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THE SUITABILITY OF BIODEGRADABLE
POLY (*dl*-LACTIDE-co-GLYCOLIDE) 75:25 MICROSPHERES
FOR THE SUSTAINED RELEASE OF ANTIBIOTICS.

DAVID JONATHAN YATES

Doctor of Philosophy

ASTON UNIVERSITY

April 1998.

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Post-operative infections resulting from total hip arthroplasty are caused by bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* entering the wound peri-operatively or by haemetogenous spread from distant loci of infection. They can endanger patient health and require expensive surgical revision procedures. Gentamicin impregnated poly (methyl methacrylate) bone cement is traditionally used for treatment but is often removed due to harbouring bacterial growth, while bacterial resistance to gentamicin is increasing. The aim of this work was to encapsulate the antibiotics vancomycin, ciprofloxacin and rifampicin within sustained release microspheres composed of the biodegradable polymer poly (*dl*-lactide-co-glycolide) [PLCG] 75:25. Topical administration to the wound in hydroxypropylmethylcellulose gel should achieve high local antibiotic concentrations while the two week *in vivo* half life of PLCG 75:25 removes the need for expensive surgical retrieval operations.

Unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres were fabricated using a Water in Oil emulsification with solvent evaporation technique. Microspheres were spherical in shape with a honeycomb-like internal matrix and showed reproducible physical properties. The kinetics of *in vitro* antibiotic release into newborn calf serum (NCS) and Hank's balanced salt solution (HBSS) at 37°C were measured using a radial diffusion assay. Generally, the day to day concentration of each antibiotic released into NCS over a 30 day period was in excess of that required to kill *St. aureus* and *Ps. auruginosa*. Only limited microsphere biodegradation had occurred after 30 days of *in vitro* incubation in NCS and HBSS at 37°C. The moderate *in vitro* cytotoxicity of 20% w/w antibiotic loaded microspheres to cultured 3T3-L1 cells was antibiotic induced.

In conclusion, generated data indicate the potential for 20% w/w antibiotic loaded microspheres to improve the present treatment regimens for infections occurring after total hip arthroplasty such that future work should focus on gaining industrial collaboration for commercial exploitation.

Key words: Total hip arthroplasty; post-operative infection; water in oil emulsification with solvent evaporation; release kinetics; cytotoxicity.

Life is an accident - learn how to handle it.

New fast Automatic Daffodils, 1994.

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A handwritten signature in black ink, appearing to read 'Dave Yates', with a large, stylized flourish at the end.

Dave Yates,

January 1998.

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List of Abbreviations.

% w/w	Percentage weight for weight
CMC	Carboxymethylcellulose
CPI	Cell proliferation inhibition
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DSC	Differential scanning calorimetry/ calorimeter
DST	Diagnostic sensitivity agar
EM	Electron microscopy
ERGATT	European research group for alternatives in toxicity testing
ESR	Erythrocyte sedimentation rate
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FRAME	Fund for the replacement of animals in medical experiments
GLC	Gas liquid chromatography/ chromatograph
GPC	Gel permeation chromatography/ chromatograph
HBSS	Hank's balanced salt solution
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
ICH	International Committee for Harmonisation
ID ₅₀	Inhibitory dose 50%
IDMD	Iscove's modification of Dulbecco's medium
INVITTOX	<i>In vitro</i> techniques in toxicology
IVIV	<i>In vitro-in vivo</i>
MC	Methylcellulose
MIC	Minimum inhibitory concentration

Mn	Number average molecular weight
Mp	Peak molecular weight
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
Mw	Weight average molecular weight
NCS	Newborn calf serum
NET	Norethisterone
O/W	Oil in Water
Pd	Polydispersity
PGA	Poly (glycolide) or (glycolic acid)
PLA	Poly (lactide) or (lactic acid)
PLCG	Poly (lactide-co-glycolide)
PMMA	Poly (methyl methacrylate)
ppm	Parts per million
PVA	Poly (vinyl alcohol)
RDA	Radial diffusion assay
SD	Standard deviation
SEM	Standard error of the mean
SPAN	Sorbitan monopalmitate
Tg	Glass transition temperature
THA	Total hip arthroplasty
W/O	Water in oil
W/O/O	Water-in oil-in oil
W/O/O/O	Water-in oil in oil-in oil
W/O/W	Water-in oil-in Water

Chapter 1: Total Hip Arthroplasty: Procedures and Problems associated with the surgical replacement of hip joints.

1.1. Total hip arthroplasty: Reasons for use, applications and surgical details.

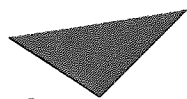
Total hip arthroplasty (THA) involving surgical replacement of the hip joint with a prosthetic implant has a proven track record in relieving pain and improving limb function in over 90% of recipients with osteoarthritis and rheumatoid arthritis (Poss *et al*, 1984; Schulte *et al*, 1993; Siopack and Jergesen, 1995). One of the most popular THA techniques in use today is low-friction arthroplasty, developed by the British surgeon Sir John Charnley in the late 1960's, which utilises the biomechanical principles of human hip joint function (Siopack and Jergesen, 1995). The procedure involves surgical exposure of the femur by trochanteric osteotomy (Schulte *et al*, 1993; Wroblewski and Siney, 1993) followed by excision of the head and proximal neck of the femur (the "ball" of the femur and associated bone) together with the acetabular cartilage and subchondral bone (the "socket" portion of the joint). A prosthesis, constructed using an alloy of either stainless steel, titanium or cobalt-chrome and composed of a stem and a small diameter head (22 mm) made of either metal or ceramic, is inserted into the medulla of the femur by first creating a canal through the bone marrow. Adjusting the length of the head portion allows correction for leg length. The acetabular component, constructed using either ultra-high or high molecular weight polyethylene with a low-friction surface and an approximate diameter of between 32 and 44 mm (Pritchett and Bortel, 1991; Schulte *et al*, 1993) is implanted into the enlarged space created by the removal of the acetabular cartilage and associated structures. Both femoral and acetabular components are fixed to the bone using self-curing poly (methyl methacrylate) [PMMA] bone cement. The processes of microlocking (where cement infiltrates the bone crevices lining the prosthesis-bone

interface) and macrolocking (where cement fills all the large spaces in the bone surrounding the implant) help prevent the prosthesis loosening. The preparation of PMMA under vacuum or involving centrifugation reduces the formation of air bubbles in the polymer which can weaken its structure (Siopack and Jergesen, 1995). Cemented arthroplasty provides early pain relief and a rapid return of function but cement, polyethylene and acrylic debris can cause osteolysis. This destruction of bone is caused by phagocytosis of these wear particles by macrophages which leads to local resorption of bone and subsequent loosening of the prosthesis (Siopack and Jergesen, 1995). This problem has been circumvented by the development of cementless THA in which the femoral component is either “press-fitted” into the bone or coated with a porous material, typically metal beads between 250 μm and 400 μm in diameter, which allows bone fibres to penetrate the surface of the prosthesis holding it in place (biologic fixation). The acetabular component is attached to the bone using cancellous spikes or screws. The potential 15 year life span of cementless prostheses and the lower incidence of bone loss during their implantation makes this operation popular with younger, active patients. However, cementless THA can cause more low-grade thigh pain than its cemented counterpart; requires a more specialised surgical procedure, increases the frequency of intra-operative femoral fractures, can lead to loosening of the prosthesis because of osteolysis and sometimes leads to the release of carcinogenic metal ions into tissues. These factors have increased the popularity of combining a cemented femoral component with an un-cemented acetabular component for patients over the age of 60 years (Pritchett and Bortel, 1991; Siopack and Jergesen, 1995; Hearn *et al*, 1995). The anatomy of the hip before and after surgery for THA is shown in figure 1.1. below.

Figure 1.1. Anatomical representation of the right hip before and after Charley low-friction total hip arthroplasty.

(Siopack and Jergesen, 1995).

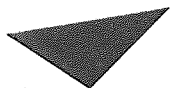
Figure 1.1.a) Anatomy of the right hip before THA showing the site of femoral neck excision (-----).



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Figure 1.1.b) Anatomy of the right hip after THA showing cemented femoral and acetabular components.



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1.2. Management of post-operative infections occurring as a result of total hip arthroplasty.

Post-operative infection of the implant site, usually of the prosthesis itself, is a major problem arising from THA operations and occurs in <1% to 3% of cases depending upon the surgical procedure employed (Charnley, 1972; Fitzgerald *et al*, 1977; Gristina and Kolkin, 1983; Canner *et al*, 1984; Poss *et al*, 1984; Hamblen, 1993; Schulte *et al*, 1993; Wroblewski and Siney, 1993; Siopack and Jergesen, 1995). At first glance, these figures appear to be acceptably low, but when one considers that an estimated 300,000 THA procedures are performed each year world-wide, (Siopack and Jergesen, 1995) and that, in 1995, the cost of surgical revision of a single infected hip was quoted as being US\$29,666 (approximately £18,500) including hospital services, surgeon's fee, and anaesthesia (Barrack, 1995) the seriousness and scale of the problem becomes apparent. The three main time-scale categories for post-operative THA infection are acute, sub-acute and late (Gristina and Kolkin, 1983). Acute infections tend to present themselves within 12 weeks of operation, usually within four weeks, and can cause warming and reddening of the skin around the wound, pus seepage from the unhealed wound, oedema at the operation site, low-grade fever and an increase in the erythrocyte sedimentation rate (ESR). Sub-acute infections occur within a year of operation and are characterised by transient and immediate post-operative symptoms such as a mildly elevated ESR, increased temperature and a reddening of the wound area, all of which disappear spontaneously. It is only, sometimes months, later that pain develops in the hip. Late infections occur at least a year after surgery and are characterised by a gradual increase in constant hip pain that is not relieved by changes in postural position. Associated fever and chills have also been reported (Fitzgerald *et al*, 1977). The administration of antibiotics will decrease this pain and discomfort which indicates that bacteria are the cause, rather than mechanical (aseptic) loosening of the prosthesis (Gristina and Kolkin, 1983). Infections of the hip have been further categorised as being either suprafascial or deep. A suprafascial condition is defined as an acute infection of the surgical incision skin or tissues above the fascial layer (the

layer of fibrous connective tissue and fat separating the skin from the underlying tissues) (Fitzgerald *et al*, 1977; Sanderson, 1983; Beck-Sague *et al*, 1992) whereas deep infections involve tissues beneath the fascial layer and often the implant itself (Charnley, 1972; Sanderson, 1983). Suprafascial infections can be caused by post-operative decreases in the supply of blood to the tissues occurring as a result of blood vessel strangulation by skin sutures together with the nosocomial acquisition of skin pathogens (Charnley, 1972). However, there is general agreement that acute and sub-acute deep infections of the hip wound area (roughly the area of approximately five or six petri dishes each with a diameter of three and a 1/4 inches (Charnley, 1972)) can be caused by intra- and peri- operative contamination of the wound by skin scales shedded from surgical staff, the bacterial flora on the patient's own skin, an ineffective aseptic technique and bacteria carried on dust particles floating in the air (Poss *et al*, 1984; Siopack and Jergesen, 1995; Gristina and Kolkin, 1983; Charnley, 1972; Sanderson, 1983; Inman *et al*, 1984). Late infections are thought to occur either as a result of the growth of latent bacteria present in the wound at the time of surgery, or the haemetogenous spread of bacteria from other sites of infection such as the skin (including dermatitis and pressure sores) or the urinary and gastrointestinal tracts and mouth (Fitzgerald *et al*, 1977; Gristina and Kolkin, 1983; Sanderson, 1983; Inman *et al*, 1984; Poss *et al*, 1984; Siopack and Jergesen, 1995). Treatment regimens for suprafascial and deep infections of the hip vary. Suprafascial infections can be eradicated using appropriate antibiotics (subsequent to bacterial culturing and identification), drainage to remove haematomas and abscesses, cleansing by frequent changes of dressings and, if possible, early closure of the wound (with skin grafts if necessary) to avoid secondary contamination (Wilson *et al*, 1973). However, the surgical procedures required for the treatment of deep infections are more complex. For sub-acute and acute deep infections, immediate re-operation is necessary. The entire wound should be cleared of blood clots and abscesses with thorough débridement of devitalised and necrotic tissue. Prosthesis removal is not essential for the eradication of the infection unless it is hindering débridement (Wilson *et al*, 1973; Tsukayama *et al*, 1996). Subsequent closure of the wound over a saline suction-irrigation system is then advisable

until samples including tissue, blood and tissue fluid taken from the wound are negative for bacterial growth (by culturing for at least 4 days) after which time the tubes can be converted to suction only and removed a day later (Wilson *et al*, 1973). Management of a late-onset deep infection involves removal of the prosthesis followed by extensive débridement of the wound area to ensure the retrieval of all cement (if used), infected tissue and bone as well as devascularised scar tissue, thereby eliminating dead space. Frequent topical administration of high dose antibiotics (according to the nature and sensitivity of the infecting organism) either by irrigating or packing the wound is also a necessity. Immediate re-implantation of the prosthesis may be possible if inflammation is minimal (one-stage procedure). However, if there is evidence of gross wound tissue inflammation, reconstruction must be delayed until the wound has healed (two-stage procedure) during which time the patient is kept in skeletal traction. A minimum of two weeks of traction is usually required to ensure total wound healing. This will, of course greatly inflate the costs of treatment. After 2-4 weeks of irrigation therapy, antibiotics are, if suitable, then administered orally. For surgical revisions where the prosthesis is not re-implanted, 6-8 weeks of oral antibiotic therapy is usually sufficient as a treatment measure but this can be prolonged, possibly indefinitely, if a new prosthesis is being fitted (Wilson *et al*, 1973; Buchholz *et al*, 1981; Cherney and Amstutz, 1983; Siopack and Jergesen, 1995; Mulcahy *et al*, 1996; Tsukayama *et al*, 1996). For incurable hip infections involving prostheses, the Girdlestone procedure of excision arthroplasty is the last remaining surgical option (Hamblen, 1993). It is used, patient's health permitting, only when there is inadequate bone stock for fixation of the prosthetic components, extensive infection of the soft tissues by mixed bacteria which may well be antibiotic resistant, and gross scarring and muscle depletion as a result of previous surgery (Hamblen, 1993; Mulcahy *et al*, 1996). The Girdlestone procedure involves the removal of the lateral acetabular rim and the proximal part of the femur (at the inter-trochanteric line) to produce two closely fitting articulating surfaces (Canner *et al*, 1984). Again, post-operative traction and antibiotics are used (Canner *et al*, 1984; Hamblen, 1993). However, the majority of patients cannot walk long distances even after total recovery from excision arthroplasty and

often require either two walking sticks or a frame for support purposes. The mobility and function of the limb are both reduced as the leg on the side of the excision arthroplasty acts as little more than a semi-rigid prop. Some shortening of the affected leg does occur and lack of muscle power leads to the adoption of a Trendelenburg gait where the pelvis tilts downwards instead of upwards when the affected leg is raised (Hamblen 1993). Therefore, to prevent surgical revision procedures, especially the use of the Girdlestone operation, it is imperative that incidences of infection are reduced to a minimum and that, if they do occur, they are quickly diagnosed and effectively controlled. In order to help reduce the rate of infection, the recognition of high risk patients is important. Generally, factors such as advanced age, obesity, current distant infections or skin lesions, steroid therapy, rheumatoid arthritis, diabetes mellitus, poor nutrition, depressed immunological defences, prolonged hospitalisation and a previous THA operation can increase the occurrence of post-operative infections (Fitzgerald *et al*, 1977; Cherney and Amstutz, 1983; Gristina and Kolkin, 1983; Poss *et al*, 1984; Wroblewski and Siney, 1993). Before entering the operating theatre, the patients should first be washed with antiseptic and the thigh and adjacent perineum electric-shaved before again being washed with antiseptic and sterile-draped. Modern day orthopaedic operation suites often incorporate laminar flow enclosures in which the surgeons work and/or 1-2 μm filtered air systems which regularly replace the air in the operating room with fresh, sterile air (up to 300 times an hour) and create a positive pressure inside the operating room such that bacteria cannot enter it in air from the outside (Charnley 1972; Learmonth, 1993; Hearn *et al*, 1995; Siopack and Jergesen, 1995). Ultra-violet light has also been employed to sterilise the operation area prior to surgery (Poss *et al*, 1984). Each member of the operating staff should wear a surgical hood that leaves no hair exposed and a mask to prevent potentially pathogenic bacteria from their naso-pharyngeal tract reaching the wound. The use of complete elasticated tight-fitting gowns made, for example of gortex, are both comfortable for the members of staff wearing them and prevent passage of bacteria from their skin to the patient. Double-gloving subsequent to hand washing with benzalkonium (for ten minutes before the first operation of the day and for five minutes between each successive

operation) will also help prevent bacterial transfer, since surgical gloves tend to become punctured in 48% of orthopaedic operations (Gristina and Kolkin, 1983; Learmonth, 1993; Siopack and Jergesen, 1995). Alternatively, the use of total body exhaust suits by the surgical staff will prevent their bacterial flora from contaminating the wound whilst decreasing the airborne bacterial count (Charnley, 1972; Learmonth, 1993). Longer operation times also increase the incidence of infections involving the hip prosthesis. In a study of 3,215 THA procedures the incidence of deep sepsis was 0.9% for operations lasting less than 140 minutes whereas this figure rose to 1.7% for operations lasting over 140 minutes (Fitzgerald *et al*, 1977). It is thought that the longer the time the wound and prosthesis are exposed to bacteria, the greater is the amount of tissue breakdown and ischaemia both of which lengthen recovery time and depress immunological resistance to infection (Gristina and Kolkin, 1983). Diagnosis of infection can be achieved by using bone scans; measuring the ESR (which is raised above approximately 30 mm/h for a prolonged period of time in 75% of infections (Kamme and Lindberg, 1981; Gristina and Kolkin, 1983), performing hip aspirations with subsequent culturing of fluid (although it must be remembered that between 7% and 15% of infected arthroplasties show no positive culture from hip aspirates (Gristina and Kolkin, 1983), histological examination of blood, tissue or tissue fluid samples removed from the wound site at the time of operation (for bacteria or inflammatory cells such as neutrophils) (Gristina and Kolkin, 1983), consistently recording a temperature above 38°C and examining blood samples for signs of leukocytosis (Buchholz *et al*, 1981; Gristina and Kolkin, 1983; Canner *et al*, 1984). The use of antibiotics to eradicate pathogenic organisms from the hip joint is fundamental in the treatment of THA infections. Details of the types of antibiotics commonly utilised during THA procedures and their means of administration are documented below.

1.3. THA infections: causative bacterial species and attempts to eradicate them by using specific means of antibiotic administration.

Bacteria responsible for infections in THA include *Staphylococcus aureus*, *S. epidermidis*, *S. albus*, β -haemolytic *Streptococci*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*; *Proteus mirabilis*; *Corynebacterium* spp.; *Peptococcus* group; *Propionibacterium acnes*; *Clostridium* spp., *Micrococcus* spp., *Salmonella choleraesuis*, *Diplococcus pneumoniae* and *Moraxella nonliquefaciens*. (Charnley, 1972; Wilson *et al*, 1973; Fitzgerald *et al*, 1977; Buchholz, 1981; Kamme and Lindberg, 1981; Cherney and Amstutz, 1983; Gristina and Kolkin, 1983; Sanderson, 1983; Canner *et al*, 1984; Inman *et al*, 1984; Poss, *et al* 1984; Learmonth, 1990; Beck-Sague *et al*, 1992; Padgett *et al*, 1995; Mulcahy *et al*, 1996; Tsukayama *et al*, 1996). While the majority of these organisms are common to the skin, species such as *E. coli* and *Ps. aeruginosa* are bowel flora thought to enter the wound via the THA incision of the perineum in the perianal region (Sanderson, 1983). The sheer number of potential pathogens pre-empt the need for wide spectrum antibiotics in the prevention of infection. Documented systemic pre- and peri- operative prophylactic antibiotics, usually administered immediately before operation and up to 48 hours afterwards, include oxycillin, streptomycin and more recently, cephalosporins (such as cephalothin, cefazolin and cephapirin), ciprofloxacin, gentamicin, vancomycin, ampicillin, tetracycline, erythromycin, flucloxacillin, methicillin, cefuroxime, rifampicin and amoxycillin as well combinations of several of these antibiotics (Poss *et al*, 1984; Pritchett and Bortel, 1991; Kelman *et al*, 1993; Meißner and Borner, 1993; Aagard *et al*, 1994; Ochsner *et al*, 1995; Raut *et al*, 1995). The use of either vancomycin or clindamycin has been recommended for patients who are either allergic to penicillin or hypersensitive to cephalosporins (Poss *et al*, 1984). Antibiotics such as bacitracin (25 units/ml) and polymyxin B (350 units/ml) dissolved in saline have also been used for frequent post-operative irrigation of THA wounds (Poss *et al*, 1984). Irrigation fluid also removes blood clots and debris as well as keeping soft tissues moist but the

establishment of regular flow pathways across the surface of tissues can direct antibiotic away from areas of infection (Sanderson, 1983; Learmonth, 1993). Secondary contamination of the wound, especially at entrance and exit ports, by bacteria carried in irrigation fluid and feeding tubes also casts doubt on this method as a treatment for infections and as an effective means of prophylaxis (Sanderson, 1983). A current popular method for topical administration of antibiotics to the hip involves the incorporation of water soluble, heat stable, non-hypersensitive antibiotics in the bone cement used to fix the implanted prosthetic components. This technique was originally developed by Buchholz and co-workers in 1972 for use in exchange arthroplasties after deep infections and utilised Palacos R acrylic cement loaded with gentamicin (Buchholz *et al*, 1981). Gentamicin impregnated cement is prepared at the time of surgery (as pre-manufactured antibiotic-cement mixtures had not, at least in 1993, received approval from the Food and Drug Administration (FDA) (Brien *et al*, 1993)) by adding antibiotic powder to the polymer, stirring for approximately five minutes and then adding the mixture to the monomer liquid. However, voids in the polymerised cement, created as a result of non-homogeneous mixing, caused a reduction in mechanical strength as did the addition of liquid antibiotics to the cement (Buchholz *et al*, 1981, Heck *et al*, 1995). With bacteria free wounds, 1g of gentamicin was added to 40g of cement as a prophylactic measure whereas a further 2-4 g of either lincomycin or ampicillin was added when either *S. aureas* and *Peptococcus spp.* or *Corynebacteria* respectively were responsible for the infection. (Buchholz *et al*, 1981). Gentamicin incorporation into bone cement is still popular (Learmonth, 1993; Heck *et al*, 1995), although tobramycin, (Brien *et al*, 1993; Heck *et al*, 1995; Masri *et al*, 1995), vancomycin (Brien *et al*, 1993; Heck *et al*, 1995; Raut *et al*, 1995) cephalosporins (Heck *et al*, 1995), erythromycin, flucloxacillin and cephadrine (Raut *et al*, 1995) have also been used. A further refinement of the PMMA-antibiotic mixtures, developed by Dr. Klaus Klemm, was to fashion bone cement into beads after gentamicin impregnation and string them onto steel surgical wire in order to deliver high local concentrations of antibiotic and to fill dead space (Nelson *et al*, 1993; Henry and Galloway, 1995). Beads have also been generated containing tobramycin, (Kelman *et al*, 1993; Henry and Galloway, 1995);

vancomycin, cephalosporins, erythromycin, lincomycin and colistin (Henry and Galloway, 1995). Local topical delivery of antibiotics to the hip has many advantages over systemic delivery and is preferred in the management of deep THA infections (Buchholz *et al*, 1984). Parenteral administration can lead to high, sometimes toxic, concentrations of antibiotic in the circulation and, because of poor vascularisation in the infected necrotic area, an antibiotic may not even reach the region where it is needed. However, topical delivery from antibiotic impregnated bone cement and PMMA bead chains provides local extra-circulatory concentrations of an antibiotic which are 5-10 times greater than achieved using systemic administration (Henry and Galloway, 1995). High concentrations of antibiotics are required for the eradication of organisms such as *Staphylococci* and *Pseudomonas* species due to the ability of these bacteria to form biofilms composed of exopolysaccharide glyocalyx polymers (Gristina and Kolkin, 1983; Anwar *et al*, 1992). This meshwork (or slime layer) forms a matrix within which the bacteria can grow, divide and adhere to the implanted prosthetic device. Biofilm bacteria are more able to withstand host defences and are more resistant to antibiotics than when in the planktonic form. Once established, sessile bacterial cells can only be killed by concentrations of antibiotic which would be lethal to patients. Long-term exposure of sessile bacteria to sub-inhibitory doses of antibiotic is thought to be the mechanism by which resistance is conferred. The immediate removal of potential biofilm bacteria while in their planktonic form using antibiotics is thus essential (Gristina and Kolkin, 1983; Anwar *et al*, 1992).

Improvements have recently been made to the technique of using antibiotic impregnated cement in THA surgery. Masri and colleagues have created three different preparations of tobramycin impregnated Simplex B cement each with a distinctive surface pattern consisting of either a smooth surface, four rows of hemispheric ridges, or 8 rows of hemispheric ridges (Masri *et al*, 1995). It was established that increasing the surface area of cement (by increasing the number of surface ridges) enhanced the rate of antibiotic elution. Large surface area preparations were also found to release a burst concentration of tobramycin which was thought to be useful for the treatment of biofilm bacteria and could

potentially improve the release of vancomycin from similarly prepared bone cement (Masri *et al*, 1995). Bunetel, and co-workers showed that the use of low-viscosity gentamicin loaded cement conferred greater mechanical strength to the prosthesis without affecting antibiotic release (Bunetel *et al*, 1990). Custom made, immediate-fit, temporary hip-joint prostheses and spacers have also been fashioned, using antibiotic impregnated bone cement, for implantation between stages one and two of the THA procedure. The immediate fit prosthesis was found to stabilise and increase the mobility of the joint and maintain limb length. The use of a spacer reduced the occurrence of fractures and preserved alignment of the joint (Kraay *et al*, 1992; Duncan and Beauchamp, 1993). Despite all its advantages, Buchholz, Elson and Heinert (1984) have stated that the mechanical properties of bone cement (in this case Palacos R) are altered by the addition of any amount of antibiotic powder such that the stability of a 40g preparation of cement containing 5g antibiotic was uncertain. Therefore, fixation using antibiotic loaded cement could eventually lead to loosening of the prosthesis and the subsequent requirement for revision procedures. To strengthen antibiotic impregnated bone cement, modifications to its constituents have been made. Otsuka and colleagues have incorporated cephalexin into a bioactive bone cement consisting of 15% bisphenol- α -glycidyl methacrylate, 15% triethylene-glycol dimethacrylate resin and 70% apatite- and wollstonite-containing glass ceramic (Otsuka *et al*, 1997). With a loading of 5% cephalexin, the compression strength of this modified bone cement was approximately twice that of PMMA bone cement (Otsuka *et al*, 1997). After prosthesis removal in a two-stage procedure, dense fibrous scar tissue can obliterate the joint space (Kraay *et al*, 1992) Implants such as poly (lactide-co-glycolide) discs have been shown to become covered in a collagen matrix when implanted in the pectoralis muscles of rats (Zislis *et al*, 1989). Therefore, removal of PMMA-antibiotic beads used during two-stage revision surgery and as a prophylactic measure in the joint space after initial prosthesis implantation would require extensive débridement during revision procedures. PMMA has also been shown to harbour and support bacterial growth such that its use in THA procedures should be limited (Zhang *et al*, 1993, Gristina

et al, 1983). An alternative to using preparations of antibiotic impregnated bone cement PMMA-antibiotic preparations, especially beads, is therefore clearly needed.

Zhang, Mattheus and Goosen (1993) have discussed the possible use of biodegradable polymers, such as polyesters, for use in topical antibiotic delivery to joints. These polymers are generally biocompatible, non-antigenic, non-carcinogenic, non-toxic, non-teratogenic and non-mutagenic and, once implanted, are broken down naturally by the body to metabolites of low toxicity obviating the need for surgical retrieval procedures (Zhang *et al*, 1993, Vainionpää *et al*, 1989). Hydrophobic polymers such as polyesters can be utilised to prevent the rapid release of water-soluble antibiotics. Their use for the delivery of antibiotics in the present work was designed to meet the criteria for the treatment of post-operative THA infections which involves the continuous release of antibiotic for at least 30 days (Zhang *et al*, 1993). Candidate polymers used to construct such a delivery device include the synthetic polyesters (including poly (lactide), poly (glycolide) and their copolymer poly (lactide-co-glycolide), poly (ϵ -caprolactone), poly (orthoesters), poly (phosphazenes) and poly (amino acids) together with natural biodegradable polymers such as collagen and chitosan (Zhang *et al*, 1993). Polyesters are homogeneously biodegraded by simple hydrolytic cleavage. In the case of the copolymer poly (lactide-co-glycolide) the end products of biodegradation are lactic and glycolic acids, both of which can be metabolised via the Tri-Carboxylic Acid cycle during respiration (Setterstrom *et al*, 1984; Dunn *et al*, 1988; Jalil and Nixon, 1990d; Whateley, 1993). In addition, polyesters such as poly (lactide) and poly (glycolide) have been chemically biodegraded *in vitro* by enzymes such as ficin, carboxypeptidase A, alpha-chymotrypsin, clostridiopeptidase A, esterase, leucine aminopeptidase and bromelain. Also lipids such as butyric acid, caproic acid, heptanoic acid and stearic acid as well as demineralised bone, hydroxyapatite and the adsorption of plasma proteins have all been shown to be capable of biodegrading polyesters (Zislis *et al*, 1989; Holland and Tighe, 1986; Makino *et al*, 1987).

Poly (glycolide) [PGA] has been used in the commercial production of biodegradable Dexon® sutures since 1970 (Reed and Gilding, 1981; Vainionpää *et al*, 1989) whereas a copolymer of poly (glycolide) and (lactide) (90%:10% respectively) has been used to fabricate Vicryl® sutures (Holland and Tighe, 1986). Poly (lactide-co-glycolide) [PLCG] has received approval from the Food and Drug Administration (FDA) for long-term parenteral use and is currently used commercially to manufacture the sub-cutaneous implant ‘Zoladex®’ for the controlled release of goserelin (a synthetic analogue of luteinizing hormone releasing hormone) as well as Prostag SR for the treatment of prostatic cancer (Whateley, 1993). Polyesters have also been employed to fabricate antibiotic delivery devices. Poly (lactide) [PLA] microspheres loaded with ciprofloxacin have been fabricated using a phase separation technique with the aim of treating peritoneal implanted biofilms of *Ps. aeruginosa* (Owuso-Ababio *et al*, 1995) whereas gentamicin loaded PLA microcapsules have been produced by coacervation for the treatment of osteomyelitis (Sampath *et al*, 1992). Cephalexin monohydrate, a first generation cephalosporin, has been encapsulated within PLA microspheres by coacervation with the aim of treating intraperitoneal infections (Owuso-Ababio and Rogers, 1996) while PLA microspheres loaded with mitomycin C (an anticancer drug) and fabricated using a W/O emulsification with solvent evaporation process have been produced to lower the systemic toxicity, while increasing the stability and biological half life, of mitomycin C (Tsai *et al*, 1986). Microcapsules composed of PLA and loaded with oxytetracycline as a model antibiotic have been fabricated using coacervation with the aim of reducing the need for patient compliance and strict dosing regimens (Vidmar *et al*, 1984). PLCG has also been exploited in the production of controlled release preparations. PLCG nanoparticles containing roxithromycin have been produced using an O/W emulsification with solvent evaporation process with the aim of eradicating *Toxoplasma gondii* which causes toxoplasmosis (Julienne *et al*, 1989). An emulsification with solvent evaporation procedure has also been employed to individually encapsulate ampicillin and gentamicin within PLCG microcapsules while coacervation has been used for the fabrication of polymixin B and chloramphenicol loaded PLCG microcapsules for the treatment of open

wounds on the battle field (Lewis *et al.*, 1980). Ciprofloxacin has recently been encapsulated within PLCG microspheres using an O/W emulsification with solvent evaporation process in order to decrease the large concentrations of unchanged drug excreted after an oral dose (Martinez *et al.*, 1997). Ampicillin has been incorporated into PLCG microcapsules produced by phase separation for the treatment of wound infections (Setterstrom *et al.*, 1984). In addition, an O/W emulsification with solvent evaporation technique has been used to encapsulate cisplatin within PLCG microspheres with the aim of providing fast and adjustable antibiotic release over a period of a few days to one month for the treatment of cancer (Spenehauer *et al.*, 1988). Cisplatin has also been incorporated into a delivery system in which *in-situ* solidification of PLCG brought about the encapsulation of the antibiotic after sub-cutaneous injection into rats (Dunn *et al.*, 1997). Ofloxacin (related to ciprofloxacin) loaded PLCG microspheres (fabricated using an O/W emulsification with solvent evaporation process) and gentamicin loaded PLCG and PLA films have been recently fabricated with the aim of developing controlled local delivery devices for the treatment of osteomyelitis (Owuso-Ababio *et al.*, 1997; Lee *et al.*, 1997). Similarly, both cefazolin and tobramycin microspheres composed of PLCG 50:50 and fabricated using solvent extraction have been developed with the prospect of treating open wound infections (Pak *et al.*, 1997; van Hamont *et al.*, 1997). PLCG has also been utilised to fabricate depot microspheres using either solvent evaporation, solvent extraction or a combination of both techniques and containing the antibacterial agent metronidazol in order to reduce both its systemic toxicity and dosing frequency (Vook *et al.*, 1997). Histatin peptide (found in saliva) has been encapsulated within PLCG microspheres, using a double emulsion technique, for the topical treatment of periodontitis (Jeyanthi *et al.*, 1997).

On a more general note, the antibiotic cefadroxil has been incorporated into microcapsules composed of hydroxypropylmethylcellulose (HPMC) phthalate and acetate succinate by a solvent evaporation process in order to reduce the rate of its elimination from the body (Uchida *et al.*, 1992). HPMC acetate succinate tablets have also been used for the controlled release of amoxicillin trihydrate, the most widely prescribed oral semi-synthetic

penicillin in the world (Hilton and Deasy, 1993). Further to this, amoxicillin, used for the treatment of ulcers caused by *Helicobacter pylori* infection of the stomach, has been encapsulated within carboxymethylcellulose (CMC) and Carbopol® microcapsules produced by coacervation and in spray-dried microspheres composed of muco-adhesive polymers including sodium alginate (Ertan *et al*, 1997; Ferrari *et al*, 1997). Nakhare and Vyas (1995) have documented the release of rifampicin (used in the treatment of tuberculosis) from a water-in oil-in water emulsion while Jain and Vyas (1995) have described a method of producing niosomes (uni- or multi- lamellar vesicles) composed of Sorbitan monopalmitate-85 and cholesterol, both of which are surfactants of polyglycerol ethers, and containing rifampicin. Ciprofloxacin has also been incorporated into liposomes for the treatment of both murine salmonellosis (caused by the intracellular pathogen *Salmonella typhimurium*) (Magallanes *et al*, 1993) and tularaemia (caused by *Francisella tularensis* passage from animals to humans via a tick vector) (Di Ninno *et al*, 1993) while aclarubicin (an anticancer antibiotic) has been entrapped within microspheres composed of poly- β -hydroxybutyric acid and fabricated using an O/W solvent evaporation process for parenteral administration (Juni *et al*, 1986). The antibiotic cefotiam has been encapsulated within hydroxyapatite beads for the treatment of local infections at the operating site after orthopaedic reconstructive surgery (Yamamara *et al*, 1992). Taste masking of bitter drugs, such as clarithromycin, by encapsulation within gelatin microspheres further coated with Eudragit® polymers (a collection of water soluble methacrylate based resins) has been achieved by using coacervation (Friend, 1992). Methacrylate polymers such as ethyl methacrylate have been used to fabricate slow-release discs containing chlorohexidine for use in dentistry to control periodontitis and the build up of plaque in the mouth (Swai *et al*, 1997). An ophthalmic insert christened Cronologic® and composed of a mixture of three polymers (Klucel®, Ethocel® and Carbopol®) containing both gentamicin and dexamethosone has been fabricated by extrusion to reduce the dosing frequency. These drugs are used to treat ocular infections (gentamicin) and reduce the concomitant inflammatory response (dexamethosone) (Baeyens *et al*, 1997). Gentamicin sulphate has been incorporated into a hydraulic calcium phosphate cement (composed of β -tricalcium

phosphate, monocalcium phosphate monohydrate and water) for the treatment of bone infections (Bohner *et al*, 1997). β -tricalcium phosphate mixed with the amino acids cystine or cysteine has been used to produce a ceramic cement containing erythromycin for the treatment of orthopaedic related infections (Willoughby *et al*, 1989).

Microparticulates suspended within injectable vehicles have been routinely administered to laboratory animals in order to monitor the *in vivo* release of drugs and the biodegradation of the delivery device (Mason *et al*, 1976; Beck *et al*, 1983; Visscher *et al*, 1988; Bodmer *et al*, 1992; Zhou *et al*, 1993; Whateley, 1993). Microparticulates under $200\mu\text{m}$ in diameter are suitable for subcutaneous or intramuscular administration. The volume of the injection vehicle for these preparations should be below 2ml to avoid discomfort to the patient and therefore microsphere loadings have to be high (a reason why the microspheres used in this practical work were loaded with 20% w/w antibiotic) (Linhardt, 1989). However, intravenous administration requires that the diameter of a delivery device such as microspheres is below $8\mu\text{m}$ so that capillary clogging is prevented (Linhardt, 1989). Poly (*dl*-lactic acid) and microcapsules containing the narcotic antagonist cyclazocine have been suspended in sesame seed oil at a concentration of drug to suspending medium of 25 mg/ml (Mason *et al*, 1976). This preparation was injected subcutaneously between the shoulder blades of male Holtzman rats using either 18 gauge or 16 gauge needles depending on microsphere diameter ($177\text{-}595\mu\text{m}$). Beck and co-workers suspended 40mg of PLCG microcapsules ($90\text{-}106\mu\text{m}$ in diameter) containing norethisterone (NET) in a 1 ml of 2% w/w CMC and 1% w/w Tween (polysorbate) 20 in sterile USP water (Beck *et al*, 1983). These preparations were injected into the femoral muscle of female Sprague-Dawley rats using 3ml disposable syringes fitted with 1 inch, 18 gauge needles in order to assess their effectiveness as a contraceptive measure. Similar preparations were also injected into the thigh muscle of baboons using a conventional syringe fitted with an 18 gauge needle (Beck *et al*, 1983). HPMC has also been utilised as an injection vehicle for the *in vivo* administration of PLCG 50:50 microcapsules containing an ergot alkaloid (Visscher *et al*, 1988). 10mg of these microcapsules (<45 and $177\mu\text{m}$ in diameter) were

suspended in 0.2ml of 0.5% w/v HPMC and injected into the gastocnemius muscle of male Charles River CD rats using 1.0cc syringes to determine the effect of particle size on the *in vivo* degradation rate (Visscher *et al*, 1988). PLCG-D-glucose microspheres with a mean diameter of 50 μ m and loaded with the somatostatin analogue octreotide have been suspended in a 1.8% w/v solution of CMC and subsequently administered intramuscularly into rabbits using 1mm diameter needles to assess the potential for increasing the half life of polypeptides by encapsulation within microspheres (Bodmer *et al*, 1992). Zhou and colleagues have employed CMC (1% w/v in a solution also containing 2% w/v Tween 80) for the injection of PLCG 90:10 microcapsules 65-100 μ m in diameter and containing NET into the thigh muscle of Sprague-Dawley rats using a 20 gauge syringe to determine the effectiveness of this preparation as a long-term contraceptive device (Zhou *et al*, 1993). The *in vivo* administration of "Zoladex" and Prostrap SR as suspensions in CMC using a 16 gauge needle and 23 gauge needle respectively has also been reported (Whateley, 1993). Different concentrations of solutions containing HPMC, CMC and hydroxypropylethylcellulose (of various apparent viscosities measured in centipoises (cps) for a 2% solution at 37°C) together with Carbomer 934 all dissolved in double distilled water were tested for their ability (related to their apparent viscosity) to suspend microspheres, their optical clarity, ease of gelling (without production of too many air bubbles) and the maintenance of structural integrity during *in vitro* incubation in both newborn calf serum (NCS) and Hank's balanced salt solution (HBSS) at 37°C. Out of all the preparations tested, 10% w/v HPMC (4000 cps), an ether of cellulose with methoxyl and 2-hydroxylpropyl groups, was chosen as the injection vehicle for the antibiotic loaded microspheres fabricated in the present work since it suspended microspheres for an approximate period of 3 days in both NCS and HBSS, was optically clear and remained intact during 30 days of *in vitro* incubation. The viscosity of 10% w/v HPMC (4,000cps) containing 20% w/w antibiotic loaded microspheres (0.1g/ml gel w/v) was such that *in vitro* administration into NCS and HBSS was possible using a disposable 5ml Gillette syringe barrel with a nozzle diameter of 2mm.

1.4. Candidate antibiotics for the management and prevention of orthopaedic infections of the hip.

Gentamicin is still widely used to treat infections encountered in orthopaedics by incorporation into PMMA bone cement topically administered to the hip joint even though nosocomially acquired bacteria are becoming more resistant to it (Schaberg *et al*, 1991). Whereas only 3% of *Ps. aeruginosa* isolates were gentamicin resistant in 1982, 12% of isolates had become resistant by 1989. Likewise, *Enterococcal* resistance to gentamicin is also on the increase as is the percentage of *S. aureus* isolates that are methicillin resistant (1% in 1982 to 17% in 1989) (Schaeberg *et al*, 1991). However, comparatively little attention has been paid to the possible use of antibiotics such as vancomycin, ciprofloxacin and rifampicin for the treatment of THA infections. Vancomycin was isolated from the fermentation broth of *Streptomyces orientalis* cultures in the early 1950's at Eli Lilly and Company and originally called Compound 05865. It was found to be very potent against all *Staphylococcal* species (including methicillin resistant *S. aureus* [MRSA]) and nicknamed 'Mississippi mud' because of its many impurities. However, better purification techniques using ion-exchange resins and ionic precipitation, which also eliminated many of the adverse reactions that sometimes occurred with the original vancomycin preparation, were later employed to create vancomycin hydrochloride (Griffith, 1984). Vancomycin HCl is an odourless glycopeptide, tan/brown in colour and free-flowing with a bitter taste (Remington's, 1990). Proprietary names include Vancocin HCl, Vancoled and Vancocina (Martindale, 1993). It is freely soluble in water (0.10 g will dissolve completely in 1ml of water and a 5% w/v solution produces a pH of 2.8-4.5) but is only slightly soluble in ethanol (96%). The antibacterial potency of vancomycin is not less than 900 Units per mg (British Pharmacopaeia, 1993). It is highly active against Gram +ve cocci as well as *Neisseria* and *Clostridia* species where it inhibits the synthesis of peptidoglycan during cell-wall formation. However, Gram -ve organisms, mycobacteria and fungi are not susceptible (Daum *et al*, 1992; Remington's, 1993). Development of bacterial resistance to

vancomycin is presently rare (it has occurred, in the case of *Enterococcus faecalis* for example (Daum *et al*, 1992)) but vancomycin still remains an ideal treatment for methicillin resistant *S. aureus* and *S. epidermidis* infections. Normally, 7.5 mg/kg or 500 mg for adults and 11 mg/kg for children is slowly infused intravenously every 6 hours (Remington's, 1993). The elimination half life for vancomycin is, on average, approximately 6 hours with 80-90% of a dose being excreted unchanged in the urine within 24 hours. However, increases in alpha₁-acid glycoprotein levels as a result of MRSA infection can lower the available serum concentration of vancomycin due an increase in protein binding, which can vary in healthy patients from <10% to 64% (Morita and Yamaji, 1995). Oral administration is rarely used since vancomycin is absorbed poorly from the gastro-intestinal tract (Remington's, 1993). Side effects of vancomycin therapy include thrombophlebitis at the site of injection, chills, fever, nephrotoxicity, ototoxicity and Red man's syndrome (involving rashes and hypotension) (Martindale, 1993; Remington's, 1993). The minimum inhibitory concentration (MIC) of vancomycin for *Staphylococci* is in the region of 0.1-2.0 µg/ml with bactericidal concentrations being close to this range. Organisms with intermediate-sensitivity to vancomycin, such as some *Clostridia* species, have a vancomycin MIC of between 4 and 16 µg/ml (Martindale, 1993). Vancomycin has been successfully used, in combination with rifampicin and fleroxacin, to successfully treat chronic foreign body infection due to MRSA (Chuard *et al*, 1991). Vancomycin in combination with rifampicin has also been used in the treatment of experimental aortic-valve endocarditis caused by MRSA (Bayer and Lam, 1985). In addition to this, vancomycin has shown impressive anti-*S. aureus* activity in an experimental model of wound infection (Kernodle and Kaiser, 1994) as well as in experimental chronic osteomyelitis after joint administration with rifampicin (Norden and Shaffer, 1983). *In vitro* antagonism between vancomycin and rifampicin has been experienced using time-kill curve measurements, while other studies using the "checkerboard" technique show indifference or synergy between the actions of vancomycin and rifampicin (Watanakunakorn and Guerriero, 1981; Norden and Shaffer, 1983; Bayer and Lam, 1985; Van der Auwara and Joly, 1987). A decrease in bacterial

resistance to rifampicin when combined with vancomycin *in vivo* has been documented (Norden and Shaffer, 1983; Bayer and Lam, 1985; Van der Auwara and Joly, 1987; Chuard *et al.*, 1991). This phenomenon was thought to be the result of vancomycin eliminating strains resistant to rifampicin while rifampicin was responsible for the major part of killing (Norden and Shaffer, 1983; Van der Auwara and Joly, 1987; Chuard *et al.*, 1991). To widen the spectrum of antibacterial activity, it has been suggested that vancomycin should be administered with other antibiotics, especially those effective against Gram -ve bacteria such as ciprofloxacin, in the treatment of joint infections (Kernodle and Kaiser, 1994).

Ciprofloxacin, with proprietary names including Baycip, Ciproxin and Cetraxal (Martindale, 1993), has received FDA approval for the treatment of post-operative wound infections in adults, including those of bone. Its wide spectrum of activity covers Gram -ve organisms such as *E. cloacae* and *Ps. aeruginosa* as well as Gram +ve bacteria such as *Staphylococci* including some methicillin resistant (MRSA) strains (Ramirez *et al.*, 1985; Limon, 1989; Martindale, 1993; Remington's, 1993). Ciprofloxacin is a flouro-4-quinolone originally developed to replace the less active quinolones such as nalidixic acid and cinoxacin which had a narrow spectrum of activity and showed a rapid build up of resistance (Limon, 1989). Ciprofloxacin is yellowish in colour and slightly soluble in both water (where a 2.5% w/v solution has a pH of 3.0-4.5) and in methanol (Martindale, 1993). Its bactericidal mode of action involves inhibition of DNA gyrase and hence DNA replication and transcription although lower concentrations tend to be bacteriostatic (Remington's, 1993). Sensitive organisms, with *in vitro* MIC₉₀ concentrations of ≤ 1 $\mu\text{g/ml}$, include *E. coli*, *Enterobacter*, *Klebsiella* and *Proteus*, *S. aureus* (including MRSA) and *S. epidermidis*, whereas the MIC₉₀ for *Ps. aeruginosa* is in the region of 2.0 $\mu\text{g/ml}$. *Neisseria* species are also very susceptible to ciprofloxacin while fungi tend to be resistant (Limon, 1989; Martindale, 1993). Ciprofloxacin has also been shown to inhibit the *in vitro* growth of bacteria resistant to gentamicin (Chin and Neu, 1984). Relatively rapid resistance to ciprofloxacin by some *Staphylococcal* species (including MRSA),

Pseudomonal species and *E. coli* strains has occurred. Some cross-resistance to other unrelated antibacterials, as well as to other fluoroquinolones has also been documented (Eliopoulos *et al*, 1984; Martindale, 1993; Kernodle and Kaiser, 1994). Ciprofloxacin can be administered orally (oral bioavailability is 70%) and intravenously, and has a plasma half-life of 3.5-4.5 hours. Roughly 40-50% of an oral dose and up to 70% of a parenteral dose is excreted unchanged in urine within 24 hours with 15% and 10% of an oral and a parenteral dose respectively are excreted as metabolites of ciprofloxacin (principally oxociprofloxacin). Faecal excretion of ciprofloxacin and its metabolites (such as sulphociprofloxacin) over five days accounts for 20-35% of an oral dose and 15% of an intravenous dose (Martindale, 1993; Remington's, 1993). The oral dose of ciprofloxacin given to adults presenting bone infections is 500 mg, in tablet form, every 12 hours. The usual intravenous dose is between 100mg and 400 mg twice daily (Martindale, 1993; Remington's 1993). Common adverse effects of ciprofloxacin therapy include nausea, vomiting, abdominal pain and dyspepsia along with dizziness, headaches and restlessness (Martindale, 1993). Ciprofloxacin has been successfully used to treat experimental osteomyelitis in a rabbit model by eradicating *Ps. aeruginosa* infections when administered over a period of one month. The successful outcome was due to good bone penetration and high anti-*pseudomonal* activity, although some antibiotic resistance was encountered (Norden and Shinnors, 1985). Oral ciprofloxacin therapy is clinically effective in the treatment of osteomyelitis when accompanied by débridement although, again, some resistance particularly from *S. aureus* and *Ps. aeruginosa* has been experienced (Greenberg *et al*, 1987). In combination with the antibiotic ceftazidime, ciprofloxacin has eradicated infections associated with prostheses and caused by *Pseudomonas aeruginosa* without the need for prosthesis removal (Brouqui *et al*, 1995). Ciprofloxacin has also been used in combination with fosfomycin for the more successful treatment of experimental endocarditis caused by *Ps. aeruginosa* in a rabbit model (Xiong *et al*, 1995). The actions of ciprofloxacin and rifampicin have been shown to be antagonistic (time-kill curve method) in the *in vitro* treatment of experimental endocarditis caused by methicillin susceptible *S. aureus* (Kaatz *et al*, 1989). However, in rabbits, the addition of rifampicin

significantly reduced bacterial resistance to ciprofloxacin by killing or inhibiting resistant sub-populations. This combination therapy was judged to be as effective as vancomycin for the treatment of *Staphylococcal* induced endocarditis in a rabbit model (Kaatz *et al*, 1989).

Rifampicin, with proprietary names of Rifampin, Rifadin and Rimactane, is a red/brown crystalline powder belonging to the rifamycin group of antibiotics (all isolated from strains of *Streptomyces mediterranei*) and commonly used to treat meningitis, tuberculosis and leprosy. It is slightly soluble in water (where a 1% w/v suspension gives a pH of 4.5-6.5) and ethanol (96%) and freely soluble in organic solvents such as chloroform and methanol (Martindale, 1993; British Pharmacopoeia, 1993). Its mode of action is bactericidal and involves the inhibition of DNA-dependent RNA polymerase thereby interrupting nucleic acid synthesis (Martindale, 1993). Intracellular and semi-dormant/persisting organisms can be eliminated in this way as can mycobacteria such as *Mycobacterium tuberculosis* and *M. leprae* (MIC, 0.1-0.2 $\mu\text{g/ml}$). Gram +ve organisms such as *Staphylococci* are very susceptible (MIC, 0.01-0.02 $\mu\text{g/ml}$) but rifampicin possesses only intermediate activity against Gram -ve organisms such as *N. gonorrhoeae*. At high concentrations some activity against viruses has been seen and, although not directly effective against fungi, rifampicin can potentiate the activity of the anti-fungal compound amphotericin (Martindale, 1993). For adults, intravenous administration involves the infusion of a total dose of 600 mg of rifampicin over a 3 hour period whereas children receive 11.5 mg/kg body weight infused over 30 minutes (Martindale, 1993). Oral doses are provided in the form of either 600 mg rifampicin capsules or oral suspensions (British Pharmacopoeia, 1993). The half life of rifampicin is approximately 2-5 hours but in the early stages of treatment may decrease to 2-3 hours as rifampicin can induce its own enzymatic metabolism. Rifampicin, unlike its metabolites including formyrifampicin, is subject to intra-hepatic circulation such that roughly 60% of a dose eventually appears in the faeces. Rifampicin and its metabolites are also excreted in urine (Martindale, 1993). Adverse effects of rifampicin usage include gastrointestinal bleeding, gastritis, renal failure, liver abnormalities and contact dermatitis.

Initial worries that rifampicin could have a detrimental effect on bone calcium metabolism due to its ability to lower serum concentrations of hydroxycholecalciferol appear to be unfounded (Martindale, 1993). Rifampicin is usually administered with other antimicrobials since susceptible organisms can rapidly develop *in vivo* and *in vitro* resistance to rifampicin (Martindale, 1993). Examples of such combinations include rifampicin with vancomycin, methicillin, cephalothin, cefotiam, fusidic acid, erythromycin, minocycline, clindamycin, trimethoprim and gentamicin to treat isolates of MRSA and methicillin susceptible *S. aureus* (MSSA) (Zinner *et al*, 1981). Rifampicin has also been combined with the glycopeptide antibiotic teicoplanin for the treatment of experimental foreign body infection by MRSA during which antagonism *in vitro* turned to synergy *in vivo* (Schaad *et al*, 1994). Urinary infections involving *Ps. aeruginosa*, *E. coli*, *P. miribalis* and *K. aerogenes* were also treated by combining rifampicin with the quinolone nalidixic acid. Both antibiotics worked in harmony to eradicate resistant bacterial strains leading to the conclusion that combining rifampicin and nalidixic acid considerably widened the spectrum of antibacterial activity (Greenwood and Andrew, 1978). Chuard and associates state that there is no existing regimen combining vancomycin, a quinolone and rifampicin for the treatment of infections caused by *S. aureus*. Further to this, they extol the usefulness of such a combination for the treatment of infections associated with prosthetic devices where conventional therapies tend to be ineffective (Chuard *et al*, 1991).

1.5. Current techniques for the microencapsulation of medicaments within controlled release devices.

Microencapsulation has been defined as the entrapment of solids, droplets of liquids and dispersions within thin polymeric coatings to produce particles with an approximate size range of between $5\mu\text{m}$ and 2mm (Watts *et al*, 1990; Donbrow, 1992; Whateley, 1993). Microencapsulation can be employed to fabricate microcapsules and microspheres. Microcapsules contain a reservoir of encapsulated agent within a shell of polymer whereas microspheres are monolithic matrices throughout which the entrapped agent is homogeneously dispersed either molecularly or particularly (Whateley, 1993). The most popular method for producing drug loaded microparticulate systems is emulsification with solvent evaporation (Whateley, 1993). This process involves the mixing of two immiscible liquids at a high shear speed. One of these liquids, or phases, usually consists of a small volume of volatile solvent containing dissolved polymer and solubilised or particulate drug. This is added to a larger volume of liquid containing a stabiliser or emulsifier to maintain and stabilise the interface between the two phases. The polymer phase will form droplets in the larger phase thereby entrapping the drug and, by allowing the solvent to evaporate, microparticles are subsequently precipitated. The oil in water (O/W) emulsification with solvent evaporation technique incorporates an oil phase usually composed of a polymer and drug in dichloromethane (DCM) and a water phase composed of distilled water containing the stabiliser poly (vinyl alcohol) [PVA] (Donbrow, 1992; Whateley, 1993). The O/W technique produces microspheres with hydrophilic surface properties and diameters in the range of $\leq 1\mu\text{m}$ - $100\mu\text{m}$. However, although this is a good method for encapsulating lipophilic drugs, water soluble drugs tend to partition into the aqueous phase where they can crystallise thereby drastically reducing the concentration of these drugs encapsulated within the final microspheres. This problem has been partially solved by pre-saturating the aqueous phase with drug (although only when the drug is sparingly soluble in the aqueous phase); buffering the aqueous phase to lower drug solubility in this phase; using a concentrated inorganic salt solution (such as 37% w/w

ammonium sulphate) as the water phase coupled with the employment of acetone (water soluble) as the organic solvent; changing the pH of the aqueous phase; and the addition of Pluronic L101 to the organic solvent to increase drug solubility in the oil phase (Donbrow, 1992; Whateley, 1993). However, the common use of DCM and other halogenated solvents in the O/W fabrication process has caused concern because of their carcinogenic properties (Whateley, 1993). The O/W technique has been superseded by the W/O technique for the encapsulation of water soluble drugs within microspheres (Tsai *et al*, 1986; Jalil and Nixon, 1990d). The W/O method of emulsification with solvent evaporation utilises a polymer such as PLA or PLGA which is dissolved in acetonitrile (also containing the drug to be encapsulated) to create the water phase. The oil phase typically consists of light white mineral oil containing an emulsifier such as sorbitan monopalmitate (SPAN) 40. Pre-heating the phases to 55°C with subsequent emulsification and solvent evaporation at this temperature is followed by a period of microsphere hardening at 35°C. This temperature is used to keep SPAN 40 in solution during the hardening process. Subsequently, microspheres are harvested using vacuum filtration or centrifugation (Tsai *et al*, 1986; Jalil and Nixon, 1990d). Microspheres smaller than approximately 50µm in diameter tend to be difficult to produce using the W/O protocol (presumably due to the relatively high viscosity of mineral oil compared with distilled water used in the O/W protocol) and can aggregate in aqueous vehicles because of the hydrophobic nature of the polymers used in their fabrication. However, drug encapsulation efficiencies for microspheres generated by the W/O technique tend to be high (Whateley, 1993). A more complicated method of encapsulating highly water soluble drugs, such as proteins and polypeptides, is the W/O/W double emulsion with solvent evaporation technique (Whateley, 1993). The drug is dispersed in a small volume of DCM containing the polymer and mixed to form a W/O emulsion. This is dispersed into an aqueous phase of larger volume containing PVA (W/O/W) and the DCM allowed to evaporate to produce microcapsules. Gelatin in distilled water can also be used as the inner water phase (Jalil and Nixon, 1990d). However, residual gelatin within the final microcapsules may cause immunological reactions. Jeffery and associates have

circumvented this problem by using distilled water only as the inner water phase (Jeffery *et al.*, 1993). However, all these methods can result in residual water encapsulation which decreases microcapsule stability and increases the rate of polymer biodegradation when implanted *in vivo* (Whateley, 1993). Microspheres can also be formed using a triple emulsion process whereby a W/O emulsion of aqueous drug solution in soya bean oil is dispersed in a solution of acetonitrile and polymer (W/O/O) which is subsequently dispersed into paraffin oil (W/O/O/O) and the acetonitrile allowed to evaporate. Although large microspheres ($\geq 200 \mu\text{m}$ in diameter) have been produced by this method it is useful for the encapsulation of proteins since it avoids any contact between organic solvent and the proteins which are hence spared possible denaturation (Whateley, 1993).

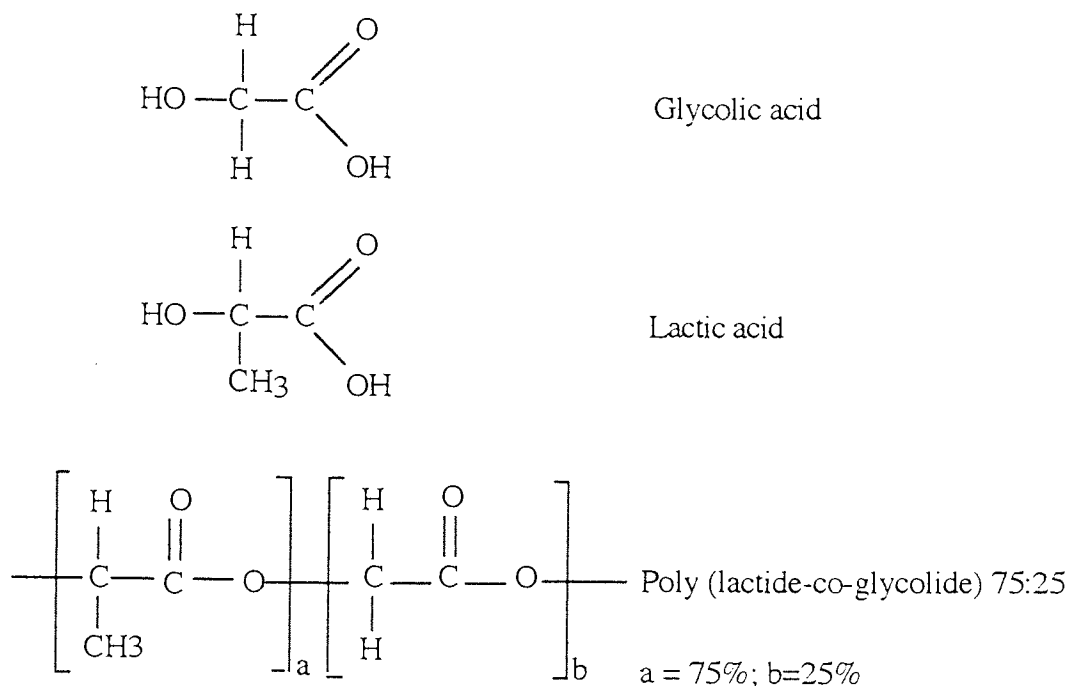
Other methods of medicament encapsulation include pan coating, spray coating and spray drying (Jalil and Nixon, 1990d; Donbrow, 1992; Whateley, 1993; Pavanetto, 1993). Pan coating involves rotating a mixture of solid drug-containing cores and molten or dissolved wall material within coating pans (Donbrow, 1992). The cores can be produced by granulation or spheronization of solid or liquid drugs. Spray coating is facilitated by suspending either solid core particles or frozen liquids in a rising air stream. These particles are then sprayed with coating material dissolved in a volatile organic solvent such as chloroform. Chloramphenicol has been coated with PLA in this way (Jalil and Nixon, 1990d; Donbrow, 1992). Spray drying involves spraying either a suspension or solution of lyophilised drug together with dissolved polymer in organic solvent through an atomiser nozzle into an evaporation or cooling chamber containing a layer of liquid nitrogen covering a layer of ethanol. As the nitrogen evaporates, the organic solvent is taken up by the ethanol leaving solid polymer/drug structures typically in the form of membrane coated or matrix-embedded microparticles. Vitamin D₃ and proteins have been encapsulated within PLA dissolved in a number of chlorinated solvents using this method even though drug properties such as polymorphism can be altered by the extreme temperatures and the particular organic solvent employed in the procedure (Jalil and Nixon, 1990d; Whateley, 1993, Pavanetto, 1993, Donbrow, 1992). Coacervation is another popular technique for

encapsulating therapeutic agents and involves the addition of a non-solvent (for the polymer) to a pre-stirred emulsion of an organic solvent (containing polymer and drug) and an aqueous solution. The non-solvent induces phase separation by taking up the organic solvent thus precipitating the emulsion droplets, containing encapsulated drug, either as microspheres or microcapsules depending on the precise technique used (Jalil and Nixon, 1990d; Donbrow, 1992; Arica *et al*, 1993; Nihant *et al*, 1993; Whateley, 1993). In this regard, diclofenac sodium has been encapsulated within microcapsules composed of the polymer Eudragit® using chloroform as the organic solvent and deionised water containing TWEEN 80 and 0.1N hydrochloric acid as the non-solvent (Arica *et al*, 1993). Interfacial deposition has been employed to produce unloaded microspheres composed of PLA. The technique involved drop-wise addition of DCM containing dissolved PLA into an O/W emulsion of an aqueous solution containing n-haptane and the emulsifier Pluronic P68. Stirring under vacuum facilitated solvent evaporation and polymer precipitation at the surface of the n-haptane droplets. Subsequent evaporation of n-haptane created small (1-5 μ m diameter) microspheres. However, the use of an aqueous phase rules out this method for the microencapsulation of water soluble drugs (Jalil and Nixon, 1990d). The technique of droplet extrusion has been utilised to create cellulose-based gel capsules by ejection of core drug (liquid, molten or in solution) together with cellulose gel, from a syringe nozzle as microdroplets, into an acid or salt bath. Passage of cellulose through this medium caused it to harden, thus entrapping drug within solid particles. Cells have also been encapsulated in this way (Donbrow, 1992). Industrial use of this technique produces particles ranging from 400-7,000 μ m in diameter (Donbrow, 1992). *In situ* polymerisation provides a means of producing microspheres by heating a polymer such as PLA in dried and degreased silicone oil until it melts. Addition of a drug which melts at or below this temperature (such as estrone) causes the drug to dissolve in the polymer. Subsequent to agitation, catalyst induced polymerisation of the PLA produces matrix like microspheres (100-1,000 μ m in diameter). However, the presence of a drug can make the polymerisation procedure unpredictable (Jalil and Nixon, 1990d). Freeze-drying has also been employed to fabricate microspheres composed of PGA and containing calcitonin. A

mixture of polymer and hormone in hexafluoroacetone was dispersed in carbon tetrachloride and the suspension freeze-dried and washed with carbon tetrachloride. However, although sub- $5\mu\text{m}$ particles were produced, calcitonin loading was less than 7.5% w/w (Whateley, 1993).

In the present work, the employment of a suitable microencapsulation technique for the fabrication of vancomycin, ciprofloxacin and rifampicin loaded microspheres was based upon three criteria: a) suitability of the method for the encapsulation of water soluble drugs; b) ability of the method to produce high encapsulation efficiencies; and c) ease of fabrication. Evaluation of the fabrication methods described above confirmed that the W/O emulsification with solvent evaporation technique fitted the required criteria and, with modifications to the protocols proposed by both Tsai *et al* (1986) and Jalil and Nixon (1989) was used for all subsequent experimental work. The polyester *dl*-PLCG 75:25 was employed in the production of these microspheres because of its excellent *in vivo* biocompatibility (Cutright *et al*, 1974), relatively short *in vivo* half life of 0.6 months (Miller *et al*, 1977) and regulatory approval for use in surgical procedures involving humans (Whateley, 1993). The PLCG 75:25 copolymer, composed of a ratio of 75% lactide units to 25% glycolide units, has the structure shown in Figure 1.2 below:

Figure 1.2 Chemical Structures of glycolic and lactic acid monomers and their copolymer poly (lactide-co-glycolide) 75:25. (Whateley, 1993; Shen *et al*, 1993)



PLCG can be synthesised by polycondensation and, more popularly, by ring opening polymerisation of cyclic dimers which occurs at temperatures in the region of 130-180°C over a time span of 5 to 18 hours (Wood, 1980; Setterstrom *et al*, 1984; Cha and Pitt, 1990; Dahlmann *et al*, 1990; Hutchinson and Furr, 1990; Nihant *et al*, 1993; Shen *et al*, 1993; Zhang *et al*, 1993). Efficient ring opening polymerisation requires the employment of catalysts in the melt such as stannous octoate (which has FDA approval), stannous chloride and organo-tin compounds such as tetraphenyl tin (0.02 % wt) together with chain initiators such as lauryl alcohol which control copolymer molecular weight (Schindler *et al*, 1977; Setterstrom *et al*, 1984; Cha and Pitt, 1990; Dahlmann *et al*, 1990; Hutchinson and Furr, 1990; Beck *et al*, 1993; Shen *et al*, 1993; Zhang *et al*, 1993). A reaction scheme for the production of PLCG has been documented by Shen *et al* (1993) and is shown in Figure 1.3 below:

pure poly (lactide) and pure poly (glycolide) but biased towards the degradation characteristics of PLA due to the higher content of PLA within a given quantity of PLCG 75:25 copolymer. The ratio of lactide to glycolide units will also affect the glass transition temperature (T_g) of the copolymer. The T_g of a polymer is the temperature at which it turns from a glassy state into a rubbery state and is a function of chain mobility. Therefore, the more crystalline a polymer, or the higher its molecular weight (indicating longer chain lengths) then the higher the T_g since more energy is required to mobilise and unpack the polymer chains. PGA has a lower T_g (36°C) than PLA (57°C) due to its simpler structure (Fredericks *et al*, 1984). The effect of PLCG copolymer composition on T_g has been well documented in the literature. PLCG 10:90 has a T_g of 37°C (Fredericks *et al*, 1984) while *dl*-PLCG 50:50 has a T_g of 50°C (Menei *et al*, 1993). Polymer molecular weight is defined according to the weight average (M_w) that is the average weight of all the molecules, or number average (M_n) which relates to the number of molecules within different weight classes. The ratio between the two (M_w/M_n) is the polymer polydispersity (pd) which gives an indication of the spread of molecular weights within the polymer (Wood, 1980). Not only are higher molecular weight polymers less soluble in aqueous media, they also take longer to biodegrade due to the extended chain lengths within their structure. Eventually, hydrolysis of ester linkages will create oligomers soluble within the aqueous continuum leading to mass loss from the polymer.

1.6. Aims

In order to develop a therapeutically useful antibiotic delivery device for the treatment of orthopaedic infections many different avenues of research need to be explored. Validation of the fabrication procedure in terms of the reproducibility of microsphere physico-chemical characteristics is important not only to ensure accurate results for *in vitro* dissolution studies but also for the continuous production of the delivery devices on a commercial scale to a recognised pharmaceutical standard and quality. Therefore, examination of such parameters as microsphere percentage yield, encapsulation efficiency

together with size distribution and diameter (to assess the injectability of microspheres through syringes), morphology (microsphere surface topography and internal architecture to assess structural integrity), residual microencapsulated solvent concentrations (which could be toxic *in vivo*), changes in polymer molecular weight as a result of PLCG incorporation into microspheres, and changes in polymer glass transition temperature (as a result of loading microspheres with antibiotics) will be determined. The *in vitro* release of antibiotics from the delivery device will also be monitored in physiological media. Release studies, when used as a quality control tool, are routinely conducted by the incubation of microspheres in simple solutions such as phosphate buffered saline or other salt solutions isotonic with serum. In keeping with this practice, microspheres will be incubated in Hank's balanced salt solution (HBSS, pH 7.0-7.4) which contains physiological concentrations of buffer salts. Incubation of 20% w/w antibiotic loaded microspheres in non heat treated newborn calf serum (NCS, pH 7.0-7.2) will also be carried out to provide information on the potential effects of serum enzymes and serum protein adsorption on both the release of antibiotics and microsphere biodegradation and to generate data to aid in future *in vitro/in vivo* (IVIV) correlations. Any changes in the kinetics of antibiotic release from loaded microspheres into NCS and HBSS after entrapment within an aqueous preparation of 10% w/v HPMC and subsequent incubation in NCS and HBSS at 37°C will also be monitored. Data from the release profiles will be fitted to both first order and Higuchi kinetic models of encapsulated material release over time in order to elucidate the mechanism by which antibiotics are released into NCS and HBSS from 20% w/w loaded PLCG 75:25 microspheres. Since the *in vitro* release of antibiotics will be monitored for a period of up to 30 days (the minimum period of time generally required to suppress post-operative hip infections in the surgical environment) the *in vitro* biodegradation of microspheres incubated in NCS and HBSS will also be monitored over 30 days. Microsphere morphology will be monitored at regular intervals using scanning electron microscopy to assess gross changes in both their surface topography and internal architecture (after embedding microspheres in gelatin and sectioning using a microtome). Percentage mass loss occurring during biodegradation as a result of solubilisation of the

polymer backbone will also be assessed using gravimetry. Changes in molecular mass will be determined using gel permeation chromatography to give an indication of the point at which the polymer chains become short enough, as a result of hydrolytic scission, to solubilise. Determination of the changes in microsphere glass transition temperature with incubation time using thermal analysis (differential scanning calorimetry) will also provide information on the changing state of polymer crystallinity and chain length. Finally, it will be important to assess the cytotoxicity of both unloaded and antibiotic loaded microspheres so that biomaterial/tissue interactions can be quantified and potential risks of implanting the devices into humans can be evaluated. This will be achieved by exposing microspheres to cultured 3T3-L1 cells in order to generate both ID_{50} and Cell Proliferation Inhibition index values for each microsphere preparation as described in INVITTOX protocol 3b and protocol 104 produced by The Fund for the Replacement of Animals in Medical Experiments (FRAME). The overall suitability of 20% w/w antibiotic loaded PLCG 75:25 microspheres fabricated for the treatment of hip replacement infections in terms of their ease and reproducibility of fabrication, encapsulation efficiency, release of antibiotics, biodegradation, and toxicity will then be assessed. Further to this, the usefulness of these controlled delivery devices to treat infections arising after THA procedures will be objectively evaluated and future goals of research in this area highlighted.

Chapter 2: Materials and Methods.

2.1. Materials.

Poly (*d,l*-lactide-co-glycolide) [PLCG] 75:25, Resomer 752, MW>39kD, inherent viscosity 0.22 dl/g, medical grade, was manufactured by Boehringer Ingelheim and purchased through ALFA Chemicals, Bracknell, UK. Vancomycin hydrochloride (approximately 1,000 µg/ml by bioassay), rifampicin (crystalline, approximately 95% by HPLC) sorbitan monopalmitate (SPAN 40), anhydrous sodium carbonate and Duraseal™ laboratory stretch film, manufactured by Diversified Biotech, Boston, Massachusetts, were all obtained from Sigma Chemical Co., Poole, UK. Ciprofloxacin (potency 848 mcg/mg) was obtained through Bayer, Leverkusen, Germany. Acetonitrile (HPLC grade), dichloromethane (DCM, HPLC grade), petroleum ether (40-60°C fraction, analytical reagent grade) ethanol (95% v/v, analytical reagent grade) and glacial acetic acid were all purchased from Fisher Scientific UK, Loughborough, Leicestershire, England. Hydroxypropylmethylcellulose (HPMC, 4,000cps) and light white mineral oil (density 0.838) were obtained from Aldrich Chemical Co., Gillingham, UK. Hanks' balanced salt solution (HBSS 1x sterile liquid, pH 7.0-7.4); non-heat treated newborn calf serum (NCS, origin New Zealand, batches 30Q8355D, 30Q2626D, 30Q1653D and 30G2343D, mycoplasma and virus screened, pH 7.0-7.2); phosphate buffered saline (PBS 1x liquid pH 7.4 ± 0.05); Dulbecco's Modified Eagles Medium (DMEM, 1x liquid with Glutamax II™ 4,500 mg/l D-glucose without sodium pyruvate); Iscove's Modification of Dulbecco's Medium (IMDM) powder; Trypsin-EDTA (1x liquid containing 2.5 g/l trypsin (1:250) in Gibco solution A); antibiotic/antimycotic solution (100x, containing 10,000 units penicillin (base), 10,000µg streptomycin (base), 25µg amphotericin B/ml and utilising penicillin G (sodium salt), streptomycin sulphate and amphotericin B as Fungizone® in 0.85% saline, diluted to 1x with PBS); cell culture freezing medium (containing DMSO and prepared using DMEM supplemented with FBS/calf serum); and Fungizone® (250µg amphotericin

B/ml water) were all supplied by GIBCO, Life Technologies Ltd., Paisley, Scotland. Nutrient broth, nutrient agar and diagnostic sensitivity test (DST) agar were obtained from Oxoid through Unipath Ltd., Basingstoke, Hampshire, England. Methocel high viscosity methylcellulose (MC) powder 3,000-5,000 mPascal was obtained from Fluka Biochemika, Buchs, Switzerland. Tissue-culture equipment such as flasks, tubes, pipettes and 96 well plates, manufactured by Corning Laboratory Sciences, together with Coomassie brilliant blue R250 and sodium hydroxide powder were purchased from Merck Ltd, Lutterworth, Leicestershire, England. Potassium acetate was supplied by FLUKA Chemicals, Gillingham, Dorset, England. Syringes and 0.2 μ m filters for tissue culture, filter papers (Whatman 50 hardened, 7cm diameter) and solvent resistant polypropylene backed PTFE filters (diameter 47mm, pore size 1 μ m) were obtained from Whatman International Ltd., Maidstone, Kent, England. Sterile screw-capped polypropylene tubes (15ml volume, conical base) were supplied by Sarstedt Ltd., Leicester, England.

2.2. Methods.

2.2.1. Fabrication of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres.

A water in oil (W/O) emulsification with solvent evaporation technique (Tsai *et al*, 1986; Jalil and Nixon, 1989) was employed to fabricate unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres. An oil phase was created by heating a solution of 125g of light white mineral oil containing either 2.5g (2% w/w) or 3.75g (3% w/w) SPAN 40 to 55°C within a squat-form 250ml beaker (Fisher Scientific UK) placed in a water bath. A water phase was prepared in a glass beaker by dissolving 1g of PLCG 75:25 in 30ml of acetonitrile using ultrasonication for 5 minutes at ambient temperature (Lucas-Dawe Ultrasonics sonicleaner) and heating to 55°C in a water bath. When fabricating antibiotic loaded microspheres, 0.25g of either vancomycin, ciprofloxacin or rifampicin powder was

also added to the water phase prior to sonication to produce a 20% w/w concentration of antibiotic by weight of polymer. The oil phase was stirred at a high sheer speed (Emulsifier model L4RT, maximum speed 9,000rpm, Silverson Machines Ltd., Chesham, UK), typically 4,000 rpm, and the water phase quickly added to create an emulsion. Any residual antibiotic solid was washed into this emulsion with 10ml acetonitrile. Emulsification progressed for 30 minutes in a fume hood during which time droplet formation was monitored using light microscopy and representative 35mm photographs taken. A three bladed propeller attached to a Heidolph stirrer (Heidolph, Lab-plant Ltd., Huddersfield, UK) and set in an off centre position was then employed to mix the emulsion to facilitate solvent evaporation (see table 2.1.). This was carried out at 55°C in a fume hood. Microspheres were subsequently hardened by incubation in a water bath at 35°C for 60 minutes before being harvested by vacuum filtration using solvent resistant polypropylene backed PTFE filters and washed with excess petroleum ether at 55°C to remove SPAN 40. The resultant free-flowing microspheres were air dried, weighed in order to calculate percentage yield and stored desiccated at room temperature. The calculation for microsphere percentage yield is shown in Equation 2.1. below:

Equation 2.1. Calculation of the percentage yield of freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres.

$$\frac{\text{Actual yield of fabricated microspheres (g)}}{\text{Theoretical maximum yield of fabricated microspheres (1.25g)}} \times 100\%$$

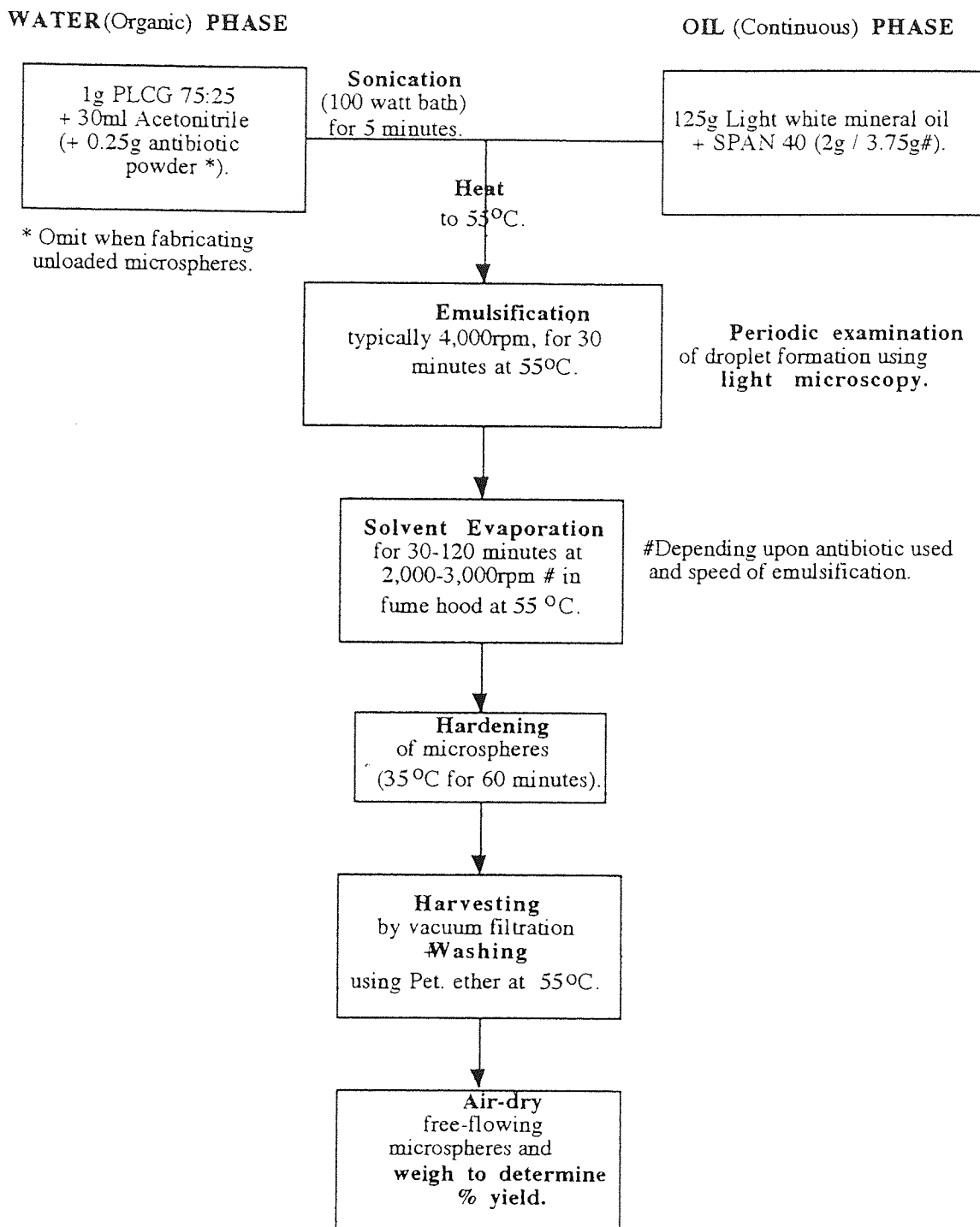
Table 2.1. below shows the conditions employed during the fabrication of unloaded microspheres and 20% w/w antibiotic loaded microspheres.

Table 2.1. The conditions employed during the fabrication of unloaded and 20% w/w antibiotic loaded microspheres.

Microsphere type	Span 40 concentration (% w/w)	Emulsification speed (rpm)	Speed of solvent evaporation (rpm)	Time length of solvent evaporation (minutes)
20% w/w vancomycin loaded	2	1,000	1,000	120
	2	2,000	2,000	60
	2	4,000	2,000	60
	2	6,000	2,000	60
	2	8,000	2,000	60
20% w/w ciprofloxacin loaded	2	4,000	2,000	45
20% w/w rifampicin loaded	3	4,000	3,000	30
Unloaded	2	4,000	2,000	60

A flow-diagram summarising the W/O protocol is shown in Figure 2.1.

Figure 2.1. Flow Diagram for the fabrication of microspheres using W/O Emulsification with Solvent Evaporation.



2.2.2. Determination of the encapsulation efficiency for 20% w/w antibiotic loaded microspheres.

The extent of antibiotic encapsulation within freshly fabricated microspheres theoretically loaded with 20% w/w vancomycin or 20% w/w ciprofloxacin (the encapsulation efficiency) was determined by first combining 0.25g of 20% w/w antibiotic loaded microspheres with 5ml of dichloromethane and 5ml double distilled water in a screw-top glass vial sealed with Duraseal™ laboratory stretch film. Agitation of the preparation overnight at 37°C (Griffin orbital shaker, level 4) caused the PLCG to dissolve in the DCM releasing antibiotic into the aqueous phase. The aqueous phase was carefully removed, split into four 1ml aliquots in separate sterile plastic tubes, covered with permeated Duraseal™ and freeze-dried (Edward's Modulyo 4 litre Freeze-drier, Edward's High Vacuum International, Crawley, West Sussex, England) for 24 hours to lyophilise the antibiotic. The lyophilised antibiotic was weighed and the encapsulation efficiency calculated using equations 2.2 - 2.4.:

Equation 2.2. Calculation of the mass of vancomycin and ciprofloxacin residue equivalent to an encapsulation efficiency of 100% (g/ml).

$$\frac{\text{Mass of microspheres (0.25g)} \times \text{fraction of microsphere mass theoretically antibiotic (20\%/100\%)}}{\text{Volume of distilled water used (5ml)}}$$

The value obtained from Equation 2.2. was corrected to account for the solubility of vancomycin and ciprofloxacin in dichloromethane. This was achieved by adding 0.05g of antibiotic to 5ml of DCM and agitating this mixture in a screw-top glass vial sealed with Duraseal™ at 37°C for 24 hours thereby reproducing the conditions documented above for the determination of encapsulation efficiency. After 24 hours of incubation, the volume of the preparation was measured to account for the loss of solvent by evaporation and the preparation filtered (Whatman 50 hardened filter paper, 7cm diameter) to remove

undissolved antibiotic particles. The filtrate was then left to evaporate on a covered pre-weighed watch glass in a fume hood. The mass of precipitated vancomycin and ciprofloxacin per ml of DCM and the percentage solubility of these antibiotics in DCM were calculated using Equation 2.3.1 and 2.3.2 below:

Equation 2.3.1. Calculation of the mass of vancomycin and ciprofloxacin precipitated from 1ml of evaporated DCM (g/ml).

$$\frac{\text{Mass of precipitated antibiotic and watch glass (g)} - \text{mass of pre-weighed watch glass (g)}}{\text{Volume of solvent remaining in vial after incubation (ml)}}$$

Equation 2.3.2. Calculation of the percentage solubility of vancomycin and ciprofloxacin in DCM.

$$\frac{[\text{Mass of antibiotic dissolved in 1ml DCM (from eq. 2.3.1)} \times \text{original solvent volume (5ml)}]}{\text{Mass of antibiotic added to original solvent volume (0.05g)}} \times 100\%$$

A control composed of 5ml of DCM was allowed to evaporate under the same experimental conditions and the mass of the resultant precipitate was subtracted from the value obtained in the top half of Equation 2.3.2. before completing calculating equation 2.3.2.. Equation 2.4. below was then used to calculate the actual encapsulation efficiency of both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres:

Equation 2.4. Calculation of the actual encapsulation efficiency of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres.

$$\frac{\text{Actual mass of antibiotic precipitate (g/ml DCM)}}{\text{Mass of antibiotic precipitate equivalent to 100% encapsulation efficiency (g/ml) (from eq. 2.2)}} \times 100\%$$

No residue was produced as a result of freeze-drying the aqueous water fraction from a preparation of unloaded microspheres previously incubated in 5ml of DCM and 5ml of double distilled water. This acted as a control to show that antibiotic, and not PLCG, was precipitated as a result of freeze-drying.

As rifampicin is soluble in DCM, the encapsulation efficiency of 20% w/w rifampicin loaded microspheres was measured using a modification of the technique described by Hora *et al* (1990). 0.1g of loaded microspheres was placed in a sterile plastic screw-top tube and digested by the addition of 10ml 1N NaOH with overnight agitation at 37°C. The resultant solution was then filtered through a Whatman number 1 filter paper to remove any residual polymer. The absorbance of the filtrate was measured at 472nm against a reference blank generated using unloaded microspheres exposed to the same experimental conditions. A standard curve of absorbance versus a range of known concentrations of rifampicin in 1N NaOH at 472nm was used to determine the concentration of microencapsulated rifampicin (figure 2.2.). Encapsulation efficiencies were subsequently calculated using Equations 2.5.1. and 2.5.2. below:

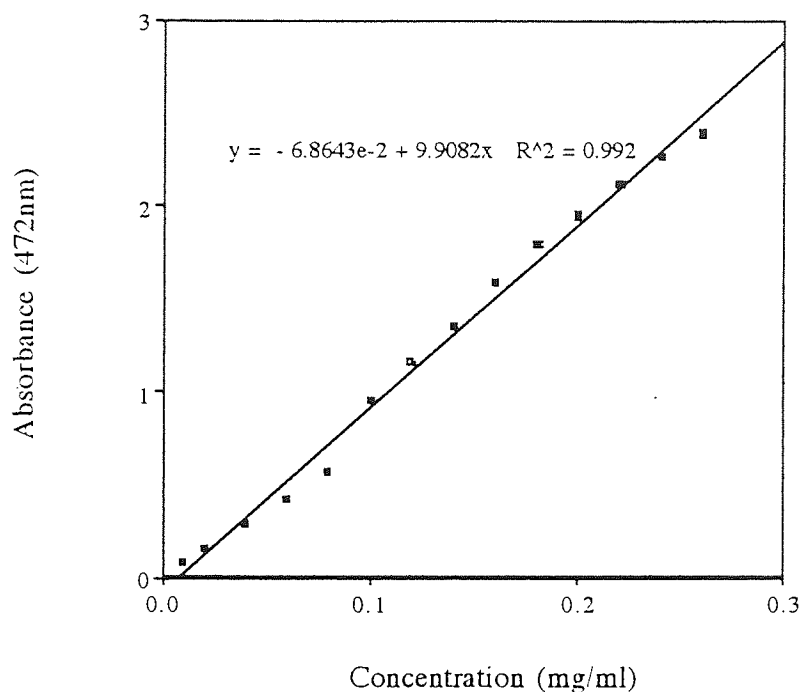
Equation 2.5.1. Calculation of the concentration of rifampicin equivalent to an encapsulation efficiency of 100% (g/ml).

$$\frac{\text{Mass of microspheres (0.1g)} \times \text{fraction of microsphere mass theoretically rifampicin (20\%/100\%)}}{\text{Volume of 0.1N NaOH used (10ml)}}$$

Equation 2.5.2. Calculation of the encapsulation efficiency of 20% w/w rifampicin loaded microspheres.

$$\frac{\text{Actual concentration of encapsulated rifampicin (g/ml)}}{\text{Concentration of rifampicin equivalent to 100\% encapsulation efficiency (g/ml) (from eq. 2.5.1)}} \times 100\%$$

Figure 2.2. Standard curve of rifampicin in 1N NaOH (mean values \pm SEM, n=6 for each point)



2.2.3. Determination of mean volume diameters and size distribution profiles for unloaded and 20% w/w antibiotic loaded microspheres.

Mean volume diameter measurements and size distribution profiles for unloaded and 20% w/w antibiotic loaded microspheres were generated using a Mastersizer/E laser diffractor (Malvern Instruments Ltd, Malvern, UK). 0.1 g of either unloaded microspheres or 20% w/w antibiotic loaded microspheres was suspended in 10 ml double distilled water which had been previously filtered through a 0.2 μ m porous membrane to remove particulate impurities. This preparation was sonicated for 5 minutes to dissociate microsphere aggregates and a 5ml aliquot removed for injection into the laser diffraction equipment. Microsphere mean volume diameter and size distribution data were generated using a P.C. compatible computer interfaced with the laser diffraction equipment.

2.2.4. Examination of the surface topography and internal architecture of unloaded microspheres and 20% antibiotic loaded microspheres.

The surface topography of freshly fabricated unloaded microspheres and 20% w/w antibiotic loaded microspheres was characterised using scanning electron microscopy (E/M). Thoroughly air-dried microspheres were mounted on aluminium stubs with double sided adhesive and gold/palladium sputter coated for 90 seconds under argon gas vacuum (Emscope SC500 coater). A Cambridge Instruments Stereoscan 90 SEM with a Polaroid facility was then used to examine the microspheres and take representative 35mm photographs of the microspheres on each stub. 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres, which had been previously immersed in double distilled water for approximately one minute to remove surface antibiotic crystals and subsequently freeze-dried, were also examined using the same scanning E/M technique.

In order to examine the internal architecture of freshly fabricated unloaded microspheres and 20% w/w antibiotic loaded microspheres, small samples of microspheres were added to a warm solution of 10% w/v gelatin and 1% w/w glycerine (as a plasticiser) in double distilled water and dried under high vacuum for 24 hours (Iwata and McGinity, 1993). Samples were examined using a Jeol 1200 EX Temscan electron microscope fitted with a cryostage. This work was carried out under the supervision of Lesley Tompkins at the Department of Biochemistry, University of Birmingham, Birmingham, England.

2.2.5. Residual solvent and glass transition temperature measurements for unloaded microspheres and 20% w/w antibiotic loaded microspheres.

The concentration of residual acetonitrile remaining within unloaded microspheres and 20% w/w antibiotic loaded microspheres after solvent evaporation was measured using

Gas-liquid chromatography (GLC). The concentration of petroleum ether that penetrated the internal matrix of these microspheres during the washing stage of the microsphere fabrication process was also measured using GLC. Approximately 20mg of unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres were accurately weighed and separately dissolved in 10ml of DCM. Three aliquots of 1.5 μ l were then removed from each stock using a 10 μ l glass syringe and individually introduced into a Pye Unicam 304, GL chromatograph (injector temperature 210°C, oven temperature 200°C, detector temperature 230°C) coupled to a Pye-Unicam trace recorder. A 2 metre glass column containing Porapak Q was used with pressures of Nitrogen (N₂, the carrier gas), Hydrogen (H₂), and air of 20mm Hg; 1.05 kg/cm₂; and 0.4kg/cm₂ respectively. Standard solutions composed of known concentrations of acetonitrile and petroleum ether separately dissolved in DCM were analysed in the same way and standard curves generated for both these solvents so that the concentration of residual solvent liberated from the microspheres could be obtained by interpolation.

The glass transition temperatures (T_g) for unloaded microspheres, 20% w/w antibiotic loaded microspheres and virgin PLCG 75:25 copolymer (as supplied by the manufacturer) were measured using a Perkin-Elmer-4 series differential scanning calorimeter (DSC) with nitrogen ambient accessory as described by Bodmeier *et al* (1989). The DSC equipment was coupled to a Perkin-Elmer thermal analysis computerised interface. Duplicate 5-10 mg samples of air-dried microspheres as well as vancomycin, ciprofloxacin and rifampicin powders and virgin PLCG 75:25 copolymer were accurately weighed and individually scanned in sealed aluminium pans at a heating rate of 10°C per minute to produce thermograms over the range of 10°C to 200°C. Shifts in the point of the T_g peak onset for 20% w/w antibiotic loaded microspheres were recorded and compared with the T_g onset for unloaded microspheres. Changes in the appearance or the disappearance of the melting points for antibiotic powders before and after incorporation into microspheres as well as the T_g peak onset for virgin PLCG 75:25 copolymer were also recorded. Any shifts in the T_g peak onset for PLCG incorporated into loaded microspheres when compared with

unloaded microspheres provides an indication of whether antibiotics have become encapsulated within loaded microspheres and their dispersion (either molecular or particulate) within the matrix of loaded microspheres. Comparison of the onset of T_g for unloaded microspheres and virgin PLCG copolymer provides information on how the structure of PLCG might change as a result of being used to fabricate microspheres using the W/O technique.

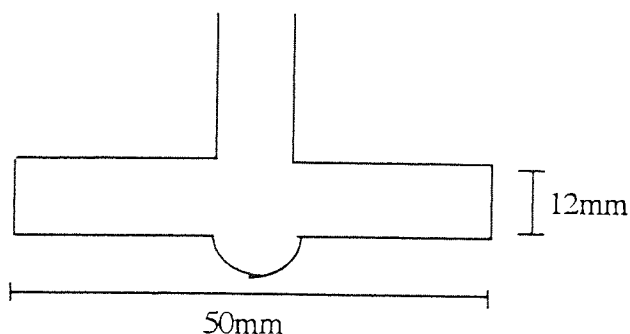
2.2.6. Determination of the molecular mass of unloaded microspheres, 20% w/w antibiotic loaded microspheres and PLCG 75:25 virgin copolymer.

PLCG 75:25 weight average (M_w), number average (M_n), and peak (M_p) molecular mass and polydispersity (pd) were determined using gel permeation chromatography (GPC) at ambient temperature using a Plgel 2 x mixed bed-B 30cm length column with a diameter of 10μm. A 20mg sample of either unloaded microspheres, 20% w/w antibiotic loaded microspheres or virgin PLCG 75:25 copolymer was dissolved overnight in chloroform stabilised with ethanol or amylene before being warmed in a water bath for 30 minutes. An internal marker of 1,2-dichlorobenzene was added and, after thorough mixing, an aliquot of the mixture was filtered through a 0.2μm polyamide membrane. This mixture was then placed into an autosampler with a nominal flow rate of 1.0 ml/minute. Effluent from the column was passed through a refractive index detector. The GPC apparatus was calibrated with polystyrene and each determination expressed as a polystyrene equivalent molecular mass. This work was carried out at Rapra Technology Ltd, Shawbury, Shrewsbury, Shropshire, UK with funding from EPSRC research grant GR/J 39014.

2.2.7. The incorporation of unloaded microspheres and 20% w/w antibiotic loaded microspheres into 10% w/v HPMC (4,000 cps) gel.

In order to generate an injectable gel vehicle for unloaded microspheres and 20% w/w antibiotic loaded microspheres, 1g of disaggregated microspheres was added to a 10ml solution of double distilled water (9 parts) and Amphotericin B (1 part) in a tall-form 250ml beaker (Fisher Scientific UK). This preparation was then stirred at approximately 35rpm with a 50mm x 12mm paddle obtained from Fisher Scientific UK (Figure 2.3.) attached to a Heidolph stirrer while 1g of HPMC (4,000 cps) powder was added slowly (in an attempt to reduce the formation of air bubbles). A homogeneous dispersion of microspheres within the HPMC was created as a result of 30 minutes stirring so that an approximate w/v concentration of microspheres to gel of 0.1g/ml was produced. Unloaded microspheres and 20% w/w antibiotic loaded microspheres fabricated using an emulsification speed of 4,000 rpm were incorporated into HPMC gel vehicles as described above. 20% w/w vancomycin loaded microspheres originally fabricated using an emulsification speed of 1,000 rpm and 8,000 rpm were also incorporated into HPMC gel. The release of antibiotics from 20% w/w antibiotic loaded microsphere-HPMC preparations were monitored using the protocol described in section 2.2.9..

Figure 2.3. Schematic diagram of the paddle employed to mix HPMC gel preparations containing unloaded microspheres and 20% w/w antibiotic loaded microspheres.



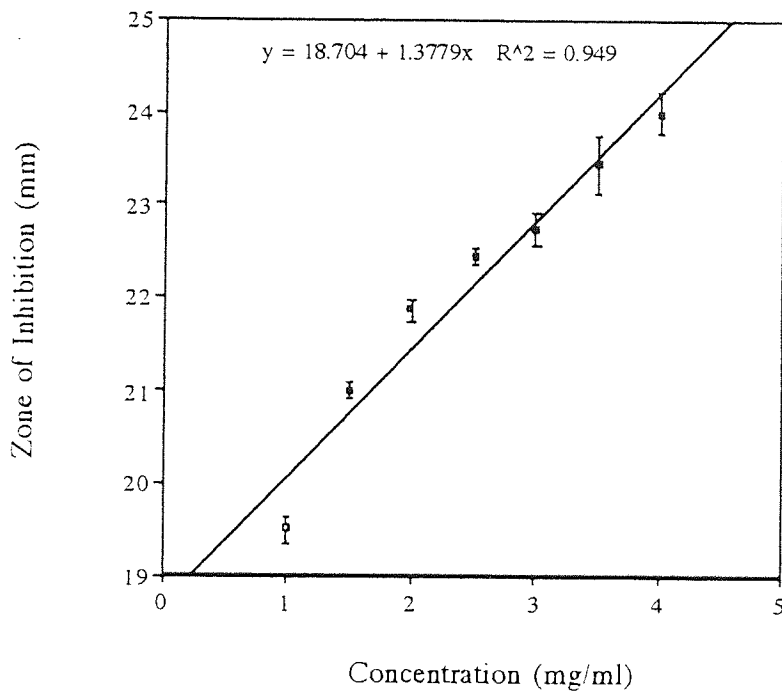
2.2.8. The release of antibiotics from 20% w/w loaded microspheres incubated in NCS and HBSS at 37°C.

In order to investigate the kinetics of *in vitro* antibiotic release from 20% w/w loaded PLCG 75:25 microspheres, 2ml of either NCS or HBSS was added to 0.1g of 20% w/w antibiotic loaded microspheres in sterile plastic screw-capped tubes. Each tube was vortex mixed for 10 seconds before being incubated at 37°C without agitation to mimic an *in vivo* situation (Park, 1994, Vert *et al*, 1991). 24 hours later, the medium in each tube was aspirated and replaced with fresh NCS or HBSS using sterile pipettes and incubation resumed. This practice was repeated every 24 hours typically for the first 14 days of the release study and every 48 hours thereafter which was thought to provide sink conditions for release while also discouraging microbial growth in the medium. The rate of antibiotic release from all 20% w/w loaded microspheres was monitored for up to 30 days. Release samples were stored at -20°C immediately after collection and thawed in a warm room, diluted if necessary, and their antibiotic concentration determined by radial diffusion (section 2.2.10.). A range of standard antibiotic concentrations dissolved in NCS and HBSS were assayed in the same manner (n=12) in order to construct standard curves of the clearing zone diameter (mm) versus antibiotic concentration (mg/ml) (Figure 2.4.). The antibiotic concentration in each release sample was determined by interpolation from the appropriate standard curve. Antibiotic release profiles were expressed as the day to day, cumulative and percentage release of antibiotic with time. Student's two-tailed unpaired t-test was used to compare the concentrations of antibiotic released into NCS or HBSS at appropriate points on the release profiles. The kinetics of *in vitro* antibiotic release were analysed kinetically using first order and "Higuchi" (square-root time) plots to ascertain the mechanism of antibiotic release. Controls of 0.1g of unloaded were incubated and assayed in an identical fashion to 20% w/w antibiotic loaded microspheres. NCS and HBSS samples obtained during incubation of unloaded microspheres had no effect on the growth of *Sarcina lutea* in the radial diffusion assay (section 2.2.10.).

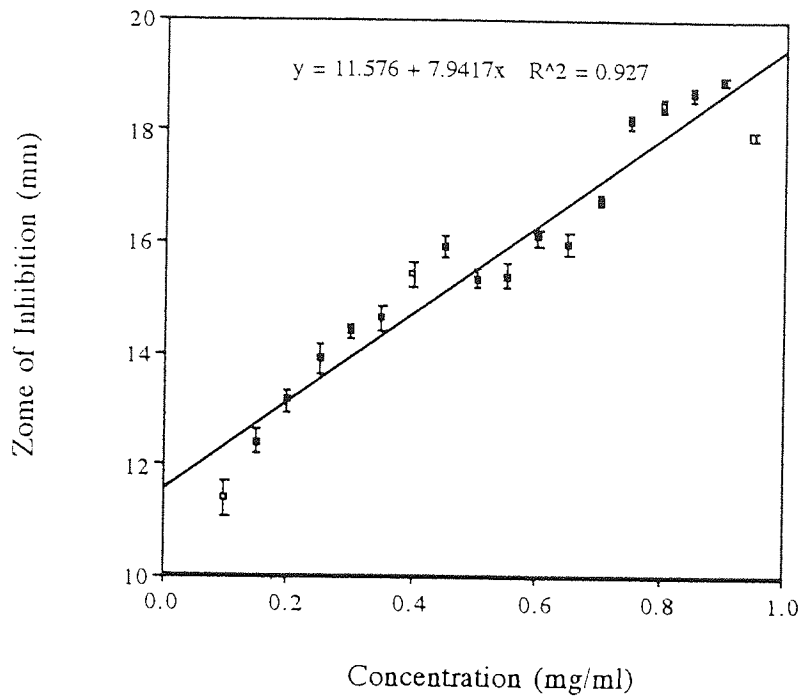
Figure 2.4. Standard curves of known concentrations of antibiotic dissolved in NCS and HBSS (mean values \pm SEM, n=12 for each point).

Figure 2.4.a) Vancomycin standard curve in NCS.

1) Concentration range between 1.00mg/ml and 4.00mg/ml.



- 2) Concentration range between 0.10mg/ml and 0.95mg/ml.



- 3) Concentration range between 0.02mg/ml and 0.08mg/ml.

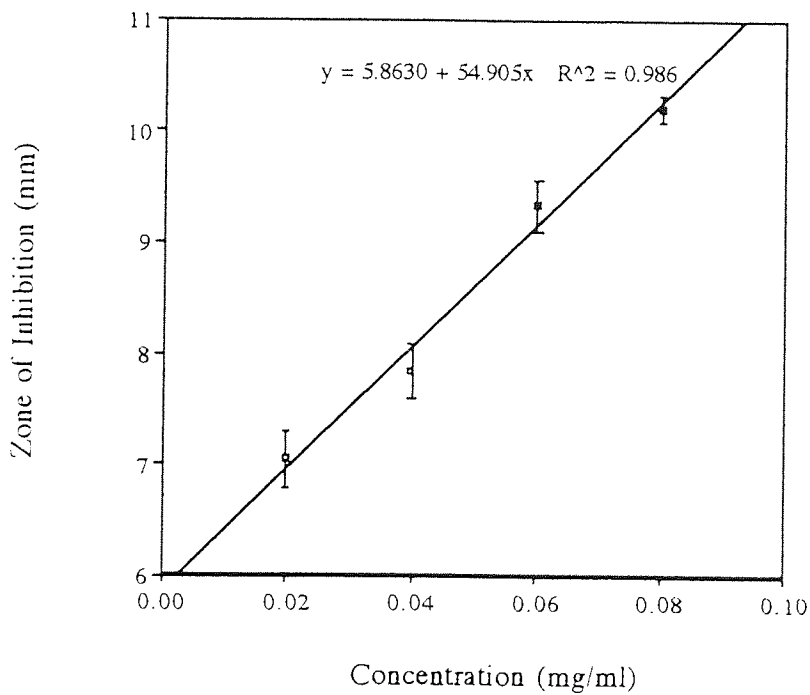


Figure 2.4.b) Vancomycin Standard Curve in HBSS

(0.01mg/ml to 5.00mg/ml).

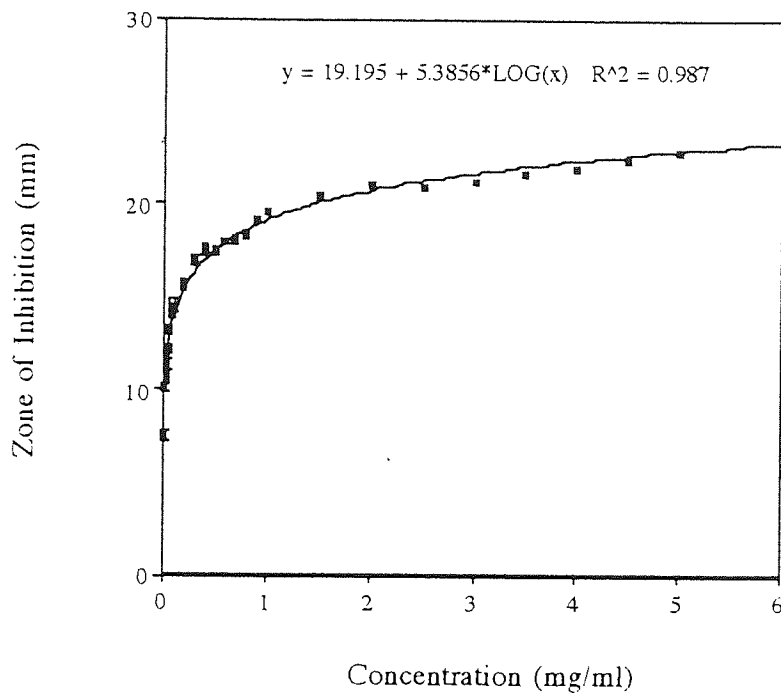


Figure 2.4.c) Ciprofloxacin Standard Curve in NCS

(0.01mg/ml to 0.30mg/ml).

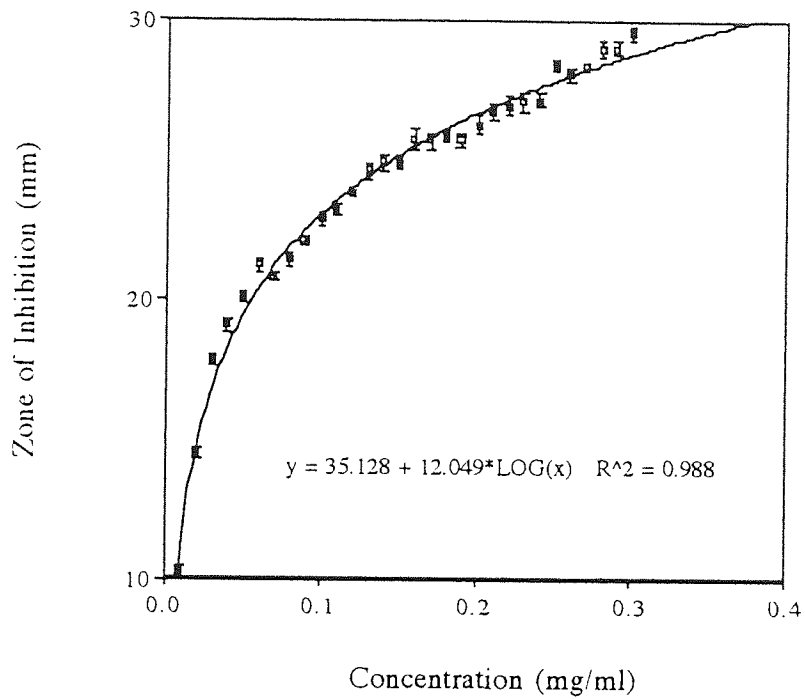


Figure 2.4.d) Ciprofloxacin Standard Curve in HBSS

(0.02mg/ml to 0.60mg/ml).

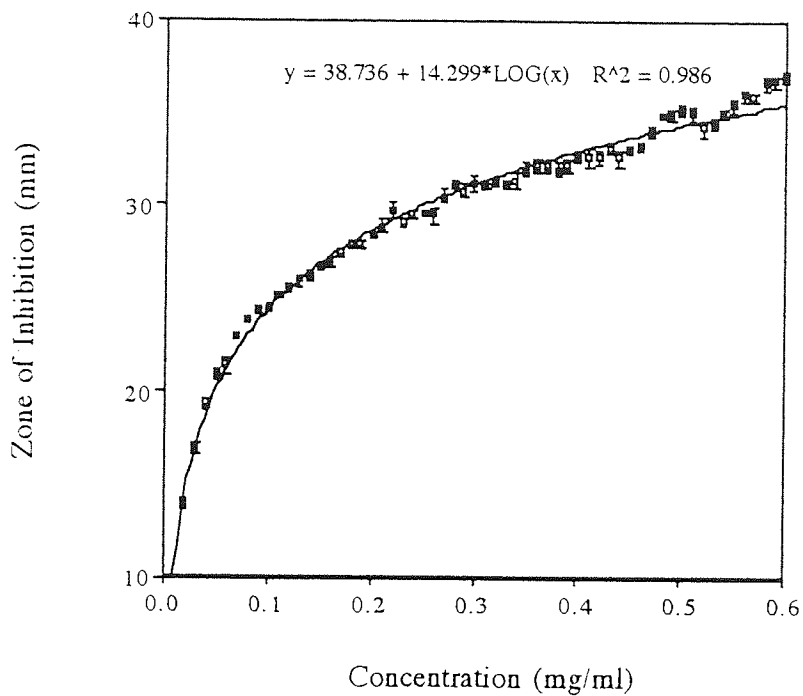


Figure 2.4.e) Rifampicin Standard Curve in NCS

(2×10^{-4} mg/ml to 0.18 mg/ml).

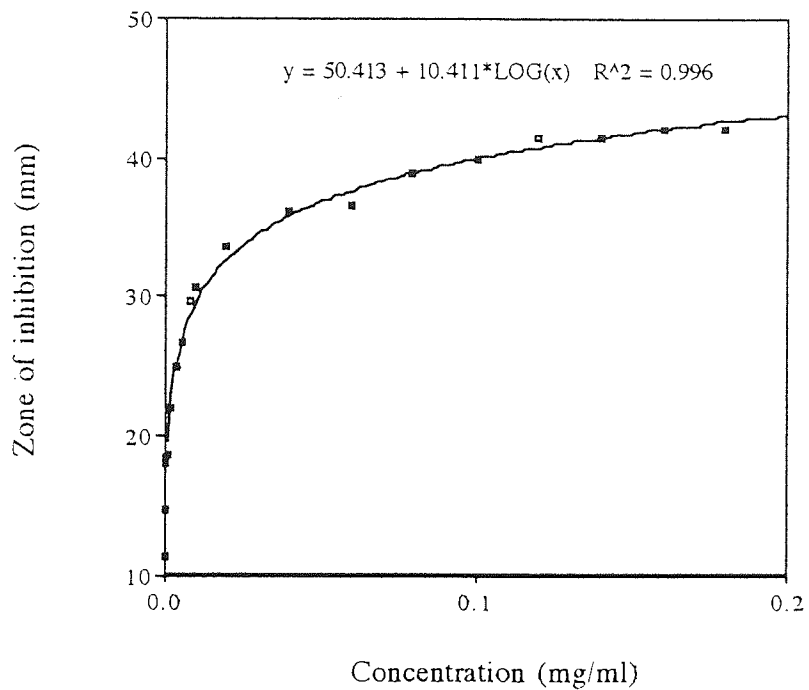
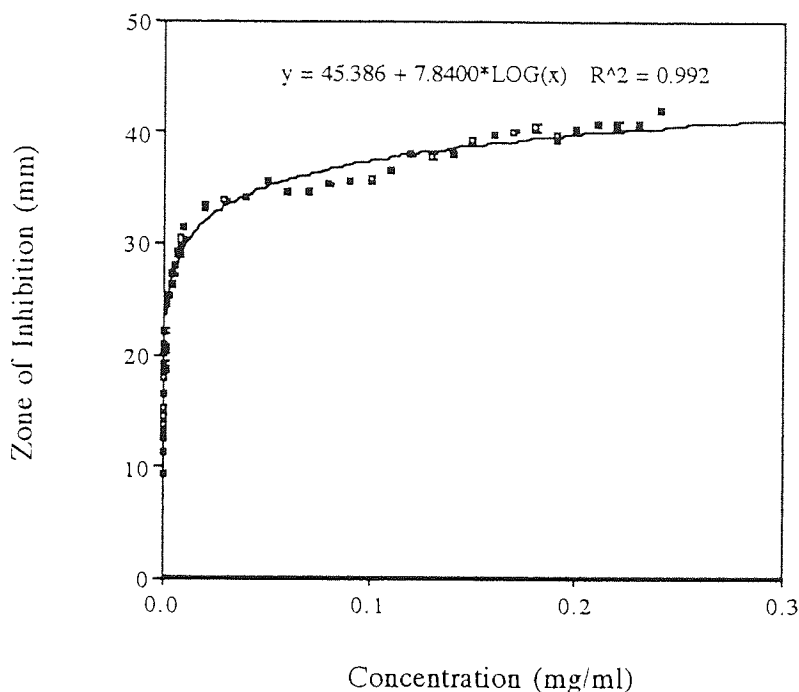


Figure 2.4.f) Rifampicin Standard Curve in HBSS

(3×10^{-5} mg/ml to 0.60 mg/ml).



2.2.9. The release of antibiotics from 20% w/w loaded microspheres suspended in a 10% w/v HPMC injection vehicle into NCS and HBSS at 37°C.

1ml aliquots of 20% w/w antibiotic loaded microspheres suspended in 10% w/v HPMC gel were injected into plastic sterile screw-capped tubes using a 5ml syringe barrel with a 2mm diameter nozzle. 2ml of either NCS or HBSS was added to each tube before incubation at 37°C without agitation. Gel samples were not vortex mixed before incubation which avoided premature microsphere precipitation. Antibiotic release from HPMC-microsphere preparations was monitored using the protocol documented in section 2.2.8. above. Controls composed of unloaded microspheres suspended in 1ml of 10% w/v HPMC at a concentration of 0.1g/ml gel w/v and 1ml of 10% w/v HPMC gel were

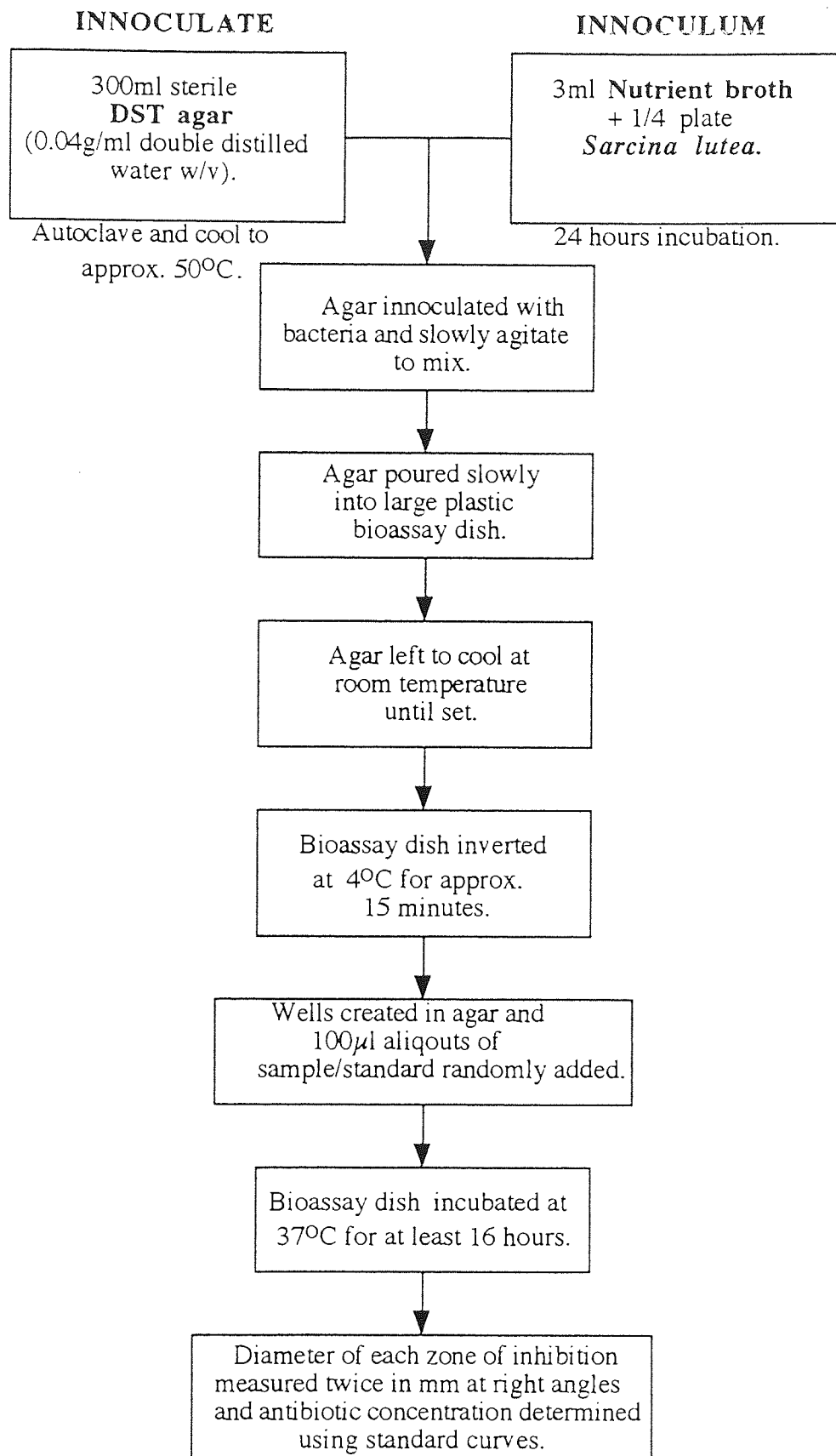
incubated and assayed in an identical fashion to HPMC gel suspended 20% w/w antibiotic loaded microspheres. NCS and HBSS samples obtained during incubation of control preparations had no effect on the growth of *Sarcina lutea* in the radial diffusion assay (section 2.2.10.). Day to day, cumulative, and percentage release profiles together with kinetic plots of *in vitro* antibiotic release from 20% w/w antibiotic loaded microspheres suspended in HPMC gel were constructed after interpolation of data from standard curves (see section 2.3.8). Student's unpaired, two-tailed t-test was employed to compare the differences in the concentration of antibiotic release from dispersed 20% w/w loaded microspheres and HPMC gel suspended 20% w/w loaded microspheres at appropriate points on the release profiles.

2.2.10. Determination of *in vitro* concentration of antibiotic released from 20% w/w loaded microspheres using a radial diffusion assay.

A radial diffusion assay (RDA) was used to determine the concentration of vancomycin, ciprofloxacin and rifampicin in NCS and HBSS release samples after incubation at 37°C by utilising the susceptible bacterium *Sarcina lutea* (in-house strain 6BV, ATCC 9341). Cultures of *S. lutea* were plated out on nutrient agar (0.028 g/ml double distilled water w/v) and incubated for 3 days at 37°C before being stored at 4°C. When required, a quarter plate of bacteria was taken up in 3ml nutrient broth (0.012 g/ml double distilled water w/v), incubated with agitation for 24 hours at 37°C and subsequently homogeneously dispersed by gentle shaking in 300ml of DST agar (0.04 g/ml double distilled water w/v) which had been previously autoclaved at 120°C for 15 minutes and left to cool to approximately 50°C. Dispersion in agar produced an estimated bacterial concentration of 2.35×10^7 organisms/ml. Once inoculated, the agar was slowly poured into pre-sterilised (70% v/v industrial methylated spirit in double distilled water or a 1% v/v solution of Virkon in water) large square plastic bioassay dishes (245x245x29mm) fitted with lids (Nunc, Roskilde, Denmark). The agar was allowed to set at room temperature

before each plate was inverted and kept at 4°C for approximately 15 minutes to allow the agar to harden. After removal of the agar from storage at 4°C and when the bioassay dish lids had been wiped clear of condensation, thirty six 6mm diameter wells were cut into the agar using the teat-end of a Pasteur pipette and each agar plug removed using the nozzle end. 100µl aliquots of standards in NCS or HBSS and release samples were then pipetted into triplicate wells in a recorded but random sequence. Subsequently, the top bioassay dish of each stack was sealed with sticky tape in order to prevent the so-called “edge effect” where drying along the edges of agar plates creates erroneously large zone diameters (Bennett *et al*, 1966). The plates were incubated for at least 16 hours at 37°C to ensure the satisfactory development of clearing zones (Bennett *et al*, 1966). After incubation the diameter (mm) of the clearing zone around each well, also known as the zone of inhibition (created by antibiotic diffusion from the well into the agar killing bacteria in that area) was measured twice at right angles. The RDA method is summarised in figure 2.5. below.

Figure 2.5. Flow diagram for the radial diffusion assay.



2.2.11. Biodegradation of unloaded microspheres and 20% w/w antibiotic loaded microspheres incubated in NCS and HBSS at 37°C.

The biodegradation of PLCG 75:25 microspheres incubated in NCS and HBSS was monitored by measuring changes in polymer molecular mass (M_w , M_n , M_p and p_d) with incubation time; polymer T_g changes with incubation time; percentage mass loss from microspheres with incubation time and changes in microsphere morphology (surface topography and internal architecture) with incubation time. A decrease in polymer weight average molecular mass with the progression of incubation will signal the start of microsphere biodegradation. Concomitant changes in both polymer M_n and p_d will also occur which will effect the T_g of the polymer. If molecular weight decreases to a point where water soluble oligomers and monomers are formed then mass loss from the microspheres will occur. These physico-chemical phenomena will affect the morphology of incubating microspheres both at their surface and within the internal matrix. Monitoring the changes in these physico-chemical parameters will provide information on the extent of microsphere biodegradation resulting from incubation in NCS and HBSS at 37°C for 30 days.

2.2.11. a) The effect of incubation in NCS and HBSS at 37°C on the polymer molecular mass of unloaded microspheres and 20% w/w antibiotic loaded microspheres.

Gel-permeation chromatography was used in an identical fashion to that documented in section 2.3.6. to measure any changes in polymer molecular mass with the progression of microsphere biodegradation as a consequence of incubation in either NCS or HBSS at 37°C. Microspheres from *in vitro* antibiotic release studies were utilised for this study. Profiles were constructed to show changes in polymer molecular mass with incubation time, typically at 6 day intervals, over a period of 30 days. After each time period, any changes in the distribution of molecular weights for both unloaded and 20% w/w antibiotic loaded microspheres were also recorded.

- 2.2.11. b) The effect of incubation in NCS and HBSS at 37°C on the glass transition temperature (T_g) of unloaded microspheres and 20% w/w antibiotic loaded microspheres.

Changes in the T_g of unloaded microspheres and 20% w/w antibiotic loaded microspheres after incubation in NCS and HBSS at 37°C for 6, 12, 18, 24 and 30 days were determined in an identical fashion to that documented in section 2.3.5.. The onset of the T_g for microspheres exposed to NCS and HBSS for various lengths of time were compared with that of freshly fabricated microspheres (see section 2.3.5.). This was used to determine whether changes in the onset of polymer glass transition temperature with incubation time could be taken as an accurate measure of the rate of microsphere degradation.

- 2.2.11. c) Determination of w/w % mass loss from unloaded microspheres and 20% w/w antibiotic loaded microspheres by gravimetry after 30 days of incubation in HBSS at 37°C.

Samples of unloaded microspheres and 20% w/w antibiotic loaded microspheres (all fabricated using an emulsification speed of 4,000 rpm) weighing approximately 0.05g were accurately weighed into sterile plastic screw-top tubes and 2ml of HBSS (supplemented with 2% v/v Fungizone®) added to each tube. The tubes were then incubated at 37°C in a warm room without agitation to reduce fragmentation of the microspheres to a minimum and mimic the *in vivo* situation where the flow rate of blood and tissue fluid in soft and hard tissues is slow (Vert *et al*, 1991). The incubation medium was replaced with fresh HBSS every three days in order to reduce the likelihood of microbial contamination. However, on days 6, 12, 18, 24 and 30 of incubation, HBSS was removed from designated sample tubes and the contents washed with 2ml of 96% v/v ethanol. Following centrifugation at 2,000rpm for 2 minutes the ethanol was decanted off and the microspheres freeze-dried and stored desiccated over silica gel at ambient temperature. Each microsphere sample was then weighed and its mass compared with the original starting mass (approximately 0.05g) at the commencement of incubation.

Computation of the w/w % microsphere mass loss with time was carried out using equation 2.6. below.

Equation 2.6. To determine w/w % mass loss from unloaded microspheres and 20% w/w antibiotic loaded microspheres incubated in HBSS.

$$100\% - \frac{\text{Mass of microspheres after incubation}}{\text{Original Mass of microspheres}} \times 100\%$$

Percentage w/w mass loss profiles were constructed to illustrate the rate of microsphere weight loss in HBSS with time.

2.2.11. d) Changes in the morphology of unloaded microspheres and 20% w/w antibiotic loaded microspheres after incubation in NCS and HBSS at 37°C.

Scanning electron microscopy was employed in an identical fashion to that documented in section 2.3.4. to examine the surface topography and internal architecture of unloaded and 20% w/w antibiotic loaded microspheres after 30 days of incubation in NCS and HBSS at 37°C.

2.2.12. *In vitro* cytotoxicity of unloaded microspheres and 20% w/w antibiotic loaded microspheres using INVITTOX protocols 3b and 104.

2.2.12. a) Maintenance and culture of 3T3-L1 murine fibroblast-like cells (ATCC CCL92.1).

A growing culture of adherent 3T3-L1 cells (derived from the 3T3 mouse fibroblast line created from disaggregated Swiss mouse embryos) was purchased from the European Collection of Animal Cell Cultures, Porton Down, UK and maintained as follows. Upon receipt from Porton Down, the old culture medium was immediately removed from the 15cm² tissue-culture flask under aseptic conditions and replaced with 5ml of fresh

complete DMEM (containing 10% v/v heat inactivated foetal bovine serum and 1% v/v solution of antibiotic/antimycotic solution). The flask was then incubated at 37°C in a humid atmosphere of 5% CO₂/95% air. When the cells had obtained near confluence they were passaged by first washing with 5ml of complete DMEM before exposure to 5ml of trypsin-EDTA solution. Both these liquids had been previously warmed to 37°C in a water bath. Following 5 minutes of incubation in a 37°C room, the cells were detached from the base of the flask using a few sharp taps and suspended in 8ml of fresh complete DMEM in a plastic sterile screw-capped tube. Subsequent to centrifugation (1200rpm for 5 minutes) the medium was decanted off and the cells suspended in fresh complete DMEM. 1ml aliquots of this cell suspension were then dispensed into each of three tissue-culture flasks (75cm² culture area, with canted neck and 0.22µm vented cap) containing 29ml fresh complete DMEM. The cells were maintained at 37°C in 5% CO₂/95% air. Every three or four days (depending on the extent of cell growth) the cells were passaged and occasionally, when an excess number of cells had been harvested, frozen down for low temperature storage. This involved isolating the cells from a 75cm² flask by trypsinisation and centrifugation as described above before the addition of a 1ml aliquot of cell culture freezing medium containing 10% v/v DMSO (to prevent cell freeze-fracture). The cells were then stored in a 2ml cryovial in liquid nitrogen (-196°C). When required, frozen cells were quickly thawed in a 37°C water bath before being added to 29ml of fresh complete DMEM in a 75cm² tissue culture flask. This ensured that the DMSO in the cell culture freezing medium had been diluted to a final concentration of less than 0.1% v/v. Subsequent to overnight incubation at 37°C, (5% CO₂/95% air) the medium was removed and replaced with fresh complete DMEM. Once incubation had been resumed the cells were passaged when near confluence and every 3-4 days thereafter. A 35 mm photograph of a confluent culture of 3T3-L1 is shown in figure 2.6. below. There was no discernable change in cell phenotype over the experimental period used.

Figure 2.6. 35mm photograph of 3T3-L1 cells during log phase growth (a)
and at confluence (b) (x150).

Figure 2.6.a) 3T3-L1 cells during log phase growth.

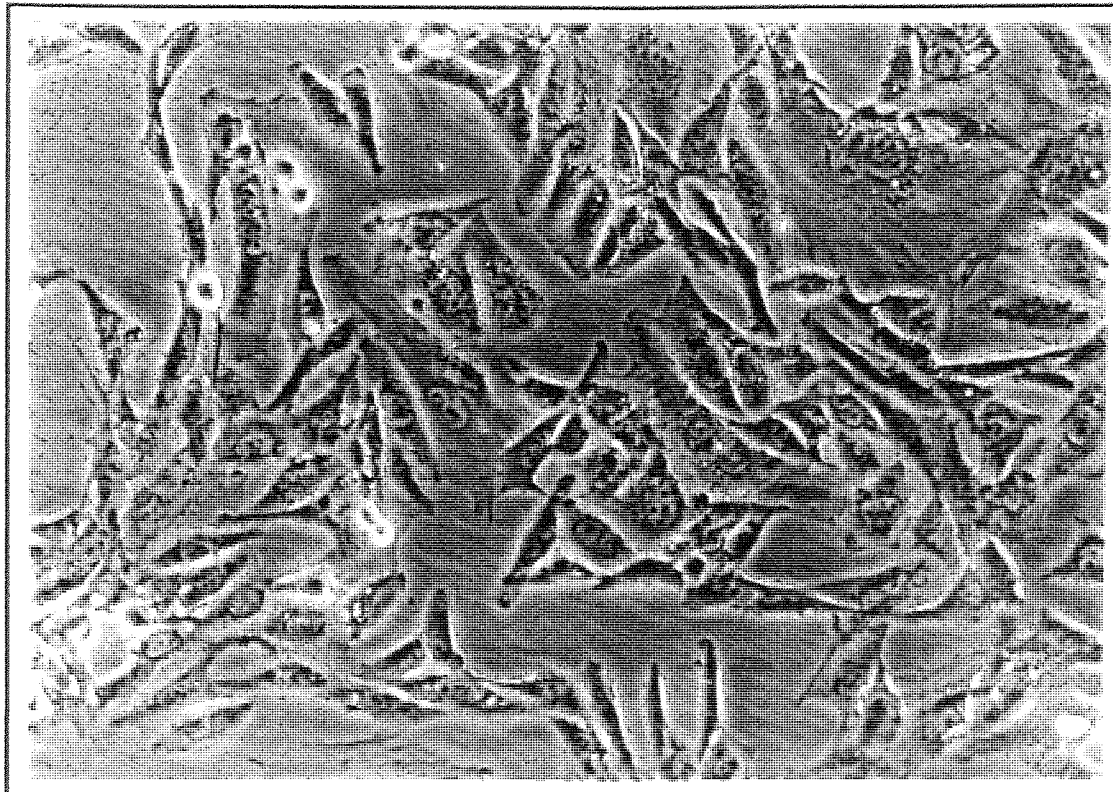
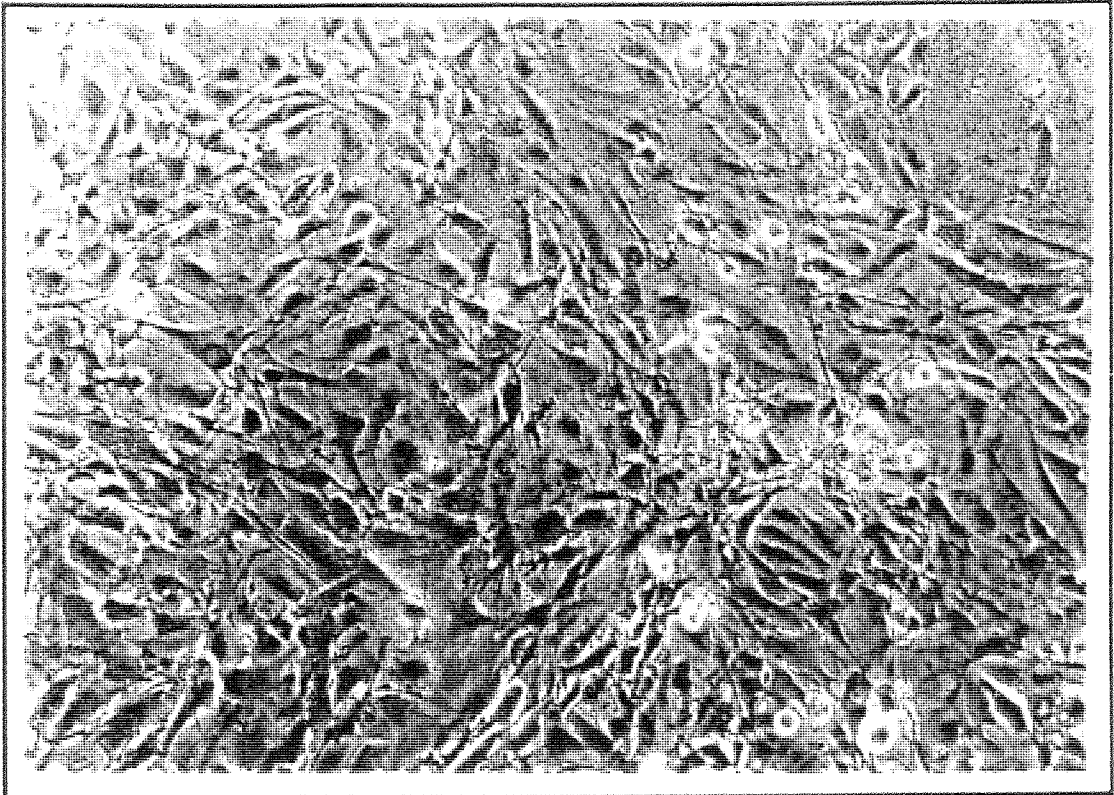
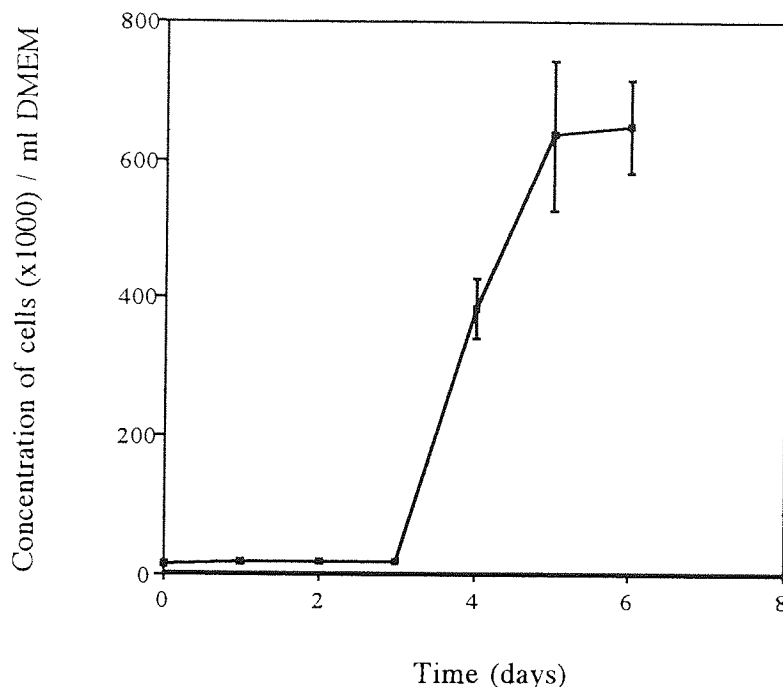


Figure 2.6.b) 3T3-L1 cells at confluence.



A growth curve for 3T3-L1 cells incubated in complete DMEM was constructed by seeding six tissue culture flasks, each containing 30ml of complete DMEM, with $1.542 \times 10^4 \pm 1.210 \times 10^3$ cells per ml. The flasks were incubated at 37°C and the following day an allotted flask was removed and, after trypsinisation, the cells were counted four times using a haemocytometer. This process was repeated every day for the next five days. The resultant growth curve is shown in figure 2.7. below.

Figure 2.7. Growth curve for 3T3-L1 cells in complete DMEM (mean values \pm SEM, n=4 for each point).



During the log (exponential) phase of growth (between days 3 and 5), the doubling time for 3T3-L1 cells in complete DMEM was approximately 0.65 days (15.53 hours). 3T3-L1 cells with passage numbers between 5 and 15 were used for the experiments documented in section 2.2.12. b) and c) below.

2.2.12. b) The FRAME Kenacid Blue dye binding method (INVITTOX protocol 3b, 1989).

The Fund for the Replacement of Animals in Medical Experiments (FRAME) Kenacid Blue dye binding method provides a means for assessing the cytotoxicity of chemicals when exposed to cultured cells. The division and multiplication of cells will be effected by a cytotoxic agent such that the cellular growth rate will be inhibited leading to a decrease in cell number (Knox *et al*, 1986, Invittox protocol 3b, 1989). The rate of cell growth can be assessed by measuring total cellular protein which should decrease with any increase in the

concentration of the cytotoxic agent exposed to the cells. The agent will either kill the cells or decrease their rate of proliferation.

The Kenacid Blue dye binding method was used to assess the cytotoxicity of unloaded microspheres and 20% w/w antibiotic loaded PLCG 75:25 microspheres. Firstly, 3T3-L1 cells were suspended using trypsinisation and centrifugation (see 2.3.12.a). After agitation of the medium to ensure the formation of a single cell suspension, the concentration of cells per ml of complete DMEM was ascertained with a haemocytometer. Appropriate dilution of the cell suspension was then carried out to provide a concentration of approximately 2.0×10^4 cells/ml of complete DMEM. A $150 \mu\text{l}$ aliquot of this suspension was then added to each of 95 wells in a 96 well plate. The remaining well was filled with $150 \mu\text{l}$ of complete DMEM to account for the adsorption of proteins in the medium to the base of each well. Plates were then incubated at 37°C overnight as previously described to allow for the attachment of cells to the base of each well (day 0). Also on day 0, 0.5g of either unloaded microspheres or 20% w/w antibiotic loaded microspheres was added to 5ml of complete DMEM in sterile a plastic screw-capped tube generating a w/v concentration of 0.1g of microspheres/ 1ml of complete DMEM. The microspheres were incubated for 24 hours at 37°C with agitation (Griffin orbital shaker, level 4) to allow both antibiotics and any residual microsphere fabrication components to elute into the culture medium.

After 24 hours of incubation (day 1), the tubes were centrifuged (2,000rpm for 5 minutes) and the supernatant separated from the microspheres. The supernatant was then diluted with complete DMEM. Given that microspheres were originally incubated in complete DMEM at a concentration of 0.1 g/ml, dilutions to the supernatant were made using fresh complete DMEM such that the w/v concentration of microspheres in their original incubation medium would have been 0.08g/ml, 0.06g/ml, 0.04g/ml, 0.02g/ml, 0.01g/ml and 0.005g/ml. The 96 well plates were then removed from incubation and the medium contained within each well aspirated and discarded. The wells of each 96 well plate were

then filled with 150 μ l of either complete DMEM or eluant samples. Ten wells were left without medium which allowed the cells in these wells to dry out. This enabled the protein content of the cells to be measured before the addition of antibiotic supernatants. Incubation of the cells at 37°C was then resumed for 72 hours. The experimental layout of each 96 well plate is shown in figure 2.8.

Figure 2.8. Typical layout of 96 well plate on day 1 of the Kenacid Blue dye binding method.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Key:

- = outer wells containing cells + complete DMEM (not used in assay as decreased cell growth can occur due to evaporation of DMEM (INVITTOX protocol 3b, 1989)).
- = control wells containing cells + complete DMEM.
- = Sample wells containing eluant dilutions of DMEM previously incubated with microspheres (column 2-9: 0.1-0.05 mg/ml).
- = Control well of complete DMEM only (A1).
- = control wells containing cells left to dry out.

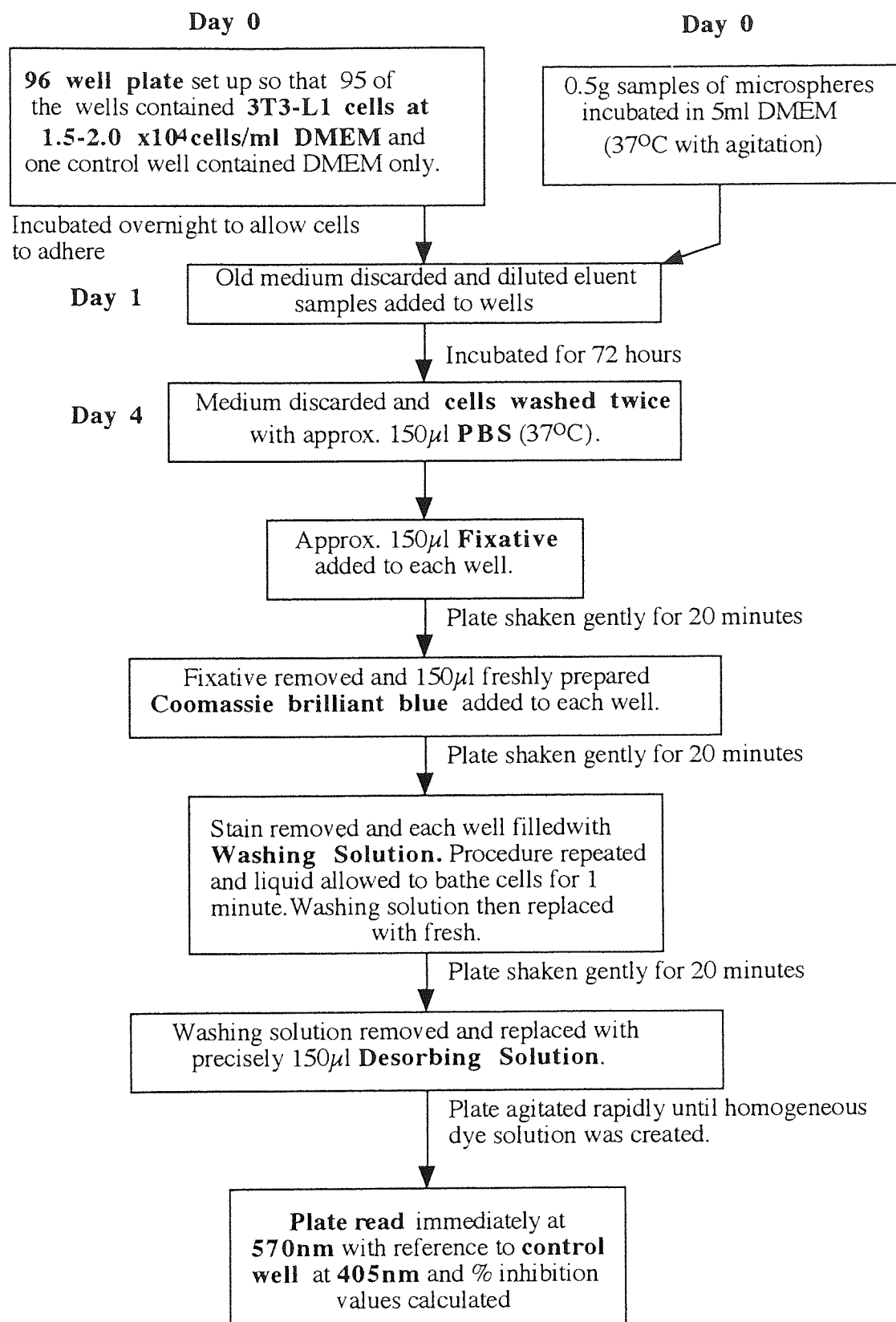
After 72 hours incubation (day 4) the liquid was aspirated from each well and the cells washed twice with phosphate buffered saline (PBS) at 37°C. Once the PBS had been

aspirated, 150 μ l of fixative (1% glacial acetic acid, 50% ethanol, 49% double distilled water) was added to each well and the plates shaken for 20 minutes on a dry rotary water bath (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, England). The fixative was then aspirated from the plates and replaced with 150 μ l of Coomassie brilliant blue solution. Coomassie brilliant blue solution had been prepared immediately before use by the addition of 12ml of glacial acetic acid to 88ml of Coomassie brilliant blue stock solution (0.4g of Coomassie brilliant blue R stain, 250 ml industrial ethanol, 630ml double distilled water). After a further 20 minutes shaking, the Coomassie brilliant blue solution was removed from each well and the cells washed with 150 μ l of washing solution (10% industrial ethanol, 5% glacial acetic acid, 85% double distilled water). Washing was then repeated and, after the solution had bathed the cells for one minute, the aspirated wells were each filled with 150 μ l of fresh washing solution. The plates were then shaken for another 20 minutes. Finally, residual washing solution was removed from each well and exactly 150 μ l of desorbing solution (98.15g of 1 M potassium acetate, 300ml double distilled water, 700ml industrial ethanol) added to each well. The plates were then shaken rapidly to liberate Coomassie brilliant blue stain from the cells such that a homogeneous blue solution was created in each well. Each plate was then read at 570nm and referenced against the control well at 405nm using an ANTHOS 2001 plate reader (ANTHOS Labtel. Instruments) attached to an EPSON LX-850 printer. The data from each plate was deemed acceptable if the absorbance value for the control well containing DMEM alone was similar at both 405 and 570nm and the absorbance values for control wells containing cells and DMEM on day 4 were at least 1.8 times the original protein values (day 1 dry wells).

Each of the microsphere eluant samples tested were usually run in triplicate. The values for percentage inhibition of cell growth caused by their presence (with respect to the appropriate control values) were meaned before plotting against diluted concentrations of microspheres originally incubated in DMEM. The percentage inhibition caused by an original concentration of 0.05g of microspheres/ml of DMEM, the same mass of microspheres per ml of incubation medium used in both release and biodegradation

studies, could then be calculated. Samples for unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres were assayed on more than one separate occasion which took into account any variations in the weighing of microspheres and the plating of cells and allowed the calculation of mean % inhibition values to be made. Comparisons between the relative cytotoxicities of individual antibiotics coupled with any cytotoxicity mediated by leached residual microsphere fabrication components was made possible by calculating the ID_{50} values (concentration of antibiotic that will inhibit 50% of normal cell growth) for unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres. A flow-diagram illustrating the protocol for the Kenacid Blue dye binding method is shown in figure 2.9..

Figure 2.9. Flow-diagram illustrating the Kenacid Blue dye binding Method.



2.2.12. c) The Methylcellulose Cell Culture Test System (INVITTOX protocol 104).

The Methylcellulose (MC) test system was used to evaluate the cytotoxicity of unloaded microspheres and 20% w/w antibiotic loaded microspheres placed in indirect contact with growing 3T3-L1 cells. This was achieved by adding cells to cell culture medium-rich MC. The cells migrated through the gel over time before adhering to the base of the wells of a 6-well plate. The placement of microspheres on the surface of the gel allowed eluted material to diffuse through the gel and reach the cells but the gel also acted as a passive barrier against the cells coming in to direct contact with the microspheres. Therefore, as in INVITTOX protocol 3b, the cytotoxicity of leachables from the microspheres could be assessed in terms of their ability to decrease cell growth and proliferation. This work was conducted by final year project students on the Applied and Human Biology Degree course at the University of Aston in Birmingham under the supervision of Dr Terry Atkins and Dave Yates.

A stock of MC gel was prepared by first mixing together 17.66g of Iscove's modification of Dulbecco's medium (IMDM) powder, 2.25g of sodium carbonate and 500ml sterile double distilled water. Once stirred to dissolve the powders, this solution was vacuum filtered through a disposable sterile 0.22 μ m filter unit. 22.5g of MC powder was then weighed into a one litre Erlenmeyer flask and 500ml of boiling double distilled water added. This mixture was stirred under aseptic conditions and swirled to disaggregate clumps of MC powder before being quickly heated to just below boiling point to dissolve the MC powder. The solution was then immediately cooled on ice to 4°C. Combination of the gel and IMDM preparations produced one litre of MC gel stock which was rotated at 4°C for 24 hours to ensure adequate mixing. The MC stock gel was then separated into 40 ml aliquots which were stored at -20°C until required. Aliquots of stock MC gel were thawed at 4°C for 24 hours prior to use which allowed residual unhydrated MC to enter the solution thereby conferring maximum viscosity to the gel. Immediately before use (within four days of thawing) the gel was heated to 37°C in a water bath. 20 ml of the stock MC

gel was then added to 8ml of FCS and 11ml of normal strength IMDM (containing penicillin, streptomycin and Fungizone®) to give v/v ratios for the three liquids of 50%, 20% and 30% respectively. This preparation was maintained at 37°C and a 1ml suspension of 0.4×10^6 3T3-L1 cells added to produce an overall concentration of 1×10^4 cells/ml. The MC-gel mixture was mixed thoroughly by inversion to distribute the cells homogeneously before 4ml aliquots were removed and added to each well of a 6-well plate. The 6-well plates were then incubated for 24 hours at 37°C in an incubator, as previously described, which allowed time for the cells to migrate through the gel and adhere to the base of each well. When incubation was complete, samples containing 200mg of microspheres (approximating to a surface contact area of 100mm^2) dispersed in 1ml of normal strength IMDM were poured onto the surface of the gel in each well. Positive and negative controls of silicone rubber tubing and polystyrene respectively were also used. Two discs, each with a radius of 4mm, were cut from samples of the controls using an 8mm corer so that their combined total surface area was 100.5mm^2 (the minimum requirement for the assay being a total surface area of 100mm^2). Discs of a particular control were ethanol sterilised before being laid on the surface of the gel in a single well. Samples of each type of microsphere were assayed in triplicate as were both negative and positive controls. Incubation of the 6-well plates at 37°C was then resumed and continued for 7 days. At the end of the incubation period, the gel was removed from each well and the cells which had adhered to the base of each well were washed four times with PBS to remove residual gel. The cells were then removed from the wells by trypsinisation and harvested by centrifugation before re-suspension in 1ml of DMEM. Four cell counts were performed per well using a haemocytometer and the data meaned. Each mean value was inserted into equation 2.7 for computation of the Cell Proliferation Inhibition Index (CPI Index) shown below. This is the percentage cell proliferation relative to the negative control cultures and gives an indication of the degree of cytotoxicity of microsphere and control preparations.

Equation 2.7. To calculate the Cell Proliferation Inhibition (CPI) Index values.

$$\text{CPI Index (\%)} = 100\% - (\text{Test culture mean cell count} / \text{Control culture mean cell count} \times 100\%)$$

Triplicate assays of each biomaterial produced a CPI Index \pm SD, n=3. Comparisons of the relative cytotoxicity of each microsphere preparation together with their cytotoxicity compared with both the negative and positive controls were then made.

2.13. Statistical Analysis.

Where relevant, mean data obtained from the present experimental work were compared with each other statistically using Student's unpaired two-tailed t-test at 95% confidence limits (Statworks® for the Macintosh). Sets of data derived from three experimental determinations (n=3) were accompanied by the standard deviation (SD) whereas sets of data derived from four or more experimental determinations (n \geq 4) were accompanied by the standard error of the mean (SEM).

Chapter 3: The physico-chemical properties of unloaded microspheres, 20% w/w antibiotic loaded microspheres and the raw materials used in their fabrication.

3.1. Introduction.

During the development of a pharmaceutical drug delivery device such as microspheres, it is important to choose a manufacturing method which ensures that key physico-chemical parameters such as drug encapsulation efficiency and mean microsphere diameter have batch to batch reproducibility. Reducing any inter-batch variation in the physico-chemical characteristics of the device will help to ensure the development of reproducible *in vitro* and *in vivo* drug release kinetics and will ease the transition from bench manufacture to pilot scale-up manufacture.

3.2. Methods.

The physico-chemical characteristics of unloaded microspheres and 20% w/w antibiotic loaded microspheres and, to a limited extent, the raw materials used in their fabrication were determined using methods documented in chapter 2. Microsphere % yield (as well as the examination of emulsion droplet formation (section 2.2.1.); microencapsulation efficiency (section 2.2.2.); microsphere mean diameter and size distribution (section 2.2.3); microsphere morphology (surface topography and internal architecture) (section 2.2.4.); the concentration of residual solvents in freshly fabricated microspheres (section 2.2.5.) and changes in antibiotic melting point, polymer weight average molecular mass and glass transition temperature before and after incorporation into microspheres (sections 2.2.5. and 2.2.6.) were all measured. Typically, unloaded microspheres and 20% w/w antibiotic loaded microspheres were fabricated using an emulsification speed of 4,000 rpm. However, 20% w/w vancomycin loaded microspheres were also fabricated using an

emulsification speed of 1,000rpm, 2,000rpm, 6,000 rpm and 8,000rpm in order to assess the effect of changing the emulsification speed on microsphere mean percentage yield, microencapsulation efficiency, and mean volume diameter.

3.3. Results.

3.3.1. Mean percentage yield of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres.

Microspheres, whether unloaded or 20% w/w antibiotic loaded, were routinely fabricated in four separate Batches and then pooled to make a single Group. Validation of the reproducibility of the percentage yield of microspheres fabricated using the W/O emulsification with solvent evaporation technique was confirmed using statistical analysis. Representative mean percentage yields for individual batches and pooled groups of unloaded and 20% w/w antibiotic loaded microspheres, including 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds ranging from 1,000 rpm to 8,000 rpm, are shown in tables 3.1. to 3.5. below.

Table 3.1. Mean percentage yields of 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 4,000rpm (mean values \pm SEM, n=4).

Group	Mean % yield
A (4 batches)	97.30 \pm 0.38a
B (4 batches)	96.99 \pm 2.84a
C (4 batches)	94.72 \pm 4.32
D (4 batches)	91.42 \pm 5.15

a Yields were significantly greater ($P < 0.05$) than for 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of either 6,000 rpm or 8,000 rpm (group A) (see table 3.2. below).

Table 3.2. Mean percentage yields of 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds between 1,000 rpm and 8,000 rpm (mean values \pm SEM, n=4).

Speed of emulsification (rpm)	% Yield
1,000 (4 batches)	97.11 \pm 2.45a
2,000 (4 batches)	98.50 \pm 1.24a
6,000 (4 batches)	91.17 \pm 0.40b
8,000 Group A (4 batches)	83.27 \pm 1.97
8,000 Group B (4 batches)	89.34 \pm 2.93

a Yields were significantly greater ($P < 0.05$) than for 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of either 6,000 rpm or 8,000 rpm.

b Yield was significantly greater ($P < 0.05$) than for 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 8,000 rpm (group A).

Table 3.3. Mean percentage yields of 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SEM, n=4).

Group	% Yield
A (4 batches)	96.57 \pm 2.25
B (4 batches)	96.90 \pm 0.33
C (4 batches)	98.22 \pm 1.61
D (4 batches)	96.09 \pm 0.25

Table 3.4. Mean percentage yields of 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SEM, n=4 unless stated otherwise).

Group	% Yield
A (6 batches)	81.18 \pm 1.14 (n=6)
B (4 batches)	77.90 \pm 3.04
C (4 batches)	75.92 \pm 5.10
D (4 batches)	76.22 \pm 0.78

Table 3.5. Mean percentage yields of unloaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SEM, n=4).

Group	% Yield
A (4 batches)	94.18 \pm 2.78
B (4 batches)	90.17 \pm 3.85
C (4 batches)	98.57 \pm 1.94
D (4 batches)	88.99 \pm 0.96

In general terms, both unloaded and 20% w/w antibiotic loaded microspheres were fabricated in reproducibly high mean percentage yields (75-99%) using the W/O emulsification with solvent evaporation technique. 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000rpm were produced in very similar mean percentage yields (approximately 96.5%) which were greater than those of both unloaded microspheres (approximately 93%) and 20% w/w rifampicin loaded microspheres (approximately 78%) fabricated using an emulsification speed of 4,000rpm. It is clear that increasing the emulsification speed during the fabrication of 20% w/w vancomycin loaded microspheres

caused a significant decrease in the mean percentage yield of microspheres. This trend will be discussed later.

3.3.2. Mean encapsulation efficiencies of 20% w/w antibiotic loaded PLCG 75:25 microspheres.

The determination of mean microencapsulation efficiency (see section 2.2.2.) was carried out after pooling four Batches of like-microspheres to form a Group. A 0.25g sample of microspheres was removed from the group and assayed to provide four determinations of microsphere encapsulation efficiency. The encapsulation efficiency of some unpooled batches were assayed in an identical fashion. In order to validate the W/O protocol in terms of the encapsulation of a reproducible concentration of antibiotic within each type of microsphere, and to determine whether changes in emulsification speed affected the encapsulation efficiency of 20% w/w vancomycin loaded microspheres, the encapsulation efficiencies obtained from assaying individual batches and groups of microspheres loaded with a particular antibiotic were analysed statistically. Mean encapsulation efficiency data for 20% w/w vancomycin loaded microspheres are shown in tables 3.6. below.

Table 3.6. Mean encapsulation efficiencies for 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds between 1,000rpm and 8,000 rpm (mean values \pm SEM, n=4 unless stated otherwise).

Emulsification speed (rpm)	Group	Mean Encapsulation Efficiency (%)
1,000	A (4 batches)	97.20 \pm 5.26
2,000	A (4 batches)	96.95 \pm 4.25
4,000	A (4 batches)	92.47 \pm 1.00, n=12
	B (4 batches)	71.08 \pm 1.99a
	C (4 batches)	88.16 \pm 7.58
	D (4 batches)	90.54 \pm 4.56
6,000	A (4 batches)	89.91 \pm 1.74
8,000	A (4 batches)	94.15 \pm 1.83
	B (4 batches)	96.63 \pm 0.96

a Mean encapsulation efficiency was significantly lower ($P < 0.05$) than for other groups of 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed ranging from 1,000 rpm to 8,000 rpm.

Table 3.7. below contains encapsulation efficiency data for 20% w/w ciprofloxacin and 20% w/w rifampicin loaded microspheres.

Table 3.7. Mean encapsulation efficiencies for 20% w/w ciprofloxacin and 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000rpm (mean values \pm SEM, n=4 unless otherwise stated).

Encapsulated antibiotic	Group	Mean Encapsulation Efficiency (%)
Ciprofloxacin	A (4 batches)	79.39 \pm 0.99
	B (4 batches)	74.38 \pm 2.88
	C (4 batches)	76.98 \pm 2.70
	D (4 batches)	88.96 \pm 1.83, n=12a
Rifampicin	A (4 batches)	8.51 \pm 0.01, n=6b†
	B (4 batches)	8.13 \pm 0.01, n=6b†
	C (4 batches)	10.85 \pm 0.07, n=6b†
	D (4 batches)	13.37 \pm 0.19, n=12b†

a Mean encapsulation efficiency was significantly greater ($P < 0.05$) than for other groups of 20% w/w ciprofloxacin loaded microspheres.

b Significant differences ($P < 0.05$) in encapsulation efficiency with the rank order (highest encapsulation efficiency to lowest encapsulation efficiency) $D > C > A > B$.

† Mean encapsulation efficiency was significantly lower ($P < 0.05$) than for all groups of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres.

Table 3.8. below contains data for the mean encapsulation efficiency of individual batches of 20% w/w antibiotic loaded microspheres.

Table 3.8. Mean encapsulation efficiencies for individual batches of 20% w/w antibiotic loaded microspheres fabricated using an emulsification speed of 4,000rpm.

Encapsulated antibiotic	Batch number	Mean Encapsulation Efficiency (%)
Vancomycin (Group A)	1	93.61± 4.57, SD n=3
	2	89.80 ± 4.75, SD n=3
	3	93.61 ± 0.00, SD n=3
	4	92.85 ± 1.32, SD n=3
Ciprofloxacin (Group D)	1	95.833± 2.887, SD n=3 ^a
	2	88.333±5.774, SD n=3
	3	88.333±6.292, SD n=3
	4	83.333±1.443, SD n=3
Rifampicin (Group D)	1	12.61 ± 0.11, SEM n=6
	2	14.22 ± 0.04, SEM n=6 ^b
	3	12.37 ± 0.06, SEM n=6
	4	14.28 ± 0.04, SEM n=6 ^b

a Mean encapsulation efficiency was significantly greater ($P<0.05$) than for other batches of 20% w/w ciprofloxacin loaded microspheres.

b Mean encapsulation efficiency was significantly greater ($P<0.05$) than for other batches of 20% w/w rifampicin loaded microspheres.

The mean encapsulation efficiency of 20% w/w vancomycin loaded microspheres was not significantly affected ($P<0.05$) by the emulsification speed employed during their fabrication. The mean encapsulation efficiency of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000 rpm were similar. There were significant variations ($P<0.05$) in the mean encapsulation efficiency of both batches and groups of 20% w/w rifampicin loaded microspheres. However, the mean encapsulation efficiencies of batches

and groups of 20% w/w rifampicin loaded microspheres were significantly less ($P < 0.05$) than the mean encapsulation efficiencies of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres.

3.3.3. Determination of the mean volume diameter, size distribution and emulsion droplet size of unloaded microspheres and 20% w/w antibiotic loaded microspheres fabricated using a range of emulsification speeds.

3.3.2.a Determination of Microsphere Mean Volume Diameter.

Validation of the W/O emulsification with solvent evaporation technique in terms of the fabrication of unloaded and 20% w/w antibiotic loaded microspheres with reproducible mean volume diameters together with the identification of any changes in the mean volume diameter of 20% w/w vancomycin loaded microspheres resulting from alteration of the emulsification speed used in their fabrication was possible using the method for particle sizing described in section 2.2.3. Samples of microspheres were taken from both individual batches and groups (containing four batches) and were measured using the Malvern 'Mastersizer' which generated a mean volume diameter and size distribution profile for each sample. The mean volume diameter for each group of 20% w/w vancomycin loaded microspheres are shown in table 3.9. below. The mean volume diameter for groups of unloaded microspheres, 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres are shown in table 3.10. below. The mean volume diameter for individual batches of unloaded microspheres and 20% w/w antibiotic loaded microspheres are shown in table 3.11. below.

Table 3.9. The mean volume diameter for groups of 20% w/w vancomycin loaded microspheres fabricated using a range of emulsification speeds.

Emulsification speed (rpm)	Group (containing four batches)	Mean Volume Diameter \pm SD in μm , after a known number of laser passes []
1,000	A	71.00 \pm 3.69 [5,000]
2,000	A	48.59 \pm 29.91 [2,000]
4,000	A	59.53 \pm 4.73, n=4a
	B	31.90 \pm 15.42 [2,000]
	C	35.29 \pm 24.24 [5,000]
	D	53.50 \pm 27.89 [2,000]
6,000	A	42.43 \pm 30.23 [2,000]
8,000	A	42.22 \pm 29.99 [2,000]
	B	47.69 \pm 28.75 [2,000]

a The average mean volume diameter after measuring each individual batch in a group - see table 3.11.)

Table 3.10. The mean volume diameter for groups of unloaded microspheres, 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000rpm.

PLCG 75:25 microsphere type	Group (containing four batches)	Mean Volume Diameter \pm SD (μm) after a known number of laser passes []
20% w/w ciprofloxacin loaded	A	22.96 \pm 12.75 [2,000]
	B	25.78 \pm 11.23 [2,000]
	C	22.30 \pm 16.49 [2,000]
	D	45.84 \pm 4.18, n=4a
20% w/w rifampicin loaded	A	14.17 \pm 12.16 [2,000]
	B	20.98 \pm 15.73 [2,000]
	C	12.05 \pm 6.92 [5,000]
	D	34.55 \pm 2.59, n=4a
unloaded	A	47.86 \pm 3.65, n=4a
	B	37.67 \pm 29.89 [2,000]
	C	39.11 \pm 30.91 [2,000]
	D	27.18 \pm 10.21 [2,000]

a The average mean volume diameter after measuring each individual batch in a group - see table 3.11.)

b Mean volume diameter was significantly greater ($P < 0.05$) than for unloaded microspheres, 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres.

c Mean volume diameter was significantly smaller ($P > 0.05$) than for unloaded microspheres, 20% w/w rifampicin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres.

Table 3.11. The mean volume diameter for individual batches of unloaded microspheres and 20% w/w-antibiotic loaded microspheres fabricated using an emulsification speed of 4,000rpm.

Type of PLCG 75:25 microsphere (Group in parenthesis)	Batch number	Mean Volume Diameter \pm SD (μ m) after 2,000 laser passes
20% w/w vancomycin loaded (A)	1	62.31 \pm 13.76
	2	63.07 \pm 14.46
	3	60.01 \pm 17.03
	4	52.71 \pm 18.56
	Mean diameter (μm) \pmSD, n=4	59.53 \pm 4.73a
20% w/w ciprofloxacin loaded (D)	1	51.74 \pm 22.23
	2	43.28 \pm 23.07
	3	45.85 \pm 18.73
	4	42.50 \pm 19.06
	Mean diameter (μm) \pmSD, n=4	45.84 \pm 4.18
20% w/w rifampicin loaded (D)	1	31.82 \pm 10.97
	2	33.04 \pm 12.11
	3	35.81 \pm 12.23
	4	37.52 \pm 16.70
	Mean diameter (μm) \pmSD, n=4	34.55 \pm 2.59b
unloaded	1	52.99 \pm 17.01
	2	46.71 \pm 17.88
	3	44.38 \pm 16.31
	4	47.36 \pm 28.06
	Mean diameter (μm) \pmSD, n=4	47.86 \pm 3.65

a Mean volume diameter was significantly greater ($P < 0.05$) than for unloaded microspheres, 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres.

b Mean volume diameter was significantly smaller ($P > 0.05$) than for unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres.

Microspheres (figure 3.10) and 20% w/w ciprofloxacin loaded microspheres (figure 3.11) fabricated using an emulsification speed of 4,000 rpm, showed size distributions (SD) were skewed in favour of larger diameter microspheres. The size distribution of 20% w/w rifampicin loaded microspheres was somewhat bimodal with a majority of microspheres in the 12.21-52.68 μ m size range.

3.3.3.b Determination of Microsphere Size Distribution

The size distribution data suggest that the mean volume diameter of 20% w/w vancomycin loaded microspheres is inversely proportional to the speed of emulsification used in their fabrication and that the group mean volume diameters for particular preparations of microspheres fabricated using an emulsification speed of 4,000 rpm were not significantly different ($P > 0.05$). This confirmed that microspheres with a reproducible mean volume diameter could be generated using the W/O emulsification with solvent evaporation technique. Calculations of the mean volume diameter for all batches within a group (see table 3.11.) facilitated the ranking of mean microsphere diameter in order the order of largest to smallest mean volume diameter as follows: 20% w/w vancomycin loaded microspheres > 20% w/w ciprofloxacin loaded microspheres = unloaded microspheres > 20% w/w rifampicin loaded microspheres.

Typical size distribution profiles for unloaded microspheres and 20% w/w antibiotic loaded PLCG microspheres generated by the 'Mastersizer' equipment are shown in figure 3.1 a) - h). In each case, measurements of the diameter of microspheres were made over the range of approximately 1-80 μ m. During the fabrication of 20% w/w vancomycin loaded microspheres, increasing the emulsification speed from 1,000 rpm through to 4,000 rpm tended to increase the proportion of smaller diameter microspheres (figure 3.1. a) - e)). 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of 6,000 rpm and 8,000 rpm (figure 3.1. d) and e)) showed similar size distributions to 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (figure 3.1.c)). Overall, unloaded microspheres (figure 3.1.h), 20% w/w vancomycin loaded microspheres (figure 3.1.c) and 20% w/w ciprofloxacin loaded microspheres (figure 3.1.f), fabricated using an emulsification speed of 4,000 rpm, showed size distributions which were skewed in favour of larger diameter microspheres. The size distribution of 20% w/w rifampicin loaded microspheres was somewhat biphasic with a majority of microspheres in the 12.21-52.68 μ m size range.

Figure 3.1. Size distribution profiles for 20% w/w antibiotic loaded microspheres and unloaded microspheres (μm).

Figure 3.1.a) 20% w/w vancomycin loaded microspheres fabricated using an emulsified speed of 1,000 rpm (one determination).

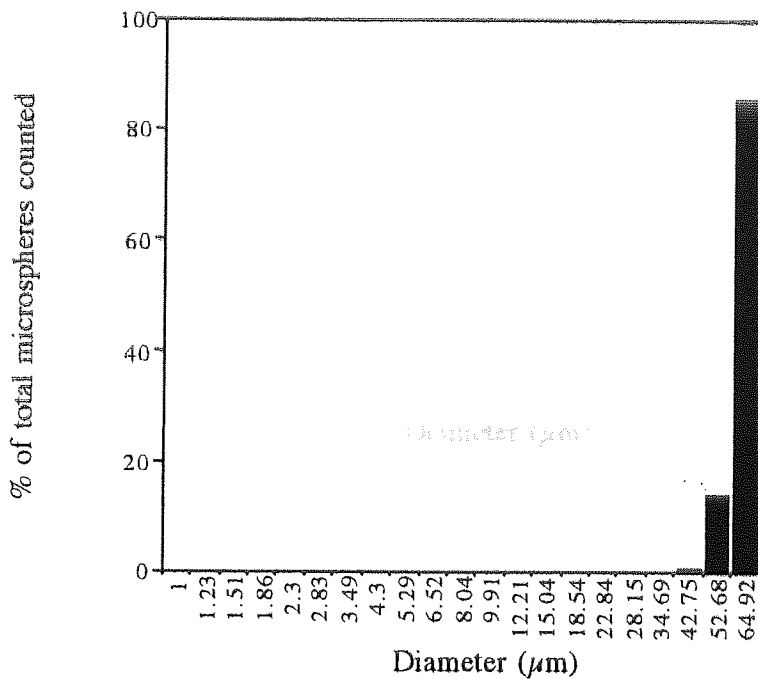


Figure 3.1.b) 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 2,000 rpm (one determination).

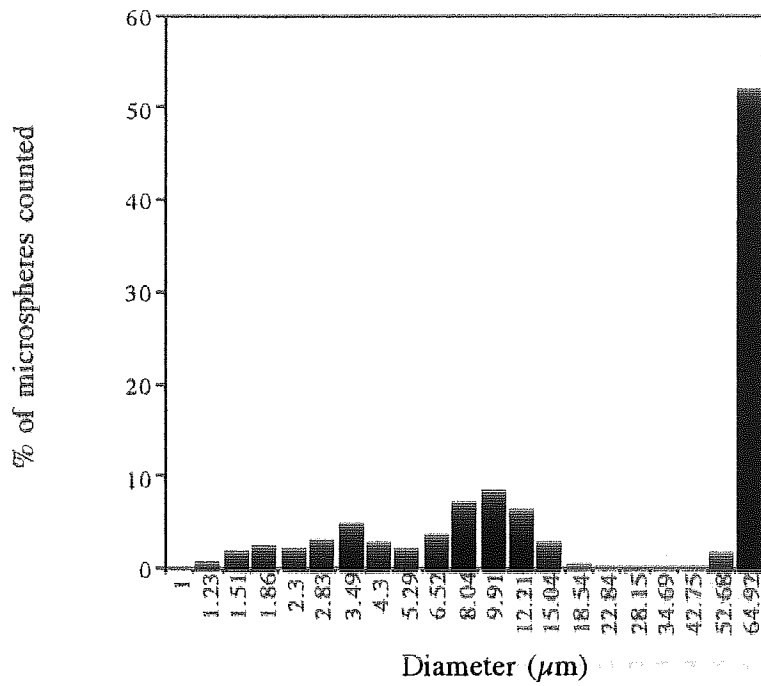


Figure 3.1.c) 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SD, n=7 for each point).

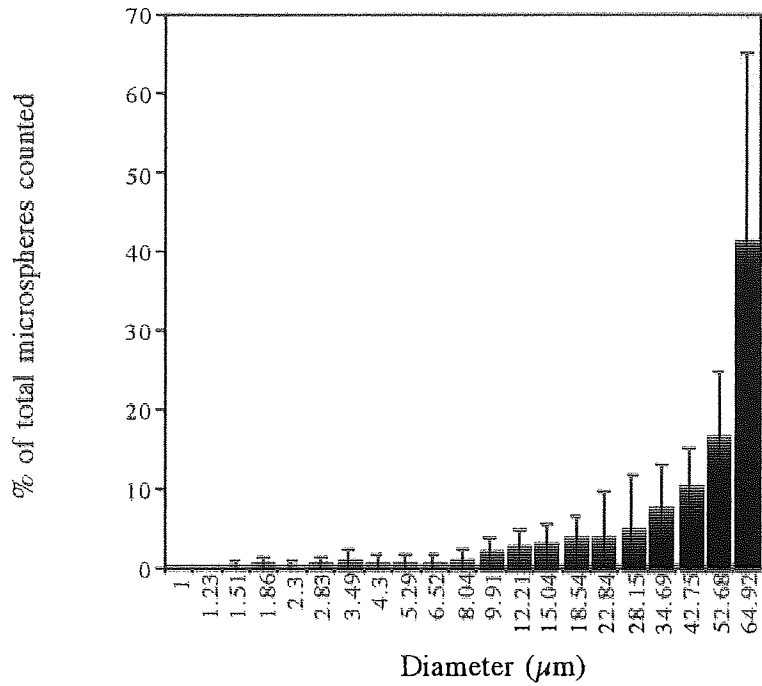


Figure 3.1.d) 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 6,000 rpm (one determination).

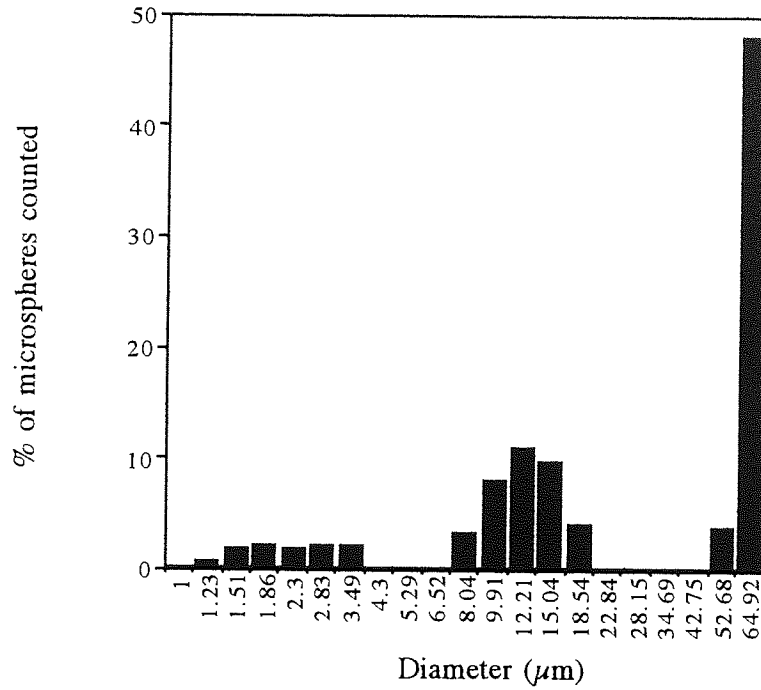


Figure 3.1.e) 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 8,000 rpm (mean values, n=2).

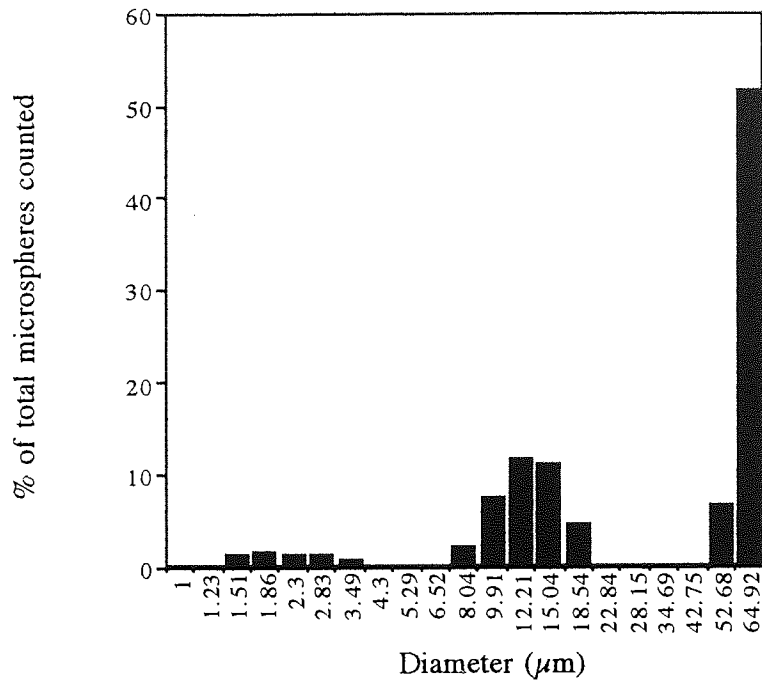


Figure 3.1. f) 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SD, n=7 for each point).

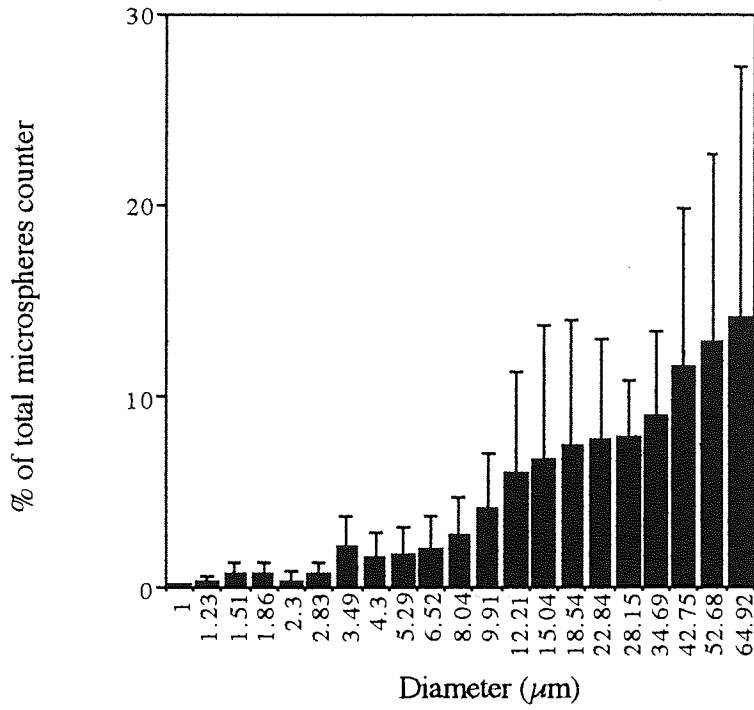
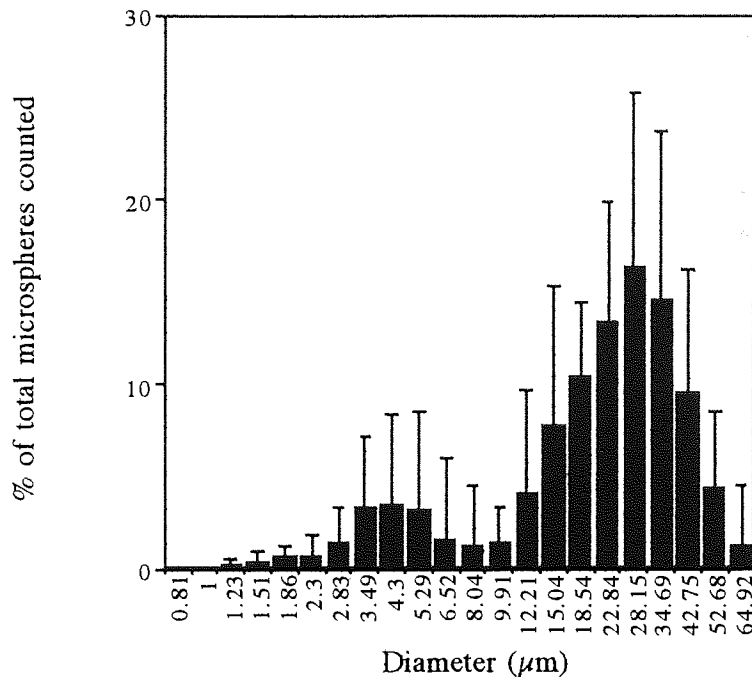
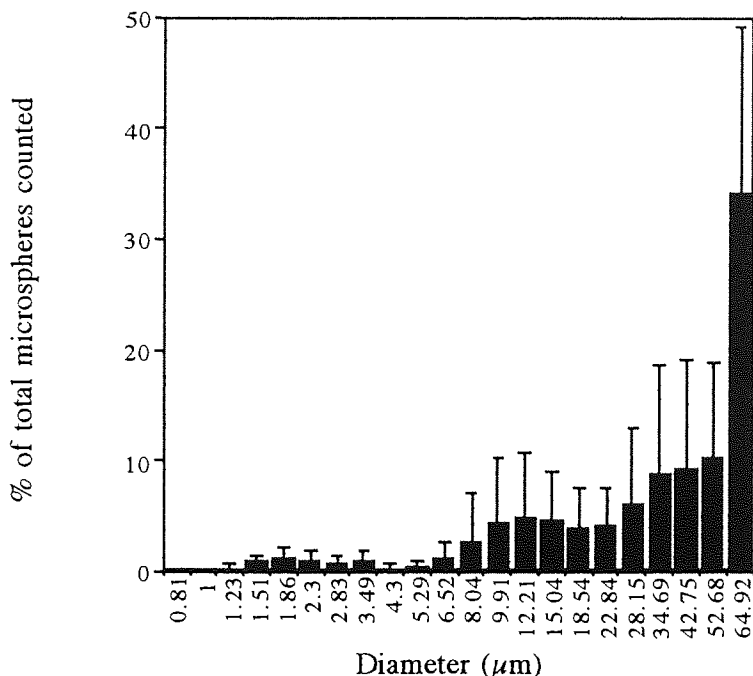


Figure 3.1.g) 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SD, n=7 for each point).



Mean diameter of particles produced during the emulsification procedure of the W/O with solvent evaporation technique was monitored using light microscopy. 3.5 μm photographs were taken during emulsification at a speed of 4,000 rpm, subsequently the emulsion was a control vial and used to calculate mean droplet diameter after microscopy for the magnification used. Enlarged copies of these photographs are shown in figure 3.2.1.) Emulsion droplet diameter decreased with an increase in the time given over to emulsification (between 5 and 20 minutes). The decrease in mean droplet diameter was associated with a narrowing of the size distribution of droplets (figure 3.2.1.). Emulsion droplet diameter was plotted against emulsification time and extrapolation of the mean emulsion droplet diameter (figure 3.2.2.) indicated that the mean theoretical evaporating droplet diameter of all microspheres would have become almost identical after 40 minutes of emulsification at a speed of 4,000 rpm. However, when emulsification at 4,000 rpm was

Figure 3.1.h) Unloaded microspheres fabricated using an emulsification speed of 4,000 rpm (mean values \pm SD., n=6 for each point).



3.3.3.c Mean emulsion droplet diameter calculations.

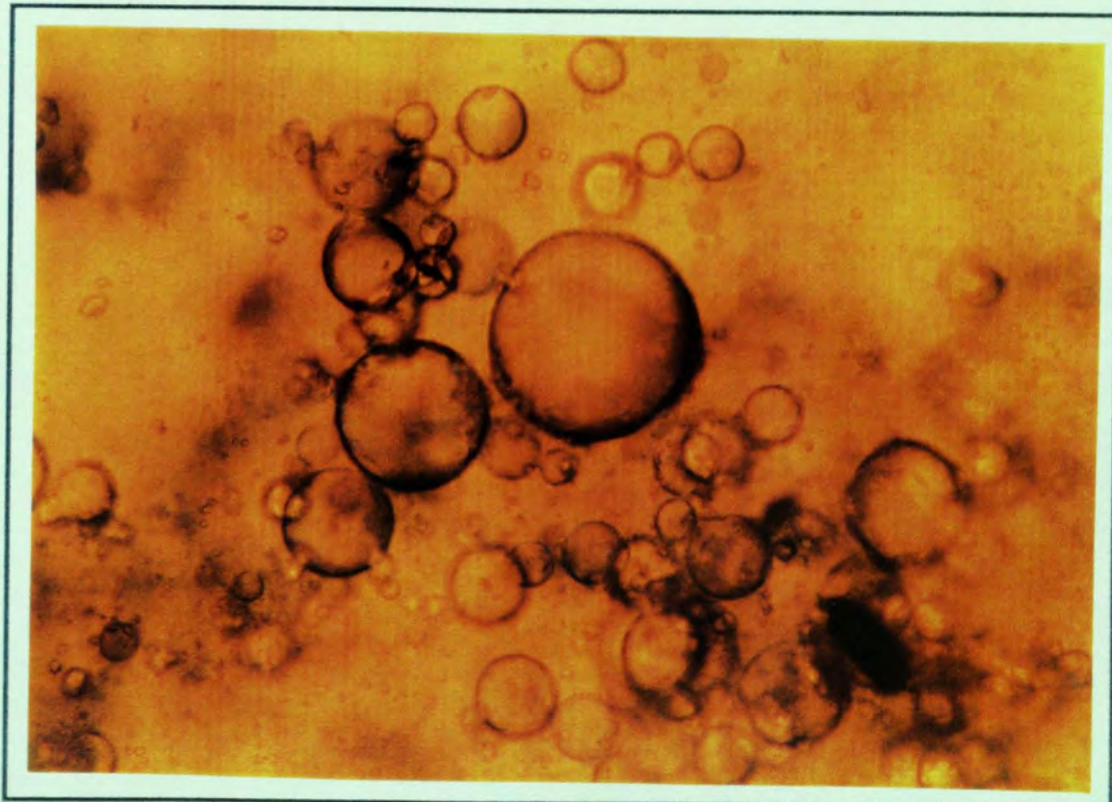
The mean diameter of droplets produced during the emulsification procedure of the W/O with solvent evaporation technique was monitored using light microscopy. 35mm photographs were taken during emulsification at a speed of 4,000 rpm, subsequently developed onto a contact sheet, and used to calculate mean droplet diameter after accounting for the magnification used (enlarged copies of these photographs are shown in figure 3.2.1.). Emulsion droplet diameter decreased with an increase in the time given over to emulsification (between 5 and 30 minutes). The decrease in mean droplet diameter was associated with a narrowing of the size distribution of droplets (figure 3.2.1.). Emulsion droplet diameter was plotted against emulsification time and extrapolation of the mean emulsion droplet diameter (figure 3.2.2.) indicated that the mean theoretical emulsion droplet diameter of all microsphere would have become almost identical after 40 minutes of emulsification at a speed of 4,000 rpm. However, when emulsification at 4,000 rpm was

stopped (after 30 minutes), the mean 20% w/w vancomycin loaded droplet diameter was significantly greater ($P < 0.05$) than that of 20% w/w ciprofloxacin loaded droplets but not significantly different ($P > 0.05$) from the mean diameter of unloaded droplets or 20% w/w rifampicin loaded emulsion droplets. 20% w/w ciprofloxacin loaded droplets were significantly smaller ($P < 0.05$) than both 20% rifampicin and unloaded droplets at this time. Although the measurements of mean droplet diameter were only approximate and only a small number of droplets were measured ($n=10$), these studies suggest that an increase in emulsification time is associated with a decrease in emulsion droplet size providing that the same emulsifier apparatus is used and the emulsification speed remains constant.

Figure 3.2.1. The gradual decrease in emulsion droplet diameter with increases in emulsification time using an emulsification speed of 4,000 rpm (original magnification x500).

Figure 3.2.1 a) 20% w/w vancomycin loaded emulsion droplets:

1) After emulsification for 5 minutes.



2) After emulsification for 30 minutes.



3) After emulsification for 30 minutes.



Figure 3.2.1.b) 20% w/w ciprofloxacin loaded emulsion droplets:

1) After emulsification for 5 minutes.



2) After emulsification for 30 minutes.

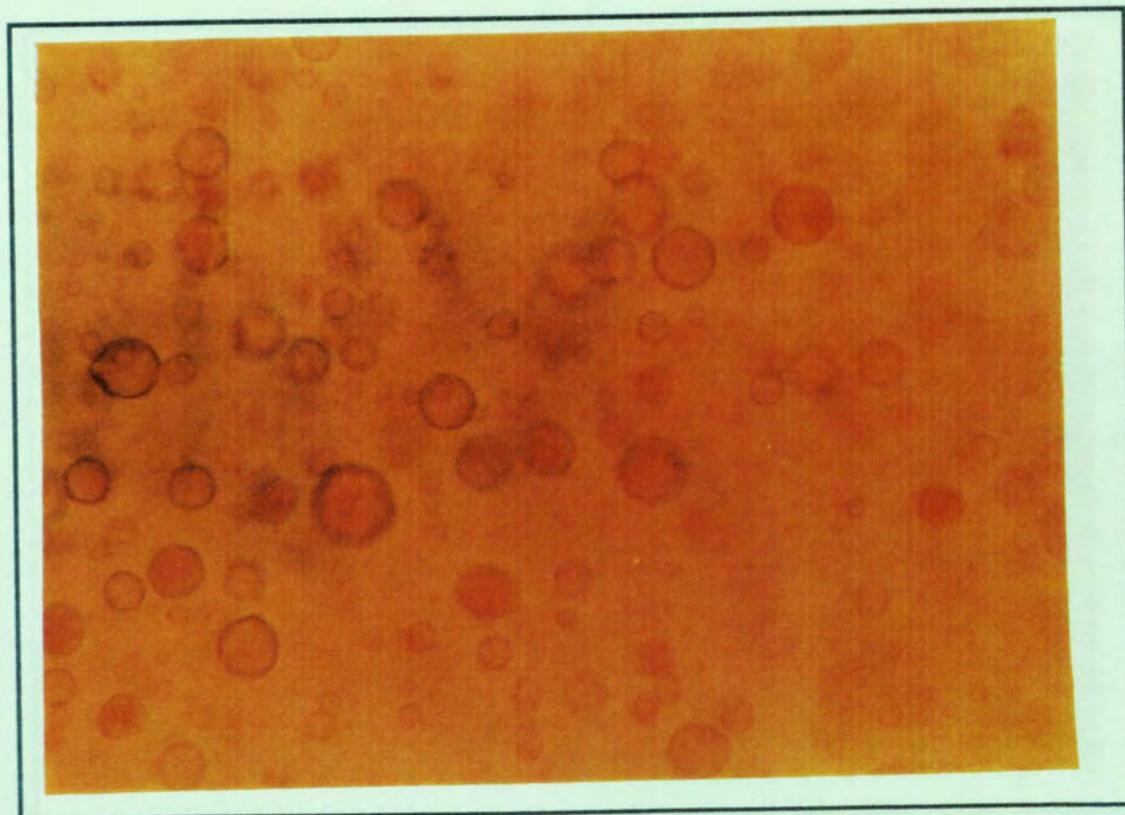


Figure 3.2.1.c) 20% w/w rifampicin loaded emulsion droplets:

1) After emulsification for 5 minutes.



2) After emulsification for 30 minutes.

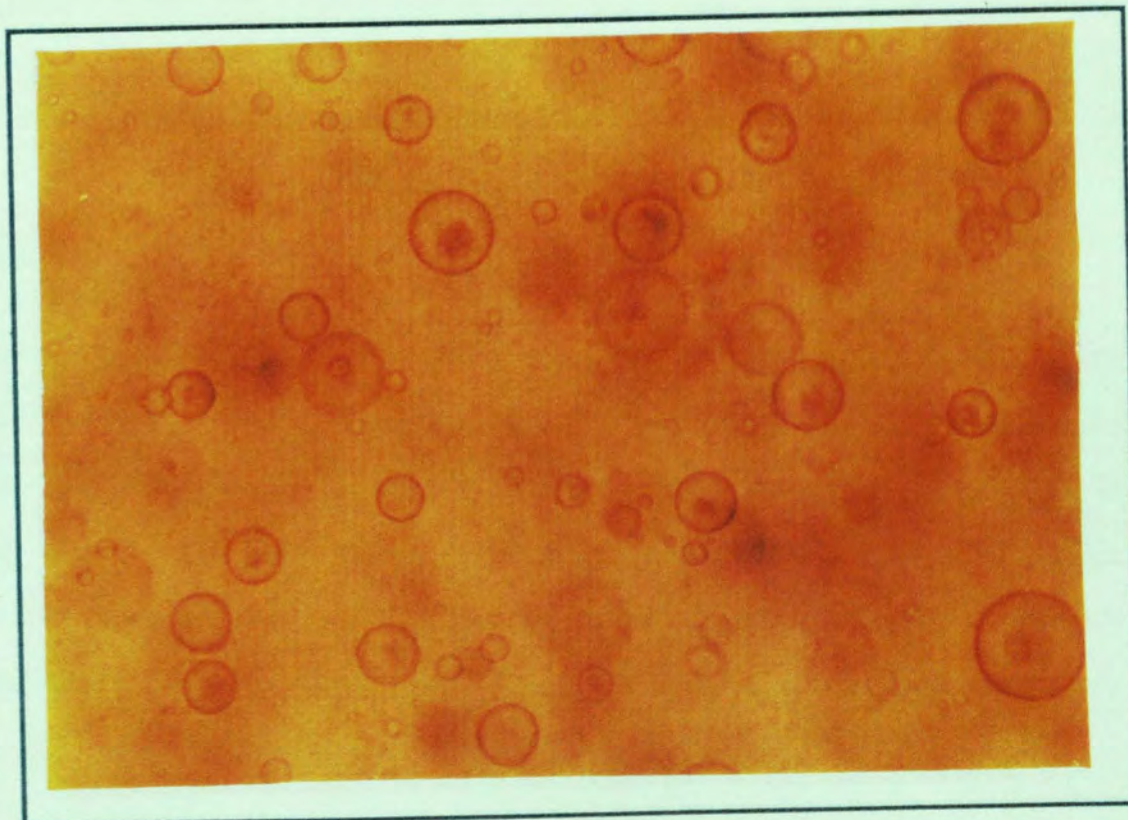
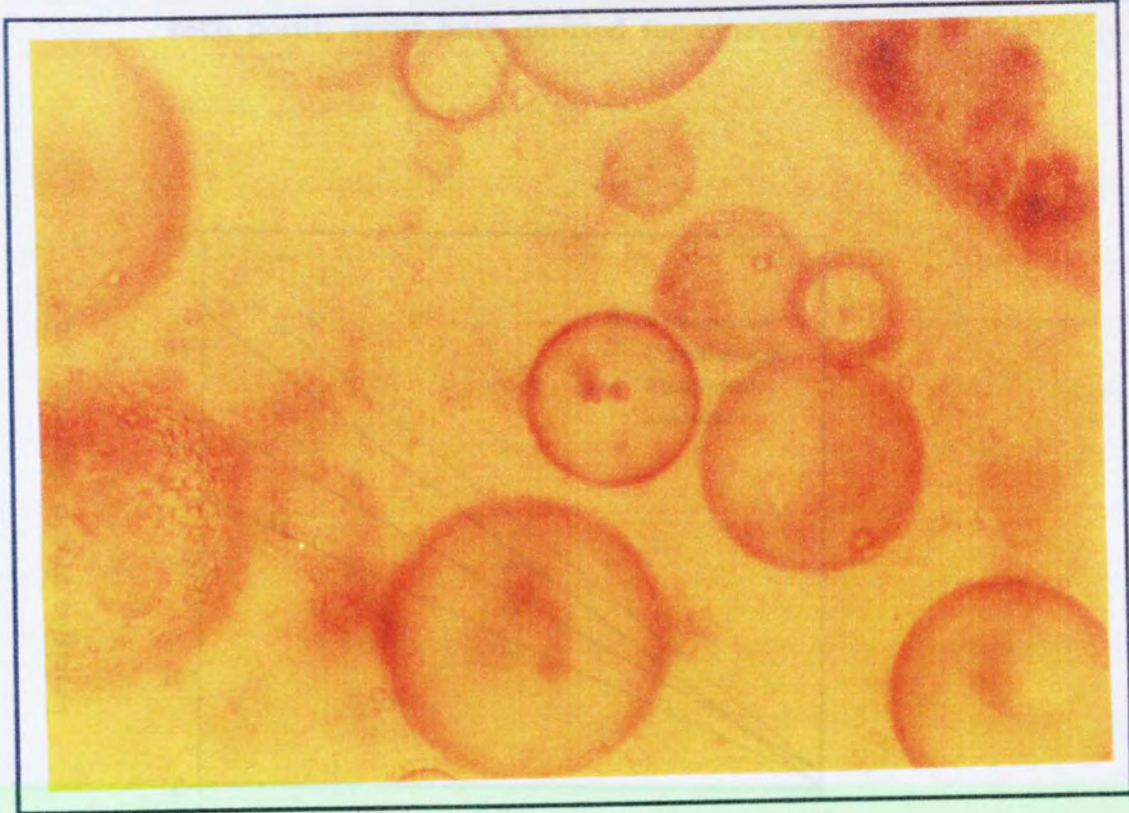


Figure 3.2.1.d) Unloaded emulsion droplets:

- 1) After emulsification for 5 minutes.



- 2) After emulsification for 30 minutes.

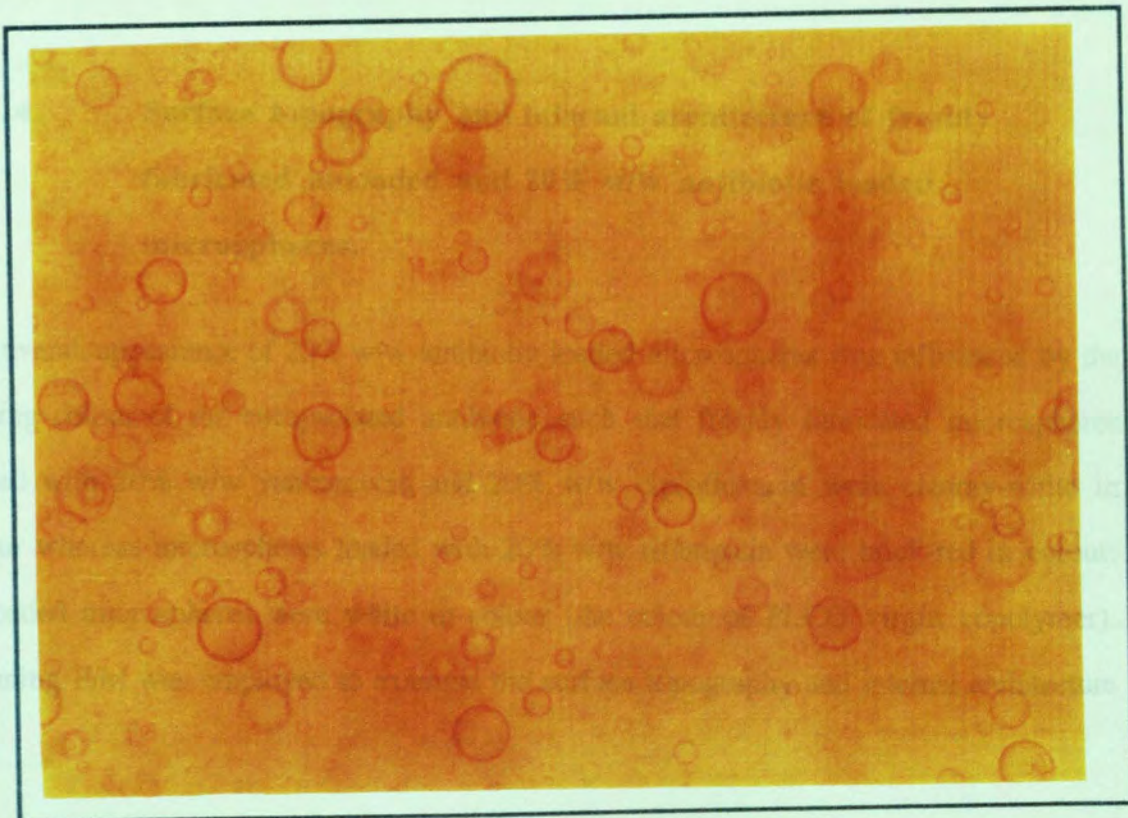
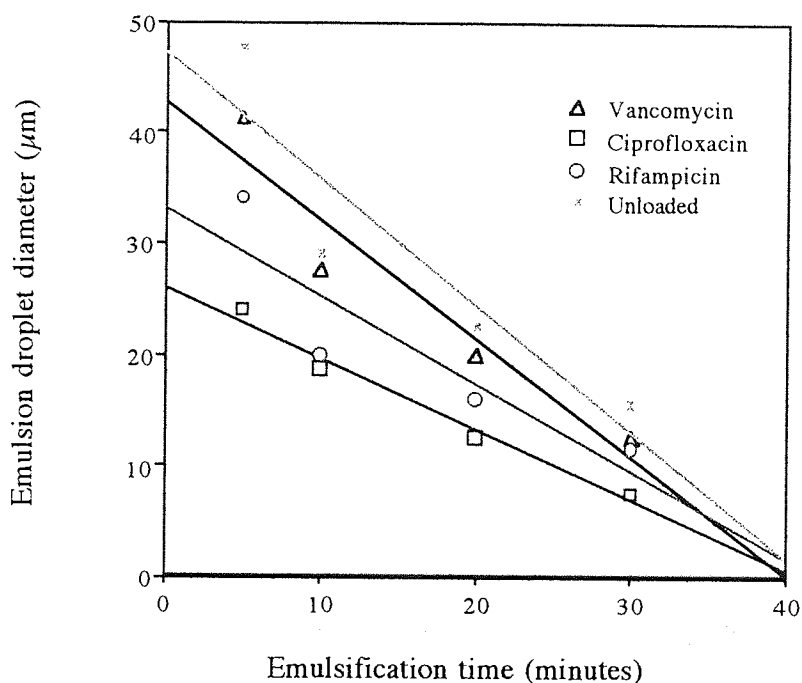


Figure 3.2.2. Changes in the mean diameter of unloaded and 20% w/w antibiotic loaded emulsion droplets during emulsification at a speed of 4,000 rpm (mean values ($\mu\text{m}/\text{minute}$) \pm SD, n=10 for each point, error bars omitted for clarity).



3.3.4. Surface topography and internal architecture of freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres.

The overall appearance of 20% w/w antibiotic loaded microspheres was influenced by the starting colour of the encapsulated antibiotic such that freshly fabricated microspheres loaded with 20% w/w vancomycin and 20% w/w ciprofloxacin were creamy-white in colour whereas microspheres loaded with 20% w/w rifampicin were brick-red in colour. Unloaded microspheres were white in colour (the colour of PLCG virgin copolymer). Scanning E/M was employed to examine the surface topography and internal architecture

of samples of unloaded microspheres and 20% w/w antibiotic loaded microspheres as described in section 2.2.4..

3.3.4.a) Surface Topography of Microspheres

Scanning E/M micrographs of samples of microspheres (figure 3.3.1. a) and b)) revealed that unloaded microspheres and 20% w/w antibiotic loaded microspheres were generally spherical in shape. The external surface of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres was covered with antibiotic crystals although the overall crystal deposition was more extensive on the surface of 20% w/w ciprofloxacin loaded microspheres. Brief washing of both 20% w/w vancomycin and 20% w/w ciprofloxacin loaded microspheres with double distilled water brought about the dissolution of these crystals and revealed pore-like depressions, especially on the surface of 20% w/w ciprofloxacin loaded microspheres (figure 3.3.1. c and d). The external surface of 20% w/w rifampicin loaded microspheres was smooth (figure 3.3.1.e) indicating that all the encapsulated rifampicin was completely sequestered within the internal matrix of the microspheres rather than being partly surface-associated. Unloaded microspheres also had smooth external surfaces.

Figure 3.3.1. The surface topography of freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres.

Figure 3.3.1.a) A 20% w/w vancomycin loaded microsphere (magnification x1,000).

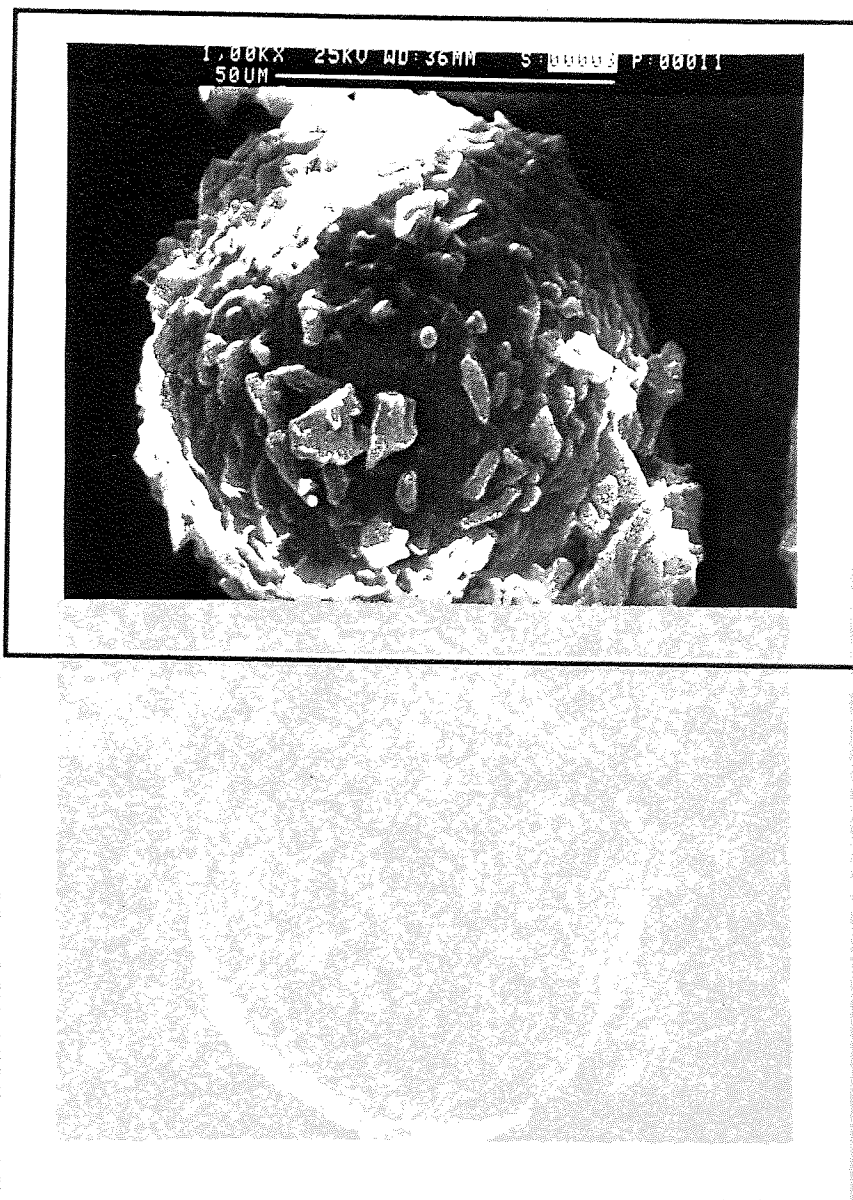


Figure 3.3.1 b) A 20% w/w ciprofloxacin loaded microspheres
(magnification x986).

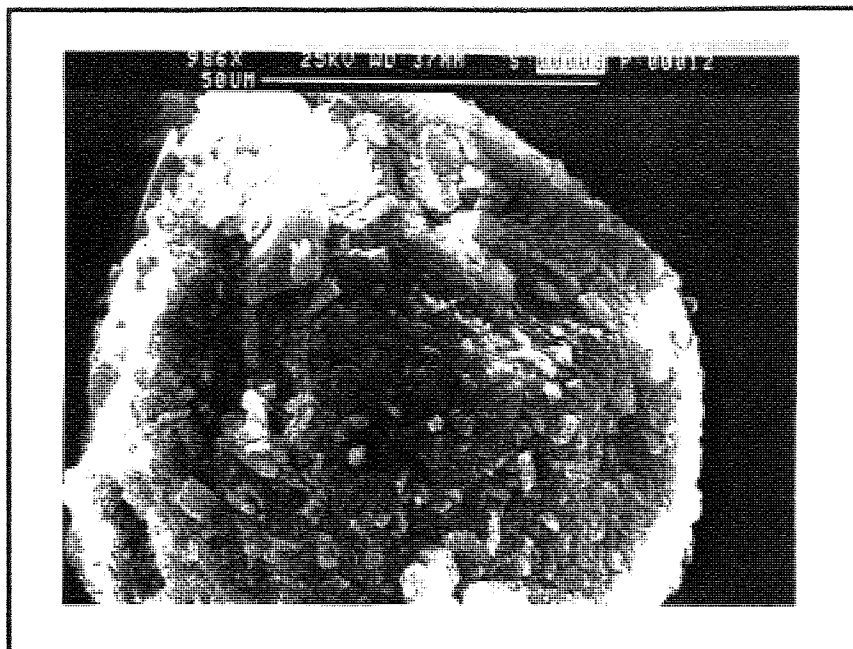


Figure 3.3.1 c) Washed 20% w/w vancomycin loaded microspheres
(magnification x966).

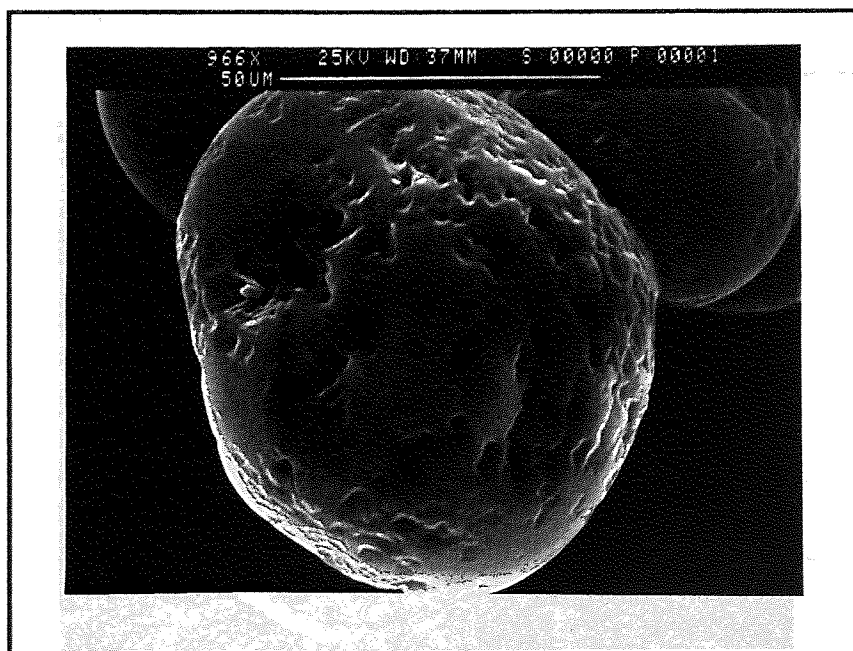


Figure 3.3.1 d) A Washed 20% w/w ciprofloxacin loaded microsphere (magnification x986).

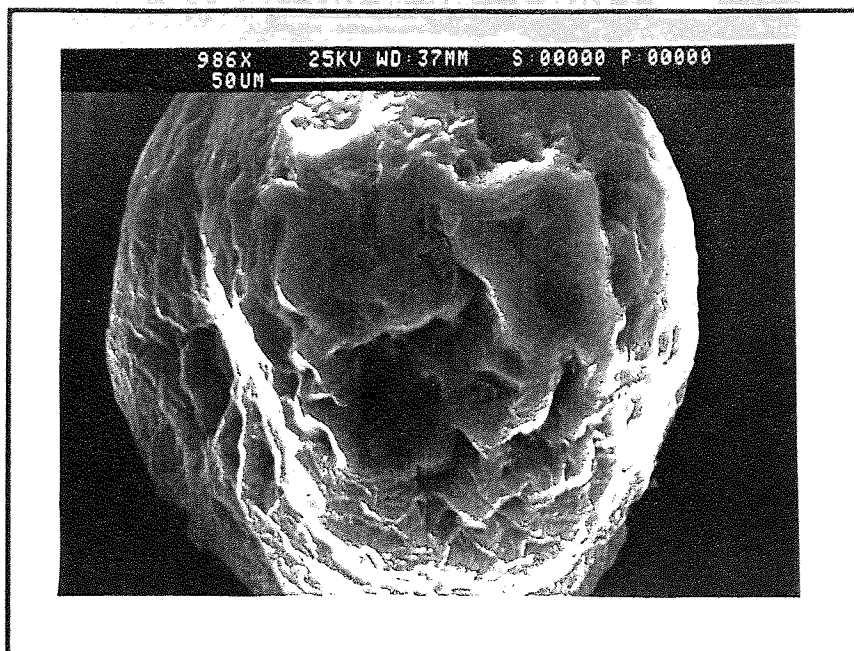


Figure 3.3.1.e) 20% w/w rifampicin loaded microspheres (magnification x1,110).

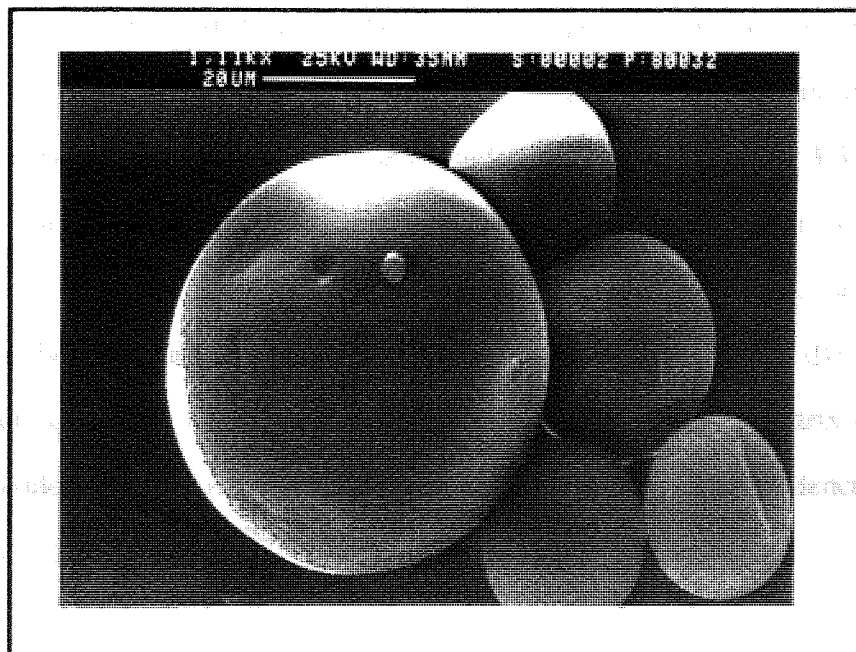
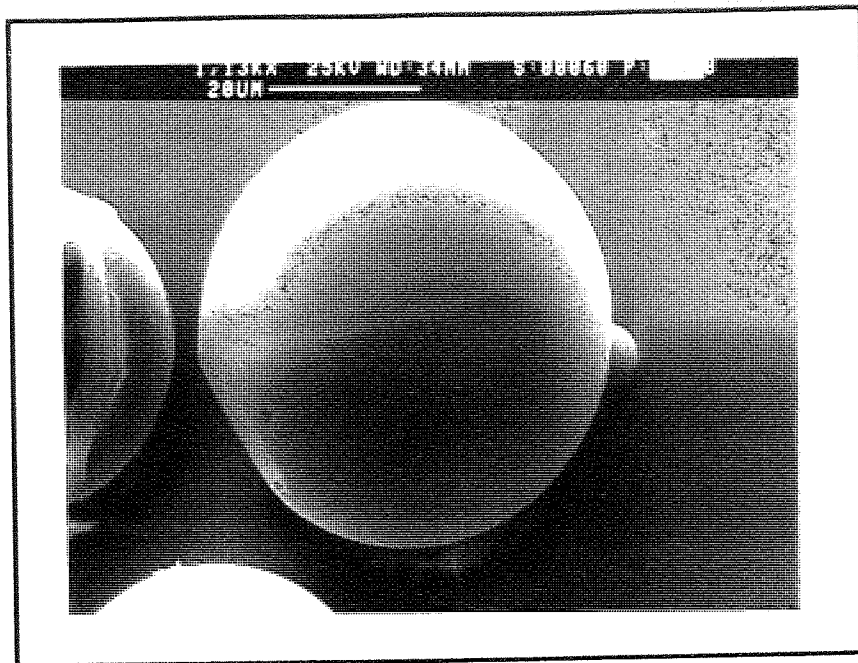


Figure 3.3.1.f) Unloaded microspheres (magnification x1,130).



3.3.4.b Internal Architecture of Microspheres

Scanning E/M was used to examine microsphere sections of unloaded microspheres and 20% w/w antibiotic loaded microspheres embedded in gelatin. The internal architecture of 20% w/w ciprofloxacin loaded microspheres was composed of a honey-comb like matrix surrounded by a peripheral crust (figure 3.3.2.b). The internal matrix of 20% w/w vancomycin loaded microspheres and unloaded microspheres (figure 3.3.2.a) and d) respectively) was less honey-combed and more homogeneous in structure than microspheres loaded with 20% w/w ciprofloxacin. The two large pores and numerous cracks inside the sectioned unloaded microsphere sample (figure 3.3.2. d)) were thought to be artefacts created during the embedding process. The internal matrix of 20% w/w rifampicin loaded microspheres was homogeneous with very little evidence of porosity (figure 3.3.2.c)).

Figure 3.3.2. The internal architecture of sectioned unloaded microspheres and 20% w/w vancomycin loaded microspheres and 20% w/w antibiotic loaded microspheres.

Figure 3.3.2. a) 20% w/w vancomycin loaded microspheres (magnification x800).

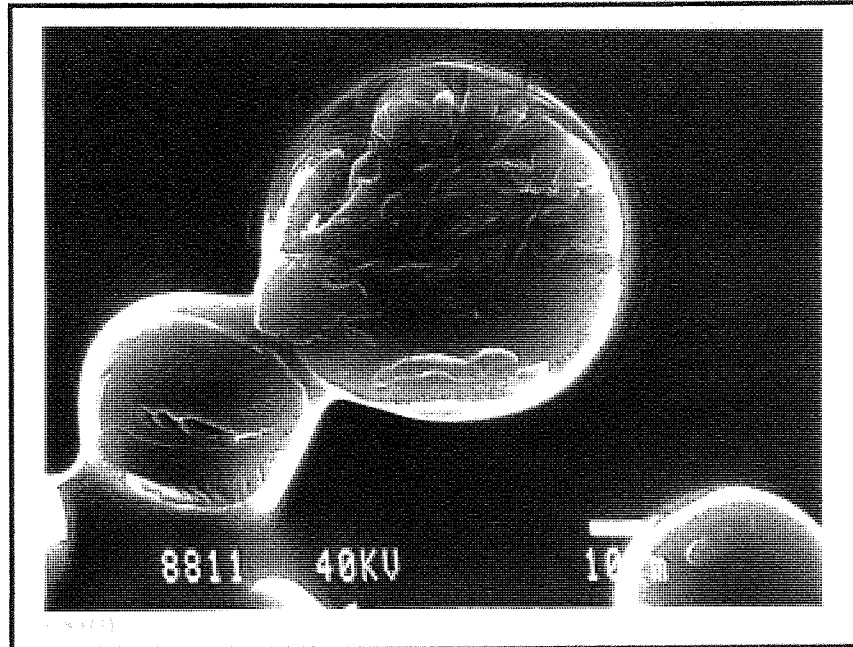


Figure 3.3.2.b) 20% w/w ciprofloxacin loaded microspheres

(magnification x800).

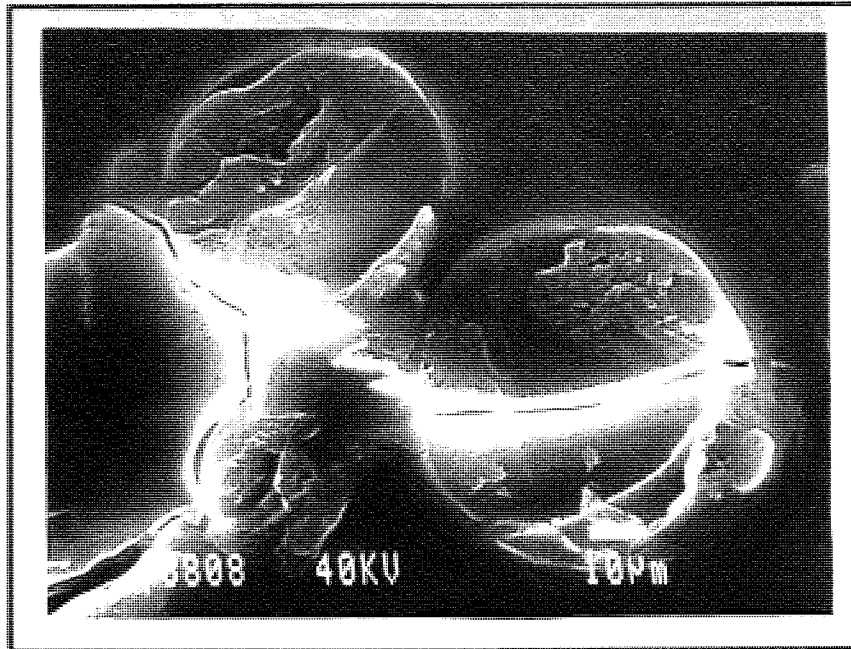


Figure 3.3.2.c) 20% w/w rifampicin loaded microspheres (magnification

x800).

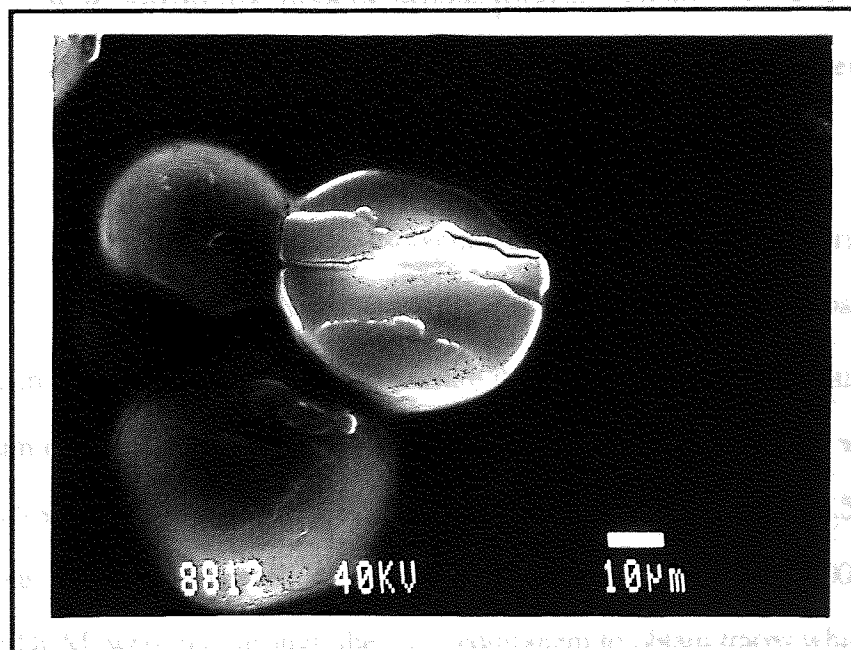
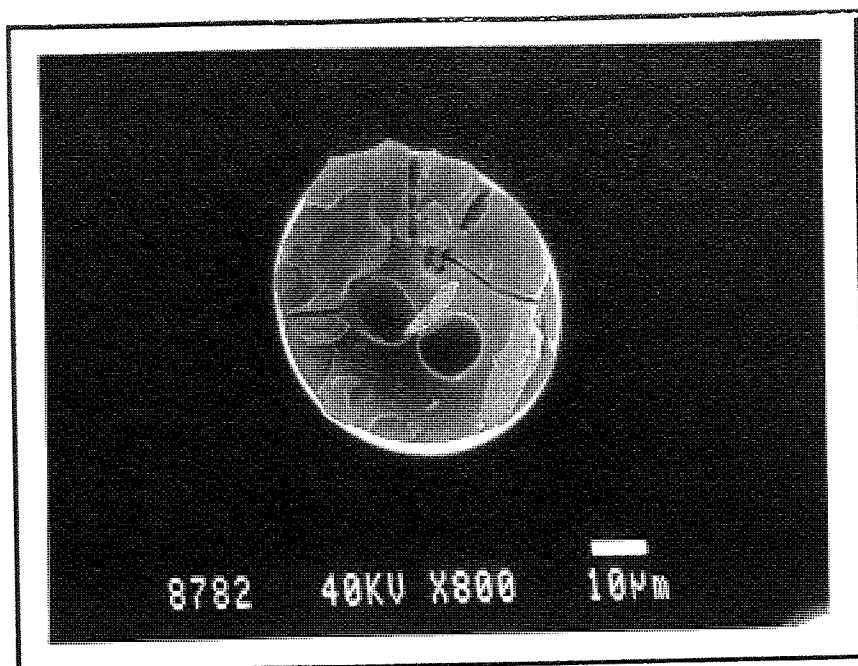


Figure 3.3.2. d) Unloaded microspheres (magnification x800).



3.3.5. Determination of residual solvent concentration in fabricated microspheres and the measurement of the glass transition temperature of freshly fabricated unloaded microspheres, 20% w/w antibiotic loaded microspheres, virgin 75:25 PLCG copolymer and the measurement of antibiotic powder melting point.

3.3.5.a Determination of residual solvent concentration in freshly fabricated unloaded microspheres and 20% w/w antibiotic loaded microspheres.

Gas-liquid chromatography was used to measure the concentrations of residual acetonitrile and petroleum ether (40-60°C) within freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres using the method described in section 2.2.5.. Standard concentrations of acetonitrile (50 -1,000 µl/l) and petroleum ether (200-1,000 µl/l), dissolved in DCM, were put through the GLC equipment to obtain traces which are shown in figure 3.4.. The data obtained from this procedure was used to generate standard curves

of solvent standard concentration against the area under the curve for that solvent, as shown in figure 3.5. below.

Samples of either unloaded microspheres or 20% w/w vancomycin loaded microspheres or 20% w/w ciprofloxacin loaded microspheres dissolved in DCM were assayed in the same way as the standards. Samples collected from each preparation of dissolved microspheres in DCM were used to detect residual concentrations of either acetonitrile or petroleum ether using the GLC apparatus. The concentration of residual solvents entrapped within the microspheres was interpolated from the standard curves for acetonitrile and petroleum ether (figure 3.5.). Representative GLC profiles for each type of microsphere are shown in figure 3.4..

GLC traces obtained for samples derived from either unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres dissolved in DCM showed no detectable peaks corresponding with even the lowest concentration of acetonitrile or petroleum ether standard in DCM. However, the Porapak Q GLC column employed to measure the concentrations of residual solvents provided a minimum sensitivity of $50\mu\text{l/l}$ (50 ppm) for acetonitrile and $200\mu\text{l/l}$ (200 ppm) for petroleum ether (see figure 3.4. a) and b) respectively). These lowest concentrations of the standards were equivalent to actual detectable v/w concentrations for acetonitrile and petroleum ether of $0.025\ \mu\text{l/mg}$ microspheres and $0.100\mu\text{l/mg}$ microspheres respectively. Based on these values, the maximum detectable parts per million concentration of each solvent remaining within the microspheres was calculated as being 25,000 ppm for acetonitrile and 100,000 ppm for petroleum ether. Therefore, results suggest that any residual solvents present in the microspheres were at low concentrations below the sensitivity of the GLC apparatus used.

Figure 3.4. Typical GLC profiles for solvent standards and microsphere samples dissolved in DCM.

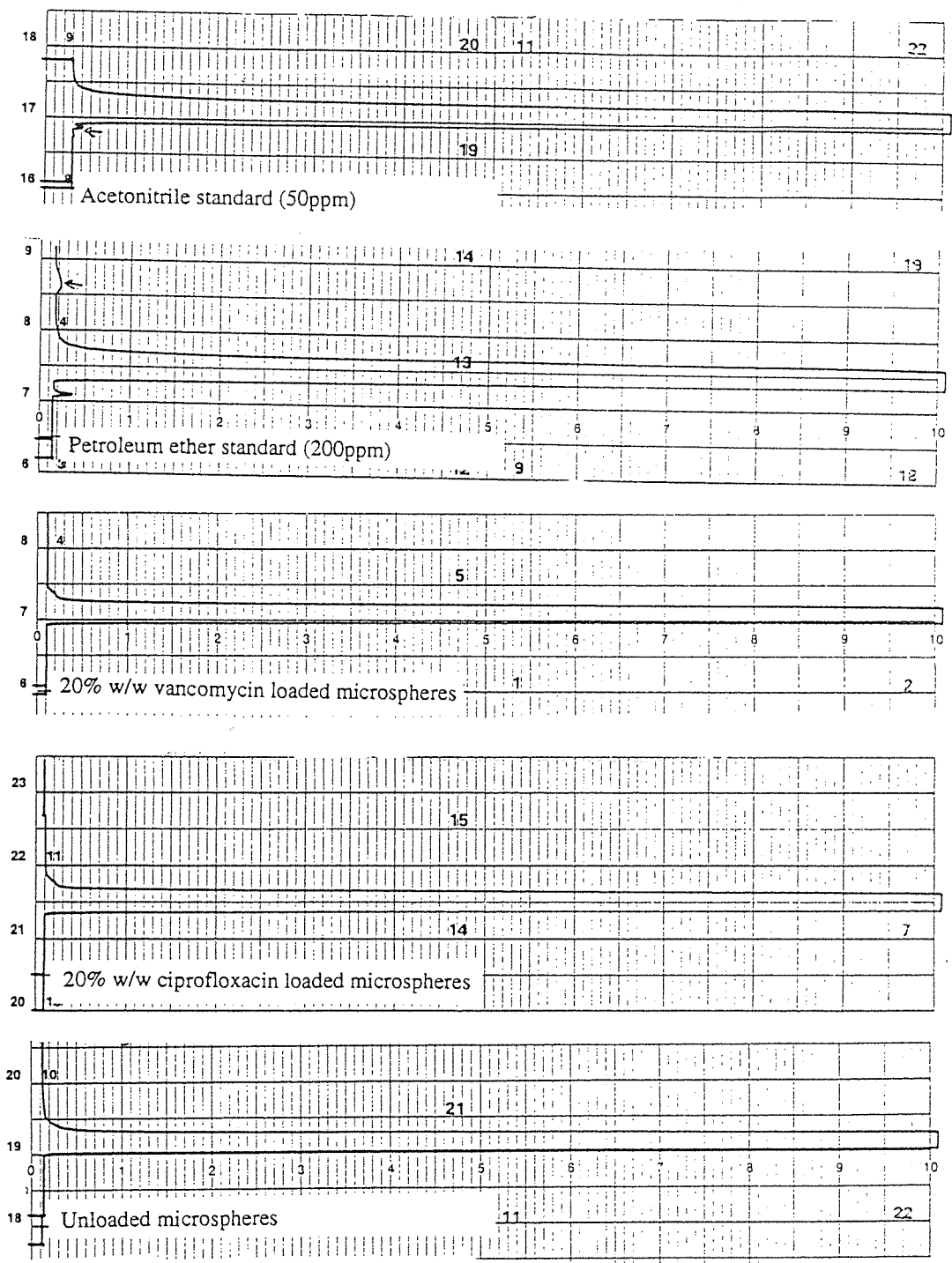
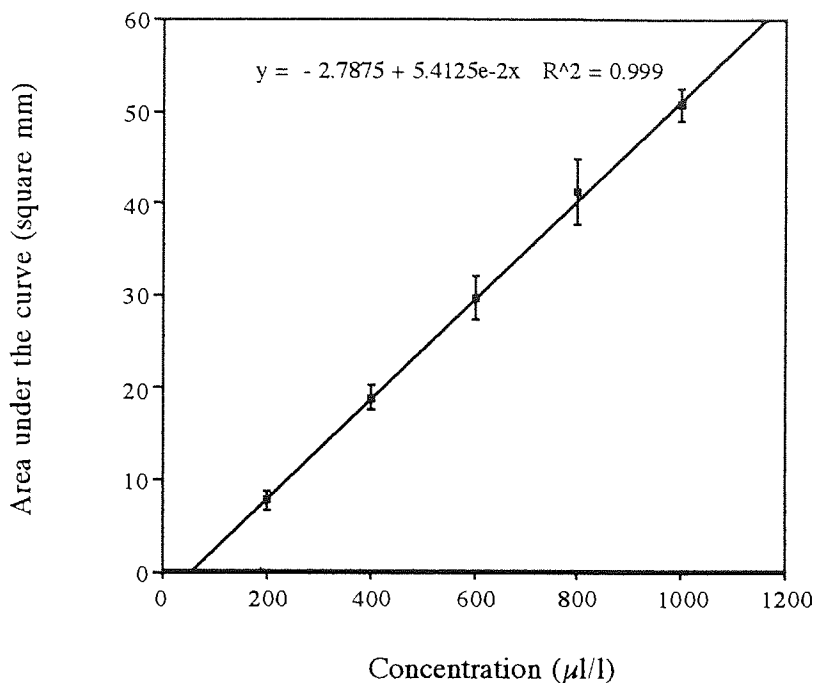


Figure 3.5.b) Petroleum ether Standard Curve.



3.3.5.b) Determination of polymer glass transition temperature of freshly fabricated unloaded, 20% w/w antibiotic loaded microspheres and virgin PLCG 75:25 copolymer together with the determination of antibiotic melting points.

DSC thermograms of freshly fabricated unloaded microspheres and 20% w/w antibiotic loaded microspheres, individual powders of vancomycin, ciprofloxacin, rifampicin and virgin PLCG 75:25 copolymer are shown in figure 3.6.. Analysis of the endothermic peaks created by each of these samples revealed that virgin PLCG 75:25 copolymer had a mean T_g (measured as the mean T_g peak onset) of 50.74°C. However, when the copolymer was used to fabricate unloaded microspheres the point of the mean polymer T_g peak onset had decreased to 43.86°C. Vancomycin, ciprofloxacin and rifampicin powders produced endothermic peaks at approximately 130°C, 140°C and 250°C respectively. These peaks were thought to indicate the melting point of each antibiotic. These melting point endotherms were not observed on DSC traces for 20% w/w antibiotic loaded microspheres presumably because the concentration of each microencapsulated antibiotic

was below the detection limit of the apparatus. When PLCG 75:25 was used to fabricate 20% w/w vancomycin loaded microspheres a mean Tg peak onset at 41.78°C was produced which was very similar to the mean Tg peak onset for 20% w/w ciprofloxacin loaded microspheres (41.92°C). However, 20% w/w rifampicin loaded microspheres exhibited an mean Tg peak onset at a higher temperature of 43.56°C. Therefore, generation of both unloaded and 20% w/w antibiotic loaded microspheres tended to decrease the Tg of the fabrication polymer PLCG 75:25 with respect to the Tg of the virgin copolymer. It is clear that the W/O with solvent evaporation technique and the encapsulation of antibiotic within polymeric microspheres both change the Tg of PLCG 75:25 with respect to the Tg of virgin copolymer. The reasons for the observed changes will be discussed later.

Figure 3.6. Typical DSC thermograms for freshly fabricated unloaded microspheres, 20% w/w antibiotic loaded microspheres, antibiotic powders and virgin PLCG 75:25 copolymer.

Figure 3.6.a) DSC thermogram for freshly fabricated 20% w/w vancomycin loaded microspheres.

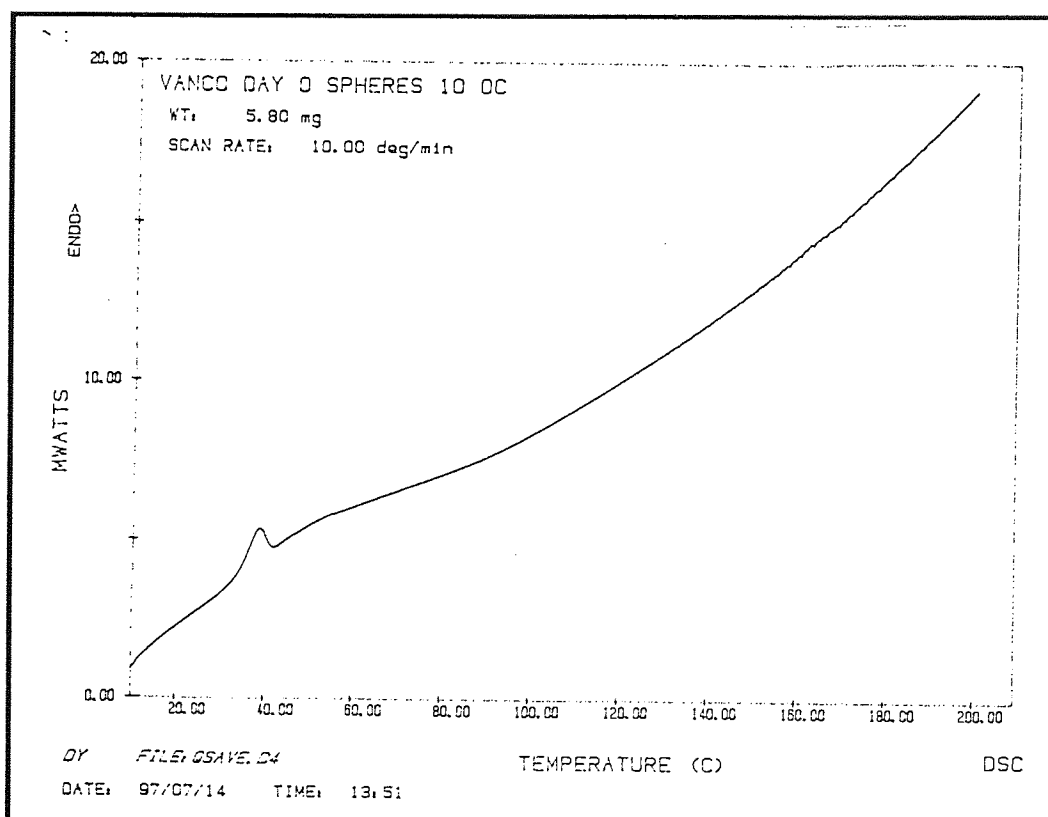


Figure 3.6.b) DSC thermogram for freshly fabricated 20% w/w ciprofloxacin loaded microspheres.

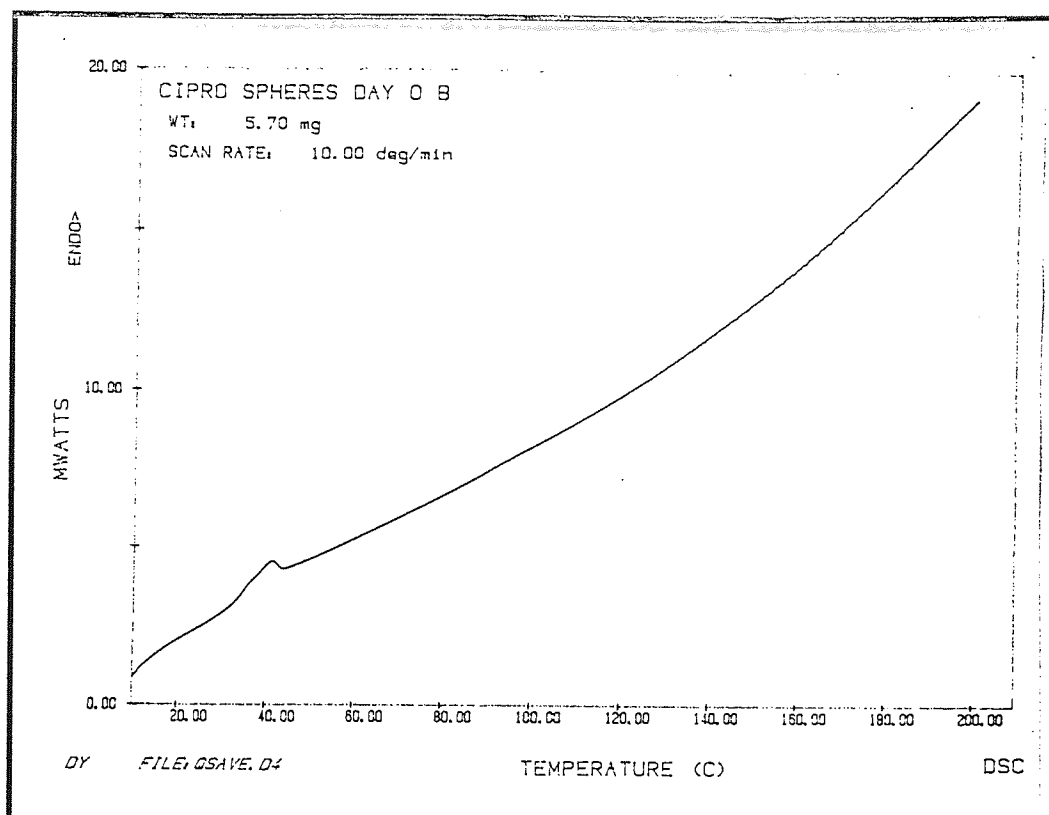


Figure 3.6.c) DSC thermogram for freshly fabricated 20% w/w rifampicin loaded microspheres.

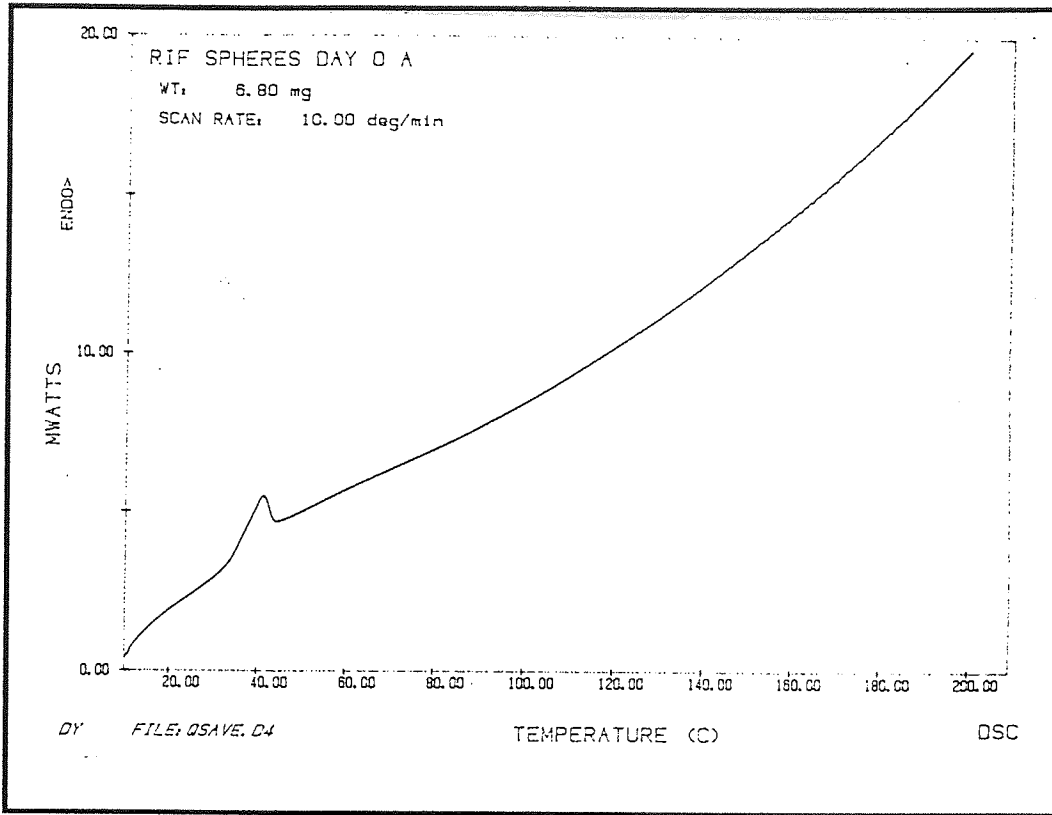


Figure 3.6.d) DSC thermogram for freshly fabricated unloaded microspheres.

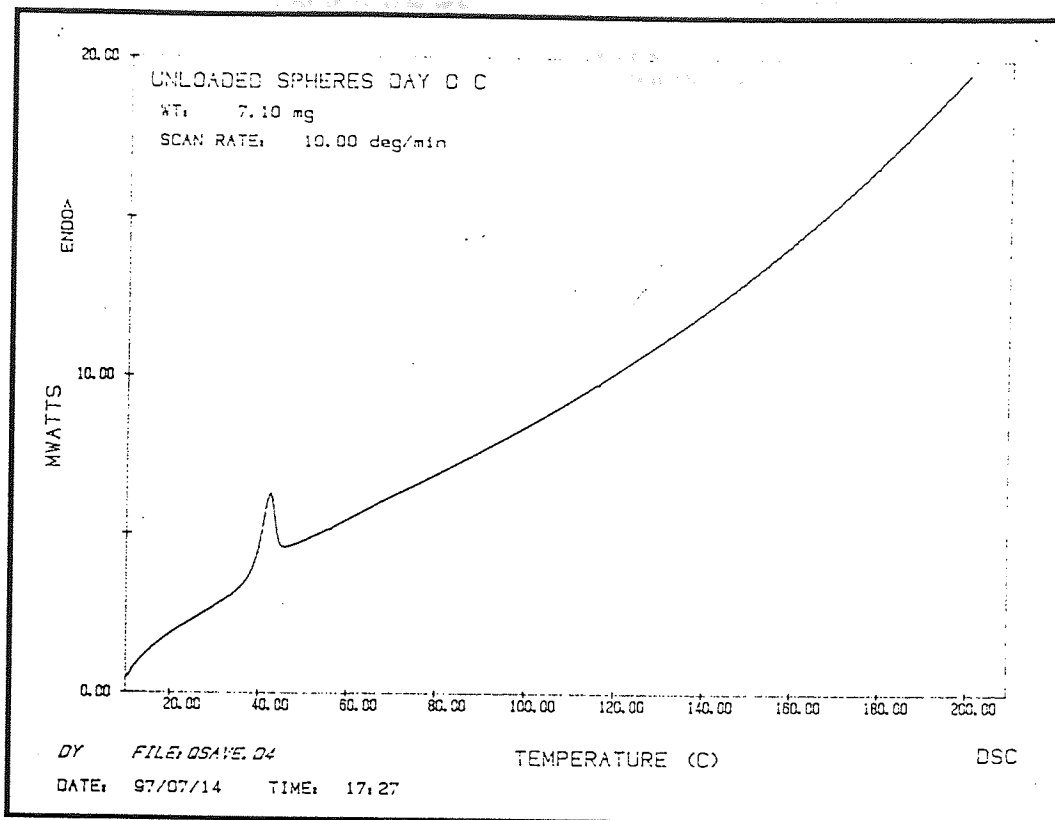


Figure 3.6.e) DSC thermogram for vancomycin powder.

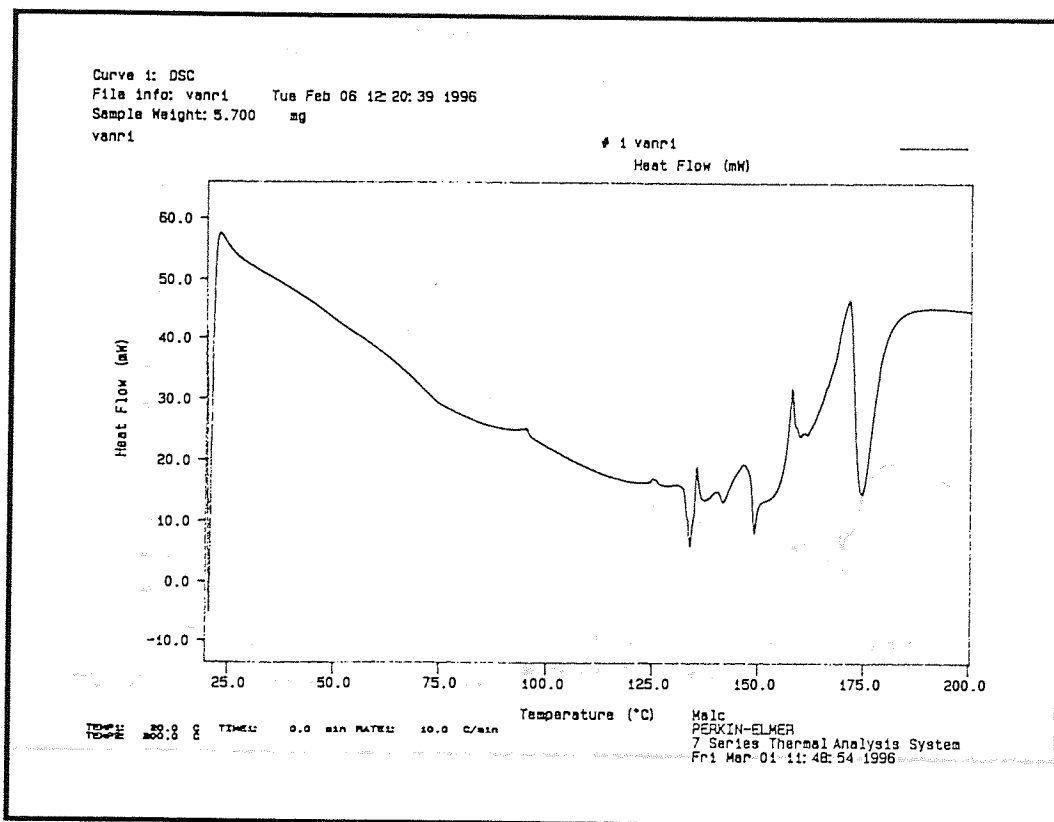


Figure 3.6.f) DSC thermogram for ciprofloxacin powder.

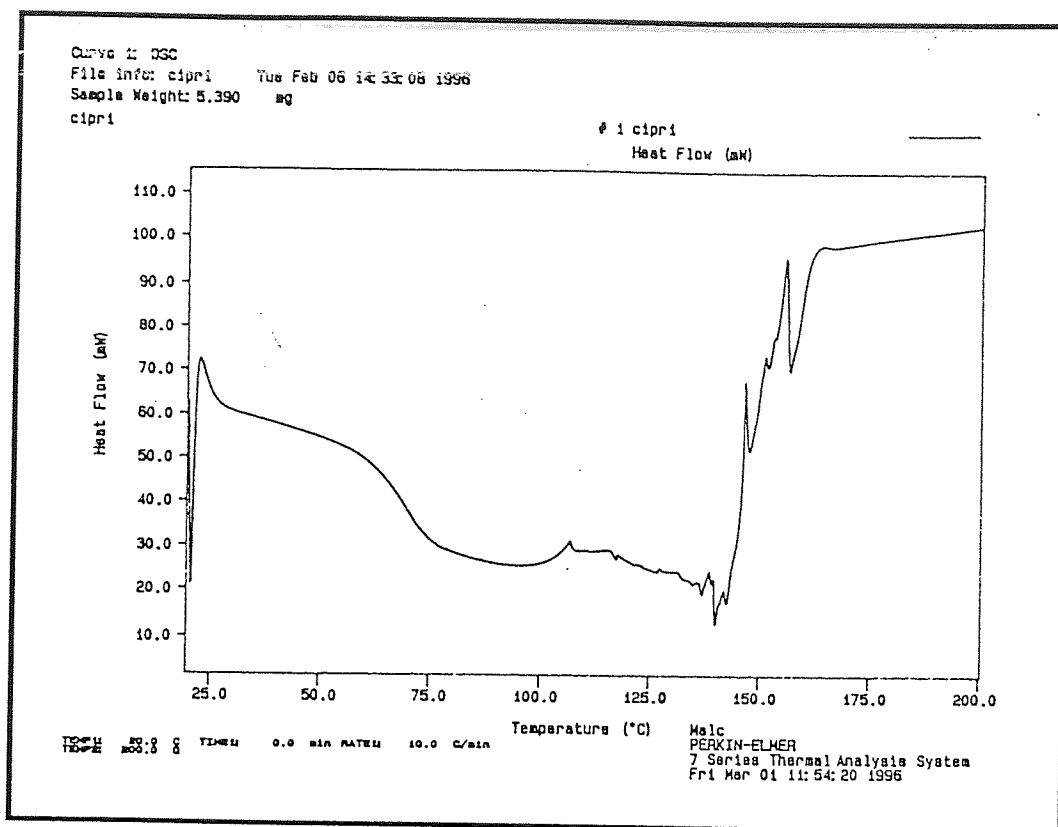


Figure 3.6.g) DSC thermogram for rifampicin powder.

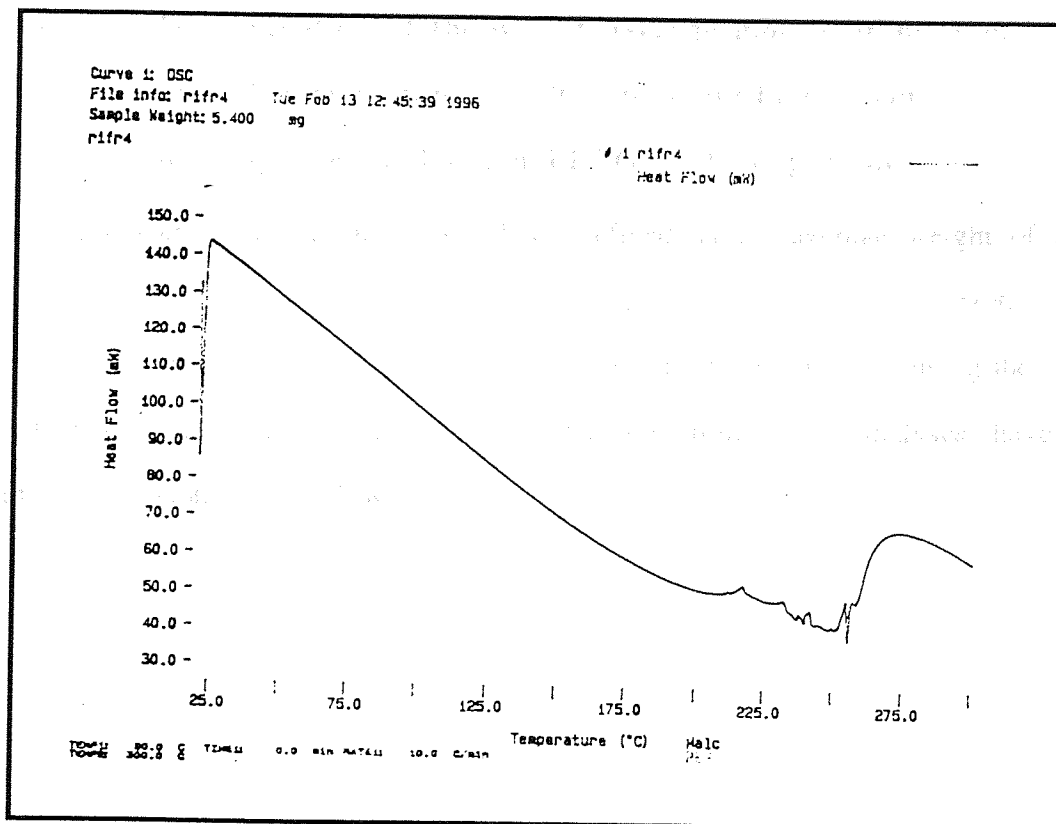
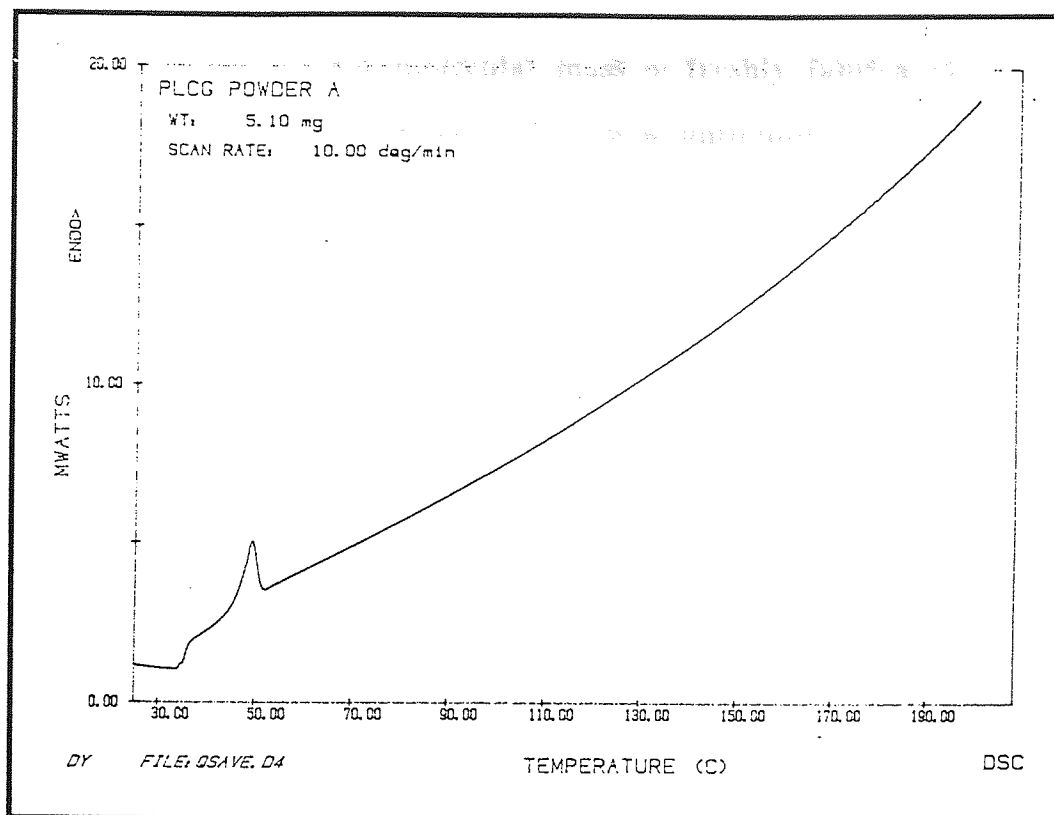


Figure 3.6.h) DSC thermogram for virgin PLCG copolymer.



3.3.6. Determination of the weight average molecular mass of unloaded microspheres, 20% w/w antibiotic loaded microspheres and virgin PLCG 75:25 copolymer.

The weight average molecular mass (M_w , defined as the average weight of all the molecules in a polymer) of unloaded microspheres, 20% w/w antibiotic loaded microspheres and PLCG 75:25 virgin copolymer was measured by GPC using the method described in section 2.2.6. The results obtained from these analyses have been summarised in table 3.12. below.

Table 3.12. Weight average molecular mass of freshly fabricated unloaded microspheres, 20% w/w antibiotic loaded microspheres and virgin PLCG 75:25 copolymer.

Sample analysed by GPC	Polystyrene equivalent Mw (mean values in Daltons \pm SEM, n=4 unless otherwise stated)
Vancomycin loaded microspheres	20,000 \pm 182.57
Ciprofloxacin loaded microspheres	19,275 \pm 624.28
Rifampicin loaded microspheres	18,275 \pm 375.28
Unloaded microspheres	19,175 \pm 172.40
Virgin PLCG 75:25 copolymer	38,200 / 52,800, mean = 45,500, n=2.

The mean Mw of freshly fabricated 20% w/w vancomycin loaded microspheres was significantly greater ($P < 0.05$) than that of both 20% w/w rifampicin loaded microspheres and unloaded microspheres but not significantly different from that of 20% w/w ciprofloxacin loaded microspheres. In addition, the mean Mw of unloaded microspheres was significantly greater ($P < 0.05$) than that of 20% w/w rifampicin loaded microspheres. The mean Mw of freshly fabricated microspheres, irrespective of the encapsulated antibiotic, was in the region of 20 kDaltons whereas the mean Mw of virgin PLCG 75:25 copolymer, as supplied by the manufacturer, was 45.5 kDaltons. This difference suggests that utilisation of the copolymer for the fabrication of microspheres using the W/O technique resulted in a 50-60% decrease in polymer Mw. Possible reasons for this will be discussed later.

3.4. Discussion.

The pharmaceutical effectiveness of a drug delivery device relies heavily on the reproducibility of the methods employed to produce it which in turn will effect the reproducibility of its performance *in vitro* and *in vivo*. For example, extreme variation in

batch to batch physico-chemical characteristics of 20% w/w antibiotic loaded microspheres such as microencapsulation efficiency and mean diameter will make the extent and duration of antibiotic release unpredictable and therefore difficult to validate. More importantly, variations in the kinetics of antibiotic release could profoundly effect the ability of antibiotic loaded microspheres to suppress and prevent infections by eradicating pathogens. Therefore, characterising the physico-chemical properties of the 20% w/w antibiotic loaded microspheres generated in this work and assessing the reproducibility of these properties are vital, especially since the bench-scale Silverson emulsifier used to produce the microspheres in the laboratory can be directly correlated with larger plant equipment applicable for use in pilot scale-up manufacturing operations. The results described in this section relate to the physico-chemical characteristics of unloaded and 20% w/w antibiotic loaded microspheres and, to a limited extent, their fabrication components. Unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres were harvested in high, reproducible yields. However, batches of 20% w/w rifampicin loaded microspheres were fabricated in reduced yields. Vancomycin and ciprofloxacin loaded microspheres theoretically loaded with an antibiotic concentration of 20% w/w by weight of polymer actually encapsulated at least three quarters of the available antibiotic (approximately 15% w/w by weight of polymer). Those microspheres theoretically loaded with 20% w/w rifampicin always encapsulated less than 15% of the available antibiotic (3% w/w antibiotic by weight of polymer). Low rifampicin microencapsulation efficiencies were caused by the partitioning of rifampicin into the subsequently discarded oil phase during emulsification while some rifampicin was leached from the microspheres into petroleum ether during the washing procedure. Loss of rifampicin by these routes led to a decrease in the microsphere mean percentage yield which was based upon the total mass of solid (antibiotic + polymer) initially used compared with the final mass of microspheres. The unpredictable partitioning of rifampicin into the oil phase during microsphere fabrication was assumed to be influenced by slight changes in the rate of solvent evaporation and emulsion temperature and these changes could have caused the variations in rifampicin encapsulation efficiency. The encapsulation

efficiency obtained for 20% w/w vancomycin loaded microspheres (ranging from 71-97%) was not significantly affected by the emulsification speed used in their fabrication, an observation consistent with the results obtained from previous studies (Benita *et al.*, 1984; Jalil and Nixon, 1990a). However, the mean volume diameter of 20% w/w vancomycin loaded microspheres was significantly reduced by increasing the emulsification speed (from 71 μ m using an emulsification speed of 1,000rpm to 42 μ m using an emulsification speed of 8,000rpm). Other workers (Benita *et al.*, 1984; Jalil and Nixon, 1990a and b; Watts *et al.*, 1990; Conti *et al.*, 1995) have made similar observations. The relationship between mean microsphere diameter and emulsification speed is thought to be dependent upon the size of droplets produced during the emulsification process (Arshady, 1991, Julienne *et al.*, 1992). Although a decrease in the mean diameter of 20% w/w vancomycin loaded microspheres with an increase in the emulsification speed used in their fabrication could be confirmed, more effective ways of decreasing microsphere mean diameter have been demonstrated by other workers. Such manoeuvres include increasing the concentration of surfactant in the oil phase (Jalil and Nixon, 1990a; Scholes *et al.*, 1993; Conti *et al.*, 1995) although using poly (vinyl alcohol) for this purpose can actually bring about an increase in microsphere mean diameter by increasing the emulsion viscosity (Benita *et al.*, 1984, Scholes *et al.*, 1993). Decreasing the mean microsphere diameter can also be brought about by decreasing the concentration of polymer in the organic phase (Watts *et al.*, 1990, Julienne *et al.*, 1992, Scholes *et al.*, 1993, Pavanetto *et al.*, 1993). Jalil and Nixon achieved this by decreasing the total amount of solid in the system (Jalil and Nixon, 1990a) and decreasing the polymer molecular weight (Jalil and Nixon, 1990b). These manoeuvres caused a decrease in polymer viscosity which increased the stirring efficiency and decreased droplet coalescence (Arshady, 1991). Other ways of decreasing microsphere mean diameter include decreasing the microsphere core loading (Jalil and Nixon, 1990b); decreasing the volume of the organic phase relative to the continuous phase (Julienne *et al.*, 1992, Scholes *et al.*, 1993); and brief sonication of the microspheres during or following the emulsification process (Jalil and Nixon, 1989; Scholes *et al.*, 1993, Park, 1994). The design of the apparatus employed in microsphere and microcapsule

fabrication can also effect the final mean diameter. Using a cylindrical emulsification vessel with attached side baffles, as opposed to a standard reactor vessel without baffles, was found to decrease microsphere mean diameter while increasing microsphere percentage yield (Arshady, 1991). Contrary to the work of other researchers (Benita *et al*, 1984; Watts *et al*, 1990), in the present study the size distribution of 20% w/w vancomycin loaded microspheres increased with increasing emulsification speed from 1,000 rpm to 4,000 rpm. Increasing the emulsification speed increased the mixing of the emulsion and quickened the rate of solvent evaporation from the droplets. Although higher rates of solvent evaporation can lead to an increase in the viscosity of the water phase which, in turn, can increase microsphere size as explained earlier, microspheres fabricated using an emulsification speed of 4,000 rpm would also harden before those fabricated using an emulsification speed 1,000 rpm due to the faster removal of solvent from the water phase. Therefore the potential for emulsion droplets to coalesce and the resultant “tacky” microspheres to aggregate would be reduced at an emulsification speed of 4,000 rpm compared with fabrication using an emulsification speed of 1,000 rpm. Using an emulsification speed of 4,000rpm would have the effect of increasing the proportion of smaller 20% w/w vancomycin loaded microspheres produced thereby widening the mean size distribution. The fact that increasing the emulsification speed from 4,000 rpm up to 8,000 rpm had no significant effect on the size distribution of 20% w/w vancomycin loaded microspheres suggests that the rate of solvent evaporation during emulsification was only increased by increasing the emulsification speed up to a maximum of 4,000 rpm. Comparison of the size distributions obtained for unloaded microspheres together with 20% w/w vancomycin loaded microspheres, 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000 rpm confirmed that 20% w/w vancomycin loaded microspheres were significantly larger than 20% w/w ciprofloxacin loaded microspheres. However, when examined morphologically, 20% w/w ciprofloxacin loaded microspheres showed a more extensive surface antibiotic crystal deposition. Jalil and Nixon have indicated that the presence of microsphere surface crystals aid in the prevention of “tacky” microsphere aggregation prior

to complete hardening by acting as a physical barrier between microspheres as they touch (Jalil and Nixon, 1990b). Therefore, in the present study it is likely that 20% w/w vancomycin loaded microspheres were more prone to aggregation than 20% w/w ciprofloxacin loaded microspheres by virtue of the fewer antibiotic crystals deposited on their surface. Assuming that the majority of antibiotic added to the system and not bound to the surface of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres became microencapsulated (as indicated by their high encapsulation efficiencies), and that both the antibiotics are virtually insoluble in acetonitrile, antibiotic crystal formation and retention within the internal matrix of both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres should have occurred. Crystal formation within microspheres can destabilise the structure of microspheres and promote microsphere fusion during fabrication (Jalil and Nixon, 1990b). As 20% w/w ciprofloxacin loaded microspheres were completely covered in antibiotic crystals, the concentration of antibiotic crystals incorporated into the internal matrix would have been fairly low compared with 20% w/w vancomycin loaded microspheres. Therefore, structural destabilisation of 20% w/w ciprofloxacin loaded microspheres and an over-enlargement of the internal matrix would be limited. This might explain why the mean diameter of 20% w/w ciprofloxacin loaded microspheres was smaller than that of 20% w/w vancomycin loaded microspheres but similar to that of unloaded microspheres. 20% w/w rifampicin loaded microspheres had a significantly ($P < 0.05$) smaller mean diameter than unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres. Jalil and Nixon showed that the mean diameter of microspheres encapsulating phenobarbitone was not significantly decreased by concentrations of SPAN 40 (an emulsifier used to prevent emulsion droplet coalescence) above 2% w/w (Jalil and Nixon, 1990a). However, in the present study, a higher SPAN 40 concentration (3% w/w) was required to stabilise emulsion droplets during the fabrication of 20% w/w rifampicin loaded microspheres. This indicates that the mean diameter of 20% w/w rifampicin loaded microspheres might be decreased by increasing the concentration of SPAN 40. The biphasic size distribution for

20% w/w rifampicin loaded microspheres may have been caused by the relatively high mixing speed (3,000rpm) used during solvent evaporation which could have led to the creation of more microspheres with small diameters. For all microspheres prepared using an emulsification speed of 4,000 rpm, decreases in mean droplet diameter were obtained by extending the emulsification time. This phenomenon was facilitated by the head stage attached to the Silverson mixer which is designed by the manufacturer to continually draw in and circulate emulsion droplets to decrease their size. Therefore, in theory, extending the emulsification time from 30 minutes to 40 minutes may have been sufficient to create a steady-state droplet diameter thereby eliminating the differences in the final size distributions obtained for differentially loaded microspheres.

Ultrastructurally, both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres were covered in antibiotic crystals. When crystals were removed by briefly washing in double distilled water, the surface of the microspheres was found to be rugose and pitted. It has been suggested that microsphere surface crystal deposition occurs when the material to be encapsulated is not soluble in the water phase solvent (Donbrow, 1992). This was confirmed in the present study since both vancomycin and ciprofloxacin formed suspensions in acetonitrile. However, the polymer T_g for both 20% w/w vancomycin and 20w/w ciprofloxacin loaded microspheres was lower than that obtained for unloaded microspheres. Decreases in the T_g caused by the encapsulation of materials within polymeric microspheres can indicate that the material has formed a molecular dispersion within the microsphere matrix by chemically interacting with the polymer (Benoit *et al*, 1986). However, the insolubility of vancomycin and ciprofloxacin in acetonitrile does not support this theory. An alternate explanation might involve the acidity of both vancomycin and ciprofloxacin. A solution of vancomycin in water (5% w/v) gives a pH of 2.8-4.5 whereas a solution of ciprofloxacin in water (2.5% w/v) gives a pH of 3.0-4.5 (Martindale, 1993). Therefore, any water vapour taken up by the microspheres after fabrication may have caused the limited solubilisation of microencapsulated vancomycin and ciprofloxacin crystals. This could have resulted in

small-scale acid hydrolysis of ester bonds within the polymer. Hydrolysis of polymer chains shortens their length and this leads to a concomitant reduction in polymer Tg (Park, 1994). Changes in polymer Tg as a consequence of microsphere biodegradation will be discussed in greater length later (chapter 5). The smooth external appearance of 20% w/w rifampicin loaded microspheres was the result of antibiotic forming a solution with acetonitrile although the possible molecular dispersion of rifampicin within the internal matrix of finished microspheres may have been partly responsible (Donbrow, 1992). However, the Tg for freshly fabricated 20% w/w rifampicin loaded microspheres was very similar to that obtained for unloaded microspheres. This would suggest that rifampicin could have re-crystallised inside the microsphere matrix during solvent evaporation rather than remaining molecularly dispersed. However, antibiotic crystals were not observed in sections of 20% w/w rifampicin loaded microspheres embedded in gelatin when examined using scanning E/M. Acid hydrolysis of the matrix of 20% w/w rifampicin loaded microspheres, caused by rifampicin solubilisation in absorbed water vapour, would have been negligible because of the low microencapsulation efficiency obtained for rifampicin. The smooth surface and spherical shape of unloaded microspheres was the result of the amorphous nature of PLCG 75:25, together with its relatively high Tg (measured as 44.07°C in the present work) relative to room temperature (Reed and Gilding, 1981; Donbrow, 1992). The internal matrix of freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres was homogeneous although some macropores were in evidence. The internal matrix was surrounded by a compressed peripheral crust and both of these structures were thought to have been formed during the solvent evaporation process. The formation of the peripheral crust during the droplet stage of emulsification was thought to occur concomitantly with the creation of a homogeneous internal matrix containing few pores. However, during solvent evaporation, while the outer shell hardened as a consequence of rapid polymer precipitation (Bodmeier and McGinity, 1987a), acetonitrile droplets would still have been diffusing out of the microspheres from the pockets of space that they occupied within the matrix leaving a number of empty pockets within the internal matrix as it hardened. It has been reported that increasing the

rate of solvent evaporation can cause local explosions inside the microsphere droplets resulting in the extension of these pores (in the form of pinholes) into the peripheral microsphere crust (Arshady, 1991).

The concentrations of residual acetonitrile and petroleum ether within unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres were below 25,000 ppm (acetonitrile) and 100,000 ppm (petroleum ether). It was not possible to measure residual solvent within rifampicin loaded microspheres since this antibiotic dissolved in the GLC injector solvent (DCM) and would therefore have blocked the GLC column. The limit for residual acetonitrile in pharmaceutical products has been set at 410 ppm by the International Committee for Harmonisation and at 50 ppm (proposed) by the European Pharmacopoeia (Witschi and Doelker, 1997). Therefore, a more sensitive GLC method than the one employed in the present work would be required to satisfy regulatory authorities that the concentrations of residual solvents within the manufactured microspheres were acceptable. Acetonitrile (methyl cyanide), which owes its toxicity to its slow *in vivo* metabolism to cyanide, has a fatal dose of 250mg in adult humans (Martindale, 1993). However, over 10 kg of microspheres would be required to provide this fatal dose of cyanide assuming that the v/w concentration of acetonitrile within each microsphere preparation was, at most, 0.025 μ l acetonitrile / mg microspheres. Petroleum ether (petroleum spirit) can be toxic due to contaminants such as lead, n-hexane and benzene (Martindale, 1993) but the v/w concentration of petroleum ether within the microspheres was less than 0.100 μ l/mg. The potential toxicity of unloaded microspheres, 20% w/w vancomycin loaded microsphere and 20% w/w ciprofloxacin loaded microspheres, in terms of their residual solvent content, was therefore negligible.

The weight average molecular mass of freshly fabricated unloaded microspheres and 20% antibiotic loaded microspheres was in the region of 20 kDaltons. Small variations in the weight average molecular mass (Mw) of unloaded microspheres and 20% w/w antibiotic

loaded microspheres were thought to have occurred as a result of variations in the baseline measurements obtained in the GPC assay rather than suggesting that the extent of antibiotic encapsulation affected microsphere molecular mass. However, the difference between the weight average molecular mass of virgin PLCG 75:25 copolymer and freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres was approximately 25 kDaltons. This may have been the result of sonicating the organic phase before the emulsification stage of the fabrication process (Park, 1994). Liu and co-workers (1992) stated that the decrease in polymer molecular weight after ultrasound treatment was due to the formation of bubbles in the solvent (cavitation). Collapse of these bubbles caused the generation of frictional forces and shock waves which exerted stresses on the polymer backbone. These stresses resulted in bond cleavage within the polymer. Shortening of polymer chains causes a reduction in polymer molecular mass and is the first stage of polymer de-esterification. However, an alternative and more likely explanation could involve the employment of a heating stage during microsphere fabrication. Emulsification was carried out at 50°C, a temperature greater than the T_g of PLCG 75:25, which would have increased bond flexibility in the polymer and may have facilitated early, possibly thermal, biodegradation of PLCG. Information concerning the mechanisms of polymer ester hydrolysis and the biodegradation of both unloaded microspheres and 20% w/w antibiotic loaded microspheres has been discussed in chapter 5. The physico-chemical characteristics of 20% w/w antibiotic loaded microspheres will influence the release kinetics of microencapsulated species. The kinetics of *in vitro* antibiotic release from dispersed and HPMC gel entrapped 20% w/w loaded PLCG 75:25 microspheres fabricated using the W/O with solvent evaporation technique have been described in the next chapter.

Chapter 4: Antibiotic release from 20% w/w loaded dispersed and hypromellose-entrapped PLCG 75:25 microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

4.1. Introduction.

Measuring the kinetics of *in vitro* antibiotic release from dispersed and HPMC-gel entrapped 20% w/w loaded microspheres will give an indication of the ability of these preparations to suppress and cure infections which develop in the hip joint after THA surgery. In order to successfully treat such infections, antibiotic should be released from microspheres at a therapeutically significant concentration for a sustained period of time.

4.2. Methods.

The release of vancomycin, ciprofloxacin and rifampicin from dispersed 20% w/w loaded microspheres and 20% w/w loaded microspheres entrapped within 10% w/v hypromellose (HPMC) gel was monitored over a period of up to 30 days of incubation in both newborn calf serum (NCS) and Hank's balanced salt solution (HBSS) at 37°C as described in section 2.3.8.. Data from these studies were used to construct profiles showing the day to day concentration of antibiotic released from 20% w/w loaded microspheres into NCS and HBSS. Student's two tailed, unpaired t-test was used to compare the mean day to day concentration as well as the total mean cumulative concentration and cumulative percentage of antibiotic released into NCS and HBSS at 37°C from both dispersed and HPMC-entrapped microspheres at specific time points on each release profile. Differences in the rate of antibiotic release from dispersed and gel entrapped microspheres in the same medium have also been compared so that the influence of entrapment of microspheres in HPMC on the kinetics of antibiotic release could be assessed. Kinetic analysis of antibiotic

release from 20% w/w loaded PLCG 75:25 microspheres has been carried out using first order and square-root (Higuchi) time plots in order to elucidate the mechanism by which antibiotic is released from 20% w/w loaded microspheres and incubated in newborn calf serum.

4.3. Results.

4.3.1. *In vitro* release of antibiotics from both dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

4.3.1.1 Release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum at 37°C.

Both dispersed and HPMC-entrapped 20% w/w vancomycin loaded PLCG 75:25 microspheres fabricated using emulsification speeds of 1,000 rpm, 4,000 rpm and 8,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of vancomycin were released into NCS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of vancomycin were released.

Data for the mean day to day concentrations of vancomycin released into NCS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm are summarised in table 4.1. below. A profile for vancomycin release from dispersed and gel entrapped microspheres over 30 days of incubation in NCS at 37°C is shown in figure 4.1.1. below.

Table 4.1. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 1,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	2	3.28 \pm 0.13	33.69 \pm 1.37	9.99 \pm 0.35c	102.76 \pm 3.58d
HPMC-entrapped	4	7.84 \pm 0.17a	80.63 \pm 1.77b	9.00 \pm 0.20	92.57 \pm 2.06

a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

b the percentage of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

c the cumulative concentration of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

d the percentage of the encapsulated load of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

The mean day to day concentration of vancomycin released from dispersed and HPMC-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm gradually decreased during the second, sustained low-level, release phase. HPMC-entrapped microspheres released 0.24 ± 0.02 mg/ml, n=6 of vancomycin on day five of incubation, which represented the first day of the second, low-level, release phase, but these microspheres only released 0.01 ± 0.01 mg/ml, n=6 of vancomycin on day 26 of incubation after which time vancomycin could not be detected in the incubation medium. However, significant concentrations of vancomycin were still being released from the

dispersed microspheres after 30 days of incubation in NCS at 37°C (figure 4.1.1.). The mean day to day concentration of vancomycin released from dispersed microspheres on day three of incubation (day one of the second, low-level, release phase) was 0.45 ± 0.04 mg/ml, n=6. However, by day 30 of incubation, the mean day to day concentration of vancomycin released from dispersed microspheres had fallen to 0.09 ± 0.003 mg/ml, n=6. Apart from on day 11, the mean day to day concentration of vancomycin released from dispersed microspheres after five days of incubation was significantly greater ($P < 0.05$) than that liberated from gel entrapped microspheres on corresponding days of the release study as shown in figure 4.1.1.. Throughout the study, the lowest mean day to day concentration of vancomycin released from dispersed and HPMC-entrapped microspheres was 0.09 ± 0.003 mg/ml, n=6 and 0.004 ± 0.004 mg/ml, n=6 respectively (figure 4.1.1.).

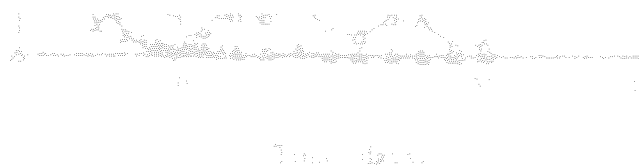
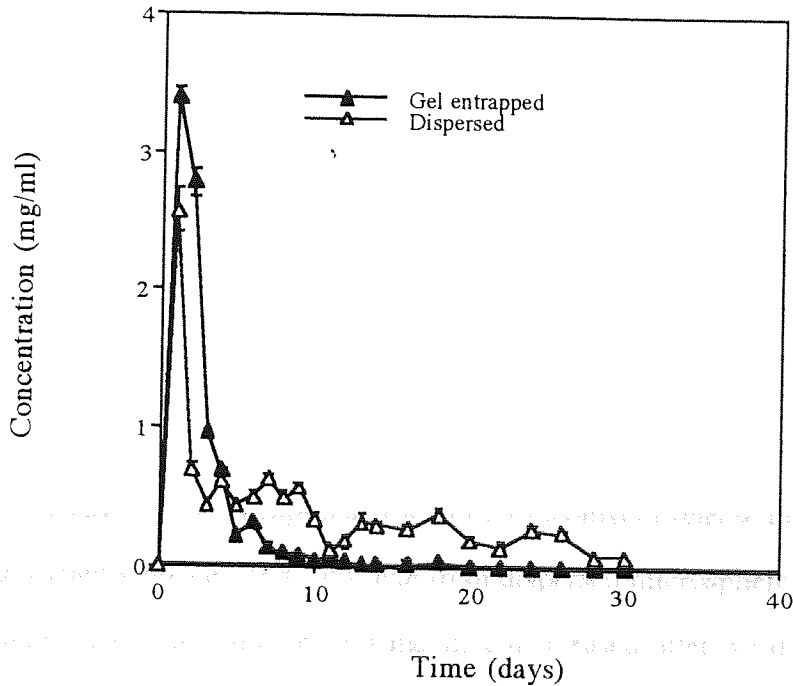


Figure 4.1.2 below shows the mean day to day concentrations of vancomycin released into NCS at 37°C from dispersed microspheres (group 1) and HPMC-entrapped microspheres (group 2) over the 30 days of incubation. Each of 4,000 ppm are contained in each 1 ml volume. A profile of vancomycin release from dispersed and HPMC-entrapped microspheres over 30 days of incubation in NCS at 37°C is shown in figure 4.1.2 below.

Figure 4.1.1. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point).



Data for the mean day to day concentrations of vancomycin released into NCS at 37°C from dispersed microspheres (group A in table 3.6) and HPMC-entrapped microspheres (group B in table 3.6) fabricated using an emulsification speed of 4,000 rpm are summarised in table 4.2. below. A profile for vancomycin release from dispersed and HPMC-entrapped microspheres over 30 days of incubation in NCS at 37°C is shown in figure 4.1.2. below:

Table 4.2. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in newborn calf serum at 37°C (mean values ± SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	3	4.85 ± 0.19	52.40 ± 2.00	8.80 ± 0.30c	95.17 ± 3.26
HPMC-entrapped	5	6.62 ± 0.34a	93.52 ± 4.75 b	7.10 ± 0.32	100.49 ± 4.58

a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

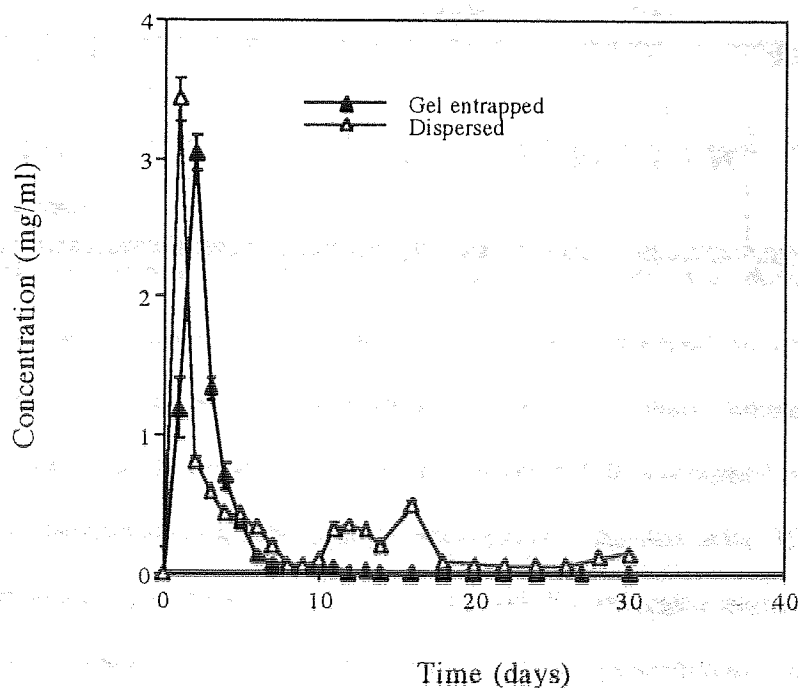
b the percentage of the encapsulated load of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

c the cumulative concentration of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

When corresponding time points on the release profiles were compared, the mean day to day concentration of vancomycin released from dispersed microspheres fabricated using an emulsification speed of 4,000 rpm was significantly greater ($P < 0.05$), apart from on days two to four of incubation, than the mean day to day concentration of vancomycin released from HPMC-gel entrapped microspheres up to and including day 13 of incubation (figure 4.1.2.). During the second, low-level, release phase, the mean day to day concentration of vancomycin released from HPMC-entrapped microspheres decreased from 0.14 ± 0.03 mg/ml, $n=6$ on day 6 of incubation, to 0.02 ± 0.01 mg/ml, $n=6$ on day 13 and vancomycin could not be detected in NCS after this time. Although the mean day to day concentration of vancomycin released from dispersed microspheres decreased in the immediate post-

burst phase period (see figure 4.1.2.), small but significant ($P < 0.05$) increases in the mean day to day concentration of vancomycin released occurred between days 11 and 15 (figure 4.1.2.). After this period, the day to day concentration of vancomycin released then fell back to previous levels of approximately 0.070 mg/ml up to day 28 and subsequently increased significantly ($P < 0.05$) up to day 30 of incubation (figure 4.1.2.). At no time did the mean day to day concentration of vancomycin released from dispersed microspheres and from HPMC-entrapped microspheres fall below 0.06 ± 0.02 mg/ml, $n=6$ and 0.01 ± 0.01 mg/ml, $n=6$ respectively over the 30 day incubation period.

Figure 4.1.2. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, $n=6$ for each point).



Data for the mean day to day concentrations of vancomycin released into NCS at 37°C from dispersed microspheres (group B in table 3.6) and HPMC-entrapped microspheres (group A in table 3.6) fabricated using an emulsification speed of 8,000 rpm are summarised in table 4.3. below. A profile for vancomycin release from dispersed and HPMC-entrapped microspheres over 30 days of incubation in NCS at 37°C is shown in figure 4.1.3. below.

Table 4.3. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 8,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	3	5.91 \pm 0.31a	61.14 \pm 3.23b	8.57 \pm 0.36c	88.71 \pm 3.72d
HPMC-entrapped	5	4.80 \pm 0.33	50.97 \pm 3.45	5.23 \pm 0.33	55.54 \pm 3.55

a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

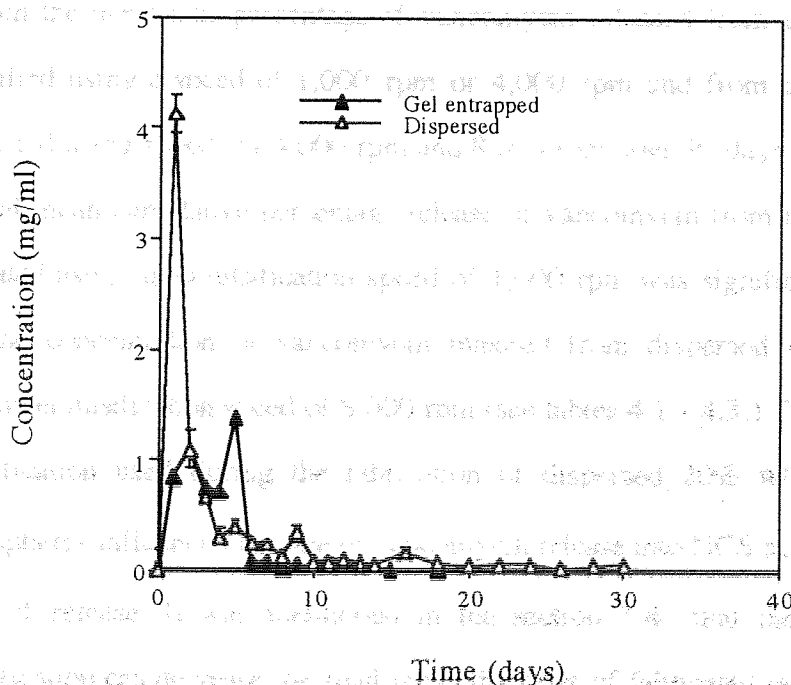
b the percentage of the encapsulated load of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

c the cumulative concentration of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

d the percentage of the encapsulated load of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

The mean day to day concentration of vancomycin released from dispersed and HPMC-entrapped microspheres gradually decreased during the second, low-level, release phase. While dispersed microspheres released 0.31 ± 0.07 mg/ml, n=6 of vancomycin on day four of incubation, they released only 0.06 ± 0.02 mg/ml, n=6 of vancomycin on day 30. On a day to day basis, HPMC-entrapped microspheres delivered 0.08 ± 0.003 mg/ml, n=6 of vancomycin on day six of incubation but only 0.02 ± 0.01 mg/ml, n=6 on day 18 after which time vancomycin could not be detected in the release medium. The lowest mean day to day concentration of vancomycin released over the whole release profile was 0.04 ± 0.01 mg/ml, n=6 from dispersed microspheres and 0.01 ± 0.01 mg/ml, n=6 from HPMC-entrapped microspheres (figure 4.1.3.).

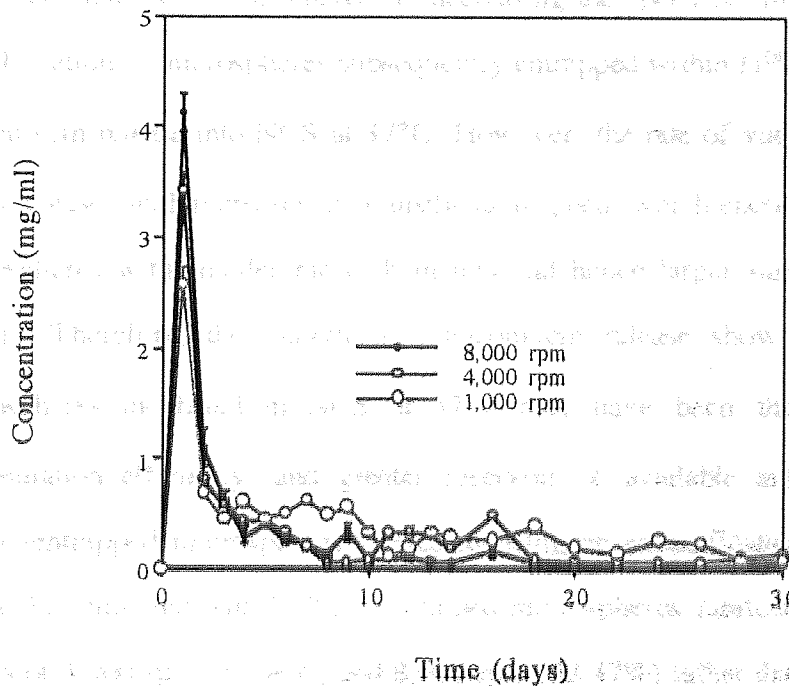
Figure 4.1.3. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 8,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point).



When the mean concentrations of vancomycin released into NCS during the burst phase from dispersed 20% w/w loaded microspheres fabricated using an emulsification speeds of 1,000 rpm, 4,000 rpm and 8,000 rpm were compared, the mean day to day concentration of vancomycin released on day one of incubation from microspheres fabricated using an emulsification speed of 8,000 rpm was significantly ($P < 0.05$) greater than that released from microspheres fabricated using an emulsification speed of 4,000 rpm or 1,000 rpm (4.13 ± 0.18 mg/ml, $n=6$ compared with 3.43 ± 0.16 mg/ml, $n=6$ and 2.58 ± 0.15 mg/ml, $n=6$ respectively - figure 4.1.4.). The total mean cumulative concentration of vancomycin released from dispersed microspheres fabricated using an emulsification speed of 8,000 rpm over the duration of the burst phase was significantly greater ($P < 0.05$) than the total mean cumulative concentration of vancomycin released from dispersed microspheres fabricated using an emulsification speed of 4,000 rpm or 1,000 rpm during their respective burst phases (see tables 4.1.- 4.3.). However, over 30 days incubation in NCS at 37°C, dispersed microspheres fabricated using an emulsification speed of 1,000 rpm released a total mean cumulative concentration of vancomycin that was significantly ($P < 0.05$) greater than that released from similar microspheres fabricated using an emulsification speed of 4,000 rpm or 8,000 rpm (see tables 4.1.- 4.3.). While there was no significant difference between the mean total percentage of vancomycin released from dispersed microspheres emulsified using a speed of 1,000 rpm or 4,000 rpm and from dispersed microspheres emulsified using speeds of 4,000 rpm and 8,000 rpm after 30 days of incubation in NCS, the total mean cumulative percentage release of vancomycin from dispersed microspheres fabricated using an emulsification speed of 1,000 rpm was significantly ($P < 0.05$) greater than the concentration of vancomycin released from dispersed microsphere fabricated using an emulsification speed of 8,000 rpm (see tables 4.1.- 4.3.). Therefore, the speed of emulsification used during the fabrication of dispersed 20% w/w vancomycin loaded microspheres influenced the rate of vancomycin release into NCS at 37°C during the burst phase of release. It was mentioned in the section 3.4. that increasing the speed of emulsification can decrease the final mean diameter of fabricated microspheres and this is confirmed by the present work and the data summarised in table 3.9.. As the mean

diameter of 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 8,000 rpm was relatively small (42.02-47.69 μm), compared with 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of 4,000 rpm (31.90-59.53 μm) and 1,000 rpm (71.00 μm), 8,000rpm microspheres had the largest surface area which explains why they liberated the largest burst concentration of antibiotic. However, it was the 20% w/w vancomycin loaded microspheres with the largest mean diameter and hence smallest surface area, fabricated using an emulsification speed of 1,000 rpm, that released the greatest total mean cumulative concentration of vancomycin into NCS over the whole 30 day period of incubation at 37°C.

Figure 4.1.4. The day to day release of vancomycin from dispersed 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm, 4,000 rpm or 8,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point).



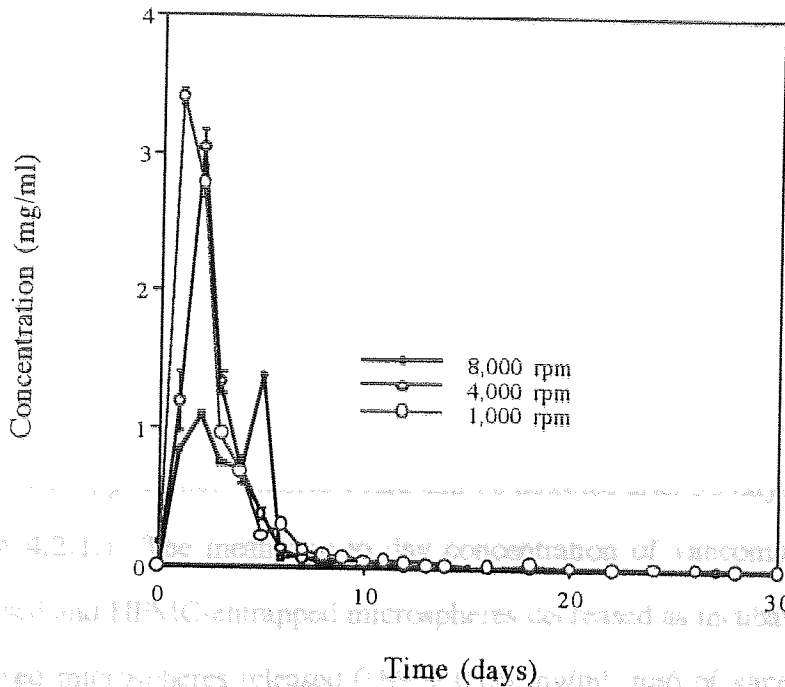
Hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of 1,000 rpm, 4,000 rpm and 8,000 rpm released 3.41 ± 0.06 mg/ml, $n=6$, 1.20 ± 0.19 mg/ml, $n=6$ and 0.85 ± 0.02 mg/ml, $n=6$ of vancomycin respectively after one day of incubation in NCS (figure 4.1.3.). The mean concentration of vancomycin released into NCS on day one of incubation, and cumulatively over the burst phase of release (see tables 4.1-4.3), was significantly greater ($P<0.05$) from HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm than from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm and 8,000 rpm. After 30 days of incubation in NCS, the mean total cumulative release of vancomycin from HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm was significantly greater ($P<0.05$) than that released from HPMC-entrapped microspheres fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm (see tables 4.1-4.3). After 30 days of incubation in NCS at 37°C, the mean total percentage of vancomycin released from HPMC-entrapped microspheres fabricated using emulsification speeds of 1,000 rpm and 4,000 rpm was significantly greater ($P<0.05$) than that released from HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm (see tables 4.1-4.3). Therefore, decreasing the speed of emulsification employed in the fabrication of microspheres subsequently entrapped within HPMC increased the rate of vancomycin release into NCS at 37°C. However, the rate of vancomycin release should have increased with increases in emulsification speed (which encourages the production of microspheres with smaller mean diameters and hence larger surface areas as explained earlier). Therefore, the patterns of vancomycin release shown by HPMC-entrapped microspheres incubated in NCS at 37°C may have been the result of the higher encapsulation efficiency (and greater reservoir of available antibiotic) shown by the HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm (97.10%) compared with HPMC-entrapped microspheres fabricated using emulsification speeds of 4,000 rpm (71.08%) and 8,000 rpm (92.47%) rather than to differences in their mean diameter.

The total mean concentration of vancomycin released into NCS at 37°C from HPMC-entrapped microspheres fabricated using emulsification speeds of 1,000 and 4,000 rpm during the initial burst phase was significantly greater ($P < 0.05$) than that released from dispersed 20% w/w vancomycin loaded microspheres (tables 4.1-4.3.). Also, the total mean cumulative release of vancomycin from dispersed microspheres fabricated using an emulsification speed of 1,000 rpm, 4,000 rpm or 8,000 rpm over 30 days of incubation in NCS at 37°C was significantly greater ($P < 0.05$) than the total mean cumulative release of vancomycin from similar microspheres entrapped in HPMC over the same time period. In summary, these data show that, compared with dispersed microspheres, the entrapment of microspheres within 10% w/v HPMC actively increased the concentration of vancomycin released during the burst phase, but reduced the extent of vancomycin release during the second phase of low-level release.



Figure 4.3. Release of vancomycin from dispersed and HPMC-entrapped 20% w/w vancomycin microspheres incubated in Hank's solution at 37°C. Each dispersed and HPMC-entrapped 20% w/w vancomycin loaded PLGA microspheres were fabricated using emulsification speeds of 1,000 rpm, 4,000 rpm and 8,000 rpm. The microspheres were relatively large (mean diameter 1.5 μm) due to the high viscosity of the HPMC solution (10% w/v). The burst phase of release was very rapid and was followed by a second slower phase which was sustained for a longer period of time.

Figure 4.1.5. The day to day release of vancomycin from hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm, 4,000 rpm or 8,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point).



(Figure 4.2.1.) The mean day to day concentration of vancomycin released from both dispersed and HPMC-entrapped microspheres decreased as incubation continued such that dispersed microspheres released 0.1 mg/ml, n=6 of vancomycin on day two of incubation but only 0.01 \pm 0.01 mg/ml, n=6 of vancomycin on day nine. HPMC-entrapped microspheres released 0.1 mg/ml, n=6 of vancomycin on day two of

4.3.1.2. Release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in Hank's balanced salt solution at 37°C.

Both dispersed and HPMC-entrapped 20% w/w vancomycin loaded PLCG 75:25 microspheres fabricated using emulsification speeds of 1,000 rpm, 4,000 rpm and 8,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of vancomycin were released into HBSS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of vancomycin were released.

The mean day to day concentrations of vancomycin released into HBSS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm are summarised in table 4.4. below. A profile of vancomycin release from dispersed and HPMC-entrapped microspheres over 30 days of incubation in HBSS at 37°C is shown in figure 4.2.1. below.

After the first two days of incubation (on day one of the second, low-level, phase of release) the concentration of vancomycin released from dispersed microspheres was not significantly different to that released from HPMC-entrapped microspheres, whereas over the period from day three to day nine, HPMC-entrapped microspheres delivered significantly greater ($P < 0.05$) concentrations of vancomycin into HBSS than dispersed microspheres (figure 4.2.1.). While vancomycin release from dispersed microspheres could not be detected beyond day nine of incubation in HBSS, vancomycin release from HPMC-entrapped microspheres could still be detected after 30 days of incubation in HBSS (figure 4.2.1.). The mean day to day concentration of vancomycin released from both dispersed and HPMC-entrapped microspheres decreased as incubation continued such that dispersed microspheres released 0.89 ± 0.06 mg/ml, $n=6$ of vancomycin on day two of incubation but only 0.001 ± 0.001 mg/ml, $n=6$ of vancomycin on day nine. HPMC-entrapped microspheres released 0.71 ± 0.11 mg/ml, $n=6$ of vancomycin on day two of incubation (figure 4.2.1.). However, no vancomycin was released from HPMC-entrapped microspheres on day 28 of incubation and only 0.003 ± 0.002 mg/ml, $n=6$ of vancomycin was released from HPMC-entrapped microspheres on day 30 of incubation after which time the study was terminated (figure 4.2.1.).

Table 4.4. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 1,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	1	7.88 \pm 0.32a	81.04 \pm 3.30b	9.38 \pm 0.34c	96.47 \pm 3.49d
HPMC-entrapped	1	3.58 \pm 0.24	36.79 \pm 2.44	8.16 \pm 0.39	83.93 \pm 3.99

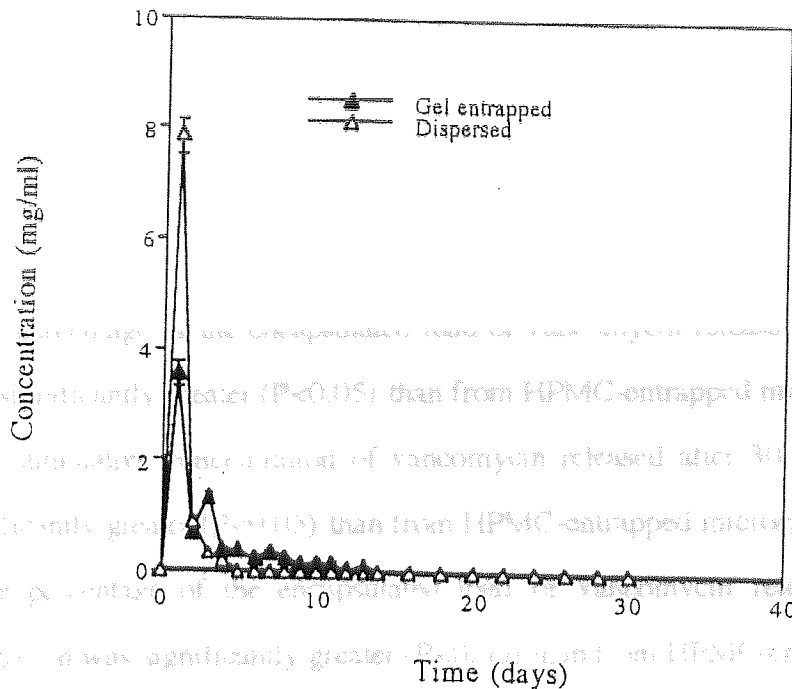
a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

b the percentage of the encapsulated load of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

c the cumulative concentration of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

d the percentage of the encapsulated load of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

Figure 4.2.1. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).



The mean day to day concentrations of vancomycin released into HBSS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (group C in table 3.6.) are summarised in table 4.5. below. A profile of vancomycin release from dispersed and HPMC-entrapped microspheres over 30 days of incubation in HBSS at 37°C is shown in figure 4.2.2. below.

Table 4.5. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	2	8.35 \pm 0.17a	94.73 \pm 1.87b	8.52 \pm 0.17c	96.61 \pm 1.95d
HPMC-entrapped	4	4.24 \pm 0.15	48.07 \pm 1.67	5.89 \pm 0.31	66.78 \pm 3.54

a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

b the percentage of the encapsulated load of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

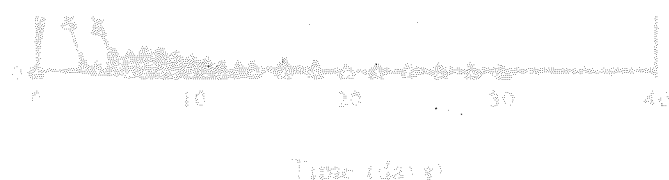
c the cumulative concentration of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

d the percentage of the encapsulated load of vancomycin released after 30 days of incubation was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

The mean day to day concentration of vancomycin released into HBSS from both dispersed and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (table 4.5. and figure 4.2.2) was similar to the mean day to day concentration of vancomycin released into HBSS from both dispersed and HPMC-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 1,000 rpm (table 4.4. and figure 4.2.1.). There was no significant difference ($P > 0.05$) between the total mean cumulative concentration of vancomycin released into HBSS from HPMC-

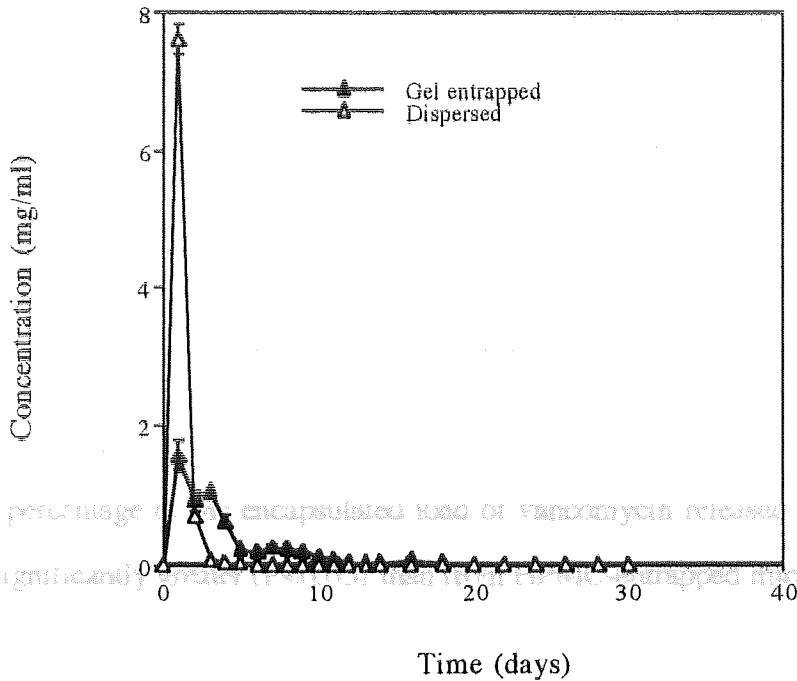
entrapped microspheres fabricated using an emulsification speed of 4,000 rpm over the four day burst release phase compared with the total mean cumulative concentrations of vancomycin released into HBSS from HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm or 8,000 rpm during their respective burst release phases (tables 4.4.-4.6.).

Day to day reductions in the mean concentration of vancomycin released from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm were such that, after seven days, vancomycin could not be detected in the HBSS incubation medium. However, vancomycin was released from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm over the whole 30 day incubation period. At no point did the day to day concentration of released vancomycin fall below 0.004 mg/ml from either dispersed or HPMC-entrapped microspheres during the period that they were actively releasing vancomycin (figure 4.2.2.).



The mean day to day release of vancomycin into HBSS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm (group B in table 3.5.) is summarised in table 4.6. below. A profile for vancomycin release dispersed and HPMC-entrapped microspheres during incubation in HBSS at 37°C is shown in figure 4.2.3. below.

Figure 4.2.2. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).



Dispersed microspheres fabricated using an emulsification speed of 8,000 rpm released a significantly greater ($P < 0.05$) concentration of vancomycin into HBSS on the first day of incubation. The mean day to day release of vancomycin into HBSS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm (group B in table 3.6.) is summarised in table 4.6. below. A profile for vancomycin release dispersed and HPMC-entrapped microspheres during incubation in HBSS at 37°C is shown in figure 4.2.3. below.

concentration of vancomycin released from these dispersed microspheres rapidly decreased following the initial incubation such that on day six of incubation, the first day of detectable vancomycin release, only 0.005 ± 0.001 mg/ml, 0.06% of vancomycin was released (figure 4.2.3). HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm released 0.006 ± 0.002 mg/ml, 0.06% of vancomycin on the first day of

Table 4.6. Release data for dispersed and hypromellose-entrapped 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 8,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [vancomycin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load	Cumulative [vancomycin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated vancomycin load
Dispersed	1	7.46 \pm 0.56a	77.21 \pm 5.78b	8.67 \pm 0.48	89.68 \pm 5.01
HPMC-entrapped	3	5.61 \pm 0.24	58.02 \pm 2.48	7.72 \pm 0.51	79.92 \pm 5.31

a the cumulative concentration of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

b the percentage of the encapsulated load of vancomycin released during the burst phase was significantly greater ($P < 0.05$) than from HPMC-entrapped microspheres.

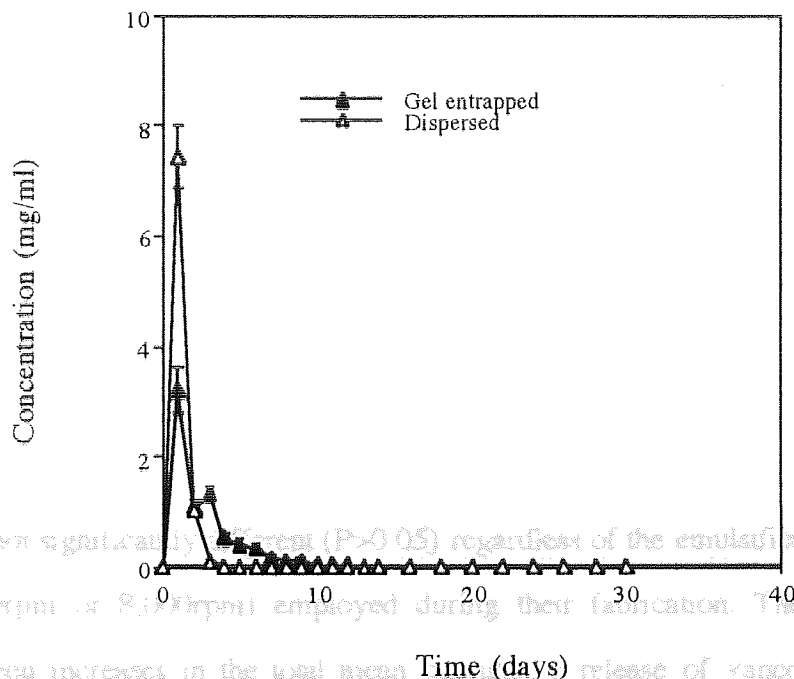
Dispersed microspheres fabricated using an emulsification speed of 8,000 rpm released a significantly greater ($P < 0.05$) concentration of vancomycin into HBSS on the first day of incubation (the burst phase of release) compared with that released during the three day burst phase produced by HPMC-entrapped microspheres (table 4.6.) Dispersed microspheres fabricated using an emulsification speed of 8,000 rpm released 1.07 ± 0.17 mg/ml, n=6 of vancomycin into HBSS on the second day of incubation, (corresponding to the first day of the second, low-level sustained release phase). However, the day to day concentration of vancomycin released from these dispersed microspheres steadily decreased following day one of incubation such that on day six of incubation, the last day of detectable vancomycin release, only 0.005 ± 0.001 mg/ml, n=6 of vancomycin was released (figure 4.2.3.). HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm released 0.56 ± 0.08 mg/ml, n=6 of vancomycin on the fourth day of

incubation in HBSS (corresponding to day one of the second, low-level sustained release phase) but only 0.007 ± 0.003 mg/ml was released on day 30 at which point the study was terminated (figure 4.2.3.). The mean day to day concentration of vancomycin released into HBSS from HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm was always greater than 0.005 mg/ml throughout the incubation period (figure 4.2.3.). During the second, low-level sustained release phase, HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm typically delivered significantly greater ($P < 0.05$) concentrations of vancomycin than dispersed microspheres fabricated using the same emulsification speed (figure 4.2.3.). Even so, the differences in the total mean cumulative and total mean cumulative percentage release of vancomycin between HPMC-entrapped and dispersed microspheres after 30 days of incubation in HBSS were not significant ($P < 0.05$) different (table 4.6.).



To summarize, vancomycin release from dispersed microspheres into HBSS on day one of incubation was related to the emulsification speed used during microsphere fabrication. The rank order of release over the time period (average concentration of vancomycin released) was 1,000 rpm > 4,000 rpm > 8,000 rpm (table 4.4-4.6 and figure 4.2.4.). Microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm provided a one day burst release of vancomycin which significantly exceeded the cumulative concentration of vancomycin released during the second, low-level release phase which was observed over 30 days. There was no significant difference in the cumulative concentration of vancomycin released during the second, low-level release phase produced by all dispersed microspheres fabricated in HBSS using the same emulsification speed. The mean cumulative concentration of vancomycin released during the second, low-level

Figure 4.2.3. The day to day release of vancomycin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 8,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).



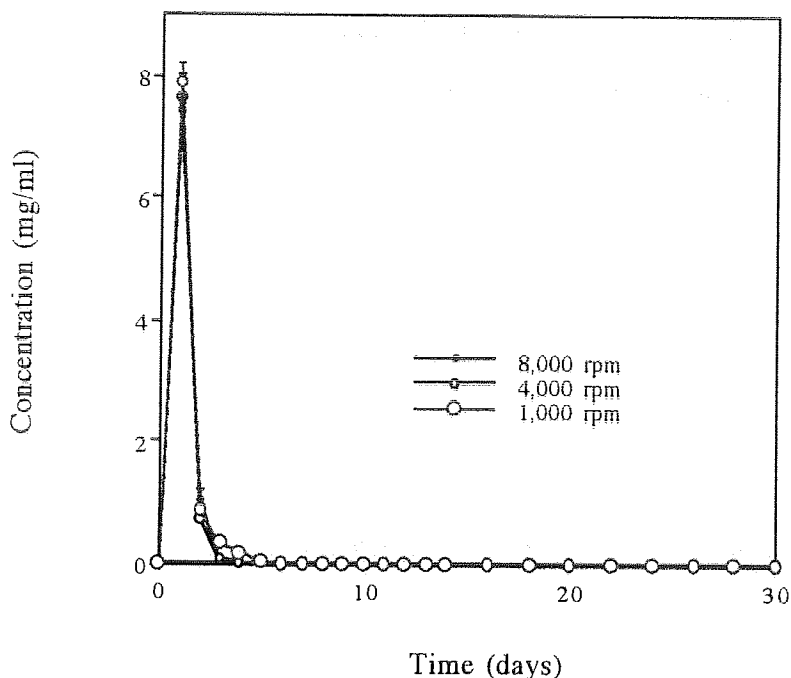
To summarise, vancomycin release from dispersed microspheres into HBSS on day one of incubation was related to the emulsification speed used during microsphere fabrication. The rank order of release over this time period (largest concentration to smallest concentration released) was 1,000 rpm > 4,000rpm > 8,000 rpm (tables 4.4-4.6 and figure 4.2.4.). Microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm produced a one day burst release of vancomycin while dispersed microspheres fabricated using an emulsification speed of 4,000 rpm produced a vancomycin burst release phase which extended over two days. There was no significant difference between the cumulative concentrations of vancomycin released during the burst release phases produced by all dispersed microspheres incubated in HBSS (tables 4.4-4.6.). The total mean cumulative concentration of vancomycin released from dispersed microspheres

fabricated using an emulsification speed of 1,000 rpm was significantly greater ($P < 0.05$) than that released from dispersed microspheres fabricated using an emulsification speed of 4,000 rpm over the 30 day incubation period (tables 4.4. and 4.5.). However, there was no significant difference between the total mean cumulative percentages of encapsulated vancomycin delivered from dispersed microspheres during incubation in HBSS regardless of the emulsification speed (1,000rpm, 4,000rpm or 8,000rpm) employed during their fabrication (tables 4.4.-4.6.).

Microsphere diameter (which has been shown in the present work to be inversely related to emulsification speed) did not significantly effect the extent of vancomycin release into HBSS during the initial burst phase (figure 4.2.4.). The cumulative concentration of vancomycin released from dispersed microspheres fabricated using an emulsification speed of 1,000 rpm was greater than that released after 30 days of incubation in HBSS from dispersed microspheres fabricated using an emulsification speed of 4,000 rpm. However, the total mean percentage release of vancomycin into HBSS from dispersed microspheres was not significantly different ($P > 0.05$) regardless of the emulsification speed (1,000rpm, 4,000rpm or 8,000rpm) employed during their fabrication. The apparent relationship between increases in the total mean cumulative release of vancomycin from dispersed microspheres as a result of decreases in the emulsification speed used in microsphere fabrication was probably influenced by encapsulation efficiency rather than indicating that increases in microsphere diameter will influence the rate of vancomycin release into HBSS.

Specifically, into HBSS from HPMC-encapsulated microspheres fabricated using an emulsification speed of 4,000 rpm (1.38 ± 0.23 µg/ml, µg of vancomycin released) (figure 4.2.5.). However, HPMC-encapsulated microspheres fabricated using an emulsification speed of 4,000 rpm produced a longer lasting vancomycin burst release than HPMC-encapsulated microspheres fabricated using an emulsification speed of 8,000 rpm or 1,000 rpm (tables 4.3-4.6). The mean cumulative concentration of vancomycin released from HPMC-encapsulated microspheres fabricated using an emulsification speed of 8,000 rpm during the initial phase was significantly greater

Figure 4.2.4. The day to day release of vancomycin from dispersed 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm, 4,000 rpm or 8,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).



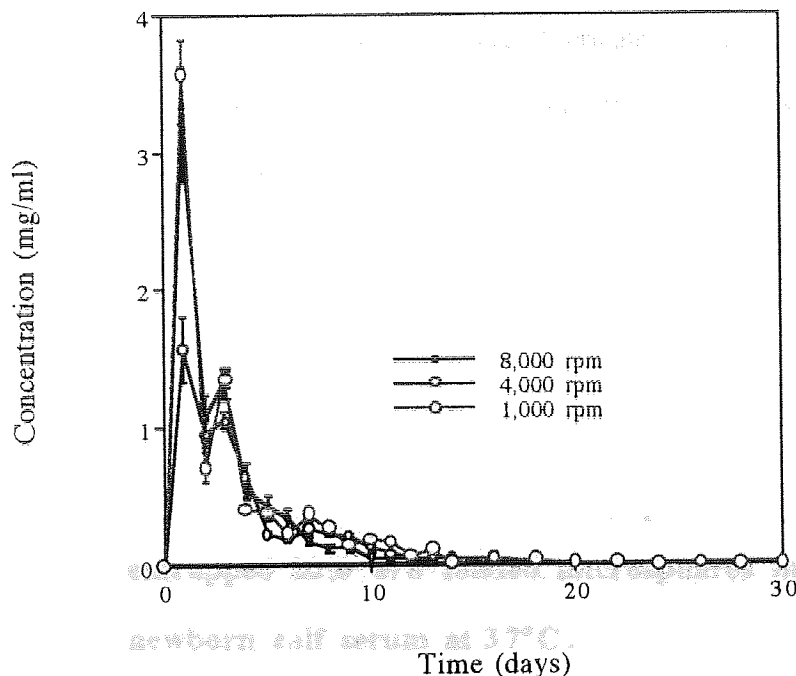
On day one of incubation in HBSS, HPMC-entrapped microspheres fabricated using an emulsification speeds of 1,000 rpm or 8,000 rpm delivered a significantly greater ($P < 0.05$) concentration of vancomycin (3.58 ± 0.24 mg/ml, $n=6$ and 3.21 ± 0.42 mg/ml, $n=6$ respectively) into HBSS than HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (1.58 ± 0.23 mg/ml, $n=6$ of vancomycin released) (figure 4.2.5.). However, HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm produced a longer lasting vancomycin burst release phase than HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm or 1,000 rpm (tables 4.3-4.6). The mean cumulative concentration of vancomycin released from HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm during the burst phase was significantly greater

($P < 0.05$) than that released from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (tables 4.3-4.6). The mean cumulative percentage concentration of vancomycin released from HPMC-entrapped microspheres fabricated using an emulsification speed of 1,000 rpm during the burst phase was significantly lower ($P < 0.05$) than that released from HPMC-entrapped microspheres fabricated using emulsification speeds of 8,000 rpm and 4,000 rpm (tables 4.3-4.6). A significantly greater ($P < 0.05$) total mean cumulative concentration of vancomycin was released into HBSS from HPMC-entrapped microspheres fabricated using an emulsification speeds of 1,000 rpm and 8,000 rpm after 30 days of incubation in HBSS compared with that released from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (tables 4.3-4.6).



The total mean cumulative concentration of vancomycin released into HBSS from HPMC-entrapped microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm were not significantly different ($P < 0.05$) probably because of the similar encapsulation efficiencies for these two preparations (around 97%). The total mean cumulative concentration of vancomycin released from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm was significantly lower ($P < 0.05$) than that released from similarly prepared microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm probably because of the lower encapsulation efficiency (around 93%) for microspheres fabricated using an emulsification speed of 4,000 rpm. The duration of the burst phase of vancomycin release was similar for all three microspheres loaded prepared and HPMC-entrapped microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm.

Figure 4.2.5. The day to day release of vancomycin from hypromellose-entrapped 20% w/w loaded microspheres fabricated using an emulsification speed of 1,000 rpm, 4,000 rpm or 8,000 rpm and incubated in Hank's balanced salt solution (mean values \pm SEM, n=6 for each point).



Both dispersed and HPMC-entrapped 20% w/w vancomycin loaded PLGA microspheres fabricated using an emulsification speed of 4,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of vancomycin were released into HBSS at 37°C. However, this burst phase of release was only transient and was followed by a period of low release. The total mean cumulative concentration of vancomycin released from HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm was significantly lower ($P < 0.05$) than that released from similarly prepared microspheres fabricated using emulsification speeds of 1,000 rpm and 8,000 rpm probably because of the lower encapsulation efficiency (around 88%) for microspheres fabricated using an emulsification speed of 4,000 rpm. Evaluation of the burst phase of vancomycin release into HBSS from both 20% w/w loaded dispersed and HPMC-entrapped microspheres showed that, irrespective of the

emulsification speed used in their fabrication, dispersed microspheres released a significantly greater ($P < 0.05$) concentration of vancomycin than their HPMC-entrapped counterparts. After 30 days of incubation in HBSS, the total mean cumulative and percentage release of vancomycin from dispersed microspheres fabricated using an emulsification speed of 1,000 rpm or 4,000 rpm was also significantly greater ($P < 0.05$) than from their HPMC-entrapped counterparts. However, there was no significant difference between the total mean cumulative and percentage release of vancomycin from dispersed and HPMC-entrapped microspheres fabricated using an emulsification speed of 8,000 rpm. In summary, it can be said that suspending 20% w/w vancomycin loaded microspheres in HPMC gel decreased the mean day to day concentration of vancomycin released into HBSS thereby extending the length of time over which encapsulated vancomycin is actively released.

4.3.2.1 Release of ciprofloxacin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum at 37°C.

Both dispersed and HPMC-entrapped 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres fabricated using an emulsification speed of 4,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of ciprofloxacin were released into NCS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of ciprofloxacin were released.

The mean day to day concentrations of ciprofloxacin released into NCS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (group B in table 3.6.) are summarised in table 4.7. below. A profile for ciprofloxacin release from dispersed and HPMC-entrapped

microspheres preparations during incubation in NCS at 37°C is shown in figure 4.3.1. below.

The mean concentration of ciprofloxacin released from dispersed microspheres on day one of incubation in NCS at 37°C (0.37 ± 0.02 mg/ml, n=6) was significantly greater ($P < 0.05$) than that released on day two (0.20 ± 0.01 mg/ml, n=6) (figure 4.3.1.). This burst phase of release was followed by a phase of sustained, low-level, ciprofloxacin release over days three to 30 of incubation. Initially, the mean day to day concentration of ciprofloxacin released from the dispersed microspheres during the second, low-level, release phase remained relatively constant at approximately 0.10 mg/ml per day (figure 4.3.1.). However, significant reductions ($P < 0.05$) in the mean day to day concentrations of ciprofloxacin released from dispersed microspheres between days 11 and 13 of incubation resulted in the release of only 0.011 ± 0.001 , n=6 mg/ml of ciprofloxacin on day 26 of incubation in NCS (figure 4.3.1.). Slight but significant ($P < 0.05$) increases in the mean day to day concentration of ciprofloxacin released into NCS occurred on days 28 and 30 after which time incubation was stopped. At no point did the mean day to day concentration of ciprofloxacin released into NCS fall below 0.011 ± 0.001 mg/ml, n=6 (figure 4.3.1.).

The cumulative concentration of ciprofloxacin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

The percentage of the encapsulated load of ciprofloxacin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

The mean day to day concentration of ciprofloxacin released from dispersed microspheres on the second day of incubation in NCS at 37°C (0.20 ± 0.01 mg/ml, n=6) was significantly greater ($P < 0.05$) than that released on day one (0.37 ± 0.02 mg/ml, n=6) (figure 4.3.1.). Both these mean concentrations were significantly greater ($P < 0.05$) than the mean day to day concentration of ciprofloxacin released from dispersed microspheres between day three and day 30 of incubation in NCS (approximately 0.10 mg/ml).

Table 4.7. Release data for dispersed and hypromellose-entrapped 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in newborn calf serum at 37°C (mean values ± SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [ciprofloxacin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated ciprofloxacin load	Cumulative [ciprofloxacin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated ciprofloxacin load
Dispersed	2	0.57 ± 0.02	7.67 ± 0.23	1.78 ± 0.06	23.88 ± 0.84
HPMC-entrapped	2	1.18 ± 0.08a	15.87 ± 1.01b	4.30 ± 0.14c	57.84 ± 1.94d

a the cumulative concentration of ciprofloxacin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

b the percentage of the encapsulated load of ciprofloxacin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

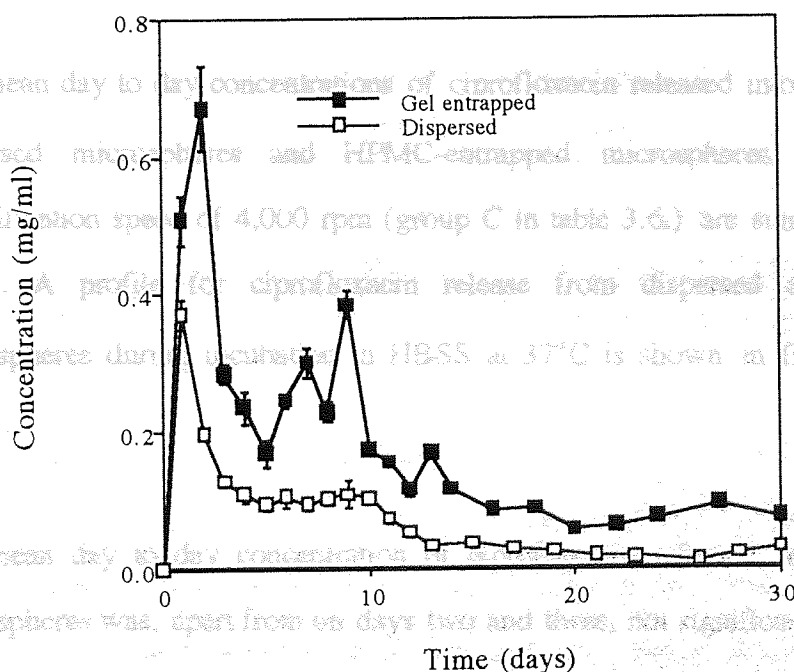
c the cumulative concentration of ciprofloxacin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

d the percentage of the encapsulated load of ciprofloxacin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

The mean day to day concentration of ciprofloxacin released from HPMC-entrapped microspheres on the second day of incubation in NCS at 37°C (0.67 ± 0.06 mg/ml, n=6) was significantly greater ($P < 0.05$) than that released on day one (0.51 ± 0.03 mg/ml, n=6) (figure 4.3.1.). Both these mean concentrations were significantly greater ($P < 0.05$) than the mean day to day concentration of ciprofloxacin released from HPMC-entrapped microspheres between day three and day nine of incubation in NCS (approximately 0.26

mg/ml per day) (figure 4.3.1.) From day 10 of incubation in NCS onwards, the mean day to day concentration of ciprofloxacin released from HPMC-entrapped microspheres dwindled significantly ($P < 0.05$) to a concentration of 0.06 ± 0.002 mg/ml, $n=6$ on day 20. A gradual but significant ($P < 0.05$) increase in ciprofloxacin release occurred between days 22-30 (figure 4.3.1.). The lowest mean day to day release of ciprofloxacin from HPMC-entrapped microspheres over the 30 day study was 0.06 ± 0.002 mg/ml, $n=6$ (figure 4.3.1.).

Figure 4.3.1. The day to day release of ciprofloxacin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum at 37°C (mean values \pm SEM, $n=6$ for each point).



While the time period of the burst phase of ciprofloxacin release from both dispersed and HPMC-entrapped microspheres was the same, the HPMC-entrapped microspheres released a significantly greater ($P < 0.05$) concentration of ciprofloxacin during this time period than dispersed microspheres. A similar situation existed after 30 days of incubation

in NCS at 37°C. Both dispersed and HPMC-entrapped microspheres were still actively releasing ciprofloxacin when the study was terminated after 30 days (figure 4.3.1.).

4.3.2.2. Release of ciprofloxacin from dispersed and HPMC-entrapped 20% w/w loaded microspheres incubated in Hank's balanced salt solution at 37°C.

Both dispersed and HPMC-entrapped 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres fabricated using an emulsification speed of 4,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of ciprofloxacin were released into HBSS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of ciprofloxacin were released.

The mean day to day concentrations of ciprofloxacin released into HBSS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (group C in table 3.6.) are summarised in table 4.8. below. A profile for ciprofloxacin release from dispersed and HPMC-entrapped microspheres during incubation in HBSS at 37°C is shown in figure 4.3.2.a) and b) below.

The mean day to day concentration of ciprofloxacin released from HPMC-entrapped microspheres was, apart from on days two and three, not significantly different ($P > 0.05$) to that released from dispersed microspheres over 30 days of incubation in HBSS at 37°C (table 4.8 and figure 4.3.2.a and b). Following the initial burst phase release of ciprofloxacin, dispersed microspheres released mean day to day concentrations of ciprofloxacin in the range of 0.002 - 0.046 mg/ml although no ciprofloxacin release was detected on day 20 of incubation in HBSS. HPMC-entrapped microspheres released mean

day to day concentrations of ciprofloxacin in the range of 0.001- 0.095 mg/ml. Between days four and 26 (when the study was terminated) of incubation in HBSS, the day to day concentration of ciprofloxacin released from both dispersed and HPMC-entrapped microspheres gradually decreased but these decreases were not significant (figure 4.3.2.a and b).

Table 4.8. Release data for dispersed and hypromellose-entrapped 20% w/w ciprofloxacin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [ciprofloxacin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated ciprofloxacin load	Cumulative [ciprofloxacin] released after 26 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated ciprofloxacin load
Dispersed	1	5.05 \pm 0.18	65.60 \pm 2.28	5.24 \pm 0.18	68.03 \pm 2.33
HPMC-entrapped	1	5.50 \pm 0.27	71.43 \pm 3.45	5.76 \pm 0.31	74.83 \pm 4.00

In summary, the total mean cumulative concentration of ciprofloxacin released from HPMC-entrapped microspheres incubated in both NCS and HBSS, and from dispersed microspheres incubated in HBSS was significantly greater ($P < 0.05$) than that released from dispersed microspheres incubated in NCS (tables 4.7-4.8). The cumulative concentration of ciprofloxacin released into NCS from HPMC-entrapped microspheres during the burst phase of release was significantly greater ($P < 0.05$) than that released during the burst phase from dispersed microspheres incubated in NCS (table 4.7). While there was no significant difference in the total mean cumulative concentration of ciprofloxacin released into HBSS from dispersed and HPMC-entrapped microspheres over

the duration of the burst phase of release, both these preparations released a significantly greater ($P < 0.05$) concentration of ciprofloxacin into HBSS than into NCS at 37°C (tables 4.7.-4.8.).

Figure 4.3.2.a) The day to day release of ciprofloxacin from dispersed and HPMC-entrapped 20% w/w loaded microspheres from day 1 to day 26 of incubation in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).

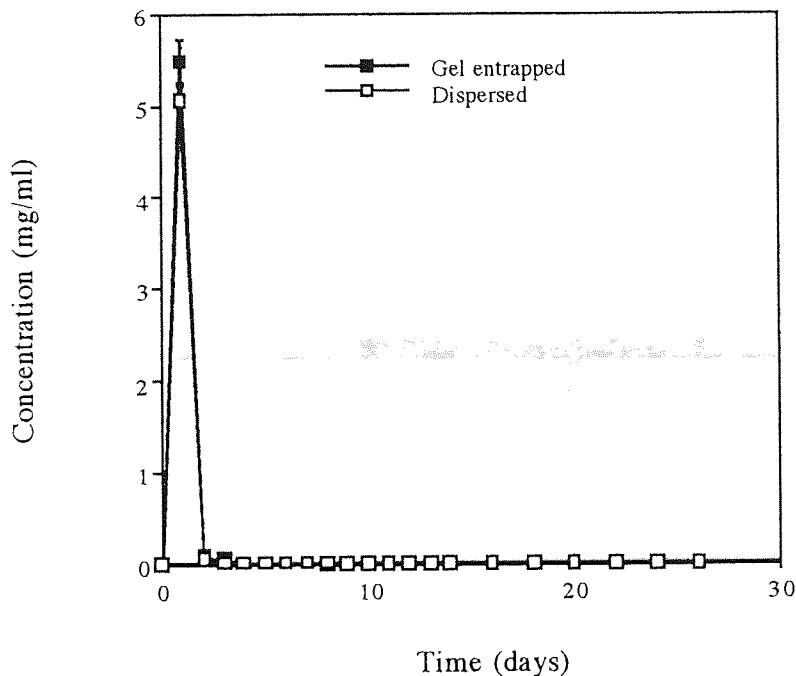
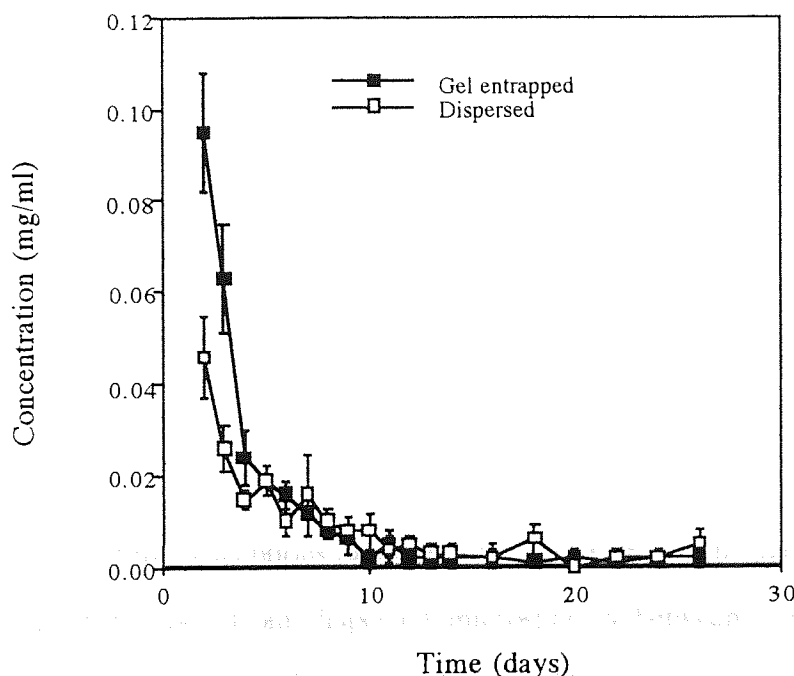


Figure 4.3.2.b) The day to day release of ciprofloxacin from dispersed and HPMC-entrapped 20% w/w loaded microspheres from day 2 to day 26 of incubation in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).



4.3.3.1 Release of rifampicin from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum at 37°C.

Both dispersed and HPMC-entrapped 20% w/w rifampicin loaded PLCG 75:25 microspheres showed a burst phase of release during which relatively large mean day to day concentrations of rifampicin were released into NCS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of rifampicin were released.

The mean day to day concentrations of rifampicin released into NCS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (group A and B respectively in table 3.6.) are summarised in table 4.9. below. A profile for rifampicin release from dispersed and HPMC-entrapped microspheres during incubation in NCS at 37°C is shown in figure 4.4.1. below.

The mean day to day concentration of rifampicin released into NCS from dispersed microspheres over the first three days of incubation decreased significantly ($P < 0.05$) with time. Subsequent to the burst phase (see table 4.9), dispersed microspheres generated a period of low-level sustained release into NCS at 37°C which lasted from day four to day 26 of incubation. Over this time, day to day concentrations of rifampicin in the range of 0.002 - 0.008 mg/ml of rifampicin were released from dispersed microspheres (figure 4.4.1.). With minor exceptions on days 7, 9, 12, 13 and 15, the day to day concentrations of rifampicin released from dispersed microspheres between day four and day 26 of incubation in NCS did not change significantly. However, a significant increase ($P < 0.05$) in the concentration of rifampicin subsequently released from dispersed microspheres into NCS was observed on day 28 (0.03 ± 0.01 mg/ml, $n=6$) and day 30 (0.01 ± 0.002 mg/ml, $n=6$) of incubation when compared with the mean day to day concentrations of rifampicin released over days 4-26 of incubation in NCS (figure 4.4.1.).

The day to day concentration of rifampicin released from HPMC-entrapped microspheres over the first two days of incubation in NCS was approximately 0.011 mg/ml. However, the mean day to day concentration of rifampicin released between days three and six became significantly smaller ($P < 0.05$) such that on day six of incubation in NCS only 0.01 ± 0.002 mg/ml, $n=6$ of rifampicin was released from HPMC-entrapped microspheres (figure 4.4.1.). The concentration of rifampicin released from HPMC-entrapped microspheres after seven, eight and nine days of incubation in NCS ranged between 0.006

and 0.009 mg/ml and was therefore significantly smaller ($P < 0.05$) than the concentrations released during the burst phase of rifampicin release. The day to day concentration of rifampicin released from day 10 of incubation onwards decreased significantly ($P < 0.05$) to between 0.001 - 0.003 mg/ml by day 30 of incubation.

Table 4.9. Release data for dispersed and hypromellose-entrapped 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6).

Microsphere preparation	Length of primary burst phase (days)	Cumulative [rifampicin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated rifampicin load	Cumulative [rifampicin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated rifampicin load
Dispersed	3	0.11 \pm 0.02	12.62 \pm 1.85	0.21 \pm 0.02	25.11 \pm 2.60
HPMC-entrapped	6	0.37 \pm 0.02a	45.27 \pm 2.07b	0.41 \pm 0.02c	50.91 \pm 2.06d

a the cumulative concentration of rifampicin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

b the percentage of the encapsulated load of rifampicin released during the burst phase was significantly greater ($P < 0.05$) than from dispersed microspheres.

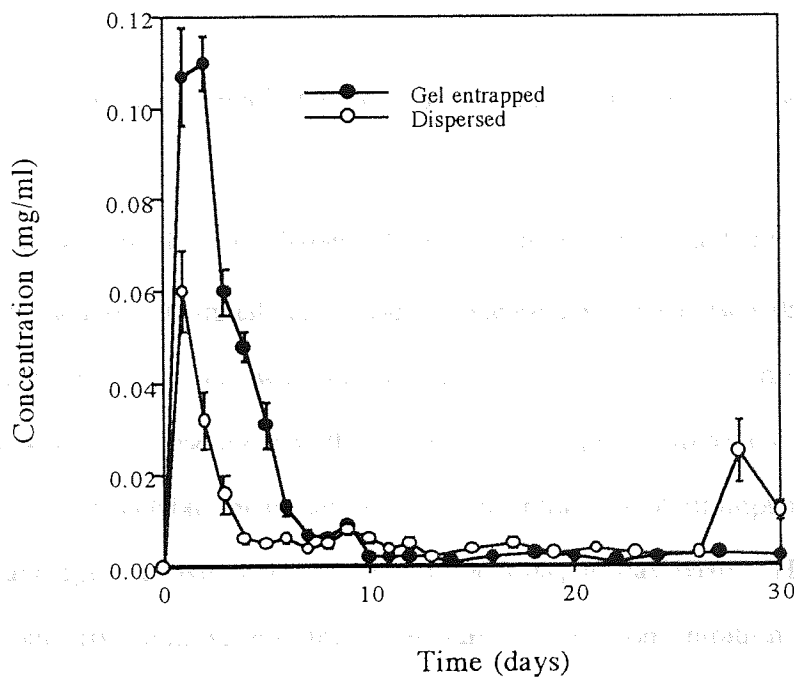
c the cumulative concentration of rifampicin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

d the percentage of the encapsulated load of rifampicin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

Apart from release on days seven, eight and 13, HPMC-entrapped microspheres released significantly greater ($P < 0.05$) concentrations of rifampicin than dispersed microspheres over the first 13 days of incubation in NCS at 37°C. The mean total cumulative

concentration of rifampicin released from HPMC-entrapped microspheres after incubation in NCS at 37°C for 30 days was almost double that released into NCS from dispersed microspheres. The total mean cumulative concentration of rifampicin released from HPMC-entrapped microspheres during the initial burst phase of release was approximately 3.5x greater than that released from dispersed microspheres even though the time period for the burst phase of rifampicin release from HPMC-entrapped microspheres was only double that of dispersed microspheres (table 4.9).

Figure 4.4.1. The day to day release of rifampicin from dispersed and HPMC-entrapped 20% loaded microspheres incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point).



From day 04 to day 30 after incubation in newborn calf serum at 37°C, the concentration of rifampicin released from dispersed microspheres was significantly lower than that released from HPMC-entrapped microspheres. The total mean cumulative concentration of rifampicin released from dispersed microspheres was 0.002 mg/ml, while that released from HPMC-entrapped microspheres was 0.007 mg/ml (table 4.9).

4.3.3.2. Release of rifampicin from dispersed and HPMC-entrapped 20% w/w loaded microspheres incubated in Hank's balanced salt solution at 37°C.

Both dispersed and HPMC-entrapped 20% w/w rifampicin loaded PLCG 75:25 microspheres fabricated using an emulsification speed 4,000 rpm produced a burst phase of release during which relatively large mean day to day concentrations of rifampicin were released into HBSS at 37°C. However, this burst phase of release was only transient and was followed by a second release phase during which sustained, low-level mean day to day concentrations of rifampicin were released.

The mean day to day concentrations of rifampicin released into NCS at 37°C from dispersed microspheres and HPMC-entrapped microspheres fabricated using an emulsification speed of 4,000 rpm (group C in table 3.6.) are summarised in table 4.10. below. A profile for rifampicin release from dispersed and gel entrapped microspheres during incubation in HBSS at 37°C is shown in figure 4.4.2. below.

The release profile for dispersed microspheres incubated in HBSS at 37°C was characterised by an initial lag phase of release lasting for two days during which time ≤ 0.001 mg/ml rifampicin was released into HBSS over each consecutive 24 hour period (figure 4.4.2.). Subsequently, the release of rifampicin, up to and including day 22, was erratic with detectable mean day to day concentrations of rifampicin ranging from 0.01 - 0.10 (average approximately 0.05 mg/ml on a day to day basis). However, apart from on days four, five and seven, the mean day to day concentration of rifampicin released between days three to 24 were not significantly different. From day 24 to day 30 after which time the study was terminated, the mean day to day concentration of rifampicin released decreased significantly ($P < 0.05$) compared with the mean day to day concentrations released between day four and 24 such that on day 30, only 0.002 ± 0.000 mg/ml, $n=6$ rifampicin was released (figure 4.4.2.).

Table 4.10. Release data for dispersed and hypromellose-entrapped 20% w/w rifampicin loaded microspheres fabricated using an emulsification speed of 4,000 rpm and incubated in Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6).

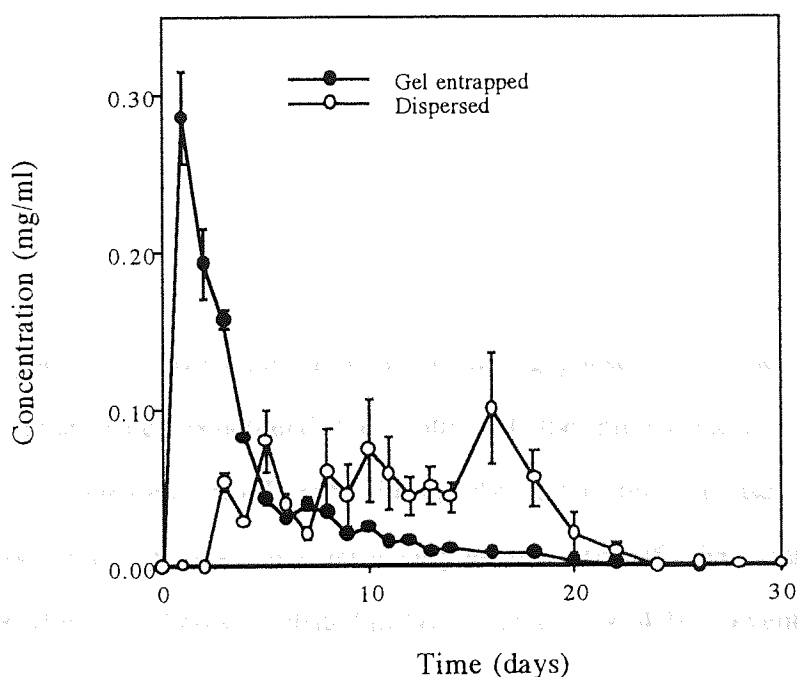
Microsphere preparation	Length of primary burst phase (days)	Cumulative [rifampicin] released during primary burst phase (mg/ml)	Equivalent % of actual total micro-encapsulated rifampicin load	Cumulative [rifampicin] released after 30 days incubation (mg/ml)	Equivalent % of actual total micro-encapsulated rifampicin load
Dispersed	N/A	N/A	N/A	0.78 \pm 0.06	72.23 \pm 5.72
HPMC-entrapped	4	0.72 \pm 0.04	66.24 \pm 3.38	0.99 \pm 0.04a	91.13 \pm 3.64b

a the cumulative concentration of rifampicin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

b the percentage of the encapsulated load of rifampicin released after 30 days of incubation was significantly greater ($P < 0.05$) than from dispersed microspheres.

The mean concentration of rifampicin released from HPMC-entrapped microspheres on day one of incubation in HBSS was significantly greater ($P < 0.05$) than that subsequently released on day two ($P < 0.05$) and after this time, a gradual decrease in the mean day to day concentration of rifampicin released was observed up to the termination of the study after 30 days of incubation. While 0.04 ± 0.004 mg/ml, n=6 of rifampicin was released on day five of incubation in HBSS, only 0.001 ± 0.000 mg/ml, n=6 of rifampicin was released on day 30. The lowest mean day to day concentration of rifampicin released into HBSS from HPMC-entrapped microspheres was 0.002 ± 0.000 μ g/ml, n=6.

Figure 4.4.2. The day to day release of rifampicin from dispersed and HPMC-entrapped 20% w/w loaded microspheres incubated in Hank's balanced salt solution at 37°C (mean values ± SEM, n=6 for each point).



The mean day to day concentration of rifampicin released from HPMC-entrapped microspheres was significantly greater ($P < 0.05$) than that released from dispersed microspheres over days one to four and day seven of incubation in HBSS. However, the mean day to day concentration of rifampicin released from dispersed microspheres was significantly greater ($P < 0.05$) than that released from HPMC-entrapped microspheres over days six, 12-22 and 28-30. Therefore, while rifampicin was released in greater mean day to day concentrations from HPMC-entrapped microspheres during the initial stages of the release profile, the dispersed microspheres were responsible for the release of greater mean

day to day concentration of rifampicin during the second, low-level sustained release phase (figure 4.4.2.).

In summary, over 30 days of incubation at 37°C, HPMC-entrapped microspheres released a significantly greater ($P < 0.05$) total mean cumulative concentration of rifampicin into HBSS than dispersed microspheres into both NCS and HBSS (tables 4.9-4.10). The total mean cumulative concentration and cumulative percentage of rifampicin released from dispersed and HPMC-entrapped microspheres incubated in HBSS at 37°C was significantly greater ($P < 0.05$) than that released from dispersed and HPMC-entrapped microspheres incubated in NCS (tables 4.9-4.10). The initial burst phase of rifampicin release produced by HPMC-entrapped microspheres incubated in both NCS and HBSS extended for a longer period of time and involved the release of a significantly greater ($P < 0.05$) concentration of rifampicin compared with the burst phase release of rifampicin produced by dispersed microspheres incubated in NCS (tables 4.9-4.10.). Dispersed microspheres in HBSS produced an initial lag phase of release (figure 4.4.2.) during which time water penetrated the matrix of the microspheres. The concentration of rifampicin released into HBSS during the initial burst phase produced by HPMC-entrapped microspheres was significantly greater ($P < 0.05$) than that produced by HPMC-entrapped microspheres incubated in NCS (tables 4.9.-4.10.) eventhough the burst phase release from HPMC-entrapped microspheres incubated in NCS extended for a longer time period than the burst phase of rifampicin release produced by HPMC-entrapped microspheres incubated in HBSS (figure 4.4.2.).

4.3.4 Kinetic analyses of *in vitro* antibiotic release from dispersed and hypromellose-entrapped 20% w/w loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

Theoretical mathematical models such as first order and square-root time plots can be applied to release data in order to characterise the release of encapsulated material *in vitro* from microspheres. Rate constants generated from the regression lines of best-fit for antibiotic release over time give an indication of how quickly antibiotics can be released from the microspheres and allow comparison of the rate of release of different antibiotics from microsphere preparations. Antibiotic release has been characterised as being either first order (where release is proportional to the amount of antibiotic present and is therefore governed by the antibiotic concentration rather than by its entrapment within a polymer matrix) or diffusional (where the microsphere polymer matrix acts as a passive barrier to antibiotic release and controls the rate of antibiotic delivery by diffusion (Higuchi, 1963; Schindler *et al*, 1977)) and depends on the value of the regression coefficient (r^2) for the line of best fit. When the regression coefficient is 1.0, the regression line fits the data points exactly but the goodness of fit decreases as the r^2 value approaches zero. A comparison of r^2 values obtained for first order and square root time plots thus makes it possible to determine which model is best able to explain the kinetics of antibiotic release from a particular microsphere preparation.

In order to calculate the first order release constant (k_1), \log_{10} values for the mean cumulative percentage of the actual total encapsulated load of antibiotic remaining within the microspheres (100% - mean cumulative percentage of antibiotic released over time) are plotted against the time in hours. The computer generated equation for the line of best fit ($y = mx + c$) is analogous to the classical equation for calculation of the first order release constant (Martinez *et al*, 1997) which is shown in Equation 4.1. below.

Equation 4.1. Calculation of a first order release constant.

$$\log W_t = \log W_0 - (k_1 t)/2.303$$

where W_t is the percentage of antibiotic remaining within microspheres at time t , W_0 is the initial percentage of antibiotic within the microspheres (100%) and k_1 is the first order release constant. Therefore, “c” in the equation for the line of best fit $y = mx+c$ represents the release constant for the microsphere preparation (Martinez *et al*, 1997).

The Higuchi release constant (K_H) is obtained by plotting the total mean cumulative percentage of the total encapsulated antibiotic load actually released from microspheres against the square root of time (\sqrt{t} hours) or $t^{1/2}$. The equation used to calculate a square-root time release constant is shown in equation 4.2. below:

Equation 4.2. Calculation of a square-root time release constant.

$$M_t / M_\infty = K_H t^{1/2}$$

where M_t / M_∞ is the fraction of antibiotic released at time t and K_H is the Higuchi release constant (Sampath *et al*, 1992). Assuming that M_∞ is equivalent to 100%, plotting the cumulative percentage of the total encapsulated load of antibiotic released against the square root of time allows rearrangement of the equation to that shown in equation 4.3 below (Malamataris and Augerinos, 1990).

Equation 4.3. Rearrangement of the square-root time release constant equation.

$$100 - M_t = K_h t^{1/2}$$

where $100 - Mt$ is the cumulative percentage of antibiotic actually released as a proportion of 100% (the initial percentage of antibiotic encapsulated within the microspheres) such that the release constant is equivalent to "c" in the standard line of best fit equation $y = mx + c$ or $y - mx = c$ (Malamataris and Augerinos, 1990).

Data obtained for the release of antibiotic from dispersed and HPMC-entrapped microspheres loaded with 20% w/w vancomycin, 20% w/w ciprofloxacin or 20% w/w rifampicin and incubated in NCS and HBSS at 37°C have been used to construct the first order and square-root time plots shown in figures 4.5.-4.7. below.

Figure 4.5.1. First order and square-root time plots for 20% w/w vancomycin loaded microspheres incubated in newborn calf serum at 37°C (mean values \pm SEM, n=6 for each point, error bars omitted for clarity). Legends refer to the emulsification speed employed during microsphere fabrication.

Figure 4.5.1.a) First order plot for vancomycin release from dispersed microspheres.

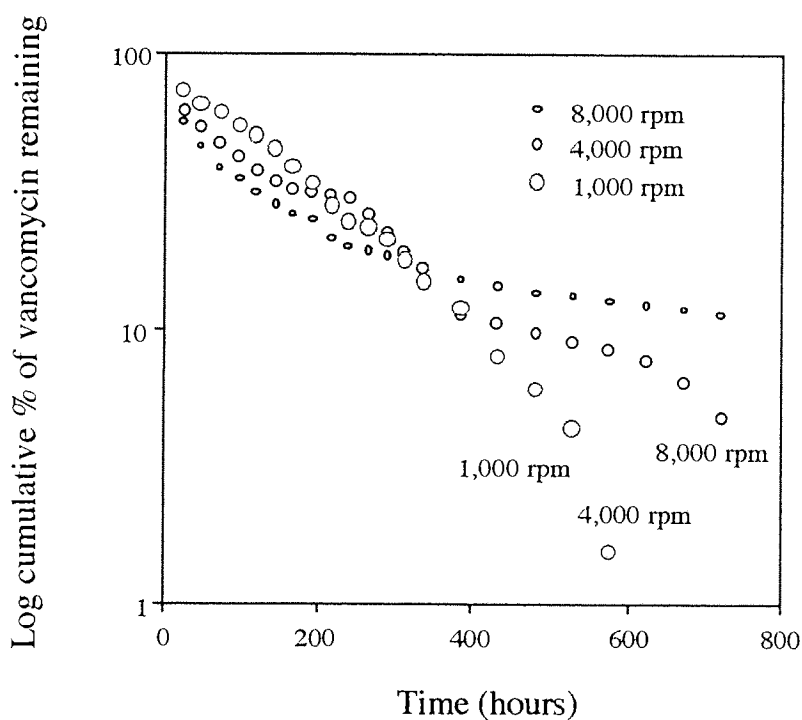


Figure 4.5.1.b) Square-root time plot for vancomycin release from dispersed microspheres.

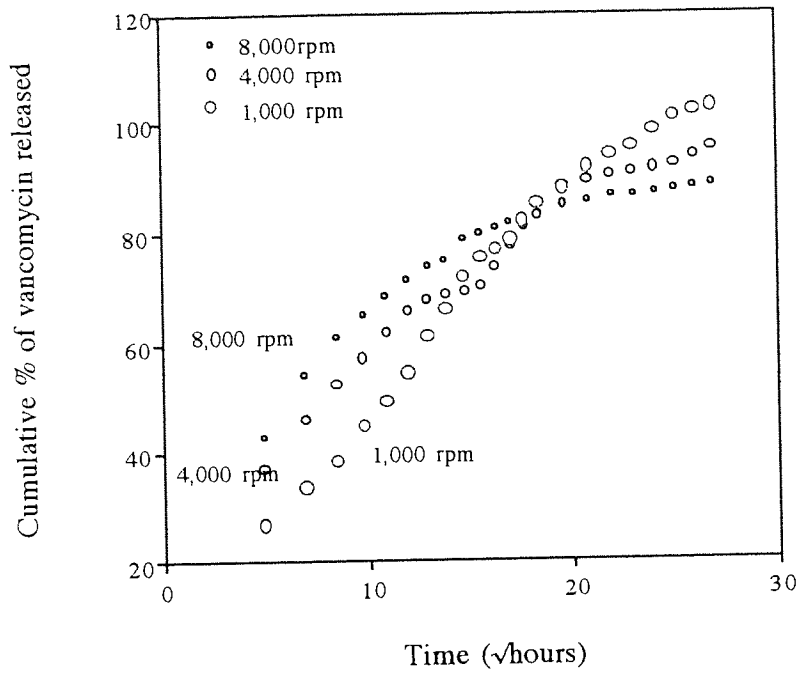


Figure 4.5.1.c) First order plot for vancomycin release from HPMC-entrapped microspheres.

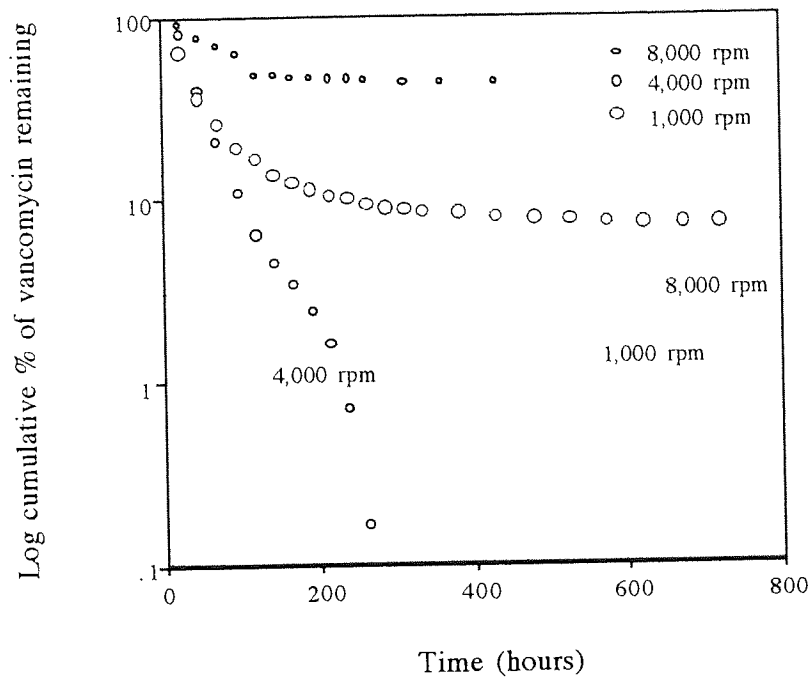


Figure 4.5.1.d) Square-root time plot for vancomycin release from HPMC-entrapped microspheres incubated in Hank's

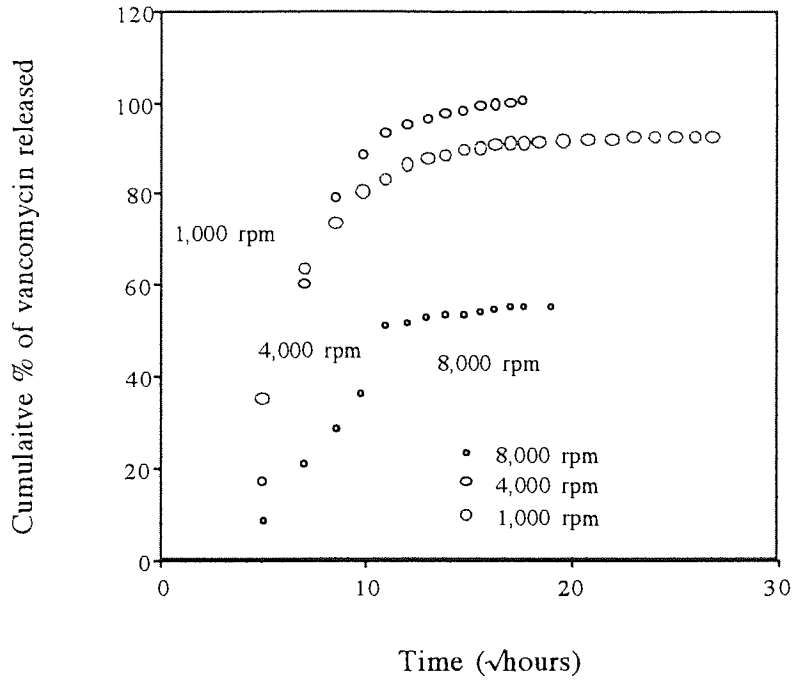


Figure 4.5.2. First order and square-root time plots for 20% w/w vancomycin loaded microspheres incubated in Hank's balanced salt solution (mean values \pm SEM, n=6 for each point, error bars omitted for clarity). Legends refer to the emulsification speed employed during microsphere fabrication.

Figure 4.5.2.a) First order plot for vancomycin release from dispersed microspheres.

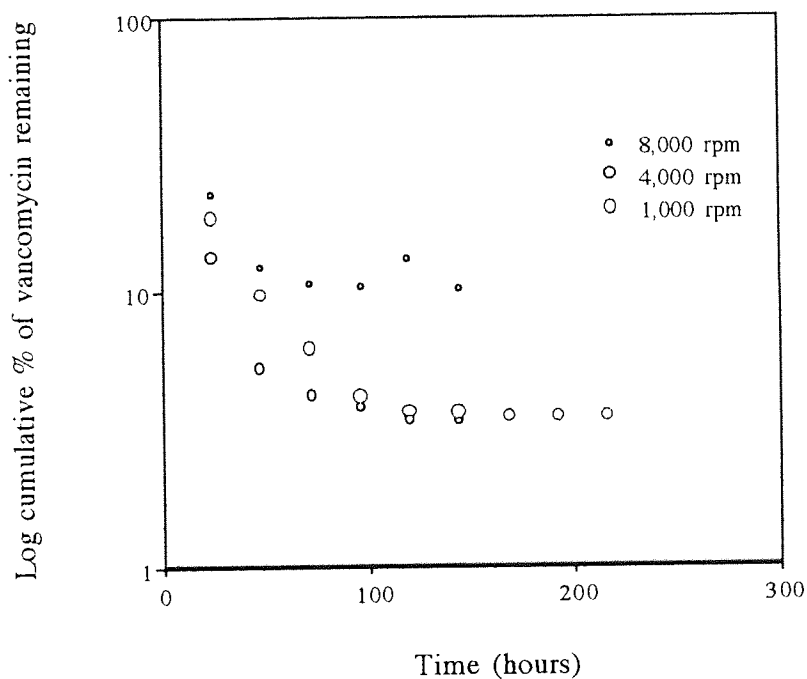


Figure 4.5.2.b) Square-root time plot for vancomycin release from dispersed microspheres.

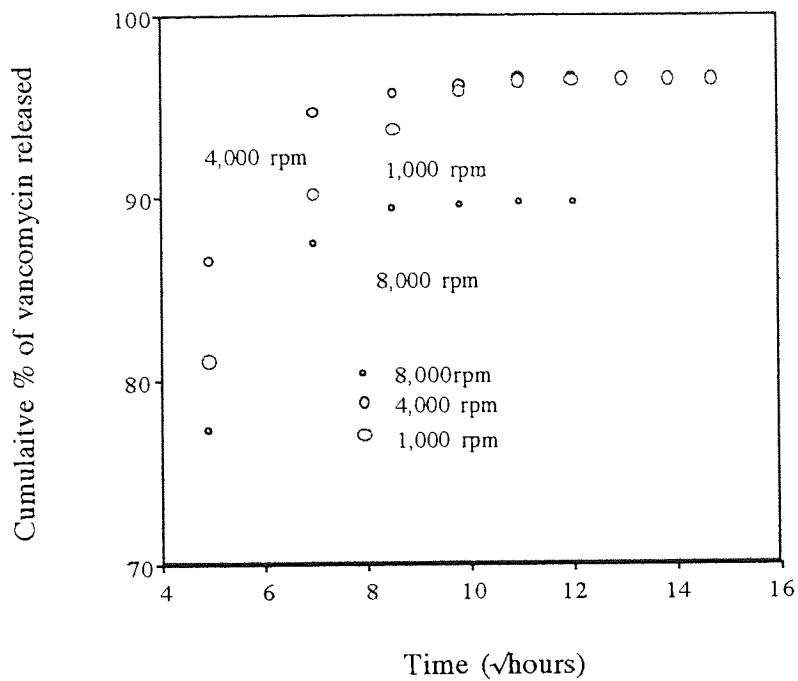
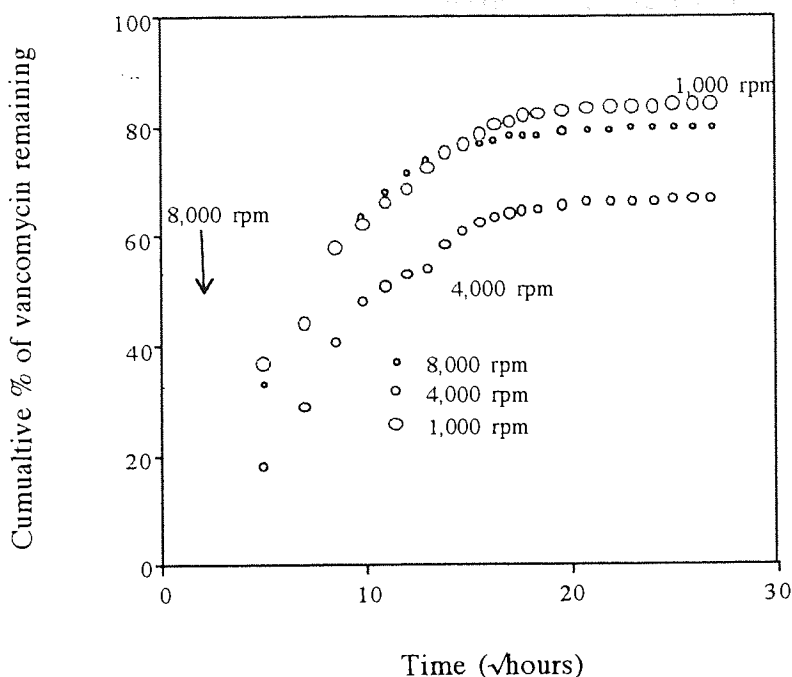


Figure 4.5.2.d) Square-root time plot for vancomycin release from HPMC-entrapped microspheres.



The release constants and regression coefficients derived from all of these kinetic plots have been summarised in Table 4.11. below.

For dispersed and HPMC-entrapped 290% w/w vancomycin loaded microspheres incubated in NCS and HBSS at 37°C, regression coefficients derived from the square-root time plots of release data provided a better fit than those derived from first order plots of the same release data (table 4.11.) suggesting that the release of vancomycin from 20% w/w loaded microspheres was essentially diffusional. The r^2 values obtained for HPMC-entrapped microspheres incubated in NCS in figure 4.5.1.c were smaller than the values obtained for dispersed microspheres over the 30 days incubation period. This indicates that HPMC gel influenced the mechanism by which vancomycin was released from HPMC-entrapped microspheres possibly by acting as a separate diffusion layer

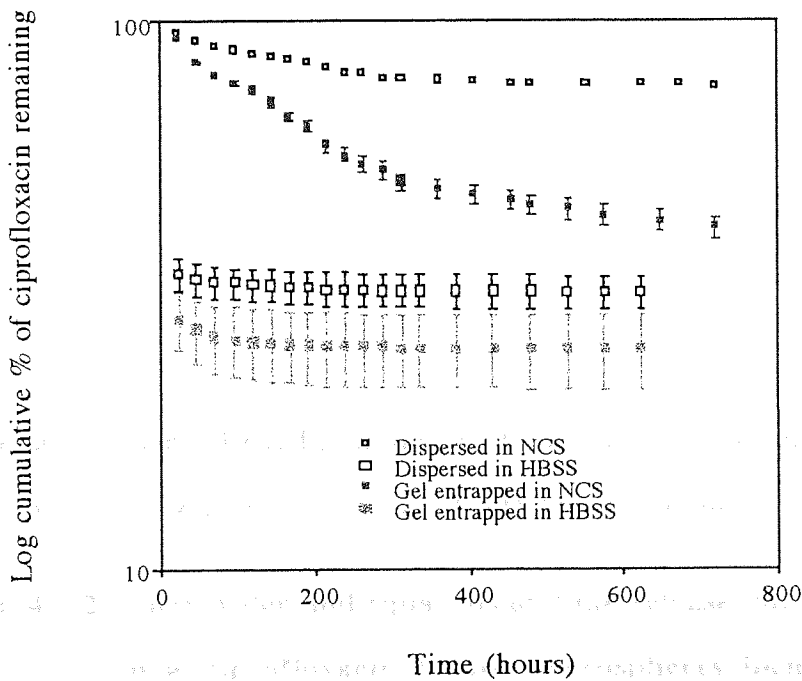
through which vancomycin had to travel before reaching NCS. The release constants obtained for HPMC-entrapped microspheres fabricated using emulsification speeds of 4,000 rpm and 8,000rpm after incubation in NCS at 37°C (figures 4.5.1.c and d) and HBSS at 37°C (figure 4.5.2.c and d) were greater than those obtained for dispersed microspheres fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm after incubation in NCS at 37°C (figures 4.5.1.a and b) and HBSS at 37°C (figures 4.5.2.a and b). This confirmed that the entrapment of microspheres in HPMC increased the rate of vancomycin release into both NCS and HBSS. This observation suggests that a more complex release model must be used to represent the kinetics of vancomycin release from HPMC-entrapped microspheres. Such a model would have to account for antibiotic release through the microsphere matrix and the HPMC gel layer into NCS and would be influenced by rheological factors such as gel viscosity and density and the rate of gel breakdown and degradation all of which are difficult to predict. The Higuchi release constants for dispersed microspheres incubated in NCS were influenced by microsphere mean diameter (as determined by the speed of emulsification used during microsphere fabrication). Microspheres fabricated using an emulsification speed of 1,000 rpm released vancomycin at a faster rate than dispersed microspheres fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm. This observation supports the finding that dispersed microspheres fabricated using an emulsification speed of 1,000 rpm released cumulatively more antibiotic into NCS after 30 days incubation than their counterparts fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm (see section 4.2.1.). There was little difference in the Higuchi rate constants obtained for dispersed 20% w/w vancomycin loaded microspheres incubated in HBSS irrespective of the emulsification speed employed during their fabrication. This was also the case for HPMC-entrapped microspheres which released vancomycin into HBSS at a faster rate than dispersed microspheres over the 30 day incubation period. This was probably because HPMC-entrapped microspheres released greater concentrations of vancomycin during the second, low-level sustained release phase than dispersed microspheres.

Table 4.11. First order and square-root time release constants for 20% w/w vancomycin loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

Microsphere Preparation and emulsification speed used	k_1 (hours ⁻¹)	r^2	K_H (hours ^{-1/2})	r^2
Dispersed in NCS:				
1,000 rpm	0.129	0.906	3.684	0.966
4,000 rpm	0.073	0.858	2.581	0.955
8,000 rpm	0.048	0.700	1.783	0.853
HPMC-entrapped in NCS:				
1,000 rpm	0.039	0.377	1.557	0.543
4,000 rpm	0.246	0.602	4.974	0.694
8,000 rpm	0.097	0.585	3.249	0.805
Dispersed in HBSS:				
1,000 rpm	0.060	0.582	1.331	0.714
4,000 rpm	0.067	0.593	1.242	0.702
8,000 rpm	0.071	0.440	1.529	0.663
HPMC-entrapped in HBSS:				
1,000 rpm	0.049	0.583	1.845	0.759
4,000 rpm	0.047	0.560	1.803	0.735
8,000 rpm	0.040	0.470	1.578	0.647

Figure 4.6. First order and square-root time plots for 20% w/w ciprofloxacin loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C (mean values \pm SEM, n=6 for each point).

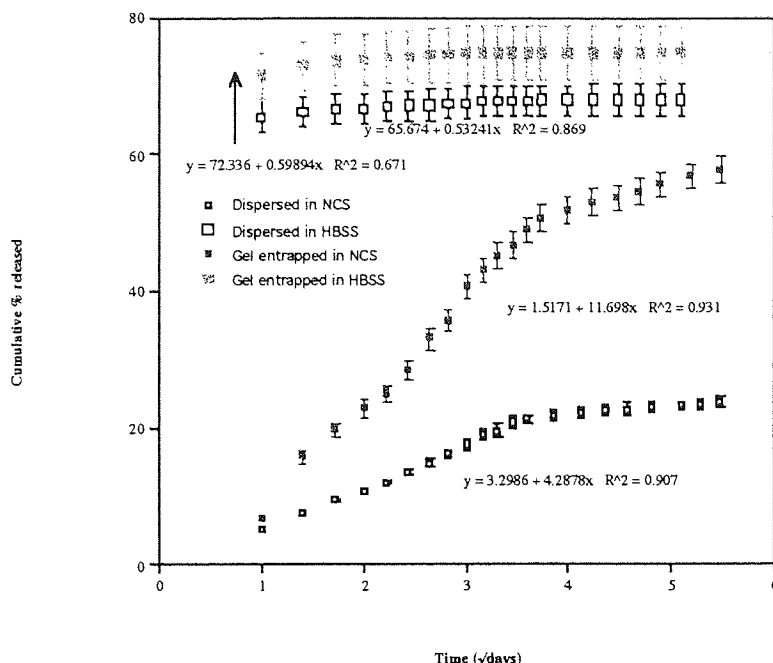
Figure 4.6.a) First order plot for ciprofloxacin release from dispersed and HPMC-entrapped microspheres.



Time (hours)	Dispersed in NCS	Dispersed in HBSS	Gel entrapped in NCS	Gel entrapped in HBSS
0	100	100	100	100
25	95	95	95	95
50	85	90	95	95
100	65	85	95	95
150	55	80	95	95
200	45	75	95	95
300	35	70	95	95
400	30	65	95	95
500	25	60	95	95
600	20	55	95	95
700	15	50	95	95
800	10	45	95	95

The first order plot for ciprofloxacin release from dispersed microspheres is shown in Figure 4.6.a, as indicated by the linear relationship between the log cumulative percentage of ciprofloxacin remaining and time (Figure 4.6.b), confirming that the release of

Figure 4.6.b) Square-root time plot for ciprofloxacin release from dispersed and HPMC-entrapped microspheres.



The release constants derived from both first order and Higuchi square root time plots of ciprofloxacin release data are summarised in table 4.12. below.

Table 4.12. First order and square-root time release constants for 20% w/w ciprofloxacin loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

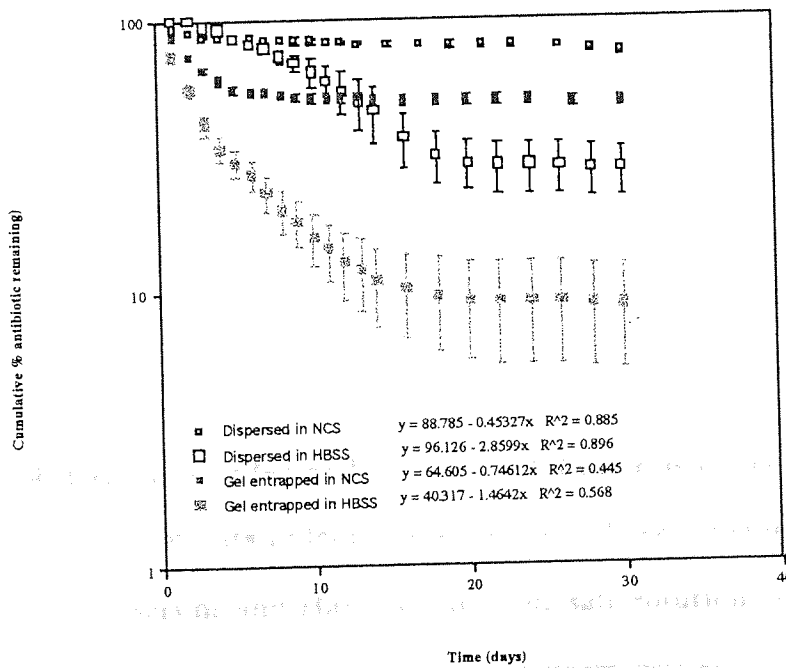
Microsphere Preparation	k_1 (hours ⁻¹)	r^2	K_H (hours ^{-1/2})	r^2
Dispersed in NCS	0.024	0.778	0.875	0.907
HPMC-entrapped in NCS	0.068	0.823	2.388	0.931
Dispersed in HBSS	0.003	0.718	0.109	0.869
HPMC-entrapped in HBSS	0.003	0.498	0.122	0.671

The Higuchi plots fitted the release data better than the First order plots, as indicated by the values for the regression coefficients shown in table 4.12, confirming that the release of

ciprofloxacin from both dispersed and HPMC-entrapped microspheres was diffusional. The Higuchi release constants obtained for HPMC-entrapped microspheres incubated in both NCS and HBSS at 37°C were greater than those for dispersed microspheres albeit only slightly greater for microspheres incubated in HBSS (figure 4.6.a and b and table 4.12.). It may be that HPMC encourages the mobilisation of ciprofloxacin encapsulated within the microsphere matrix by acting as a hydrocolloid and taking up the incubation medium during swelling thereby facilitating the penetration of the incubation medium into the microsphere matrix. This could account for the enhanced total mean cumulative release of ciprofloxacin from HPMC-entrapped microspheres compared with the release from dispersed microspheres. The K_H values computed for dispersed and HPMC-entrapped microspheres incubated in NCS at 37°C were both greater in magnitude than the K_H values obtained for similar preparations incubated in HBSS at 37°C. This may be explained by the large burst phase of ciprofloxacin release from microspheres incubated in HBSS leaving relatively little ciprofloxacin available for release during the second, low-level sustained release phase and hence the rate of ciprofloxacin release slowed over this later period of incubation.

Figure 4.7. First order and square-root time plots for 20% w/w rifampicin loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C (mean values ± SEM, n=6 for each point).

Figure 4.7.a) First order plot for rifampicin release from dispersed and HPMC-entrapped microspheres.



...the release data, as ... This suggests that release of ... HPMC-entrapped microspheres was ... faster rate of transport release into NCS than their

Figure 4.7.b) Square-root time plot for rifampicin release from dispersed and HPMC-entrapped microspheres.

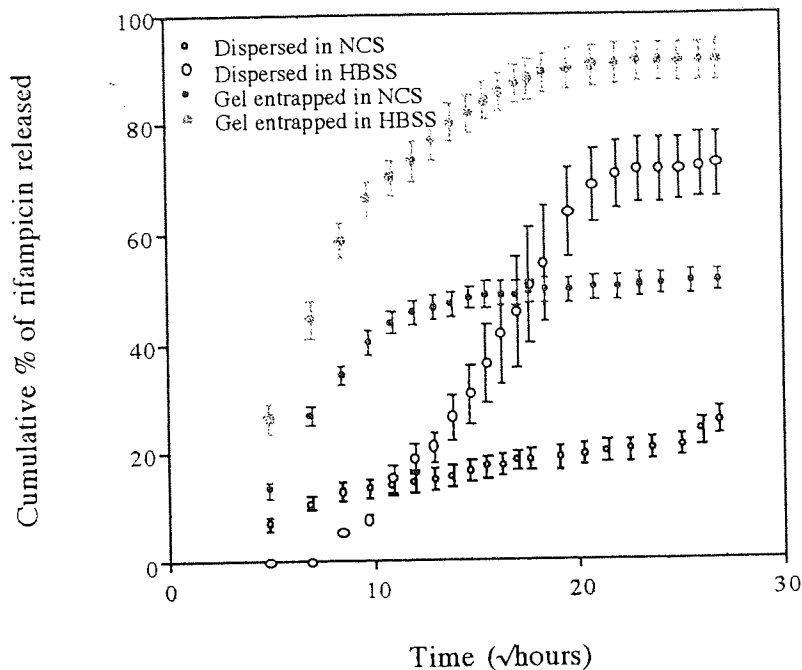


Table 4.13. First order and square-root time release constants for 20% w/w rifampicin loaded microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C.

Microsphere Preparation	k_1 (hours ⁻¹)	r^2	K_H (hours ^{-1/2})	r^2
Dispersed in NCS	0.019	0.885	0.652	0.952
HPMC-entrapped in NCS	0.031	0.445	1.207	0.623
Dispersed in HBSS	0.119	0.896	4.099	0.953
HPMC-entrapped in HBSS	0.061	0.568	2.326	0.741

The Higuchi plot provides a better goodness of fit for the rifampicin release data, as indicated by the regression coefficients shown in table 4.13. This suggests that release of rifampicin from dispersed and HPMC-entrapped microspheres was diffusional. HPMC-entrapped microspheres showed a faster rate of rifampicin release into NCS than their

dispersed counterparts (figure 4.7.a and b and table 4.13.), and was probably due to increased aqueous penetration of the microsphere matrix by NCS and HBSS mediated by the swelling of HPMC as suggested earlier. However, the rate of rifampicin release from dispersed microspheres into HBSS was greater than that from HPMC-entrapped microspheres incubated in HBSS at 37°C (figure 4.7. and table 4.13.). This suggests that the passage of rifampicin through HPMC gel after release from the entrapped microspheres slowed its entry into HBSS. The observation that Higuchi constants for rifampicin release into HBSS from both dispersed and HPMC-entrapped microspheres were larger than the Higuchi constants obtained for dispersed and HPMC-entrapped microspheres incubated in NCS are consistent with rifampicin being more soluble in HBSS than in NCS.

4.4. Discussion

The work presented here has dealt with characterising the release of vancomycin, ciprofloxacin and rifampicin from dispersed and gel entrapped 20% w/w antibiotic loaded microspheres. Hutchinson and Furr (1990) showed that the release of a microencapsulated drug from microspheres is influenced by the properties of the polymer used in microsphere fabrication. Factors such as polymer composition, molecular weight, polydispersity and crystallinity play a crucial role in deciding which polymer to utilise when constructing a delivery device. The rate of polymer degradation will also be affected by these physicochemical properties. For example, microspheres composed of a low molecular weight polymer will degrade at a faster rate than microspheres fabricated using the same polymer with a higher molecular weight, and will hence release microencapsulated drug at a faster rate (Ogawa *et al*, 1988). Likewise, changing the composition of a copolymer such as PLCG by increasing the glycolide content at the expense of the lactide content will also increase the rate of drug release by speeding up the rate of biodegradation (Beck *et al*, 1983). The rate of microsphere biodegradation has also been increased by increasing the core loading of the microencapsulated drug, as demonstrated by Wakiyama and colleagues using dibucaine (Wakiyama *et al*, 1982). The properties of the microencapsulated material

such as solubility and particle size will also have an effect on its subsequent release into the external medium. The diffusion of a drug through the polymer membrane is vital for diffusional release to occur and is dependent upon its solubility in the polymer (Jalil and Nixon, 1990c). Jalil and Nixon have indicated that if the drug cannot negotiate passage through the polymer matrix to the external medium then release will be controlled only by the rate of polymer biodegradation (Jalil and Nixon, 1990d). The diffusion of large molecules through the polymer matrix will be slow such that their release into the external medium will take longer even if the molecules in question are close to the external surface of the microspheres (Jalil and Nixon, 1990d). Also, knowing the degree of solubility of the drug in the external medium also helps to define release kinetics. This is because drugs which are soluble in the external medium are more likely to be released at a faster rate than drugs which are relatively insoluble in the same medium as there will be more impetus for the release of soluble drugs to occur down the concentration gradient (Jalil and Nixon, 1990d). Finally, the distribution of the core material within the microsphere matrix influences subsequent release since deep-seated drug will obviously take longer to be released by diffusion than drug closer to the external surface of microspheres (Jalil and Nixon, 1990d). Other factors influencing the release of microencapsulated material include the concentration of polymer used in fabrication and the extent of microsphere drug loading and microsphere diameter. Increasing the concentration of polymer in the water phase during fabrication will decrease the release rate by decreasing the matrix porosity of the final microspheres (Sato *et al*, 1988), whereas higher drug loadings increase the rate of release by increasing drug crystallinity in the polymer matrix which increases matrix porosity after crystal dissolution (Tsai *et al*, 1986). There is also evidence to suggest that the duration over which a drug can be released decreases with an increase in drug loading while the overall percentage of the microencapsulated concentration of the drug released increases with drug loading due to leaching and pore diffusion of the drug (Bodmeier and McGinity, 1987a). Microsphere diameter also influences release properties. The rate of diffusional release of material from a microsphere is directly proportional to its surface area (Heller, 1980) such that, when compared with larger microspheres, smaller microspheres

will release drug at a faster rate due to the larger collective surface area that they present to the incubation medium (Mason *et al.*, 1976; Beck *et al.*, 1983; Vidmar *et al.*, 1984; Suzuki and Price, 1985; Watts *et al.*, 1990; Pavanetto *et al.*, 1993). The methods that can be used to engineer the final diameter of microspheres, which include changes in the surfactant concentration, the emulsification speed, and the emulsion viscosity as well as sonication of the emulsion have already been discussed in chapter 3. Ultrasound treatment (Liu *et al.*, 1992, Park, 1994) and gamma-irradiation sterilisation (Setterstrom *et al.*, 1984) have been employed to quicken the rate of biodegradation and hence increase the release rate of encapsulated substances such as p-nitroaniline from poly lactic acid tablet devices (into phosphate buffer, pH 7.4) and ampicillin from PLCG microcapsules (into 0.1M potassium phosphate, pH 7.4).

It is generally accepted that the release of material from microspheres occurs in three distinct stages or phases (Beck *et al.*, 1983; Suzuki and Price, 1985; Holland *et al.*, 1986; Tsai *et al.*, 1986; Bodmeier and McGinity, 1987b; Ogawa *et al.*, 1988; Hora *et al.*, 1990; Hutchinson and Furr, 1990; Bodmer *et al.*, 21, 1992; Schmitt *et al.*, 1993; Sturesson *et al.*, 1993; O'Hagan *et al.*, 1994; Martinez *et al.*, 1997). Phase one (the burst phase) involves the release of relatively large concentrations of microencapsulated material over a short period of time. This burst phase is usually caused by the liberation of drug from the outer layers or from the surface of the microspheres. Usually, this drug is only semi-encapsulated or is in the form of embedded crystals which quickly dissolve when microspheres are placed into a conventional aqueous incubation medium. An initial burst phase release from PLCG microspheres has been documented for drugs such as octreotide (released into phosphate buffer pH 8, acetate buffer, pH 4 and horse serum) (Bodmer *et al.*, 1992); leuprolide acetate (released into phosphate buffer, pH 7 containing 0.05% Tween-80) (Ogawa *et al.*, 1988); horse serum albumin (released into sodium phosphate buffer, pH 7.5 and 154 mM/l phosphate buffered saline) (Hora *et al.*, 1990); ciprofloxacin (released into phosphate buffer, pH 7.4) (Martinez *et al.*, 1997); timolol maleate (released into phosphate buffer, pH 7.3) (Sturesson *et al.*, 1993); ovalbumin (released into

phosphate buffered saline) (O'Hagan *et al.*, 1994) and Zoladex (released into an aqueous medium at pH 7.4) (Hutchinson and Furr, 1990). Following the initial burst phase, lower concentrations of microencapsulated drug are subsequently released over an extended period of time. This second, low-level sustained release phase usually occurs as a result of the diffusion of deeper-seated drug from the microsphere matrix into the external medium and is characterised by a gradual decrease in the detectable concentrations of released drug (Beck *et al.*, 1983). Second phase low-level sustained drug release is mediated by the formation of pores and channels within the superstructure and internal matrix of the microsphere. If embedded surface and matrix drug crystals were present at the start of incubation, the indentations and pores left in the microsphere matrix subsequent to their dissolution provide an avenue through which internally sequestered drug can diffuse. Water uptake (swelling) by microspheres, especially those composed of PLCG, causes these pores and channels to become filled with incubation medium. Once an equilibrium between the internal and external concentration of medium has been reached, medium moving out of the microspheres can carry dissolved drug to the external environment (Suzuki and Price, 1985). The lower concentration of drug released during the second, low-level sustained release phase was probably the result of an even distribution of drug throughout the internal microsphere matrix (Tsai *et al.*, 1986). It has been shown by Bodmer and co-workers (1992) that pores and channels can appear in microspheres which do not exhibit surface crystal deposition. This phenomenon is thought to occur as a result of polymer hydrolysis. The network of aqueous pores and channels within the microsphere surface, however created, will also be subject to simple chemical hydrolysis during the second, low-level release phase causing them to both deepen and enlarge. This enables additional pockets of drug to come into contact with the channels which ensures prolonged drug diffusion into the external environment and an extension of the second, low-level sustained release phase. However, for macromolecules such as proteins that are mechanically trapped within the microsphere matrix due to their insolubility in the polymer and large molecular size, the second low-level release phase can take the form of a lag phase (Sturesson *et al.*, 1993; O'Hagan *et al.*, 1994) which has also been termed an

induction period (Hora *et al.*, 1990). During this phase, water still infiltrates the microspheres via the network of pores and channels in the matrix and begins to hydrolyse the polymer. However, it is only when sufficient degradation has occurred to enable proteins to actively move out of the matrix (phase three) that release actually occurs. The time taken for sufficient biodegradation to occur is the time scale of the lag phase. So, while diffusional release after the initial burst release phase creates an overall period of continuous release, the lag phase required for the further release of some encapsulated materials creates a situation known as discontinuous release (Bodmer *et al.*, 1992). The third recognised phase of release from polymeric microspheres involves biodegradation and bioerosion of the polymer matrix. While this occurs to a limited extent during the second, low-level sustained release phase, some deep seated encapsulated drug may remain isolated from the network of pores and channels for some time. Therefore gross degradation of the internal architecture is required for the release of this material. A more detailed analysis of the polymer degradation process with respect to PLCG 75:25 microspheres has been documented in chapter 5.

Occasionally, a quite different pattern of release from polymer based microspheres is experienced whereby, initially, virtually none of the encapsulated drug is liberated from the microspheres. It has been suggested that this lag phase is produced by the absence of drug crystals on or at the surface of the microspheres which eliminates the burst phase of release which would normally be expected as a result of crystal dissolution (Bodmeier and McGinity, 1987b; Iwata *et al.*, 1993). A limited burst release of drug sometimes occurs when sufficient water has penetrated the microsphere matrix to allow the formation of pores and channels and the subsequent diffusion of drug through them but this is soon replaced by a sustained, low-level, release consistent with the "normal" second, low-level sustained release phase of encapsulated material (Bodmeier and McGinity, 1987b; Iwata *et al.*, 1993). Subsequent phase three release would of course then occur depending on the rate of polymer biodegradation.

Using this information, it is possible to interpret the profiles of antibiotic release from 20% w/w loaded PLCG 75:25 microspheres. All preparations of antibiotic loaded microspheres incubated in NCS produced an initial transient burst phase where relatively large concentrations of antibiotic were released. During the burst phase, 20% w/w vancomycin loaded microspheres released the greatest cumulative concentration of antibiotic followed by 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres. Examination of the surface topography of freshly fabricated antibiotic loaded microspheres (section 3.2.4.) revealed the presence of antibiotic crystals embedded within the surface layers of both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres whereas 20% w/w rifampicin loaded microspheres were smooth in appearance with no surface crystal deposition. The early dissolution of these surface crystals would explain why both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres released significantly greater concentrations of antibiotic than 20% w/w rifampicin loaded microspheres during the burst phase. However, the fact that the rifampicin loaded microspheres did exhibit a limited burst phase of release in NCS indicates that NCS penetrated the microspheres very quickly to mobilise internal matrix stores of antibiotic. The relative differences between the magnitude of antibiotic release from 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres during the burst phase of release compared with that released from 20% w/w rifampicin loaded microspheres can be explained in a number of ways. For instance, the high solubility of vancomycin in NCS (in the region of 4.0 mg/ml according to the standard curve of vancomycin in NCS shown in section 2.2.8.) would account for the large total mean cumulative concentration and mean cumulative percentage of vancomycin released from all dispersed microsphere preparations incubated in NCS. Ciprofloxacin is sparingly soluble in water and is presumably less soluble in NCS. This is supported by the observation of ciprofloxacin crystals in NCS during release studies involving 20% w/w ciprofloxacin loaded microspheres. This suggests that some ciprofloxacin released from the microsphere matrix was not dissolving in NCS and therefore was not detected by the radial diffusion assay (the samples for which were low level release phase microspheres fabricated by surface hydrolysis. Although the

centrifuged to separate particulates). Low concentrations of ciprofloxacin are known to be bacteriostatic such that concentrations of around ≤ 0.009 mg/ml in NCS produced indistinct zones of inhibition where limited amounts of bacterial growth had obviously occurred during radial diffusion. These indistinct zones were not quantified so bacteriostatic concentrations of ciprofloxacin released from 20% w/w antibiotic loaded microspheres were not subsequently recorded. This factor may explain why proportionately less ciprofloxacin than vancomycin was released from 20% w/w loaded microspheres into NCS at 37°C over the same time period. The total mean cumulative concentration of rifampicin released from dispersed 20% w/w loaded microspheres was very low compared with that released from 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres. However, it must be remembered that the mean encapsulation efficiency of dispersed 20% w/w rifampicin loaded microspheres in NCS was approximately 10% of that observed for dispersed 20% w/w vancomycin loaded microspheres and dispersed 20% w/w ciprofloxacin loaded microspheres incubated in NCS at 37°C. Therefore, the concentration of rifampicin released would be relatively small because of the limited reservoir of antibiotic encapsulated within these microspheres. Kinetic analysis of antibiotic release from dispersed microspheres loaded with either 20% w/w vancomycin, ciprofloxacin or rifampicin and incubated in NCS at 37°C confirmed that the mechanism of antibiotic release from all three preparations was diffusional (Higuchi kinetics), especially the second, low-level sustained release phase since this period of antibiotic delivery lasted for the majority of each release study and would therefore have the greatest influence on Higuchi plots. This observation was supported by the fact that the mean day to day concentration of antibiotic delivered by microspheres loaded with 20% w/w vancomycin, ciprofloxacin and rifampicin generally decreased over this period which is characteristic of a diffusional release pattern. The second, low-level sustained phase of vancomycin and ciprofloxacin release was thought to be mediated via the network of pores and channels created in the surface of microspheres by the dissolution of embedded antibiotic crystals. The release of rifampicin during the second, low-level, release phase was probably facilitated by surface hydrolysis. Although not

apparent for dispersed 20% w/w vancomycin loaded microspheres incubated in NCS, the concentrations of ciprofloxacin and rifampicin released from dispersed 20% w/w loaded microspheres incubated in NCS increased significantly towards the end of their respective release studies. This phenomenon possibly represented phase three of the release process and was thought to be the result of the homogeneous biodegradation of the microspheres causing the release of previously entrapped antibiotic.

In summary, it can be said that the release of antibiotic from dispersed 20% w/w loaded microspheres incubated in NCS at 37°C followed the characteristic sequential phasic pattern of drug release from microparticulate systems in so far as the primary burst phase of antibiotic release was followed by a period of low level sustained release and eventually, in the case of ciprofloxacin and rifampicin, a phase of antibiotic release possibly mediated by biodegradation of the microsphere structure.

The release profile for dispersed 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres incubated in HBSS at 37°C was characterised by an initial burst release of antibiotic. Again, this burst phase of release was thought to be the result of the dissolution of antibiotic crystals on the microsphere surface. 20% w/w vancomycin loaded microspheres released a total mean cumulative concentration of antibiotic during the burst phase of release that was greater than the cumulative burst phase concentration of antibiotic released from 20% w/w ciprofloxacin loaded microspheres simply because of the greater relative solubility of vancomycin than ciprofloxacin in HBSS. However, the profile of rifampicin release from 20% w/w rifampicin loaded microspheres dispersed in HBSS showed an initial lag phase where only a very small concentration of antibiotic was released. This was followed by a phase of release in which the mean day to day concentration of rifampicin released into HBSS oscillated but gradually decreased over time. Since 20% w/w rifampicin loaded microspheres had smooth surfaces containing no embedded antibiotic crystals, the lag phase was thought to be the time taken for the incubation medium to penetrate the microsphere matrix and for

rifampicin to diffuse out of the matrix. Higuchi plots of rifampicin release data, together with the gradual decrease in the mean day to day concentration of rifampicin released into HBSS over time, confirm that the release of rifampicin during the second, low-level sustained release phase was diffusional. However, a typical burst phase of release following the lag phase and prior to the low-level, sustained release phase, which had been noted by Bodmeier and McGinity, 1987b and Iwata *et al*, 1993, was not seen in the case of rifampicin. Dispersed microspheres 20% w/w loaded with either vancomycin or ciprofloxacin and incubated in HBSS at 37°C displayed the typical diffusional release pattern throughout the second, low level sustained release phase of antibiotic. However, vancomycin release from dispersed 20% w/w loaded microspheres ceased after ten days of incubation presumably due to the exhaustion of microencapsulated antibiotic. This observation confirmed that vancomycin was more soluble in HBSS compared with ciprofloxacin which was released from dispersed 20% w/w loaded microspheres for at least 26 days.

A burst phase release of antibiotic was also shown by HPMC-entrapped 20% w/w loaded microspheres incubated in NCS at 37°C. However, the burst release phases obtained were different from the profiles observed for dispersed microspheres in that the concentration of antibiotic liberated on the second day of the burst phase tended to be significantly greater than or approximately equal to that released during the first day of the release profile. This situation might have been caused by the initial incomplete submersion of HPMC-entrapped microspheres in NCS. The HPMC gel plug sometimes adhered to the side of the incubation tube such that microspheres entrapped within the exposed portion of the HPMC gel would not be totally immersed in the release medium at the start of incubation. Therefore, the optimum release of antibiotic would not occur until the total submersion of HPMC-entrapped microspheres had occurred, typically one to two hours after administration. However, the consistently greater release of antibiotic on the second day of incubation suggested that the swelling of HPMC could be influencing the subsequent rate of antibiotic release. It is well known that HPMC swells when placed in an aqueous

medium. This property increases the length of the diffusional path along which incorporated drugs have to travel to reach the external medium (Xu and Sunada, 1995). In the present work, even though the HPMC used for entrapping microspheres already had an aqueous content (10% w/v HPMC powder in double distilled water), the hygroscopic nature of the gel still caused it to swell visibly when incubated in NCS. However, the maximal swelling of 10% w/v HPMC did not occur immediately upon immersion in NCS but gradually increased with time causing a gradual increase in the length of the gel/microsphere plug from an initial length of approximately 14 mm to approximately 21 mm over the first 10 days of incubation. Therefore, a relatively short initial diffusional pathway in HPMC for any antibiotic leached from 20% w/w loaded microspheres after incorporation into HPMC allowed the antibiotic to diffuse into NCS almost immediately. However, the release of remaining surface vancomycin and ciprofloxacin crystals and rifampicin sequestered in near-surface domains and normally released during the first day of incubation in NCS at 37°C, would be retarded by the HPMC-gel barrier as it swelled causing the diffusional path to lengthen. The net influx of incubation medium into HPMC during swelling would compete with the efflux of solubilised antibiotic, liberated from HPMC-entrapped 20% w/w loaded microspheres by diffusion. However, diffusion of antibiotic from entrapped 20% w/w loaded microspheres into HPMC as it swelled would still occur because of the creation of a favourable aqueous environment within the gel. Therefore, the antibiotic concentration within the HPMC gel would increase until water influx and efflux equilibrated thereby allowing the majority of the antibiotic stored in the gel to enter the incubation medium on day two of the burst release phase. The swelling of HPMC may also explain why HPMC-entrapped microspheres released a greater total mean cumulative concentration of vancomycin, ciprofloxacin and rifampicin into NCS than dispersed microspheres during their respective burst release phases. It is possible that entrapped microspheres could have become coated in gel during their incorporation into HPMC and when swelling occurred, the gel surrounding the microspheres would also have swelled. The close proximity of this HPMC gel to the surface of the microspheres might have assisted the uptake of incubation medium by the fabrication polymer (PLCG)

thereby facilitating the mobilisation of surface, near surface and deeper seated matrix-bound antibiotic. This mobilisation would have the effect of increasing the cumulative concentration of antibiotic released over the burst phase of release and actually causing a greater overlap of the burst phase of release with the second, low-level sustained release phase. All preparations of HPMC-entrapped microspheres incubated in NCS at 37°C displayed typical second phase diffusional release profiles for vancomycin, ciprofloxacin and rifampicin. HPMC-entrapped 20% w/w ciprofloxacin loaded microspheres also showed a significant increase in the mean day to day concentration of antibiotic released in the latter part of the release profile. This might have represented the start of the third phase of release mediated by gross biodegradation of the microsphere matrix. The method employed to insert HPMC-entrapped microspheres into incubation tubes could have significantly influenced the total mean cumulative concentration and cumulative percentage of vancomycin, ciprofloxacin and rifampicin released from HPMC-entrapped microspheres during incubation in NCS and HBSS at 37°C. 1ml portions of homogeneously mixed HPMC-entrapped microspheres were administered using a disposable 5 ml volume syringe barrel and any air incorporated into HPMC during preparation would have been compressed when pressure was applied to the syringe plunger. Therefore, although every possible care was taken, the administration of exactly 1ml HPMC could not be absolutely guaranteed and this may have resulted in marginally too little or perhaps too much gel being administered to each incubation tube. This possible error would of course alter the total mean cumulative concentration of antibiotic released from HPMC-entrapped microspheres and would also affect calculation of total actual mean cumulative percentage release of antibiotic which are based on 100% release being the encapsulated concentration of antibiotic contained within 0.05g of 20% w/w loaded microspheres per ml of incubation fluid (0.1g of microspheres were assumed to be contained within 1ml of HPMC which was incubated in 2ml of medium).

HPMC-entrapped 20% w/w vancomycin, ciprofloxacin and rifampicin loaded microsphere preparations all released a smaller total mean cumulative concentration of antibiotic during

the burst phase of release than their dispersed counterparts while increasing the concentration of antibiotic subsequently delivered during the second, low-level sustained release phase. It was assumed that the same possible mechanism of antibiotic sequestration by HPMC gel leading to a subsequently greater concentration of antibiotic being released occurred during the burst phase of release. However, in the case of dispersed microspheres incubated in HBSS, a larger concentration of antibiotic was released during the burst phase of release compared with that released from HPMC-entrapped microspheres by virtue of the greater solubility of vancomycin, ciprofloxacin and rifampicin in HBSS than in NCS. Depletion of the reservoir of encapsulated antibiotic contained in dispersed microspheres during the burst phase release would leave less antibiotic for subsequent release during the second, low-level sustained release phase. However, a substantial core load of antibiotic would remain encapsulated and be available for delivery from HPMC-entrapped microspheres after the burst phase. This situation might have accounted for the greater concentration of antibiotic released from HPMC-entrapped microspheres compared with that released from dispersed microspheres during the second, low-level sustained release phase. Increased mobilisation of deep-seated matrix-associated antibiotic due to HPMC-induced PLCG water uptake may have accounted for the larger overall total mean cumulative concentration and cumulative percentage release of both ciprofloxacin and rifampicin from HPMC-entrapped microspheres compared with release dispersed microspheres after incubation in HBSS.

Vancomycin loaded microspheres were fabricated using three different emulsification speeds, namely 1,000 rpm, 4,000 rpm and 8,000 rpm. Previous work (chapter 3) confirmed that increasing the emulsification speed during fabrication could significantly decrease the final diameter of harvested microspheres and indeed this was observed for vancomycin loaded microspheres. In addition, microsphere diameter was also shown to affect the release of encapsulated vancomycin such that smaller microspheres released more vancomycin than larger microspheres. This was because small microspheres had a collectively greater surface area in contact with the incubation medium than larger

microspheres thereby allowing greater diffusion of antibiotic into the incubation medium. The diameter of 20% w/w vancomycin loaded microspheres most influenced the concentration of vancomycin released during the initial burst phase of release since this was the period during which the majority of encapsulated vancomycin was released. Dispersed 20% w/w vancomycin loaded microspheres incubated in NCS delivered a large concentration of antibiotic on day one of incubation with microspheres fabricated using an emulsification speed of 8,000 rpm releasing a significantly greater concentration of antibiotic than microspheres fabricated using emulsification speeds of 4,000 rpm and 1,000 rpm. It is clear that microsphere diameter and the concentration of vancomycin released into NCS during the initial phase were inversely related. However, 20% w/w vancomycin loaded microspheres entrapped within HPMC gel did not show this relationship since microspheres fabricated using an emulsification speed of 1,000 rpm released a higher concentration of vancomycin on day one of incubation than those fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm. It may be that the larger microspheres (fabricated using an emulsification speed of 1,000 rpm) sedimented out from HPMC more quickly than their counterparts fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm because of their greater mass. Therefore, HPMC gel entrapment would appear to have less influence on the burst phase release kinetics of microspheres fabricated using an emulsification speed of 1,000 rpm than on HPMC-entrapped 20% w/w vancomycin loaded microspheres fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm. The concentration of vancomycin released into HBSS from dispersed 20% w/w loaded microspheres on day one of incubation was not influenced by microsphere diameter. It was assumed that the high solubility of vancomycin in HBSS outweighed any possible alteration of antibiotic release induced by differences in microsphere diameter.

The therapeutic minimum inhibitory concentration (MIC) for vancomycin is between 0.0001 - 0.016 mg/ml (Martindale, 1993). The mean day to day concentration of vancomycin released from all dispersed and HPMC-entrapped microsphere preparations

incubated in NCS at 37°C and all dispersed and HPMC-entrapped microsphere preparations incubated in HBSS at 37°C that had been fabricated using emulsification speeds of 4,000 rpm and 8,000 rpm was well in excess of the upper MIC limit. However, while dispersed 20% w/w vancomycin loaded microspheres fabricated using an emulsification speed of 1,000 rpm and incubated in HBSS at 37°C exhibited a nadir concentration of vancomycin within the MIC range, their HPMC entrapped counterparts failed to release a detectable concentration of vancomycin into HBSS at 37°C over one 24 hour sample period towards the end of the release study which could have affected the ongoing antibacterial action of the preparation. Dispersed and HPMC-entrapped 20% w/w ciprofloxacin loaded microspheres delivered mean day to day concentrations of ciprofloxacin into NCS at 37°C far in excess of those required to inhibit bacterial growth ($\leq 0.001 - 0.002$ mg/ml (Limon, 1989; Martindale, 1993)). However, this was not apparent for dispersed and HPMC-entrapped 20% w/w ciprofloxacin loaded microspheres incubated in HBSS at 37°C. While HPMC-entrapped microspheres released a rather low nadir day to day concentration of ciprofloxacin of 0.001 mg/ml into HBSS, dispersed microspheres failed to release a detectable concentration of ciprofloxacin into HBSS at 37°C over one 24 hour sample period. The MIC of rifampicin required for the eradication of bacteria such as *Staphylococcus spp.* ranges between 0.00001 - 0.0002 mg/ml (Martindale, 1993). The lowest mean day to day concentration of rifampicin released from both dispersed and HPMC-entrapped microspheres incubated in NCS at 37°C was far greater than the upper limit of the MIC range. However, the mean day to day concentration of rifampicin released from dispersed and HPMC-entrapped 20% w/w rifampicin loaded microspheres incubated in HBSS at 37°C was only marginally greater than the upper MIC limit and might have some difficulty in effectively eradicating more resistant bacterial strains. It would seem that dispersed and HPMC-entrapped 20% w/w antibiotic loaded microspheres incubated in NCS at 37°C have the potential to provide more effective long term *in vitro* antibacterial activity than dispersed and HPMC-entrapped 20% w/w antibiotic

loaded microspheres incubated in HBSS at 37°C. The importance of these kinetics of antibiotic release from dispersed and HPMC-entrapped antibiotic loaded microspheres in relation to their potential usefulness in the topical treatment of orthopaedic infections will be discussed later.

Chapter 5: The *in vitro* biodegradation of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres after incubation in Newborn calf serum and Hank's balanced salt solution at 37°C.

5.1. Introduction

The biodegradation of unloaded and 20% w/w antibiotic loaded poly (*dl*-lactide-co-glycolide) [PLCG] 75:25 microspheres as a result of incubation in newborn calf serum (NCS) and Hank's balanced salt solution (HBSS) at 37°C for 30 days has been characterised by using a number of analytical techniques.

5.2. Methods

Microsphere biodegradation was measured in a number of ways. The change in the molecular mass of PLCG was measured using gel permeation chromatography (GPC) (Rapra Technology Limited under the direction of Steve Holding) while differential scanning calorimetry (DSC) was used to measure changes in the glass transition temperature of PLCG at various time points during 30 days of microsphere incubation in both NCS and HBSS at 37°C. Gravimetric analysis provided a measurement of mass loss from microspheres during incubation in HBSS at 37°C over 30 days. Examination of the surface topography and internal architecture of unloaded microspheres and 20% w/w antibiotic loaded microspheres after incubation in NCS and HBSS at 37°C for 30 days was carried out using scanning E/M to provide extra information on the extent of microsphere biodegradation (section 2.3.11.).

5.3.

Results on the mean molecular mass (M_n) and mean

5.3.1.

Measurement of the mean molecular mass and polydispersity of unloaded microspheres and 20% w/w antibiotic loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C for 30 days.

Gel permeation chromatography was used to measure the extent of the reduction in the mean weight average (M_w), number average (M_n) and peak (M_p) molecular mass and polydispersity (pd) of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C for 30 days. These studies provided information on the extent to which PLCG microspheres were hydrolysed and the results are summarised in figure 5.1. a) and b) shown below.



Figure 5.1.1.Changes in the mean molecular mass (a) and mean polydispersity (b) of 20% w/w vancomycin loaded PLCG 75:25 microspheres incubated in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2; where error bars are present, mean values \pm SEM, n=4).

Figure 5.1.1.a) Changes in the mean molecular mass of 20% w/w vancomycin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.

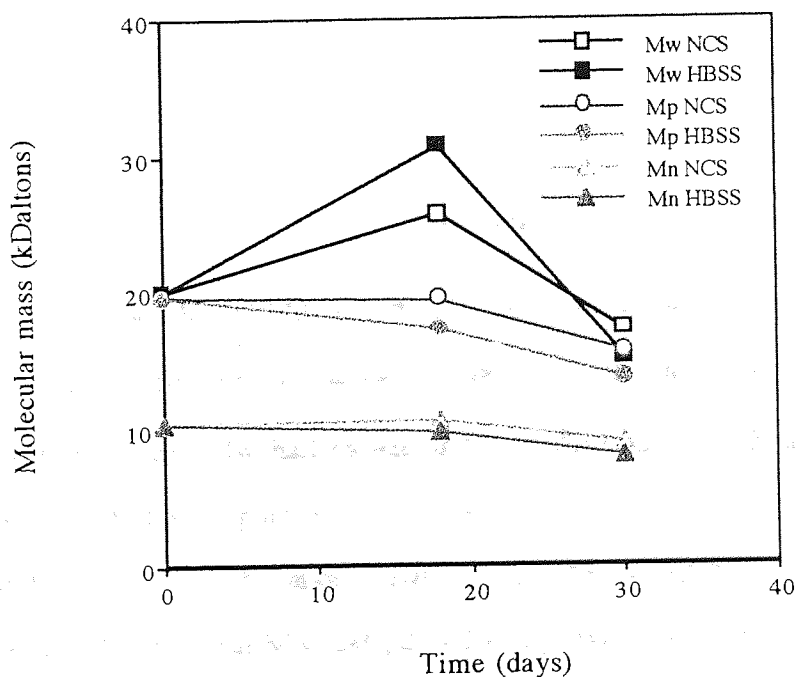
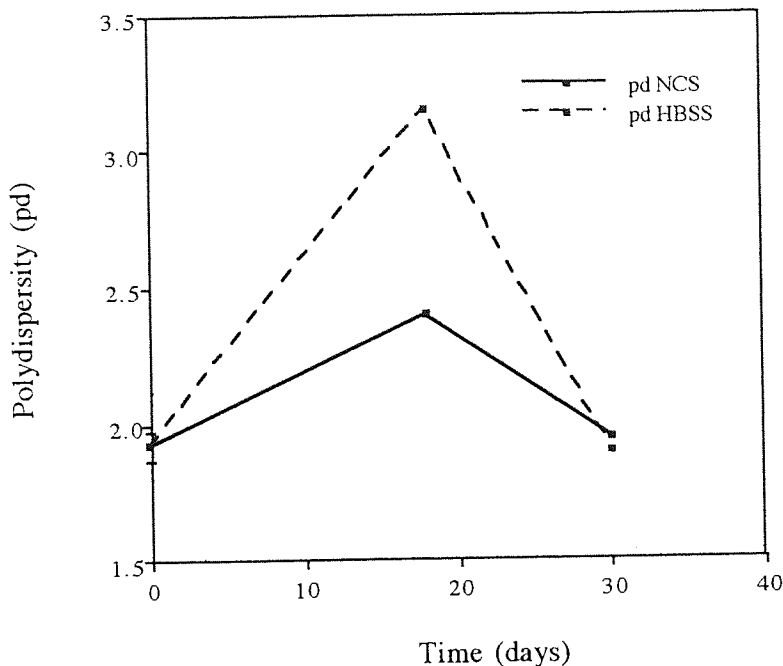


Figure 5.1.1.b) Changes in the mean polydispersity (pd) of 20% w/w vancomycin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.



Overall, the mean Mn and Mp of 20% w/w vancomycin loaded PLCG 75:25 microspheres gradually decreased with incubation in both NCS and HBSS at 37°C (figure 5.1.1.a). However, the mean Mw had increased by 5 kDaltons in NCS and by 10 kDaltons in HBSS after 18 days (figure 5.1.1.a). The mean pd had also increased by approximately 0.5 units in NCS and by approximately 1.2 units in HBSS over the same time scale compared with the mean Mw and pd of freshly fabricated 20% w/w vancomycin loaded microspheres (figure 5.1.1. b). The mean Mw for 20% w/w vancomycin loaded microspheres had fallen to approximately 15.5-17.5 kDaltons after 30 days of incubation in NCS and HBSS which was 2.5-4.5 kDaltons lower than the mean Mw before the start of incubation. After 30 days of incubation in NCS and HBSS, the mean pd value of 20% w/w vancomycin loaded microspheres was approximately 1.95 units and therefore almost identical to that observed for freshly fabricated microspheres (figure 5.1.1.b). The Mp for 20% w/w vancomycin loaded microspheres fell from 19.75 kDaltons to around 14-16

kDaltons after incubation in NCS and HBSS at 37°C for 30 days. However, the mean Mn for these microspheres remained relatively constant at approximately 10 kDaltons throughout the 30 day incubation period in both NCS and HBSS (figures 5.1.1.a).

mean Mn of microspheres in solution at 37°C over 30 days in NCS and HBSS

Figure 5.1.1.a

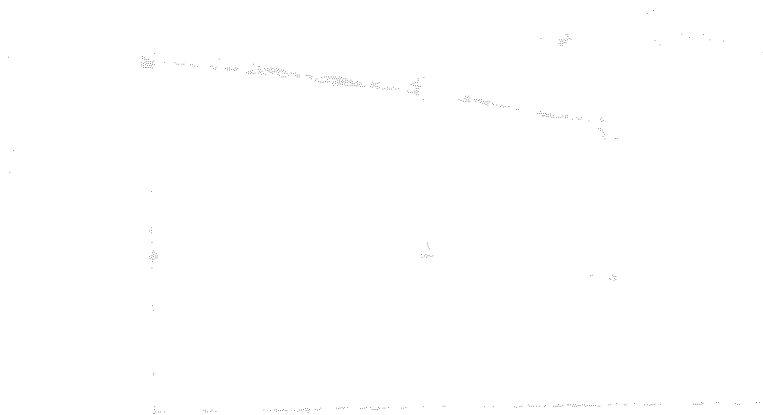


Figure 5.1.2. Changes in the mean molecular mass (a) and mean polydispersity (b) of 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2; where error bars are present, mean values \pm SEM, n=4).

Figure 5.1.2.a) Changes in the mean molecular mass of 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.

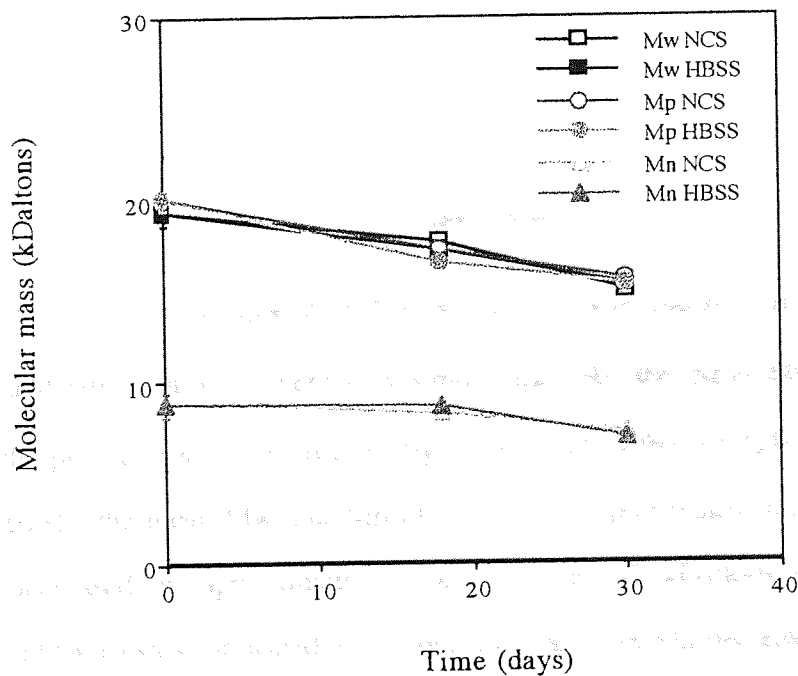
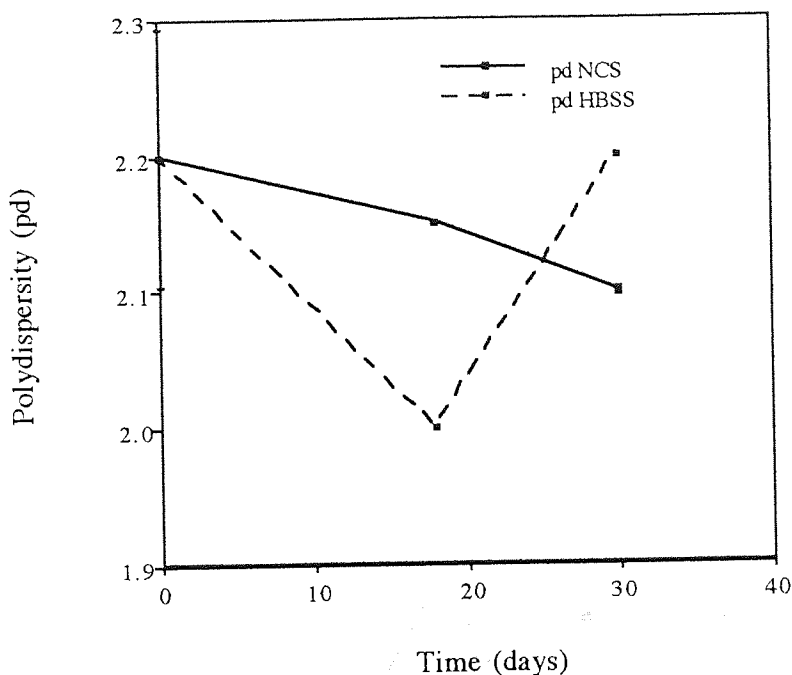


Figure 5.1.2.b) Changes in the mean polydispersity of 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.



Mean Mw and Mp profiles of 20% w/w ciprofloxacin loaded microspheres incubated in NCS and HBSS at 37°C were very similar and, like the mean Mn, gradually decreased with the progression of incubation (figure 5.1.2.a). After 30 days of incubation in NCS and HBSS, the mean Mw and Mp of 20% w/w ciprofloxacin loaded microspheres had both decreased by approximately 5 kDaltons to 15 kDaltons compared with values obtained for freshly fabricated microspheres. The mean Mn decreased from approximately 9 kDaltons at the start of incubation to approximately 7 kDaltons after 30 days of incubation in both NCS and HBSS (figure 5.1.2.a). In addition, the mean Pd decreased slightly with incubation time in NCS but in HBSS, the pd after 18 days had markedly decreased by day 24 of incubation before increasing back again to its previous level after 30 days of incubation (figure 5.1.2.b).

Figure 5.1.3. Changes in the mean molecular mass (a) and mean polydispersity (b) of 20% w/w rifampicin loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2; where error bars are present, mean values \pm SEM, n=4).

Figure 5.1.3.a) Changes in the mean molecular mass of 20% w/w rifampicin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.

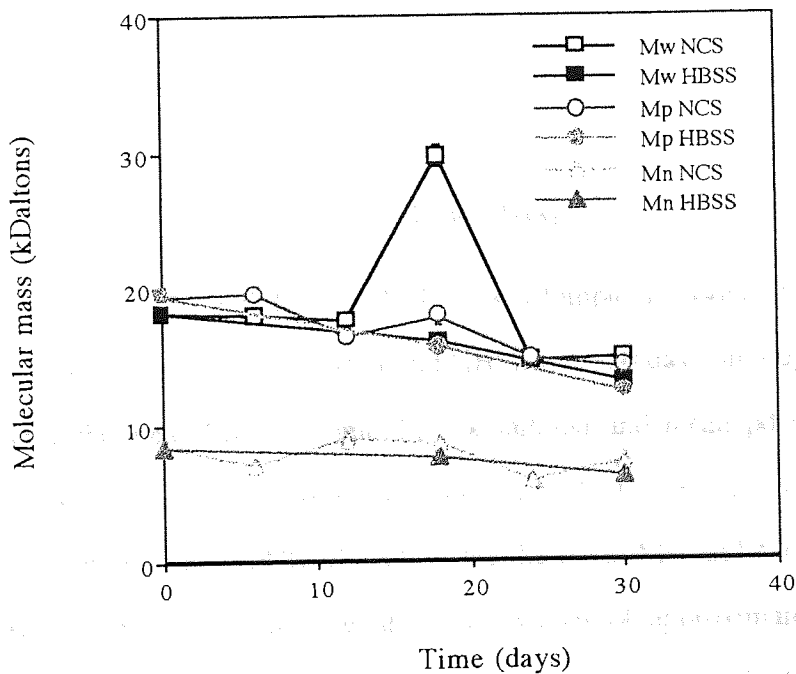
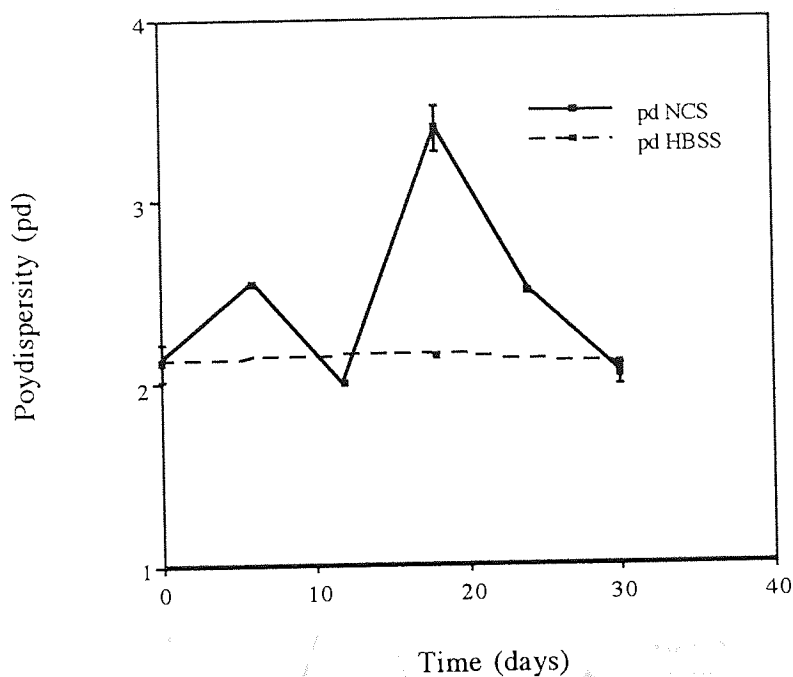


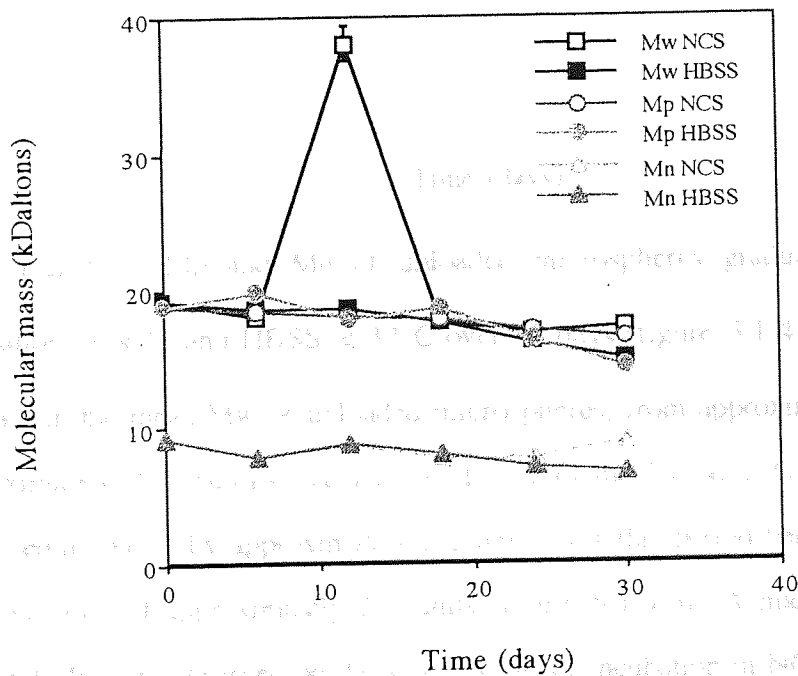
Figure 5.1.3.b) Changes in the mean polydispersity of 20% w/w rifampicin loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.



The mean Mw, Mp and Mn of 20% w/w rifampicin loaded microspheres gradually decreased during incubation in NCS and HBSS over 30 days although there was a marked increase in the mean Mw (of some 11.5 kDaltons) and mean pd (by approximately 3.3 units) between days 12 and 18 of incubation in NCS (figure 5.1.3.a and b). After 30 days of incubation in NCS and HBSS, values for the mean Mw and Mp were between 12 and 14 kDaltons and somewhat lower than their values of approximately 19 kDaltons at the start of incubation. Mean values for the Mn were approximately 6.5 kDaltons compared with 8.5 kDaltons at the start of incubation (figure 5.1.3.a). The mean pd values for 20% w/w rifampicin loaded microspheres after 30 days of incubation in NCS and HBSS were approximately 2.05 units and therefore similar to that of freshly fabricated microspheres (figure 5.1.3.b).

Figure 5.1.4. Changes in the mean molecular mass (a) and mean polydispersity (b) of unloaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days (mean values, n=2; where error bars are present, mean values \pm SEM, n=4).

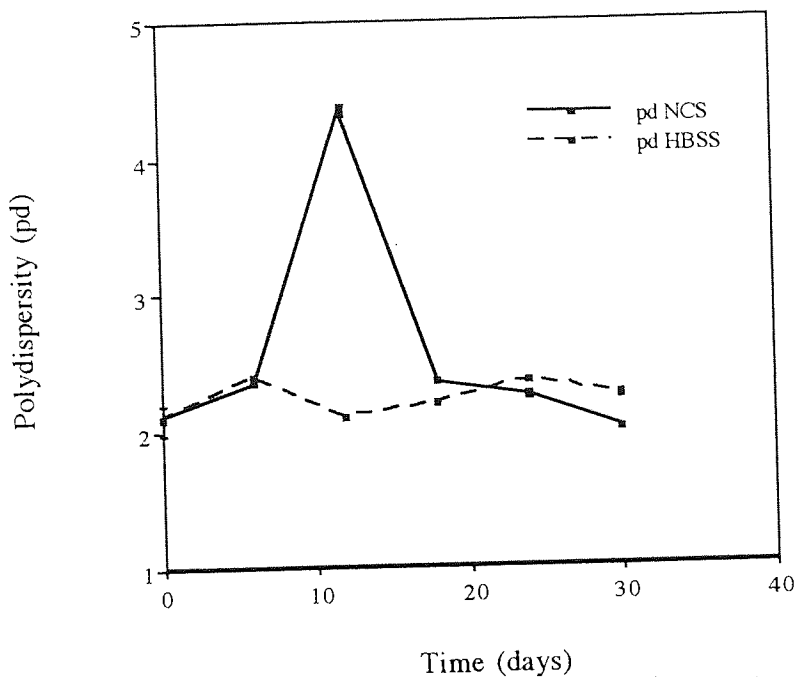
Figure 5.1.4.a) Changes in the mean molecular mass of unloaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.



incubation in NCS (figure 5.1.4.a). In HBSS, the mean molecular mass gradually decreased over the 30 day incubation period.

Figure 5.1.4.b) shows the changes in the polydispersity index (PDI) of unloaded microspheres and 20% w/w gentamycin loaded microspheres incubated in NCS and HBSS at 37°C over 30 days. The PDI of unloaded microspheres incubated in NCS increased from 1.05 to 1.15 over 30 days, while the PDI of unloaded microspheres incubated in HBSS decreased from 1.05 to 1.02 over 30 days. The PDI of 20% w/w gentamycin loaded microspheres incubated in NCS increased from 1.05 to 1.15 over 30 days, while the PDI of 20% w/w gentamycin loaded microspheres incubated in HBSS decreased from 1.05 to 1.02 over 30 days.

Figure 5.1.4.b) Changes in the mean polydispersity of unloaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C over 30 days.



The mean Mw, Mp and Mn of unloaded microspheres gradually decreased during incubation in NCS and HBSS at 37°C over 30 days (figure 5.1.4.a). However, a large increase in the mean Mw of unloaded microspheres, from approximately 19 kDaltons to approximately 38 kDaltons, occurred on day 12 of incubation in NCS. The mean pd also increased markedly by approximately 2.2 units over this period before falling back to the previous level of approximately 2.0 units (figure 5.1.4.b). A marginal increase in both Mw and Mn were observed on days 24 and 30 of incubation in NCS (figure 5.1.4.a). In HBSS, the mean microsphere pd remained relatively constant over the 30 day incubation period (figure 5.1.4.b).

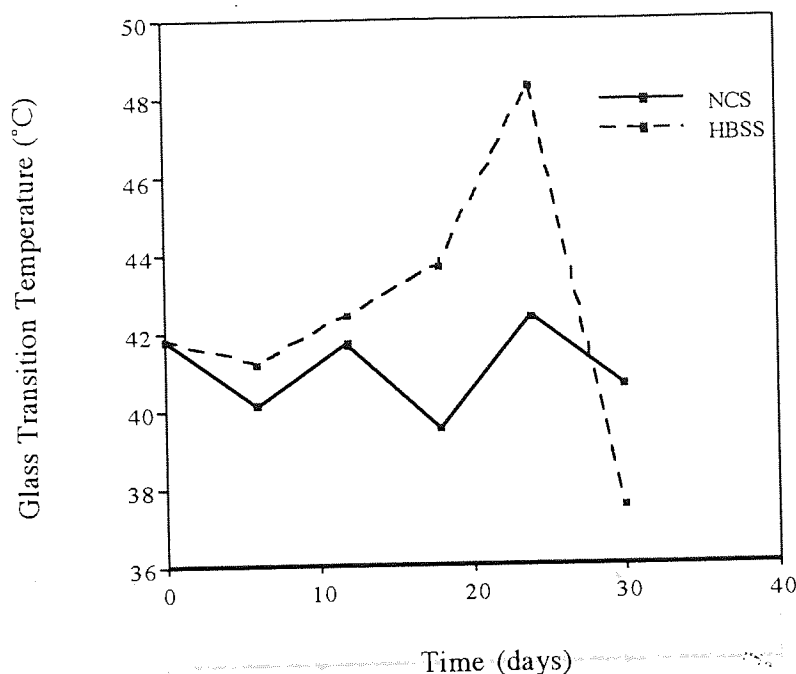
In general, the reduction in molecular mass (Mw) was marginally greater in HBSS than in NCS. In terms of the observed reduction in mean Mw and Mn resulting over 30 days of incubation in NCS at 37°C, unloaded microspheres and 20% w/w vancomycin loaded

microspheres suffered the greatest reduction in molecular mass, followed by 20% w/w ciprofloxacin loaded microspheres and 20% w/w rifampicin loaded microspheres. After incubation in HBSS at 37°C for 30 days, the rank order for the reduction in Mw and Mn was 20% w/w vancomycin loaded microspheres followed by 20% w/w ciprofloxacin loaded microspheres, unloaded microspheres and finally 20% w/w rifampicin loaded microspheres.

5.3.2. Measurement of the mean glass transition temperature of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days.

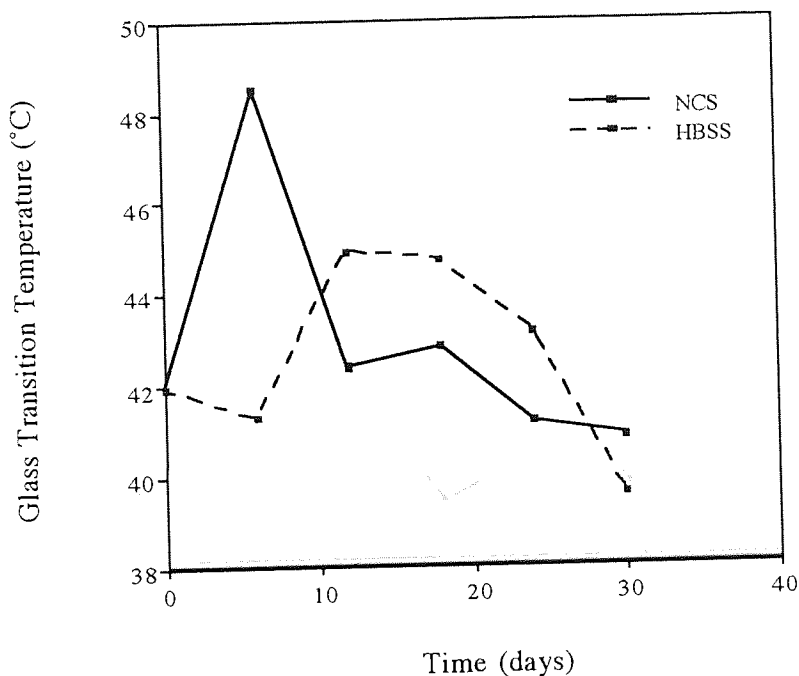
The mean glass transition temperature (T_g) of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C for 30 days was monitored using DSC. Changes in the thermal profile for microspheres will provide information on the structural re-arrangement or loss of lactide and glycolide units in PLCG which occur during incubation. T_g measurements for the various microsphere preparations have been summarised in figure 5.2. shown below.

Figure 5.2.1.a) Changes in the mean glass transition temperature of 20% w/w vancomycin loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2).



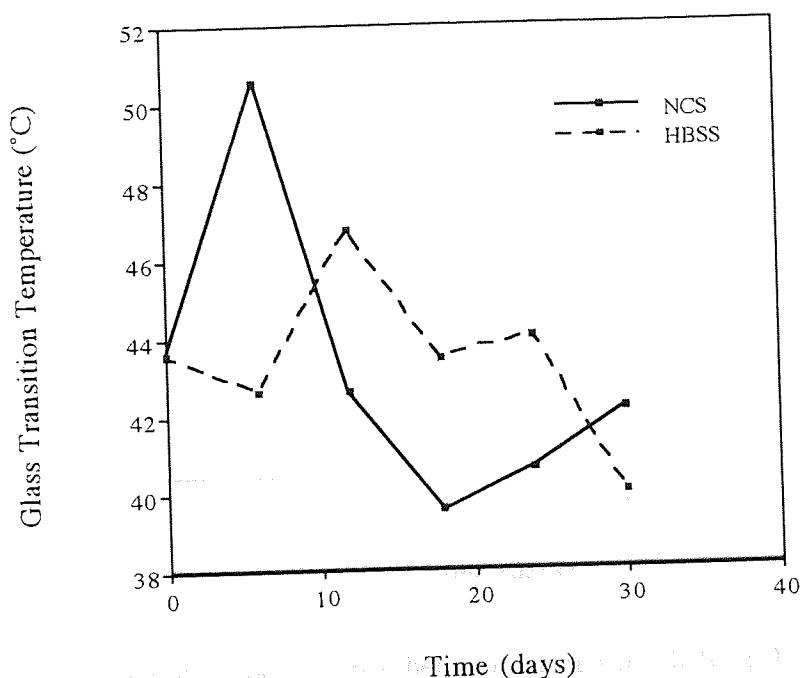
The mean Tg observed for 20% w/w vancomycin loaded microspheres incubated in NCS at 37°C had decreased from 41.78°C at the start of incubation to 40.57°C after 30 days. Over this time period, the Tg fluctuated from a minimum value of 40.11°C on day six to a maximum value of 42.36°C on day 24 (figure 5.2.1.a). During incubation in HBSS at 37°C, the mean Tg of 20% w/w vancomycin loaded microspheres increased from 41.78°C to 42.27°C after 12 days and continued to increase such that, after 24 days the mean Tg was 48.28°C. However, from day 24 of incubation onwards, the mean Tg of these microspheres decreased markedly to 37.46°C by day 30 of incubation (figure 5.2.1.a).

Figure 5.2.1.b) Changes in the mean glass transition temperature of 20% w/w ciprofloxacin loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2).



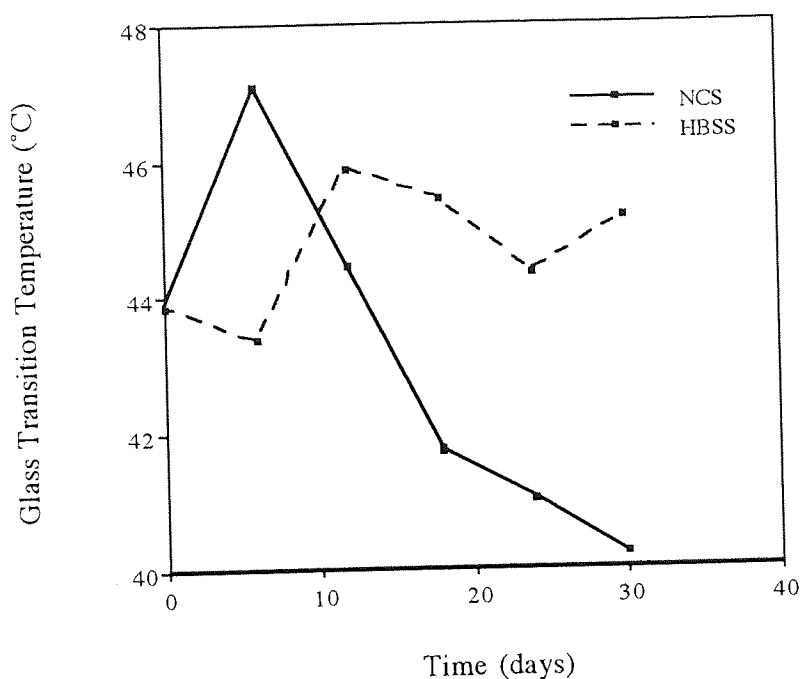
The mean Tg for freshly fabricated 20% w/w ciprofloxacin loaded microspheres was 41.92°C and this increased markedly to 48.53°C after six days of incubation in NCS at 37°C. After this time, the Tg gradually decreased to a mean value of 40.84°C after 30 days of incubation (figure 5.2.1.b). The mean Tg of 20% w/w ciprofloxacin loaded microspheres incubated in HBSS at 37°C increased from 41.92°C at the start of incubation to 44.93°C after 12 days and remained elevated until day 24 of incubation. The mean Tg then gradually decreased to 35.33°C after 30 days of incubation in HBSS (figure 5.2.1.b)

Figure 5.2.1.c) Changes in the mean glass transition temperature of 20% w/w rifampicin loaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2).



The mean Tg of 20% w/w rifampicin loaded microspheres incubated in NCS at 37°C increased from 43.56°C at the start of incubation to 50.58°C after six days. The mean Tg gradually decreased from this time point onwards such that, after 30 days of incubation in NCS at 37°C, the mean Tg had fallen to 42.15°C (figure 5.2.1.c). The mean Tg for 20% w/w rifampicin loaded microspheres incubated in HBSS at 37°C decreased slightly from 43.56°C at the start of incubation to 42.62°C after six days and then increased markedly to 46.73°C after 12 days of incubation. The mean Tg for these microspheres subsequently fell to 40.00°C after 30 days of incubation in HBSS at 37°C (figure 5.2.1.c).

Figure 5.2.1.d) Changes in the mean glass transition temperature of unloaded PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days (mean values, n=2).



The mean Tg for unloaded microspheres incubated in NCS at 37°C had increased from 43.86°C at the start of incubation to 47.09°C after six days and then gradually decreased over time to 40.22°C after 30 days (figure 5.2.1.d). However, the mean Tg for unloaded microspheres incubated in HBSS at 37°C increased slightly from 43.86°C at the start of incubation to 45.88°C by day 12 of incubation and stabilised at around 48°C for the remainder of the 30 day incubation period (figure 5.2.1.d).

Typical DSC thermograms showing the Tg of a sample of PLCG 75:25 microspheres after incubation in NCS and HBSS at 37°C over 30 days are displayed in figure 5.2.2.a) and b) below. In some cases, the Tg was characterised by two peaks and was thought to

represent a heterogeneous rate of polymer biodegradation. A DSC thermogram of a sample of PLCG microspheres displaying this occurrence is shown in figure 5.2.2.c). For all unloaded and 20% w/w antibiotic loaded PLCG 75:25 microsphere preparations, the mean Tg tended to decrease over the 30 day incubation period in both NCS and HBSS and was not accompanied by a broadening of the Tg peak. The Tg values obtained for 20% w/w antibiotic loaded microspheres incubated in HBSS at 37°C for 30 days were lower than values obtained for 20% w/w antibiotic loaded microspheres incubated in NCS at 37°C over the same time period. However, unloaded microspheres incubated in NCS at 37°C showed a lower mean Tg than unloaded microspheres incubated in HBSS at 37°C over 30 days (figure 5.2.1.d). Apart from 20% w/w vancomycin loaded microspheres, all microspheres incubated in NCS showed a marked increase in the mean Tg after six days of incubation. For microspheres incubated in HBSS, a decrease in the mean Tg after six days was followed by an increase in the mean Tg by day 12 of incubation to a level in excess of the starting mean Tg.

Figure 5.2.2. Typical DSC thermograms showing the glass transition temperature of a sample of PLCG 75:25 microspheres after incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days.

Figure 5.2.2.a) DSC thermogram showing the glass transition temperature of a sample of PLCG 75:25 microspheres after incubation in NCS at 37°C over 30 days.

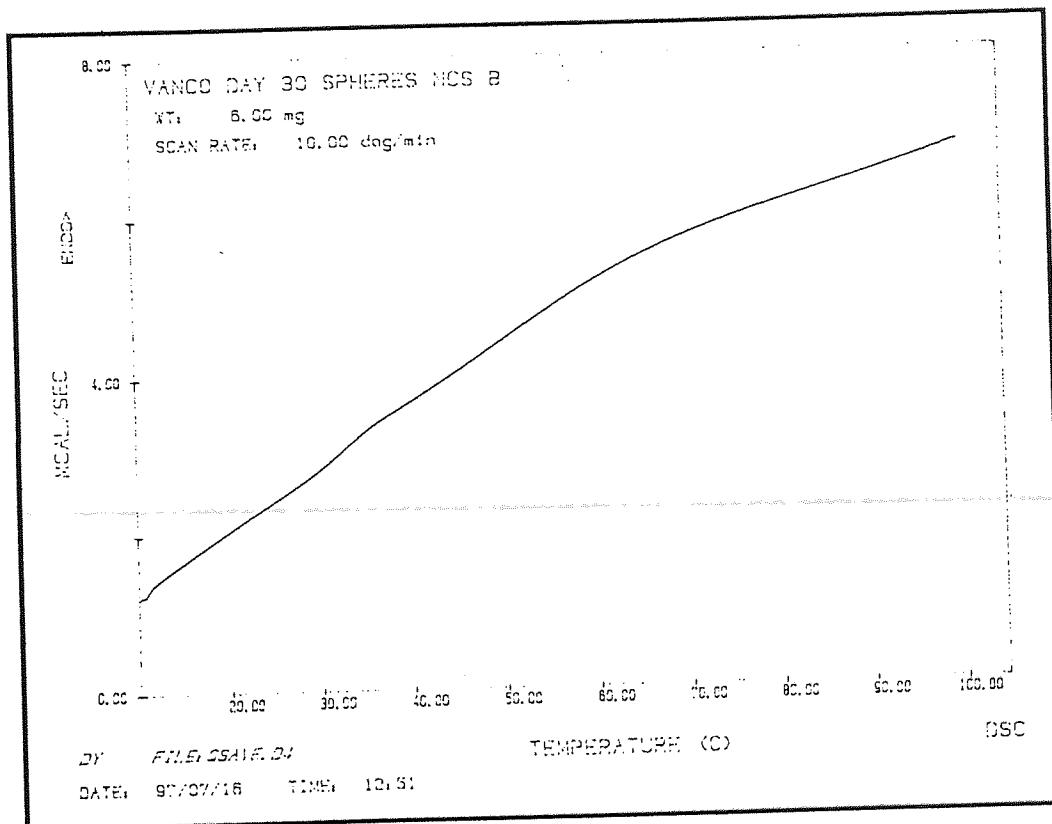


Figure 5.2.2.b) DSC thermogram showing the glass transition temperature of a sample of PLCG 75:25 microspheres after incubation in HBSS at 37°C over 30 days.

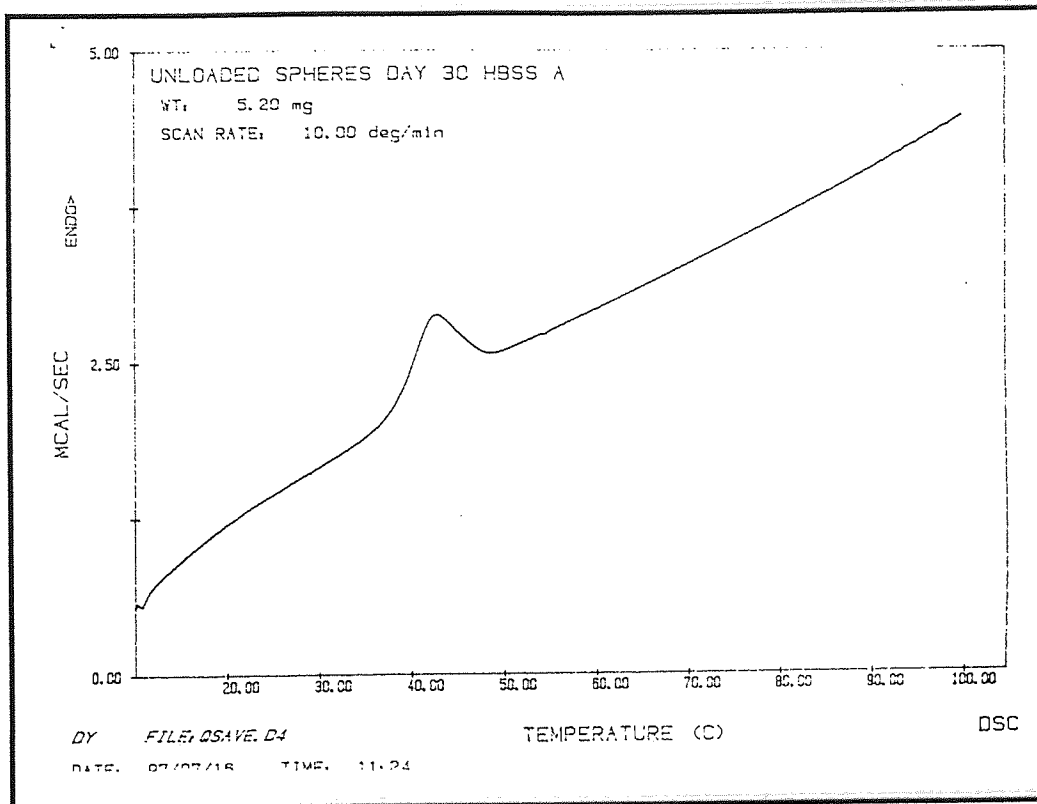
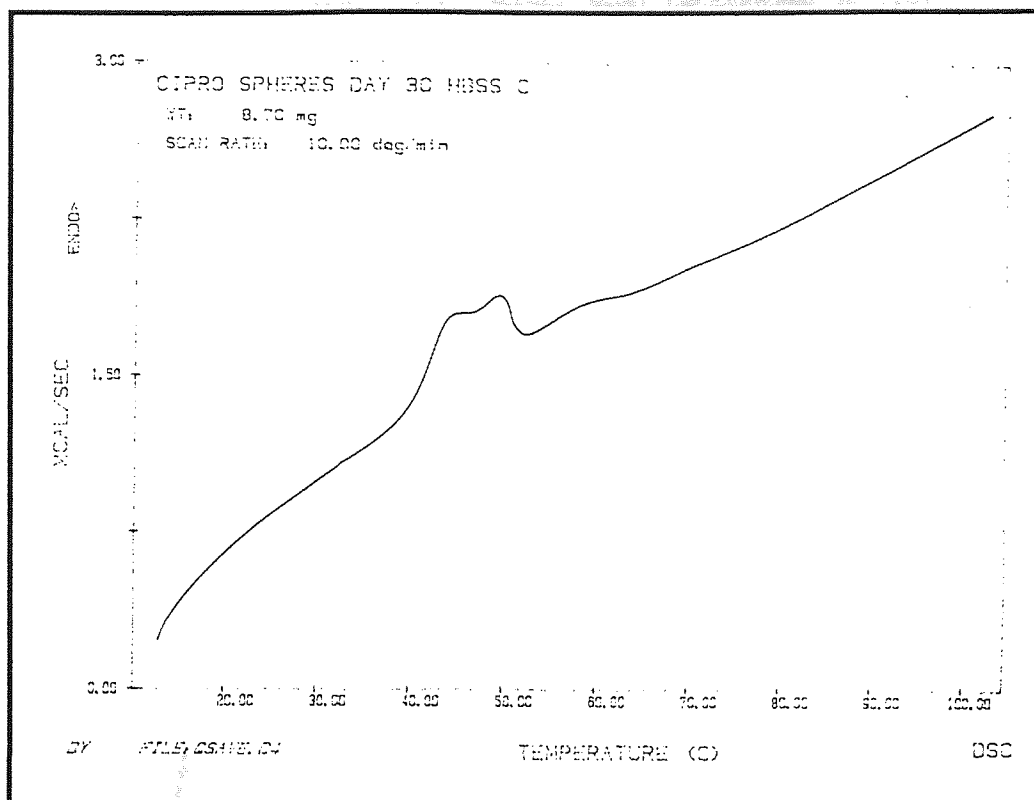


Figure 5.2.2.b) DSC thermogram showing the glass transition temperature of a sample of PLCG 75:25 microspheres after incubation in HBSS at 37°C over 30 days. The plot shows a glass transition region between 35°C and 50°C. Metadata includes: UNLOADED SPHERES DAY 30 HBSS A, WT: 5.20 mg, SCAN RATE: 10.00 deg/min, FILE: DSAVE.D4, DATE: 07/07/18, TIME: 11:24.

Figure 5.2.2.c) DSC thermogram showing the glass transition temperature of a sample of PLCG 75:25 microspheres after incubation in HBSS at 37°C over 30 days. The plot shows a glass transition region between 35°C and 50°C. Metadata includes: UNLOADED SPHERES DAY 30 HBSS A, WT: 5.20 mg, SCAN RATE: 10.00 deg/min, FILE: DSAVE.D4, DATE: 07/07/18, TIME: 11:24.

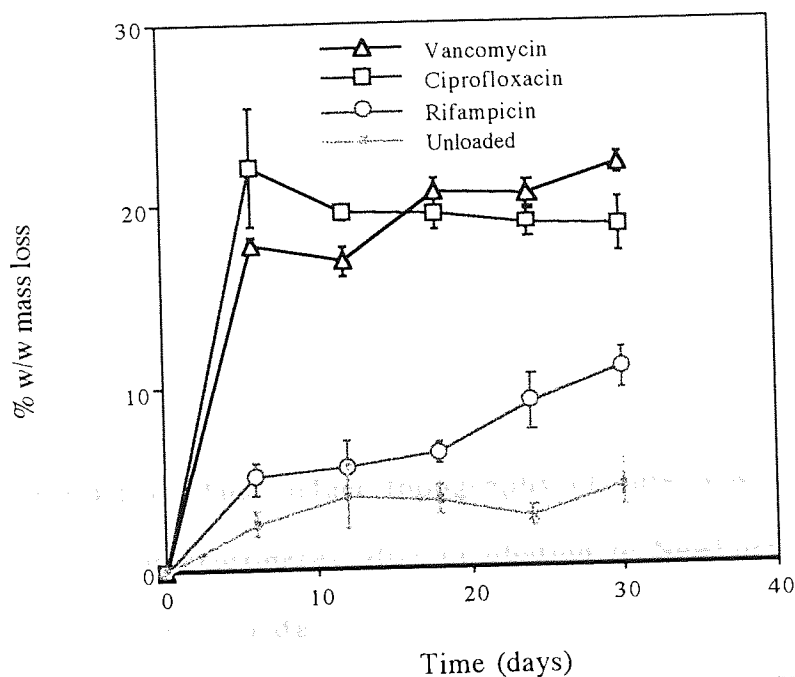
Figure 5.2.2.c) DSC thermogram showing a double peaked glass transition temperature for a sample of PLCG 75:25 microspheres (after incubation in HBSS at 37°C over 30 days).



5.3.3. Determination of the percentage w/w mass loss from unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres by gravimetry after incubation in Hank's balanced salt solution at 37°C over 30 days.

Mass loss from unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres has been monitored over 30 days of incubation in HBSS at 37°C and the results of this study are summarised in figure 5.3. below.

Figure 5.3. The percentage w/w mass loss from unloaded and 20% w/w antibiotic loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days (mean values \pm SEM, n=4 for each point).



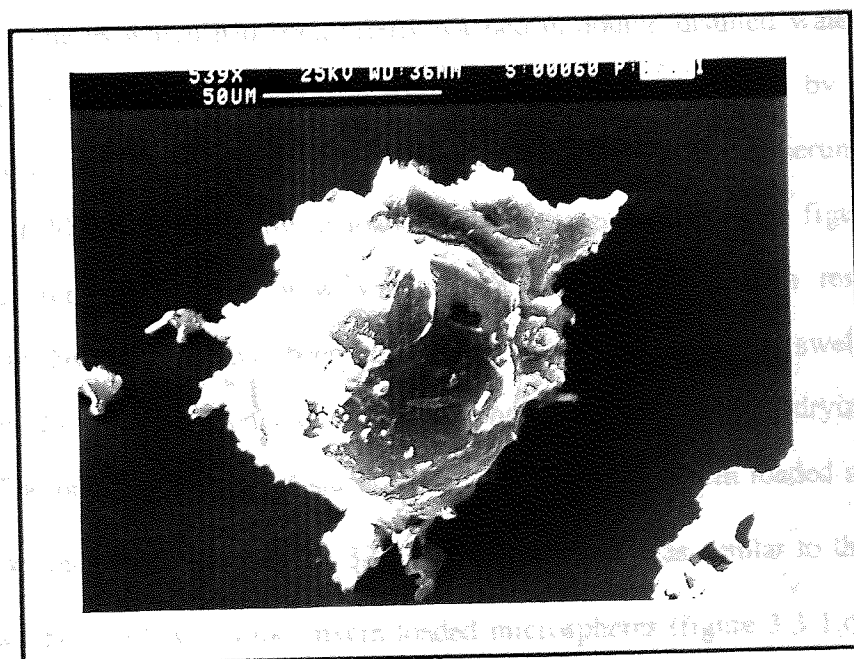
After 30 days of incubation in HBSS at 37°C, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres had both lost approximately 80% of their mass w/w whereas unloaded microspheres had lost approximately 5% w/w of their mass and 20% w/w rifampicin loaded microspheres had lost approximately 10% w/w of their mass over the same time period (figure 5.3.). 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres lost a significant ($P < 0.05$) percentage of their mass between days six and 30 of incubation of some 4.3% and 5.8% respectively (figure 5.3.). The final percentage w/w mass loss from both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres was significantly greater ($P < 0.05$) than that from 20% w/w rifampicin loaded microspheres and unloaded

microspheres after 30 days of incubation (figure 5.3.). In addition, other than on day 12 of incubation, the percentage w/w mass loss from 20% w/w rifampicin loaded microspheres was significantly greater ($P < 0.05$) than that from unloaded microspheres during the 30 day incubation period (figure 5.3.).

5.3.4. Morphological examination of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres following incubation in newborn calf serum and Hank's balanced salt solution at 37°C over 30 days.

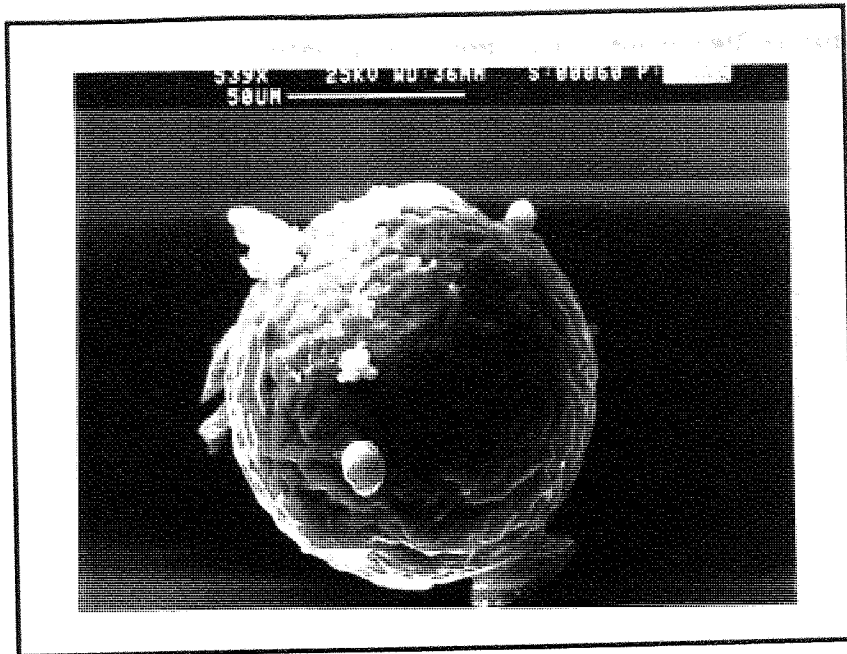
Scanning EM was employed to examine any changes in the surface topography (figure 5.4.1-5.4.4) and internal architecture (figure 5.5.1-5.5.4.) of unloaded microspheres and 20% w/w antibiotic loaded PLCG 75:25 microspheres as a result of biodegradation after 30 days of incubation in NCS and HBSS at 37°C.

Figure 5.4.1.a) The surface topography of 20% w/w vancomycin loaded microspheres after incubation in Newborn calf serum at 37°C over 30 days.



material thought to be removed from HBSS was clearly visible on the surface of 20%

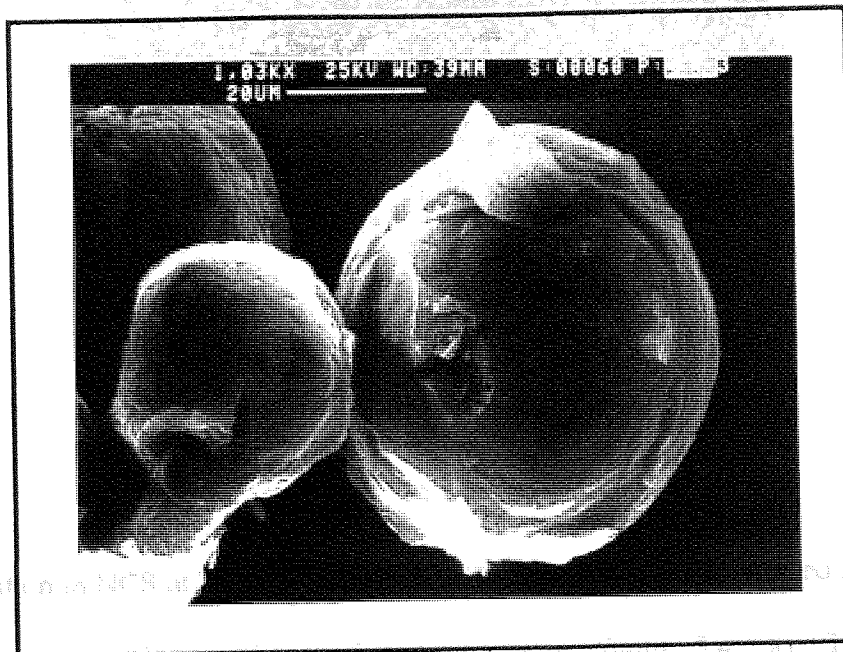
Figure 5.4.1.b) The surface topography of 20% w/w vancomycin loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days.



After 30 days of incubation NCS at 37°C, 20% w/w vancomycin loaded microspheres had undergone slight surface biodegradation as shown by the development of a pitted and crenated surface (figure 5.4.1.a) compared with freshly fabricated 20% w/w vancomycin loaded microspheres which had been briefly washed in double distilled water to remove surface vancomycin crystals to reveal indentations previously occupied by vancomycin crystals (figure 3.3.1.c). Incubation in NCS had led to the adsorption of serum proteins to the surface of 20% w/w vancomycin loaded microspheres as shown in figure 5.4.1.a). The surface crenation of 20% w/w vancomycin loaded microspheres resulting from incubation in NCS may have been an artefact resulting from the swelling of the microspheres during incubation and subsequent shrinkage during freeze-drying (Cohen *et al*, 1991). The pitted and crenated surface of 20% w/w vancomycin loaded microspheres resulting from incubation in HBSS at 37°C (figure 5.4.1.b) was similar to that of freshly fabricated washed 20% w/w vancomycin loaded microspheres (figure 3.3.1.c). Adsorbed material, thought to be buffer salts from HBSS, was clearly visible on the surface of 20%

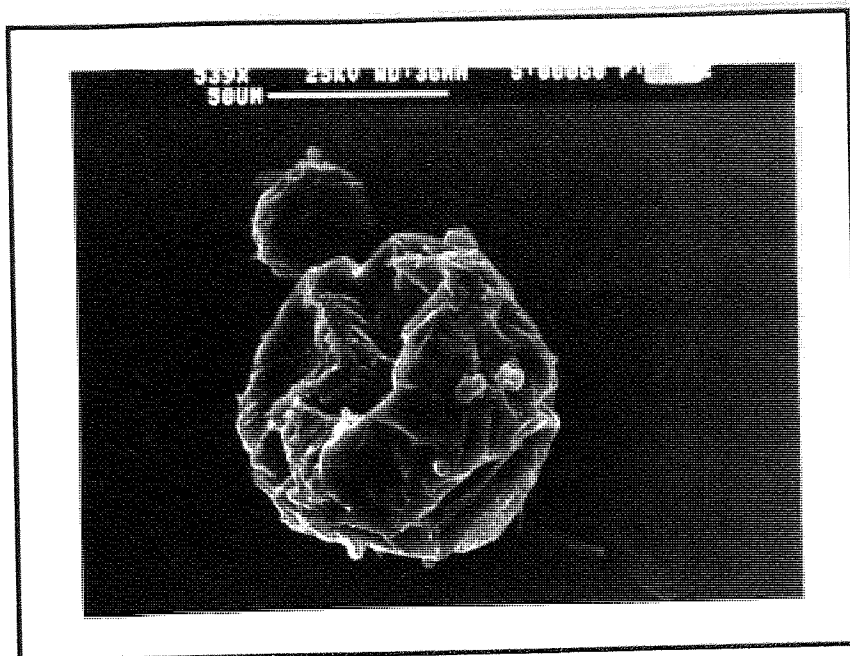
w/w vancomycin loaded microspheres after incubation in HBSS for 30 days (figure 5.4.1.b)

Figure 5.4.2.a) The surface topography of 20% w/w ciprofloxacin loaded microspheres after incubation in newborn calf serum at 37°C over 30 days.



well covered in an amorphous layer of serum protein. This gave the impression that cracks had occurred in the surface layers of these microspheres during incubation but it was assumed to be artefactual resulting from the contraction of the outer surface layers of the microspheres during scanning EM sample preparation. Areas of the internal crack porous matrix were revealed during incubation (figure 5.4.2 a) and this was thought to have been caused by mechanical stresses possibly resulting from the strain during incubation tubes during sampling. Although the surface of 20% w/w ciprofloxacin loaded microspheres incubated in HBSS at 37°C was extremely rough and porous, this characteristic was also shown by freshly fabricated 20% w/w ciprofloxacin loaded microspheres after being washed with double distilled water as routine surface treatment (figure 5.4.2 b). Material thought to be buffer salts from HBSS, had become adsorbed to the surface of 20% w/w ciprofloxacin loaded microspheres after 30 days of incubation in HBSS at 37°C (figure 5.4.2 b).

Figure 5.4.2.b) The surface topography of 20% w/w ciprofloxacin loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days.



After incubation in NCS at 37°C for 30 days, 20% w/w ciprofloxacin loaded microspheres were covered in an adsorbed layer of serum proteins (figure 5.4.2.a). This gave the impression that cracks had occurred in the surface layers of these microspheres during incubation but was assumed to be artefactual resulting from the contraction of the outer surface layers of the microspheres during scanning E/M sample preparation. Areas of the internal macroporous matrix were revealed during incubation (figure 5.4.2.a) and this was thought to have been caused by mechanical abrasion, possibly resulting from the centrifugation of incubation tubes during sampling. Although the surface of 20% w/w ciprofloxacin loaded microspheres incubated in HBSS at 37°C was extremely rough and pitted, this characteristic was also shown by freshly fabricated 20% w/w ciprofloxacin loaded microspheres after brief washing with double distilled water to remove surface antibiotic crystals (figure 3.3.1.d). Material, thought to be buffer salts from HBSS, had become adsorbed to the surface of 20% w/w ciprofloxacin loaded microspheres after 30 days of incubation in HBSS at 37°C (figure 5.4.2.b).

Figure 5.4.3.a) The surface topography of 20% w/w rifampicin loaded microspheres after incubation in newborn calf serum at 37°C for 30 days.

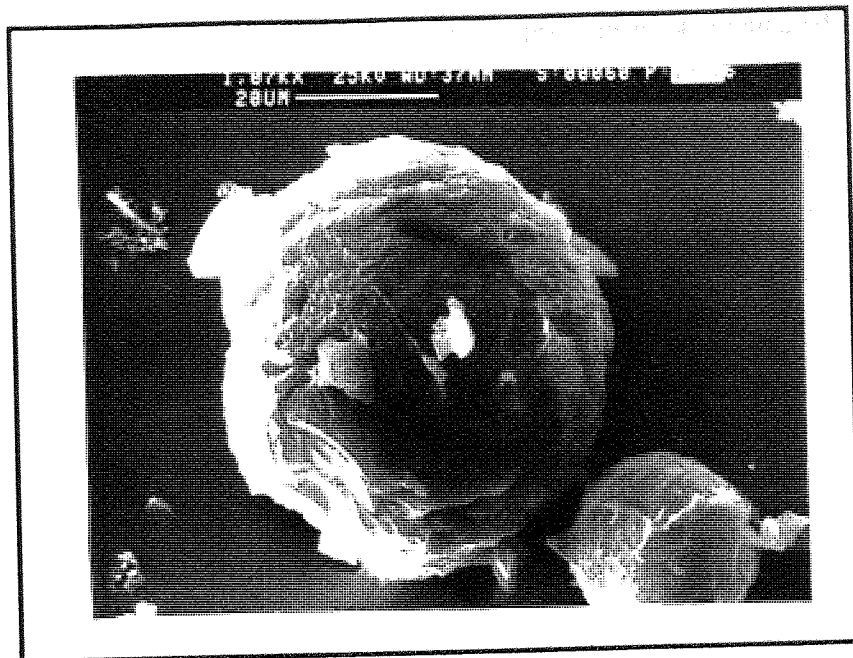
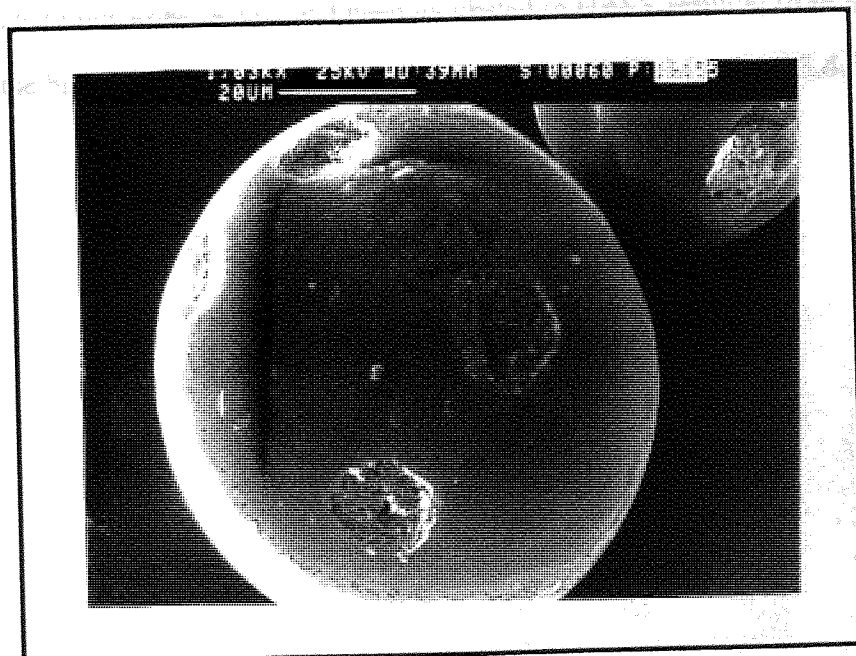


Figure 5.4.3.b) The surface topography of 20% w/w rifampicin loaded microspheres after incubation in Hank's balanced salt solution at 37°C for 30 days.



After 30 days of incubation in NCS at 37°C, the surface of 20% w/w rifampicin loaded microspheres had become pitted and rough (figure 5.4.3.a) compared with the smooth surface of freshly fabricated 20% w/w rifampicin loaded microspheres which showed no antibiotic crystal deposition (figure 3.3.1.e). The appearance of incubated 20% w/w rifampicin loaded microspheres may have resulted from limited surface hydrolysis or could have been created by swelling of the microspheres during incubation and subsequent shrinkage during freeze-drying, an observation noted by Cohen *et al*, 1991. Serum protein adsorption to the surface of 20% w/w rifampicin loaded microspheres occurred after 30 days of incubation in NCS (figure 5.4.3.a). The smooth surface of 20% w/w rifampicin loaded microspheres was not affected by incubation for 30 days in HBSS at 37°C. However, damage to the surface of 20% w/w rifampicin loaded microspheres incubated in HBSS for 30 days had occurred (figure 5.4.3.b). Parts of the outer layer had been stripped away at certain points on the microsphere surface to reveal the internal macroporous matrix. This damage was thought to have been created by mechanical abrasion rather than biodegradation since small pieces of debris were scattered over the surface of the microspheres (figure 5.4.3.b). The cracks in the surface of 20% w/w rifampicin loaded microspheres that had been incubated in HBSS resulted from the melting of PLCG by the highly focused electron beam during scanning E/M (figure 5.4.3.b).

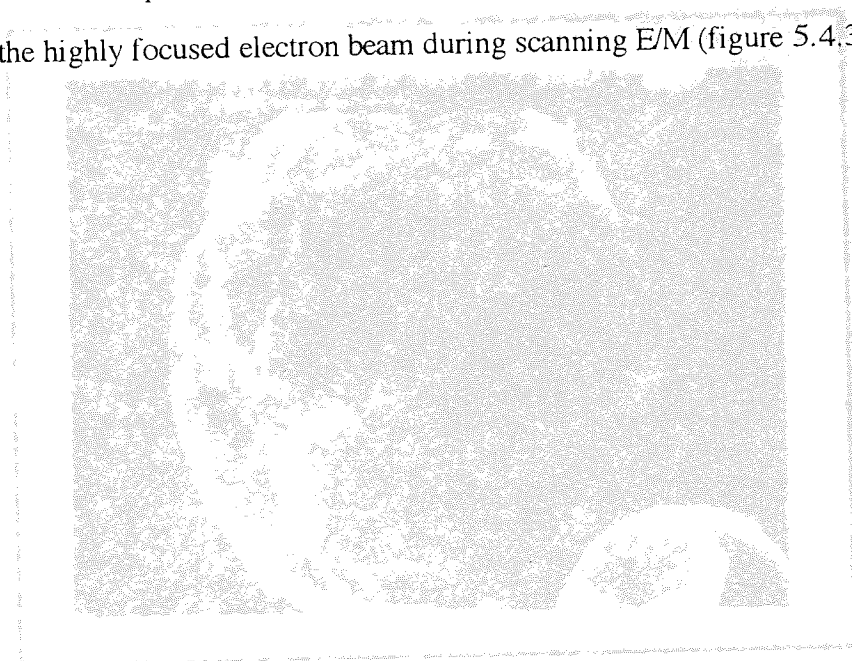


Figure 5.4.4.a) The surface topography of unloaded microspheres after incubation in newborn calf serum at 37°C for 30 days.

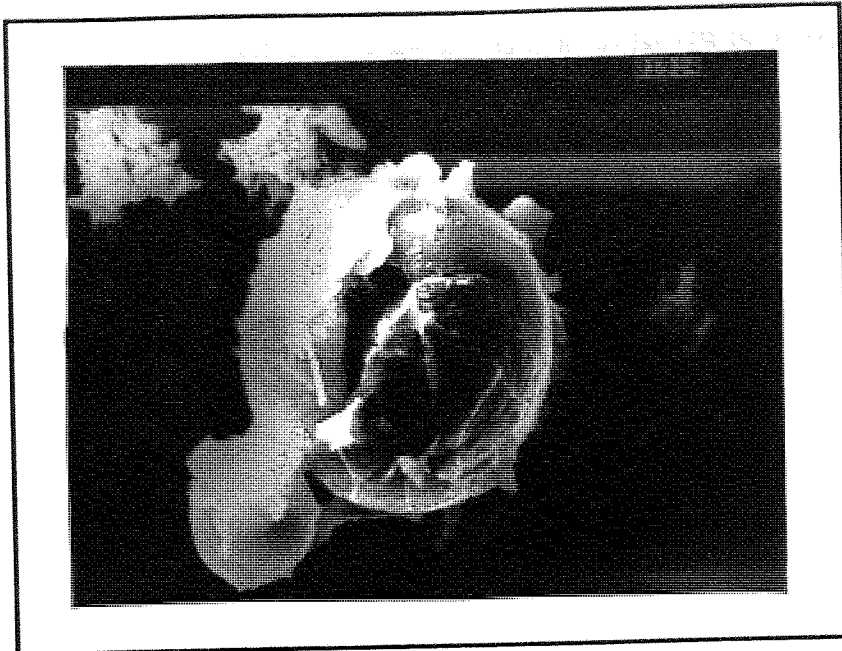
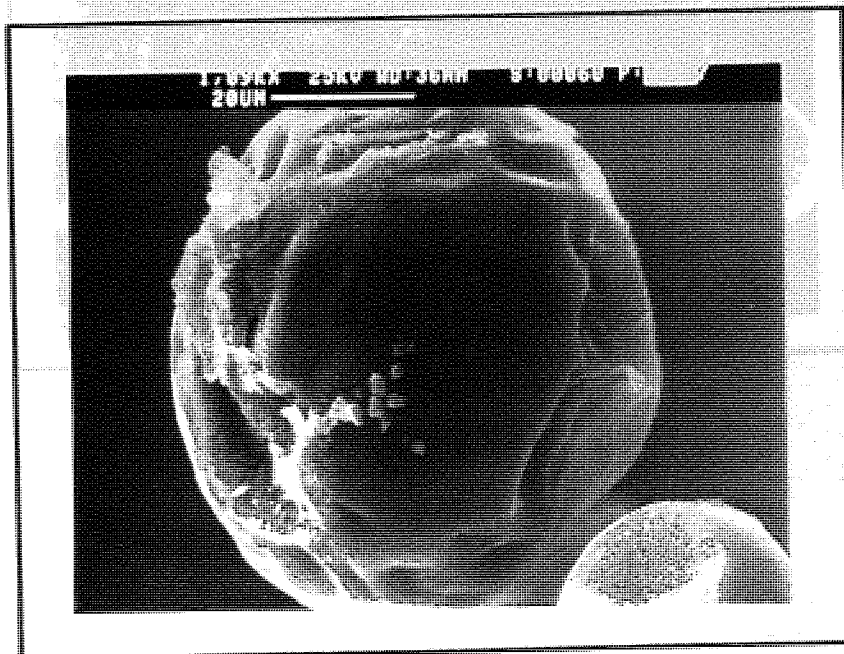


Figure 5.4.4.b) The surface topography of unloaded microspheres after incubation in Hank's balanced salt solution at 37°C for 30 days.



After incubation in both NCS and HBSS at 37°C for 30 days, the surface of unloaded microspheres had become significantly more rough and pitted (figure 5.4.4. a and b) than freshly fabricated unloaded microspheres (figure 3.3.1.f). Extensive serum protein adsorption was observed on the surface of unloaded microspheres as a result of incubation in NCS for 30 days (figure 5.4.4.a). Material, thought to be HBSS buffer salts, had become adsorbed to the surface of unloaded microspheres incubated in HBSS for 30 days (figure 5.4.4.b).

There was little visual difference in the extent of the very limited surface biodegradation of all microspheres incubated in NCS and HBSS.

Figure 5.5.1.a) The internal architecture of 20% w/w vancomycin at 37°C for 30 days (magnification x800).

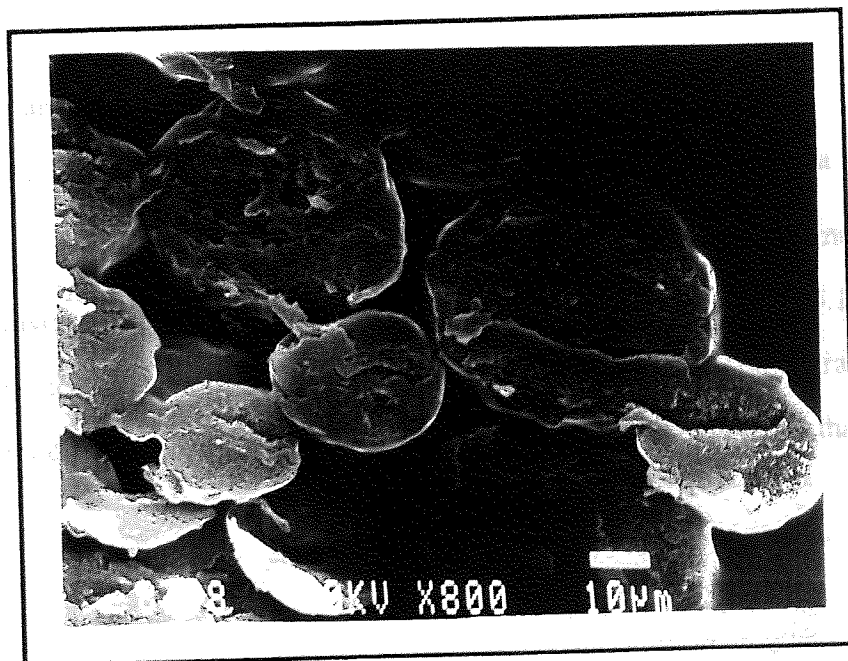
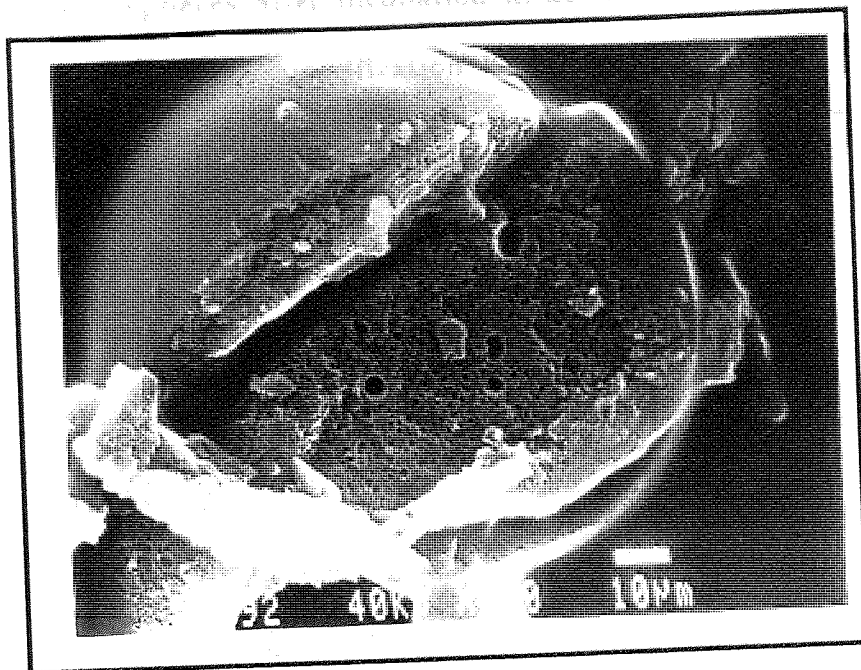


Figure 5.5.1.b) The internal architecture of 20% w/w vancomycin loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days (magnification x800).



20% w/w vancomycin loaded microspheres had developed an extensive internal macroporous matrix after 30 days of incubation in both NCS and HBSS at 37°C (figure 5.5.1 a and b). However, larger macropores were visible in the internal matrix of 20% w/w vancomycin loaded microspheres incubated in HBSS (figure 5.5.1.b) than in microspheres incubation in NCS (figure 5.5.1.a). This indicated that biodegradation of the internal matrix of the microspheres had progressed at a faster rate in HBSS than in NCS.

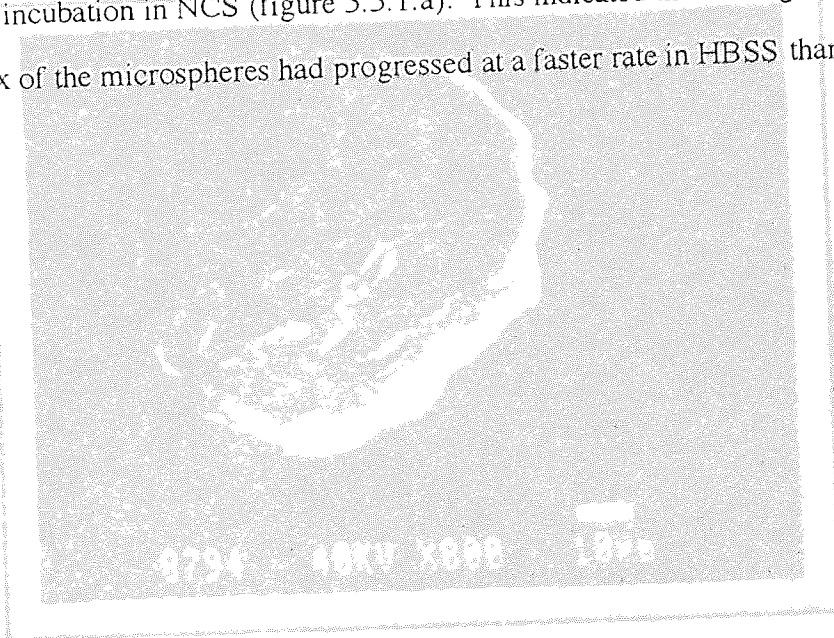


Figure 5.5.2.a) The internal architecture of 20% w/w ciprofloxacin loaded microspheres after incubation in newborn calf serum at 37°C over 30 days (magnification x800).

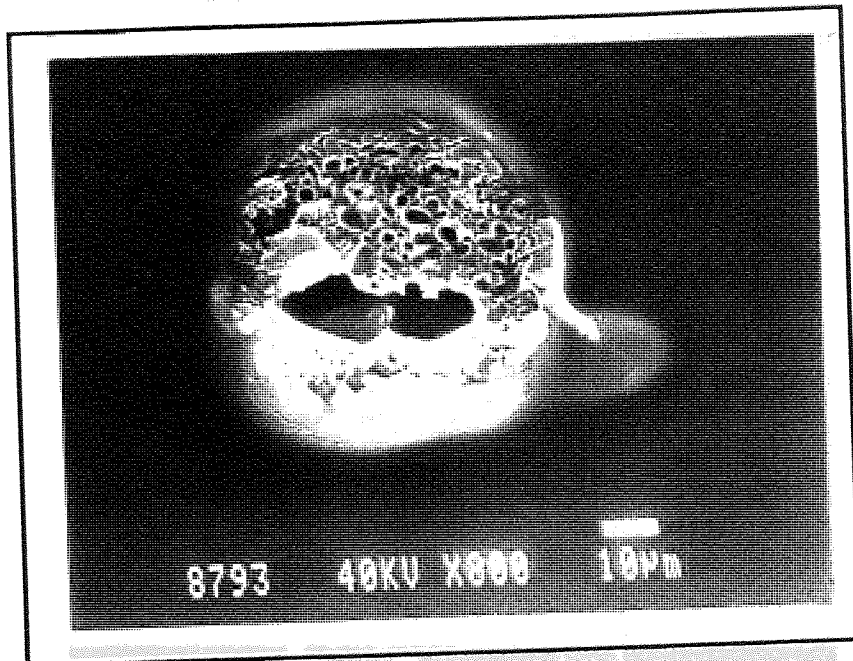
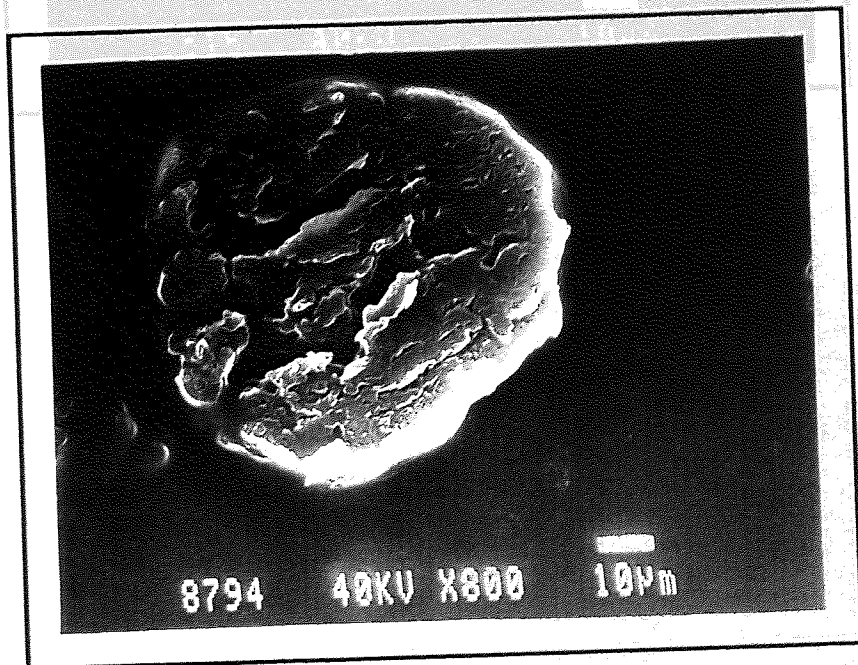


Figure 5.5.2.b) The internal architecture of 20% w/w ciprofloxacin loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days (magnification x800).



Internal architecture of 20% w/w rifampicin loaded microspheres

The incubation of 20% w/w ciprofloxacin loaded microspheres in both NCS and HBSS at 37°C caused the formation of extensive macropores within the internal matrix and the development of channels linking together macropores in different areas of the matrix (figure 5.5.2.a and b). Visually, the extent of the internal matrix biodegradation of 20% w/w ciprofloxacin loaded microspheres incubated in NCS and HBSS was similar.

Figure 5.5.3.a) The internal architecture of 20% w/w rifampicin loaded microspheres after incubation in newborn calf serum at 37°C over 30 days (magnification x800).

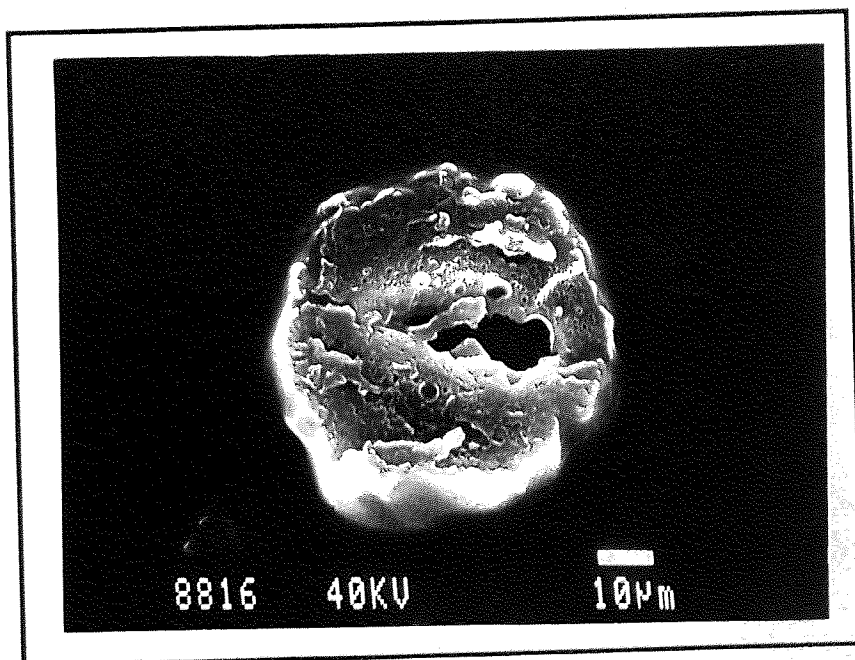


Figure 5.5.3.b) The internal architecture of 20% w/w rifampicin loaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days (magnification x800).



The incubation of 20% w/w rifampicin loaded microspheres in both NCS and HBSS at 37°C caused the formation of extensive macropores throughout the internal matrix (figure 5.5.3.a and b). The huge pore inside the microsphere incubated in NCS (figure 5.5.3.a) was thought to be an artefact created by the electron beam melting the polymer during SEM focusing. Visually, the extent of biodegradation of 20% w/w rifampicin loaded microspheres after incubation in NCS and HBSS was similar.

Figure 5.5.4.a) The internal architecture of unloaded microspheres after incubation in newborn calf serum at 37°C over 30 days (magnification x800).

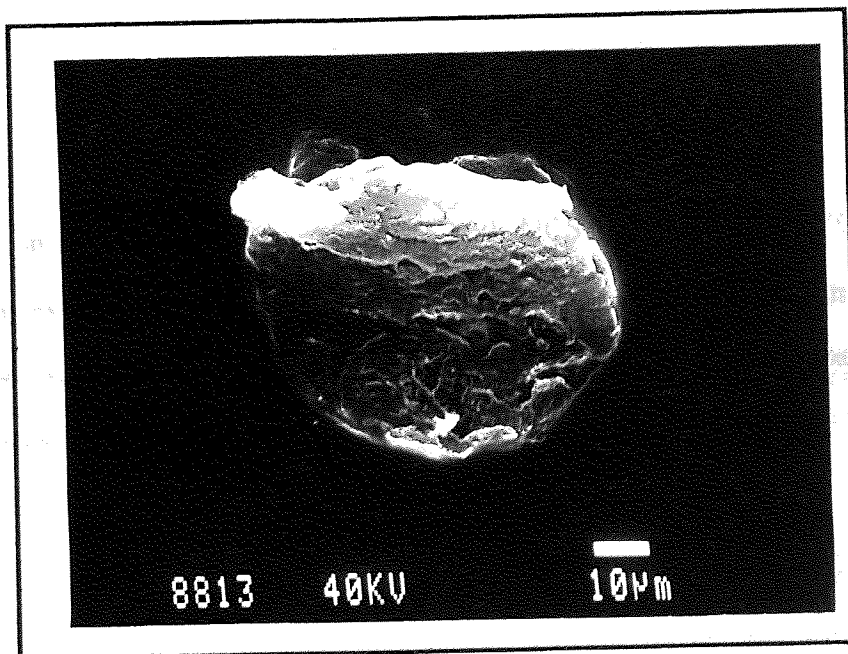
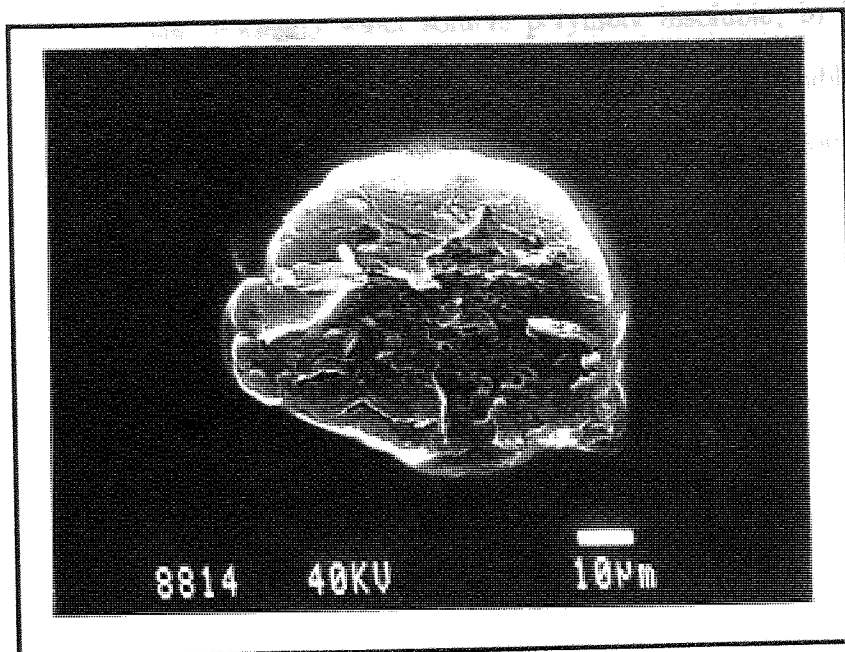


Figure 5.5.4.b) The internal architecture of unloaded microspheres after incubation in Hank's balanced salt solution at 37°C over 30 days (magnification x800).



The incubation of unloaded microspheres in both NCS and HBSS at 37°C caused the formation of extensive macropores throughout the internal matrix of the microspheres. Visually, the extent of biodegradation of unloaded microspheres after incubation in NCS and HBSS was similar.

The biodegradation of a polymer is dependent of a hydrolytic polymer such as PLGA 35:65 in an aqueous solution at physiological pH involves polymer hydration followed by

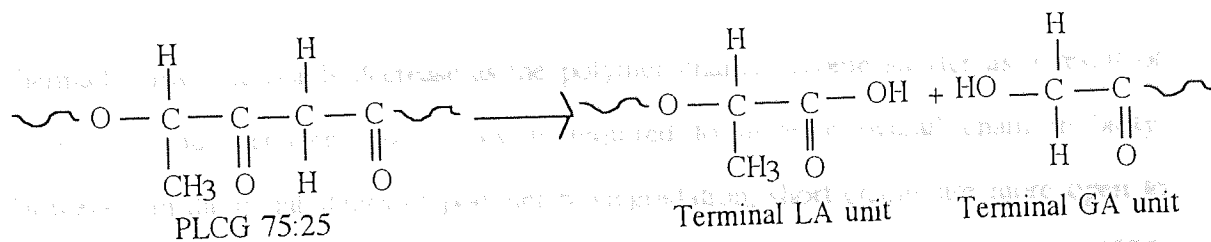
5.4. Discussion.

Biodegradation has been defined by Holland *et al.*, (1986) as the hydrolytic, enzymatic or bacteriological degradation processes occurring in a polymer which do not necessarily proceed to a stage where the physical form of the polymer is altered, as opposed to bioerosion which was defined as physical loss from the polymer matrix by physical and chemical processes (Holland *et al.*, 1986). Biodegradation of polymers can be caused by two mechanisms. Firstly, absorption of energy from X-rays and γ radiation can disrupt

primary covalent bonds in the polymer and result in the formation of free radicals which propagate molecular degradation (Williams, 1982). Secondly, hydrolysis can cause polymer biodegradation by one of three chemical mechanisms: a) hydrolytic degradation of the cross-links which make normally water soluble polymers insoluble; b) hydrolysis, ionisation or protonisation of pendant side chains to solubilise water insoluble polymers and c) random cleavage of an insoluble polymer backbone to produce aqueous oligomers (Holland *et al*, 1986, Williams, 1982). Polyesters such as poly (*dl*-lactide-co-glycolide) [PLCG] 75:25 undergo biodegradation as a result of backbone cleavage (process c). The ester linkages between monomer units are the points of hydrolytic attack, by OH⁻ and H₃O⁺ ions, such that polymers with one terminal carboxyl (COOH) group are generated (Hutchinson and Furr, 1987 and 1990; Makino *et al*, 1986). The reaction is shown in figure 5.6. below:

Figure 5.6. Hydrolysis of an ester linkage in PLCG 75:25.

(Williams, 1982).



The hydrolysis of microspheres composed of a hydrophobic polymer such as PLCG 75:25 in an aqueous medium at physiological pH involves polymer hydration followed by random chain scission of ester bonds which decreases the molecular mass of the polymer (Hutchinson and Furr, 1987 and 1990; Wang *et al*, 1990; Oh *et al*, 1993). Actual mass loss from the microspheres occurs when the size of the polymer oligomers is reduced to the extent that they solubilise in the aqueous continuum. This solubilisation signals the start of polymer disintegration. Finally, bulk solubilisation of the polymer matrix causes complete dissolution of the microspheres (Hutchinson and Furr, 1987 and 1990; Wang *et al*, 1990; Oh *et al*, 1993). Primary hydration of the polymer initiates immediate chain

hydrolysis to create more alkoxylic and carboxylic end groups which in turn increases polymer hydrophilicity. Immediate chain hydrolysis prevents equilibrium swelling of the polymer and water uptake continues unabated (Hutchinson and Furr, 1987 and 1990). Hydrolysis of polymer chains in the microsphere matrix will eventually decrease the overall molecular mass of the polymer. A decrease in the weight average molecular mass of the polymer (M_w) will occur during hydrolysis as a result of a shortening in the length of all the hydrolysed chains in the polymer. A reduction in the number average molecular mass (M_n) will occur with an increase in the number of shorter polymer chains being produced as a result of hydrolysis (Makino *et al*, 1986). Random chain scission of PLCG is assumed to be occurring if the weight average molecular mass falls more quickly than the number average molecular mass (Hausberger, 1995). Polydispersity (pd) is related to both the weight average molecular mass and the number average molecular mass and is calculated by dividing M_w by M_n . Pd will therefore decrease with reductions in M_w and M_n . Generally, a reduction in the glass transition temperature (T_g) of a biodegradable polymer will occur during incubation in an aqueous medium because the number of thermally cleavable bonds decrease as the polymer chains become shorter as a result of hydrolysis and therefore less energy is required to increase overall chain mobility. However, in the initial stages of polymer biodegradation, short chains are more open to hydrolytic attack than long chains and are preferentially hydrolysed (Makino *et al*, 1986; Park, 1994). This preferential hydrolysis increases the proportion of longer chains in the polymer and serves to increase the polymer weight average molecular mass (which is the average mass of all the molecules in the polymer), polymer polydispersity, and polymer T_g (since long chains number require more thermal energy to facilitate their decomposition than short chains) (Makino *et al*, 1986; Park, 1994). There is evidence in the literature to suggest that preferential cleavage of glycolide units occurs in PLCG during its biodegradation in both 150mM PBS plus NaCl (0.9% w/v), pH 7.2 at 37°C (Wang *et al*, 1990) and 0.01M PBS (pH 7.4) at 37°C (O'Hagan *et al*, 1994). This preferential cleavage occurs as a result of the hydrophilic PGA encouraging water uptake into PLCG in the early stages of biodegradation which subsequent initiates hydrolysis of the PGA component.

Therefore, biodegradation of PLCG can occur at two different rates depending on the distribution of lactide and glycolide units within the polymer. If PLCG is composed of repeating lactide and glycolide units then the rate of biodegradation will be constant throughout the polymer. However, if there is an isotactic distribution of monomeric units (blocks of either lactide or glycolide in defined regions of the polymer) then those regions of the polymer containing high concentrations of glycolide units will be degraded faster than regions containing high concentrations of lactide units. Further to this, Fredericks and co-workers noted that the rate of biodegradation inside Vicryl® (GA:LA 90%:10%) sutures was quicker than on the outside such that, even after 98% of the total mass of the suture was lost, the external shape of the suture remained intact (Fredericks *et al*, 1984). Kenley and associates suggested that the formation of an acidic micro-environment within rods of PLCG resulting from carboxylic acid formation could have enhanced polymer biodegradation within the internal matrix (Kenley *et al*, 1987). Li and co-workers (1990) introduced a theory for the spatial heterogeneous rate of bulk PLCG degradation of both PLA and PLCG to explain the previous observations. It was suggested that, initially, biodegradation occurred at a faster rate at the surface of a round PLCG plate than at its centre due to the primary contact of incubation medium with the surface prior to uptake into the polymer matrix. However, while the acidic polymer degradation products generated at the surface of the plate were able to diffuse into the external medium, those inside the matrix were prevented from doing so by the intact polymer membrane. Increasing concentrations of carboxylic end groups attached to the shortened polymer chains within the matrix were thought to act autocatalytically to elevate the rate of internal polymer chain acid hydrolysis above that occurring at the surface. It was suggested that, eventually, hydrolysis within the matrix extended towards the surface of the plate and together with continuing surface hydrolysis of the plate, caused a thinning of the membranous crust. The resultant increase in membrane permeability allowed oligomers to leave the plate leading to mass loss from the structure (Li *et al*, 1990). The development of an acidic micro-environment explained the observation that the internal matrix was hydrolysed at a faster rate than the surface and that no soluble products were released from

the plate before seven weeks of incubation in aqueous buffer (Li *et al.*, 1990) and may account for the faster rate of internal biodegradation observed in the microspheres used in the present work. The rate of polymer biodegradation in an aqueous medium is also affected by the constituents of the medium such as plasma proteins, enzymes and lipids. Makino (1987) demonstrated that the addition of plasma proteins such as albumin, γ -globulins and fibrinogen to solutions of PBS (ionic strength 0.154, pH 7.6, containing 1% w/w of sodium azide) and the subsequent incubation of poly (*l*-lactide) microcapsules in this medium at 37°C accelerated polymer chain scission (Makino *et al.*, 1987). The mechanism by which this occurred was termed the "Cage Effect" and resulted from the adsorption of plasma proteins onto the surface of the microcapsules. It was suggested that the outer area of the adsorbed proteins (closest to the incubation medium) contained an overall positive charge whereas the inner area of the proteins, in contact with the microcapsule surface, was both negatively charged and hydrophobic. The low partition coefficient for electrolyte ions between the inner, hydrophobic, region and the external medium was thought to result in a low concentration of electrolytes in the inner region and the build up of a negative charge in this area with respect to the external medium. This negative electrical potential induced a negative charge in the membrane of the microcapsule in contact with the hydrophobic protein region attracting hydrogen ions in the medium to the surface of the microcapsule. It was postulated that hydrogen ion accumulation at the microcapsule surface-protein interface enhanced polymer biodegradation in this region. The formation of a cage of water molecules around the hydrophobic regions of the proteins and hence around the surface of the microcapsules due to hydrogen bonding ("Cage Effect") was thought to further increase the rate of biodegradation by increasing the solubility of the polymer in the incubation medium (Makino *et al.*, 1987). The development of a "Cage Effect" around microspheres used in the present work could also have occurred due to protein adsorption after incubation in NCS. Certain enzymes have been shown to increase the rate of PLA and PGA biodegradation by catalysing the attack by hydroxyl (OH⁻) ions on +ve carbonyl C-atoms (CO⁺) causing ester hydrolysis. (Williams and Mort,

1977; Williams, 1982). α -chymotrypsin, carboxypeptidase A, chlostridiopeptidase A and ficin were all found to increase the rate of *in vitro* biodegradation of Dexon® sutures composed of poly (glycolic acid) [PGA]. As expected, the esterases bromelain, esterase and leucine aminopeptidase (a lysosomal enzyme released from macrophages in response to implanted foreign bodies) had particularly marked effects on the rate of Dexon® degradation although this may have been partly due to the preparation of enzymes in a solution of ammonium sulphate (Williams and Mort, 1977). Enzymes responsible for enhancement of the *in vitro* rate of PLA biodegradation included pronase, proteinase K and bromelain, while ficin and esterase only slightly increased the rate of biodegradation (Williams, 1982). The rate of *in vivo* enzyme-induced polyester biodegradation is dependant upon the susceptibility of the polyester to the enzymes released during a foreign body reaction. If the polyester is affected by enzymes released from cells, such as polymorphonuclear leukocytes, activated in response to the trauma of implantation then the initial rate of biodegradation will be rapid and will slow with time. However, if the polyester is susceptible to enzymes secreted by macrophages as part of the chronic immune response then the rate of enzyme-induced biodegradation will increase over time (Williams, 1982). PLCG 75:25 in the microspheres used in the present work was in the glassy state at 37°C (see section 3.2.5.b) and there would therefore be little enzymatic involvement in the early stages of incubation as the polymer chains are "frozen" into a single conformational structure. However, with the progression of hydrolytic biodegradation, increases in chain mobility would cause re-arrangement of the polymer structure and increase the chances of the formation of the correct conformation for enzymatic attack (Holland *et al*, 1986). It has been demonstrated that, for some unknown reason, bacteria such as *Streptococcus mitis*, *Escherichia coli* and *Staphylococcus albus* can decrease the rate of *in vitro* and *in vivo* Dexon® biodegradation suggesting that bacterial enzymes do not play a part in the breakdown of polyesters (Williams, 1982). The lipids butyric acid, stearic acid, caproic acid and heptonic acid in phosphate buffer at pH 7.0 have all been shown to enhance the *in vitro* rate of biodegradation of Dexon® sutures

(as shown by the loss of suture strength after incubation) as a result of the ability of lipids to act as plasticisers (Menei *et al.*, 1993; Williams, 1982; Sharma and Williams, 1981). The potential for biodegradation of polyesters by proteins, enzymes and lipids provides an explanation for the observed discrepancies in their rate of *in vitro* and *in vivo* biodegradation (Williams, 1982). The properties of the material encapsulated within microspheres will also affect the rate of polymer biodegradation. The encapsulation of a water soluble drug will increase the rate of initial water uptake by microspheres and hence quicken the rate of polymer hydrolysis whereas the encapsulation of an acidic drug will increase the rate of biodegradation of the internal matrix of the microspheres by acting as a hydrolytic catalyst (Vert *et al.*, 1991).

The above observations can be used to interpret the results obtained from biodegradation studies involving unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres incubated in NCS and HBSS at 37°C. When incubated in both NCS and HBSS at 37°C, the mean Mw, Mp, Mn, Tg and pd of PLCG in unloaded microspheres and 20% w/w antibiotic loaded microspheres tended to decrease over 30 days. The large increase in Mw on day 18 of incubation shown by microspheres incubated in NCS was thought to be caused by protein contaminants adsorbed to the surface of these microspheres. The mean Mp of 20% w/w vancomycin loaded microspheres remained relatively constant over 30 days of incubation in HBSS and NCS at 37°C suggesting that the polymer contained more long chains than short chains. The mean Mn of these microspheres underwent small decreases over 30 days of incubation. This was assumed to be because of preferential glycolide hydrolysis which indicated that the majority of the copolymer was composed of lactide. The gradual increase in polymer Tg of 20% w/w antibiotic loaded microspheres incubated in HBSS indicated that, by day 18 of incubation, the proportion of lactide units remaining in the polymer was increasing relative to the number of glycolide units. Overall, the rate of biodegradation of 20% w/w vancomycin loaded microspheres, in terms of the gradual decrease in copolymer molecular weight, polydispersity and Tg, was slightly faster in HBSS than in NCS over 30 days of incubation at 37°C. The decrease in copolymer

Mw, Mp and Mn of 20% w/w ciprofloxacin loaded microspheres was very similar in NCS and HBSS after incubation for 30 days at 37°C although the polymer pd fluctuated within a narrow range over this period. The sharp increase in the Tg of 20% w/w ciprofloxacin loaded microspheres on day 6 of incubation in NCS was again thought to have been influenced by proteins adsorbed to the surface of the microspheres. Although fluctuations in the pattern of Tg recorded for 20% w/w ciprofloxacin loaded microspheres in HBSS occurred six days later than that for microspheres incubated in NCS, after 30 days of incubation the polymer Tg of 20% w/w ciprofloxacin microspheres incubated in HBSS was lower than that recorded for similar microspheres incubated in NCS. Overall, the rate of biodegradation of 20% w/w ciprofloxacin loaded microspheres incubated in NCS and HBSS, in terms of the gradual decrease in copolymer molecular weight, polydispersity and Tg, were approximately the same. In the initial stages of the hydrolysis of 20% w/w rifampicin loaded microspheres in NCS, polymer Mn decreased while polymer pd and polymer Tg all increased. These observations suggest that short copolymer chains in 20% w/w rifampicin loaded microspheres were being further shortened by hydrolysis. In a similar fashion to 20% w/w vancomycin loaded microspheres incubated in NCS, 20% w/w rifampicin loaded microspheres incubated in NCS showed a large increase in both Mw and pd after 18 days as well as a slight increase in Mp while the Mn remained constant. However, the polymer Tg was relatively low after 18 days of incubation in NCS and this was thought to represent the thermal profile of the glycolide component of the copolymer. An increase in the polymer Tg of 20% w/w rifampicin loaded microspheres between days 24 and 30 of incubation in NCS suggested that the concentration of glycolide in the copolymer was decreasing over time. After 12 days of incubation in HBSS at 37°C, the sharp increase in the polymer Tg of 20% w/w rifampicin loaded microspheres was thought to be due to the lactide component of the copolymer suggesting that the glycolide component had been either extensively or fully hydrolysed by this time. After 30 days of incubation, the rate of biodegradation of 20% w/w rifampicin loaded microspheres, in terms of the gradual decrease in copolymer molecular weight, polydispersity and Tg, was faster in HBSS than in NCS. The Mw and pd of unloaded

microspheres increased sharply after 12 days of incubation in NCS at 37°C and this was thought to be because of contaminant proteins adsorbed to the surface of the microspheres. The polymer Tg for unloaded microspheres had increased by day 6 of incubation which indicated that glycolide chains were already becoming shorter by this time. This assumption was supported by the observed increase in the polymer Tg after 6 days of incubation in NCS. Unloaded microspheres incubated in HBSS at 37°C showed an increase in their polymer Tg after 12 days which was thought to represent the thermal profile of the lactide component of the polymer. After 30 days of incubation, the rate of biodegradation of unloaded microspheres, in terms of the gradual decrease in copolymer molecular weight, polydispersity and Tg, seemed faster in HBSS than in NCS. The assumption that glycolide units were preferentially hydrolysed within the microspheres during incubation in both NCS and HBSS at 37°C suggested that the PLCG 75:25 used in the present work contained isotactic domains of lactide and glycolide. Initial hydrolysis of the glycolide domains would produce the observed increases in polymer Mw and polymer pd and these changes are consistent with PLCG exhibiting a heterogeneous rate of biodegradation. A heterogeneous rate of biodegradation for both unloaded and 20% w/w antibiotic loaded microspheres was confirmed by the existence of double Tg peaks on DSC thermograms where the lower temperature peak was thought to represent the glycolide component (due to the lower Tg of PGA of 36°C compared with PLA of 57°C (Fredericks *et al.*, 1984)) while the higher temperature peak was thought to represent the lactide component. The lower temperature peak was often smaller in amplitude than the peak observed at the higher temperature. This would be expected since the initial proportion of lactide was greater than that of glycolide by a ratio of 3:1. It should be noted that Park (1994) proposed a different theory to account for the double Tg peaks created by biodegrading poly (*dl*-lactic acid) in which he suggested that PLA at the surface of microspheres was responsible for the higher Tg endotherm since it was biodegraded at a slower rate than polymer deep within the internal matrix which was represented by the lower Tg endotherm (Park, 1994). Both theories provide a plausible explanation for the double peak endotherms seen for biodegrading PLA even though, to date, neither theory

has been proved. Ranking in terms of the rate of biodegradation of unloaded microspheres and 20% w/w antibiotic loaded microspheres incubated in NCS at 37°C was different from the ranking of these microspheres after incubation in HBSS at 37°C. Unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres incubated in NCS all showed very similar rates of biodegradation. The majority (approximately 75% w/w) of the encapsulated load of ciprofloxacin and rifampicin remained within their respective dispersed 20% w/w loaded microspheres throughout the 30 day incubation period in NCS due to the relatively low solubility of these antibiotics in NCS. Ciprofloxacin and rifampicin both form acidic solutions in water (Martindale, 1993). Therefore, when these antibiotics started to dissolve within the microsphere matrix they might have created an acidic environment and brought about acid hydrolysis of the internal matrix thereby enhancing the rate of the internal biodegradation of the polymer (decreasing both Mw and Mn). This would provide further support for the existence of a spatial heterogeneous rate of PLCG biodegradation. If a small percentage of microencapsulated rifampicin was molecularly dispersed within the PLCG matrix (as a result of the formation of a solution of rifampicin in the water phase solvent) the antibiotic may act as a plasticiser thereby increasing the rate of polymer biodegradation. It was assumed that the release of a large proportion of the encapsulated vancomycin (approximately 34% w/w) into NCS from 20% w/w loaded microspheres during the burst phase of release prevented a similar antibiotic induced catalysis within these microspheres and would explain why the rate of biodegradation of 20% w/w vancomycin loaded microspheres was similar to that of unloaded microspheres. The similar rate of biodegradation of 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres in HBSS at 37°C, especially in terms of the changes in Mw, was thought to be due to the rapid release of vancomycin and ciprofloxacin from encapsulated stores eliminating the possibility of antibiotic-induced matrix catalysis. The rate of biodegradation of unloaded microspheres and 20% w/w rifampicin loaded microspheres, in terms of the changes in Mw, Mn and Mp, was also similar. In the case of 20% w/w rifampicin loaded microspheres, limited stores of rifampicin (approximately 28% w/w)

remained encapsulated after 30 days and might possibly have exerted some internal catalytic and plasticiser effects. In the case of unloaded microspheres, the buffering capacity of NCS and HBSS could have accelerated the rate polymer biodegradation (Makino *et al*, 1986). This acceleration can occur when the products of PLCG biodegradation (lactic acid and glycolic acid) are neutralised by the buffering capacity of the incubation medium. This displaces the biodegradation reaction in favour of further biodegradation since an equilibrium concentration of degradation products within the external medium will never be achieved with frequent changes of the incubation medium. This situation would not have occurred with 20% w/w antibiotic loaded microspheres because the buffering capacity of the incubation medium would have been utilised in neutralising the released antibiotics. The faster rate of biodegradation observed for 20% w/w antibiotic loaded microspheres incubated in HBSS compared with the biodegradation of similar microspheres incubated in NCS was thought to be due to the faster uptake of water by microspheres incubated in HBSS. This was thought to be because of the greater solubility (and hence hydrophilicity) of vancomycin, ciprofloxacin and rifampicin in NBSS compared with NCS. While it can be said that serum protein binding, serum lipids and possibly serum enzymes could potentially increase the rate of microsphere biodegradation during incubation in NCS, it is the rapid penetration of the microsphere matrix by water which is the major factor influencing the rate of polymer biodegradation, especially over 30 days incubation.

After incubation in HBSS at 37°C for 30 days, the percentage mass loss from 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres was approximately equal to the mass of each antibiotic cumulatively released into HBSS over the 30 day incubation period. Unloaded microspheres lost only approximately 5% w/w of their mass after 30 days of incubation in HBSS at 37°C. It has been suggested that bulk solubilisation of PLCG occurs when the Mn of the polymer decreases to approximately 5.20 kDaltons (Hausberger *et al*, 1995). In the present study, the Mn of unloaded microspheres after 30 days incubation in HBSS was 6.64 kDaltons. Therefore, the 5%

w/w mass loss after incubation of unloaded microspheres in HBSS could have been caused by the solubilisation of oligomers generated by hydrolytic degradation of the polymer. Unloaded and 20% w/w antibiotic-loaded microspheres showed signs of both surface and matrix biodegradation after 30 days of incubation in both NCS and HBSS although the surface and overall ultrastructural integrity of all microspheres was still intact after this time. However, the enlargement of macropores and the development of channels within microspheres suggested that the internal matrix had been degraded at a faster rate than the surface. This suggested that a heterogeneous rate of biodegradation had occurred during the 30 day incubation period and may well have been the result of initial glycolide hydrolysis leading to matrix autocatalysis. Microsphere bioerosion (indicated by mass loss from the polymer) is thought to occur after the dissolution of hydrolytically generated oligomers from the microsphere matrix. However, it was assumed that the elution of these solubilised polymer degradation products from microspheres used in the present work was not great as the peripheral compacted outer layers of these microspheres were still intact after 30 days of incubation in both NCS and HBSS.

Chemical analysis of the normal rate of cultured cell growth or alter the normal morphology of cultured cells. It is important to know the rate of cultured cell growth and/or cultured cell morphology in the presence of a drug with the rate of cultured cell growth and/or cultured cell morphology under normal culture conditions in the absence of drug (the control). The extent of the drug's cytotoxicity can be assessed. In the present work, vancomycin, a protein synthesis inhibitor, was encapsulated within microspheres composed of poly (DL-lactide-co-glycolide) (PL(G)) 75:25. Although PL(G) 75:25 shows excellent biocompatibility (Smith et al., 1974), azobutane and petroleum ether (both of which are known to be toxic - see section 2.4.1) were utilised in the W/O emulsification with active emulsification technique used to fabricate PL(G) 75:25 microspheres (section 2.2.1.). Analysis of the drug content of unloaded and 20% w/w antibiotic loaded microspheres showed an DCLH confirmed that the concentration of azobutane and petroleum ether present within freshly fabricated microspheres was below 50 ppm (the maximum safe level in pharmaceutical products according to the ICH and FDA) and 250 ppm respectively (see

Chapter 6: *In vitro* cytotoxicity of unloaded and 20% w/w antibiotic loaded PLCG 75:25 microspheres.

6.1. Introduction.

Antibiotics such as vancomycin, ciprofloxacin and rifampicin are very effective at eradicating bacteria but this property may also cause host tissue damage. It is therefore vital to know the concentration of an antibiotic that can be safely administered to a patient without compromising its ability to effectively treat an infection. Traditionally, animal studies have been used to generate drug toxicity data but are becoming increasingly unpopular with the general public because of the moral issues involved. Therefore, a number of *in vitro* drug cytotoxicity testing programmes utilising established cell lines have been developed and validated by the Fund for the Replacement of Animals in Medical Experiments (FRAME). The basic principle of these protocols is that a cytotoxic drug or chemical will inhibit the normal rate of cultured cell growth or alter the normal morphology of cultured cells. By comparing the rate of cultured cell growth and/or cultured cell morphology in the presence of a drug with the rate of cultured cell growth and/or cultured cell morphology under normal culture conditions in the absence of drug (the control), the extent of the drug's cytotoxicity can be assessed. In the present work, vancomycin, ciprofloxacin and rifampicin were encapsulated within microspheres composed of poly (*dl*-lactide-co-glycolide) [PLCG] 75:25. Although PLCG 75:25 shows excellent biocompatibility (Cutright et al, 1974), acetonitrile and petroleum ether (both of which are known to be toxic - see section 3.4.) were utilised in the W/O emulsification with solvent evaporation technique used to fabricate PLCG 75:25 microspheres (section 2.2.1.). Although GLC analysis of unloaded and 20% w/w antibiotic loaded microspheres dissolved in DCM confirmed that the concentration of acetonitrile and petroleum ether present within freshly fabricated microspheres was below 50 ppm (the maximum safe level in pharmaceutical products according to the ICH and FDA) and 250 ppm respectively (see

section 3.3.5.a), it is possible that these concentrations could be cytotoxic to a patient in the locality of the hip joint after topical administration, especially as 20% w/w antibiotic loaded microspheres have been designed with topical delivery in mind. Light white mineral oil and SPAN 40, both of which are irritants, were also involved in the microsphere fabrication process. So, by incubating an established cell line in the presence of eluates obtained by incubation of unloaded and 20% w/w antibiotic loaded microspheres in cell culture medium, the cytotoxicity of the microsphere preparations can be assessed. This cytotoxicity was assessed using INVITTOX/FRAME protocol 3b in which 3T3-L1 cells were incubated in Dulbecco's Modified Eagles Medium (DMEM) containing leached materials eluted during 24 hours of incubation in freshly fabricated microspheres (2.2.12.b). However, precipitation of ciprofloxacin, presumed to have been caused by its low solubility in DMEM, occurred during incubation of the eluant with 3T3-L1 cells and resulted in the production of falsely high rates of cell growth due to the binding of Kenacid blue dye with this precipitated ciprofloxacin. Therefore, INVITTOX protocol 104, in which materials eluted from microspheres diffuse through a layer of methylcellulose gel before reaching 3T3-L1 cells incubated in Iscove's Modification of Dulbecco's Medium (IMDM), was also employed to measure the cytotoxicity of unloaded and 20% w/w antibiotic loaded microspheres (2.2.12.c).

Figure 6.1

6.2. Methods.

3T3-L1 cells were incubated in DMEM containing eluates from unloaded and 20% w/w antibiotic loaded microspheres at 37°C and INVITTOX protocol 3b (2.2.12.b) and INVITTOX protocol 104 (section 2.2.12.c) were used to assess the cytotoxicity of unloaded and 20% w/w antibiotic loaded microspheres to cultured 3T3-L1 cells.

The results of the cytotoxicity assays were compared with control samples (3T3-L1 cells incubated in DMEM at 37°C). These observations suggest that the cytotoxicity was reduced from 20% w/w vancomycin loaded microspheres during the incubation in DMEM for 24 hours at 37°C.

6.3 Results

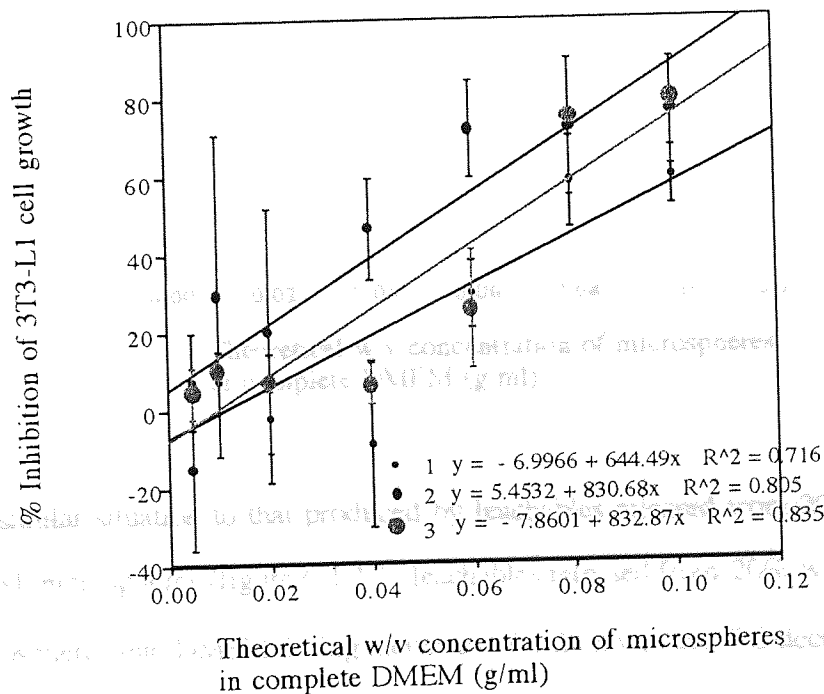
6.3.1 Cellular cytotoxicity of unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres.

The percentage inhibition of 3T3-L1 cell growth after 72 hours of incubation in the presence of increasing v/v concentrations of microsphere leachables in fresh complete DMEM is shown in figure 6.1.. Microsphere eluates were obtained by incubating either unloaded microspheres, 20% w/w vancomycin loaded microspheres or 20% w/w rifampicin loaded microspheres in complete DMEM for 24 hours at 37°C. The eluates were then diluted with fresh complete DMEM to produce a range of concentrations of microsphere leachables in DMEM. Since the actual concentrations of any leached material was unknown, each eluate were diluted according to the concentration of microspheres which had been originally incubated in it. In each case, 0.5g of microspheres had been incubated in 5ml of DMEM to produce a w/v concentration of 0.1 g/ml. Therefore, by adding fresh DMEM to the eluate, dilution of the eluant was made so that the concentration would theoretically compare with the concentration of eluate produced by incubating 0.08g, 0.06g, 0.04g, 0.02g, 0.01g and 0.05g of microspheres in 5ml of DMEM for 24 hours at 37°C (figure 6.1.).

By producing a number of dilutions (using fresh DMEM) of the eluates obtained after 24 hours of incubation of 20% w/w vancomycin loaded microspheres at 37°C and subsequently exposing 3T3-L1 cells to aliquots of progressively more concentrated eluates, an increase in the percentage inhibition of 3T3-L1 cell growth over a 72 hour period at 37°C was observed (figure 6.1.1.) when compared with control samples (3T3-L1 cells grown in fresh complete DMEM at 37°C). These observations suggest that cytotoxic leachables were released from 20% w/w vancomycin loaded microspheres during their incubation in DMEM for 24 hours at 37°C.

Figure 6.1. Percentage inhibition of growth curves for 3T3-L1 cells exposed to DMEM eluates from unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% rifampicin loaded microspheres.

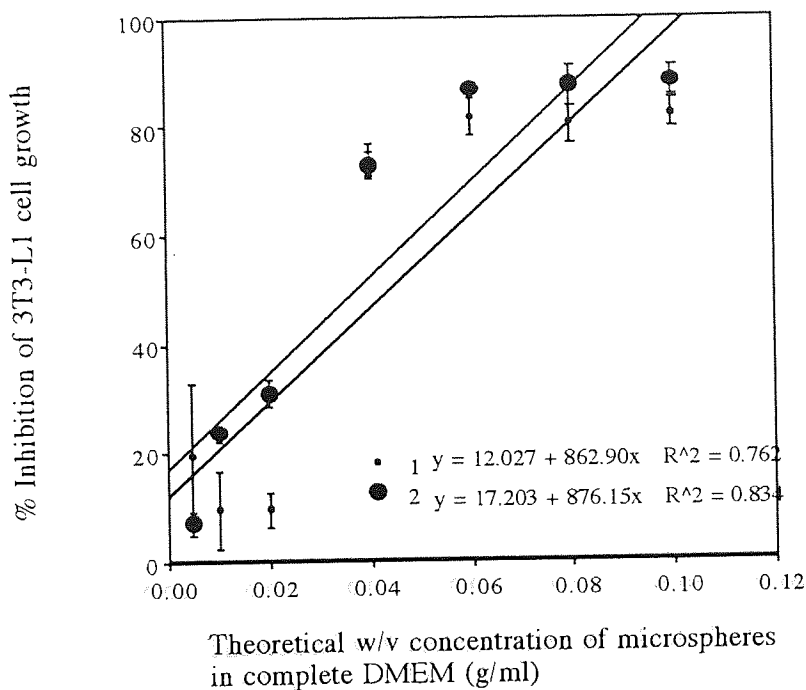
Figure 6.1.1. Percentage inhibition of growth curve for 3T3-L1 cells exposed to DMEM eluates from 20% w/w vancomycin loaded microspheres on three separate occasions (mean values \pm SD, n=3 for each point).



Theoretical w/w concentration of microspheres in complete DMEM (g/ml)

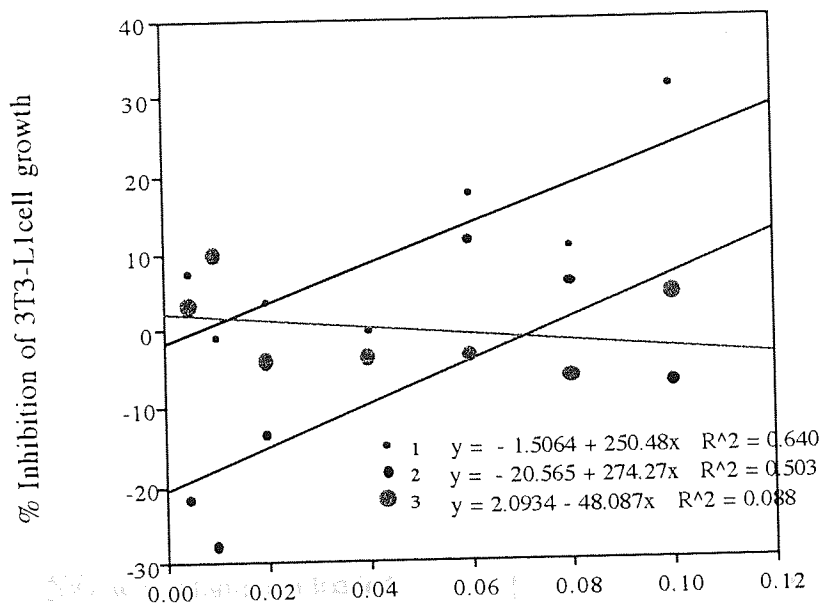
Figure 6.1.2 shows the percentage inhibition of 3T3-L1 cell growth during the 72-hour assay period compared with values for control (unloaded microspheres) in fresh complete medium at 37°C (Figure 6.1.2). Since the initial concentration of the 24-hour eluate was diluted by the addition of fresh complete DMEM, decrease in the percentage inhibition of 3T3-L1 cell growth during the 72-hour assay period was observed (Figure 6.1.2). These observations confirmed that significant amount was released from 20% w/w rifampicin loaded microspheres during their incubation in DMEM for 24 hours at 37°C.

Figure 6.1.2. Percentage inhibition of growth curve for 3T3-L1 cells exposed to DMEM eluates from 20% w/w rifampicin loaded microspheres on two separate occasions (mean values \pm SD, n=3 for each point).



In a similar situation to that produced by leachables released from 20% w/w vancomycin loaded microspheres (figure 6.1.1.), leachables released from 20% w/w rifampicin loaded microspheres into DMEM during incubation for 24 hours at 37°C decreased the percentage growth of 3T3-L1 cells during the 72 hour assay period compared with values for control preparations (3T3-L1 cells incubated in fresh complete medium at 37°C) (figure 6.1.2.). Since the original concentration of the 24 hour eluate was diluted by the addition of fresh complete DMEM a decrease in the percentage inhibition of 3T3-L1 cell growth during the 72 hour assay period was observed (figure 6.1.2.). These observations confirmed that cytotoxic material was released from 20% w/w rifampicin loaded microspheres during their incubation in DMEM for 24 hours at 37°C.

Figure 6.1.3. Percentage inhibition of growth curve for 3T3-L1 cells exposed to DMEM eluates from unloaded microspheres on three separate occasions (mean values \pm SD, n=3 for each point; error bars omitted for clarity).



There was no conclusive correlation between the theoretical concentration of unloaded microspheres in the 24 hour eluate and the percentage inhibition of 3T3-L1 cell growth during the 72 hour assay period (figure 6.1.3.). While data from two studies (figure 6.1.3., 1 and 2) suggested that decreasing the theoretical concentration of unloaded microspheres in the eluant caused a decrease in the percentage inhibition of 3T3-L1 cell growth during the assay period, a further study (figure 6.1.3., 3) contradicted this finding. In order to compare the relative cytotoxicity of the eluates obtained for unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres, the mean ID_{50} value for each these preparations was obtained from figures 6.1.1.-6.1.3. as follows. For each microsphere preparation, the regression coefficient generated for each line of best fit was used to compute the theoretical w/v

concentration of microspheres in complete DMEM (g/ml) that would generate a concentration of leachables capable of inhibiting 3T3-L1 cell growth by 50% (the ID₅₀ value) compared with controls consisting of 3T3-L1 cells incubated in fresh complete DMEM only. ID₅₀ values were calculated for each study performed using 20% w/w vancomycin loaded microspheres (three studies in total) and 20% w/w rifampicin loaded microspheres (two studies in total). ID₅₀ values generated in studies for each microsphere preparation were meaned. However, ID₅₀ values for unloaded microspheres were not computed since the inhibition of 3T3-L1 cell growth by eluates from unloaded microspheres was not conclusive.

Table 6.1. Mean ID₅₀ values for 3T3-L1 cells incubated in complete DMEM containing dissolved leachables from 20% w/w antibiotic loaded microspheres.

Microsphere type	Mean ID ₅₀ value for 3T3-L1 cells (g/ml).
20% w/w vancomycin loaded	0.07 ± 0.17 (SD, n=3)*
20% w/w rifampicin loaded	0.04, n=2*

* equivalent to the concentration of microspheres that would produce an eluate in DMEM after 24 hours at 37°C with the capacity to inhibit 3T3-L1 cell growth by 50% as determined by the Kenacid blue binding method.

The percentage inhibition of 3T3-L1 cell growth caused by eluates generated from incubating unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres for 24 hours at 37°C at a concentration of 0.05g/ml were interpolated from the inhibition of growth curves shown in figures 6.1.1-3 and are summarised in table 6.2. below.

The concentration of leachables from 20% w/w vancomycin loaded microspheres used in the present work. The concentration of 20% w/w vancomycin loaded microspheres used in the present work was lower than the actual concentration of 20% w/w vancomycin loaded microspheres (0.05g/ml) used for *in vitro* antibiotic release studies and *in vivo* biodegradation studies in the present work. 24 hour eluates generated by the

Table 6.2. The percentage inhibition of 3T3-L1 cell growth caused by leachables released by unloaded microspheres, 20% w/w vancomycin loaded microspheres and 20% w/w rifampicin loaded microspheres at a concentration of 0.05 g/ml in complete DMEM.

Microsphere type	Percentage inhibition of 3T3-L1 cell growth by DMEM eluates generated from incubating microspheres at a concentration of 0.05g/ml in complete DMEM at 37°C (± SD, n=3)
20% w/w vancomycin loaded	35.33 ± 10.69
20% w/w rifampicin loaded	58.09, n=2
Unloaded	1.27 ± 9.01

Almost half the mass of 20% w/w rifampicin loaded microspheres (0.04g), compared with the mass of 20% w/w vancomycin loaded microspheres (0.07g), was required to generate a 24 hour eluate which was capable of inhibiting the growth of 3T3-L1 cells by 50% (table 6.1.). 24 hour eluates generated from 0.05 g/ml of 20% w/w rifampicin loaded microspheres were approximately 1.7 times more toxic to 3T3-L1 cells, in terms of the percentage inhibition of 3T3-L1 cell growth, than eluates generated from 24 hour incubation of 20% w/w vancomycin loaded microspheres at a concentration of 0.05g/ml in complete DMEM (table 6.2.). The concentration of 20% w/w vancomycin loaded microspheres (0.07 ± 0.17 g/ml of complete DMEM, n=3) required to inhibit the growth of 3T3-L1 cells by 50% (table 6.1.) was somewhat greater than the actual concentration of 20% w/w vancomycin loaded microspheres (0.05g/ml) used in *in vitro* antibiotic release studies and *in vitro* biodegradation studies in the present work. The concentration of 20% w/w rifampicin loaded microspheres (0.04g/ml) causing the inhibition of 3T3-L1 cell growth by 50% (table 6.1.) was lower than the actual concentration of 20% w/w rifampicin loaded microspheres (0.05g/ml) used for *in vitro* antibiotic release studies and *in vitro* biodegradation studies in the present work. 24 hour eluates generated by the

incubation of a theoretical concentration of unloaded microspheres of 0.05 g at 37°C had little or no cytotoxic effect on the growth of 3T3-L1 cells (table 6.2.). These studies suggest that vancomycin and rifampicin eluted from 20% w/w loaded microspheres during 24 hours of incubation in DMEM at 37°C were responsible for the cytotoxicity of these preparations rather than the microsphere fabrication components such as PLCG 75:25, light white mineral oil, SPAN 40, acetonitrile and petroleum ether. However, the results only suggest that the fabrication components are non-toxic when eluted from DMEM. However, NCS and HBSS were used for *in vitro* antibiotic release and biodegradation studies but it is not envisaged that the elution of material into these media would be significantly different from that observed for DMEM at 37°C.

6.3.2. Cellular cytotoxicity of unloaded microspheres and 20% w/w antibiotic loaded microspheres measured using INVITTOX

protocol 104.

Rather like INVITTOX protocol 3b, INVITTOX protocol 104 provides a means of measuring the cytotoxicity of any material diffusing from unloaded and 20% w/w antibiotic loaded microspheres without cultured 3T3-L1 cells coming into contact with microspheres. This is achieved by depositing microspheres on the surface of a methylcellulose gel (floating on incubation medium containing 3T3-L1 cells). Any toxic material diffusing from the microspheres migrates through the gel and into incubation medium. Exposure to cultured 3T3-L1 cells over 7 day will lead to changes in the rate of cell growth and cell morphology. The degree of cytotoxicity is measured using a Cell Proliferation Inhibition (CPI) Index which indicates the rate of cell growth in the presence of a test material as a percentage of the rate of cell growth of control cells in incubation medium only. A positive cytotoxic control (silicone rubber) was included in the test to indicate the relative extent of the cytotoxicity produced by microspheres. The results are summarised in table 6.3. below.

Table 6.3. The cellular cytotoxicity of eluates released from unloaded microspheres and 20% w/w antibiotic loaded microspheres on cultured 3T3-L1 cells after incubation for seven days at 37°C.

Test preparation	Cell Proliferation Inhibition Index after 7 days incubation (% \pm SD, n=3)
20% w/w vancomycin loaded microspheres	17.4 \pm 3.1
20% w/w ciprofloxacin loaded microspheres	21.2 \pm 3.1
20% w/w rifampicin loaded microspheres	30.9 \pm 1.7
Unloaded microspheres	9.9 \pm 0.0
Positive control (silicone rubber tubing)	68.7 \pm 4.7
Control (gel and cultured cells only)	2.4 \pm 3.1

The cell proliferation inhibition index gave an indication of the extent of 3T3-L1 cell growth inhibition caused by materials diffusing from microspheres. Leachables diffusing from unloaded microspheres inhibited cell growth by less than 10% while leachables diffusing from both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres produced approximately twice this inhibition of cell growth (table 6.3.). Leachables diffusing from 20% w/w rifampicin loaded microspheres were responsible for the largest inhibition of cell growth (a CPI index value of 30.9% - table 6.3.). However, the CPI index value for 20% w/w rifampicin loaded microspheres is still less than half the CPI index value obtained for the silicone rubber positive control. The extent of the cellular cytotoxicity mediated by the different microsphere preparations relative to the control composed of MC gel and cultured 3T3-L1 cells only was determined from the CPI index data by setting ranges of CPI percentages to represent increasing levels of cytotoxicity as shown below.

<u>CPI index (%)</u>	<u>Level of Cytotoxicity</u>
0	None
15+	Low
25+	Medium / low
50+	Medium / high
>70	High

Using this ranking system, unloaded microspheres exhibited low cytotoxicity to cultured 3T3-L1 cells whereas both 20% w/w vancomycin loaded microspheres and 20% w/w ciprofloxacin loaded microspheres exhibited low to medium cytotoxicity to these cells. However, 20% w/w rifampicin loaded microspheres exhibited medium cytotoxicity to cultured 3T3-L1 cells while silicone rubber (the positive control) exhibited medium to high cytotoxicity to cultured 3T3-L1 cells. These results correlate well with data generated using INVITTOX protocol 3b which suggests that the microspheres are not cytotoxic but rather it is the antibiotics encapsulated within them which exhibit *in vitro* cytotoxicity to cultured 3T3-L1 cells. Also, both protocols confirm that rifampicin is more cytotoxic than vancomycin (and more cytotoxic than ciprofloxacin according to INVITTOX protocol 104). INVITTOX protocol 104 was successfully used to measure the relative cytotoxicity of 20% w/w ciprofloxacin loaded microspheres, after the failure of INVITTOX protocol 3b to do so. This is because protocol 104 relied on viable cell count as a determination of cytotoxicity rather than the indirect measurement of viable cell numbers, which is based upon the total concentration of protein in each incubation well as indicated by protein binding to Coomassie brilliant blue dye, used in protocol 3b. INVITTOX protocol 104 is a good indicator of relative antibiotic cytotoxicity by providing relative and comparative values for unloaded and 20% w/w antibiotic loaded microspheres used in the present work.

Microspheres containing 20% w/w vancomycin, rifampicin and ciprofloxacin were prepared in these studies. The results showed good biocompatibility. PLOO PLOO 50:50 microspheres have also been implanted into the skulls of male Wistar rats to assess the tissue reaction to this polymer (Khan et al. 1993). No behavioral changes, neurological deficits

6.4.

Discussion.

The extent to which a material is biocompatible with tissue is related to the immunological and inflammatory response it induces when implanted in an *in vivo* situation. The *in vivo* biocompatibility of PLCG has been well documented (Cutright *et al*, 1974; Visscher *et al*, 1988; Asano *et al*, 1989; Spenlehauer *et al*, 1989; Menei *et al*, 1993; Yamaguchi *et al*, 1993). Cylindrical pellets of PLCG 75:25 have been implanted into the femoral medullary space of male albino rats by Cutright and colleagues. They observed bone and connective tissue infiltration into the pellet which became surrounded by bony tissue. After 220 days, the pellet had been replaced by bony tissue. Since no inflammatory cells were present at any time, the adjacent tissues were thought to show excellent tolerance to PLCG (Cutright *et al*, 1974). Visscher and co-workers have reported that, after injection of PLCG 50:50 microcapsules into the gastrocnemius muscle of male Charles River CD rats, the tissue reaction was minimal and only involved a sharply localised acute inflammatory response together with a macrophage foreign-body giant cell and connective tissue response which diminished as the polymer biodegraded (Visscher *et al*, 1988). PLCG 70:30 cylinders have been sub-cutaneously implanted into the backs of male adult Wistar rats with no evidence of the subsequent development of a foreign-body reaction for 4 weeks after implantation (Asano *et al*, 1989). After this time, the implant was surrounded by a histiocyte capsule which was itself surrounded by both granulation and fibrous tissue (Asano *et al*, 1989). Spenlehauer and colleagues have injected PLCG 75:25 into the hepatic portal vein of male Sprague-Dawley rats before excising the livers for histological examination (Spenlehauer *et al*, 1989). They defined three stages of inflammation caused by PLCG involving a) a primary subacute inflammatory response during which macrophages and small foreign-body giant cells surrounded the microspheres; b) a subsequent increase in this inflammatory response, associated with further infiltration of foreign-body giant cells and c) the disappearance of the inflammatory response once microsphere degradation was complete. In these studies, the rats showed good biotolerance to PLCG. PLCG 50:50 microspheres have also been injected into the striata of male Wistar rats to assess the tissue reaction to this polymer (Menei *et al*, 1993). No behavioural changes, neurological deficits

or systemic/local PLCG induced toxicity were observed and it was concluded that PLCG 50:50 was biocompatible with brain tissue (Menei *et al*, 1993). Yamaguchi and co-workers have subcutaneously injected microspheres composed of Medisorb® (PLCG 65:35) into the backs of female Sprague-Dawley rats and monitored necrotic and degenerative changes at the injection site over 150 days (Yamaguchi *et al*, 1993). They found that while there was a mild to moderate inflammatory response up to 30 days after injection which was accompanied by the infiltration of some foreign-body giant cells, the inflammation decreased over time while collagen deposition around the microspheres increased with time. Complete *in vivo* degradation of the microspheres occurred after 150 days and the study concluded that PLCG showed good biocompatibility (Yamaguchi *et al*, 1993).

The INVITTOX cytotoxicity data generated in the present work indicate that PLCG, in the form of unloaded microspheres, showed minimal *in vitro* cytotoxicity to cultured 3T3-L1 cells and these observations are consistent with the *in vivo* work reported in the literature. The innate biocompatibility of PLCG is not surprising since it is biodegraded to lactic acid and glycolic acid, both of which are indigenous to human tissue and are easily metabolised by the body to carbon dioxide and water. In addition, the polymer PLCG used in the present study was pure medical grade meaning that the concentrations of potentially toxic compounds including tin, heavy metals and acetone used in PLCG synthesis were present at very low levels. The cytotoxicity work also confirmed that any residual concentrations of light white mineral oil and SPAN 40, both of which are irritants, were present within the microspheres in acceptably low concentrations; the cellular cytotoxicity of 20% w/w antibiotic loaded microspheres must have been derived almost entirely from leached antibiotics. The adverse side effects of vancomycin, ciprofloxacin and rifampicin treatment regimens have been well documented. Vancomycin has the potential to cause nephrotoxicity, ototoxicity and Red man's syndrome while common adverse effects of ciprofloxacin therapy include nausea, vomiting, abdominal pain and dyspepsia along with dizziness, headaches and restlessness (Martindale, 1993; Remington's, 1993). Adverse side effects of rifampicin usage include gastrointestinal bleeding, gastritis, renal failure,

liver abnormalities and contact dermatitis (Martindale, 1993). The determination of ID_{50} and CPI index values allows these antibiotics to be put in a rank order of relative cytotoxicity (most toxic to least toxic): rifampicin > ciprofloxacin > vancomycin. This rank order is consistent with the relative MIC values shown for these antibiotics against bacteria associated with orthopaedic infections such as *Staphylococcus aureus*. The cellular cytotoxicity of vancomycin, rifampicin and ciprofloxacin measured in the INVITTOX assays may have been caused by the burst release of antibiotic from microspheres during the 24 hour elution period of 1 day (protocol 3b) and 7 day diffusion period (protocol 104). When administered topically *in vivo*, the cytotoxicity of the microsphere preparations would be expected to gradually decrease with the release of the encapsulated antibiotic reservoir due to minor antibiotic leakages into the systemic circulation and most would be used to kill bacteria in the joint space. The burst release of antibiotic would presumably act to eradicate bacteria in the immediate post-implantation period while the subsequent low-level, sustained antibiotic release would discourage further colonisation of the joint area by bacteria entering the wound in the blood. The biodegradation of PLCG would also ameliorate any extended inflammatory response to the topically administered microspheres. Hydroxypropylmethylcellulose (HPMC) gel was used as the suspending vehicle for the microspheres in the present work. The fact that HPMC has been utilised as a vehicle for the injection of PLCG 50:50 microspheres into the gastocnemius muscle of male Charles River CD rats (Visscher et al, 1988) and as an excipient in tablet formulations (in the form of HPMC acetate succinate in tablets containing amoxicillin trihydrate) (Hilton and Deasy, 1993) shows that HPMC is non-toxic. It is assumed that the systemic cytotoxicity of vancomycin, ciprofloxacin and rifampicin after topical administration to the joint space would be very limited since poor local scar tissue neovascularisation after THA would effectively limit antibiotic entering the systemic circulation. These topically applied antibiotics should therefore only act at the local level which would presumably cause only minor cellular damage and tissue disruption in the patient. In any case, low-level local antibiotic cytotoxicity would be outweighed by the benefit of suppressing and killing the bacteria associated with hip joint infections.

Chapter 7: General Discussion.

The present work has focused on characterising 20% w/w antibiotic loaded microspheres fabricated using poly (*dl*-lactide-co-glycolide) [PLCG] 75:25 and capable of the sustained *in vitro* release of vancomycin, ciprofloxacin and rifampicin at therapeutically significant concentrations into newborn calf serum and Hank's balanced salt solution during a 30 day incubation period at 37°C. It was found that microspheres with high, reproducible mean percentage yields (>75%) could be produced using the W/O emulsification with solvent evaporation technique. The mean encapsulation efficiency of vancomycin and ciprofloxacin loaded microspheres was $\geq 70\%$ while the encapsulation efficiency of rifampicin loaded microspheres was only $\geq 8\%$ because of partition of rifampicin into light white mineral oil during the fabrication process. Vancomycin loaded microspheres fabricated using an emulsification speed of 4,000 rpm were slightly larger in diameter (approximately 45 μm mean volume diameter) than unloaded microspheres (approximately 38 μm mean volume diameter), ciprofloxacin loaded microspheres (approximately 29 μm mean volume diameter) and rifampicin loaded microspheres (approximately 20 μm mean volume diameter). This may have been because of extensive antibiotic crystal deposition within the internal matrix of vancomycin loaded microspheres. Experiments using 20% w/w vancomycin loaded microspheres showed that microsphere mean volume diameter was inversely proportional to the emulsification speed employed during fabrication. The surfaces of freshly fabricated vancomycin loaded microspheres and ciprofloxacin loaded microspheres were covered in antibiotic crystals while the surfaces of rifampicin loaded microspheres and unloaded microspheres were smooth. Microsphere antibiotic crystal deposition was thought to be the result of vancomycin and ciprofloxacin remaining in suspension in acetonitrile during the fabrication process. The internal architecture of freshly fabricated unloaded and 20% w/w antibiotic loaded microspheres was composed of a macroporous matrix and a compressed outer layer both thought to have been created during solvent evaporation. The incorporation of 20% w/w antibiotic PLCG 75:25

microspheres into an aqueous hydroxypropylmethylcellulose gel vehicle significantly influenced the kinetics of antibiotic release into NCS at 37°C. There was an increase in the day to day concentration of ciprofloxacin and rifampicin released during the second phase, low-level sustained release phase. It was hypothesised that this was the result of extensive water penetration into the microspheres, facilitated by the hygroscopic nature of the gel, which was thought to cause the mobilisation of deep seated stores of antibiotic within the internal microsphere matrix. The rate of vancomycin, ciprofloxacin and rifampicin release from both dispersed and gel entrapped microspheres during incubation at 37°C was much faster into HBSS than into NCS. This was thought to be due to the greater solubility of these antibiotics in HBSS than in NCS. Kinetic analyses of the antibiotic release data confirmed that release from microspheres was diffusional although microsphere biodegradation may have caused a slight increase in the concentration of both ciprofloxacin and rifampicin released into NCS after approximately 27-30 days of microsphere incubation at 37°C. Microsphere biodegradation in terms of changes in weight average, number average and peak molecular mass was measured. Values for these parameters tended to decrease with the progression of incubation. Likewise, mass loss from microspheres together with microsphere glass transition temperature (Tg) both decreased with continued incubation. Changes in polymer glass transition temperature also highlighted a heterogeneous rate of lactide and glycolide biodegradation when correlated with measurements of molecular mass. Morphological changes, represented by an increase in the roughness of the microsphere surface and an increase in the size of the macropores within the internal matrix of the microspheres confirmed that the extent of PLGA biodegradation during incubation in NCS and HBSS over 30 days at 37°C was limited. The *in vitro* cytotoxicity of unloaded microspheres and 20% w/w antibiotic loaded microspheres when exposed to cultured 3T3-L1 cells indicated that very little toxic material was eluted from the polymer. The moderate cytotoxicity of 20% w/w antibiotic loaded microspheres, when compared to a positive control of silicone, was therefore due to the antibiotics. Cytotoxicity testing confirmed that rifampicin was the most cytotoxic antibiotic

followed by ciprofloxacin and vancomycin respectively. The data generated in the present work form a comprehensive report cataloguing the development of a novel biodegradable antibiotic delivery device for the treatment of orthopaedic infections and a novel and convenient way for its topical administration by injection in a aqueous gel vehicle during surgery. However, potential improvements to the reproducibility and accurate measurement of some physical properties of the microspheres such as encapsulation efficiency and particle size distribution are possible. Martinez and co-workers used spectrophotometry (320nm) to measure the efficiency of ciprofloxacin encapsulation within PLCG 75:25 and PLCG 50:50 microspheres after dissolving the microspheres in DCM and extracting the ciprofloxacin in a aqueous medium (Martinez *et al*, 1997). The authors describe an indirect method of measuring the encapsulation efficiency of ciprofloxacin that is, not a measure of biological activity but a measure of antibiotic concentration. Their method may be more accurate than the precipitation method used in the present work which is also an indirect measurement and involves the solubilisation of microspheres in a 50:50 v/v DCM/distilled water mixture and subsequent isolation and evaporation of the water phase, containing dissolved antibiotic, to facilitate the precipitation of antibiotic. This method relies on the total desiccation of material containing only a small concentration (0.01g/ml) of previously microencapsulated ciprofloxacin.

The accuracy of particle size analysis using the laser sizer in the present work may have been improved by sieving microspheres prior to sizing using laser diffraction. Microsphere size distribution profiles and light microscopy of droplets during the emulsification process indicated that there was a wide distribution in both the diameter of emulsion droplets generated during emulsification and the finished microspheres. A sieve shaker or an air-jet sieve could have been used to separate fractions of microspheres according to their size (usually expressed as percentage undersize) allowing the mean diameter of microspheres in each fraction to be subsequently determined using laser diffraction. Separation of microspheres into different size fractions might have provided another way of manipulating the release kinetics for each preparation.

Measurement of the concentration of each antibiotic released into both NCS and HBSS at 37°C in the release studies was achieved using a radial diffusion assay. Although this is a recognised and well characterised method for detecting aqueous concentrations of antibiotic, the process requires facilities for the storage and culture of bacteria as well as sterilisation equipment such as autoclaves and the establishment of aseptic techniques. Other researchers have used high pressure liquid chromatography (HPLC) to determine the concentration of both rifampine (a rifampicin analogue) and ciprofloxacin in serum (Riva *et al*, 1991; Joos *et al*, 1985). Results obtained using HPLC and radial diffusion assays (utilising *Sarcina lutea* and *Escherichia coli* respectively as the susceptible organism) correlated well. Although, in the present work, the radial diffusion assay acted as a reliable developmental tool for the measurement of antibiotic concentrations in NCS and HBSS, the use of HPLC as a technique for the routine measurement of antibiotic concentrations in NCS and HBSS could prove to be more sensitive to small concentrations of antibiotic. The sensitivity of the RDA used in the present work was 20µg/ml for vancomycin in NCS which was 10 x the MIC for *S. aureus*.

In the present work, studies involving the release of antibiotics into HBSS highlighted the fact that the rate of vancomycin, ciprofloxacin and rifampicin release from microspheres into HBSS at 37°C was faster than into NCS at 37°C. Newborn calf serum was utilised because it was an inexpensive surrogate serum and contains various quantities of enzymes, proteins and lipids also found *in vivo*. HBSS, on the other hand, is a well defined buffer system which provided a means of identifying the effect of simple chemical hydrolysis on polymer degradation. The basic chemical constituents of HBSS compared with NCS make HBSS a good medium to utilise as a quality control tool. On an industrial scale, to ensure that a batch of microspheres has been fabricated satisfactorily, that is to a required specification with the correct properties, a sample from the batch of microspheres is routinely tested prior to use for other experimental work and processing. To test the release kinetics of such a sample in the laboratory may take up to 30 days in NCS but, as

antibiotic is released faster during incubation in HBSS, the process would be achieved much more quickly using HBSS. As long as the actual profile of antibiotic release into HBSS from a sample of microspheres fits the expected profile of release into HBSS for that type of microsphere then the batch of microspheres can be passed as achieving specification. The fact that release into HBSS does not resemble an *in vivo* situation is not important since the test to specification is run to check the manufacturing technique only and therefore needs to be carried out quickly and accurately. In addition, HBSS is a fraction of the price of NCS.

A final possible improvement to the accuracy of the microsphere fabrication technique is to employ factoring when calculating the mass of antibiotic to be used in the encapsulation process. Factoring involves taking into account the potency and moisture content of the antibiotic in question. Therefore, although the concentration of each antibiotic equivalent to 20% w/w was used for all microsphere preparations in the present work, the actual biological activity of each encapsulated antibiotic would depend upon its potency and moisture content.

The potential efficacy of 20% w/w vancomycin, ciprofloxacin and rifampicin loaded microspheres for the delivery of antibiotics and the control of infection in the hip joint after total hip arthroplasty can now be addressed. The best way to do this is by comparison with presently used topically administered antibiotic prophylactic regimens in orthopaedic surgery such as antibiotic loaded poly (methyl methacrylate) cement blocks and beads. Wahlig and colleagues have used 'Palacos R' bone cement containing 0.5g and 1.0 g of gentamicin in 40 g of cement powder for prosthesis fixation during hip replacement surgery (Wahlig *et al* 1984). On the same day of the operation, a gentamicin concentration of approximately 47 $\mu\text{g/ml}$ (0.5g initial gentamicin loading) and 118 $\mu\text{g/ml}$ (1.0g initial gentamicin loading) was detected in the fluid collected from a drain placed adjacent to the artificial joint. On day one post-operative, the concentration of gentamicin in the fluid collected from this drain had decreased to 13 $\mu\text{g/ml}$ (0.5g initial gentamicin load) and

41 $\mu\text{g/ml}$ (1.0g initial gentamicin load). The highest post-operative serum gentamicin concentration (venous supply) was 2.4 $\mu\text{g/ml}$ and occurred two to three hours post-operatively (Wahlig *et al*, 1984). Garvin and associates have used gentamicin impregnated 'Palacos' bone cement during hip replacement surgery after deep periprosthetic infection (Garvin *et al*, 1994). Over an average follow-up period of 5.7 years, only 5% of the patients in the study had developed a recurrent hip joint infection (Garvin *et al*, 1994). On day one post-operative, an average local gentamicin concentration of 14.9 $\mu\text{g/ml}$ was detected compared with an average concentration of 2.1 $\mu\text{g/ml}$ after intravenous gentamicin administration (Garvin *et al*, 1994). In the same study, gentamicin loaded 'Palacos' beads were also employed to deliver high local concentrations of gentamicin to the hip joint in the interim period between prosthesis removal and re-implantation. These beads released 36 $\mu\text{g/ml}$ of gentamicin on day one post-operative (Garvin *et al*, 1994). After four weeks, the local concentration of gentamicin in the joint space released from the gentamicin impregnated bone cement used for prosthesis fixation was 5 $\mu\text{g/ml}$ compared with 22.3 $\mu\text{g/ml}$ from gentamicin impregnated bone cement beads after 6 weeks suggesting that the rate of antibiotic release was quicker from cement beads than from cement blocks (Garvin *et al*, 1994). The average serum concentration of gentamicin detected seven days after the initial administration of either gentamicin impregnated bone cement blocks or beads was only 0.01 μg (Garvin *et al*, 1994). Bunetel and co-workers have used 'Palacos' bone cement and 'Cerafix Genta R' bone cement containing gentamicin at a concentration of 8.9 mg/g of cement and 9.0 mg/g of cement respectively in hip replacement surgery (Bunetel *et al*, 1990). They concluded that the biphasic pattern of gentamicin release obtained from bone cement (a rapid release phase for up to four hours after implantation followed by a slow release phase for up to 72 hours after implantation) was bacteriologically important because the rapid initial release (25.1 $\mu\text{g/ml}$ for 'Cerafix Genta R' bone cement and 31.8 $\mu\text{g/ml}$ from 'Palacos' bone cement after 4 hours post-implantation) could kill bacteria contaminating the wound during surgery (Bunetel *et al*, 1990). Also, the concentration of gentamicin entering the systemic circulation after release from either type of bone cement into the joint space was low (0.12

$\mu\text{g/ml}$ - $0.14 \mu\text{g/ml}$ after 5 hours). Such a low level would avoid systemic antibiotic toxicity but would be insufficient to provide systemic antibacterial protection for the patient (Bunetel et al, 1990). A ten year survey of 1,688 total hip arthroplasty operations carried out between 1976 and 1978 revealed that the rate of infection after operations involving systemic antibiotic administration (either 1g of cloxacillin four times a day for 7-14 days; 1g of dicloxacillin four times a day for 9-11 days; or 0.65g of phenoxypenicillin four times a day for 10 days) was 1.6% whereas only 1.1% of hips became infected after treatment using gentamicin impregnated bone cement to fix the prosthesis (Josefsson and Kolmert, 1993). A nephrotoxic reaction also occurred in one patient after systemic antibiotic administration although this was not observed in patients treated with gentamicin impregnated bone cement (Josefsson and Kolmert, 1993). Henry and Galloway have indicated that the administration of antibiotics in PMMA bone cement is safer than systemic administration and avoids the allergic, nephrotoxic and ototoxic reactions sometimes associated with parenteral antibiotic administration (Henry and Galloway, 1995). Henry and Galloway have also reported that on day one post-operative the gentamicin concentration in the fluid around the hip arthroplasty after release from antibiotic impregnated bone cement beads and blocks was 17x and 7x greater respectively than that achieved by the parenteral administration of gentamicin (Henry and Galloway, 1995). Gentamicin and tobramycin are the two most commonly used antibiotics in PMMA beads although it has been suggested that vancomycin could also be a contender for routine use in PMMA bone cement beads as it possesses similar elution characteristics to tobramycin (Henry and Galloway, 1995). Brien and colleagues have compared the kinetics of tobramycin and vancomycin release from Palacos-R cement and Simplex cement used for prosthesis fixation in total hip arthroplasty (Brien *et al*, 1993). After 24 hours, a total cumulative concentration of tobramycin of approximately $50 \mu\text{g/ml}$ was detected in fluid drained from the wound after release from 'Palacos-R' bone cement (Brien *et al*, 1993). Approximately $5.5 \mu\text{g/ml}$ of vancomycin had been cumulatively released from 'Palacos-R' bone cement into drainage fluid 24 hours post-operative and this was considered to be sub-optimal (Brien *et al*, 1993).

For the systemic treatment of infections, both ciprofloxacin and rifampicin can be administered by the oral route whereas vancomycin has to be administered intravenously due to its low oral bioavailability (Martindale, 1993). However, the topical administration of an antibiotic to the hip joint, especially when the antibiotic is encapsulated within bone cement beads placed inside the wound at the time of surgery, elevates the potential local concentration of the antibiotic compared with its systemic administration (Henry and Galloway, 1995). This suggests that the topical administration of vancomycin, ciprofloxacin and rifampicin entrapped within biodegradable microspheres suspended in a gel vehicle could be a more effective way of achieving high local antibiotic concentrations within the joint space and a useful means of antibiotic administration to treat post-operative hip prosthesis infections both after one-stage prosthesis replacement surgery and in the interim period between prosthesis removal and re-implantation during a two-stage procedure.

The present work has shown that vancomycin, ciprofloxacin and rifampicin can be released *in vitro* from dispersed and HPMC-entrapped 20% w/w antibiotic loaded PLCG 75:25 microspheres for a period of up to 30 days of incubation in NCS at 37°C. Typical day to day concentrations of the antibiotic released were significantly in excess of those required to kill common pathogens of open wounds such as *Staphylococcus aureus* (MIC <2.0 µg/ml) and *Pseudomonas aeruginosa* (MIC <2.0 µg/ml) and could therefore potentially suppress opportunistic infections caused by these pathogens. The pattern of antibiotic release into NCS at 37°C was characteristically biphasic and composed of an initial burst phase of antibiotic release which lasted for approximately 2-6 days depending on the antibiotic being released. This burst release would be useful for the possible prevention of infection caused by bacteria entering the joint space at the time of surgery. The burst phase was followed by a sustained period of low-level antibiotic release typically lasting for 24-28 days. The early depletion of vancomycin from within 20% w/w antibiotic loaded microspheres as a result of the initial burst release of a relatively large concentration

of vancomycin curtailed the duration of the second phase of vancomycin release into NCS and HBSS at 37°C.

Dispersed microspheres released the largest day to day concentration of each antibiotic during day one of incubation while microspheres entrapped within hydroxypropylmethylcellulose (HPMC) gel (10%w/v) tended to release the largest day to day concentration of each antibiotic during day two of incubation. Also, the primary burst release of antibiotic from gel entrapped 20% w/w loaded microspheres tended to be longer in duration than that released from similarly loaded dispersed microspheres during incubation in NCS at 37°C. The cumulative release of vancomycin into NCS at 37°C during the initial burst phase was significantly greater than the initial burst phase release shown by both ciprofloxacin and rifampicin into NCS at 37°C. The day to day concentrations of vancomycin, ciprofloxacin and rifampicin released from 100 mg of 20% w/w antibiotic loaded microspheres in the present work were in the mg/ml range, while other workers have shown that the day to day concentrations of gentamicin released *in vivo* from antibiotic impregnated bone cement (blocks and beads) were in the $\mu\text{g/ml}$ range (Garvin *et al*, 1994; Bunetel *et al*, 1990). This suggests that microspheres could be a more effective means of eradicating infectious bacteria in the joint space than antibiotic impregnated bone cement blocks and beads.

Measurement of *in vitro* antibiotic release in the present work has shown that HPMC gel entrapped 20% w/w antibiotic loaded microspheres can release therapeutically effective concentrations of antibiotic over a 30 day incubation period in a physiological medium (NCS). In order to establish the potential of 20% w/w antibiotic loaded PLCG 75:25 microspheres as a replacement for traditional antibiotic regimens employed during THA operations (systemic, bone cement block or bone cement bead administration of antibiotics) and in order to secure future regulatory approval (Medicines Control Agency and FDA) for the use of the microsphere in gel preparation as a novel antibiotic delivery device in man, *in vitro* - *in vivo* (IVIV) correlation studies will need to be developed and

initiated. Powers and associates have developed a rat model for total hip arthroplasty although they state that sheep, goats, dogs, geese, ponies and baboons have all been used as animal models for THA (Powers *et al*, 1995). Sprague-Dawley rats were chosen in favour of dogs (traditionally popular for functional THA studies) for full THA procedures involving a specially constructed tri-lock prosthesis because of the relatively inexpensive husbandry, ease of analysis of joint function (for example treadmill exercises), their age-related bone loss, the considerable physiological, immunological and toxicological data available and ethical considerations (Powers *et al*, 1995). Therefore, it follows that if the hip joint of a rat was deliberately infected with common open wound pathogens (such as *Pseudomonas aeruginosa* or *Staphylococcus aureus*) at the time of surgery and, following confirmation that an infection had developed in the joint space (by culturing samples of tissue, tissue fluid and blood), gel entrapped 20% w/w antibiotic loaded microspheres could be topically administered to the joint space, the effectiveness of infection suppression by delivered antibiotics could be monitored. Kaiser and colleagues have used a similar idea to develop and monitor a low-inoculum model of surgical wound infection using Guinea pigs (Kaiser *et al*, 1992). They injected a solution of PBS containing *Staphylococcus aureus* (previously recovered from a deep wound infection) into the intermuscular space in the dorsum area. Ampicillin (100mg/kg) and cefazolin (100mg/kg) were administered by subcutaneous injection into the dorsal fat pad under the scruff of the neck 15 minutes before the inoculation of *S. aureus*. After four days, the Guinea pigs were sacrificed and punch biopsies of the lesions taken. Also, the serum antimicrobial concentration was measured using bioassay and the estimated number of bacteria in an inoculum giving a 50% probability of producing an infection (ID_{50}) calculated after taking into account the *Staphylococcal* strain and the treatment regimen used (Kaiser *et al*, 1992). Kaiser and co-workers maintained that the Guinea pig model of surgical wound infection could provide a pre-clinical trial screening tool and that ID_{50} values indicated the extent of strain virulence and antibiotic efficacy (Kaiser *et al*, 1992). Therefore, future utilisation of this method for use with 20% w/w antibiotic loaded microspheres delivered topically to the site of infection in a gel vehicle could provide the means for establishing an IVIV

correlation of the therapeutic effectiveness of antibiotic release from 20% w/w antibiotic loaded PLCG 75:25 microspheres. *in vitro* studies showed that only 0.3% of the drug was released in these devices (Brady *et al.*, 1973). In the present work, the release of vancomycin, ciprofloxacin and rifampicin from dispersed and HPMC gel entrapped 20% w/w loaded microspheres into NCS at 37°C may have been influenced by PLCG 75:25 biodegradation as indicated by a dumping of ciprofloxacin and rifampicin from dispersed microspheres towards the end of the 30 day incubation period. Decreases in polymer molecular weight (weight average, number average and peak values) and the decrease in polymer polydispersity, fluctuations in polymer glass transition temperature, polymer mass loss, and morphological changes to microspheres confirmed that limited biodegradation of PLCG 75:25 had occurred. In support of this observation, Zhang and colleagues have stated that, for diffusion controlled drug release from polymer microparticulates, the rate of polymer biodegradation has to be slow so as not to effect the diffusion coefficient during release (Zhang *et al.*, 1993). First order and square-root time plots of the release data generated in the present studies have confirmed that the release of vancomycin, ciprofloxacin and rifampicin from 20% w/w loaded PLCG 75:25 microspheres was diffusional. There is some evidence in the literature to suggest that the rate of *in vivo* polyester biodegradation might be faster than the rate of *in vitro* biodegradation due to possible polymer breakdown by proteins, enzymes and lipids (Williams, 1982). On this basis, although the extent of biodegradation does not significantly affect the kinetics of antibiotic release from 20% w/w loaded PLCG 75:25 microspheres during incubation *in vitro*, it does not hold that the same will apply to microspheres implanted *in vivo* where they will be exposed to a physiological environment. Future studies will therefore need to include *in vivo* microsphere biodegradation studies. Brady and associates have implanted blocks of ¹⁴C poly (*dl*-lactic acid) in the abdominal wall of Sprague-Dawley rats in order to measure the rate of PLA biodegradation *in vivo* (Brady *et al.*, 1973). The collection of urine and faeces provided a means of measuring the activity of ¹⁴C expelled by the body and allowed a calculation to be made which indicated that the majority of ¹⁴C activity was expelled via respiration. The

liver, kidneys and lungs were also removed from each rat after sacrifice in order to measure the activity of ^{14}C after incorporation into tissues which showed that only 0.3% of the activity lost by the implant was found in these tissues (Brady *et al*, 1973). In the same study, histological observation of the implant site using light microscopy confirmed that the implant was well tolerated by the host tissue (Brady *et al*, 1973). Wakiyama and co-workers have measured the *in vivo* rate of poly (lactic acid) microsphere biodegradation after the dorsal subcutaneous implantation of microspheres into Guinea pigs. 72 hours and 120 hours after implantation, the microspheres were removed, dried and examined using scanning E/M. The disintegration rate of microspheres *in vivo* (total biodegradation after 72 hours) was much faster than *in vitro* (only slight biodegradation after 120 hours) (Wakiyama *et al*, 1982). Kenley and co-workers have implanted cylindrical rods of PLCG 50:50 subcutaneously into the nape of the neck of rats and monitored polymer molecular weight loss using size-exclusion chromatography and weight loss (indicative of polymer erosion) after drying the samples to a constant weight. Their studies showed that polymer molecular mass decreased before the onset of polymer erosion and that significant polymer erosion had occurred only three weeks after implantation (Kenley *et al*, 1987). Similar studies could also be carried out using the unloaded and 20% w/w antibiotic loaded microspheres generated in the present work in order to determine the rate of PLCG 75:25 biodegradation *in vivo* perhaps with concomitant IVIV correlation studies. The potential biodegradability of PLCG in physiological environments is an added complication when trying to establish reproducible and therapeutically useful *in vivo* antibiotic release kinetics. Studies by Miller and colleagues on the biodegradation of PLCG 75:25 gave it an approximate *in vivo* half life of two weeks (0.6 months) after implantation in the tibia or abdominal wall of Sprague-Dawley rats (Miller *et al*, 1977). On this basis, 6 months after *in vivo* implantation of the 20% w/w antibiotic loaded microspheres generated in the present work (containing 80% w/w PLCG 75:25), 99.99% of the polymer would have been biodegraded. Hence, compared with the use of large amounts of poly (methyl methacrylate) bone cement blocks or beads, both of which are known to harbour and support bacterial growth (Zhang *et al*, 1993), the use of PLCG 75:25 microspheres loaded

with antibiotic would be extremely advantageous in suppressing both secondary infections involving the prosthesis and avoiding surgery for implant retrieval.

In the present work, the *in vitro* cytotoxicity of 20% w/w antibiotic loaded PLCG 75:25 microspheres when exposed to cultured 3T3-L1 cells was moderate (compared with a positive control of silicone) according to the Cell Proliferation Inhibition index (INVITTOX Protocol 104) and toxicity was as a result of the release of the antibiotics themselves rather than to the potential cytotoxicity of PLCG 75:25 and any residual fabrication components such as DCM, petroleum ether, SPAN 40 and light white mineral oil associated with the microspheres. Initially, there was some concern that the extent of the *in vitro* burst release of antibiotic from the dispersed and gel entrapped 20% w/w loaded PLCG 75:25 microspheres might generate a measure of systemic and local cytotoxicity when used *in vivo*. However, *in vivo* studies conducted by Garvin *et al* (1994), Josefsson and Kolmert (1993), Bunetel *et al* (1990) and Wahlig *et al* (1984) have confirmed that systemic toxicity after topical antibiotic administration to the hip joint did not occur, presumably because there is poor vascularisation in infected hip joints (Henry and Galloway, 1995). The moderate *in vitro* toxicity of vancomycin, ciprofloxacin and rifampicin demonstrated in the present work suggests that a similar degree of cytotoxicity might occur *in vivo* but will need to be accepted as a necessary consequence of their use especially when compared with the well documented toxic effects such as nephrotoxicity, ototoxicity and Red man's syndrome (vancomycin) dizziness, headaches and restlessness (ciprofloxacin) and renal failure together with liver abnormalities (rifampicin) (Martindale, 1993; Remington's, 1993) of these antibiotics when administered systemically at high concentrations (required for the accumulation of therapeutically useful antibiotic concentrations in the hip joint). It has also been shown that gentamicin may exhibit *in vivo* cellular cytotoxicity when present in high concentrations and can increase the phase one tissue response to an implant containing gentamicin (Anderson and Shive, 1997). In the future, the use of an animal model for *in vivo* cytotoxicity / biocompatibility studies involving unloaded and 20% w/w vancomycin, ciprofloxacin or rifampicin loaded 75:25

PLCG microspheres would prove useful in extending the *in vitro* cytotoxicity data generated in the present work. (1973). Biocompatibility studies have also been conducted

in which microspheres composed of PLCG (50:50)

The tissue response to implanted microspheres, indicating the biocompatibility of the microspheres, has been categorised into three distinct phases (Anderson and Shive, 1997). Phase one, the initiation of the acute and chronic immune responses, occurs within the first two weeks of implantation. Over this period, polymorphonuclear leukocytes, lymphocytes, plasma cells and monocytes accumulate at the implant site. After a few days, monocytes are the primary cell type at the site of implantation. These monocytes soon differentiate into macrophages (tissue histiocytes) which signals the start of the second phase of the tissue response to the implanted microspheres. During phase two of the tissue reaction, the differentiation of monocytes into macrophages continues. In turn, some macrophages fuse together to form foreign body giant cells which collect, with macrophages, at the microsphere/tissue interface. A fibrous capsule also begins to develop around the implanted microspheres during the second phase of the tissue response. Infiltration of fibroblasts into this fibrous capsule allows the deposition of collagen around and between the microspheres to occur. Neoangiogenesis (the formation of blood capillaries) within the fibrous capsule is also initiated at this time. The length of time given over to the second phase of the tissue response to implanted microspheres is dependent upon the rate of biodegradation of the polymer from which the microspheres were fabricated such that the second phase of the tissue response to 50:50 PLCG microspheres is approximately 60 days whereas the second phase of the tissue response to PLA microspheres lasts for some 400+ days. As the microspheres biodegrade, they break down into particles which are phagocytosed by macrophages. This is the third phase of the tissue reaction to implanted microspheres and culminates in fibroblasts and neovascularisation filling the volume within the fibrous capsule left by degraded microspheres. The time period for the third phase of the tissue reaction to implanted microspheres is also dependent on the rate of microsphere biodegradation and can last anywhere from a few weeks to a few months (Anderson and Shive, 1997). Histological studies after the

implantation of ^{14}C PLA in rats has confirmed the good tissue tolerance of rats to polyesters *in vivo* (Brady *et al*, 1973). Biocompatibility studies have also been conducted by Yamaguchi and colleagues in which microspheres composed of PLCG 65:35 (Medisorb®) have been injected into the right and left side of the back of female Sprague-Dawley rats. These animals were subsequently killed at 30, 45, 60, 75, 90, 120 or 150 days after microsphere implantation and gross examination and microscopic analysis of the implantation sites was achieved by removing and fixing the tissue from the implantation sites, sectioning and subsequently embedding the sections in paraffin (Yamaguchi *et al*, 1993). Each section was then stained with haematoxylin and eosin to determine cellularity and the number of cells at the tissue/implant interface which showed that macrophages were the predominant cells in the early and later stages after implantation while fibroblasts became the predominant cell type between 60 and 75 days after implantation (Yamaguchi *et al*, 1993). Masson's Tri-chrome and Picro-Sirius Red stain was also used to show fibrous capsule formation and collagen deposition and alignment which, 150 days after implantation, was comparable with intact tissue (Yamaguchi *et al*, 1993). Evaluation of the tissue reaction of the implant site was also assessed by examining the degeneration and necrotic changes of the tissues and no changes were seen after 150 days. Inflammation of the immediate area surrounding the implant was mild to moderate up to 120 days after implantation and then minimal up to 150 days after implantation. Foreign body giant cell formation and minimal to moderate foreign body giant cell infiltration was noted (Yamaguchi *et al*, 1993).

In conclusion, there is scope in the present work for the use of different analytical methods for antibiotic assays and particle sizing in order to optimise the measurement of microsphere physical characteristics increase the sensitivity of the antibiotic detection technique. Future preparative work should be geared towards the use of both Pharmacopoeia approved equipment (such as a United States Pharmacopoeia dissolution bath) and materials so as to meet industrial standards and help secure a patent for the delivery device. Hopefully, this could facilitate an academic-industrial collaboration

leading to further development and refinement of the device for eventual commercial exploitation. Certainly the development of an *in vitro-in vivo* correlation protocol will confirm the potential usefulness of antibiotic loaded microspheres for the treatment of infections in hip replacements and ensure that *in vitro* release kinetics reliably reflect *in vivo* release kinetics. Providing these criteria are fulfilled, 20% w/w antibiotic loaded PLCG 75:25 microspheres will certainly have a future role to play in providing effective antibacterial prophylaxis after hip replacement surgery and the suppression of existing infection in the treatment of open fractures and the replacement of hip prostheses. A combined preparation of microspheres containing vancomycin ciprofloxacin and rifampicin will also serve to widen the spectrum of antibacterial activity and suppress the emergence of antibiotic resistant bacterial strains thereby superseding gentamicin impregnated PMMA bone cement as the most effective treatment for orthopaedic wound infections.

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