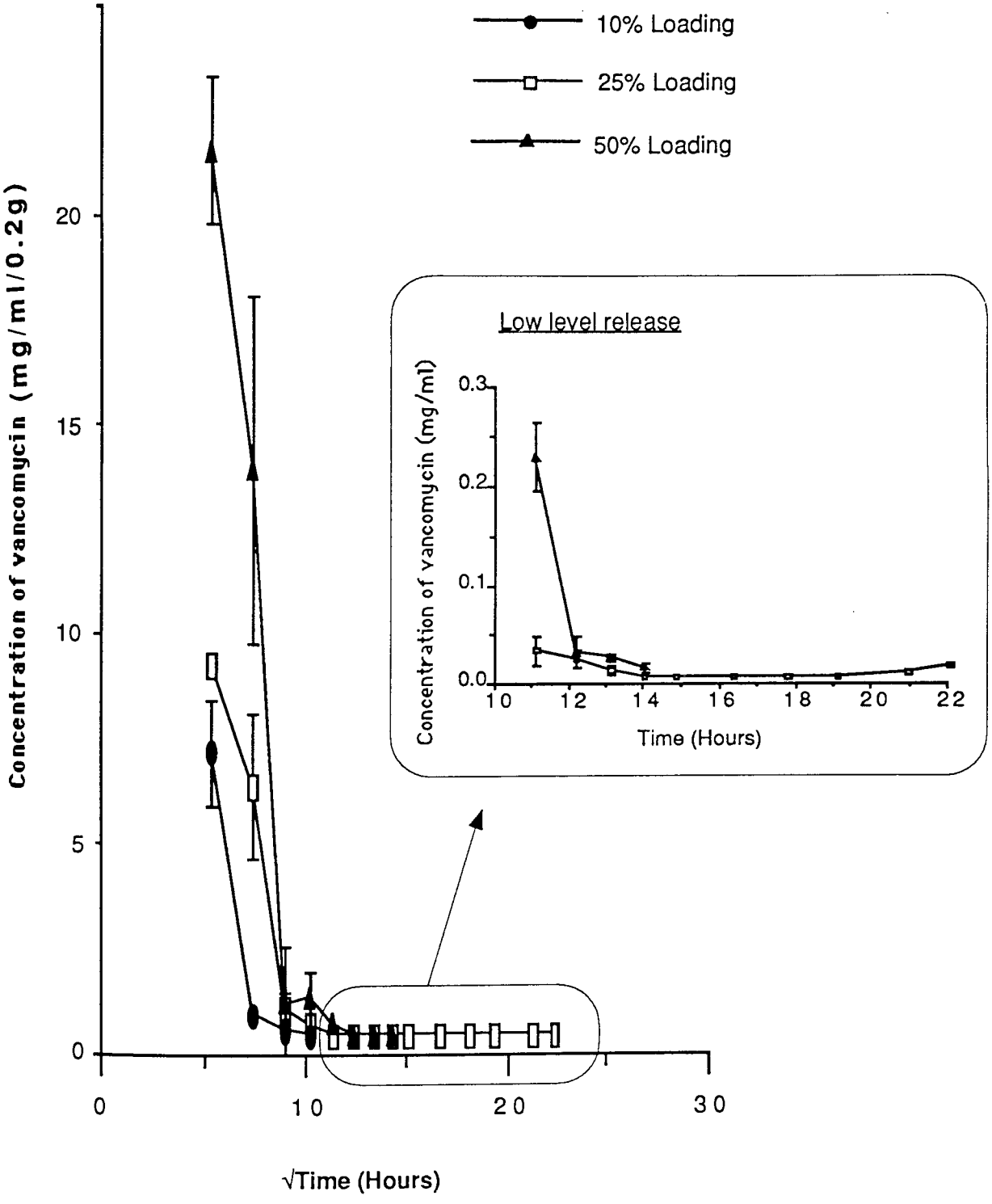


Figure 5.3.0 The effect of percentage loading on the total mean cumulative release of vancomycin from PLCG 75:25 microspheres in Hank's buffer at 37°C. (mean values ± SEM, n=12)



Increasing the percentage vancomycin loading of PLCG 75:25 microspheres incubated in Hank's buffer at 37°C significantly increased the total mean cumulative release of vancomycin ($p<0.05$), figure 5.3.0. 25% and 50% loaded microspheres released therapeutically significant levels of vancomycin for 24 and 8 days respectively. However, vancomycin release from 10% loaded PLCG 75:25 microspheres incubated in Hank's buffer could not be detected beyond 4 days, figure 5.3.0. Incubation in Hank's buffer reduced the burst release seen in newborn calf serum, figure 5.2.9 from 5 days for 50% loaded microspheres and 4 days for 25% loaded microspheres to 3 days for both loadings, figure 5.3.1.

Figure 5.3.1 **The effect of percentage loading on the daily release of vancomycin from PLCG 75:25 microspheres incubated in Hank's buffer, (mean values ± SEM, n=12)**



Examination of the total cumulative percentage release profile for vancomycin from 50% loaded microspheres revealed that 99.39% of the total vancomycin release occurred within the first 3 days of incubation with the remaining 0.66% being released over the subsequent 5 days, figure 5.3.2. 25% vancomycin loaded microspheres released 99.11% of their vancomycin content, again within 3 days, with the remaining 0.89% being released over the subsequent 4 days, figure 5.3.2.

The total concentration of vancomycin released by 50% and 25% loaded microspheres into Hank's buffer was only 53.48% and 62.68% of that released when microspheres from the same fabrication batch were incubated in newborn calf serum at 37°C for the same length of time, figure 5.2.8.

Vancomycin release from PEA and 50% PEA/50% PLCG 50:50 blend microspheres.

The total cumulative vancomycin release profiles for PEA and 50% PEA/50% PLCG 50:50 microspheres in Hank's buffer were very similar, figure 5.3.3. In both cases the initial burst release phase lasted 2-3 days, with therapeutically significant levels of vancomycin being released for up to 8 days from both types of microsphere, figures 5.3.4.and 5.3.5.

The total mean cumulative release of vancomycin from both PEA and 50% PEA/50% PLCG 50:50 microspheres into new born calf serum was significantly higher ($p<0.05$) than that released into Hank's buffer, figures 5.3.6 and 5.3.3, and therapeutically significant levels (20± 0.5μg/ml and 17 ± 0.01μg/ml for PEA and 50% PEA 50% PLCG 50:50 microspheres respectively) were maintained for up to 22 days, figure 5.3.7 and 5.3.8.

Figure 5.3.2 The total cumulative percentage release of vancomycin from PLCG 75:25 microspheres in Hank's buffer, pH 7.4 at 37°C. (mean values ± SEM, n=12)

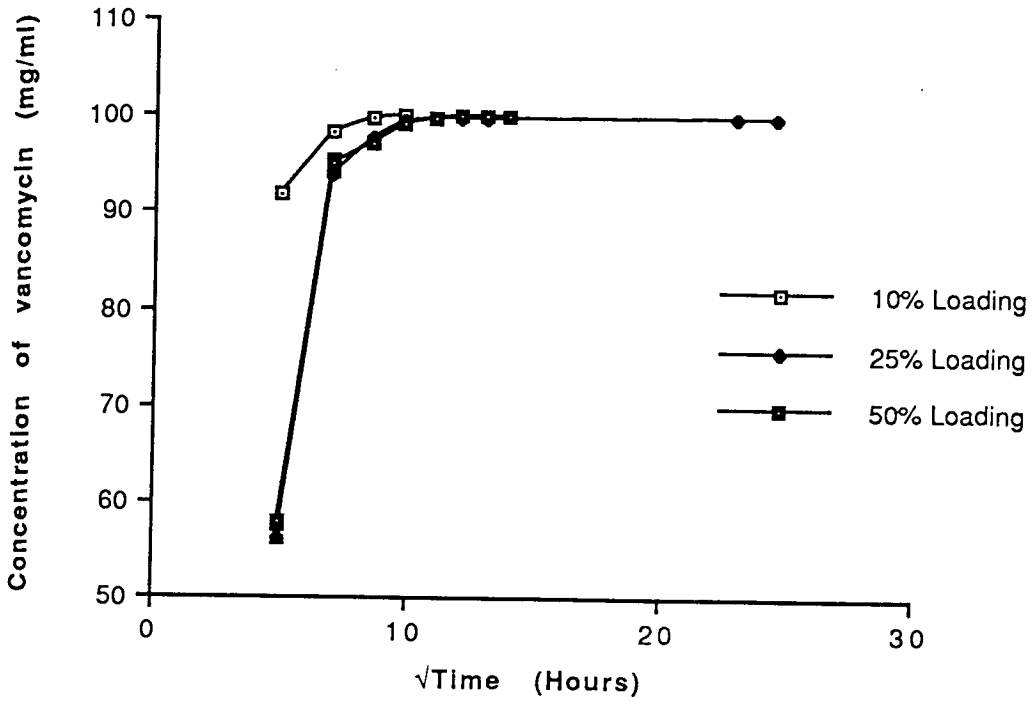


Figure 5.3.3 The total mean cumulative release of vancomycin from 25% loaded PEA and PEA/PLCG 50:50 blend microspheres incubated in Hank's buffer pH 7.4 at 37°C (mean values ± SEM, n=12)

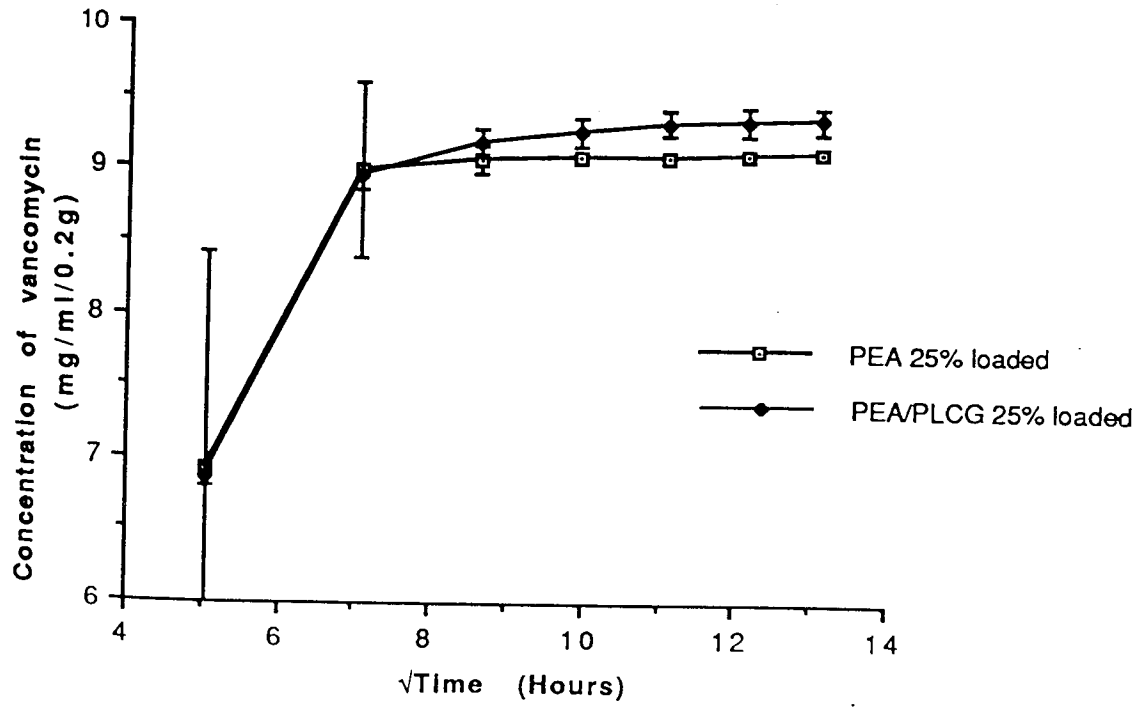


Figure 5.3.4 The daily release of vancomycin from 25% loaded PEA and PEA/PLCG microspheres incubated in Hank's buffer, pH 7.4 at 37°C (mean values ± SEM, n=12)

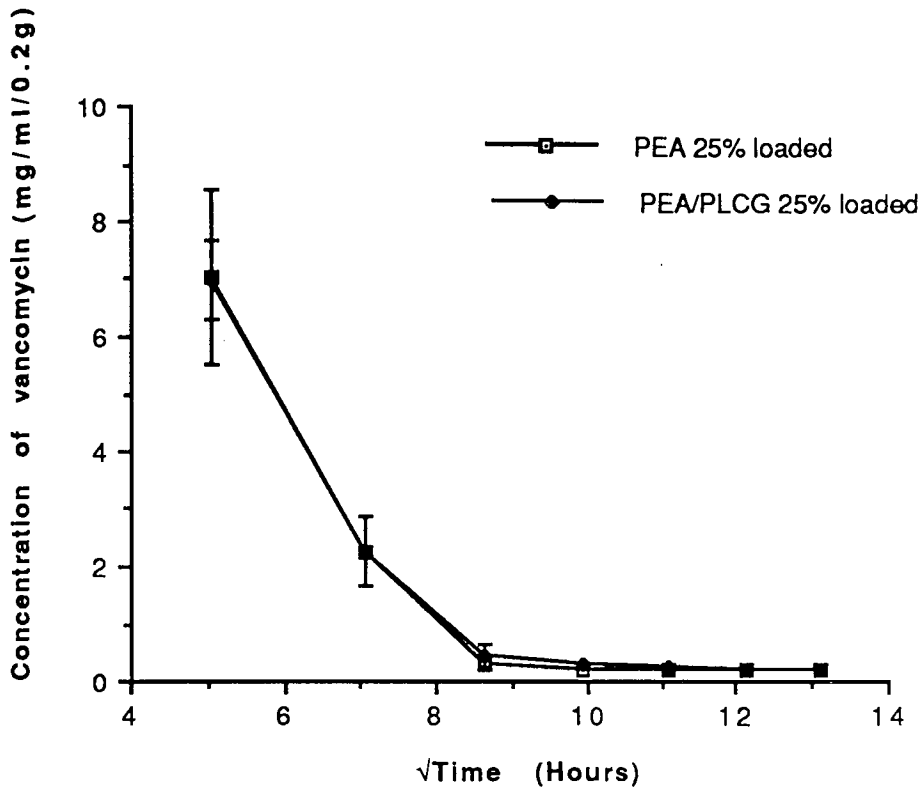


Figure 5.3.5 Closer examination of the low level release from PEA and PEA/PLCG microspheres incubated in Hank's buffer, pH 7.4 at 37°C (mean values ± SEM, n=12)

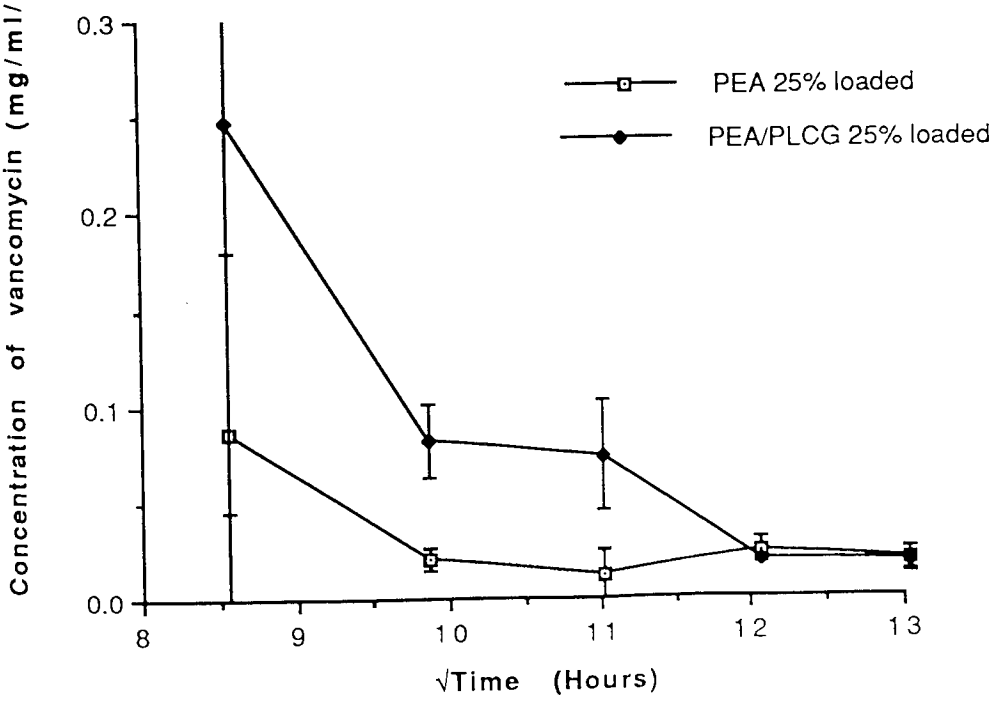


Figure 5.3.6 The total cumulative release of vancomycin from 25% loaded PEA and PEA/PLCG 50:50 blend microspheres incubated in newborn calf serum at 37°C (mean values ± SEM, n=12).

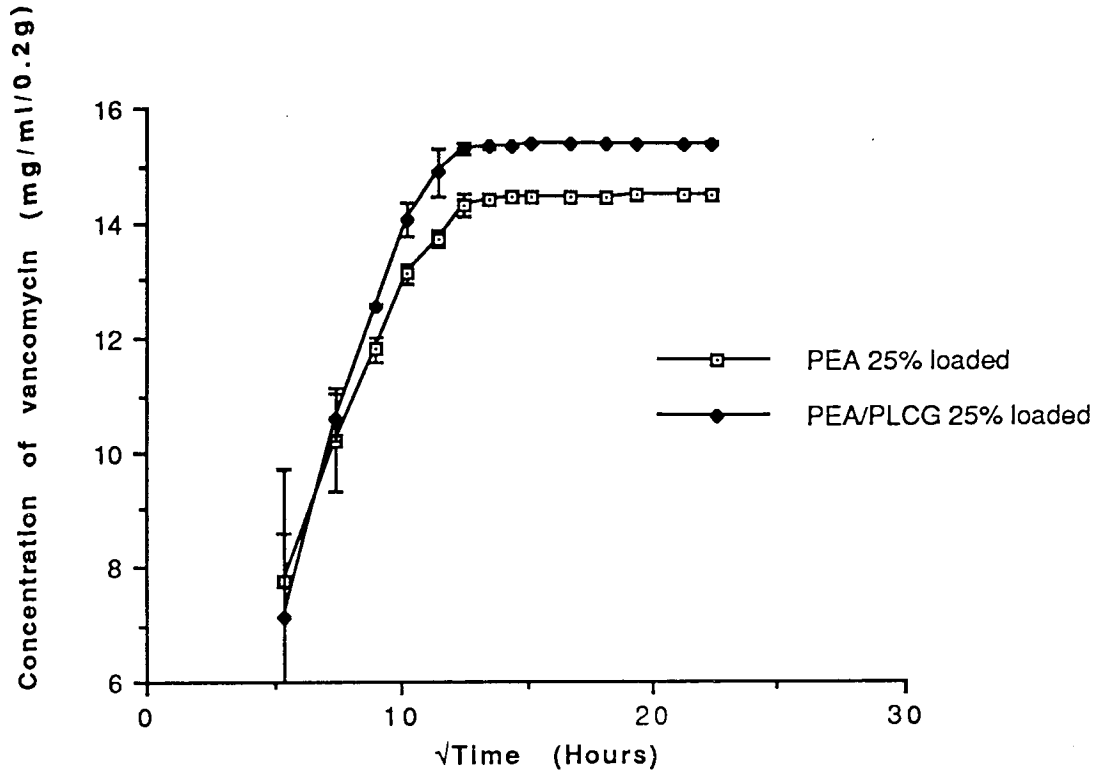


Figure 5.3.7 The daily release of vancomycin from 25% loaded PEA and PEA/PLCG 50:50 blend microspheres incubated in newborn calf serum at 37°C, (mean values ± SEM, n=12)

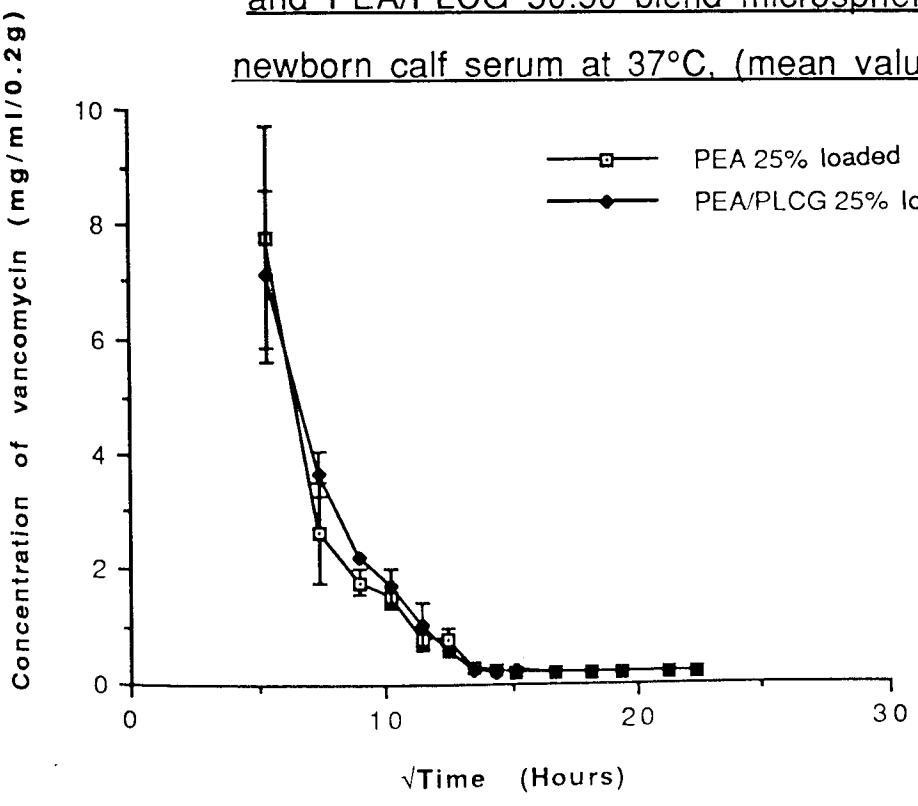


Figure 5.3.8 Closer examination of the low level release of vancomycin from PEA and PEA/PLCG microspheres incubated in newborn calf serum at 37°C (mean values ± SEM, n=12).

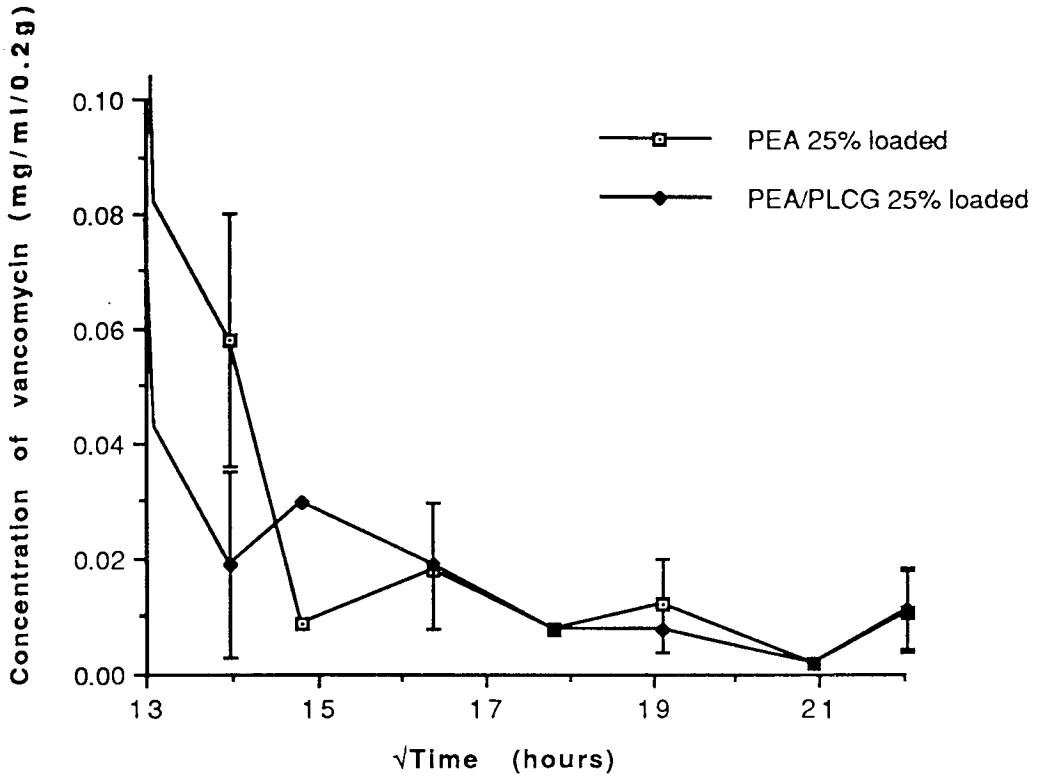
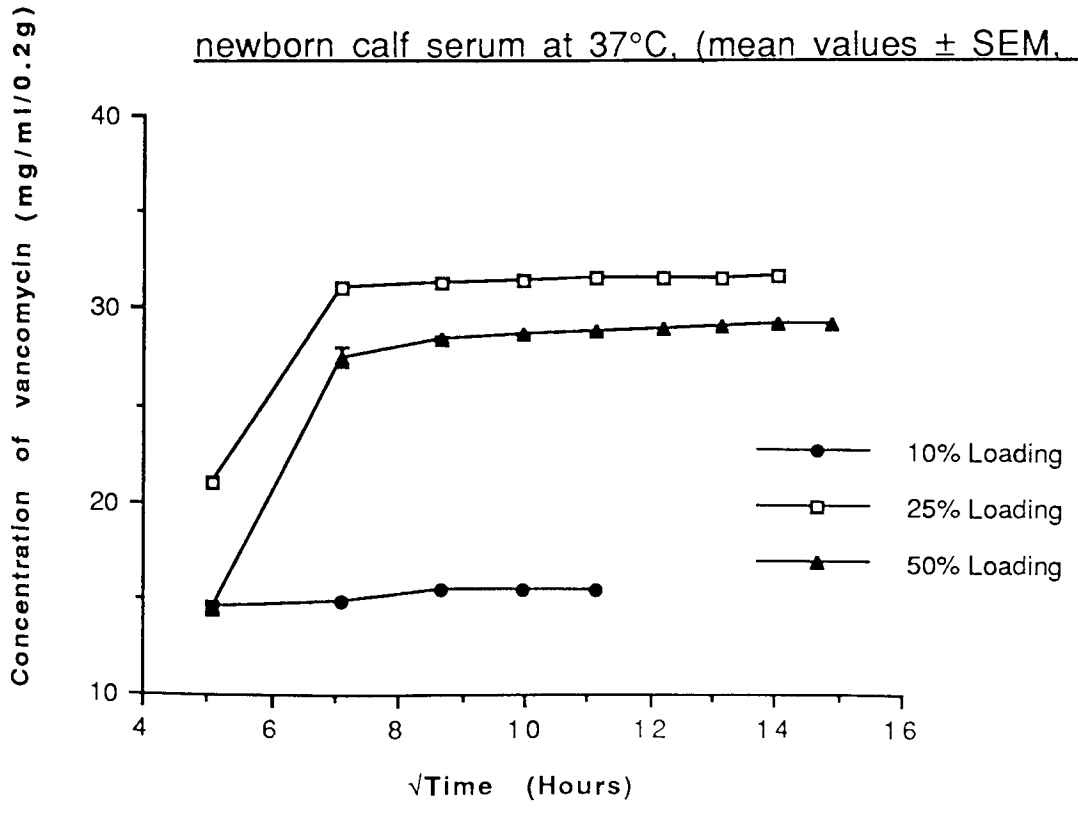


Figure 5.3.9 The total mean cumulative release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres incubated in newborn calf serum at 37°C, (mean values ± SEM, n=12)



Vancomycin release from P(HB-HV)/PLCG 50:50 blend microspheres.

The total cumulative release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres in new born calf serum was characterized by an initial burst release lasting 2 days followed by a much lower sustained release which lasted for up to 8 days, figure 5.3.9. Detectable vancomycin ceased after 5 days from 10% loaded microspheres, figure 5.4.0 and 5.4.1. The total mean cumulative release of vancomycin in Hank's buffer was similar to that seen with newborn calf serum, although significantly elevated, and vancomycin release increased with increasing percentage loading, figure 5.4.2. Closer examination of the daily low level release of vancomcyin, figure 5.4.3 indicated that it was maintained for up to 7 days from both 25% and 50% loaded microspheres and even 10% loaded microspheres continued to release detectable vancomycin for up to 6 days in Hank's buffer, figure 5.4.4.

5.3 Discussion.

The present work confirms that it is possible to incorporate a water soluble macromolecule such as vancomycin HCL into monolithic microspheres fabricated with a selection of biodegradable polymers and their blends using W/O emulsification with solvent extraction.

Average percentage yields were generally high with mean microsphere diameters ranging from 17.39 ± 6.89μm, n=74 to 56.5 ± 13.8μm, n=70. Percentage loading had no significant effect on either percentage yield or microsphere size. However, the type of polymer and polymer blend were found to influence microsphere size. The smallest microspheres were fabricated using PLCG 50:50, and had a

Figure 5.4.0 The daily release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres incubated in newborn calf serum at 37°C. (mean values ± SEM, n=12).

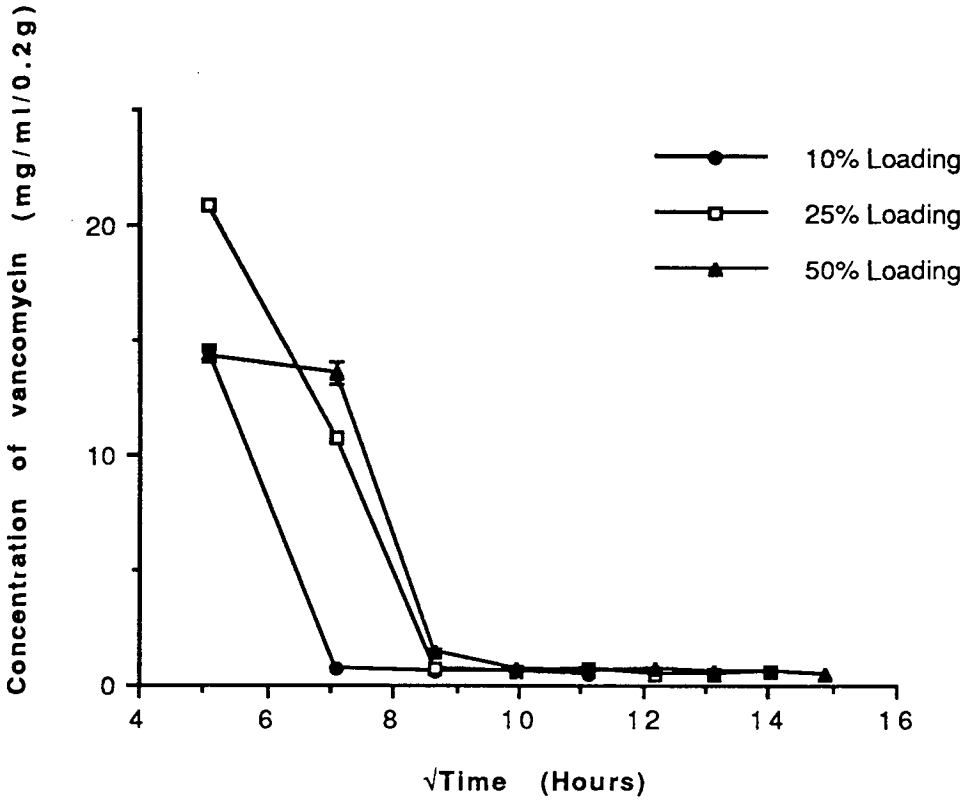


Figure 5.4.1 Closer examination of the daily low level release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres incubated in new born calf serum at 37°C. (mean values ± SEM< n=12).

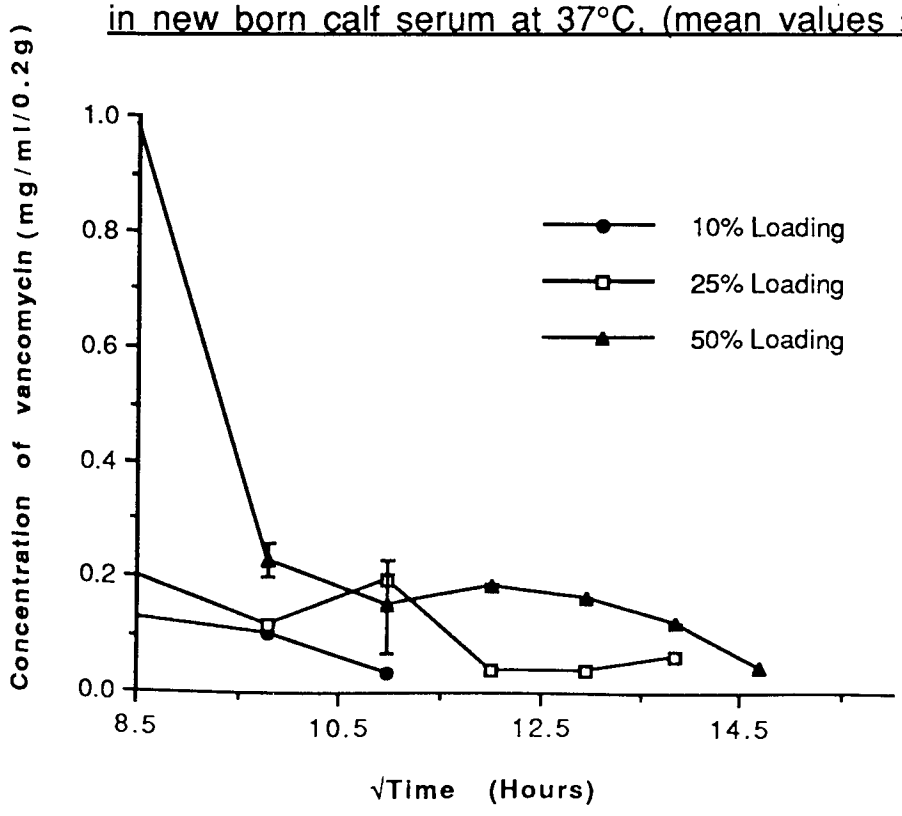


Figure 5.4.2 The total mean cumulative release of vancomycin from P(HB-HB)/PLCG 50:50 microspheres incubated in Hank's buffer pH 7.4 at 37°C. (mean values ± SEM, n=12).

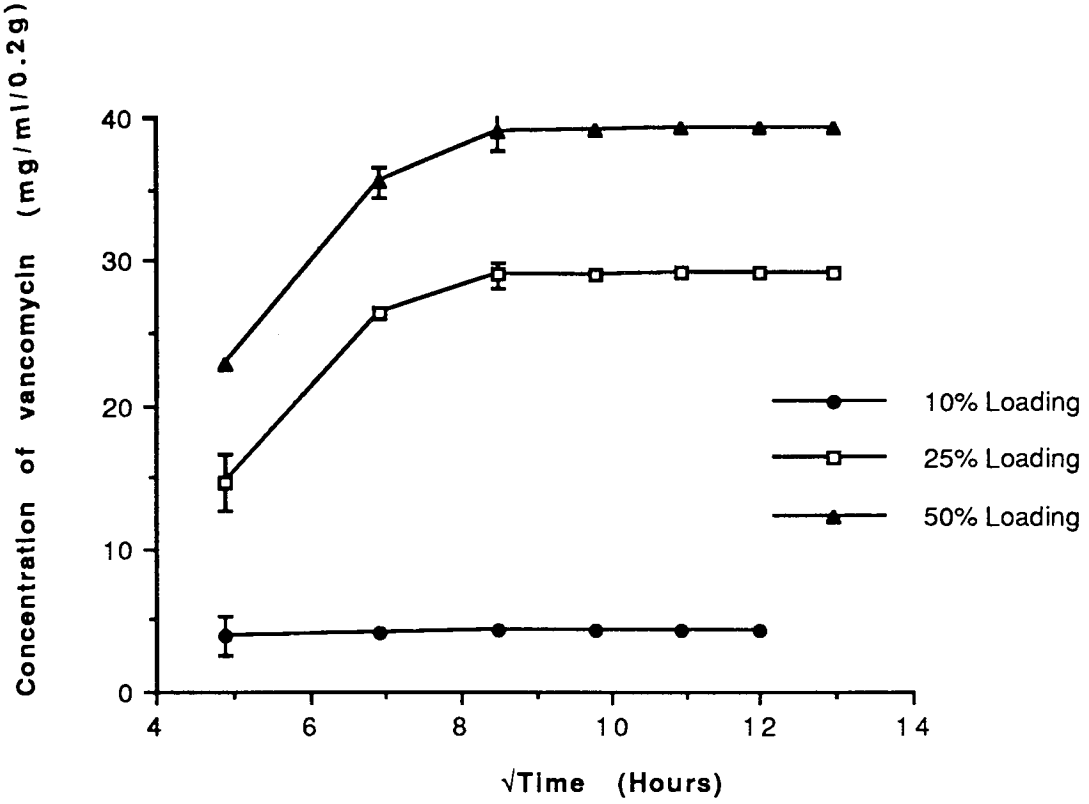


Figure 5.4.3 The daily release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres incubated in Hank's buffer, pH 7.4 at 37°C. (mean values ± SEM, n=12).

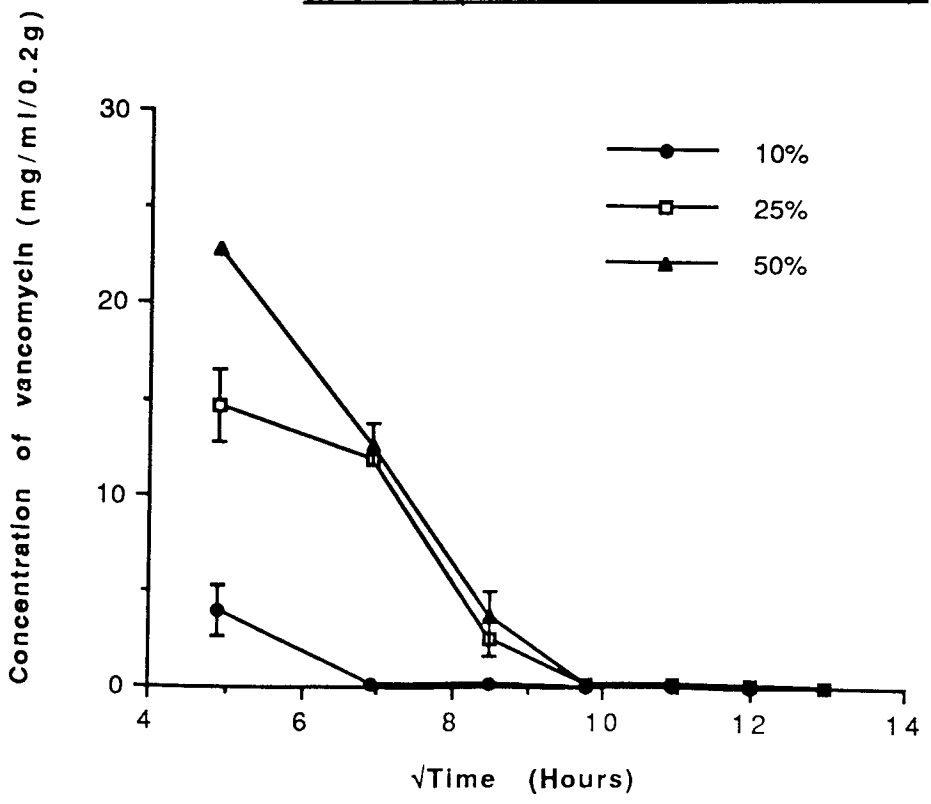
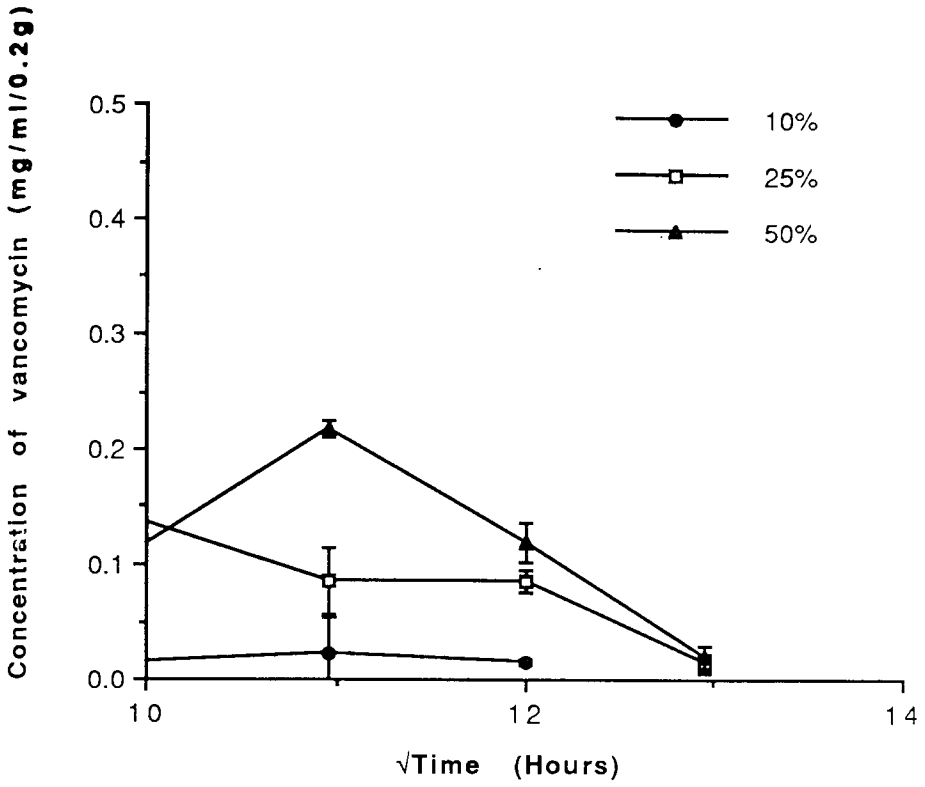


Figure 5.4.4 Closer examination of the daily low level release of vancomycin from P(HB-HV)/PLCG 50:50 microspheres incubated in Hank's buffer, pH 7.4 at 37° C. (mean values ± SEM, n=12).



mean diameter of 17.39 ± 6.89μm, n=74. Microspheres fabricated using PLCG 75:25 were marginally larger with a mean diameter of 26.34 ± 6.4μm, n=65 and P(HB-HV)/PLCG 50:50 microspheres had a mean diameter of 33.4 ± 6.7μm, n=72. Microspheres fabricated using PEA had the largest mean diameter (56.5 ± 13.8μm, n=70) although blending PEA with PLCG 50:50 reduced this to 38.9 ± 10.4 μm, n=70. Using the W/O emulsion with solvent extraction technique Jalil and Nixon found a direct relationship between polymer molecular weight and average microsphere diameter whereby an increase in the molecular weight of poly(L-lactic acid) from 2,400 to 61,300 resulted in an increase in mean microsphere diameter from 29 ± 1.55μm to 53.33 ± 2.13μm with a core (phenobarbitone) to polymer ratio of 1:1[146]. Similarly, the same workers found that the use of poly(DL-lactic acid) (M_W 20,500) resulted in the fabrication of

microspheres with a mean diameter of 42.11 ± 1.77μm, compared with a lower mean diameter of 39.19 ± 1.65μm when poly(DL-lactic acid) (M_W 5,200) was used in fabrication, again a 1:1 core (phenobarbitone) to polymer ratio was used[147]. Jalil and Nixon indicated that, in general, high molecular weight polymers produce larger microspheres due to the higher viscosity of the polymer solution. Furthermore, these workers stated that microspheres fabricated from high molecular weight polymers tended to aggregate due to their inherent tackiness and the thermoplastic nature of the fabrication polymer.

A similar pattern of microsphere size was observed in the present work where the mean microsphere diameter increased with increasing molecular weight for PLCG 50:50 (M_W 14,810), PLCG 75:25 (M_W 18,640) and P(HB-HV)/PLCG 50:50 (M_W 330,000). PEA however had a relatively low M_W of 17,900 and yet theuse of this polymer resulted in the fabrication of the largest microspheres (56.5 ± 13.8μm). It may be that PEA microspheres underwent aggregation during the fabrication process. Indeed, PEA microspheres always took longer to harden than other microspheres during fabrication. PEA is a common plasticisor and the soft nature of this polymer may result in increased microsphere aggregation and consequently larger microspheres.

A feature of the W/O emulsion solvent extraction technique was the high encapsulation efficiencies achieved with mean values ranging from 47.75 ± 9.0% to 96.74 ± 13.2%, n=12.

With all microspheres irrespective of the fabrication polymer used the release profile was characterised by two distinct phases, especially with the higher percentage vancomycin loadings. An initial burst release, which tended to increase with percentage loading, was thought to be largely but not entirely due to the

deposition of vancomycin on the microsphere surface and within the peripheral matrix of the microspheres. In retrospect, it might have been more useful to employ percentage loadings (20%) in an attempt to reduce the deposition of vancomycin on the microsphere surface and thus reduce the extent of the burst release phase. However, although 10% loaded microspheres showed a reduced burst phase, their reservoir capacity was also reduced and this was reflected in their shorter and much reduced release profile; such that a sustained and a therapeutically significant (15-25μg/ml) level of release could not be achieved. In addition, some systematic inaccuracies had to be tolerated as a consequence of the large dilutions required to reduce the concentration of vancomycin in a sample sufficiently to fit the standard curves of the radial diffusion assay. The second phase of release was low level and maintained at a rate of 20-25μg/ml for 11 days from 25% vancomycin loaded PEA/PLCG 50:50 microspheres, and for 20-27 days from 50% vancomycin loaded PLCG 50:50 microspheres and 25% and 50% vancomycin loaded PLCG 75:25 microspheres in newborn calf serum. PLCG 75:25 microspheres maintained therapeutically significant vancomycin levels for 24 days in Hank's buffer.

The results of the present work indicated that microspheres fabricated using PLCG75:25 appeared to offer the most suitable characteristics for the sustained delivery of vancomycin. The highest percentage yield (97.16 ± 4.3%, n=3) and the highest encapsulation efficiency of 96.74 ± 13.2%, n=12 was obtained with this polymer. Furthermore, 25% and 50% vancomycin loaded PLCG 75:25 microspheres maintained therapeutically significant levels of vancomycin release for up to 27 days in newborn calf serum and 24 days in Hanks buffer. In subsequent work, however, it may prove beneficial to reduce the percentage loading to approximately 20% in

an attempt to reduce the surface deposition of vancomycin crystals and consequently eliminate the potentially overwhelming burst release of vancomcyin which occurrs during the first four days of incubation.

CHAPTER SIX

IN VITRO BIODEGRADATION OF VANCOMYCIN HCL LOADED POLY [D,L-LACTIDE-CO-GLYCOLIDE], POLY[ETHYLENE-ADIPATE] AND POLY-ß-HYDROXYBUTYRATE/ HYDROXYVALERATE MICROSPHERES PREPARED USING W/O SINGLE EMULSION WITH SOLVENT EVAPORATION.

6.0 *Introduction*

Previous work confirmed that microspheres could be successfully fabricated from PLCG (75:25), PLCG (50:50), PEA and P(HB-HV) and their blends using the W/O single emulsion solvent extraction technique described in section 2.4, p80. Since the microspheres are to be used for the delivery of antibiotics in a variety of orthopaedic applications, it became clear that only the well characterised biodegradable polymers PLCG 75:25 and 50:50 could be utilised in the present studies. It is well established that poly(D,L-Lactide-co-glycolide) degrades in the body to the metabolites lactic and glycolic acid and their presence produces minimal local or systemic toxicity[13,148,149]. The physico-chemical properties and rate of biodegradation of PLCG depends upon the molar ratio of the two monomers within the polymer chain, and on the polymer molecular weight[13]. Thus by varying the proportions of PLA and PGA the biodegradation rate of the polymer in aqueous buffers can be varied from under 100 days to 220 days[13,150]. The rate of biodegradation, both *in vitro*, and *in vivo,* is fastest when the proportion of lactide present is low. The problem with medical grade PLCG is that it is a very expensive polymer and there is a continual need to synthesis cheaper alternatives with the same biodegradability and biocompatibility. With this mind it was decided to blend rapidly biodegrading PLCG 50:50 with less expensive but fairly well characterised and slowly biodegrading polymers, PEA and P(HB-HV) with a view to accelerating the rate of biodegradation of these polymers with a view to reducing microsphere residence time *in vivo* .

The present work was carried out to monitor the extent of biodegradation and the changes in both gross and ultrastructural morphology which occurred over a period of 28 to 35 days incubation

Plates 6.1.1 Biodegradation of 25% vancomycin loaded PLCG 50:50 microspheres incubated in new born calf serum.

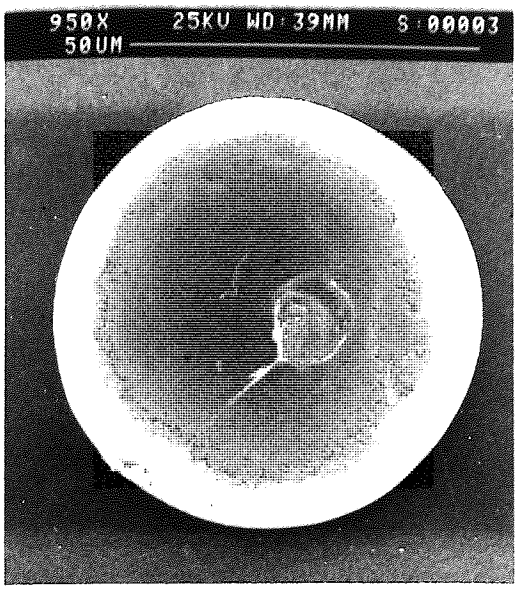


Plate 6.1.1a
Appearance after
7 days incubation (X950).

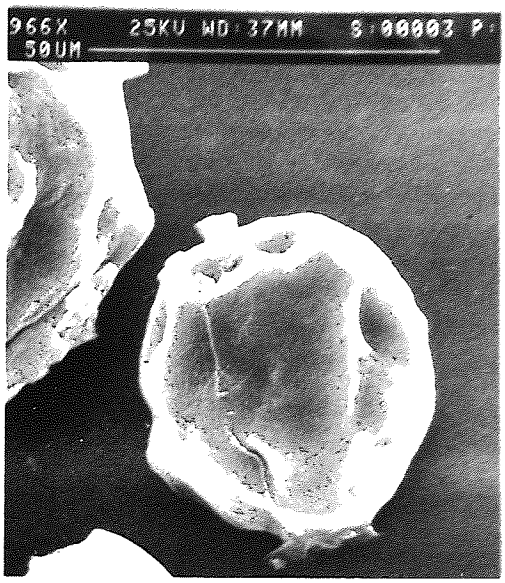


Plate 6.1.1b
Appearance after 14
days incubation (X966)
showing surface pitting.

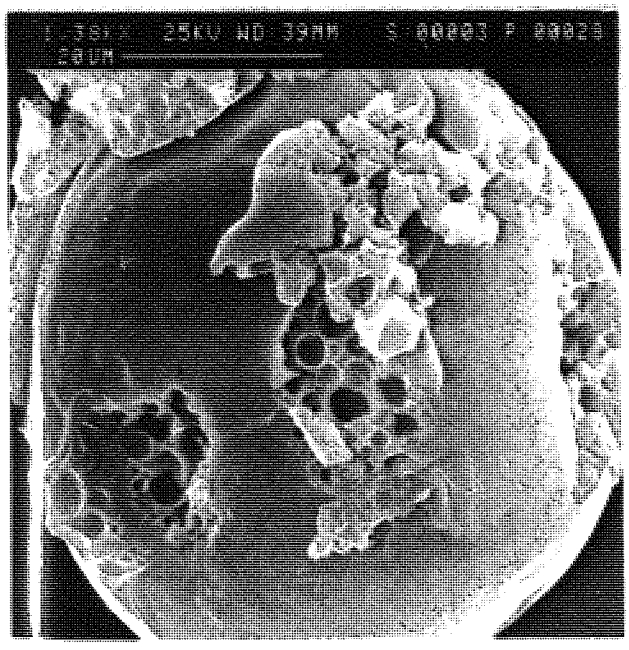


Plate 6.1.1c
Appearance after 21
days incubation (KX1,38).

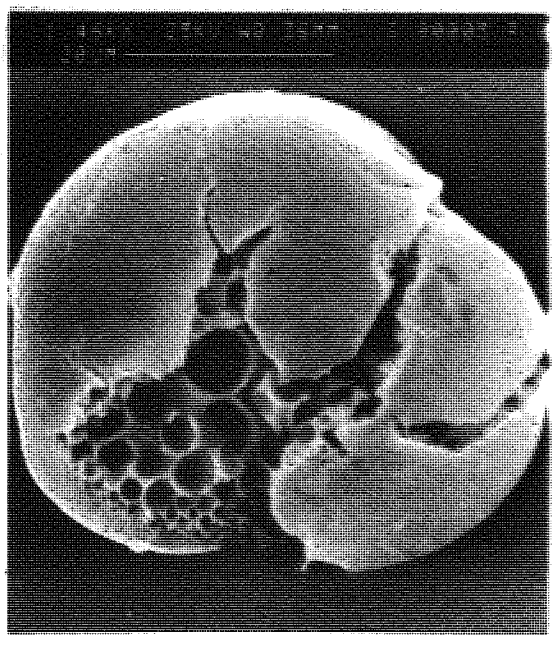


Plate 6.1.1d
Appearance after 28
days incubation (KX1,46)
showing extensive
macroporosity.

Plates 6.1.2 **Biodegradation of 50% vancomycin loaded PLCG 50:50 microspheres incubated in new-born calf serum.**

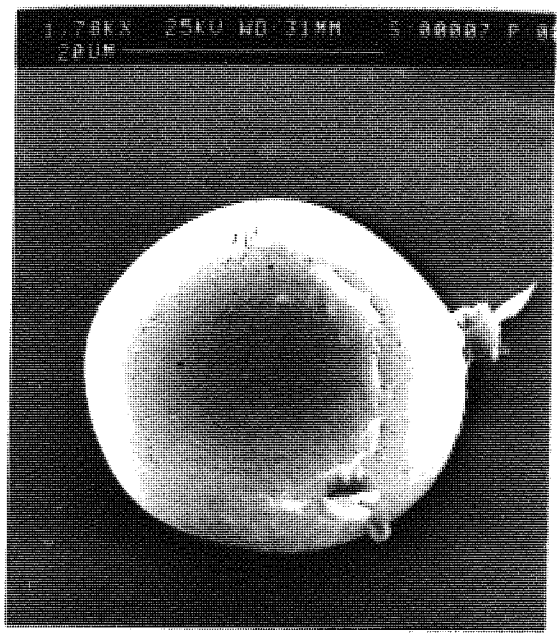


Plate 6.1.2a
Appearance after 7 days incubation (KX1,78).

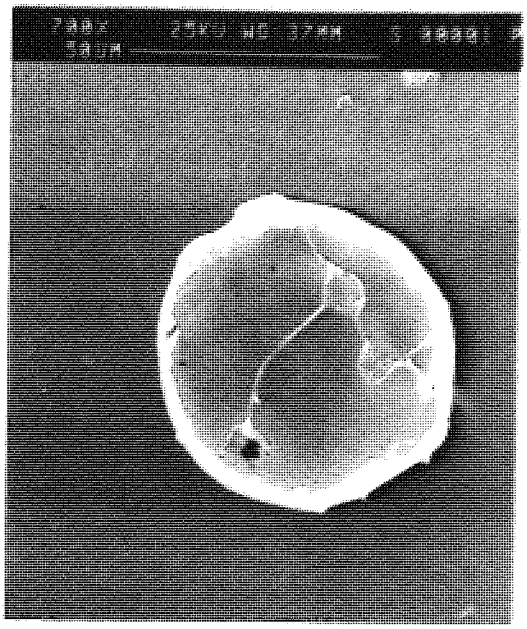


Plate 6.1.2b
Appearance after 14 days incubation (X700)

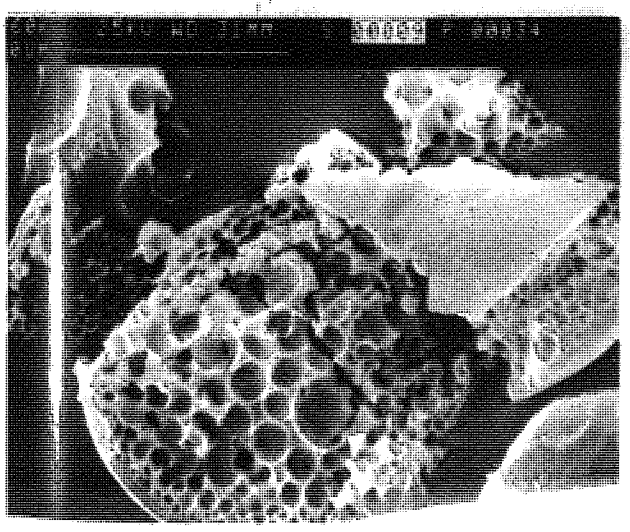


Plate 6.1.2c
Appearance after 21 days incubation (KX1,60).

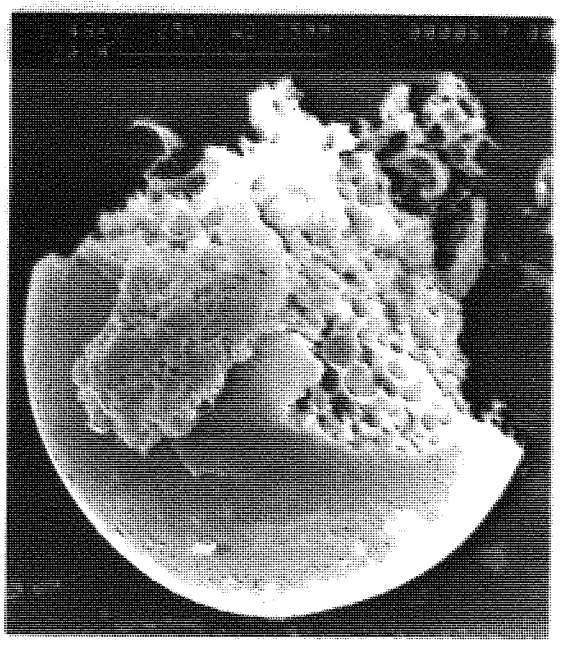


Plate 6.1.2d
Appearance after 28 days incubation (1,48KX).

Plates 6.1.3 **Biodegradation of 25% vancomycin loaded PLCG 50:50 microspheres incubated in Hank's buffer.**

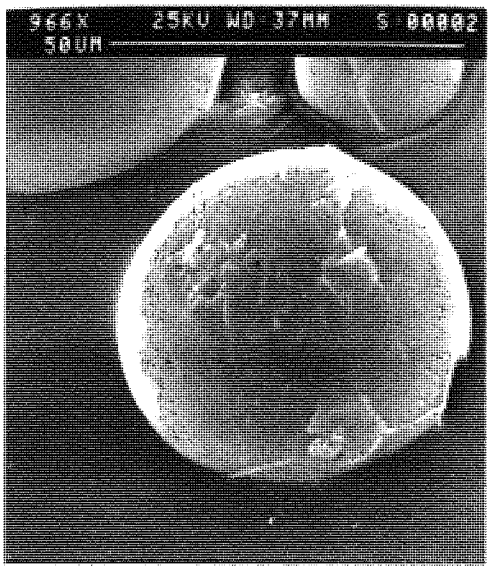


Plate 6.1.3a
Appearance after 7 days incubation (X966).

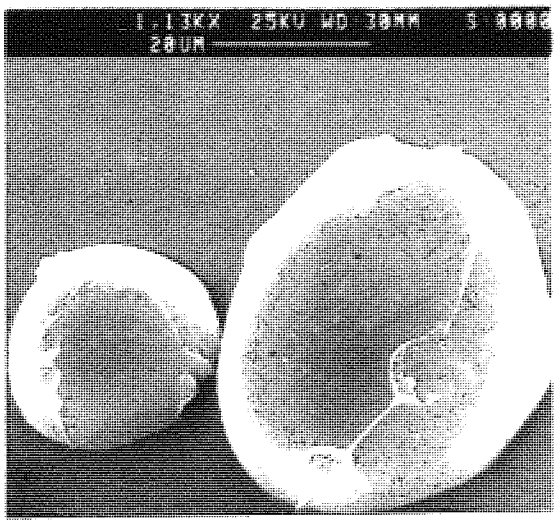


Plate 6.1.3b
Appearance after 14 days incubation (KX1,13).

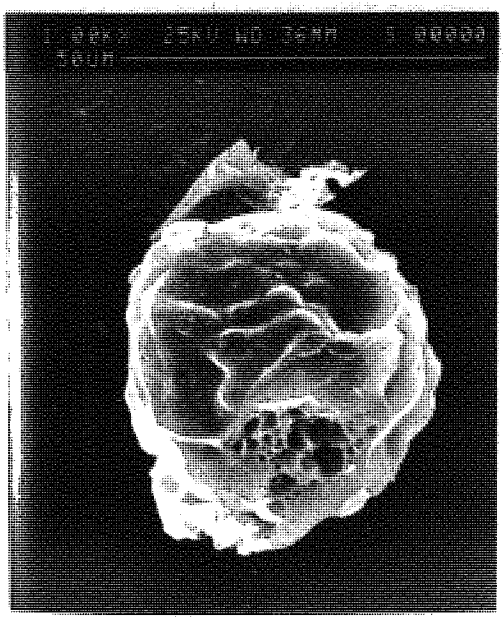


Plate 6.1.3c
Appearance after 21 days incubation (KX1,00)

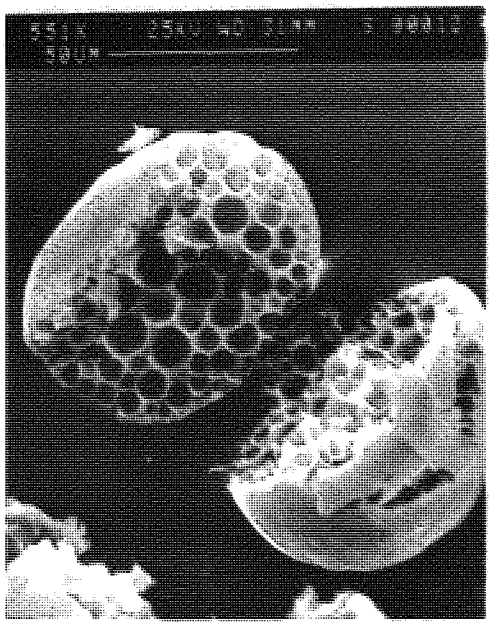


Plate 6.1.3d
Appearance after 28 days incubation (X551)

Plates 6.1.4 **Biodegradation of 50% vancomycin loaded PLCG 50:50 microspheres incubated in Hank's buffer.**

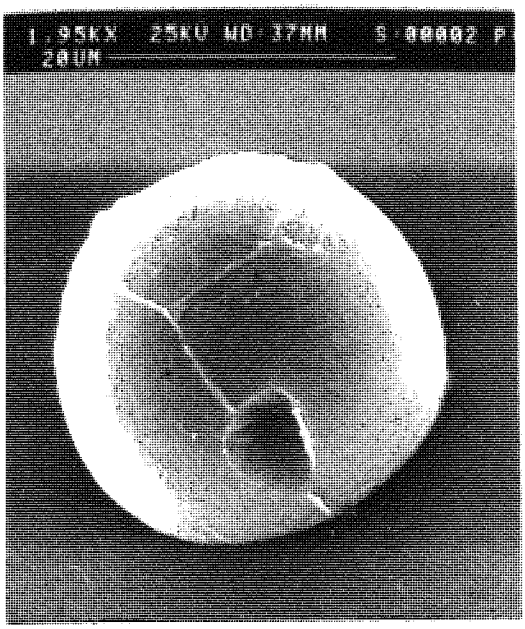


Plate 6.1.4a
Appearance after 7 days incubation (KX1,95)

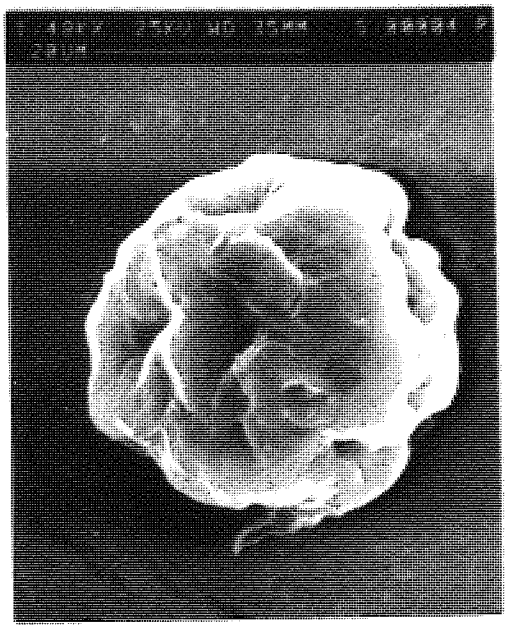


Plate 6.1.4b
Appearance after 14 days incubation (KX1,48)

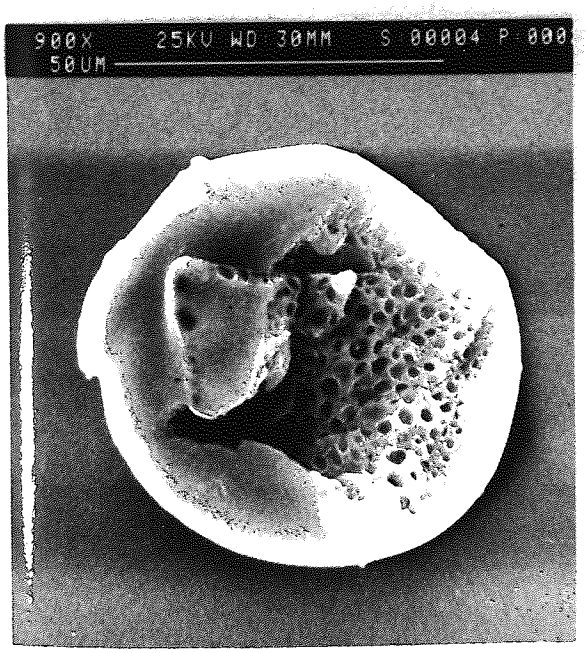


Plate 6.1.4c
Appearance after 21 days incubation (X900)

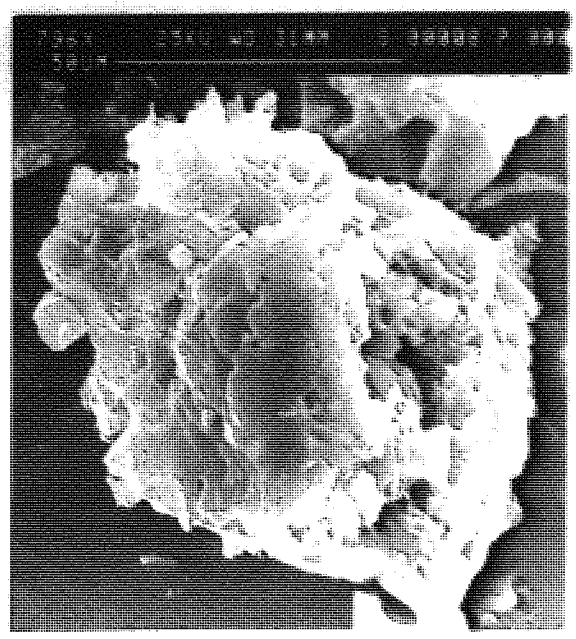


Plate 6.1.4d
Appearance after 28 days incubation (X796)

in both Hank's buffer and newborn calf serum. Changes in morphology were monitored using scanning electron microscopy and the extent and pattern of biodegradation recorded was used to provide some indication of the potential residence time *in vivo* and the way changes in structure over time might result in the vancomycin release profiles previously recorded for PLCG microspheres.

6.1 Methodology.

Microspheres were fabricated from PLCG (75:25), PLCG (50:50), PEA, 50% PEA/50% PLCG (50:50) and 75% P(HB-HV)/25% PLCG (50:50) using the W/O single emulsion solvent extraction technique as previously described, p80. Biodegradation studies were carried out using 25% and 50% vancomycin loaded microspheres incubated in Hank's buffer, pH 7.4 and newborn calf serum at 37°C as described, p84. Structural changes related to biodegradation were monitored at weekly intervals over a period of 28 to 35 days.

6.2 Results.

6.2.1 Biodegradation of PLCG 50:50 microspheres loaded with 25% and 50% vancomycin.

Biodegradation of these microspheres in newborn calf serum, irrespective of percentage loading, was characterized by initial surface cracking, plate 6.1.1a and the subsequent appearance of large smooth edged pits by day 14, plate 6.1.1b. These pits acted as focci for further erosion, without any significant reduction in microsphere diameter. 25% loaded microspheres were still relatively intact at day 21, plate 6.1.1c, but had begun to break-up and fracture after 28 days, plate 6.1.1d. The internal architecture

was macroporous with the macropores being compressed at the periphery of the microsphere, plate 6.1.1d. A similar pattern of biodegradation was shown by 50% vancomycin loaded microspheres in newborn calf serum, with some surface pitting appearing after 14 days, plate 6.1.2b. Microsphere fragmentation began after 21 days, plate 6.1.2c and the 50% loaded microspheres had almost totally disintegrated by day 28, plate 6.1.2d. Serum protein adsorption was not evident on the surface of the microspheres, neither was there any vancomycin crystal deposition, but there was some indication that increasing the percentage vancomycin loading to 50% did accelerate microsphere degradation, presumably as a result of increased pore generation and matrix destablization by dissolving vancomycin particles, plates 6.1.2c and d. A similar sequence of structural changes was observed for 25% and 50% vancomycin loaded microspheres in Hank's buffer, except that the occurrence of smooth pits appeared at around day 21 for 25% and day 14 for 50% loaded microspheres as shown in plates 6.1.3c and 6.1.4b respectively. Both 25% and 50% loaded microspheres had disintegrated by day 28 in Hank's buffer, plate 6.1.3d and 6.1.4d.

6.2.2 Biodegradation of PLCG 75:25 microspheres loaded with 25% and 50% vancomycin.

Embedded vancomycin crystals were quickly lost from the surface of 25% loaded microspheres incubated in Hank's buffer and by day 7 had developed a rugose flaky surface, plate 6.1.5a. By day 14 quite extensive fragmentation of the microsphere had occurred, revealing the macroporous interior, plate 6.1.5b. Crumbling fragmentation was almost complete at day 28, plate 6.1.5c, with large pores becoming evident in the internal matrix of the microsphere by day 35, plate 6.1.5d. In Hank's buffer, therefore,

Plates 6.1.5 **Biodegradation of 25% vancomycin loaded PLCG 75:25 microspheres incubated in Hank's buffer.**

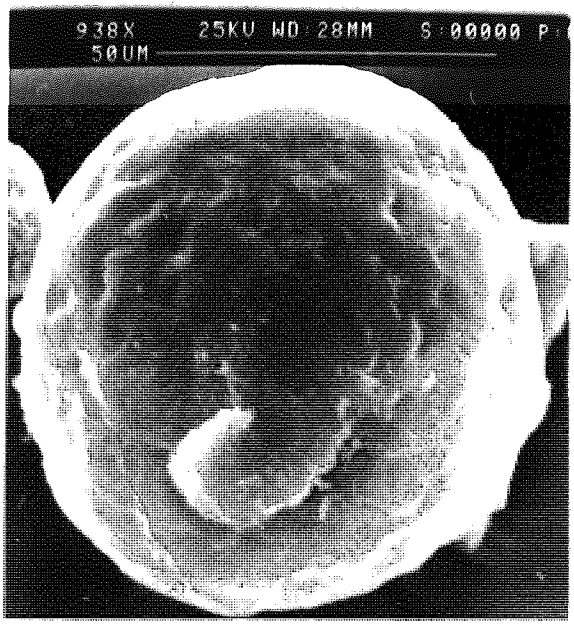


Plate 6.1.5a
Appearance after 7 days incubation (x938)

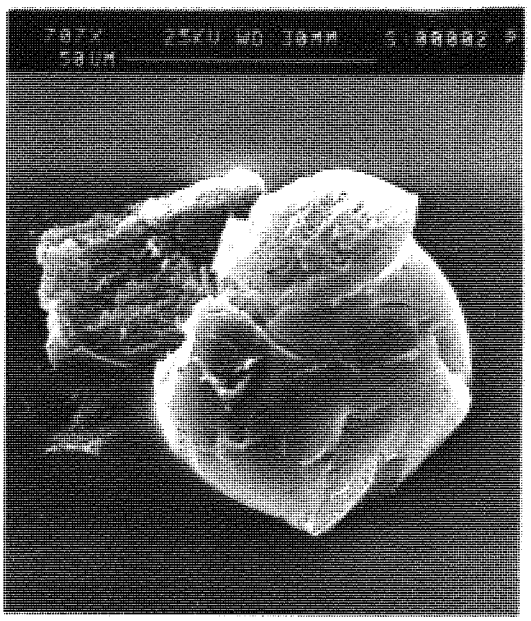


Plate 6.1.5b
Appearance after 14 days incubation (x707)

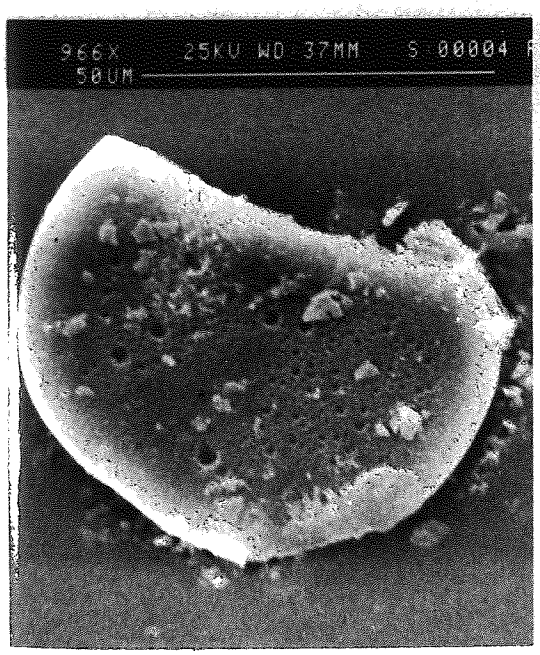


Plate 6.1.5c
Appearance after 28 days incubation (x966)

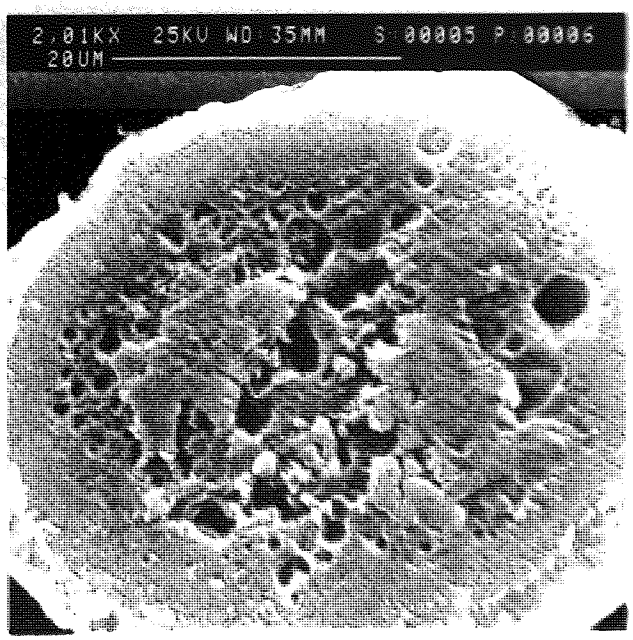


Plate 6.1.5d
Appearance after 35 days incubation (Kx2,01)

Plates 6.1.6 **Biodegradation of 25% vancomycin loaded PLCG 75:25 microspheres incubated in new-born calf serum.**

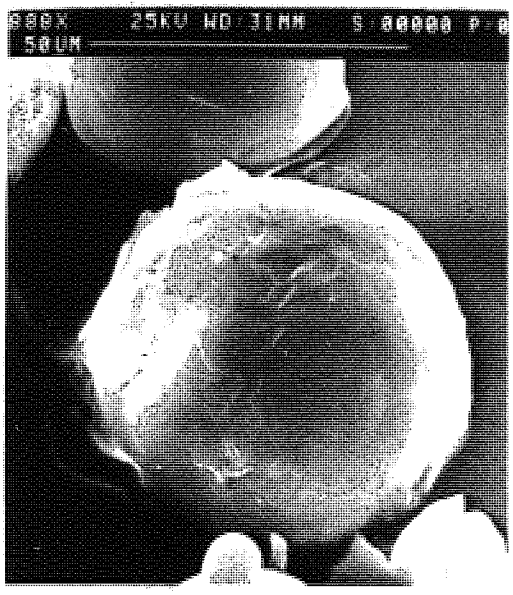


Plate 6.1.6a
Appearance after 7 days incubation (X888)

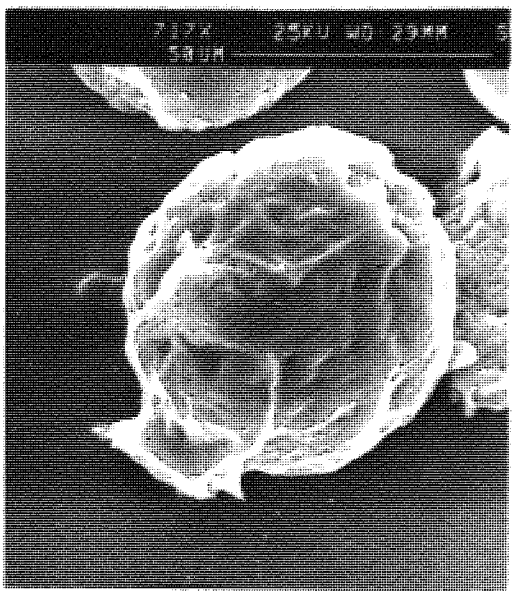


Plate 6.1.6b
Appearance after 14 days incubation (x717)

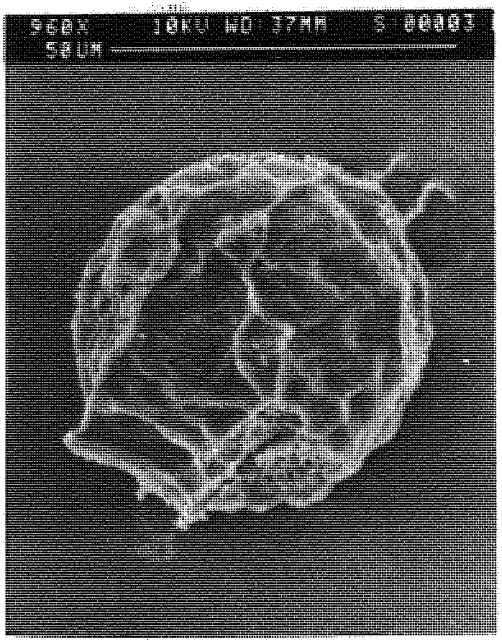


Plate 6.1.6c
Appearance after 21 days incubation (X960)

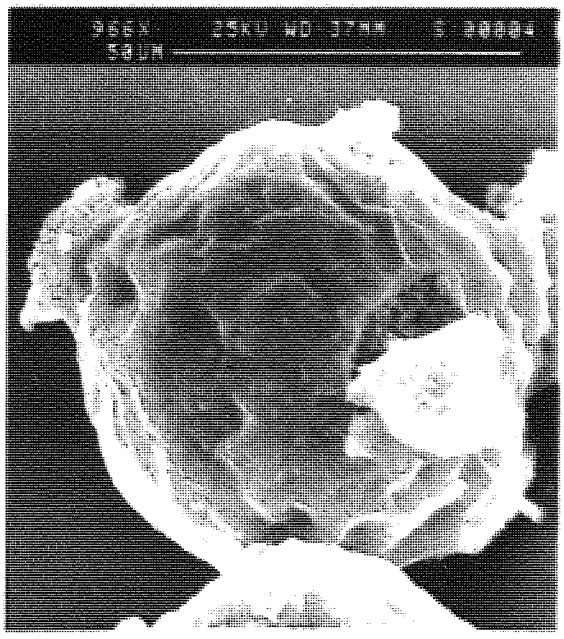


Plate 6.1.6d
Appearance after 28 days incubation (x966)

these microspheres were fracturing and breaking up rather than showing surface erosion. After 7 days incubation in newborn calf serum 25% loaded microspheres had lost their surface deposit of vancomycin crystals and appeared flaky with surface dents and some loss of shape, plate 6.1.6a. Fractured microspheres revealed a macroporous interior matrix which by day 14 had begun to break down with an increase in the diameter of the macroporous domains, plate 6.1.6b. There was a further increase in surface rugosity and a progressive increase in the diameter of matrix pores and dents from day 21 to day 28, plates 6.1.6c and 6.1.6d respectively. These observations might be accounted for by serum enzyme activity eroding the external surface of the microspheres viz increased surface rugosity and decrease in size, and at the same time accessing the internal architecture via macropores to accelerate matrix erosion. The biodegradation of 50% vancomycin loaded microspheres in newborn calf serum, plate 6.1.7a-d, followed a similar pattern of degradation to that shown by 25% loaded microspheres except that the rate of biodegradation was slower, plate 6.1.6a-d. A significant increase in surface rugosity was not observed until day 21 with 50% loaded microspheres, plate 6.1.7b and a spherical shape was maintained until day 28 with some degradation of the macroporous interior, plate 6.1.7c. By day 35 the microspheres were misshapen, reduced in diameter and characterized by well defined domains of surface erosion, 6.1.7d. However, indications were that the rate of matrix erosion in 50% vancomycin loaded microspheres was somewhat slower than that in 25% loaded microspheres. The pattern of biodegradation shown by 50% vancomycin loaded microspheres in Hank's buffer, plates 6.1.8a-d, was not dissimilar to that shown by 25% loaded microspheres and characterised by internal macroporosity, the appearance of surface

Plates 6.1.7 **Biodegradation of 50% vancomycin loaded PLCG 75:25 microspheres incubated in new born calf serum.**

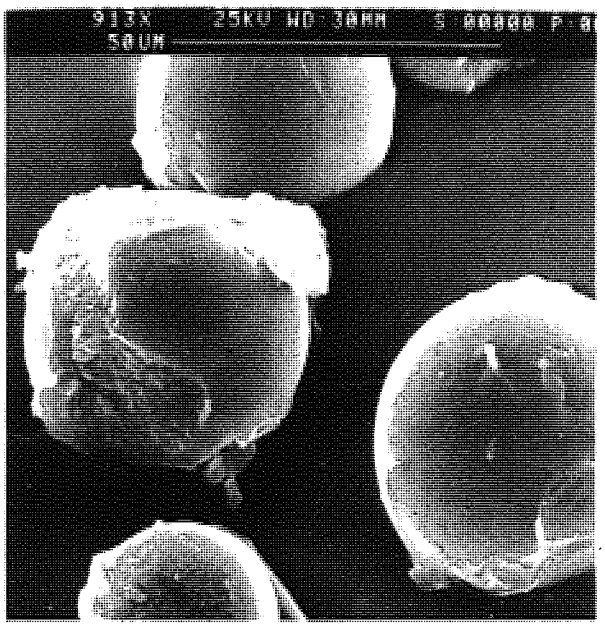


Plate 6.1.7a
Appearance after 7 days incubation (X913)

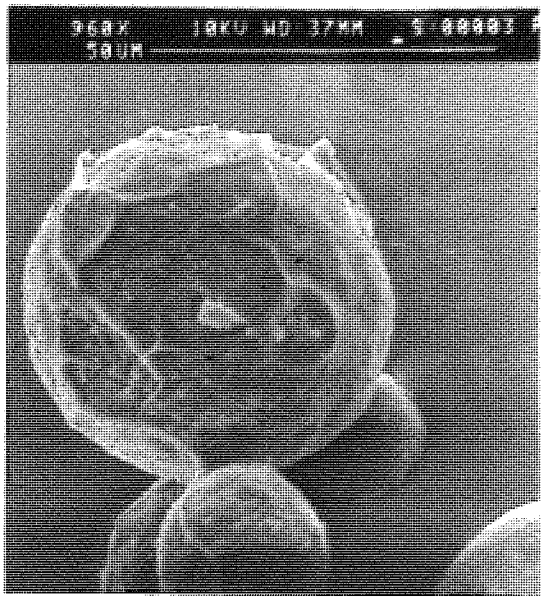


Plate 6.1.7b
Appearance after 21 days incubation (x960)

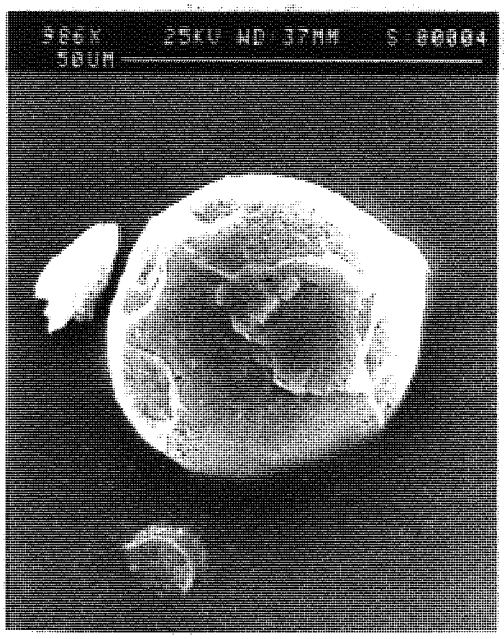


Plate 6.1.7c
Appearance after 28 days incubation (X968)

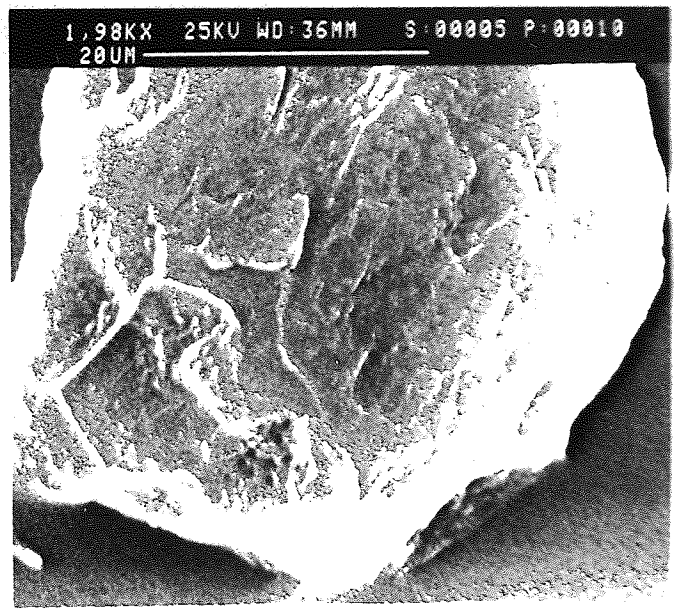


Plate 6.1.7d
Appearance after 35 days incubation (KX1,98)

Plates 6.1.8 **Biodegradation of 50% vancomycin loaded PLCG 75:25 microspheres incubated in Hank's buffer.**

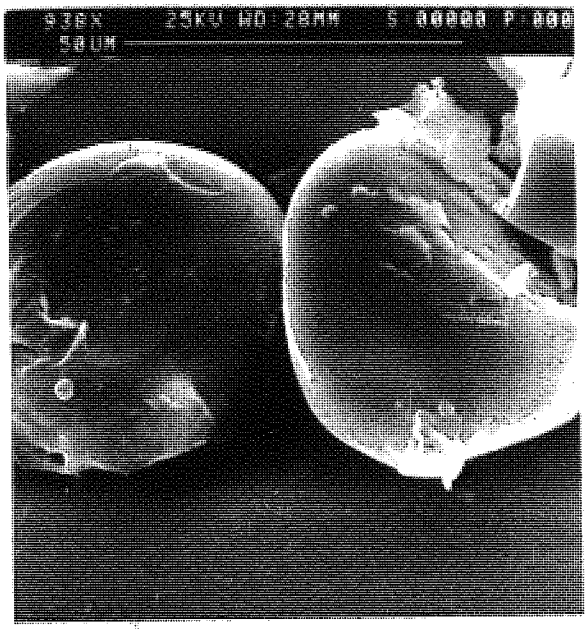


Plate 6.1.8a
Appearance after 7 days incubation (X938)

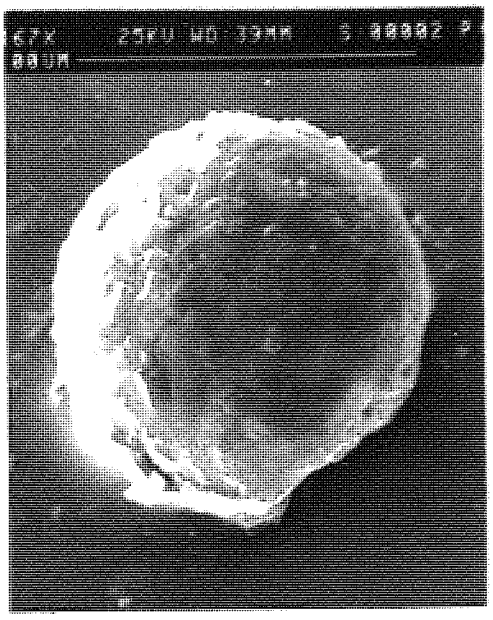


Plate 6.1.8b
Appearance after 14 days incubation (x467)

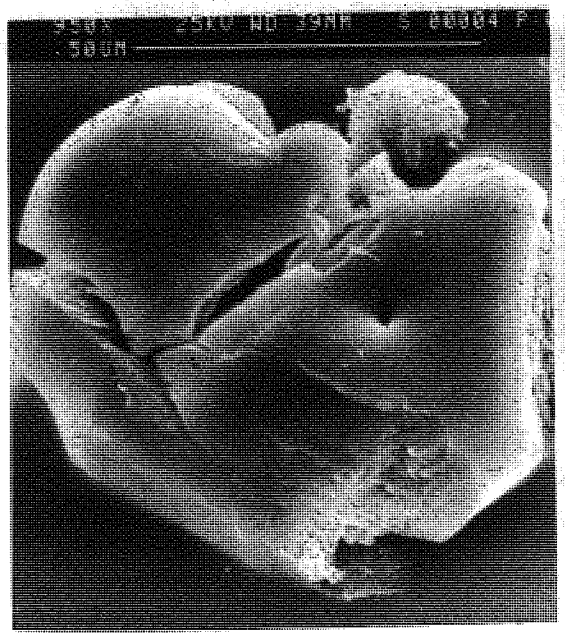


Plate 6.1.8c
Appearance after 21 days incubation (X960)

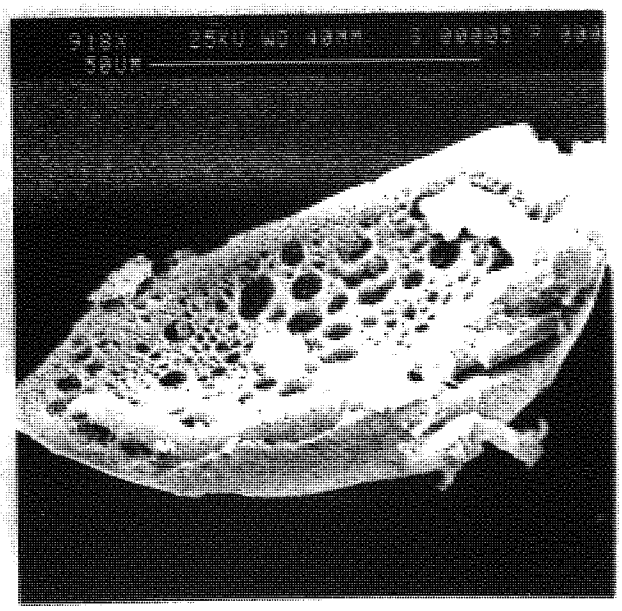


Plate 6.1.8d
Appearance after 35 days incubation (X918)

rugosity and fragmentation by day 21, plate 6.1.8c, revealed the comprehensive macroporosity of the microsphere interior.

6.2.3 Biodegradation of PEA and PEA/PLCG 50:50 blend microspheres loaded with vancomycin.

The biodegradation of 25% vancomycin loaded PEA and blend microspheres in the two different media was characterised by gradual erosion and increasing porosity. Biodegradation was faster in newborn calf serum than in Hank's buffer. Blend microspheres showed more extensive biodegradation than PEA microspheres in both calf serum and Hank's buffer, but despite this, all microspheres were relatively intact after 35 days with varying degrees of porosity. PEA microspheres in newborn calf serum showed surface cracks and flaking by day 28, plate 6.1.9d. The surface of PEA microspheres in Hank's buffer gradually eroded to reveal small pores by day 28, plate 6.2c, followed by surface erosion and reduction in size by day 35, plate 6.2d. In newborn calf serum blend microspheres became increasingly irregular and dented by day 28, plate 6.2.1c, and by day 35 substantial numbers of surface macropores had appeared, associated with surface erosion and a reduction in size, plate 6.2.1d. In Hank's buffer blend microspheres were characterised by the presence of an outer coating of surface bubbles after 14 days. This bubbly layer gradually eroded to reveal comprehensive surface pore domains and internal macroporosity after 28 and 35 days, plate 6.2.2c and 6.2.2d respectively.

Plates 6.1.9 **Biodegradation of 25% vancomycin loaded PEA microspheres incubated in new born calf serum.**

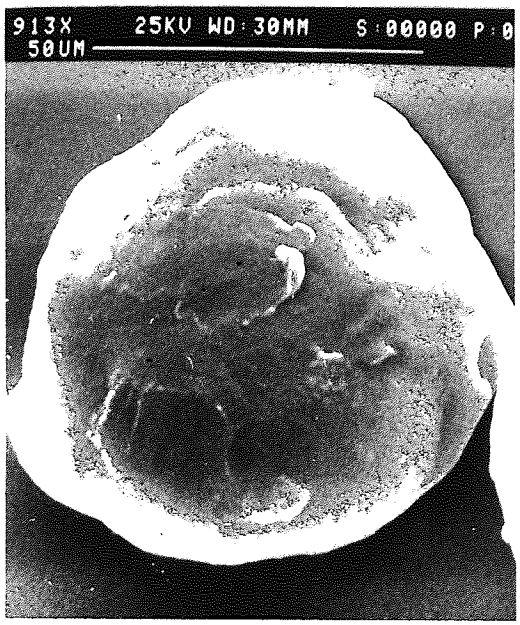


Plate 6.1.9a
Appearance after 7 days incubation (X913)

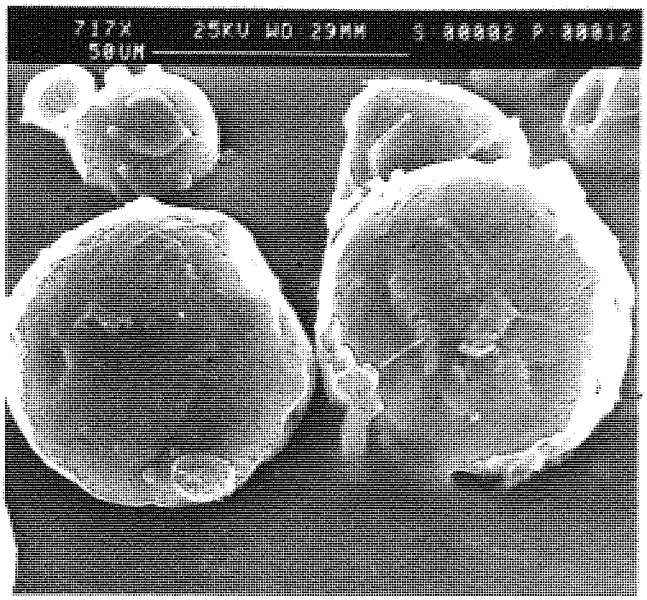


Plate 6.1.9b
Appearance after 14 days incubation (x717)

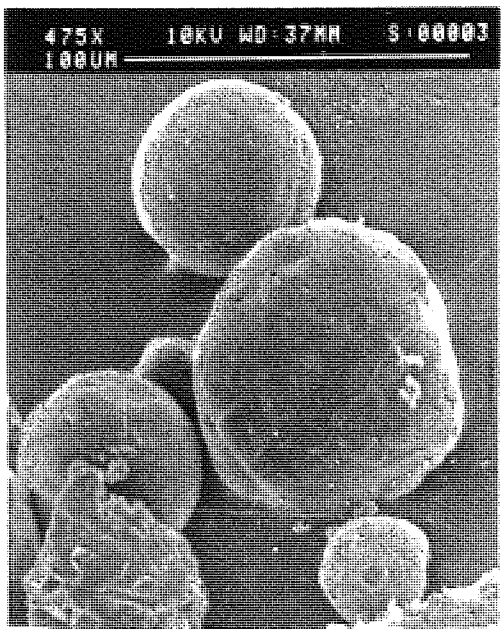


Plate 6.1.9c
Appearance after 21 days incubation (X475)

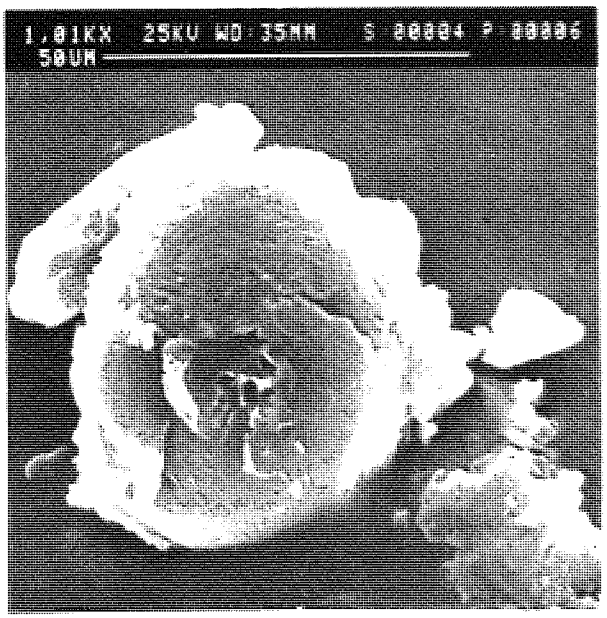


Plate 6.1.9d
Appearance after 28 days incubation (KX1,01)

Plates 6.2 **Biodegradation of 25% vancomycin loaded PEA microspheres incubated in Hank's buffer.**

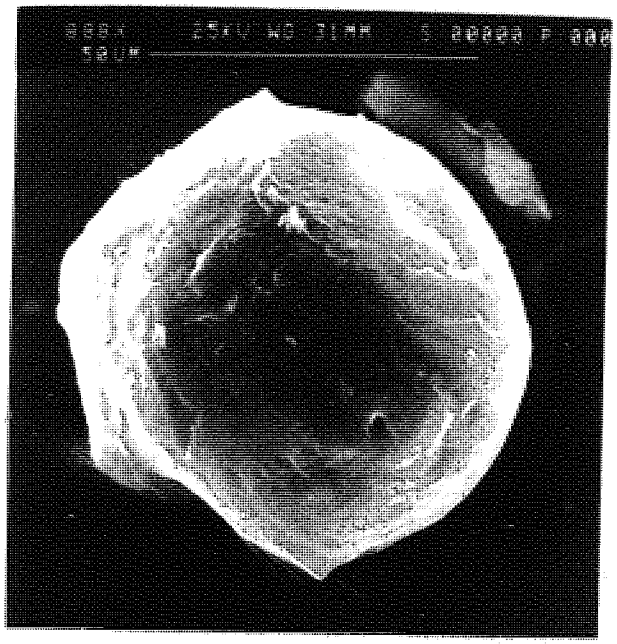


Plate 6.2a
Appearance after 7 days incubation (X888)

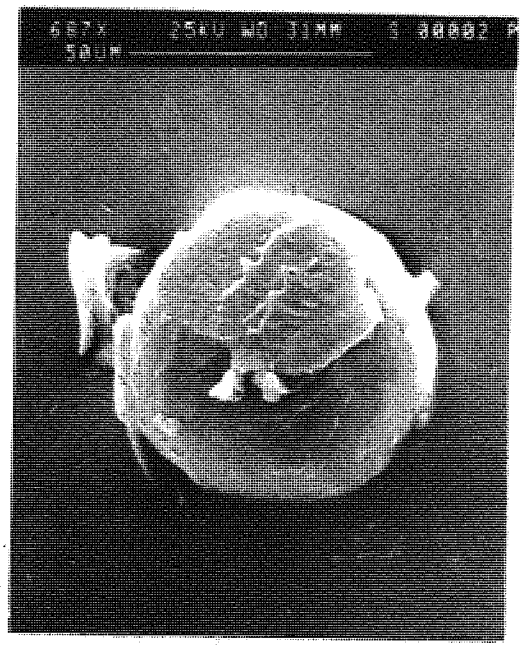


Plate 6.2b
Appearance after 21 days incubation (KX1,93)

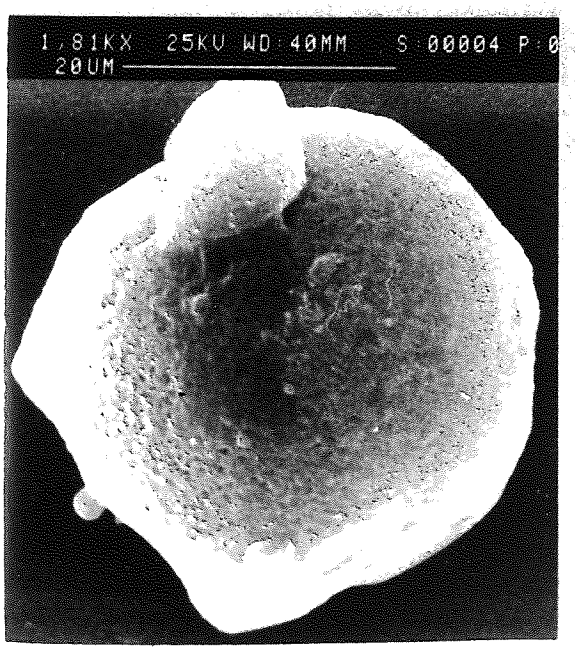


Plate 6.2c
Appearance after 28 days incubation (KX1,81)

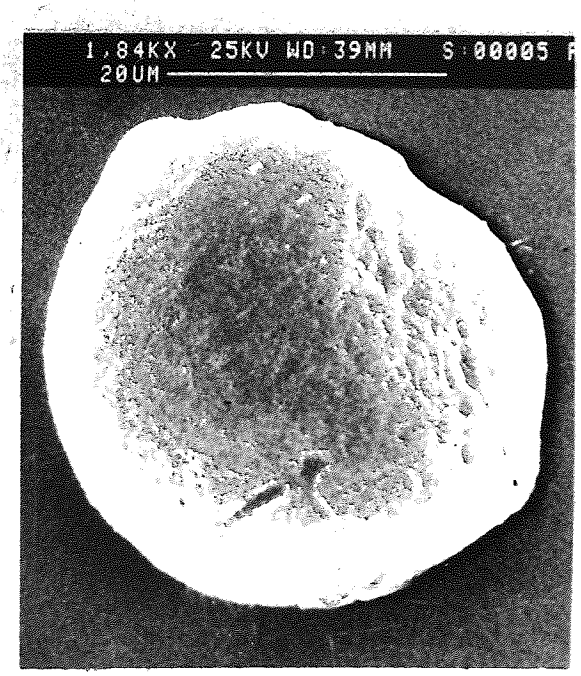


Plate 6.2d
Appearance after 35 days incubation (KX1,84)

Plates 6.2.1 **Biodegradation of 25% vancomycin loaded PEA/PLCG 50:50 blend microspheres incubated in new born calf serum.**

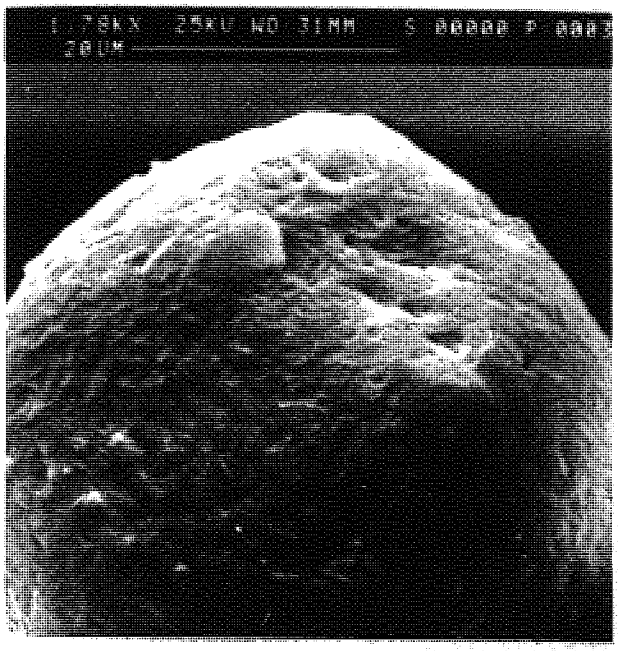


Plate 6.2.1a
Appearance after 7 days incubation (KX1,78)

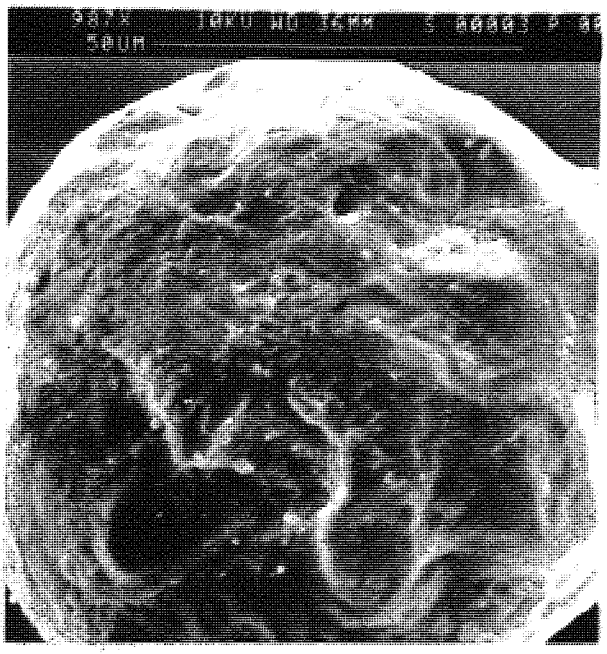


Plate 6.2.1b
Appearance after 21 days incubation (X987)

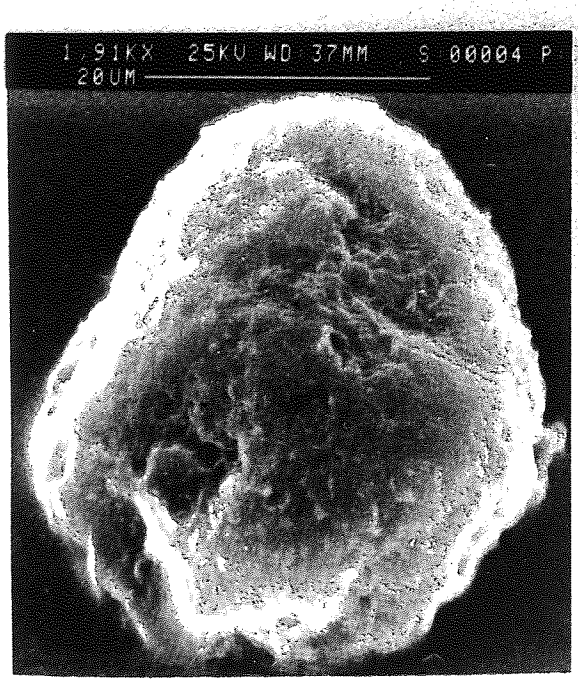


Plate 6.2.1c
Appearance after 28 days incubation (1,91KX)

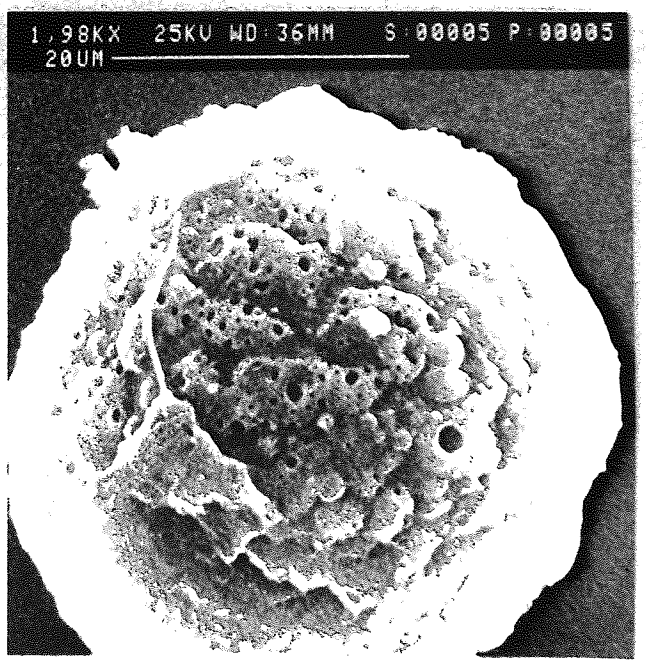


Plate 6.2.1d
Appearance after 35 days incubation (1,98KX)

Plates 6.2.2 **Biodegradation of 25% vancomycin loaded PEA/PLCG 50:50 blend microspheres incubated in Hank's buffer.**

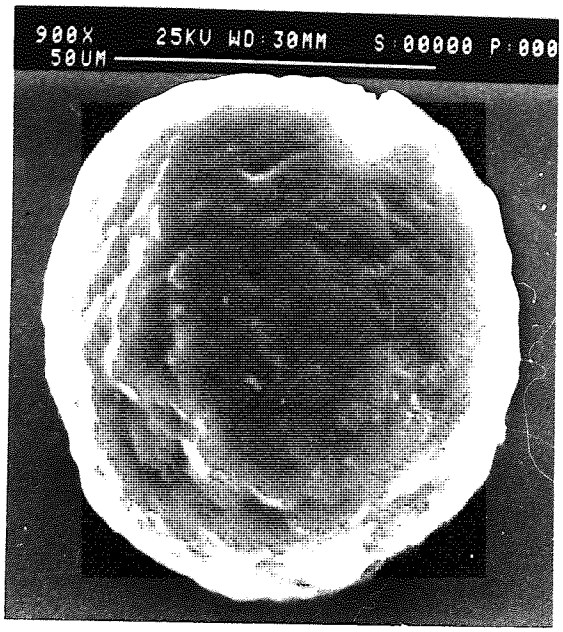


Plate 6.2.2a
Appearance after 7 days incubation (X900)

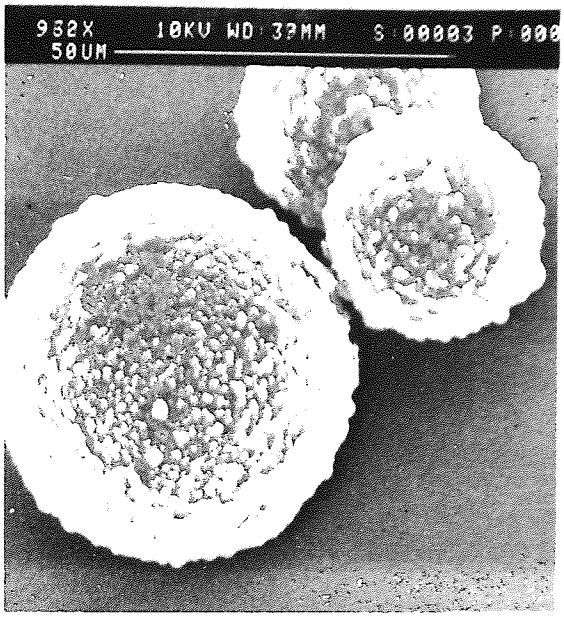


Plate 6.2.2b
Appearance after 21 days incubation (X932)

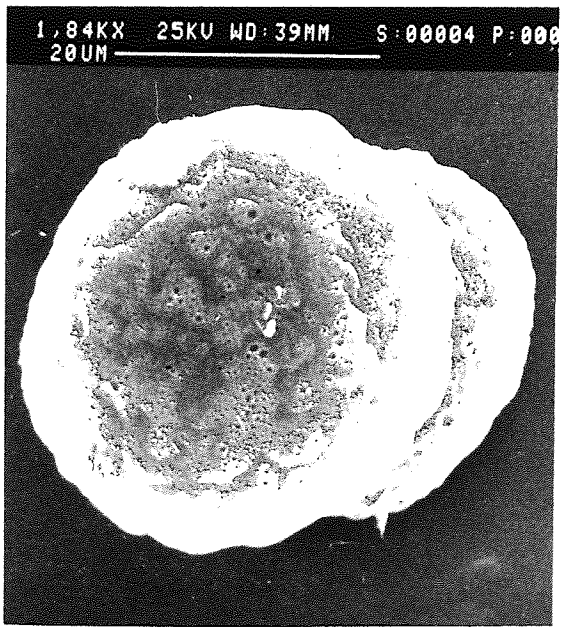


Plate 6.2.2c
Appearance after 28 days incubation (KX1,84)

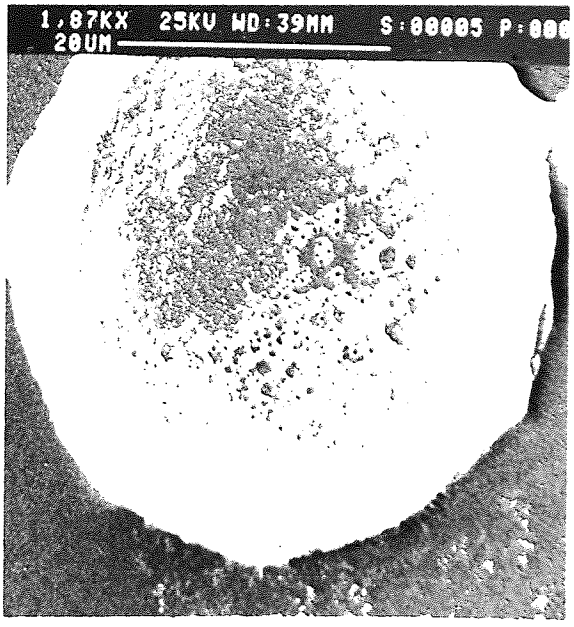


Plate 6.2.2d
Appearance after 35 days incubation (KX1,87)

Plates 6.2.3 **Biodegradation of 25% vancomycin loaded P(HB-HV)/PLCG 50:50 blend microspheres incubated in Hank's buffer.**

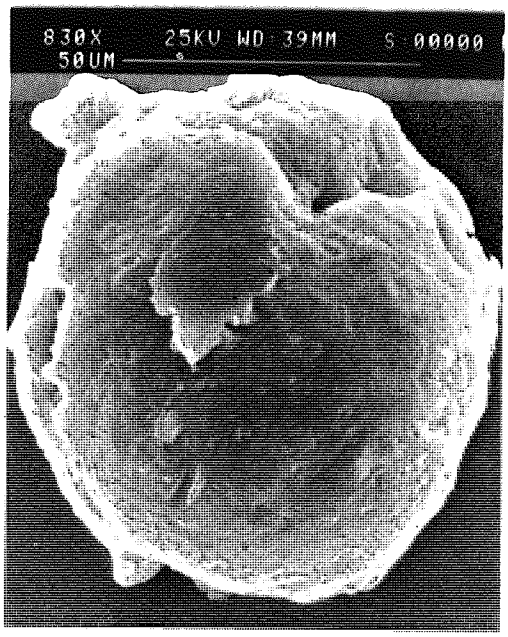


Plate 6.2.3a
Appearance after 7 days incubation (x830)

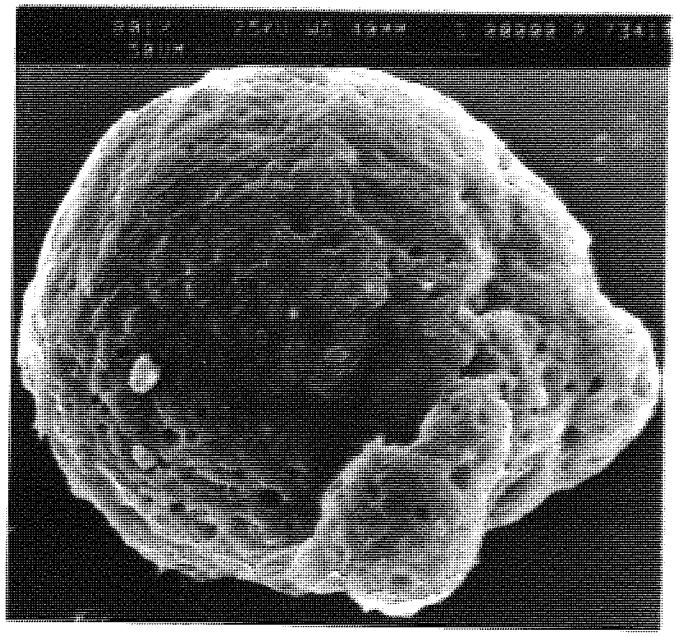


Plate 6.2.3b
Appearance after 14 days incubation (x801)

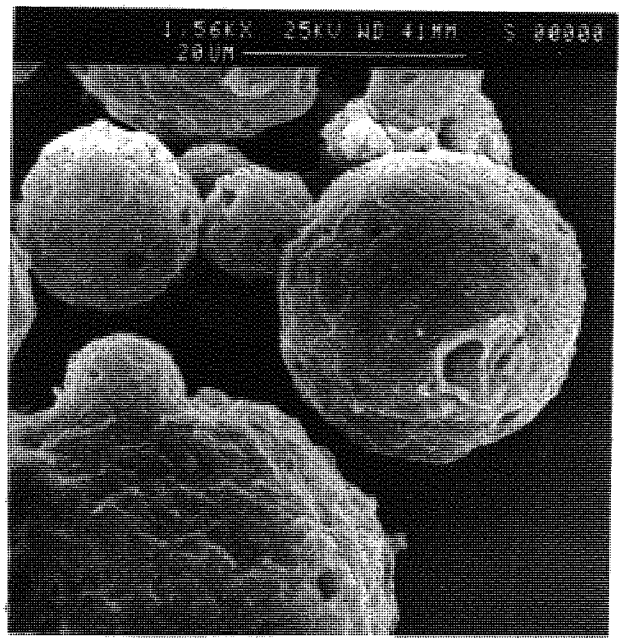


Plate 6.2.3c
Appearance after 21 days incubation (KX1,56)

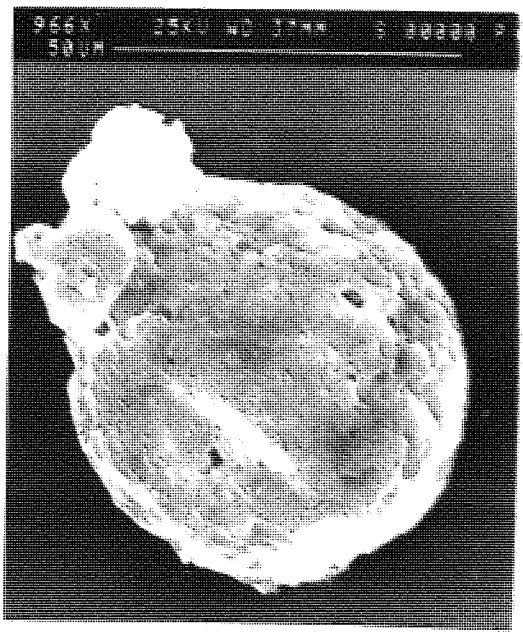


Plate 6.2.3d
Appearance after 28 days incubation (x966)

Plates 6.2.4 **Biodegradation of 25% vancomycin loaded P(HB-HV)/PLCG 50:50 blend microspheres in newborn calf serum.**

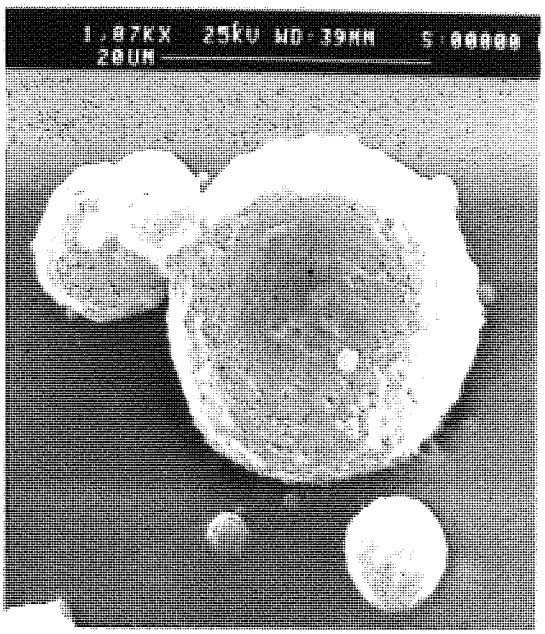


Plate 6.2.4a
Appearance after 7 days incubation (KX1,87)

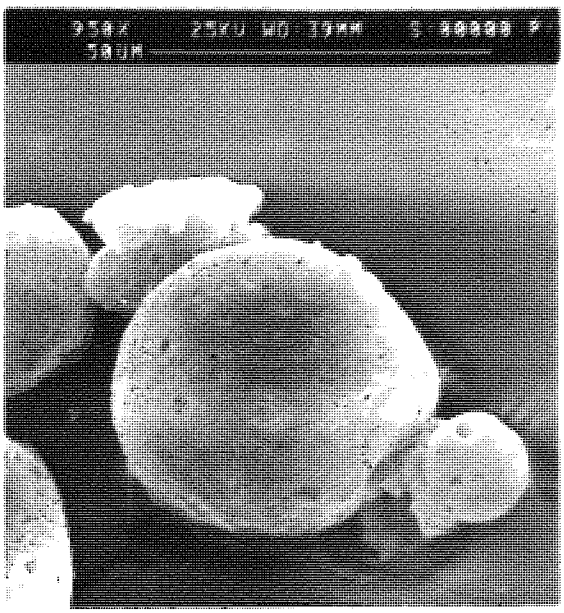


Plate 6.2.4b
Appearance after 14 days incubation (x950)

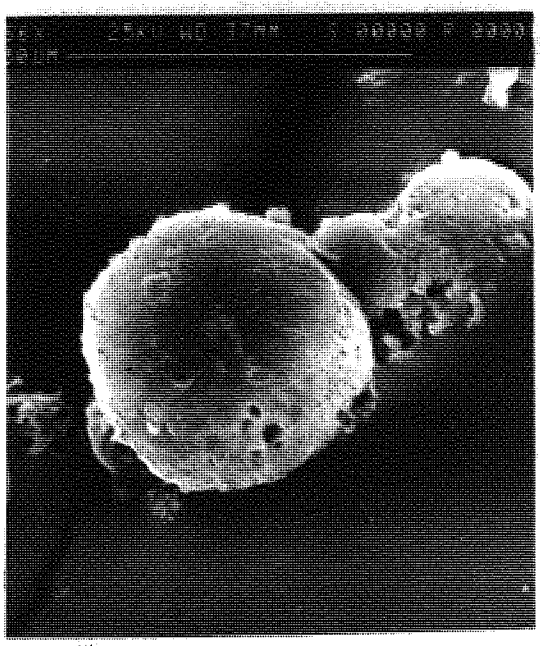


Plate 6.2.4c
Appearance after 21 days incubation (X966)

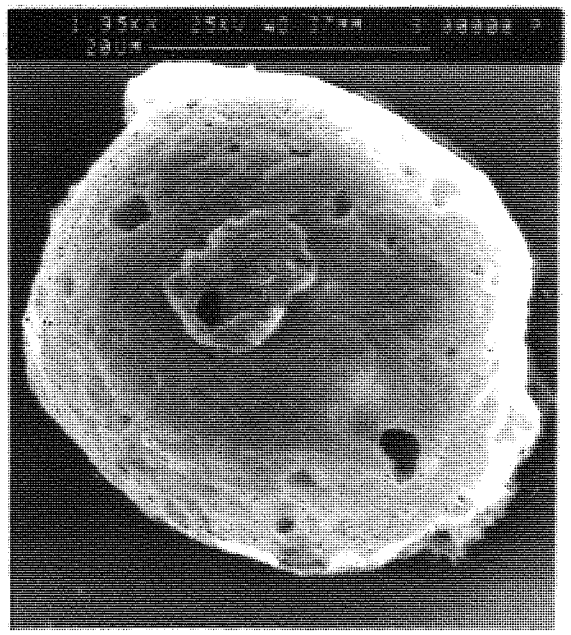


Plate 6.2.4d
Appearance after 28 days incubation (KX1,95)

6.2.4 Biodegradation of P(HB-HV)/PLCG 50:50 blend microspheres loaded with vancomycin.

Biodegradation in Hank's buffer was characterised by the development of well defined macropores by day 14, plate 6.2.3b. By day 28 these macropores had increased in size by erosion although the microspheres remained essentially intact and spherical at this time, plate 6.2.3d. Microsphere biodegradation in newborn calf serum showed a similar trend with surface macropores developing after 7 days incubation, plate 6.2.4a. By day 21 the microspheres had begun to lose their spherical shape, plate 6.2.4c, and were somewhat reduced in size. Although the microspheres were intact after 28 days, large macropores had appeared at the surface suggesting extensive internal macroporosity and biodegradation, plate 6.2.4d.

6.3 Discussion.

The *in vitro* biodegradation of microspheres fabricated using the W/O single emulsion solvent extraction technique was influenced by both the fabrication polymer and incubation medium used. Microspheres composed of PLCG 50:50 had disintegrated by day 28 in Hank's buffer but were still relatively intact after that time in newborn calf serum. This would suggest that enzymatic activity, which would be greater in newborn calf serum than Hank's buffer, did not play a significant role in the biodegradation of these microspheres. In Hank's buffer PLCG 50:50 is thought to undergo biodegradation, principally, by a process of hydrolysis, which is more marked in alkaline environments[6]. There was some indication that increasing the vancomycin percentage loading accelerated the disintegration of PLCG 50:50 microspheres, presumably as a result of increased pore generation and matrix disruption by dissolving vancomycin crystals. In Hank's buffer microspheres composed of

PLCG 75:25 began to fragment after 14 days and with a gradual increase in internal pore size, had crumbled into fragments by day 28. In newborn calf serum, these microspheres showed an increase in the diameter of internal pores with erosion of the matrix. There was a gradual reduction in microsphere diameter and loss of spherical shape although overall integrity was maintained, demonstrating again that biodegradation was greatest in Hank's buffer. In addition, there was some indication that the rate of matrix erosion in 50% loaded microspheres was somewhat slower than that of 25% loaded microspheres.

The biodegradation of PEA and blend microspheres in both Hank's buffer and newborn calf serum was characterised by a gradual erosion and increased porosity, although overall structural integrity was maintained for up to 35 days. Biodegradation was more rapid in newborn calf serum than in Hank's buffer and enzymatic degradation afforded by the presence of low levels of esterase and some protease activity in newborn calf serum may have been partly responsible. Blend microspheres showed more extensive biodegradation than PEA microspheres, revealing comprehensive surface pore domains and internal macroporosity. Clearly, the addition of PEA as a plasticiser represents a means for slowing down the biodegradation of more vulnerable polyesters such as PLCG 50:50. The biodegradation of P(HB-HV)/PLCG 50:50 microspheres was characterised by the appearance of macropores in the microsphere surface when incubated in either Hank's buffer or newborn calf serum. Although the microspheres remained intact and retained their shape and macroporosity in both incubation media for up to 28 days, surface erosion appeared to be slightly faster in newborn calf serum than Hank's buffer. This would suggest that although the principle process of P(HB-HV) biodegradation is

undoubtedly hydrolysis, a very low level of enzymatic (esterase and protease) activity may be exerted by newborn calf serum.

By monitoring biodegradation in terms of changes in gross and ultrastructural morphology it was hoped that some insight could be obtained into the relationship between changes in structure induced by temporal biodegradation and vancomycin release. All microspheres, regardless of the fabrication polymer or incubation medium used, exhibited an initial burst of vancomycin release lasting from 2 to 8 days. This burst release was attributed to the early dissolution of surface deposited vancomycin crystals into the incubation media. This conclusion is supported by the fact that no vancomycin crystals were observed on the surface of microspheres after 7 days incubation. In all cases the period of vancomycin burst release was followed by a period of low level sustained release, varying from 7 to 26 days. During the period over which microspheres released vancomycin they remained structurally intact regardless of fabrication polymer or incubation medium used. For example, in the case of 25% and 50% vancomycin loaded PLCG 50:50 microspheres, low level vancomycin release was maintained for 15 days in Hank's buffer and 11 days in newborn calf serum. At both percentage loadings these microspheres remained relatively intact for up to 21 days in Hank's buffer and 14 days in newborn calf serum. This was also true for PLCG 75:25 microspheres. In Hank's buffer 25% vancomycin loaded PLCG 75:25 microspheres released vancomycin for 7 days and remained intact, while 50% vancomycin loaded PLCG 75:25 microspheres released vancomycin for 8 days and remained intact for at least 14 days in Hank's buffer. In newborn calf serum 25% and 50% vancomycin loaded PLCG 75:25 microspheres released vancomycin for 26 days and 25 days

respectively. At both percentage loadings these microspheres remained intact for up to 28 days. This was also found to be true for microspheres fabricated using PEA, PEA /PLCG 50:50 and P(HB-HV)/PLCG 50:50. This would suggest that in Hank's buffer and newborn calf serum, vancomycin release occurs by a process of diffusion through aqueous channels created by the previous dissolution of vancomycin and not by either the bioerosion or disintegration of the polymer matrix.

From the observations made here it is quite clear that microspheres fabricated using PEA, PEA/PLCG 50:50 and P(HB-HV)/PLCG 50:50 would have a long residence time *in vivo* . After 28-35 days incubation in either Hank's buffer or newborn calf serum all three types of microsphere remained relatively intact, although in the case of PEA/PLCG 50:50 and P(HB-HV)/PLCG 50:50 begun to show quite extensive macroporosity. Although PEA and PEA/PLCG 50:50 microspheres released vancomycin for up to 22 days in newborn calf serum, P(HB-HV)/PLCG 50:50 microspheres in both Hank's buffer and newborn calf serum and PEA and PEA/PLCG 50:50 microspheres in Hank's buffer released vancomycin for only 8 days. The long residence time and short release profiles exhibited by the microspheres fabricated using these three polymers have limited potential as implant devices for orthopaedic use. Microspheres fabricated from PLCG 50:50 appear to offer a more suitable residence time in both Hank's buffer and newborn calf serum than microspheres fabricated using PEA, PEA/PLCG 50:50 or P(HB-HV)/PLCG 50:50, with microsphere disintegration occurring around 28 days in both media. However, vancomycin release from PLCG 50:50 microspheres was not detectable after 15 days incubation. This is a limiting factor since vancomycin release must be maintained for 3-4 weeks at physiologically meaningful levels to be

of use as an orthopaedic drug delivery device. Only PLCG 75:25 microspheres released physiologically useful levels of vancomycin for up to 25-26 days in newborn calf serum and these microspheres would appear to offer the most appropriate characteristics for the delivery of antibiotics in orthopaedic applications.

CHAPTER SEVEN

GENERAL DISCUSSION.

7.0 General Discussion.

Work presented in this thesis has been directed towards the utilisation and evaluation of techniques for the microencapsulation of the biologically significant macromolecules bovine serum albumin V and vancomycin HCl into microspheres and microcapsules fabricated using the synthetic biodegradable polyesters P(HB-HV), PEA, PLCG 75:25 and 50:50 and their blends. In addition the gross morphological and ultrastructural changes that occur when these microparticulates biodegrade *in vivo* have been monitored both in Hank's buffer, newborn calf serum, synthetic gastric juice and pancreatic solution with a view to understanding the potential biological and physio-chemical mechanisms involved in biodegradation and the structural changes which might be expected to subsequently occur *in vivo*. Finally, the sum of this knowledge has been employed in the development of a prototype vehicle for the sustained delivery of the water soluble antibiotic vancomycin. This antibiotic has great potential for the control of infection in orthopaedic surgery, especially hip joint replacement and the treatment of open fractures.

In initial work, the polymer blends P(HB-HV)/20% PCL II; PEA/20% PCL II and 50% P(HB-HV)/50% PEA were used to encapsulate the surrogate macromolecule bovine serum albumin V within microspheres fabricated using the O/W single emulsion with solvent extraction. In addition, microcapsules were fabricated from the same polymers using a W/O/W double emulsion with solvent extraction. P(HB-HV) was utilised in the early work because of its particular interest to the Speciality Materials Group at Aston, who

are presently synthesizing new polyesters for use in biomedical applications. Using the two emulsification techniques described, spherical microspheres and microcapsules were successfully fabricated. Loading both microspheres and microcapsules with 10% to 50% BSA by dry weight of polymer had no significant effect on the gross shape or the extent of macroporosity in either the matrix of the microsphere or the outer membrane of microcapsules. The mean diameter of 20% BSA loaded P(HB-HV)/20% PCL II microspheres was 26.28 ± 2.34 μm, n=25 with a diameter range of 6 to 50μm. The microsphere surface was comprehensively punctuated with micropores of about 0.2μm in diameter. 20% BSA loaded PEA/20% PCL II microspheres were significantly smaller than those of P(HB-HV)/20% PCL II with a mean diameter of 2.88 ± 0.23μm, n=25 (diameter range of 0.6 to 5.0μm) and showed no evidence of surface micropores or the surface deposition of BSA. Microspheres composed of a 50% P(HB-HV)/50% PEA blend and loaded with 20% BSA showed a significant increase in the mean diameter to 8.86 ± 11μm (diameter range 2.1-50μm) presumably as a result of the addition of 50% P(HB-HV). Ultrastructural examination of these microspheres revealed that the product consisted of an equal mixture of P(HB-HV)/20% PCL II microspheres, characterised by having a rugose surface punctuated with macropores, and PEA/20% PCL II microspheres characterised by a smaller overall diameter and a smooth surface. This suggested that the 50/50 combination of P(HB-HV) with PEA had not favoured a stable compatible blend and the individual components has separated into discrete microdroplets during emulsification.

Microcapsules fabricated from the same polymers using double emulsification with solvent extraction were always significantly

larger than the microspheres. This was the result of the lower emulsification speed of 2,200 rpm used during microcapsule fabrication compared with 10,000 rpm for microspheres. P(HB-HV)/20% PCL II microcapsules had a mean diameter of 100.24 ± 10.11μm, n=25 (diameter range 21-200μm) and were significantly larger than microcapsules fabricated from either PEA/20% PCL II or 50% P(HB-HV)/50% PEA with mean diameters of 37.04 ± 5.47μm, n=25 and 48.57 ± 26.17μm, n=25 respectively. This suggested that the physio-chemical properties of PEA (viscosity, chain length, molecular weight) in some way resulted in microcapsules with a small diameter. P(HB-HV)/20% PCL II microcapsules showed comprehensive inherent microporosity and the appearance of micropores in the outer membrane occurred during the solvent evaporation stage of fabrication. PCL II is inherently more soluble than P(HB-HV) in dichloromethane, the organic phase. During solvent evaporation at 20-22 °C, PCL II remains in solution longer than P(HB-HV). This results in PCL II boiling out through the hardened P(HB-HV) microcapsule wall as the latter contracts forming pores of about 2μm in diameter. In microspheres, the PCL II probably boils out more slowly because of the greater path length, and its slow diffusion through the microsphere matrix probably accounts for the smaller pore size eventually generated. Although PCL II was combined with PEA during the fabrication of PEA microspheres and microcapsules there was no evidence of micropore formation. The presence of precipitated polymer during PEA microparticulate fabrication indicated that a stable emulsion had not been formed between PEA and PCL II and that PCL II remained as a separate phase during fabrication and was not involved in micropore generation.

The BSA encapsulation efficiencies for both microspheres and microcapsules using P(HB-HV)/20% PCL II, PEA/20% PCL II and 50% P(HB-HV)/50% PEA polymer blends were generally low. The highest encapsulation efficiency of 15 ± 0.5%, n=4 was achieved with P(HB-HV)/20% PCL II microspheres using a theoretical BSA loading of 50%. The BSA encapsulation efficiency was lowest in microspheres fabricated using PEA/20% PCL II. Typically, a 50% theoretical BSA loading provided the highest encapsulation efficiency of 11 ± 0.1%, n=4.

Although the O/W emulsification technique has not been used extensively to encapsulate water soluble macromolecules, it has been widely used to encapsulate low molecular weight water soluble drugs such as theophylline, caffeine, salicyclic acid phenobarbitone[141,143]. Indeed, low encapsulation efficiencies of 16.86 ± 0.8%, n=12 have been reported for phenobarbitone in microspheres fabricated using Poly(L-lactic acid)[13,137,142]. Water soluble and moderately water soluble macromolecules tend to partition out of the organic dispersed phase into the aqueous continuous phase and the successful entrapment of macromolecules within microspheres and microcapsules will thus be highly dependent upon the solubility of the macromolecule in the aqueous phase. The low encapsulation efficiency observed for quinidine sulphate loaded Poly(D,L-lactic acid) microspheres prepared using the O/W emulsification, solvent evaporation process has been attributed to the diffusion of solubilised quinidine and quinidine sulphate across the interface of the unprecipitated microdroplet[151]. The partitioning of quinidine and quinidine sulphate from the dispersed to the continuous phase by simple diffusion along a concentration gradient is indeed plausible provided that it is

thermodynamically favourable and kinetically achievable. The thermodynamic feasibility is dictated by the relative solubility of the compound in each phase and the relative volume of each phase. Work by Benoit, Courteille and Thies has indicated that poly(D,L-lactide) might affect the solubility of progesterone within the dispersed phase, whilst the presence of the emulsifier, poly(vinyl alcohol) might have affected the solubility of progesterone in the continuous phase[152]. In general terms, the flow of any solubilised drug into the continuous phase by simple diffusion is likely to remain thermodynamically favourable until equilibrium partitioning has been achieved and there is no longer a gradient in drug concentration between the two phases. However, because the experimental system is itself dynamic as a consequence of differential solvent evaporation, the continuous/dispersed phase ration will be continuously increasing. The progressive decrease in the relative volume of the dispersed phase will constantly alter the position of the partitioning equilibrium so as to favour the sustained migration of drug out of the microdroplet, at least until the continuous phase becomes saturated. An additional effect of the changing phase ratio will be reflected on the kinetics of drug partitioning. As the dispersed phase solvent is lost, microdroplet viscosity will be enhanced as the polymer starts to precipitate. This will progressively reduce the rate of drug diffusion and, to a lesser extent, solvent diffusion into the continuous phase until the viscosity becomes so high that drug partitioning is effectively inhibited. The kinetics of drug movement may therefore make the attainment of thermodynamic equilibrium unachievable within the framework of the experiment. At this stage, there may still be a significant quantity of residual solvent containing solubilised drug in the nascent microspheres. When this is removed, the drug will

solidify although the actual physical form of the drug will be dependent on the precise nature of the system. Consequently, in a single emulsion single emulsion solvent evaporation process, the rate of polymer precipitation in relation to the rate of drug partitioning may be influential in determining the encapsulation efficiency of the microsphere. Indeed, Bodmeier and McGinity have provided evidence to show that an increase in the rate of poly(D,L-lactide) precipitation, and thus a reduction in the rate of quinidine sulphate partitioning will improve the loading of the quinidine sulphate[151]. Thus, when the rate of poly(D,L-lactide) precipitation is enhanced by employing a dispersed phase solvent with a lower heat of evaporation and a higher solubility in the continuous phase, the proportion of microencapsulated drug is dramatically increased. Moreover, these same workers also found that quinidine sulphate encapsulation efficiency in microspheres could be enhanced by increasing the molecular weight of the Poly(D,L-lactic acid) although the magnitude of this effect was generally small and dependant on the underlying polymer concentration[150]. However, the increased viscosity produced by the use of more concentrated polymer solutions significantly improved the amount of progesterone encapsulated in Poly(D,L-lactic acid) microspheres regardless of the molecular weight of the fabricating polymer. The relationship between either the polymer concentration or the molecular weight and the progesterone encapsulation efficiency has been attributed, by Benoit *et al*, to both an increase in the rate of Poly(D,L-lactic acid) precipitation and a decrease in the rate of progesterone diffusion[152].

The diffusion of a solubilised drug out of a microdroplet can also be reduced by lowering the solubility of the drug in the

continuous phase. Bodmeier and Chen demonstrated that the encapsulation efficiency of three different anti-inflammatory agents: indomethacin, ibuprofen and ketoprofen in ethylcellulose microspheres was inversely related to the solubility of each agent in poly(vinyl alcohol), the continuous phase[75]. Additionally, Bodmeier and Mcginity improved the encapsulation efficiency of quinidine sulphate on poly(lactic acid) microspheres by increasing the pH of the external aqueous phase which lowered the solubility of the quinidine sulphate in the continuous phase[151]. Consistent with these manoeuvres, a commonly applied method for increasing the proportion of encapsulated drug in microspheres involves the presaturation of the continuous phase with drug, thereby eliminating the concentration gradient[17,25]. However, this technique can prove non-economic and costly since it is difficult to to recycle expensive macromolecular drugs from the large continuous phase. Although it can be shown that kinetic considerations may result in less macromolecule partitioning into the continuous phase than would be expected on a purely thermodynamic basis, there are reports that, in some systems, more drug partitions into the aqueous phase than is required to saturate it[17]. Indeed, whilst Jalil and Nixon found that presaturation of the aqueous phase with phenobarbitone enhanced the encapsulation of this drug in microspheres prepared using the O/W emulsification solvent evaporation process, 15.5% of the original dispersed phase phenobarbitone still ended up in the continuous phase[17]. This observation suggested that simple diffusion was not the only mechanism by which drug could be lost from the microdroplets prior to microsphere formation. An alternative mechanism might involve the simultaneous loss of both drug and dispersed phase solvent from the microdroplets. However, Jalil and Nixon attributed the

phenobarbitone partitioning to spontaneous crystal growth, which occured via a stage of supersaturation and precipitation of drug crystals whilst the polymer was still in solution, followed by the shedding of the crystals from the droplet surface. Indeed, the crystallisation of initially solubilised drug within the microdroplets is not necessarily restricted to systems where the continuous phase has been presaturated with drug although a number of conditions would have to be met for this to occur[17]. Firstly, thermodynamic considerations should favour the retention of drug in the microdroplet but the drug must also have little miscibility with the polymer otherwise it is likely to remain molecularly dispersed throughout the process of precipitation by the polymer and be present as a solid in solution within the final microsphere[152]. Secondly, the drug should have a lower degree of solubility in the dispersed phase solvent than the polymer and, ideally, the initial concentration of the drug in the dispersed phase solvent should be close to its saturation level. These features should ensure that the drug starts to precipitate within the microdroplet whilst the polymer is still fully solvated. Thus, if drug crystallisation within the microsphere is to occur, it is essential that, as the drug precipitates, the viscosity is sufficiently low to enable the drug molecules to diffuse together to nucleate and develop crystal domains. If the microdroplet is to viscous when the saturation of the drug is reached, drug crystallisation may be effectively prevented. In this manner, drug that has little mutual miscibility with the polymer could be in a molecularly dispersed state within the final microsphere although this does not necessarily mean that all the drug molecules are individually dispersed within the polymer[152]. Clusters of drug molecules may be associated within the polymer phase but at concentrations too low to lead to

crystallisation. Indeed, indications are that, in the present work, when BSA was incorporated into microspheres and microcapsules fabricated from P(HB-HV)/20% PCL II, PEA/20% PCL II and 50% P(HB-HV)/50% PEA the macromolecule was limited to domains within the polymer matrix with little evidence of BSA crystal deposition at or on the surface of microspheres or microcapsules. The high aqueous solubility of crystalline BSA V would suggest that its partitioning from the organic to the continuous phase was responsible for the low microspheres encapsulation efficiencies observed in the present study.

Low levels of BSA entrapment were also obtained with P(HB-HV)/20% PCL II, PEA/20% PCL II and 50% P(HB-HV)/50% PEA microcapsules fabricated using the W/O/W double emulsion technique with solvent evaporation. The highest encapsulation efficiency of 15 ± 0.05%, n=4 was achieved using PEA/20% PCL II microcapsules and a 40% theoretical BSA loading. Bodmeier, Chen, Tyle and Jarosz have reported a very creditable encapsulation efficiency of 30.1% for the highly water soluble, low molecular weight drug pseudoephedrine HCL when incorporated into Poly(D,L-lactide) microcapsules using the W/O/W double emulsion technique[34]. However, microspheres fabricated with the same polymer but using the O/W single emulsion technique contained only 14.3% of the initial drug loading[34]. Bodmeier and colleagues explained this difference by suggesting that the organic solvent phase, methylene chloride/poly (D,L-lactide), in the W/O/W double emulsion technique acted as a barrier between the pseudoephedrine HCL containing internal phase and the external aqueous phase, thus minimising drug loss via partitioning down the concentration gradient. On the other hand, work carried out by Embleton involving

P(HB-HV)/20% PCL II microcapsules containing crystalline BSA V and crystalline bovine insulin in a low temperature gelling agarose inner aqueous phase, fabricated using the W/O/W double emulsion technique and an initial drug loading of 1.7%, generated encapsulation efficiencies of approximately 11.39% for BSA and 2.85% for bovine insulin[153]. Embleton offered two possible explanations for these low encapsulation efficiencies. The first suggested that during the process of dichloromethane extraction, and especially as the P(HB-HV) precipitated from the organic phase solution, the organic phase experienced contractile forces caused by the substantial volume decrease which accompanied the precipitation of P(HB-HV) from solution. The intense extraction of the polymer phase was accompanied by a substantial reduction in the volume of the macromolecule containing inner aqueous phase. The second explanation suggested that the water soluble macromolecule (BSA or Insulin) partitioned into the continuous phase by simple diffusion along a concentration gradient before the onset of polymer precipitation. Theoretically, there are two possible mechanisms by which this diffusion induced macromolecule partitioning could have occurred. Firstly, direct contact between the inner aqueous phase and the continuous phase could have arisen as a result of a local demulsification produced by vigorous stirring although the purpose of adding agarose was to prevent this by increasing the viscosity of the inner water phase. The second possibility was that the macromolecule could diffuse along a concentration gradient from the inner aqueous phase through the organic phase into the continuous phase. Overall, Embleton favoured the explanation that contractile forces were responsible for the encapsulation efficiencies experienced with the W/O/W double emulsion technique[153]. It may be that a combination of contractile

forces and simple diffusion best accounts for the low encapsulation efficiencies obtained for BSA using the W/O/W double emulsion technique.

BSA V release from microspheres fabricated using P(HB-HV)/20% PCL II, and microspheres and microcapsules fabricated from PEA/20% PCL II and 50% P(HB-HV)/50% PEA blends were characterised by an initial lag phase lasting some 6-9 days, subsequently followed by an exponential release covering some 6-11 days whose extent was increased with percentage BSA loading. This exponential phase was followed by an extended period of sustained low level release which decreased gradually with time until either the BSA could no longer be detected in the sample or sampling was discontinued at 30 days. P(HB-HV)/20% PCL II microcapsules maintained a steady state BSA release for up to 24 days. It is unlikely that the release of BSA from either P(HB-HV)/20% PCL II, PEA/20% PCL II or 50% P(HB-HV)/50% PEA microspheres and microcapsules occurred solely by partition dependant diffusion which involves the migration of individual macromolecules through polymer dense regions of microsphere matrix or microcapsule outer membrane[47]. For partition dependant diffusion to occur, the BSA would have to exhibit appreciable solubility in the polymer phase which it does not[47]. Macromolecules are likely to be released from poly(α, β ester) matrices such as P(HB-HV) or PEA by one of two mechanisms[54]. Initial release of macromolecules from a non-biodegradable or slowly biodegrading microsphere matrix such as P(HB-HV) may involve the rapid leaching of exposed macromolecules directly into the dissolution medium and this may be reflected by a significant "burst release effect" or rapid initial discharge of entrapped (but not fully encapsulated) macromolecule. The same

"burst release effect" can be seen from microcapsules providing the membrane is macroporous or the membrane becomes saturated with drug from the central reservoir. The macromolecule will then be rapidly lost from the membrane when immersed in liquid. However, in the present studies, burst release properties were not observed for microspheres and microcapsules fabricated from either PEA/20% PCL II or P(HB-HV)/50% PEA and from microspheres fabricated from P(HB-HV)/20% PCL II. It is likely that the BSA was released from the microparticulates via channels created in the microcapsule membrane or the microsphere matrix by the ingress of incubation medium and the subsequent dissolution of BSA. The macromolecule would then diffuse down the concentration gradient from the interior to the exterior environment according to Fickian diffusion via pores and channels created by previously dissolved BSA. Clearly the distribution of BSA within a microsphere matrix could significantly influence the release kinetics. BSA crystals were not associated with the surface of microspheres fabricated using P(HB-HV)/20% PCL II or the surface of microspheres and microcapsules fabricated using P(HB-HV)/20% PCL II and PEA/20% PCL II. As a consequence, a burst release profile was not observed for these microparticulates. The initial lag phase of the release profile may have resulted from the poor initial penetration of incubation medium into the compressed periphery of the microspheres. Also, BSA might have been sparsely distributed in this region. As the incubation medium (Hanks' buffer) gradually penetrated the matrix of the microsphere, it began to dissolve small quantities of peripherally located BSA which subsequently leached out of the polymer matrix leaving behind hydrated pores and channels. At a critical point around 6-9 days the penetrating buffer began to dissolve centrally located BSA, which subsequently

diffused out through the preformed pores and channels. In the case of BSA loaded P(HB-HV)/20% PCL 11 microcapsules, release of the macromolecule was by diffusion through inherent micropores in the capsule connecting the hollow central reservoir with the external environment and no lag phase was present in the release profile. The formation of interconnecting pore spaces in the matrix of microspheres as a consequence of macromolecule leaching has been described by Seigal, Kost and Langer. These workers have examined the internal architecture of microspheres matrices before and after the release of molecules and have confirmed that as the macromolecule content increased, the individual particles became more closely associated and a greater number came into direct contact with either adjacent microsphere surface or an open pore or channel[153]. A consequence of this distribution was that the percentage loading of the macromolecule had a significant effect on the rate of macromolecule discharge from the polymer matrix. Thus at high loadings, when the initially exposed particle leached into the penetrating front of the dissolution medium, more interconnecting pore spaces were created and a greater proportion of the entrapped macromolecule was able to escape by aqueous diffusion. Since this is a more rapid mechanism of macromolecule release than would be available via biodegradation of the polymer matrix, an increase in the rate of release (or more precisely an increase in the proportion of macromolecule that is released rapidly) with an increase in the percentage loading of the macromolecule is a common characteristic of polymer controlled release and one which was observed in the present studies.

A second possible mechanism of macromolecule release from polymer based microsphere matrices and microcapsule

membranes involves release of the entrapped species as a consequence of a penetrating chemical hydrolysis of the polymer matrix. Holland and colleagues demonstrated that such a chemical hydrolysis might facilitate the release of an entrapped macromolecule from a poly(α,β ester) matrix such as P(HB-HV) by a mechanism related to biodegradation [ie a reduction in the molecular weight and a diminution in the level of crystallinity] and/or surface bioerosion [ie, a gradual physical breakdown of the polymer matrix and the generation of porosity]. In the case of biodegradation, partition dependant diffusion coefficients are expected to increase as the molecular weight and chain entanglements of a poly(α, β ester) decreases[55]. Alternatively, bioerosion related macromolecule release is thought to typically involve the diffusion of the macromolecule through water filled pores which arise either from the erosion of solubilised low M_w polymer fractions into the dissolution medium, or total collapse of the polymer matrix[55].

In the present work incubation of P(HB-HV)/20% PCL 11 and PEA/20% PCL 11 microcapsules and microspheres in Hank's buffer over the relatively short release period used confirmed that neither biodegradation nor bioerosion processes contributed significantly to the release of BSA. However, an increase in the diameter of surface micropores in P(HB-HV)/20% PCL 11 microcapsules and micropores was evident after 30 days incubation in Hank's buffer although BSA release from these microparticulates had ceased after 26 days. Ultrastructural studies revealed some surface pitting of P(HB-HV)/20% PCL 11 microspheres and microcapsules after 30 days incubation in Hank's which was more marked in microcapsules than microspheres and suggested the occurrence of limited surface

erosion. Such limited bioerosion might account for the significant reduction in weight ie 25.78 ± 0.8%, n=4, and 28.11 ± 0.92%, n=4 shown by P(HB-HV)/20% PCL 11 microspheres and microcapsules respectively after 30 days incubation in Hank's buffer. On the other hand, PEA/20% PCL 11 microparticulates showed little evidence of biodegradation/bioerosion after 30 days incubation in Hank's buffer with low percentage weight loss values of 12.9 ± 0.79%, n=4 and 12.5 ± 0.5%, n=4 for PEA microcapsules and microspheres respectively. Such low values, suggested that microparticulates fabricated using PEA/20% PCL 11 were less susceptible to hydrolytic degradation in Hank's buffer, than either P(HB-HV)/20% PCL 11 microcapsules or microspheres. An observation which may be due to the presence of susceptible ester bonds in PEA. However, it is also likely that the singular lack of micropores in the surface membrane of microcapsules and the absence of microporosity in the matrix of microspheres could have contributed significantly to the biodegradation of PEA microparticulates.

When P(HB-HV)/20% PCL 11 and PEA/20% PCL 11 microspheres and microcapsules were incubated in newborn calf serum, porcine pancreatin solution and synthetic gastric juice, in and attempt to mimic the parenteral and gastrointestinal biodegradation of microparticulates, microcapsules, irrespective of polymer, consistently showed a significantly greater weight loss than microspheres. This observation was almost certainly due to the difference in internal architecture, ie the presence of a susceptible membrane bounded internal reservoir compared with a solid monolithic matrix. Microcapsule architecture would encourage greater polymer/aqueous phase interaction and facilitate enhanced enzymic and/or hydrolytic biodegradation. Substantial weight loss

by both P(HB-HV)/20% PCL 11, (65.6 ± 0.6%, n=4) and PEA/20% PCL 11 (69.78 ± 0.43%, n=4) was observed from microcapsules after incubation in newborn calf serum. Low level tissue derived esterase activity and protease activity associated with the presence of α and β globulin fractions of serum proteins may account for the potential enzymic activity of newborn calf serum. Incubation of P(HB-HV)/20% PCL 11 and PEA/20% PCL 11 microparticulates in enzyme rich porcine pancreatin solution produced substantial weight loss, significantly greater than that seen after incubation in either Hank's buffer or synthetic gastric juice. Porcine pancreatin contains numerous active enzymes many of which have wide range of substrate specificities, ie trypsin (3.4.21.4), chymotrypsin (3.4.21.2), elastase (3.4.21.11), carboxypeptidase A (3.4.12.2), cholesterol esterase hydrolase (3.1.1.13), phospholipse A_2 (3.1.1.4), lipase (3.1.1.3) and amylase (3.2.1.1, 3.2.1.2.). Many of them have the potential to contribute to the enzymatic biodegradation of PLCG, P(HB-HV) and PEA microparticulates. Certainly, in the present work PEA/20% PCL 11 microparticulates appeared to be more susceptible than P(HB-HV)/20% PCL 11 microparticulates to the enzymic activity of newborn calf serum and porcine pancreatin.

A distillation of the preliminary work represented in this thesis established that while microcapsules and microspheres could be readily manufactured using the O/W single emulsion solvent extraction and W/O/W double emulsion with solvent extraction techniques, the low encapsulation efficiencies achieved for the surrogate macromolecule, BSA limited the potential of the vehicles for the sustained and controlled delivery of water soluble macromolecules. In response to this, attention was focused on a technique introduced by Tsai and co-workers, in which a W/O single

emulsion system with solvent extraction was employed to microencapsulate water soluble mitomycin c with high encapsulation efficiency[35]. This technique was adapted for the microencapsulation of the water soluble antibiotic vancomycin hydrochloride, in an attempt to generate a dosage form which would be more effective than gentamicin in combating infections commonly seen in orthopaedic surgery especially those associated with the treatment of open fractures and total hip arthroplasty. Poly(lactide-co-glycolide) with copolymer ratios of 50:50 and 75:25 were chosen as candidate biodegradable fabrication polymers because of their well established biocompatibility[13,148,149] and acceptance by the majority of the pharmaceutical regulatory bodies ie Food and Drug Administration. PLCG 50:50 (MW 31,000) and PLCG 75:25 (MW 47,600) survive for about two and six weeks respectively in both rat tissue (abdominal wall) and bone (tibia)[150]. PEA (MW 17,900) and P(HB-HV) (MW 330,000) were also included in the study because of the interests of the speciality materails group at Aston University and the research sponsor ICI. Both P(HB-HV) and PEA were blended with PLCG 50:50 in an attempt to speed up their rate of biodegradation *in vitro*.

The average percentage yield obtained during W/O fabrication, using PLCG 75:25; PLCG 50:50; PEA; 50% PEA/50% PLCG 50:50 and 75% P(HB-HV)/25% PLCG 50:50 was generally high, between 55% and 90%, with mean microsphere diameters ranging from 17.39 ± 6.89μm, n=74 to 56.7 ± 13.8μm, n=70. Neither the average % yield nor the microsphere diameter were significantly influenced by the percentage vancomycin loading over the range used (10-50% theoretical loading). At higher percentage loadings vancomycin crystals were comprehensively deposited both on and in the surface

layers of the macroporous microspheres. This exaggerated crystal deposition may have resulted from the fact that vancomycin is poorly soluble in petroleum ether and crystals of the antibiotic would not be dissolved away during the washing procedure designed to remove span 40. Also when using high loadings all the vancomycin could not be accommodated within the matrix of the microspheres without some destabilisation. Jalil and Nixon used a similar explanation to account for the excessive deposition of phenobarbitone crystals on poly(L-lactic acid) microspheres fabricated using the W/O technique[17]. These workers also indicated that acetonitrile does not give a smooth film after precipitation and phenobarbitone crystals become embedded in the microsphere surface[17].

With the W/O technique, vancomycin HCL encapsulation efficiencies were generally high, ranging from 47.7 ± 4.0%, N=3 for 25% loaded 75% P(HB-HV)/25% PLCG 50:50 microspheres up to 96.74 ± 13.2%, n=3 for 50% loaded PLCG 75:25 microspheres, with only the latter showing significant increase in mean encapsulation efficiency with increasing percentage loading.

Vancomycin release profiles, irrespective of the fabrication polymer used, were characterised by an initial burst release phase extending over 2 to 8 days followed by a much reduced but generally sustained release phase extending up to 27 days. The total cumulative release of vancomycin increased proportionally with percentage loading (up to 50%) and therapeutically significant level of vancomycin HCL release (20-25μg/ml) could be monitored for up to 20 days from 25% and 50% loaded PLCG 50:50 microspheres and up to 27 days from 50% loaded PLCG 75:25 microspheres in newborn

calf serum. 75% P(HB-HV)/25% PLCG 50:50 microspheres released detectable concentrations of vancomycin for up to 8 days in newborn calf serum and up to 7 days in Hank's buffer at 37°C. The substantial burst release phase observed in the majority of the release profiles was the result of vancomycin crystal deposition on the surface and the release of vancomycin associated with the peripheral compressed macroporous matrix of loaded microspheres. In retrospect, it might have been of value to use lower percentage vancomycin loadings in the study ie 20%, in an attempt to reduce the level of vancomycin crystal deposition and the extent of the burst release phase. 10% loading did reduce the burst release phase but did not provide a sufficiently large enough reservoir of vancomycin for subsequent sustained release. Indeed, an initial modest burst release of vancomycin from microspheres may be desirable when considering their potential use in topical applications. The toxic side effects associated with high systemic concentrations of vancomycin will be less important when the antibiotic is applied topically to infected scar tissue in the hip joint cavity. The subsequent low level sustained release of vancomycin would help to suppress and reduce bed occupancy time prior to the fitting of a replacement joint prosthesis.

The burst release phase described above for vancomycin is a characteristic component of drug release profiles from a variety of polymer based delivery vehicles. A biphasic release of mitomycin c from poly(D,L-lactic acid) microspheres, fabricated using the W/O single emulsion with solvent extraction technique has been described by Tsai and colleagues[35]. The initial burst release of the drug was thought to be associated with surface located mitomycin c and followed by a low level sustained release from a depot of finely

dispersed drug in the microsphere matrix. Jalil and Nixon have also reported an initial burst release of phenobarbitone from both poly(D, L-lactic acid) and poly(L-lactic acid) microspheres fabricated using the W/O single emulsion system[145,155]. However, these workers also mention the presence of a subsequent post burst, lag phase of phenobarbitone release. They attributed this "lag phase" to the process of hydration and penetration of the microsphere by the dissolution fluid (phosphate buffer, pH 2 and 9) prior to core leaching. Once core leaching had commence, the subsequent low level release of phenobarbitone was sustained[145,155]. No such "post burst, lag phase" was observed with vancomycin HCL loaded microspheres in the present studies, irrespective of either the type of polymer, incubation medium or percentage loading used. This might suggest that the vancomycin was evenly distributed through out the microsphere matrix rather than being concentrated at the microsphere core.

Biodegradation studies carried out in Hank's buffer and newborn calf serum confirmed that vancomycin release occurred by diffusion through aqueous pores and channels created by the previous dissolution of the antibiotic rather than by either bioerosion or disintegration of the fabrication polymer. 25% and 50% vancomycin loaded PLCG 50:50 microspheres when incubated in newborn calf serum showed initial surface cracking, followed by the appearance of large smooth edged pits which acted as focci for further erosion and by day 28 fragmentation of PLCG 50:50 microspheres had begun. A similar sequence of structural changes was observed for 25% and 50% vancomycin loaded PLCG 50:50 microspheres in Hank's buffer, except that the occurrence of smooth pits appeared around day 21 for 25% and day 14 for 50% loaded

microspheres. In Hank's buffer, PLCG 50:50 microspheres had disintegrated by day 28 and there was some indication that increasing the vancomycin percentage loading accelerated the dintergration process of PLCG 50:50 microspheres, presumably as a result of increased pore generation and matrix disruption by dissolving vancomycin crystals. The pattern of biodegradation shown by 25% and 50% vancomycin loaded PLCG 75:25 microspheres in Hank's buffer was characterised by the appearance of surface flaking, with a gradual increase in pore diameter and eventual fragmentation by day 28. In newborn calf serum, these microspheres showed an increased in the diameter of internal pores with erosion of the matrix. There was a gradual reduction in microsphere diameter although overall integrity was maintained for up to 35 days, demonstrating again that biodegradation was greatest in Hank's buffer. PEA, 50% PEA/50% PLCG 50:50 and 75% P(HB-HV)/25% PLCG 50:50, 25% vancomycin loaded microspheres remained structurally intact for between 28 and 35 days in both Hank's buffer and newborn calf serum, suggesting that they would have a substantial residence time *in vivo*. Despite maintaining their structural integrity PEA and 50% PEA/PLCG 50:50 microspheres only released detectable amounts of vancomycin for 22 days in newborn calf serum and for only 8 days in Hank's buffer. In addition, 75% P(HB-HV)/25% PLCG 50:50 microspheres released detectable amounts of vancomycin for only 8 days in both Hank's buffer and newborn calf serum. This would suggest that microspheres fabricated using these polymers have limited potential as antibiotic delivery vehicles in orthopaedic applications.

On the other hand PLCG 50:50 microspheres demonstrated a more satisfactory residence time in both Hank's buffer and newborn

calf serum, with microspheres persisting for up to 28 days in both incubation media. However, these microspheres ceased releasing detectable amounts of vancomycin after 15 days incubation. Only 25% and 50% PLCG 75:25 microspheres released physiologically meaningful concentrations of vancomycin (20-25μg/ml) for up to 25 and 26 days respectively in newborn calf serum, making these microspheres the most appropriate candidates for use in orthopaedic surgery.

The future development of antibiotic delivery vehicles must address the increasing problem of antibiotic resistance. It may be that the development of a vehicle which would release a spectrum of antibiotics ie vancomycin HCL, rifampicin and ciprofloxacin would be effective in the treatment of infections due to *stapholococcus aureus* and *Pseudomonas sp* in orthopaedic surgery, especially hip replacement.

The acceptance of such a device by the pharmaceutical regulatory bodies will involve the fabrication of a sterile, biocompatible product. A possible solution to sterilization may be to treat the vehicle with 70% alcohol or ethylene oxide. It may be significant that gamma radiation appears to effect the integrity of poly(α,β esters) like PLCG[156]. The biocompatibilty will need to determined in terms of *in vitro* cytotoxity[157,158], genotoxicity[159] implant material toxicity[160] and articular chondrocyte functional toxicity[161] followed by a rigorous assessment of *in vivo* whole animal toxicity prior to surgical trials and commercial exploitation.

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Appendices

Appendix 1

Sterile Hank's balanced salt solution.

Sodium Chloride (A.R Grade)	8.00g/l
Potassium Chloride (A.R Grade)	0.40g/l
Magnesium Sulphate (A.R Grade)	0.20 g/l
Calcium Chloride (A.R Grade)	0.14g/l
Disodium Hydrogen Phosphate (A.R Grade)	0.06g/l
Potassium Dihydrogen Phosphate (A.R Grade)	0.06g/l
Sodium Hydrogen Carbonate (A.R Grade)	0.35g/l

Dissolved in 1litre of double distilled water.

The pH was adjusted to pH 7.4 with sodium hydroxide.

Appendix 2

Reagent A of BCA protein Assay.

1% Bicinchoninic acid,

2% Sodium carbonate,

0.4% Sodium hydroxide,

0.16% Sodium tartrate

0.95% Sodium hydrogen carbonate.

Appendix 3

Reagent B of BCA protein Assay

4% Copper sulphate in double distilled water.

Publications.

Presented at: 8th International Symposium on microencapsulation 16-18th Sept 1992, Dublin, Ireland.

THE INCORPORATION AND RELEASE OF BSA FROM PHB/PHV AND POLYETHYLENE-ADIPATE FABRICATED MICROCAPSULES.

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Presented at: 8th International Symposium on microencapsulation
16-18th Sept 1992, Dublin, Ireland.

IN VITRO BIODEGRADATION OF PHB/PHV AND POLYETHYLENE-ADIPATE MICROPARTICULATES.

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Presented at: 9th International Symposium on microencapsulation

September 1993, Ankora, Turkey.

THE INCORPORATION AND RELEASE OF VANCOMYCIN FROM MICROSPHERES FABRICATED USING POLY[D,L-LACTIDE-CO-GLYCOLIDE] AND BLENDS WITH PHB/PHV AND POLY[ETHYLENE-ADIPATE].

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Presented: at Monte Venta Conference on Biocompatible Materials Systems, October 11-14th 1993, Ascona, Switzerland.

IN VITRO BIODEGRADATION OF VANCOMYCIN LOADED MICROSPHERES FABRICATED USING POLY[D.L-LACTIDE-CO-GYCOLIDE] AND BLENDS WITH PHB/PHV AND POLY[ETHYLENE-ADIPATE].

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