BIOCOMPATIBILITY TESTING OF RESORBABLE MATERIALS USING IMPROVED *IN-VITRO* TECHNIQUES

by

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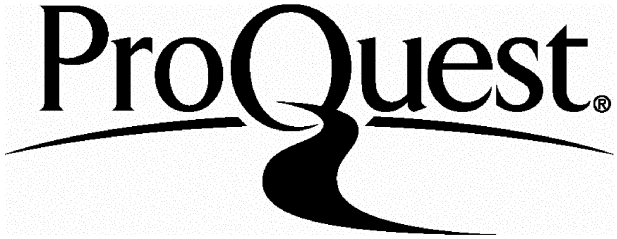
Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science at the University of London.

June 1997



Acknowledgement

This thesis is dedicated to my parents

I would like to thank Professor Sandra Downes for all her help and support throughout this study and being very patient with me. I would also like to thank the Dept of Human Morphology at the University of Nottingham.

Special thanks for help above and beyond the call of duty go to:

Mike Kayser for teaching me everything about microscopy, electron microscopy and photography and giving me lots of advise and help.
Dr Sanjukta Deb for starting me out on degradable polymers and Dr Mary Anstice for introducing me to the joys of gel permeation chromatography (GPC) and being a big help with a subject I knew nothing about. A big thankyou also to Paul Clarke at Viscotek for his help with the GPC equipment.
Padmini Sarathachandra my pal, for helping me whenever I was stuck which was quite often.
Dr Lucy Di Silvio for proof reading the thesis and giving me helpful hints during the writing of the thesis and sharing the office with me without going potty.
Mrs Caroline Clifford for proof reading the thesis without whose input the thesis would have been in any other language but English.
Dept of Morbid Anatomy and in particular Dr Ann Sandison for putting up with me and giving me time off to finish writing up. Professor Yousuf Ali, Dr David Lee, Professor George Bently and everyone at the Institute of Orthopaedics who has put up with me during the last few years and let me be part of the team.

Finally I would like to thank my long suffering husband who has helped and supported me throughout and without whose help I definitely would not have finished writing up.

Abstract

Interest in degradable polymer systems for use in various biomedical applications has been increasing since their development in the 1960s. The first polymer to be used was polyglycolic acid, marketed under the trade name Dexon, as a resorbable suture. Although the success of resorbable polymers has been mainly in the form of sutures and small pins, their potential use as materials for bone fixation, bone regeneration and drug delivery vehicles is vast. These materials have obvious advantages in that retrieval of the implant can be avoided causing minimal inconvenience to the patient. Extensive *in-vivo* biocompatibility testing has been performed on a range of resorbable polymers but there is a lack of information available on events occurring at the cellular level specially during long term implantation. This can be investigated in depth using *in-vitro* assays and utilising methods which mimic the long term *in-vivo* degradation of polymers.

The aim of this study was to determine the short and long term biocompatibility of degradable polymers using *in-vitro* cell culture methods. The morphology and proliferation of osteoblast-like cells and monocyte/macrophages on the polymer surfaces was investigated by light microscopy, electron microscopy and biochemical assays. Polymer degradation by enzymes and other degradation methods was investigated using gel permeation chromatography and the biocompatibility of polymers at stages of degradation was studied. The effect of the acidic pH caused by the monomers and the monomers themselves on the viability of osteoblast-like cells and monocyte/macrophages was studied. Evidence for HOS cells undergoing apoptosis was investigated by transmission electron microscopy and by utilising stains specific for apoptosis when cultured in the presence of monomers.

This study demonstrated that the accelerated degradation of polymers by heat and gamma irradiation provides a good method for obtaining polymers for "long-term" biocompatibility testing. Enzyme solutions also influenced the degradation of the polymers in particular the polymer surfaces. The morphology and proliferation of osteoblast-like cells varied on the different polymer surfaces depending on surface structure, crystallinity and the release of degradation products; and in addition the presence of monomers caused a decrease in the mitochondrial activity of both the cell types tested. The monocyte/macrophages also had varying morphologies on the different polymers and were stimulated by some of the polymers to a greater extent. By using the *in-vitro* methods described the difficulties associated in determining the biocompatibility of many resorbable materials can be overcome.

Contents

Abbreviations

APC	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
MCF	Macrophage Chemotactic Factor
LIF	Lymphocyte Inhibitory Factor
MIF	Macrophage Inhibiting Factor
PDMS	Polydimethylsiloxane
^{3}H-Thymidine	Tritiated thymidine
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
CPMs	Counts Per Minute
D3-HB	D3-hydroxybutyric acid
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ECACC	European Collection of Animal and Cell Cultures
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Foetal Calf Serum
GA	Glycolic Acid
GPC	Gel Permeation Chromatography
HMDS	Hexamethyldisilazane
HOS	Human Osteosarcoma (TE85) cell line
LA	Lactic acid
Mn	Number average molecular weight
mosm	milliosmoles
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide
Mw	Weight average molecular weight
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NGA	Neutralised Glycolic acid
NLA	Neutralised Lactic acid
NNGA	Non-neutralised Glycolic acid
NNLA	Non-neutralised Lactic acid
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone

PDLA	Poly D-Lactic acid
PG910	Polyglactin 910 - (90% PLA:10% PGA copolymer)
PGA	Polyglycolic acid
PHB	Polyhydroxybutyrate
PHB-PHV	Polyhydroxybutyrate-Polyhydroxyvalerate copolymer
PLA	Polylactic acid
PLLA	Poly L-Lactic acid
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoroethylene
SEM	Scanning Electron Microscopy
SR-PGA	Self Reinforced - Poly glycolic acid
TCA	Trichloroacetic acid
TCP	Tissue Culture Plastic
TEM	Transmission Electron Microscopy
THF	Tetrahydrofuran
THP-1	Monocyte/Macrophage cell line
TK-HSD	Tukey Kramer-Honestly Significantly Different test
TNF	Tumour Necrosis Factor
UV	Ultraviolet light

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ELGA, High Wycombe, Bucks, UK
Johnson and Johnson (J & J), (Orthopaedics R&D), Raynham, MA, USA.
Life technologies, Paisley, UK
Marlborough Biopolymers, Stockton-on-Tees, UK.
Merck, Poole, UK
Millipore, Watford, Herts, UK
Philip Harris Scientific, London, UK
Sigma, Poole, UK

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CHAPTER I

General Introduction

Biomaterials have been in use for many centuries but it is only in the last three decades that their use has intensified and their use for novel clinical applications has increased. A biomaterial is any material of biological or chemical origin which can be used for repairing or reconstructing tissues of the body. Biomaterials generally fall into two main classes, degradable and non-degradable. Non-degradable materials consist of various metals, ceramics and cements. They are used for applications that require high mechanical strength and long term stability. The most common uses of non-degradable materials include hip replacements and vascular prostheses. Synthetic degradable materials, however, are relatively new and their clinical use, so far, has been restricted to materials for drug delivery and as sutures. One of the first degradable materials used in suture form was catgut, a collagen based material which is still in use today. There were, however, problems encountered with catgut such as non-uniformity in its mechanical properties and rates of degradation. The material also induced inflammatory responses due to its sheep origin (Albertsson and Ljungquist, 1981) this led to the development of synthetic degradable polymers which did not have the problems associated with materials of animal origin. There is now interest in these materials for fracture fixation and tissue regeneration which has been brought about by the development of polymers that can be manufactured to produce devices with high mechanical strength and "controlled" degradation characteristics.

Polymers are composed of molecules in long sequences which are linked to each other by covalent bonds. The long sequences are also termed macromolecules which can be linear or branched (Young and Lovell, 1991). The branched polymers are basically linear chains which have side chains and the degree of branching can be simple or complex depending on the length of the side chains and the degree of branching. As well as linear and branched polymers, there are network polymers which are three-dimensional structures in which chains are connected to other chains. These are also termed crosslinked polymers and are characterised by the degree of crosslinking. Macromolecules are formed by linking monomer molecules via the process of polymerisation where a monomer is the basic component of the polymer. Polymers can be homopolymers or copolymers. A homopolymer is a polymer made up from only one type of monomer whereas a copolymer is made up of more than one type of monomer. An example of a homopolymer is polylactic acid (PLA), which is made from the polymerisation of lactide and the final degradation product of this polymer is lactic acid, the monomer component. Several copolymers of polylactic acid can be formed with polyglycolic acid (PGA) and one of these is polyglactin which is 90% PLA and 10% PGA; the final degradation products of this copolymer are lactic acid and glycolic acid. The properties of the various degradable polymers are discussed in greater depth later in this chapter.

Biocompatibility testing: *in-vitro* and *in-vivo*

The definition of biocompatibility as it currently stands is "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1987). This definition came about when materials were developed that were required to have specific functions as opposed to being totally inert in the body. Biocompatibility assessment is essential for pre-clinical testing of materials intended for use as implantable devices. The testing procedures available for the assessment of biocompatibility should give quantitative data using appropriate cell types. Assessment of biodegradable polymers, however, is complex due to the degradation of the materials during testing and the need to artificially age the materials in order to assess longer term effects. Due to the wide variety of methods for material formulation which have different molecular weights the biocompatibility of many resorbable materials in the long term remains unclear. Kirkpatrick *et al* (1997) in a review states that "combinative techniques" involving morphological analysis and molecular biology present a useful set of methods for the biocompatibility testing of materials.

Biocompatible in the *in-vitro* situation means that the material or any leachable products from it do not cause cell death or impair cellular functions. Biomaterials used *in-vivo* should be biocompatible in both the short term and the long term during degradation of the material and the release of degradation products. These include the bioresorbable materials, and bioactive materials. For example, a material that is intended for use as a drug delivery vehicle will require different properties to one that will be used for tissue replacement. A biomaterial designed for hip replacement will require high mechanical strength, have low corrosion and degradation in addition to having good bone induction, protein adsorption and cell adhesive capabilities. However a material used for use for vascular reconstruction will need to have good stability, low degradation and low protein and cell adsorption properties.

In determining the suitability of a biomaterial for a specific application the material and site of implantation play a major role. Degradable biomaterials are constantly changing with time as the biomaterial undergoes bulk and surface degradation which leads to changes in the environment around the material. Non degradable materials also undergo changes in their chemical and structural nature but this change is not as dramatic and the time involved for physical and chemical changes to occur is longer. Appropriate biocompatibility testing is an essential process in the development of implantable devices. A material must be proved "safe" before it can be implanted into the body.

The Medical Device Directive states that a material to be used for medical devices should comply with toxicity tests (Haustveit *et al* 1984). The International Organisation for

Standardisation (ISO) is a world wide federation of national standards bodies. The ISO 10993 consists of a number of parts under the general title of "Biological evaluation of medical devices". The international standard ISO 10993-5:1992(E), tests for *in-vitro* cytotoxicity defines a series of guidelines that can help in selection of the most appropriate test for biocompatibility screening. The three categories are extract tests, direct contact and indirect contact tests (Wilsnack *et al* 1973). The other directives of ISO 10993 deal with other aspects of testing such as *in-vivo* testing, which include systemic injection tests, intracutaneous tests and the implantation test in animal models.

The definition of biocompatibility as defined by Williams is widely accepted as the general definition which is applied to all materials used for implantation purposes. However the designation of "biocompatibility" for each current application and newly developing applications needs to be determined and test methods need to be standardised. This is inevitably a long and difficult task, but one that is necessary, as currently a whole range of tests are performed which cannot be compared due to the differences in the methodologies used. It is first necessary to determine what the requirements of each medical device are and then accordingly choose a material that will fulfil the criteria. Applications generally fall into the headings; dental, orthopaedic, vascular implants, drug delivery, transplantation, nerve regeneration, and soft tissue implants. A major requirement for all these applications is that the materials must be non-immunogenic, that is they should not induce gross inflammatory or immune responses.

There is evidence, to suggest that toxicity at the cellular level is not easy to detect on experimental animals (Guess *et al* 1967). They stated that "subtle toxicity" which developed slowly at the cellular level was difficult to demonstrate in animals. It could, however, be detected by immunological studies using tissue culture models as these allow the investigation of single effects without interferences from the whole immune system.

Adverse responses *in-vivo* include decreased cell viability and proliferation of cells around the site of implantation or any reaction not normal for that tissue. An inflammatory reaction can also occur due to the influx of inflammatory cells to the area. Although some inflammation is necessary for the wound healing process to proceed normally, if this inflammatory reaction continues it can lead to chronic inflammation and granuloma. Biocompatibility testing must address all of these parameters using both *in-vivo* and *in-vitro* tests.

In-vivo testing usually involves the implantation of a test device (which has been sterilised by an appropriate method) into an animal model. Such studies yield information on the long term biocompatibility of materials and the effect of the material on the immune system. The problems associated with *in-vivo* biocompatibility testing are usually due to

the difficulty in quantifying specific cellular and biological events. However several methods have been developed which aim to isolate cell-biomaterial interactions *in-vivo* one of which is the cage implant system described by Marchant *et al* (1983). This allowed for the evaluation of the effect of cellular and humoral components of the exudate which surround polymeric materials following implantation. In this method, the biomaterial was placed inside a stainless steel cylindrical cage which was then implanted subcutaneously into a rat model. Aliquots of the exudate surrounding the implant were removed using a syringe and analysed. The types of cells present in the sample can then be identified.

Polymorphonuclear leukocytes (PMNs) and macrophages become phagocytic when cellular debris or foreign particles are present. When phagocytosis is initiated, the cell becomes metabolically active and there is a respiratory burst resulting in the production of hydrogen peroxide and oxygen free radicals and anions. These free radicals are effective in killing many types of bacteria and have been linked with initiating the degradation of some polymers. The role of the macrophage in the inflammatory process is very important because it is the longest surviving and most active of the inflammatory cells.

Although *in-vivo* testing cannot be completely replace *in-vitro* tests steps are being taken to move away from animal models through the development of better *in-vitro* tests. *In-vivo* testing is also more expensive, lacks control due to animal and species differences and problems are frequently encountered with obtaining ethical permission to carry out tests.

In-vitro biocompatibility testing involves studying the behaviour of various cell types in response to a particular test agent. The simplest of these can be carried out by monitoring cell viability and proliferation when in contact with a biomaterial. Cell viability can be monitored by the incorporation of various dyes such as trypan blue and erythromycin red which are exclusion or inclusion dyes. Trypan blue stains dying cells blue and erythromycin red stains non viable cells pink. These are tests which can give rapid estimations of the number of viable cells present. Coulter counters and cell sorters utilising fluorescent dyes can be used to distinguish dead cells from live ones to give a more accurate cell count.

In-vitro testing using cell culture methods has a number of advantages over *in-vivo* testing, in that the response of a variety of cells can be compared and evaluated over a shorter time period in a reproducible and controlled manner as compared to *in-vivo* tests. *In-vitro* testing is usually carried out before *in-vivo* testing and involves the culture of appropriate cell types on the material concerned.

The "appropriate" cell type is determined according to the application of the material in question. If the device is designed for orthopaedic fixation osteoblasts will usually be cultured on the surface and if the material is going to be used for vascular reconstruction blood compatibility will be investigated. Different material characteristics are needed depending on the final application. For the two applications the material is expected to behave differently; for example, in orthopaedic fixation, the material will need to have good protein adsorption characteristics to aid cell adhesion and proliferation, while for the blood contact material it needs to have low protein adsorption and low cell attachment.

Although the time points involved for *in-vitro* testing are short as compared to *in-vivo* testing, *in-vitro* testing does yield information on the cellular morphology and retention of phenotype of the individual cells. *In-vivo* testing is usually long term and can last several years. The use of *in-vitro* and methods which yield polymers that have been artificially degraded the time periods involved for *in-vivo* testing can be significantly reduced. Methods such as storage of polymers at high temperature, and in enzyme solutions which accelerate their degradation compared to *in-vivo* are termed artificial degradation. This has been demonstrated by several groups who have artificially degraded polymers before implantation into animals (Bergsma, 1995). In this study PLLA was predegraded at elevated temperatures and it was found that the follow-up times could be reduced; the results were comparable with the *in-vivo* findings which had not utilised pre-degraded particles. Long term biocompatibility testing is particularly important for degradable materials which are constantly changing their chemical and physical structures and releasing degradation products which may be detrimental to the cells adjacent to the implant.

Before carrying out *in-vitro* testing it is important to determine the cell type(s) that will be used for the study. It is important to use an appropriate cell type for that particular application. For bone implant materials the cells in contact with the device will be osteoblasts, osteocytes and osteoclasts. For blood contact materials none of these cells will be in contact with the material in the long term. It is therefore important to use cells that are most representative of those encountered in the *in-vivo* situation. Primary cells are a good choice but obtaining sufficient cells to carry out large numbers of tests can be problematic as well as obtaining normal human tissue for a particular cell type. Cell lines are therefore more commonly used, and sometimes more useful as they can replicate faster and have a well defined phenotype. For other devices fibroblasts, epithelial cells and hepatocytes can be used while studies of the immune response can utilise monocyte/macrophages and other cells of the immune system. *In-vitro* testing allows the standardisation of test methods and reduces the need for large numbers of experimental animals. It also allows qualitative determination of signs of cell damage, detected by light or electron microscopy.

Although quantitative methods are valuable for direct comparisons for biocompatibility testing, qualitative analysis of cells on materials is crucial for determining the morphology and behaviour of cells. Differences in the morphology of various cell types can be observed and cytotoxic effects which lead to dramatic changes in morphology of the cells can be examined at the light microscope level or in more detail at the electron microscope level. Microscopy is a vital and often underused tool in determining the biocompatibility of biomaterials. Light microscopy allows any damage occurring to the nucleus and cytoplasm to be viewed. The cells can be lysed due to damage caused by high concentrations of degradation products or due to the acidic pH. Scanning electron microscopy in combination with light microscopy can give valuable information on the morphology of the cells on the polymer surfaces. Most cells have a characteristic shape but this can be influenced by the substrate to which they are attached. Transmission electron microscopy is a useful method that can be used to determine changes occurring at the ultrastructural level such as damage to cellular organelles, for example the Golgi apparatus and the mitochondria.

A major problem with *in-vitro* cell culture systems is dedifferentiation of cells in culture which should not be confused with differentiation. A cell differentiates when it stops proliferating and matures to express its phenotype; for example an osteoblast becomes a mature osteoblast and starts producing alkaline phosphatase, osteopontin and osteonectin. An osteogenic cell line MC3T3-E1, for example was found to be able to differentiate into osteoblasts and osteocytes and formed calcified bone tissue *in-vitro* (Sudo *et al* 1983). A cell may dedifferentiate when it has been passaged in culture a number of times and its phenotype is lost. Several cells have been found to de-differentiate *in-vitro* such as chondrocytes from chick embryos which, when cultured *in-vitro* for long periods, ceased to synthesize chondroitin sulphate (Holtzer *et al* 1970). This de-differentitaion of cells is one of the disadvantages of *in-vitro* testing and precautions must be taken to monitor cell dedifferentiation. In our system cells over a passage of 14 were not used for the biochemical or qualitative studies.

The majority of *in-vitro* biocompatibility tests described in the literature involve the morphological assessment of cells on materials, usually by light or scanning electron microscopy. The time courses for cell exposure are usually short term and at the most last for a few days. The types of cells used for these tests are different in source and species. This can give conflicting results and makes comparisons between different laboratories difficult. It has been demonstrated that different cells will behave differently on the same material surface. Differences were observed between periodontal ligament (PDL) fibroblasts and L929 cells, (a mouse fibroblast cell line) when measuring toxicity of a dental material (Al-Nazhan and Spangberg, 1990). They found that the PDL fibroblasts

cells were less sensitive than the L929 cells, and ultrastructural differences between the two cell types were observed. Different cell types, for example fibroblasts and osteoblasts, were found to respond differently to a range of biomaterials (Hunter *et al* 1995).

Despite all the problems associated with this type of screening of materials, *in-vitro* tests can provide fast, reproducible and useful data on the biocompatibility of a material. This is possible for *in-vivo* tests due to the differences in site and species and the size and type of materials used. *In-vitro* tests can be used to determine initial attachment of different cell types on various material surfaces and for carrying out "long term" biocompatibility testing. Cytotoxicity of leachable compounds released from certain materials such as resorbable polymers can also be measured.

The applications for *in-vitro* tests are vast, but what is required is a standardised method of carrying out these tests. A standardised protocol needs to be developed that sets out tests which provide meaningful results, that can then be used for comparison within groups carrying out similar tests. It is not only the cell types that vary but also the test materials and cell seeding density. For example polylactic acid may be the material under investigation but the method of formulation and sterilisation may be different. A report by Engelberg & Kohn (1991) stated that new applications of materials require that they be fully characterised and have defined material properties which is currently not the case. They also stated that there was a lack of reliable data and tests carried out and materials could not be compared due to the variability in the samples.

Cells death and apoptosis

Cell death is a major concern with regard to biocompatibility testing. If cells die on a device or a material under investigation it is obvious that something leachable from the biomaterial or the material is killing the cells. There are however different types of cell death, notably necrosis and apoptosis.

Necrotic cell death usually occurs as a result of injury in the presence of excessive toxins. Apoptosis, also termed cell suicide or programmed cell death, was first described by Kerr *et al* (1972) as a mechanism for the natural elimination of cells from the body and is an important mechanism for tissue homeostasis. When a cell is undergoing apoptosis various morphological and ultrastructural changes occur in the cell that can be detected by light microscopy or TEM. In apoptosis the cell first detaches from adjacent cells and then looses surface structures such as microvilli. The organelles within the cytoplasm shrink and, as a result, the cell also shrinks. Cytoplasmic, plasma and nuclear membrane blebbing occurs and the cell fragments into many membrane-bound pieces. The cell

organelles, however, remain intact and the mitochondria remain viable late into the process. The chromatin condenses and the nucleus can break up. The condensed chromatin is one of the major features utilised in TEM to detect apoptotic cells. The methods for detecting cells undergoing apoptosis are still being developed. There is also the problem in obtaining cells that are undergoing apoptosis *in-vivo* as apoptotic cells are rapidly phagocytosed.

Biodegradable polymers

There has been increasing interest in degradable polymer systems for use in biomedical applications such as drug delivery (koosha *et al* 1989; Wagenaar and Miller 1994), fracture repair (Ewers 1990; Illi *et al* 1992), tissue remodelling (Freed *et al* 1993; Gilbert *et al* 1993; Puelacher *et al* 1994; Chaput *et al* 1996) and soft tissue implants (Mooney *et al* 1996). Degradable materials have certain advantages that make them desirable for some orthopaedic applications. Their degradation rates and tensile strength can be controlled by varying molecular weight and, for copolymers, varying the ratio of the components can also dramatically affect their degradation rates (Nakamura *et al* (1989). The majority of degradable materials have been poorly characterised using *in-vitro* methods which have usually involved the assessment of fibroblast, and some osteoblast and hepatocyte, growth on them. There has been considerable work done with degradable polymers *in-vivo* but the mechanisms of cell attachment and proliferation on these polymers have not been fully investigated. Moreover the effect of the cellular activity on the degradation of the polymer and the effect of the degradation products on the cells are not well understood.

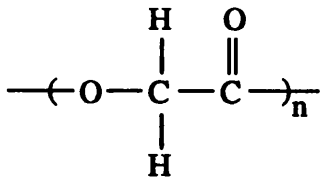
Polymers can be separated into three groups namely thermoplastics, elastomers, and thermosets. The thermoplastics, which constitute most of the degradable polymers, are further divided into crystalline and amorphous (non crystalline) polymers. Thermoplastic polymers are the most commonly used polymers and can be moulded into any shape by extrusion or injection moulding. Thermoplastics do not crystallise easily upon cooling as the polymer chains have to form highly ordered structures from their entangled state in order to become crystalline. What usually occurs with most polymers is some crystallisation in certain regions of the polymers resulting in polymers with crystalline regions and amorphous regions, thus termed semicrystalline polymers. The melting point (Tm) is used to characterise the crystalline regions and the glass transition temperature (Tg) is used to characterise the amorphous regions. The Tg is the temperature at which the polymer transforms from the glassy state to the rubbery state. Tm and Tg increase with an increase in chain stiffness and with increasing forces of intermolecular attraction.

Biodegradable polymers are categorised under several generic names some of which include polyesters, polyurethanes, polyamides, polyureas, polyanhydrides, polyphosphazanes, polyacrylates and polycyanoacrylates. The polymers most studied studied and those under intensive research currently are the polyesters which include PLA and PGA. The first synthetic absorbable material was developed in the 1962 by the Cyanamid Corporation using polyglycolic acid which was marketed under the trade name Dexon in the late 1960s (Gilding and Reed, 1979). This was followed by Medfit, a homopolymer of glycolic acid and Vicryl and polyglactin 910, copolymers of polyglycolic acid and polylactic acid as sutures.

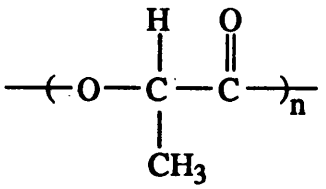
Aliphatic polyesters:

The biodegradable polymers studied belong to a group of compounds known as aliphatic polyesters. These include polylactic acid, polyglycolic acid, polyhydroxybutyric acid and poly ε caprolactone. These are the most extensively studied of the degradable polymers which also include polymalic acid and poly dioxanone. They all belong to the sub group poly α hydroxyacids for which the general formula is -(-O-CHR-CO-)n.

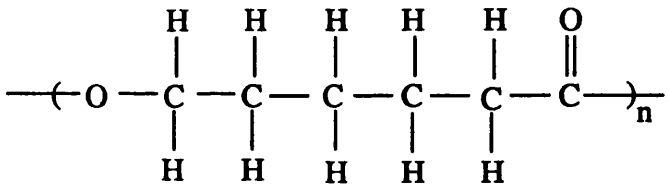
The structures of some of the most commonly used degradable polymers are shown below.



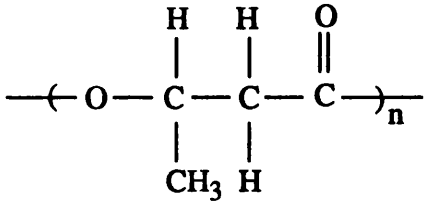
Poly (glycolide) (PGA) -[- O - CH_2 - CO -]-



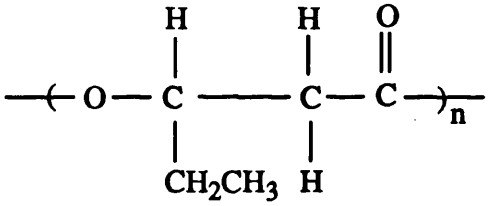
Poly (L-lactide) (PLA) -[- O - $CH(CH_3)$ - CO -]-



Poly (ε- caprolactone) (PCL) -[- O - $(CH_2)_5$ - CO -]-



Poly (β-hydroxybutyrate) PHB -[- O - CH_2 CH (CH_3) - CO -]-

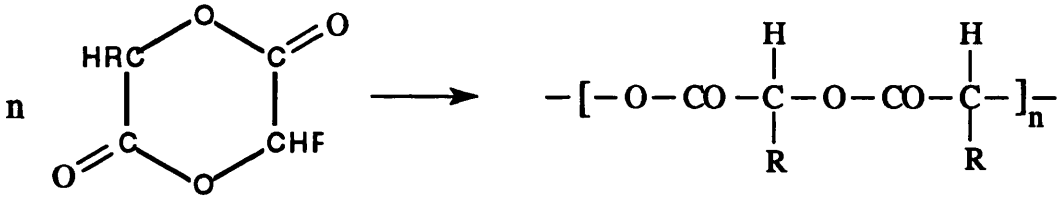


Polyhydroxyvalerate (PHV) -[- O - CH_2 CH $(CH_2 CH_3)$ CO -]-

Poly(lactic) and Poly(glycolic) acids

Polylactic acid and Polyglycolic acid are the most widely studied and clinically used of the degradable polymers available. They have been used mainly in the form of sutures, plates, screws and as vehicles for drug delivery. PGA and PLA, made by simple polycondensation, yield low molecular weight polymers which are not suitable for devices that are required to have high mechanical strength. The preferred method of polymer formation is ring opening polymerisation of cyclic diesters using catalysts, which yields high molecular weight polymers with longer degradation times (Gilding, 1979).

Diagram of Ring opening polymerisation

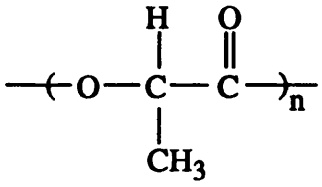


Polyglycolic acid is a highly crystalline linear aliphatic polyester with a melting point of 224-226°C and a glass transition temperature (Tg) of 36°C. PGA is not easily soluble in organic solvents but is easily degraded *in-vivo* or under physiological conditions *in-vitro*. Due to its hydrophilicity it has a rapid degradation rate and complete resorption *in-vivo* usually occurs between 2-14 weeks depending on the initial molecular weight of the polymer. The methods of formulation and sterilisation influence its crystallinity and permeability which, in turn, affect its degradation. PGA can be copolymerised with other polymers such as PLA to yield copolymers which have a slower degradation rate and greater mechanical strength (Gilding and Reed 1979; Athanasiou *et al* 1996).

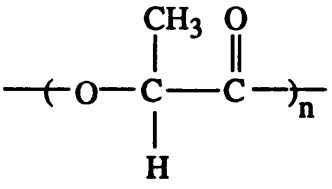
PGA was the first synthetic polymer to be used as a suture, however its low mechanical strength and rapid degradation rates have not allowed it to be considered for uses which require greater stability such as fracture fixation. Recently, however, methods which yield self reinforced PGA rods have been studied for use as osteosynthetic materials due to the higher mechanical stability achieved for these polymers. (Tormala *et al* 1991; 1993)

PLA can exist in two stereoisomeric forms, D-PLA and L-PLA as it is formed from the polycondensation of lactide which is a chiral molecule.

D-PLA



L-PLA



A racemic mixture of D and L PLA yields D, L-PLA which is amorphous although D-PLA and L-PLA are semicrystalline. L-PLA is the most widely studied of the forms as it is hydrolysed into L(+) lactic acid which can be metabolised by the body and is eliminated by the Krebs cycle to CO_2 and water (Bazile *et al* 1992). D, L-PLA is usually considered

for applications in drug delivery where it can form a homogeneous mixture with a protein. L-PLA is used more for orthopaedic fixation devices where high mechanical strength is required (Engelberg, 1991). D-PLA is not as extensively used as the other forms due to its low mechanical strength; as this form is more amorphous it is used in drug delivery applications.

There has been a large amount of work carried out on the degradation mechanisms of PLA and PGA and it is understood that degradation is faster *in-vivo* than *in-vitro* (Claes 1992). Strength retention of self-reinforced poly L-lactide screws and plates was investigated *in-vivo* and *in-vitro* and loss of strength was faster *in-vivo* than *in-vitro* (Suuronen *et al* 1992). Further work demonstrated that degradation was faster *in-vivo* and that hydrolytic degradation in semi crystalline poly L-Lactic acid occurs preferentially at the amorphous regions resulting in increased brittleness both *in-vivo* and *in-vitro* (Laitinen *et al* 1992). The porosity of the material was also found to affect the degradation rates where Kobayashi *et al* (1991) investigated the degradation of three types of films porous, non porous and a combination film *in-vitro* and *in-vivo*. The non-porous film degraded faster than the porous film over a 180 day period and the presence of hydrophilic units in the main polymer chain also resulted in increased degradation rates.

The degradation of PGA occurs mainly as a result of hydrolytic scission and to a lesser extent by enzymatic degradation (Bostman *et al* 1992). Kulkarni *et al* (1971) demonstrated that poly L(+) lactic acid of high molecular weight could be used in fibre film or coat form and, when implanted into pigs and rats, was completely metabolised without accumulation in the body organs. It was also found that as D,L-lactic acid is less ordered than L(+) lactic acid, and it degrades faster (Maduit *et al* 1996; Gerlach and Eitenmuller 1987). Cutright *et al* (1971) tested sutures for internal fixation of fractures of the mandible of rhesus monkeys, degradation was slow and complete degradation was not complete even after 12 weeks. Further work by Cutright *et al* (1974) on PLA and PGA showed that degradation was initiated in growth of capillaries and/or phagocytes. The cylindrical polymer fragments were replaced by a fibrous connection and the resorption times were dependent on the ratio of PGA to PLA. A mixture of 25% PLA and 75% PGA was degraded most rapidly followed by 50% PLA and 50% PGA, 75% PLA and 25% PGA with 100% PLA taking the longest. Paivarinta *et al* (1993) studied degradation *in-vivo* and found that there was no PGA remaining after 36 weeks, whereas PLA was virtually intact at 48 weeks; the materials were used as screws to fix femoral osteotomies in rabbits.

Two types of microbeads of PLA were prepared by solvent evaporation and implanted into dog mandible and studied at 6 and 18 weeks. No bone regeneration was seen at either time period with either of the types and the failure was attributed to the presence of poly

(vinyl alcohol) as a residue from the evaporation process at the surface of the beads (Anselme *et al* 1993).

Composites of PLLA which are resorbable have good initial mechanical properties but these are quickly lost with exposure to an aqueous environment (Andriano *et al* 1992). PLLA poly (L-lactide) mesh, sheets, microfilaments and mesh cylinders with fresh autogenic particulate cancellous bone and marrow (PCBM) were implanted subcutaniously into dogs. The inflammatory response to PLLA was similar to a polypropylene (PP) control. Three months after implantation the number of histiocytes and mononucleate giant cells increased in number as the monofilaments degraded. Copolymers with a higher lactic acid content and purified polymers such as poly L-Lactide have been shown to have a lower rate of degradation (Kinoshita, 1993).

Polycaprolactone

Poly ε caprolactone (PCL) is a semicrystalline aliphatic polyester that has a glass transition temperature of -60°C and a melting point of 60°C which is low compared to the other polymers. The monomer of PCL, ε-caprolactone, is manufactured by oxidation of cyclohexane with peracetic acid. The polymerisation of ε-caprolactone can be by four different methods: anionic, cationic, co-ordination and radical. Each method has a different degree of control over the molecular weight, molecular weight distribution and chemical structure which in turn determine the permeability and degradability of the polymer (Pitt *et al* 1990). The crystallinity of the polymer varies with its molecular weight; usually polymers with molecular weights of over 100,000 have a low crystallinity of 40% which rises to 80%, with a decrease in molecular weight down to 5000. The crystallinity is important in determining permeability and degradability as the bulk crystalline regions are inaccessible to water. PCL is readily soluble in organic solvents such as tetrahydrofuran (THF), chloroform, toluene, benzene, cyclohexane and dihydropyran at room temperature. It is readily degraded by microorganisms and is hydrolysed under physiological conditions. Enzymatic surface erosion is also thought to occur although PCL degrades more slowly than PLA and PGA (Pitt *et al* 1984; Jarret *et al* (1984).

Because of its slow degradation rate PCL is widely considered for long term implantable drug delivery systems and as degradable packaging and bottles. PCL is currently undergoing clinical trails as a one year contraceptive device by Capronor in Europe and Asia. PCL readily forms blends with other polymers such as PLA, cellulose propionate and cellulose acetate butyrate and can be copolymerised with PLA, and PHV which influence its degradation rate and mechanical properties (Koleske, 1978; Feng *et al* 1983). Due to its poor mechanical properties PCL is unsuitable for use in load bearing situations

but it can be used in drug delivery applications where biomechanical considerations are not of such crucial importance. The exact mechanism of degradation of PCL is not understood, but chemical hydrolysis is thought to play a large part and workers have reported that the molecular weights of polymers determine the rate of degradation (Albertsson and Ljungquist 1981; Pitt *et al* 1981; Piskin 1994). Current research in the field of degradation points towards the role of enzymes, peroxides, free radicals, phagocytic cells and lipids in the degradation of polymers, but there is no standardised method of measuring or preventing this degradation. Heterochain polymers, in particular those containing oxygen and nitrogen atoms in the main chain, are generally more susceptible to hydrolysis. Polymers susceptible to hydrolysis are those in the polyamide, poly amino acid, some polyurethane and polyester groups (Ali *et al* 1992; Ali *et al* 1993). PCL is slower to degrade than PLA but it has better permeability.

Upon implantation degradation of the polymer begins by random hydrolytic chain scission of the ester bonds in the polymer backbone. This reduces the viscosity and the molecular weight of the polymer (Pitt *et al* 1981). The same rate is observed in water at 40°C and is not altered with a ten-fold change in surface to volume ratio, indicating a bulk process. There is no weight loss during the first stage suggesting an autocatalytic process where the liberated carboxylic end groups catalyse the cleavage of additional ester groups. The second stage is a decrease in rate of chain scission and the initiation of weight loss. There is also an increase in crystallinity leading to brittleness and possibly the break-up of the polymer producing small particles which can be phagocytosed. The process is autocatalytic and, for polymers with molecular weights of over 5000, weight loss is not significant but once degradation is initiated weight loss rate depends on particle size. Enzymatic degradation is thought to play a role in the surface degradation of polymers as rapid surface erosion is observed following implantation. Increasing the crosslinking reduces the susceptibility of these polymers to enzymatic attack, by restricting the mobility of the chains; the ester groups cannot assume a configuration able to interact with the active site of the enzyme (Pitt *et al* 1981). Feng *et al* (1983) combined the two polymers to modify and vary their properties. *In-vitro* degradation of the polymer films was investigated in deionised water for up to 70 days and degradation of the copolymers was found to be faster than for PCL and slower than for PLA homopolymers corresponding to the ratio of PLA to PCL.

Pitt *et al* (1981) looked at the degradation of PCL *in-vivo* and concluded that the degradation mechanism involves random chain scission by hydrolytic cleavage of ester groups. The rate was not dependent on geometry and enzyme degradation was excluded as the same rate of degradation was observed in water at 40°C. The kinetics of the chain scission indicated an autocatalytic process i.e. the generation of carboxylic acid ends by ester hydrolysis. Further work by Pitt *et al* (1981) looked at the enzymatic degradation of

PCL and concluded that uncrosslinked PLA and PCL degraded *in-vivo* by a random chain scission process, aqueous hydrolysis of ester links. Also, long induction periods were required before any bioerosion occurred (Pitt *et al* 1984). Crosslinked polyesters are affected by the same hydrolytic process, but also undergo attack by enzymes at the surface. This is because the mobility of polymer chains for a non crystalline polymer allows the ester group to change its orientation in order to interact with the enzyme. This enzymatic attack becomes negligible with an increase in cross linking.

Poly(hydroxybutyrate)

Poly(hydroxybutyrate) (PHB) is a linear, thermoplastic polyester of 3-hydroxybutyric acid produced by the industrial fermentation of glucose by *Alcaligenes eutrophus* (Doyle *et al* 1991). In bacteria, its purpose is to provide intracellular storage of carbon and energy. In the fermentation process the growth medium has limited phosphorous thus arresting bacterial growth. Once this has happened, glucose is added to the growth medium and, as the cells cannot convert glucose to protein, PHB is formed instead and accumulates in the cells. The level of PHB can be as high as 80% of the dry mass of cellular material. The polymer is extracted by aqueous or solvent extraction and polymers with molecular weight of 5×10^5 - 1.5×10^6 can be produced (Vert *et al* 1986). The solvent extraction method yields polymers of higher molecular weight compared to the aqueous method which is cheaper and safer but yields a lower molecular weight polymer (Luzier, 1992). PHB-PHV is formed if an organic acid such as propionic acid is added with the glucose. This results in the incorporation of HV units within the HB segments to form the PHB-PHV copolymer.

PHB can be degraded by soil bacteria and *in-vivo* it degrades into D-3-hydroxybutyric acid which is found in normal blood. PHB is available both as a homopolymer and copolymer with polyhydroxyvalerate under the trade name Biopol. The homopolymer is crystalline, but the copolymers are less crystalline and thus readily processed. The polyester undergoes hydrolytic and enzymatic degradation in a physiological environment It is currently used in the production of plastic bottles, mouldings, fibres and films. PHB has also been blended with plasticizers and found to be degraded by PHB depolymerases and lipases although the enzymes degraded the polymer films to varying degrees suggesting specificity of the enzymes (Abe *et al* 1994).

A study by Boeree *et al* (1993) concluded that injection moulding of PHB resulted in a material with satisfactory mechanical properties for use in orthopaedic use. PHB/HA composite has been found to be bioactive and have good bonding with the surrounding tissue (Yasin *et al* 1990, 1992, Knowles and Hastings 1992). Yasin *et al* (1989) studied the degradation of PHB-PHV copolymer blends with amylose, dextran, dextrin and

sodium alginate at various pH values with temperatures at 37°C and 70°C. The hydrolytic degradation was found to be affected by the presence of polysaccharides, pH and temperature. The highest rate of degradation was found to be in pH 10.6 buffer at 70°C. The degradation of PHB homopolymer is still not fully established. Work by Miller and Williams (1987) showed that *in-vivo* degradation only occurred if the polymer was pre-degraded by 10.0 Mrad of γ irradiation and the rate was faster *in-vivo* than *in-vitro.* Copolymer additions of PHV 8% and 17% did not increase degradation and at high temperatures retarded the rate of degradation. They concluded that biodegradation of PHB and its copolymers could not be predicted with confidence and that more work was needed to establish the correct polymer properties for degradation to take place. Work by Knowles and Hastings (1991) concluded that the slower degradation characteristics of PHB as compared to PLA and PGA, would make this polymer more suitable for use in applications where longer resorption times were needed; however, more work is needed to determine the degradability of the polymer. pH has a considerable effect on the degradation of PHB and its copolymers. Changes in surface gloss were measured as an indication of polymer degradation, using a technique called goniophotometry which is the measurement of reflected light as a function of viewing angle. The surface roughness or gloss factor are measured by analysing the reflectance pattern from an incident beam of light. Solutions with pH 3, 6, 7, 9, 10 and 11 were used to degrade the polymers. A decrease in molecular weight was observed for all polymers irrespective of pH, but the alkaline solutions produced a more aggressive degradation resulting in areas with deep surface erosions. The degradation of PHB-PHV was investigated by Holland *et al* (1987; 1990). The polymer was resistant to degradation for a period of one year after which accelerated degradation was observed and an increase in porosity increased the diffusion, of the degradation products more effectively.

Immune response

One of the first things encountered by a material following implantation in the body is blood, which contains cells of the immune system. The immune system is the body's defence system and its primary aim is to defend the body against invasion by foreign organisms, such as bacteria and viruses. Before dealing with the invasion it has to first recognise foreign organisms and then eliminate them. This may lead to hypersensitive reactions characterised by an inflammatory response. This is characterised by increased blood flow to the affected area, with an increase in capillary permeability allowing molecules and cells to cross the endothelium and leukocytes to migrate from the blood into the surrounding tissue.

The immune system may be divided into two categories; specific and non-specific (Roitt, 1988). Both act differently but can be mediated by the same agents. The non-specific or

innate mechanisms can be further subdivided into humoral and cellular defence mechanisms. A number of plasma proteins are involved in the humoral defence mechanism. These include lysozymes, interferons, and the complement system. Activation of the complement cascade of proteins leads to the generation of a membrane attack complex, C3bBb which lyses, bacteria. C3bBb attaches to foreign bodies labelling them for phagocytosis and other leukocytes, causing them to move into the area where the chemoattractant is concentrated. In addition to being chemoattractants, both molecules are powerful anaphylatoxins, that is, they bind to and cause degranulation of the mast cells and basophils with release of vasoactive amines, histamine, leukotrienes and other inflammatory mediators. The net effect of the innate response is contraction of vascular smooth muscle, increased vascular permeability and emigration of neutrophils and monocytes from the blood vessels. These blood cells engage in phagocytosis of the complement-bound foreign body or alternatively, degranulate with further production of inflammatory mediators with the overall result being the production of a local inflammatory response.

The protection offered by the specific or acquired immune system is due to a group of cells known as lymphocytes: B lymphocytes and T lymphocytes. Each individual cell has a receptor on its surface which is specific for a particular antigen. In the specific immune response the antigen is processed by antigen presenting cells (APC). These are a heterogeneous population of leukocytes, which include B cells and macrophages, that can take up antigen and express part of it on their surface. A sub-set of the T cell population, helper T cells (T_H), recognise the antigen in combination with class II MHC. This causes them to secrete the T cell growth factor Interleukin 2 (IL2), which stimulates T cell proliferation. T_H also "help" the B cells which recognise the same antigen by stimulating them to differentiate into plasma cells which secrete antibodies. The stimuli for this differentiation are the IL1 from the APC and IL4 from the T cells. Antibodies bind to the foreign body marking it for killing by phagocytosis or by some other mechanism.

Delayed-type hypersensitivity occurs when antigen becomes trapped in the macrophage and cannot be cleared, and is mediated by the specific immune system. This causes T_H cell activation and proliferation which produce a number of lymphokines, for example macrophage activating factor (MAF), which induce inflammatory reactions, attract and activate macrophages with the further release of inflammatory mediators. T cells are also activated by class I MHC to become cytotoxic to the cells carrying the antigen. Continuous stimulation by persisting antigen leads to further influx of macrophages, their differentiation into giant cells and eventually the formation of chronic granuloma.

Biomaterials when implanted into the body, are essentially foreign bodies, which are expected to elicit an immune response. The severity and nature of this response will

depend on the material under investigation. Therefore the initial host response must encompass a repair process. Neutrophils are the first cells to reach the site of infection, attracted by chemotactic factors such as complement, with their predominant role being phagocytosis. The phagocytosed body is fused with a lysosome and destroyed by lytic enzymes. The neutrophil respiratory burst involves a sharp uptake of oxygen and results in the generation of toxic oxygen products, such as superoxide anions and hydroxyl radicals. If the material cannot be phagocytosed the neutrophil will release destructive substances into the extracellular environment in a process known as frustrated phagocytosis. Any of these parameters can be used as an indicator of neutrophil activation by biomaterials. Once at a site of the biomaterial, there are two ways in which neutrophils could act, either by respiratory burst, or by the release of lysosomal enzymes.

The monocyte/macrophage response to biomaterials has been the most extensively studied of all the cells of the immune system (Bonfield and Anderson 1993; Benahamed *et al* 1997) and have been implicated in causing increased degradation of the materials. Macrophages have been shown to infiltrate the implant site after implantation of a biomaterial and, once activated, macrophages have a much altered metabolism. This includes altered phagocytic ability and increased lysosomal enzyme release as well as secretion of cytokines and growth factors. Macrophage activation by materials can be assessed by measuring any of the above parameters. The effect of metals on macrophage viability has been determined by a number of researchers but the effect of degradation products and the effect of degradable polymers on macrophages both *in-vivo* and *in-vitro* has not been as extensively studied.

Activated macrophages display altered morphology which can be identified microscopically. Macrophages cultured with a range of biomaterials were analysed by scanning electron microscopy for alterations in morphology, attachment and cell density (Miller *et al* 1989). After 24 hours in culture the cells displayed an activated morphology; they were elongated or flattened with numerous filopodia for attachment. The highest density of morphologically active cells were seen on Dacron and polyethylene, while the lowest were on polydimethylsiloxane (PDMS). Supernatants from these cultures were assayed for interleukin-1 (IL1). A good correlation between IL1 secretion and morphological activation was seen.

In-vivo macrophages are not the only cells which come into contact with the implanted biomaterial and it is not just the biomaterial that has an effect on the cells. There can be an indirect effect of biomaterial-stimulated macrophage secretion products on other cell types such as fibroblasts (Bonfield *et al* 1991, 1992; Greisler *et al* 1989) and endothelial cells (Miller and Anderson, 1989). Supernatants from macrophages cultured on Dacron, polyethylene, polyethyleneterephthalate (ePTFE) and PDMS all stimulated collagen

production by fibroblasts. All, except Dacron, caused fibroblast proliferation and all produced PDGF and IL1 (Murray and Rushton, 1990). Antibodies to these cytokines demonstrated that IL1 is responsible for the fibroblast stimulation (Greisler *et al* 1991). The fibroblast stimulating factors were divided into two types, depending on whether they act early in the cell cycle at Go, signalling entry into G1 (competence-like factors) or on cells in the G1 phase, signalling entry into S phase (progression-like factors) (Bonfield *et al* 1991). Protein preadsorption on polymers was shown to modulate production of these factors by monocytes/macrophages. No simple correlation was found between adsorbed proteins and polymer and fibroblast stimulation by factors released from monocytes/macrophages cultured on a range of protein-polymer combinations Bonfield *et al* (1992) but the effect of macrophage-derived growth factors on endothelialisation was more clear cut (Miller and Anderson, 1989). The materials tested were polyglactin (PG910) and Dacron which were finely shredded. Supernatants from macrophages cultured on PG910 caused endothelial cell proliferation but Dacron had no effect. Cytoplasm inclusions of PG910 were seen microscopically, including phagocytosis of the material. This is likely to be the main cause of macrophage activation. The factors responsible for endothelialisation were not altered.

Lymphocytes are the cells responsible for the specific immune response. Their reaction towards foreign bodies includes secretion of cytokines and antibodies. Activation is also seen as expression of surface antigens. Metal ions can be released into the body due to the biodegradation of metallic prostheses and other implants. Again, a large amount of work has been carried out on the effect of metal particles on these cells, but very little work has been done on the effect of degradation products from degradable polymers on these cells.

The normal healing process occurs in two phases (Williams, 1989). The first phase is inflammation, followed by repair. Immediately following injury, blood enters the area and a fibrin clot forms trapping red blood cells and activating platelets. The blood vessels in the local area dilate and white cells, mainly neutrophils initially, plus plasma proteins and other inflammatory mediators, diffuse out. The neutrophils phagocytose cellular debris generated by the injury and an acute inflammatory response is set up. Vascular regeneration begins with the formation of new capillaries in the wounded area. Fibroblasts lay down collagen in the form of scar tissue. The extent of injury determines the duration of inflammation and the time taken for the repair process.

If the source of irritation is not removed before repair begins which is what happens in an implant situation, then the inflammation process continues and may delay the repair. The extent of this will depend on the nature of the implanted material. With a single, solid, non-biodegradable material the inflammatory response will be unaffected. As before, fibroblasts will lay down collagen, but it will not be able to infiltrate the implant. Instead,

a fibrous capsule will form around the implant. This is known as classic fibrous encapsulation, and is rare. Usually there is a reaction between the implant and the tissue, involving cells of the immune system. In extreme cases this results in persistent inflammation and repair is never complete. In cases involving degradable materials the response is slightly different. Chignier *et al* (1993) introduced Dacron pieces into the peritoneal cavity of rats for six hours after which the peritoneal cells were harvested. Enzyme assays were performed and the results showed that macrophages spread around the Dacron fibres with cytoplasmic projections but no phagolysosomes. The levels of 5' nucleotidase (an enzyme triggered by the cellular activation during the inflammatory response) decreased (Therin *et al* 1992).

MC3T3-E1 cells were used by Elgendy *et al* (1993) to study the potential of degradable polymers and ceramics to support osteoblast growth for a proposed bone-polymer composite for skeletal tissue repair. Cells were seeded onto 50:50 poly (Lactide co glycolide) , HA, 50:50 HA/ poly (Lactide-co glycolide). Cell attachment and growth was highest on poly L-co glycolide and least on the HA and HA/Poly L-co glycolide was intermediate. Gogolewski *et al* (1993) implanted PLA, PHB and PHB/HV polymers into mice and studied polymer degradation and response. All the polymers were well tolerated by the tissues, but no acute inflammation or abscess formation or tissue necrosis was detected. Degradation of the polymers was accompanied by an increase in collagen deposition, mononuclear macrophages, proliferating fibroblasts and mature vascularised fibrous capsules which are typical of the tissue response. All polylactides degraded significantly, (56-99% by six months) and the degradation rate increased with a decrease in the molecular weights *in-vivo*. PHB/VA and PHB polymers degraded 15-43% less than the Polylactides following six months implantation. The greatest degradation was seen in polymers with high valerate content. The weight loss for polylactides was 0-50% where as for PHB it was 0-1.6%. Work by Suganuma and Alexander (1993) involving PLLA *in-vivo* showed good biocompatibility up to six weeks after implantation with increased bone ingrowth into the PLLA; however, after this time period the biocompatibility decreased and extensive bone resorption took place with the infiltration of inflammatory cells. This reduction was thought to be due to the start of the degradation process which may have reduced the local pH, as well as the presence of small PLLA particles. In cases where the degradation of PLA and PGA yielded molecular weight products of 10,000-20,000 an increase in inflammation was observed (Spenlehauer *et al* 1989).

Saitoh *et al* (1994) however, observed good bone formation upon implantation of PLA into skeletal tissue with complete resorption of PLA taking place in 24 weeks. No severe inflammation was observed but some phagocytic reaction was present. Getter *et al* (1972) also demonstrated that PLA was well tolerated by the tissue in fixation of mandibular

fractures in dogs. The problem associated with using the data available, to form a complete picture on the biocompatibility of degradable polymers, is the diversity of the tests performed. The time points are usually inappropriate, and usually not long enough to take into account the effect of late degradation products on the biocompatibility. The molecular weight, physical characteristics and changes in surface structure following sterilisation of the polymers are not established or stated. As the initial molecular weight influences the rate of degradation it will affect the biocompatibility, and hence the immune response. It is therefore important to determine the molecular weight of a device immediately before implantation, that is after it has been sterilised as sterilisation processess are known to influence the molecular weight of certain polymers.

Effects of different sterilisation methods

Degradable polymers are being extensively studied for their use in orthopaedic surgery and it is for these applications that the materials need to be sterilised. Different methods of sterilisation have been evaluated and their effect on the polymers investigated. The universal method of sterilisation by gamma irradiation is known to cause degradation and sometimes crosslinking of some polymers thereby increasing or decreasing their molecular weight. Ethylene oxide is thus the preferred means of sterilisation, but there are problems associated with its toxicity to tissue and cells in culture. Work by Zislis *et al* (1989) showed that materials sterilised by ethylene oxide needed to be aerated for at least 7-14 days before good fibroblast growth and attachment occurred on the surface. Rozema *et al* (1991) investigated the effect of steam sterilisation on the mechanical properties of PLLA and found that the molecular weight was affected by the moisture and the high temperatures that were used for the sterilisation. The molecular weight and the tensile strength decreased by 35% but a shorter time and higher temperature during sterilisation led to a lower molecular weight loss.

Gamma irradiation of PLA decreased the crystallinity of the material while the material underwent chain scission and cross linking simultaneously in the presence of air and nitrogen at room temperature (Gupta and Deshmukh, 1983). Recently, gamma irradiation has been used to decrease the molecular weights of the polymers, so mass loss occurs faster allowing for short term studies to be carried out *in-vitro* and *in-vivo* (Zhang *et al* 1993). Spenlehauer *et al* (1989) also used gamma irradiation to study the effect on stability and establish degradation processes *in-vivo* and *in-vitro* and found that gamma irradiation decreased the molecular weight dramatically of poly(D,L lactide/glycolide) microspheres. Ultrasound irradiation could be used for drug delivery applications as a mode for increasing the release of proteins although a reduction in molecular weight and mass occurred (Agrawal *et al* 1994). Bruck and Mueller (1988, 1989) concluded that, if polymeric implants were to be used as long term implants, their properties needed to be

evaluated fully and more work was needed to determine the long term effects of high energy irradiation on polymers.

Applications

In orthopaedic and dental surgery it is often necessary to fill in bone defects or remodel bone. Surgical reconstruction of bone defects resulting from trauma or tumour resection requires large volumes of bone graft. Repair of segmental defects that can often be up to 25cm long in longbones may involve autografts, allografts of fresh or frozen bone, freeze dried bone or demineralised freeze-dried bone, which are used as chips or in powdered form. Allograft procedures are undesirable due to the risk of infection (HIV and hepatitis) and autograft procedures are undesirable due to the increased morbidity caused by the additional surgical trauma. Biodegradable biocompatible polymers could overcome the problems associated with supply, infection and reoperation for implant removal associated with conventional grafts.

A study by *Acuna et al* (1992) used PLA in combination with calcium phosphate and calcium carbonate as degradable bone fillers. *In-vitro* degradation of composites of PLA with molecular weights of 2000 and 16000 with HA or CA was evaluated. pH change and calcium concentration were measured at 37°C in distilled water. Composites of PLA with molecular weight of 16000 released no calcium during the 120 days experimental time but HA/PLA 2000 molecular weight composites released calcium for up to 90 days. The release of calcium increased with an increase in the amount of PLA in the composites. A histological study was also performed on HA-containing composites. In this study composite rods were implanted in the tibiae of Sprague-Dawley rats which were sacrificed at 1 and 26 weeks after implantation; the tissue around HA was normal, but there was a toxic tissue reaction initially near the implant site. This necrosis of the tissue was most likely due to the acidic environment caused by the fast degradation of the polymer as well as the swelling of the polymer into the tissue.

Drug delivery

Polyesters of α hydroxyacids and other degradable polymers have and are continuing to attract interest for use as controlled drug delivery systems (Huatan *et al* 1995; Schakenraad *et al* 1988). Most common are the homo and co polymers of lactic and glycolic acid although collagen based hydrogels and gelatin in the form of microspheres have been investigated for use in delivering antibiotics and growth factors (Di Silvio *et al* 1994; Eldridge *et al* 1992). These drug delivery systems offer the same advantages as the degradable materials do to other applications. There is no need for reoperation to remove

the implant, allowing for reduced inconvenience to the patient and reduced risk of infections and complications, as well as a long term sustained delivery of the drug.

The most common delivery vehicles are microparticulate drug delivery systems which are produced by solvent evaporation, solvent extraction phase separation or spray dryings. Spray drying is the most commonly studied method as it is fast and results in the formation of particles which are 1-15 μm in diameter which are easily scaled up.

PLA was investigated as a delivery vehicle for calcitonin which is a synthetic analogue of hypocalcaemic peptide hormone used to treat osteoporosis, Paget's disease and hypercalcaemia and has to be injected regularly over a long time period (Asano *et al* 1993). The release of calcitonin from Poly(DL-Lactide) with molecular weights of 1400, 2000 and 4400 was measured *in-vitro*. The lowest molecular weight polymer released all the drug within 3 days where as the 4400 molecular weight polymer released at a constant rate for 24 days.

There are increasing demands that new vaccines be without risk and free from side effects. As the isolated antigens are sometimes of low immunogenicity there is a need for delivery vehicles which are safe and which enhance the antibody response. In a study Eldridge *et al* (1991) used microspheres of (DL-L Lactide-Co-Glycolide) as vehicles for Staphylococcal enterotoxin B toxoid which enhances the level of toxin neutralising antibodies. They concluded that due to the biocompatibility of the copolymer and the flexibility in terms of the release kinetics this has been approved for human use and is being investigated for mucosal immunisation because of its ability to protect the vaccine and enhance adsorption into associated tissues. Progesterone was loaded in microspheres of Poly (DL-Lactide-co-glycolide) and found that variations in the progesterone loading affected the structural and thermal properties (Rosilio, 1991)

Poly(D,L-lactide) and poly(D,L-latide-co-glycolide) microspheres containing Piroxicam were prepared by a spray drying method and *in-vitro* release studies were carried out. Release from the DL-PLA microspheres was slow where less that 20% of the drug had been released by day 10. The DL-PLGA microspheres however, released about 50% of the drug in the first 5 hours of the study. It was suggested that release from the DL-PLA was by diffusion through the intact polymer barrier. The release rate from DL-PLGA was much faster and this was due to the immediate water absorption by the polymer due to the glycolic acid content which is more hydrophilic that PLA. This allows the microsphere to swell thus allowing for diffusion of the drug out through pores.

In-vivo biocompatibility

In-vivo inflammatory potential of implants has been assessed by implanting the materials into experimental animals and examining the surrounding tissue histologically and biochemically. The presence of inflammatory cells has been demonstrated microscopically by immunostaining and by enzyme histochemistry. A large amount of *in-vivo* work has been carried out involving non degradable materials such as metals, ceramics and in particular bone cements such as PMMA. In all cases involving PMMA inflammatory reaction to particulate cement or powder was noted. This was in the form of foreign body giant cells (Goodman, 1989) release of TNF, PGE2, and an increase in the white blood cell count (Gelb, 1994) and an infiltration of macrophages, most of which contained cement particles into the area (Thompson, 1992). Gelb *et al* (1994) concluded that surface area of the particle was important in determining the inflammatory response. This is not only applicable to bone cements but also to degradable materials where, the material is undergoing degradation, giving rise to particles which elicit an inflammatory response. Toxicity to PLA and PGA and their copolymers was determined by implantation into the rabbit corneal pouch (Kobayashi, 1992). Macroscopic and microscopic evaluation over a fourteen day period revealed increased vascularisation and opaqueness which are indicative of cellular inflammation although a detailed study of this cellular response was not carried out. An influx of neutrophils and eosinophils on day one was followed by an influx of monocytes and macrophages on day three following subcutaneously implanted PLA as shown by enzyme staining (Schakenraad *et al* 1990). Monoclonal antibodies were used to identify neutrophils and macrophages surrounding intramuscularly implanted PLA, PGA and PLA/PGA copolymers (Vince, 1991).

A recent study by Ignatius and Claes (1996) evaluated the toxicity of PLA and PGA which were commonly studied degradable materials. Their biocompatibility has still to date been not determined and more information is needed about the effect of the degradation products and *in-vitro* cell culture methods used. There has been a large amount of work carried out in the area of degradable materials, various animal models have been used and some clinical trials have also been carried out. The table in appendix one presents a summary of some of the findings from work carried out using degradable polymers for various applications.

Clinical trials

Various clinical trials have been carried out on devices made of PLA and PGA which have yielded variable results. Bostman (1993) used absorbable PGA pins for internal fixation of fractures in children. In this study there were 71 patients and the follow up period was on average 15.8 months. The mechanical strength of the pins was found to be acceptable

for the fixation of all the fractures except supracondylar fractures of the humerous. The failure in this case was due to lack of mechanical stability at the start, thus this could not be corrected by using a more slowly degradable polymer as the initial strength of PLA and PGA are similar. It was concluded that the main advantage of using resorbable fixation was the fact that they did not remain in-situ and thus did not require removal.

A PLA expansion plug was used for the fixation of the cracked bone block in the Bristow-Latarjet operation. This procedure is used for operation for recurrent anterior dislocation of the gleno-humeral joint. Complications caused by metal devices such as osteopenia due to stress shielding has meant that they are no longer used. Biocompatibility and suitability of the PLA expansion plug for this purpose were investigated. The procedure was found to be suitable for a mean 12 month follow up period and no redislocations occurred in this time period. The shape of the implant was not affected in the 18 months degradation period and no inflammatory foreign body reactions was noted.

Sinisaari *et al* (1996) used a PLA and PGA implant for the fixation of displaced ankle fractures. They investigated the incidence of infection with the two types of implants. It was concluded that the type of implant did not have a significant effect on the wound infection rate. Kankare *et al* (1995) used self reinforced PGA screws and metallic screws. They found a slightly higher increase in the failure of PGA implants. Bostman *et al* (1992) have carried out a series of experiments where they looked at the effectiveness of SR-PGA screws. Three months after operation 24 out of 216 patients developed a local non bacterial inflammation. Over several studies they concluded that a complication in adults which was due to a foreign body reaction resulted in tissue fluid accumulation at the site of the implant. The discharging sinus had remnants of degradable implant present in it 2-4 months after implantation. In paediatric patients this was not observed, but could have been due to the sample number compared to the adult patients. The pins used were also smaller in size. They concluded that although these resorbable implants did not appear to cause any problems in children, it was still too early to rule out other possible problems such as growth disturbances, thus the report was preliminary (Bostman, 1993).

Bergsma *et al* (1993) used SR-PLLA screws and plates to treat zygomatic fractures. The (Number average molecular weight), Mn was 7.6×10^5 and 10 patients were treated. After three years some patients returned with a painful swelling at the site of implantation and on recalling the others, a similar reaction was noted. Reoperation was carried out and the material and area analysed over a period of 3.3 years to 5.7 years. It was found that there was a fibrous capsule around the implant and on molecular weight analysis at both points the Mn was approximately 5000. Very little change in the Mn value of the polymer had taken place at the two points. It was hypothesised that this was possibility due to the

high crystallinity of the PLLA fragments which take a long time to degrade. Also, remnants of PLLA particles which were needle shaped were found in cells such as macrophages and fibroblasts around the implant. This may contribute to the slow degradation of the polymer as inside the cells they were not able to degrade as the main degradation mechanism is hydrolysis and even though macrophages contain a large range of enzymes this does not have very much effect on the polymer.

This literature search has aimed to bring together all the work that has been carried out in the biocompatibility testing of degradable materials using *in-vivo* or *in-vitro* techniques. The literature search has mainly concentrated on the most commonly used degradable polymers and those that are causing the greates interest. It has been shown to date that the biocompatibility of the majority of these materials in the short term is very good *in-vitro* and *in-vivo*. The problems however lie in their long term biocompatibility which is yet to be determined. Little progress has been made in this area due to the long degradation times associated with polymers such as PLA and PGA. The biocompatibility of PGA gives some indication of what happens to the slowly degrading polymers. PGA is quick to degrade and problems have been observed with it biocompatibility both *in-vivo* and *in-vitro*. This is caused by the release of acidic degradation products which kill the cells immediately adjacent to the site of implantation or in cell culture cells in the dish. The levels of acidity and the concentrations of degradation products released are not as yet known in the *in-vivo* situation to allow for appropriate tests to be developed.

This study aimed to develop and characterise a set of controllable, meaningful *in-vitro* tests for the biocompatibility testing of resorbable polymers. The methods included qualitative and quantitative tests which yielded information on the cellular morphology, cellular adhesion and proliferation on the various polymers. The polymers used were PLA, PGA, PHB, PHB-PHV copolymer and PCL which are the most commonly studied of the resorbable polymers. The cell types used were the a human osteosarcoma (HOS) cell line an osteoblast like cell line and the THP-1 cell line a monocyte/macrophage cell line. The cells were easy to culture and had a rapid turnover. The water uptake and release of degradation products from degradable polymers was measured using biochemical assays. The degradation of the polymers in various solutions was measured using gel permeation chromatography which measured changes in molecular weight. The effect of degradation products on cells in culture was investigated using colorimetric assays.

This thesis is divided into seven chapters, where chapter one is a review of the literature regarding the biocompatibility of resorbable polymers *in-vivo* and *in-vitro*. In chapter two the polymers degradation, weight changes and release of degradation products in various solutions is discussed. Molecular weight changes were measured by gel permeation chromatography and results from these are described. Chapter three describes the

qualitative analysis of the morphology of HOS cells. HOS cells were cultured on the various polymer surfaces and analysed by light, scanning and transmission electron microscopy. In chapter four the quantitative results of HOS cells on the polymer surfaces which had been cast and stored in various solutions was examined. Tritiated thymidine incorporation was used to determine the proliferation of the cells on the polymer surfaces. Chapter five describes the effect of monomers of PLA, PGA and D3-HB on HOS cells in culture. The effect of pH on the viability of the cells was measured using quantitative assays and electron microscopy. Chapter six discusses the effect of monomers on THP-1 cells and the effect of the polymer surfaces on the morphology of the monocyte/macrophage cell line. Chapter seven is the final discussion with suggestions of further work which would allow a more complete assessment of the long term biocompatibility of a polymer under investigation.

Determination of biocompatibility of biomaterials is necessary, but sometimes a difficult, task. The choice of tests is vast and results can be misleading and difficult to interpret. For degradable materials the problem is further complicated by the changes that occur to the material as it is degrading and releasing degradation products, thus the biocompatibility of the polymer as it is degrading also need to be determined. *In-vitro* tests can provide very useful and extensive information on the biocompatibility of resorbable materials reducing both expense, time and the use of animal models. The literature review concludes that although there is sufficient data to state that resorbable materials are biocompatible there is equally contrary data which states that there are long term problems associated with biocompatibility of these polymers. It is therefore hypothesised that degradation products released from the late degradation of many resorbable polymers cause problems with biocompatibility and a range of *in-vitro* tests can be evaluated which can determine if certain materials are more "incompatible" than others and if degradation products from these polymers are cytotoxic for the *in-vitro* cell culture model used. The materials tested were found to be biocompatible in the short term, however, results from cells cultured on PGA which was the most rapidly degrading of the materials showed that there was a possibility that these materials would not be biocompatible in the long term *in-vitro*.

CHAPTER II

Polymer characterisation and degradation

Introduction

Degradable polymers have attracted much interest in recent years in particular for their use as *in-vivo* delivery vehicles for proteins and growth factors and as osteointegrating materials. Polymer degradation is an important consideration and, due to the uncertainty of degradation rates and the effect of degradation products on cells and tissues around implants, there is a great need to investigate the mechanisms involved in this process. In addition to this it is also necessary to measure the levels of breakdown products such as monomers, oligomers and other degradation products that are released from the various polymers. Having identified these degradation products, they need to be correlated with levels that would be found in a clinical situation thus allowing *in-vitro* tests to be designed. *In-vitro* cytotoxicity tests enable one to test the degradation products using comparable concentrations.

Many factors influence the degradation rate of a polymer; these include crystallinity, initial molecular weight and the morphology of the specimen, all of which play a major role in the degradation of the polymer (Vert *et al* 1984). Early data on degradation of PLA and PGA homopolymers and copolymers showed that degradation was by hydrolysis and that copolymers had a wide range of degradation rates governed by the hydrophilic and hydrophobic nature of the copolymers as well as the crystallinity (Reed and Gilding, 1981). Degradation is described as the hydrolysis of ester bonds via a bulk erosion process which is autocatalysed by the generation of carboxylic acid end groups (Vert *et al* 1992). The main polymer chains undergo cleavage resulting in smaller fragments (oligomers) which break up further to form monomers. The *in-vitro* degradation of high molecular weight aliphatic polyesters derived from glycolic acid and lactic acid is heterogeneous with a faster degradation rate in the centre compared to the surface (Li *et al* 1990a, 1990b). This can be explained by the fact that there is greater acid autocatalysis in the centre so that the degradation products from the centre of the polymer cannot be removed quickly and there is a build up of acidic degradation products which catalyse the further breakdown of the polymer. Confirmation of these findings *in-vivo* was seen in work carried out by Therin *et al* (1992). They also demonstrated that the differences were due to the fact that degradation was slightly slower *in-vitro* suggesting the role of enzymes in the degradation process *in-vivo*. Williams in 1979 and 1981 demonstrated the degradation of polymers in particular PLA by 7 different enzymes. The role of enzymes in degradation was again demonstrated in 1985 by Smith and Williams who showed that polyethylene terephthalate (an aromatic polyester) was degraded by lysosomal esterase enzymes *in-vitro*. In 1987 Smith *et al* further demonstrated the degradation of non-resorbable polymers by a number of enzyme solutions which included trypsin, papain and chymotrypsin.

It has been suggested that cells involved in the immune response are capable of influencing degradation Sutherland *et al* (1993). Macrophages produce a range of enzymes, superoxide ions and hydrogen peroxide free radicals which are able to destroy microorganisms, and it is thought that these may also be able to influence degradation of some of the polymers. This would account for the faster rate of degradation observed *in-vivo* when compared with *in-vitro* observations (Ali *et al* 1994). Zitas *et al* (1988) showed that cell to cell and cell-material interaction evoked the release of mediators such as chemotactic and growth factors that induced an inflammatory response at the implant site.

Degradation in a biological environment can occur as a result of attack by biological molecules, such as enzymes. Polyester urea-urethane has been shown to be degraded by the action of enzymes cholesterol esterase, cathepsin B or Xanthine oxidase but not collagenase. Different pH and incubation times were also shown to affect degradation of the polymer (Santerre *et al* 1993). The degradation rate of a series of bisphenol A-based poly (phosphoester) polymers was found to be affected by polymer side chain structure (Richards *et al* 1991). This was due to the increased absorption of water by the ethyl side chain polymers compared to the phenyl counterparts.

The method of processing used for the moulding of the polymers also affects the degradation rate and other properties of the polymers. For example block polymerised and injection moulded PLLA rods are chemically similar but have very different mechanical properties (Eitenmuller *et al* 1995). They demonstrated that block polymerised PLLA rods lost their bending strength and molecular weight more rapidly in comparison to injection moulded PLLA rods. Methods of formulation, and the conditions under which polymers are made, also affect the properties; for example fast cured PGA degrades more rapidly as compared to slow cured PGA (Cutright, 1972).

In another study by Vert *et al* (1994) the degradation mechanisms of various aliphatic polyesters were investigated in depth. They described the degradation of aliphatic polyesters as proceeding heterogeneously, which is faster inside the device than at the surface. This is due to the internal autocatalysis that occurs as a result of release of acidic degradation products. Water uptake is the first process to occur *in-vivo* or *in-vitro* in an aqueous environment. This initially causes a negative gradient of water concentration from the surface to the centre which disappears within a few days, due to the rapid diffusion of water molecules. This is followed by an increase in the number of carboxylic chain ends which autocatalyse ester hydrolysis (Pitt, 1992) and results in soluble oligomers escaping into the aqueous medium. As degradation proceeds, oligomers at the surface are able to leach out more easily whereas those at the centre of the device cannot, thus increasing the autocatalytic effect due to their acidic nature. This difference in the

concentration of acidic groups leads to the generation of a layer of polymer at the surface which is less degraded than the centre. Thus, when a gel permeation chromatography analysis is carried out on these samples, a bimodal chromatogram is produced, that is one that has two peaks. The two peaks represent the two different distributions of molecular weights that are present.

In another study by Helder (1990) glycine-DL-Lactic acid copolymers were investigated at 37°C, pH 7.4 in phosphate buffer. The degradation was examined using Poly (DL-Lactide) as the reference material. All the copolymers showed an immediate decrease in molecular weight; however their weight remained almost unchanged over the time period studied. This weight decrease was observed earlier in those copolymers whose glycine component was greater. Chawla (1985) showed PLA with a lower molecular weight degraded at a faster rate than the higher molecular weight polymers. This variability in degradation rates can be exploited as different degradation rates are required for different applications and in some cases these can be an advantage. Variable degradation rates can be achieved by various methods some of which include:

a) Copolymerisation.
b) Using the different sterioisomeric forms; for example DL-Lactic is less ordered than L(+) so DL degrades faster than the L(+).
c) Introducing crystallinity during the formation, casting and drying of the polymer.
d) Using different molecular weight polymers.
e) Varying formulation methods such as gel casting which shows potential for varying resorption rates as well as stability and compressive properties (Coombes *et al* 1992; 1994).
f) Blending of polymers can also effect the degradation; for example Cha and Pitt (1990) blended PLLA and PCL with PGLA and samples were degraded *in-vitro* for 3000 hours in pH 7.4 buffer at 37°C. They concluded that the rates of hydrolytic degradation for compression moulded polymers was influenced by the blending method and concentrations.

In a three part study involving PLA implantation into the dorsal muscle of rats over a 116 week period it was concluded that crystalline block PLA was stable over the period tested. Amorphous injection moulded PLA developed a rough surface within a few weeks and degraded completely within 2 years (Pistner 1993; 1993; 1994). PLA degradation is affected by molecular weight and unreacted monomer content. Pure PLA is slower to degrade and, in the above study after six months *in-vivo,* pure PLA had lost no mass and the molecular weight had been reduced by 50% (Kinoshita *et al* 1993). The PLA with unreacted monomer showed a microporous structure extending from the surface to the centre of the materials whereas, in the pure PLA, no microporous structure

could be seen (Pistner *et al* 1993). Self reinforced-PGA (SR-PGA) rods coated with PLLA and PHBA were found to degrade at a slower rate compared to PDMS. However the coatings came off and the PGA rods degraded faster than the coatings. Due to the hydrophilic nature of the PGA rods more fluid was absorbed, and as a result, they expanded (Vasenius *et al* 1990). Schakenraad *et al* (1990) using PLLA implants, concluded that hydrolysis by tissue fluids was the main mechanism of degradation. An exponential degradation was observed for PLA after one year, when the molecular weight of the polymer was less than 5000. However Schakenraad *et al* in 1989, studied the degradation of glycine/DL-lactic acid microspheres and concluded that the degradation *in-vitro* was comparable to *in-vivo* thus there was possibly no effect of enzymes.

In this chapter the degradation of polymers has been assessed by investigating bulk mass changes, monomer release and molecular weight changes (measured by gel permeation chromatography). Gel Permeation Chromatography (GPC) was developed in the mid 1960s and has now become an invaluable tool for polymer characterisation. GPC is able to separate molecules according to their size in solution, thus the method is also referred to as size exclusion chromatography (SEC). Separation of the molecules is carried out in a column packed with beads (usually about 5-6mm in diameter) made from cross-linked polystyrene gel or porous glass. The pores in the column packing vary in size and are the means by which separation occurs. When a sample is injected onto the column, it passes through the packing until it is eluted at the end of the column. As a molecule passes through the packing, it may also pass into the pores in the packing. Whether a molecule goes into a pore depends on both the size of the molecule and the size of the pore. Consequently small molecules are detained as they pass both through the packing and into the pores, large molecules which cannot pass into the pores merely pass through the packing and intermediate sized molecules pass into some but not all of the pores. Thus the sample is separated with large molecules being eluted first and small molecules being eluted later. The separation is measured by a suitable detector as the sample is eluted, and a chromatogram showing concentration, refractive index, viscosity, or laser light scatter against elution time is obtained.

Since GPC gives information of the size of the molecule in solution and not the molecular weight of a polymer, the apparatus must be calibrated in order to give the relationship between elution time and molecular weight. The conversion is straightforward if polymer standards of known molecular weights are available for the polymer under investigation. In this instance, a calibration curve for the experimental set up of molecular weight against elution time can be constructed using the standards. This then allows the molecular weight of the sample to be determined from the measured elution time. This calibration only holds if the standards and the sample have the same structure such as the degree of branching for example. However, if the sample being measured is of an

unknown polymer or a polymer for which suitable standards are not available, then this method of calibration cannot be used. Instead, a "universal calibration" curve for the experimental set up must be constructed (Benoit, 1967).

According to Einstein's viscosity law (equation 1) the hydrodynamic volume (Vh) of a molecule in solution is proportional to the product of its molecular weight and intrinsic viscosity (η).

$$V_h = (2/5)[\eta]M/N_A \qquad (1)$$

Vh is the hydrodynamic volume, M is the molar mass of the polymer (mass per mol) and N_A is the Avogadro constant.

In GPC the hydrodynamic volume of a species determines its retention on the column and is therefore related to its elution volume. A universal calibration plot for the experimental set up can be obtained by plotting the product of molecular weight and intrinsic viscosity against the elution time. The universal plot although specific for a particular experimental set up, solvent, column and temperature will hold for any polymer tested under that set-up regardless of its chemical nature or morphological structure. The need for standards specific to the polymer under test is removed, and the molecular weight of unknown or novel polymers can be determined once the elution time and intrinsic viscosity of a sample is known.

GPC yields information on the molecular weight distribution of a polymer sample. Mn and Mw values are frequently used to describe the changes occurring to a polymer. Mn is the number average molar mass and is defined as the sum of the products of the molar mass of each fraction multiplied by its mole fraction. The Mw value is the weight average molar mass and is defined as the sum of the products of the molar mass of each fraction multiplied by its weight fraction. The ratio Mw/Mn must by definition be greater than 1 for a polydispersed polymer and is known as the polydispersity or heterogeneity index. A perfectly monodispersed polymer would have a polydispersity index of 1. This value is often used as a measure of the breadth of the molar mass distribution.

Many groups have used GPC and SEM to analyse the surface changes occurring degradation and molecular weight changes occurring to the polymer during degradation. Albertsson and Ljungquist (1981) used SEM to determine changes in the polymer structure and used the Mn values from GPC to determine molecular weight changes. However a range of methods can be used for characterising polymers and their degradation, these are:

a) DSC- differential scanning calorimetry
b) IR- infrared spectroscopy to analyse the chemical composition of the bulk of the polymers.
c) MIR-FTIR, Multiple Internal Reflection Fourier Transform Infra-Red spectroscopy and ESCA, Electron Spectroscopy for Chemical Analysis, to analyse the chemical composition of the surface of the polymers.
d) Proton and carbon nuclear magnetic resonance spectroscopy, to analyse the chemical composition of the bulk of the polymers.
e) SEM- Scanning electron microscopy
f) Mechanical testing for tensile strengths and mechanical properties.

The general degradation mechanisms of a range of aliphatic polyesters have been elucidated (Vert *et al* 1992; Li, 1990; Knowles and Hastings, 1992) but the exact degradation rates for different formulations and, the concentrations of species released when these polymers degrade *in-vivo* are not known.

The aim of these experiments was to study the degradation of five resorbable biomaterials and to use the information obtained to investigate the biocompatibility of "as cast" and degraded materials *in-vitro*. Initially polymer samples were incubated in various solutions to determine water uptake and mass loss over the study period. The effect of various enzyme solutions on degradation was investigated, in order to determine the potential use of enzymes to artificially degrade polymers. Surface degradation of the samples was monitored by surface examination using scanning electron microscopy (SEM). GPC was used to analyse the molecular weight changes occurring in the polymers which had undergone storage in various solutions including the enzyme solutions. The effect of storage at room temperature without desiccation was determined over a 20 month period to measure the molecular weight loss with incorrect storage. The release of monomers from PHB and PLA was measured to test if the amounts released could be quantified and later used to determine the correct concentrations to use for *in-vitro* testing. The accelerated degradation of the polymers could be useful in the long term biocompatibility testing of biomaterials.

MATERIALS AND METHODS

The materials used in this study are shown in table 2.1

Table 2.1: Polymer specifications.

Polymer	Presentation	Supplier	Nominal molecular weight or Intrinsic viscosity
Polylactic acid (PLA)	Granules	J & J	2.01 dl/g
Polylactic acid	bars	J & J	1.88 dl/g
Polyglycolic acid (PGA)	bars	J & J	1.30 dl/g
Polycaprolactone (PCL)	pellets	Aldrich	72,000
Polyhydroxybutyrate (PHB)	powder	Aldrich	539,000
Polyhydroxybutyrate	sheets (1x1 cm)	Biopolymers	530,000
Polyhydroxybutyrate-hydroxyvalerate copolymer (PHB-PHV)	Powder	Aldrich	400,000-750,000

Preparation of polymer films

The polymers in powder form and granule form were dissolved in chloroform in the concentrations shown in table 2.2. These concentrations were chosen as they gave optimal casting solutions and also the films, upon drying, were easily cut into the disks of the correct size. PCL and PLA granules were easily soluble in chloroform at 37°C whereas PHB and PHB-PHV were not easily soluble at 37°C therefore they were refluxed at 78°C for 4 hours prior to casting. The PGA granules were not soluble in any suitable solvents and therefore could not be cast into films.

Table 2.2: Polymer concentrations for casting films

Polymer	Concentration (w/v)	Temperature	Refluxed
PLA granules	4%	37°C	No
PHB powder	3%	78°C	Yes
PHB-PHV copolymer	3%	78°C	Yes
PCL pellets	7%	37°C	No

All the polymers PLA, PCL, PHB and PHB-PHV were cast onto glass slides at room temperature. To do this, a thin film of polymer solution was pipetted uniformly, using a glass pasteur pipette, onto a clean glass slide and covered with a glass petri dish for 24 hours to allow uniform drying of the film. After 24 hours, the petri dishes were removed and the films were allowed to air dry for at least 48 hours to ensure maximum removal of chloroform. Once dry, the films were easily removed from the glass surface and cut into disks 15 mm in diameter using a cork borer. Immediately prior to use for tissue culture the films were washed in 70% and 100% ethanol and then dried.

Weight change of polymers during incubation in various solutions

Where possible bars/sheets (1 x 1 x 0.2 cm) were used in the weight change experiments as these were easy to handle and their high initial weight made changes in weight easily detectable as compared to films. Bars of PLA and PGA and squares of PHB were placed into 20ml universals with 10mls of the incubating solutions separately as listed in table 2.3. The universals were stored at 37°C on a rotating mixer for the duration of the experiment.

Table 2.3: Incubating solutions

Solution	Supplier	Activity	pH at start
Phosphate Buffered Saline (PBS)	SIGMA	NA	7.4
Distilled water	ELGA	NA	6.5
Trypsin in PBS	SIGMA	10 U/ml	7.3
Papain in PBS	SIGMA	1 U/ml	6.2
Pepsin in PBS	SIGMA	10 U/ml	5.8

After various time intervals the samples were removed from their storage solutions, dried by dabbing gently on filter paper, and then weighed immediately. After weighing the samples were replaced in fresh storage solution. The samples were out of their storage solutions for a maximum of two minutes during the weighing process.

Measurement of changes in pH of storage solutions

The storage solutions in each universal were changed every 48 hours initially and then, after the second week, once a week. Measurements of the pH of the solutions were made prior to changing using a Corning pH meter 240 which was calibrated using pH 4 and 7 buffer solutions (Philip Harris Scientific). The solutions were stored at -20°C for further analysis.

Measurement of degradation products

The solutions obtained from the degradation experiments were analysed for their lactic acid and D3-hydroxybutyric acid content using enzyme based kits. The kits were obtained from Boeringer Mannheim (Lewes, E. Sussex, UK) for the colorimetric determination of D-3-hydroxybutyric acid and a UV method for the determination of L-lactic acid. The kits were modified for use in 96 well plates allowing more samples to be analysed.

Lactic acid determination

Any L-lactic acid present in the test solution was oxidised by nicotinamide adenine dinucleotide (NAD) in the presence of L-lactate dehydrogenase (L-LDH) to pyruvate and the reduced form of nicotinamide adenine dinucleotide (NADH). The pyruvate produced is trapped in a reaction catalysed by glutamate pyruvate transaminase (GPT) in the presence of L-glutamine. The concentration of NADH was determined by the absorbance measured at 340 nm UV range. This allows the quantity of L-Lactic acid in the test solution to be determined.

D3-Hydroxybutyric acid determination

The D3-hydroxybutyric acid in the presence of the enzyme 3-hydroxybutyrate dehydrogenase (3-HBDH) is oxidised by NAD to acetoacetate and NADH. In a reaction catalysed by diaphorase the NADH formed converts iodonitrotetrazolium chloride (INT) into a formazan which is measured at 470 nm.

Degradation of PLA and PCL granules for molecular weight analysis

PLA granules and PCL pellets (0.1g ± 0.01g) were placed into universals with 10 mls of incubating solutions as listed in table 2.3 for 10 days and 20 days. After this time period the granules were dried at 37°C and the polymer molecular weight determined using the method described below. The pellets and granules were used as supplied by the manufacturers and which had been stored in a desiccator prior to analysis.

Molecular weight measurements using GPC

Samples for molecular weight analysis were dissolved in chloroform to give samples of a known concentration (± 0.001g/cm^3). The GPC experimental set up used tetrahydrofuran (THF) as the mobile phase with a flow rate of 1.0 ml/min in a column (8 x 300 mm) packed with polystyrene gel. Solutions of known concentrations of polystyrene standards

from 3K-300 MK obtained from the Commission of the European Community, dissolved in chloroform, were used to obtain the universal calibration curve. Although the standards were soluble in tetrahydrofuran, PLA and PCL were not as readily soluble, thus it was necessary to use chloroform as the sample solvent to establish the universal calibration plot. PLA and PCL samples were the only polymers that could be analysed by GPC. PHB, PGA and PHB-PHV were not soluble in any suitable solvents thus could not be included in these studies.

Detection of the samples as they passed out of the column was achieved using a differential refractometer-viscometer detector (Viscotek 200) which simultaneously measures the viscosity of a solution and its concentration (by refractive index). This detector was designed specifically for use with the universal calibration technique. In the results section the quoted molecular weight values are the Mw values and not the Mn or Mz values unless specified.

Quantitative analysis of surface changes

Degradation of the films was monitored by surface analysis. Cast films of PCL, PHB and PHB-PHV were stored in the various solutions as made up for the degradation studies. After various time intervals two films selected at random were removed from storage, rinsed briefly in water, air dried and mounted onto a stub. The mounted films were then coated with gold and viewed by a Joel SEM.

Statistics

The results obtained were analysed using appropriate statistical tests. The data sets were tested for normality using the Shapiro-Wilk W test. If p values obtained were less than 0.05 the data was not normal. If the data was normal (parametric) an analysis of variance was used, and if the data was not normal (non-parametric) the Mann-Whitney test was used. Depending on the results from these tests a multiple comparison test was carried out, the Tukey Kramer-Honestly Significant Difference test (TK-HSD). All tests were carried out using alpha values of 0.05 and 0.01 and significance was denoted * for significance at $p<0.05$ and ** for significance at $p<0.01$. Where multiple comparison was not required and comparisons could be made to a control the Dunnett's test was carried out at alpha values of 0.05 and 0.01 and significance was denoted as above. Some data sets could not be analysed due to small sample sizes.

RESULTS

The results section for this chapter is divided into five sections. The first section discusses the surface changes occurring to the polymer films after incubation in various solutions for different time periods. This is followed by a second section where molecular weight changes occurring to granules and pellets of PLA and PCL incubated in the solutions for 10 and 20 days are described. Results from a long term degradation study on the molecular weight changes occurring to PLA stored in four solutions over 20 months are also presented. Section three shows the bulk mass changes occurring to the PLA, PGA and PHB squares when stored in solutions and section four looks at the pH changes taking place in the solutions that the polymers are stored in. Finally section five measures the release of monomers into the solutions and quantifies some qualitative results observed on the solutions stored for monomer release measurements.

1. Surface degradation of polymer films

Five polymer films were stored in each universal containing 10 mls of the incubating solutions. Samples were analysed in duplicate at each test period. The scanning electron micrographs of the polymers incubated in the different solutions are shown in figure sets 2.1, 2.2 and 2.3. Figures 2.1a and 2.2a show the 'as cast" polymer surfaces. The as cast PCL surface (2.1a) consisted of spherulites ranging between 30-60 μm in diameter while the PHB surface (2.2a) was very grainy and rough with the particle sizes being less than 20 μm. Upon incubation of PCL with trypsin for six weeks (figure 2.1b) the surface became hydrated with the spherulites increasing in size and the rough surface becoming smoother. The size of the spherulites increased to 100-200μm in diameter. Following incubation of PCL in papain for six weeks (figure 2.1c) the surface was again different compared to figure 2.1a and 2.1b. The spherulites were still 100-200μm in diameter but they were no longer "swollen" and had become flat and smooth. There were also gaps prevading throughout the surface in between the spherulites.

PHB films incubated with the trypsin and papain after 6 weeks were also examined but no degradation of the surfaces was noted except a smoothing of the granular surface (SEM not included). Degradation after incubation in trypsin after 24 weeks was, however, observed (figure 2.2c). The surface of dry as cast PHB at a higher magnification was included as a control. PHB incubated with trypsin (figure 2.2c) after 24 weeks showed there were gaps present on the polymer surface giving it a lacy appearance and the smooth areas as seen in figure 2.2b were no longer prevalent. PHB incubated in papain for 24 weeks (SEM not shown) did not show significant degradation compared to PHB in trypsin.

The surfaces of PHB-PHV stored in the trypsin and papain solutions are shown in figures 2.3c-2.3f, which were compared to the "as cast' surfaces figures 2.3a and 2.3b. Two different surfaces of the "as cast" polymer were observed for this inspite of attempts to standardise the casting method. One structure was very lacy (figure 2.3a)while the other was smooth and had no gaps on the surface (figure 2.3b). Upon incubation of PHB-PHV with trypsin for 19 weeks degradation of the surface had occurred (figure 2.3c) with the surface appearing granular rather than lacy. Upon incubation with papain (figure 2.3d) for 19 weeks degradation had also occurred with a few holes appearing on the surface. The films incubated in trypsin (figure 2.3e) and papain (figure 2.3f) after 24 weeks had undergone further degradation with the surfaces becoming more porous and rough. Compaison between the different solutions could not be made as the starting surface of this polymer was so variable. Incubation in other solutions also resulted in degradation and these SEMs are not shown. Upon incubation of PHB-PHV with PBS degradation also occurred after 19 weeks where holes appearted on the surface but this degradation was minimal compared to the enzyme stored polymers.

The action of the enzymes, distilled water and PBS on the polymers varied with material and exposure time. The different surfaces observed with the different enzymes, the distilled water and PBS suggest that enzymes were able to influence the degradation of the polymer surfaces. In this study it was found that the presence of an aqueous environment affected the surfaces of all three polymers. PCL was degraded most by papain; in contrast trypsin had the greatest effect on PHB and PHB-PHV.

Figure 2.1a: The PCL surface six weeks after casting and storage in a dessicator at room temperature; note the rough apperance of the spherulites which also varied in size.

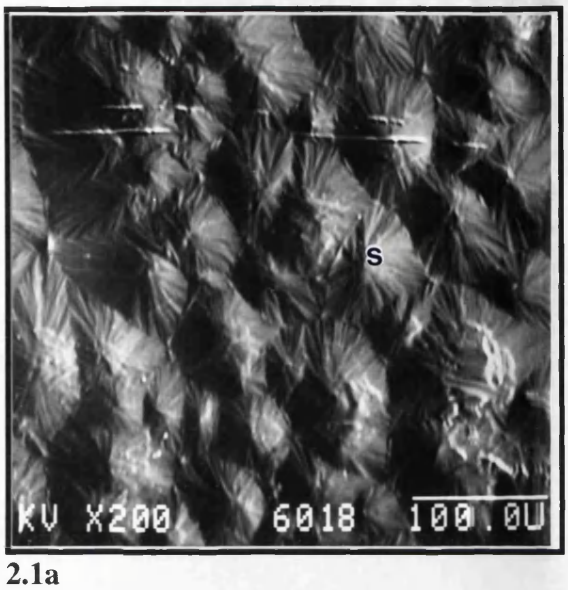
Figure 2.1b: The PCL surface after incubation in trypsin for 6 weeks; note the spherulites which had become smooth and enlarged.

Figure 2.1c: The PCL surface after 6 weeks in papain; note the spherulites which had flat surfaces and gaps in between the spherulites.

Figure 2.2a: The surface of "as cast" PHB; note the granular surface of this polymer compared to PCL.

Figure 2.2b: The surface of "as cast" PHB at a higher magnification (control for figure 2.2c).

Figure 2.2c: The surface of PHB after storage in trypsin for 24 weeks.



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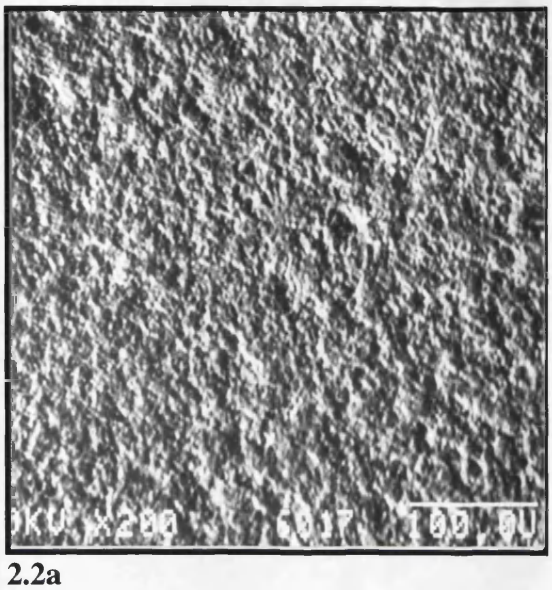

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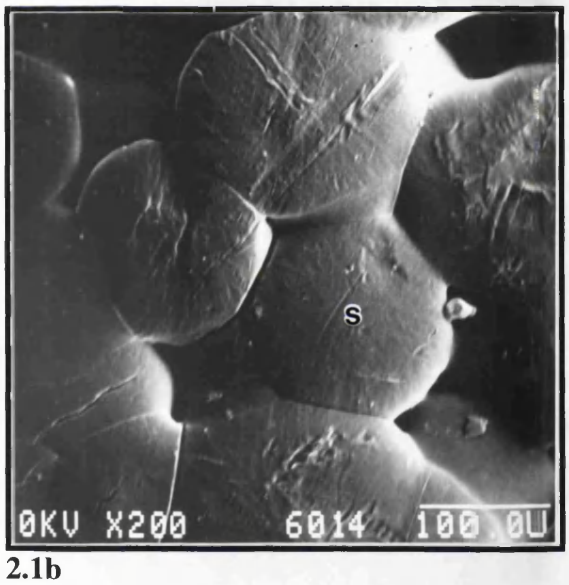
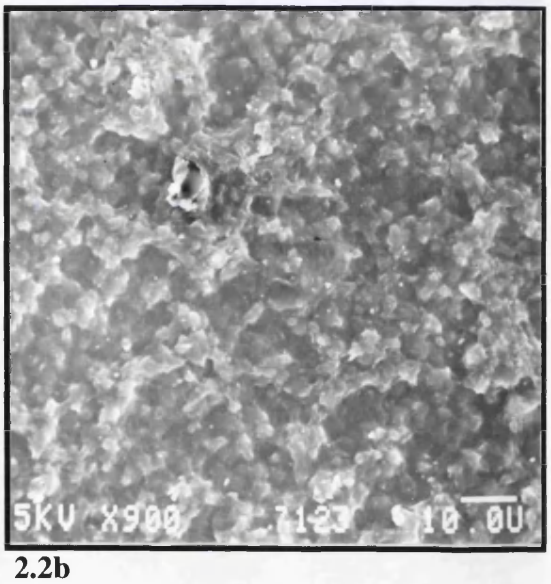
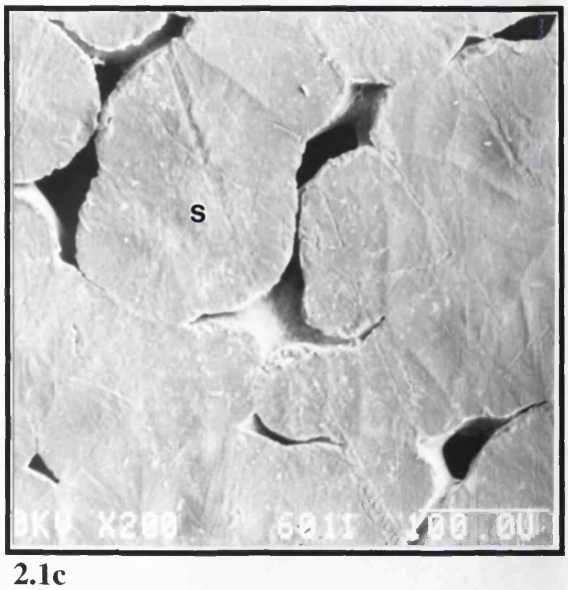


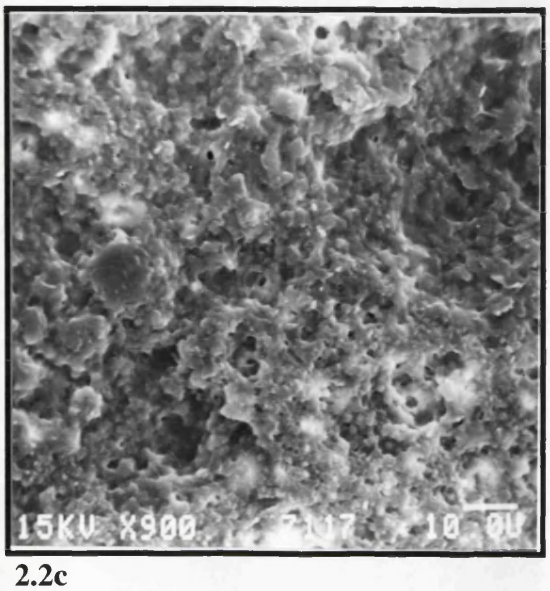
2.1b



2.2b



2.1c



2.2c

Figure 2.3a: The surface of "as cast" PHB-PHV showing a lacy surface with many gaps.

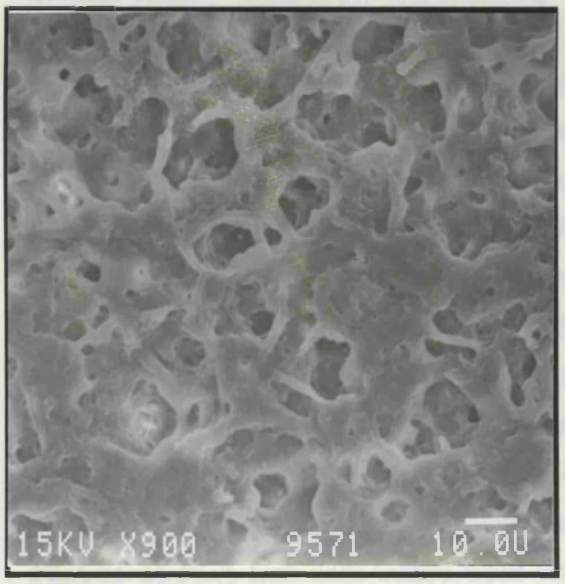
Figure 2.3b: The surface of "as cast" PHB-PHV showing a smooth surface; note the difference in the surface compared to figure 2.3a.

Figure 2.3c: PHB-PHV incubated in trypsin after 19 weeks; degradation had occurred with the surface appearing lacy.

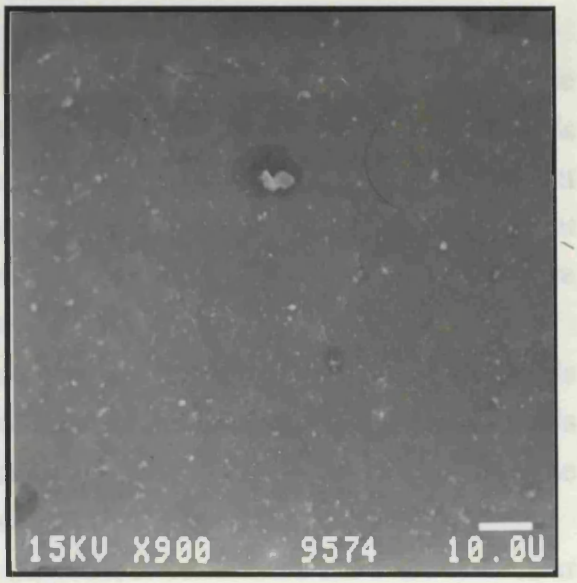
Figure 2.3d: PHB-PHV in papain after 19 weeks; note the surface which had undergone degradation with small spherulites appearing.

Figure 2.3e: PHB-PHV in trypsin after 24 weeks; very little change in surface structure had occurred compared to figure 2.3c.

Figure 2.3f: PHB-PHV in papain after 24 weeks; further degradation of the surface had occurred with a longer incubation in papain; more spherulites were visible with gaps present in between the spherulites.



2.3a



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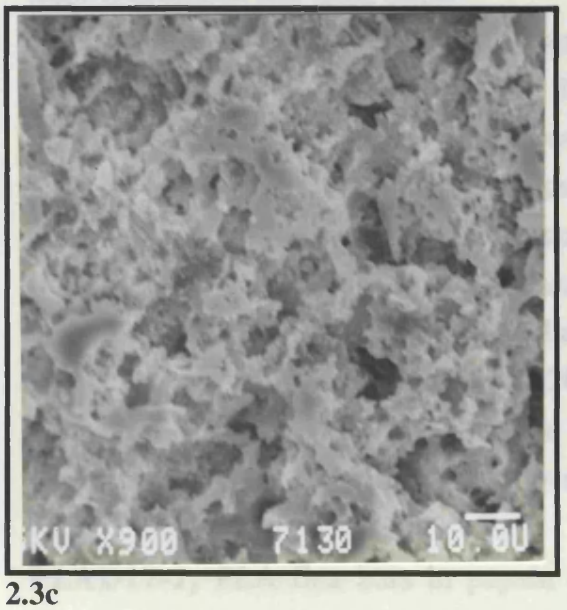

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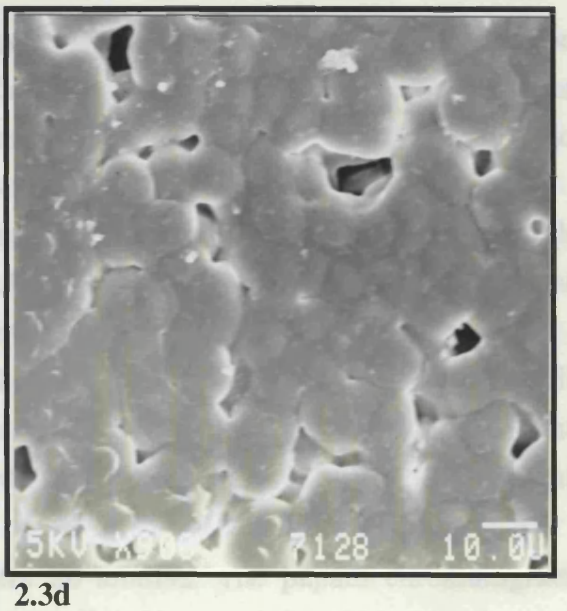

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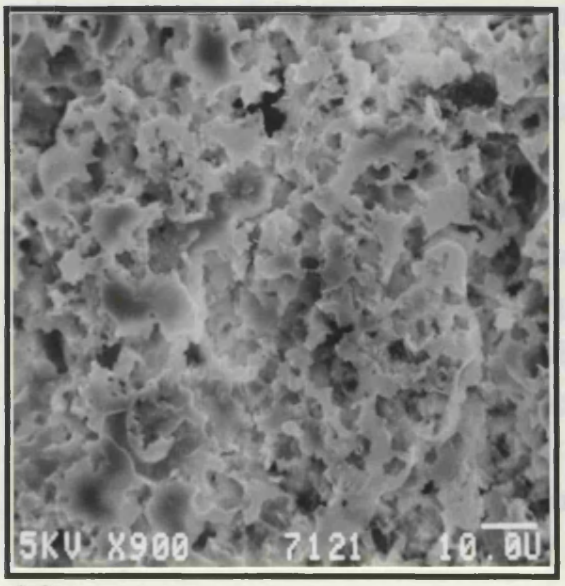

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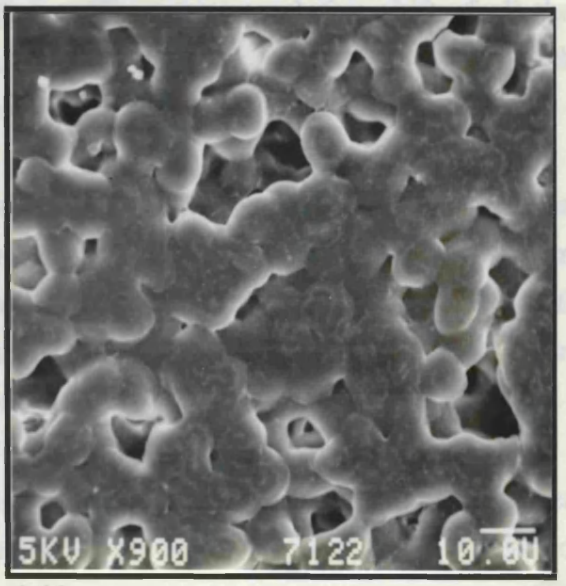


2.3f

2: Molecular weight determined by GPC.

The quoted molecular weight values in the results section are the Mw values and not the Mn or Mz values unless specified. The Mark-Houwink plot of the polystyrene standards which was used to obtain the universal calibration curve for molecular weight determinations is shown in figure 2.4a. An overlay of the different molecular weight distributions obtained for PLA after different storage conditions can be seen in figure 2.4b and individual distributions are as follows.

PLAfx-1; PLA flex bars which had been stored in a desiccator for 20 months. This chromatogram shows a uniform narrow peak. The molecular weight distribution thus is narrow with a slight tail at the lower end of the molecular weight range suggesting the presence of shorter molecular weight chains in the sample.

PLAd1-1; PLA flex bars which were stored without desiccation show the molecular weight distribution to be broader because the chromatogram is wider. This indicates that some degradation had taken place.

PLAD5; PLA flex bars in PBS for 20 months resulted in a chromatogram whose shape had changed and now showed two peaks. This may be due to the degradation mechanism proposed by Vert *et al* (1994) who showed that the bimodal degradation of aliphatic polyesters resulted in a chromatogram with two peaks.

PLAD9-1; PLA flex bars in distilled water for 20 months showed a chromatogram with a slight hint of bimodal degradation and the broadening of the molecular weight distribution of the polymer appeared similar to that of PBS (PLAD5).

PLAd14-1; PLA flex bars in trypsin for 20 months. This chromatogram showed the tail to be at the higher end of the molecular weight range. The overall molecular weight distribution, however, had shifted towards the lower end of the molecular weight indicating that degradation had occurred.

PLAD17-1; PLA flex bars in papain for 20 months. The papain chromatogram appeared similar to the trypsin chromatogram. This suggested that the mechanisms of degradation by the two enzymes were similar. There was a relative shift of all the chromatograms stored in the solutions towards the smaller molecular weight range which suggested an overall reduction in molecular weight.

The GPC results clearly demonstrate differences in the degradation following incubation in the various storage conditions. Molecular weight determinations following incubation in various solutions of untreated and post gamma irradiated PLA granules after 10 and 20 days are shown in figures 2.5 and 2.6. Each time point on the graphs represents the mean of four values. The errors bars are expressed as the percent standard error of the means.

Figure 2.5 shows the molecular weight changes occurring to PLA granules after incubation in various solutions after 10 and 20 days. The Dunnetts test was used to

determine significant differences between the test samples and the controls. PLA granules after 10 days in PBS and distilled water at 60°C showed a significant decrease in molecular weight. None of the other solutions caused a significant decrease in the molecular weight of the PLA granules after 10 days incubation. After 20 days a significant reduction in molecular weight was observed for PLA granules stored in PBS and distilled water at 60°C and for PLA granules stored in lipase at 37°C. For each individual treatments a t test was carried out to determine if there had been a significant change in molecular weight between 10 and 20 days. A significant difference was only observed for incubation in lipase where a significant decrease in molecular weight was seen after 20 days compared to 10 days ($p < 0.05$).

Gamma irradiated PLA stored in PBS and distilled water at 60°C showed the most dramatic decrease in molecular weight. Although gamma-irradiated PLA and autoclaved PLA showed a significant reduction in the molecular weight compared to the dry PLA, the reduction observed was not as large as that for the 60°C treatment. The enzyme solutions at 10 and 20 days did not show a significant difference compared to the dry control and the gamma irradiated polymers. However, a higher percent standard error of the means for all the enzyme treated polymers was observed. This suggests that, although the average molecular weight did not decreasing significantly, some degradation was occurring resulting in a spreading of the weight distribution. Increasing the exposure time of the polymers for a further 10 days resulted in a decrease in the molecular weight of the polymer with all the solutions tested following a similar pattern to that at 10 days but now with a significant decrease from PLA stored in lipase (Fig 2.6). The most dramatic decrease in molecular weight occurred with PLA granules stored at 60°C in PBS and distilled water after 10 and 20 days. There was also a significant reduction in molecular weight of the PLA granules stored in lipase after 20 days. No other significant differences were observed; even though distilled water at 37°C caused a dramatic decrease it was not significant as the sample number was too small.

The results for the long term (20 months) degradation of PLA in various solutions are shown in figure 2.7. This figure also illustrates the effect of polymer storage at room temperature without desiccation. Again each point on the graph is a mean of four values, and the error bars are the percentage standard deviation of the mean (figure 2.7). A significant reduction in molecular weight of all the samples occurred compared to the dry bars both desiccated and not desiccated. The control bars were the bars which were placed in universals at 37°C without any solutions. The dry bars, however, were stored at room temperature in a desiccator. The significant drop in molecular weight between the control bars and the dry bars could be due to storage at elevated temperature as well as storage without desiccation. The results showed a significant drop in the molecular weight of the dry polymer at 37°C stored without desiccation (Control) over the period of

the test. There was a significant difference in the molecular weight for the control as compared to the dry bars and the bars stored in the solutions. There was no significant difference, however, between the molecular weight of the sample stored in each of the four solutions. Significant differences in the molecular weight were observed if the data for the dry bars was removed, with the molecular weight for the PBS stored sample being significantly higher than those stored in distilled water or papain.

The results for molecular weight determinations for PCL pellets after storage in solutions for 10 and 20 days are shown in figures 2.8 and 2.9. These two figures show a very different profile to those for PLA granules which had been stored in the same solutions. Neither gamma irradiation nor storage in enzyme solutions decreased the molecular weight of the pellets when compared to the dry stored samples. Incubation at 60°C did decrease the molecular weight of the pellets for both 10 days and 20 days incubation. The molecular weight after storage in distilled water at 60°C for 20 days was significantly lower than after storage in PBS at 60°C for 20 days. The overall molecular weight pattern at 20 days was similar to that at 10 days, but with PCL pellets stored in water and PBS at 60°C showing a further decrease in molecular weight. Statistical analysis shows a significant decrease in molecular weight of the polymer stored in PBS and distilled water at 60°C. No other significant differences were observed. Gamma-irradiated polymers were not significantly different to the non-irradiated polymer.

Figure 2.4a: The Mark-Houwink plot used to generate the universal calibration curve for experimental set-up. Polystyrene standards were dissolved in chloroform, with the mobile phase THF, run at a flow rate of 1ml/min.

Figure 2.4b: Molecular weight chromatograms of PLA.

PLAfx-1: PLA flex bars which have been stored in a desiccator for 20 months.

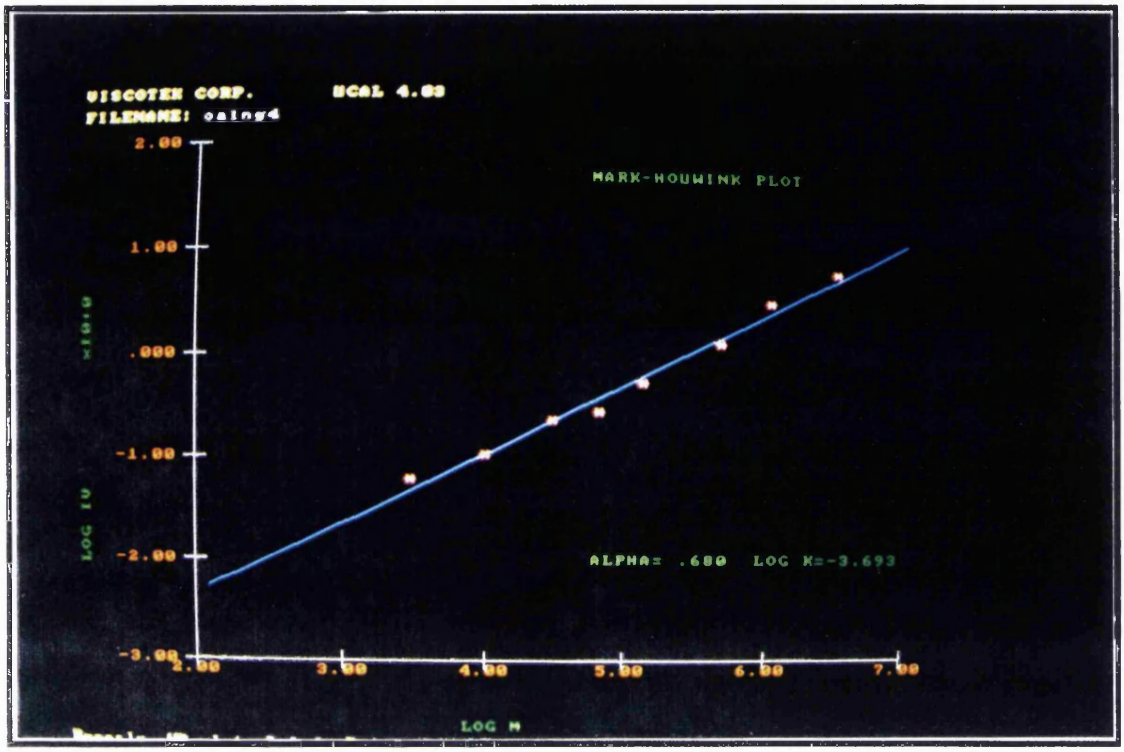
PLAd1-1: PLA flex bars in universals but without solutions at 37°C (controls).

PLAD5: PLA flex bars in PBS for 20 months.

PLAD9-1: PLA flex bars in distilled water for 20 months.

PLAd14-1: PLA flex bars in trypsin for 20 months.

PLAD17-1: PLA flex bars in papain for 20 months.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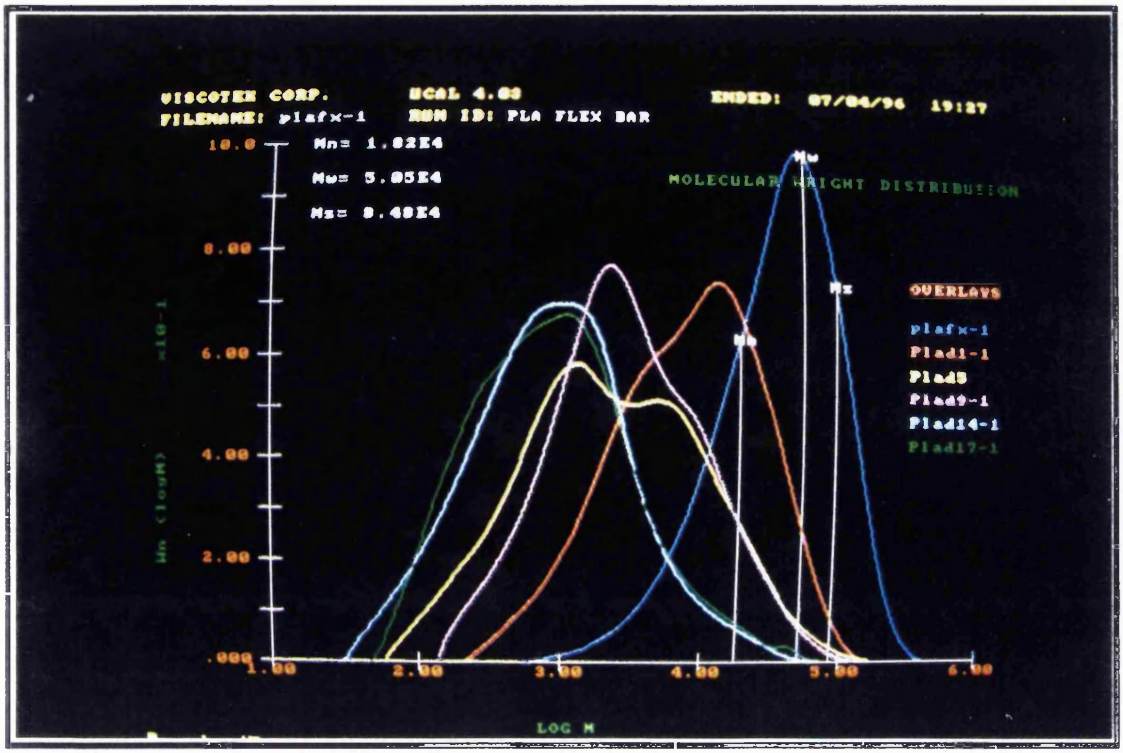
LOG IV
LOG M

2.4a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Mw= 5.85E4
Mz= 8.48E4
MOLECULAR WEIGHT DISTRIBUTION
OVERLAYS
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Pla41-1
Pla45
Pla49-1
Pla414-1
Pla417-1
LOG M

2.4b

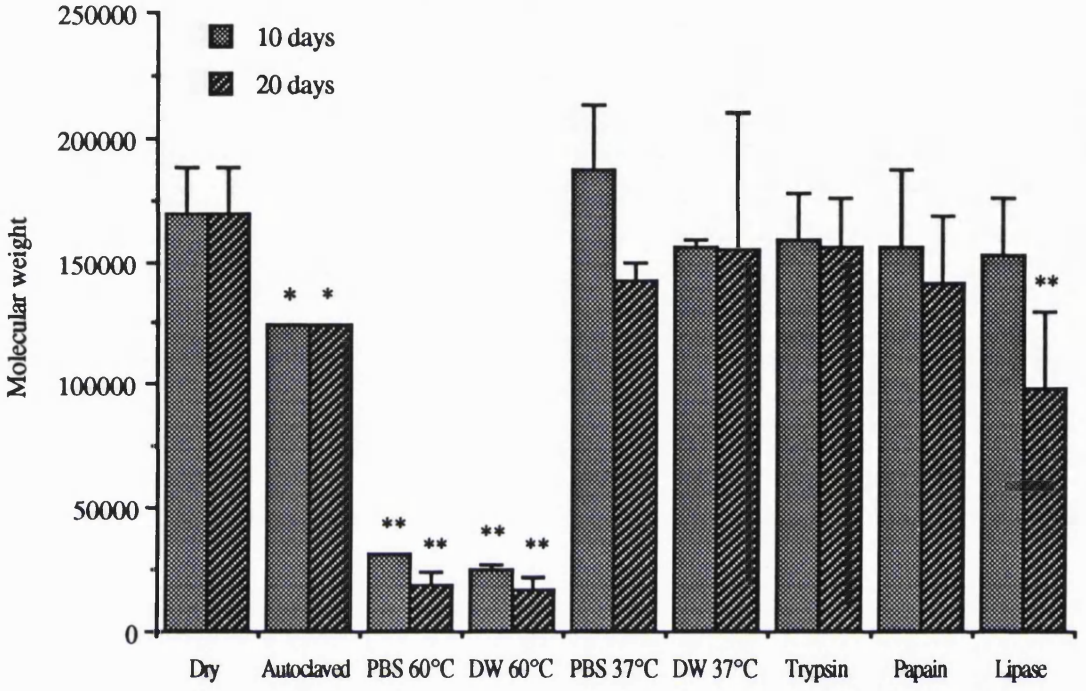


Figure 2.5 shows the molecular weights of PLA granules after incubation in various solutions for 10 days and 20 days. The Dunnetts test was used for the comparison of the test means against the mean of the control (dry), all significance is denoted by * = ($p < 0.05$) and ** = ($p < 0.01$).

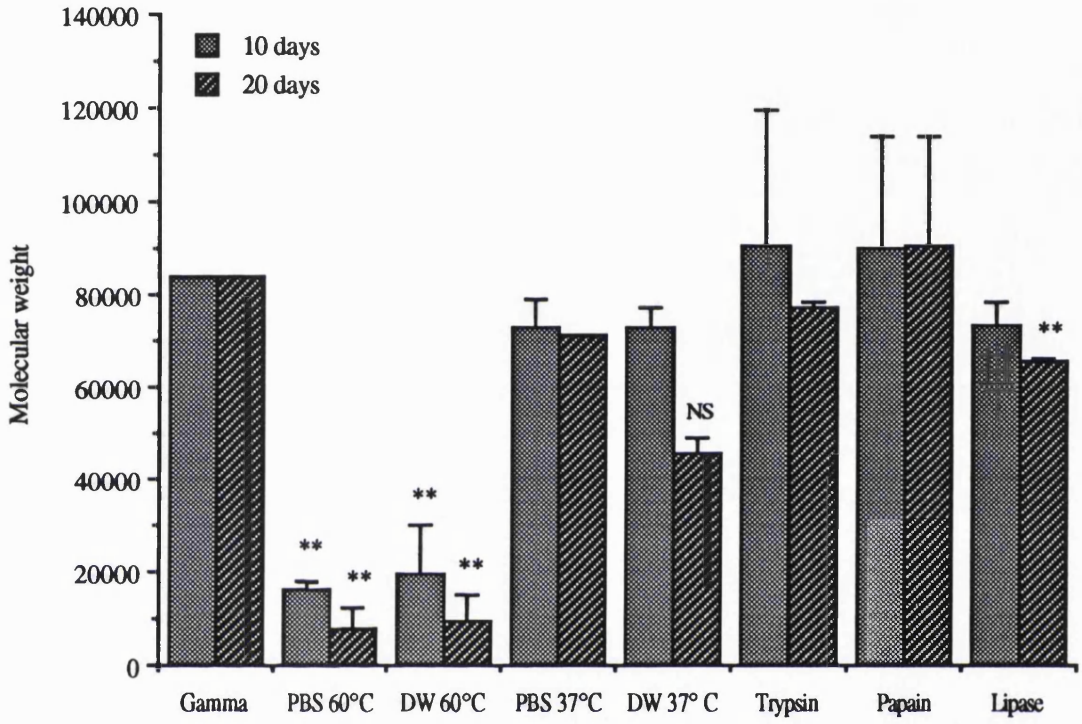


Figure 2.6 shows the molecular weight measurements of gamma-irradiated PLA granules after incubation in various solutions after 10 and 20 days. The Dunnetts test was used for the comparison of the test means against the mean of the control (gamma), all significance is denoted by * = ($p < 0.05$) and ** = ($p < 0.01$). NS = not significant due to small sample volume.

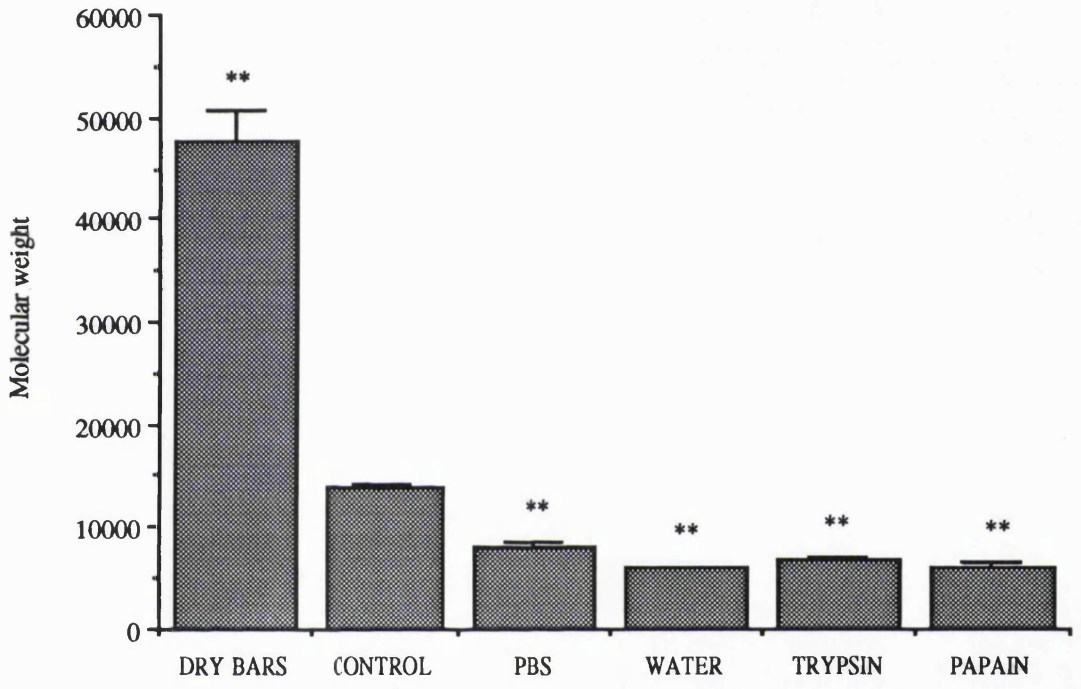


Figure 2.7 shows the change in molecular weight for PLA flex bars incubated in various solutions for 20 months. The molecular weight was measured after 20 months for the remaining polymer. The Dunnetts test was used for the comparison of the test means against the mean of the control, all significance is denoted by * = ($p < 0.05$) and ** = ($p < 0.01$).

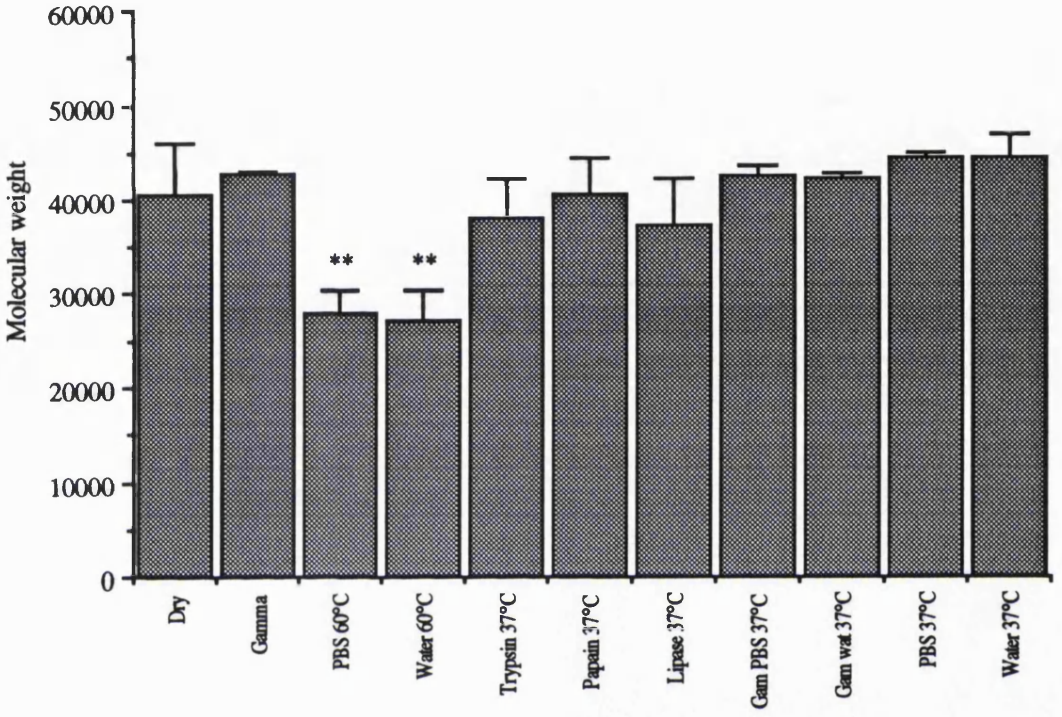


Figure 2.8 shows the changes occurring in molecular weights of PCL pellets following various treatments for 10 days. The Dunnetts test was used for the comparison of the test means against the mean of the control (dry); all significance is denoted by * = ($p < 0.05$) and ** = ($p < 0.01$).

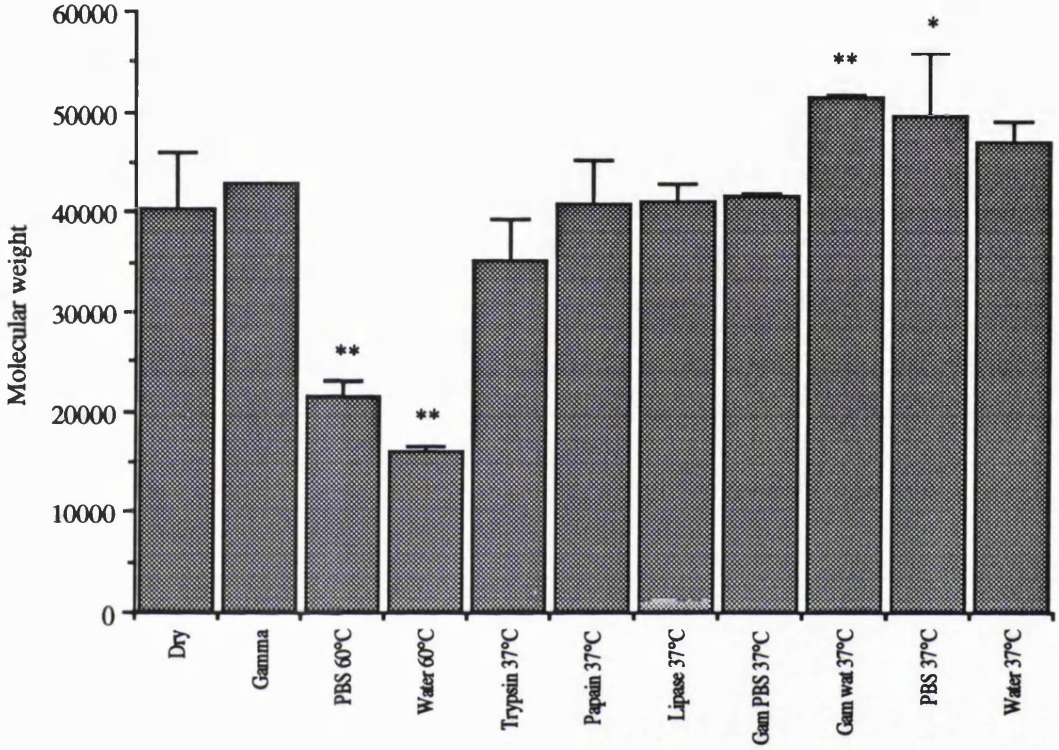


Figure 2.9 shows the changes in molecular weight changes of PCL pellets incubated in various solutions for 20 days. The Dunnetts test was used for the comparison of the test means against the mean of the control (dry); all significance is denoted by * = ($p < 0.05$) and ** = ($p < 0.01$).

3: Bulk mass changes on storage of polymers in a range of solutions.

PLA, PGA and PHB bars were stored in the various storage solutions as described previously and the change in weight of the samples was monitored over time. Each value quoted is a mean of data from three separate samples and statistical analysis was carried out using the ANOVA and the TK HSD test. The error bars are the percent standard error of the mean. The results from the weight measurements expressed as percent weight changes are shown in figures 2.10, 2.11 and 2.12.

The PLA samples stored in the solutions (figure 2.10) showed a steady increase in mass until day 157 when it appeared to equilibrate. This may not actually represent complete equilibration of the polymer but it may be the point at which degradation of the polymer commenced, so the amount of fluid absorbed was equal to the amount of polymer lost due to degradation. The dry PLA stored at 37°C had not changed in mass over the 222 day period studied. Significant differences in the measured masses started at day 15 for all the solution stored samples when compared to the dry control. No differences were observed between the PLA in the different solutions until day 222, where PLA in PBS was significantly lower than PLA stored in trypsin.

The PGA samples stored in the solutions (figure 2.11) showed an initial increase in mass but after day 15, decreased rapidly compared to the dry PGA. There was no significant weight change in the dry polymer over this time period, but PGA in all the solutions started to decrease in weight after 16 days. By day 45 the bars had lost up to 15% of their initial weight. By day 45 it became difficult to handle the material due to it breaking up, so the readings had to be stopped. The TK HSD test showed that there were no differences in weight of PGA in the different solutions until day 45. At day 45 the dry PGA was significantly higher in mass than PGA in all the solutions. There were no significant differences in behaviour in the different solutions with the exception of PGA stored with trypsin which had a smaller weight loss.

PHB (figure 2.12), in comparison, showed very little weight change on storage and no significant differences were observed between behaviour in the different solutions over the time period studied. The maximum amount of absorption was less than 2.5% of the initial polymer weight, whereas for the PLA the polymer in distilled water had absorbed up to 45% of its initial weight by day 157. For PGA it was difficult to measure this as the polymer started to degrade very quickly.

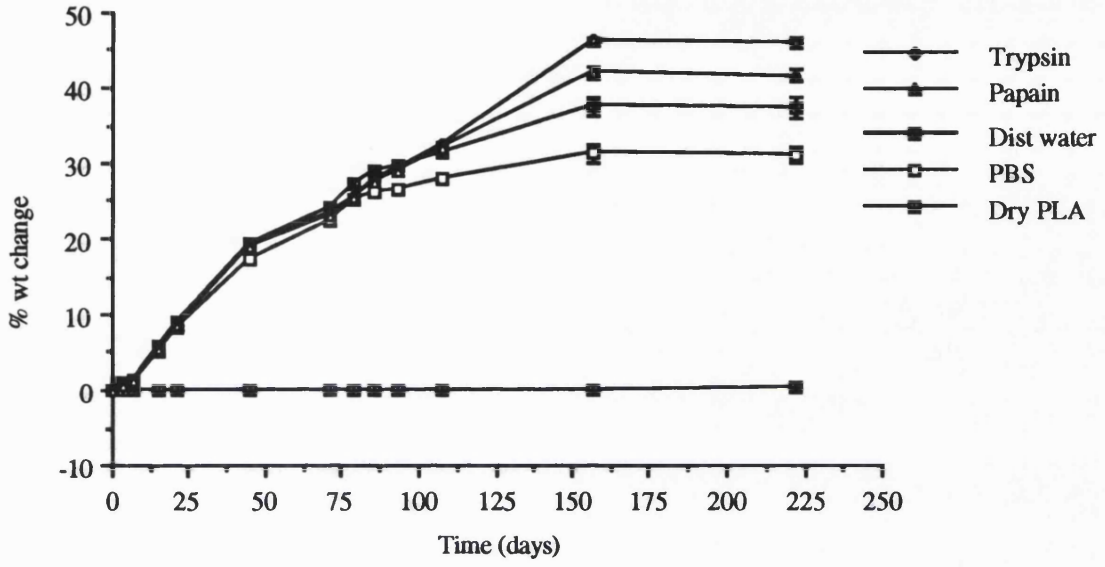


Figure 2.10 Weight change of PLA expressed as percent of initial weight in various solutions (TK-HSD P<0.05).

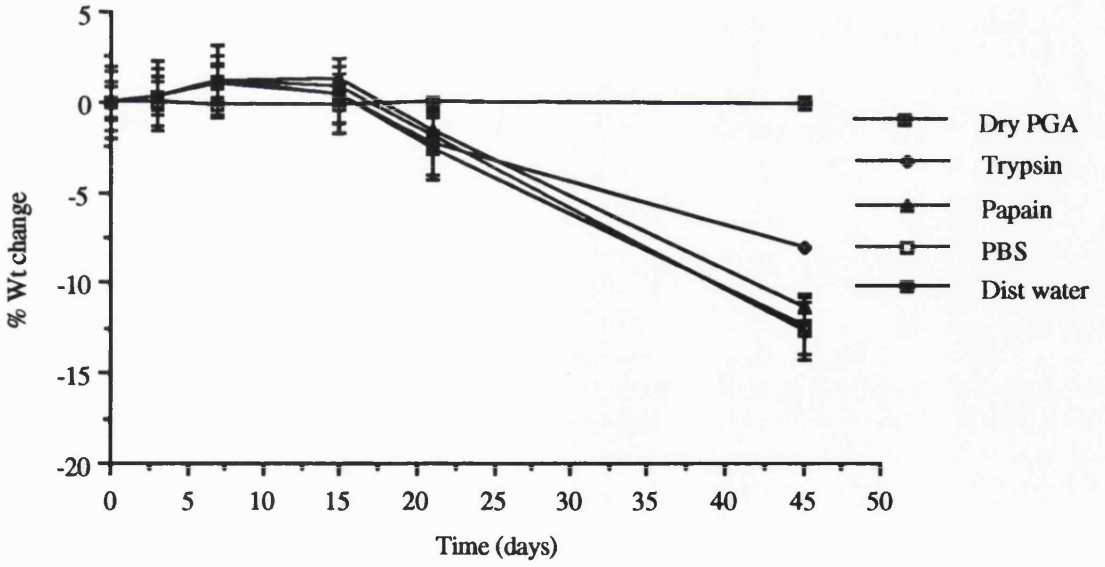


Figure 2.11 Weight change of PGA expressed as percent of initial weight in various solutions over a 45 day period TK-HSD (P < 0.05).

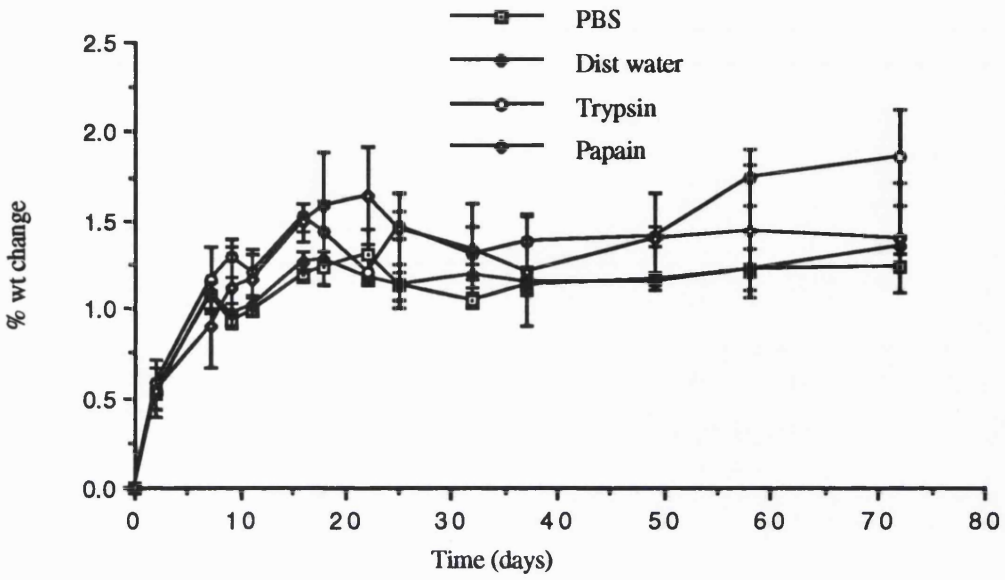


Figure 2.12 Weight change of PHB expressed as percent of initial weight in various solutions (TK-HSD $P < 0.05$).

4: pH changes of the storage solutions.

The measured pH of the incubation solutions for PLA is shown in figure 2.13. The results show a dramatic drop in pH of distilled water at day 3 which then remained at a pH of between 3 and 4 for the remainder of the study. PBS remained at approximately 7.4 for the majority of the time until day 155 where the pH of PBS dropped to between 4 and 5. The pH of the papain solution was initially about 6.4, it rose to about 7.4 for most of the study but by day 155 had also dropped to between 4 and 5. Trypsin remained at approximately 7 throughout the study.

The results for the PGA incubated in the solutions are shown in figure 2.14. It shows a profile where the pH of the distilled water dropped dramatically at day 3 and was followed by a pH drop of the other solutions at day 14. Following the rapid drop the pH remained at approximately two for the remainder of the study period.

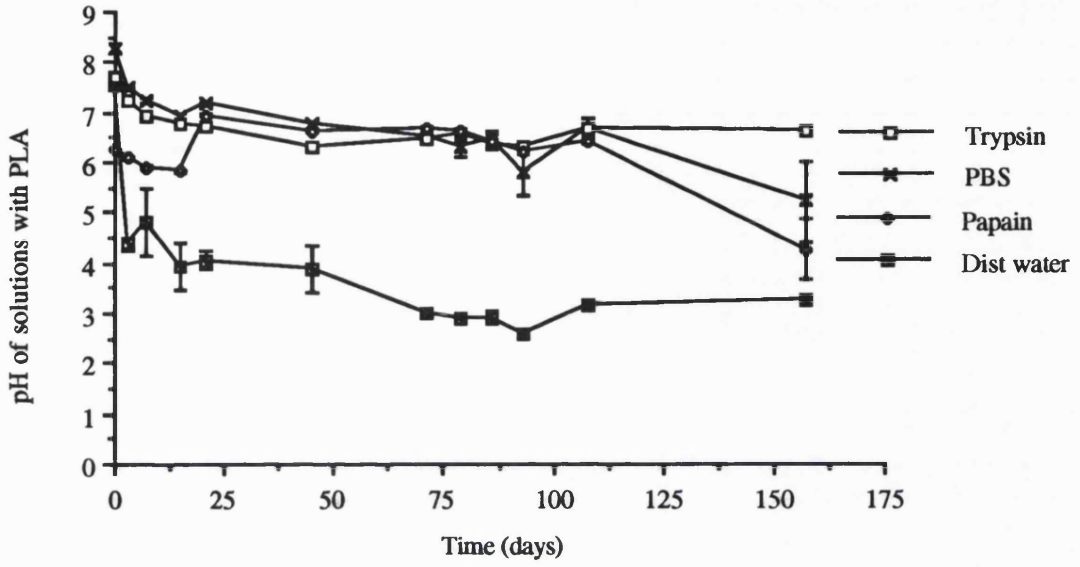


Figure 2.13 The change in pH of the solutions incubated with PLA; each point is a mean of three samples with the error bars being the percent standard error of the mean.

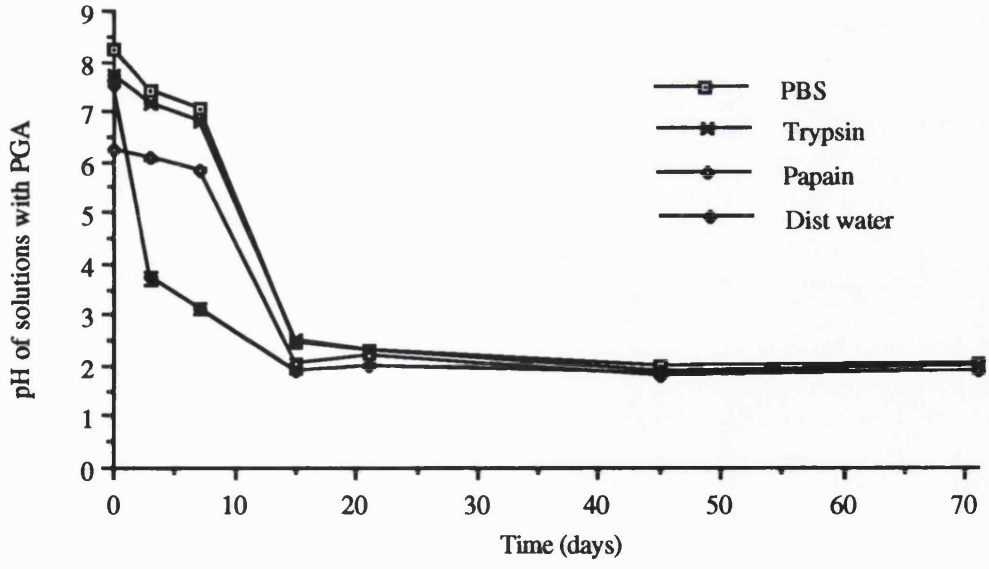


Figure 2.14 The change in pH of the solutions incubated with PGA; each point is a mean of three samples with the error bars being the percent standard error of the mean.

5: Measurement of monomer release

The release of LA or D3-HB acid from PLA and PHB was measured in all the solutions using enzyme based kits as described in the materials and methods section. The values were accumulated to give a total monomer release with time. The samples were analysed in triplicate and the results are shown in figures 2.15 and 2.16. Each point on the graph represents the mean of three separate samples. The error bars are the percent standard error of the mean. Statistical analysis could not be carried out on these results as the sample number were too small and the values could not be standardised as the initial weight, size and shape of each polymer square were not identical.

Figure 2.15 shows the accumulative release of LA from the PLA squares stored in the various incubating solutions. There was very little release from the PLA bars in any of the solutions until day 71 where some release was observed in PBS and distilled water. The release in both these solutions was the same and the release continued until day 116 when sampling stopped. With papain there was no release until day 116 after which the sampling stopped and no release was observed with incubation in trypsin.

As can be seen from figure 2.16 the release profile of D3-HB from PHB was different; PHB incubated in trypsin showed the greatest monomer release, starting at day 22 and continuing throughout the study. PHB stored in papain and distilled water remained constant after an initial release in monomer. No release was observed with incubation in PBS until day 74 where a small amount was released.

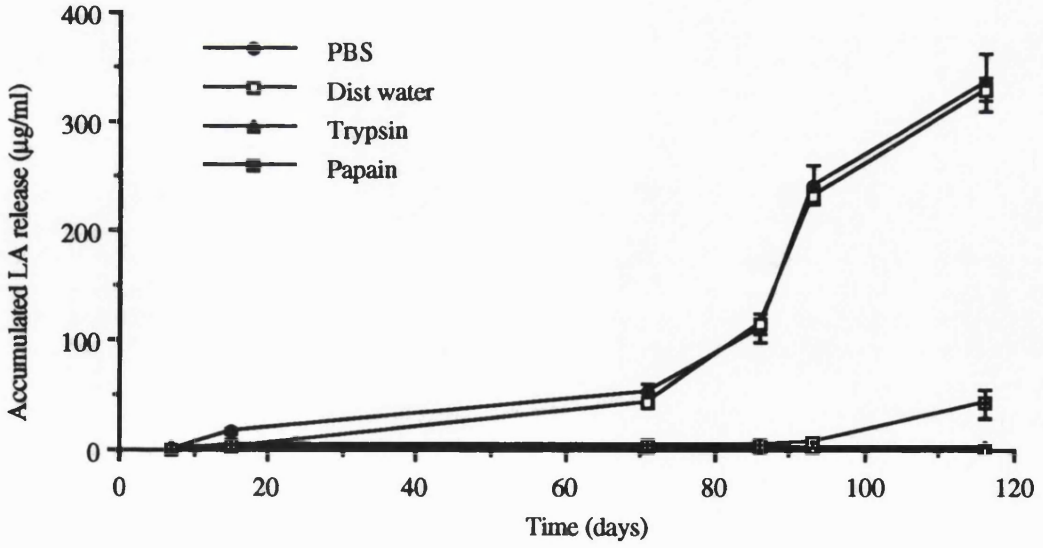


Figure 2.15 Lactic acid release from PLA into various solutions; each point is a mean of three samples analysed in triplicate.

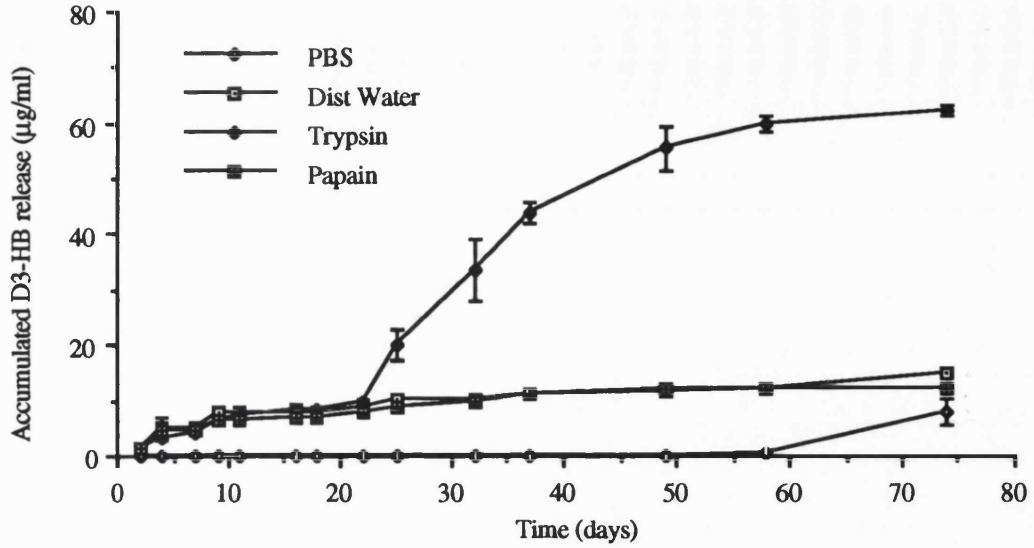


Figure 2.16 The accumulative release of D3-HB acid from PHB into various storage solutions; each point is a mean of three samples analysed in triplicate.

Qualitative observations on the degradation of PLA, PGA and PHB bars

The incubation solutions from different times of the degradation experiments were stored until required for further analysis. The analysis involved measuring the LA and D3-HB acid released from the polymers. These solutions were stored in plastic tubes at room temperature. When the solutions were tested a sediment was observed at the bottom of some of the tubes. On further examination it was seen that the sediment was present in all three tubes of a particular replicate. A qualitative evaluation was made of quantity of sediment in each tube. Tubes were scored between 0 indicating no sediment and 8 indicating the most sediment. The intermediate values were scored by eye where the highest sediment was scored a value of 8. The results of this evaluation are shown in table 2.4. It can be seen from table 2.4 that PLA in distilled water, PGA in papain and PHB in trypsin had more sediment than the other solutions. A toluidine blue stain of the particles taken from the frozen samples was carried out and no signs of bacteria were observed. The fragments were too large to be bacteria or fungi and unstained particles under polarised light microscopy showed birefringence suggesting that these were polymer fragments. The highest amount of sediment for PLA was observed for PLA stored in distilled water starting at day 21 but virtually no sediment was observed with PBS, trypsin or papain. With PGA the highest sediment release was observed with papain, starting at day 21, and with a small amount of release in PBS which increased at day 49. With PHB the highest sediment release was observed with trypsin at day 21; also negligible amounts were released with the other solutions.

Table 2.4: Sediment table

DAY	PLA PBS	PLA water	PLA Tryp	PLA Papain	PGA PBS	PGA water	PGA Tryp	PGA Papain	PHB PBS	PHB Water	PHB Tryp	PHB Papain
2	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0
22	0	8	1	0	0	0	0	8	1	1	8	2
25	1	8	1	1	1	0	0	8	1	2	8	2
32	1	8	1	1	1	0	0	8	1	2	8	2
37	0	8	0	0	1	0	0	8	1	0	8	1
49	0	8	1	1	8	0	1	8	0	0	8	0
58	0	8	0	0	3	0	0	8	1	0	8	0
74	0	8	0	0	8	0	3	8	0	0	8	0
SUM	2	56	4	3	22	0	4	56	5	5	56	7

DISCUSSION

The surface degradation of PLA, PHB and PHB-PHV films was observed over the experimental time period. Scanning micrographs of the polymer surfaces showed that surface degradation of the polymers occurred following incubation in the aqueous solutions and the enzyme solutions. Each polymer was affected to a different degree by the various solutions. PCL was degraded by papain to a greater degree than trypsin whereas PHB-PHV was affected by trypsin and papain but differences between the two could not be compared due to differences in the initial polymer surfaces. PHB was also degraded by papain and PBS after 19 weeks incubation at 37°C. Figure 2.3a showing PHB-PHV film incubated with both trypsin and PBS illustrates the degradation caused by trypsin of the surface whereas very little degradation was observed with incubation in PBS. The surface degradation of polymers is an important factor in determining the biocompatibility of the polymer. Osteoblasts are approximately 10-30 μm in diameter and when attaching and spreading over polymer surfaces undergoing degradation, they will be influenced by the presence of gaps and granules which are prevalent on the polymer surfaces. The shape and size of the polymer surfaces constantly changes and this may affect the attachment of osteoblasts and other cells. These findings were based on

qualitative observations, and molecular data could not be used to corroborate these results as PHB and PHB-PHV were insoluble in the solvents used for testing.

The data from the mass changes of PLA and PGA and PHB over various times suggests that there is a large difference in the water uptake capacities of the various polymers. Previous workers in the field have noted that reduction in the weight of the polymers lags behind loss in molecular weight (Gopferich, 1996). There is initial water absorption into the polymer causing an increase in measured bulk mass which is followed by polymer scission. This occurs in the amorphous region of the polymer resulting in a release of acidic degradation products which autocatalyse the breakdown of the bulk polymer resulting in a reduction of the bulk weight. The degradation of these polymers is affected by their hydrophilicity, which is in turn affected by their crystallinity. Differences that may occur in the water uptake would be due to the crystallinity where it would take longer for the solvent to diffuse into the crystalline regions than in the amorphous regions. The increase in mass in the uptake experiments may be attributed to water absorption or mass reduction of the polymers. Crystallinity affects the rate of polymer degradation considerably. Rozema (1992) stated that PLLA implants increased in crystallinity by 60% as a result of hydrolysis. In a semicrystalline polymer, crystalline domains are interspersed with amorphous regions; molecular chains may extend from one crystalline domain to another and are called tie molecules. In the amorphous regions the molecules are more loosely packed thus, during hydrolytic degradation, water molecules interact preferentially with the amorphous regions and, as a result, tie molecules and other molecules in the amorphous regions undergo a reduction in their chain lengths. As shorter chains are more mobile than long chains they possess a higher susceptibility for crystallisation (Li *et al* 1990). Where degradation is by hydrolysis (controlled by water uptake into the polymers) the uptake of water into the polymer is higher than the other solutions due to the osmotic pressure. This is also affected by temperature where an increase in temperature leads to an increased diffusion process which results in a faster rate of water uptake leading to faster initial degradation for polymers. Of the materials tested PGA was the fastest to disintegrate and loose its structural and mechanical integrity. PGA could not be analysed for molecular weight changes occurring in the various solutions due to its insolubility in any of the possible solvent systems.

PHB (figure 2.12) samples increase in mass by approximately 1.5% of their initial weight compared to PLA (figure 2.10) which increased by 30-43% of initial weight in the various solutions. For PGA (figure 2.11) it was difficult to determine the amount of fluid absorption because as previously stated degradation of the bulk polymer started early and the polymer started to loose weight after a few days. It is not possible to determine the point at which the polymer started to break down from mass change data as the uptake

values are a combination of both the water uptake and mass loss from the bulk polymer by degradation.

The type of buffer used for degradation studies will determine how long the pH is maintained. Therefore the quantity of degradation products produced may not necessarily be reflected by a measured change in pH. From figures 2.13 and 2.14 the pH of the PBS solutions following incubation with PLA and PGA was higher than after incubation of the polymers in distilled water. There was however, little difference in the pH of the PBS compared to the trypsin; it is unclear whether this was due to buffering or the non-release of degradation products.

The pH measurements show that PLA in distilled water released acidic degradation products as early as day 2 and maintained this release for the duration of the study. The other solutions did not show a drop in pH until day 150 but the pH still remained above 3. A reason for this could be the buffering effect of PBS against the low concentrations of monomers released. For PGA the pH drop was most rapid for the polymers incubated in distilled water. All the solutions remained at a pH of approximately two for the remaining time period of the study. The effect on pH of the degradation products and monomers was significant.

Results obtained in this laboratory study can be correlated with the sediment release to some extent. PLA showed the highest sediment release with distilled water starting at day 21 and also showed LA release in distilled water as well as PBS. PLA in PBS, however, showed no sediment accumulation. This suggests a different mode of action of the solutions on the bulk polymer. It may be that PBS was buffering the degradation process and the fluid was only able to attack the surface causing monomer release but not the breakdown of the bulk polymer causing oligomer to be released. The highest LA monomer release was observed with distilled water but a similar amount was also observed with PBS. The highest PHB monomer release was also observed with trypsin incubation, starting at day 21.

The measurement of D3-HB in serum and blood was investigated by Foster and Tighe (1995) and they concluded that there was potential for the use of this method for measuring the hydrolytic degradation of PHB. In this study it was also found that the D3-HB release from the degrading polymer was a good method for measuring the degradation rate and the effect of various solutions, including enzymes, on the degradation rates. Control groups with just the enzymes used showed that the enzymes did not interfere with the assay. As PHB is not easily soluble in the solvents used in GPC, this would be a good method for determining the degradation of PHB in many solutions *in-vitro* and also *in-vivo*.

GPC analysis was used to determine the change in molecular weight of the various polymers in different solutions; it has been used by other groups to determine molecular weight changes that occur in resorbable polymers following degradation *in-vitro* and *in-vivo*. For example, Ali *et al* (1993) studied the degradation of PCL *in-vitro* and the role of free radicals in the degradation mechanisms. They also used the Mn and Mw values as indicators of degradation. They concluded that the presence of hydrogen peroxide or metal ions alone had no effect on the hydrolytic degradation of PCL. The presence of gaps and cracks around the spherulites which extend inwards was also observed here, and was thought to be due to the initiation of degradation in the amorphous regions which then proceeds to the crystalline regions. Gamma irradiation can also induce crosslinking by recombination of free radicals but, in our study, there was no evidence of gel formation; however it could have been retained in the pre-filter. The listed molecular weight of the PCL pellets was 72,000, but when measured by GPC for the control it was 40,000. The reason for this reduction is not known; it could have been due to slow degradation which may have occurred over the storage period even though the pellets had been stored in a dessicator.

The 10 and 20 day experiments using enzymes to degrade the PLA granules and PCL pellets showed that enzymes did not have a significant effect on the degradation rates. This is contradictory to the surface degradation results measured qualitatively from the SEMs. It is possible that enzymes may have more of an influence on the overall degradation of the polymers at a lower molecular weight. Both PLA granules and PCL pellets were significantly affected by high temperature and the long term degradation of PLA flex bars showed degradation occured following storage in all the solutions.

From the long term study and the 10 and 20 day degradation study we can see that the enzymes we tested do not appear to cause degradation of the polymer. Degradation of PGA is by hydrolysis and the mechanism is the same *in-vivo* and *in-vitro*. However there are many reports of degradation being faster *in-vivo* than *in-vitro* and one of the reasons for this have been suggested to be the presence of enzymes (Ali *et al* 1994) However, our results show there was little effect of the enzymes on the overall molecular weight but there may be an effect on the Mn value and the polydispersity. Polydispersity is a measure of the spread of the molecular weight and can tell us whether degradation produced the low molecular weight fragments. The reason may be difference in diffusion rather than the mechanism of degradation.

The long term degradation studies were able to demonstrate the problems that may be encountered if a polymer is stored incorrectly. The polymers lost the majority of their molecular weight when stored at 37°C without desiccation. It is most likely that

temperature was an important contributing factor for causing the greatest degradation; this was evident for those samples stored at 60°C. Thus elevated temperatures caused significant degradation for both PLA and PCL but only PLA was affected by storage in enzyme solutions.

In a study by Gilding and Reed (1979) gamma irradiation sterilisation was found to cause the chain scission of PLA and PGA polymers, with a decrease in molecular weight observed for PGA sutures with increasing doses. They also concluded that the rate of Mn decrease was more rapid than the Mw decrease. This suggested that random chain scission was not the primary mechanism and the faster decrease in Mn implied that the effect of the radiation was greater on short molecular chains. Even though the appearance of the polymers had remained unchanged the radiation had significantly effected the properties and the useful lifetime of the polymers.

Miller *et al* (1987) concluded that PHB and PHB-PHV did not degrade unless they had been irradiated. Knowles and Hastings (1992) however, concluded that the degradation of PHB did occur although it was very slow at high molecular weights and thus was difficult to detect after short experimental time periods. The reason it appeared that only irradiation caused degradation was due to the decrease caused to the bulk polymer making it easier to detect degradation. They also concluded that pH affected the degradation of PHB and PHB-PHV(7%) copolymer and, as it was difficult to predict the degradation of PHB, *in-vivo* correlation between *in-vitro* and *in-vivo* degradation would be difficult. Yasin and Tighe (1992) found that PHB-PHV/PCL blends were more stable to hydrolytic degradation compared to PHB-PHV/polysaccharide blends. Surface degradation predominated during the early stages which was measured by goniophotometry and surface energy measurements.

Due to the many differences in results arising from the clinical use of PLA, Vert and Chabot (1981) concluded that there were many types of PLA resulting in differences in degradation times and differences in mechanical and biological behaviour. They also concluded that levels of crystallinity played a major part in these differences and should be controlled and maintained if the degradation rate was to be reproducible. The effect of differences in crystallinity for the same polymer was not investigated in this study. The factors affecting degradation are polymer structure, that is copolymers, backbone, chirality, molecular weight, processing technique, crystallinity and amorphous regions and whether the polymer is *in-vitro* or *in-vivo*. Sometimes microbial degradation proceeds much faster than enzymatic degradation using purified enzymes. In solvents there is a shift in molecular weight toward low molecular weight with the appearance of a bimodal distribution. Differences were attributed to diffusion behaviour rather than mechanisms of degradation. Crosslinking limits the accessibility to enzymes and affects

the degradability of polymers. Non crosslinked polymers are degraded uniformly in amorphous regions.

The results from these series of experiments prove that the degradation of resorbable polymers is variable. Various factors such as temperature, the nature of the solution and the presence of enzymes can affect the rates. If the long term biocompatibility of these polymers is to be determined the mechanisms involved in the degradation need to be understood. The levels of monomers and degradation products released and their effect on biocompatibility also need to be determined. The concentrations of degradation products that can be found *in-vivo* from a degrading device need to be measured in order to device meaningful and comparable *in-vitro* tests. The pH of the environment immediately surrounding an implant *in-vivo* needs to be measured so the effect of pH on the cells and tissue around the implant can be investigated and the levels of buffering occurring *in-vivo* can be determined. Martin *et al* (1996) used a microelectrode to measure the pH around eroding polylactide-polyglycolide films *in-vitro* and *in-vivo* in rabbit tibial bone chambers. Significant differences were found but these were only 0.2 pH units; this was found with incubation in PBS and in interstitial fluid subject to vascular exchanges. The shortcoming, however, of this set-up was that the size of the film was far to small to represent changes that would occur with a larger device. The film used was in the form of a washer, with an inner diameter of 0.8 mm and an outer diameter of 1.6 mm with a thickness of 100μm. The method could be utilised larger devices and the pH changes measured to determine what level of buffering was occurring, if any, *in-vivo* as compared to *in-vitro*. Vert *et al* (1990) stated that degradation is influenced by the degree of water uptake and the release of soluble oligomers depends on the solubility of the environment, which is influenced by factors such as pH, ionic strength, temperature and buffering capacity. This is in agreement with the results obtained in this study. The studies also show that PLA, PCL and PHB were slow to degrade as compared to PGA and PHB-PHV copolymers. This confirms some of the findings of Helevirta *et al* (1987) who also showed that PGA, PLA/PGA copolymers lost their mechanical strength more readily than PHB and PLA-based materials.

CHAPTER III

Qualitative Evaluation of Cells on Materials

The majority of *in-vitro* biocompatibility tests for materials involve the morphological assessment of cells which adhere to polymer surfaces (Callen *et al* 1993; Howlett *et al* 1994). Techniques such as light, scanning and transmission electron microscopy are generally used. *In-vitro* cell culture systems can be highly sensitive and reproducible and furthermore they allow quantitative as well as qualitative assessment from a series of well-designed statistically significant experiments. Other techniques which can be applied to tissue culture models include environmental SEM, confocal laser scanning microscopy and Raman spectroscopy. Environmental SEM allows cells to be observed on the material surfaces without fixation, thus cells can be observed while they are still viable and interacting with the biomaterial surface. Confocal laser scanning microscopy allows cell visualisation in a three dimensional plane so that cells within several layers can be seen and the cytoskeleton can be stained with various fluorescent dyes.

In-vitro cell and tissue culture systems are becoming increasingly popular in the field of biocompatibility testing of biomaterials. Although *in-vitro* methods cannot replace animal models they have certain advantages that allow their preferred use in certain circumstances. *In-vitro* cell models allow a more detailed study of a particular cell type without interferences from other factors, such as the immune system, which are frequently encountered with *in-vivo* testing methods. The tests performed can be simple qualitative tests, or they can be more complex and give a more in-depth assessment of the biocompatibility of the material. Qualitative assessment of cells cultured on the surfaces of biomaterials can be a useful indicator of biocompatibility. This can be achieved by examining the individual cell morphology and cellular structures, and the degree of cell attachment and spreading on the polymer surface.

In this study differences in the morphology and growth characteristics of cells cultured on various polymer surfaces were investigated using light, transmission and electron microscopy. The studies were performed using a human osteosarcoma (HOS) cell line TE85, which was obtained from the European Collection of Animal Cell Cultures (ECACC). The major advantages of using HOS cells were their reproducibility and fast turnover rate (doubling time of 21.6 hours). This allows large number of cells to be obtained for the *in-vitro* assays. The HOS cell line has been tested for its suitability as a representative cell model for primary osteoblast-like cells. Work by Clover and Gowen (1994) showed that, while the cell line was a suitable model for studying integrin subunit expression and cell adhesion, proliferation and alkaline phosphatase activity were not representative of primary bone cell cultures. However, provided these differences were taken into account, HOS cells were considered to be a valuable tool for investigating specific aspects of bone cell function.

Osteoblasts are cells found in bone which are responsible for the synthesis of the organic components of bone matrix. When they are synthesising matrix in bone they have a cuboid shape, but when the synthesising activity decreases they become more flattened. Osteoblasts are polarised cells; the chromatin in the nuclei is finely dispersed and is found at the side of the cell away from the matrix. The newly synthesised, but uncalcified, collagenous matrix is referred to as osteoid.

There are various ultrastructural markers within cells which give an indication of the state of the cell; these include the mitochondria, the Golgi body and lysosomes. The nucleus itself and the appearance of the cytoplasm can also give an indication about the state of the cell. The Golgi body is involved in the production of almost all the exocrine and endocrine secretions and it also forms lysosomes which perform their function inside the cell. Most of the products of the Golgi complex are structurally distinct, making them good ultrastructural markers for specific cell types. In general, immature undifferentiated or fast growing cells have a smaller Golgi complex than their normal mature counterparts. Lysosomes are membrane bound vesicles that function as the digestive system of the cell. They handle materials of exogenous or endogenous origin within the cell and are discharged from the cell under certain conditions. Lysosomes have been detected in all animal cells and are formed by budding off of the sacs of the Golgi complex. Lysosomes contain a whole range of enzymes which are hydrolytic in nature. The effect these enzymes have on the degradation of various biomedical polymers will depend on the type of material concerned and the physical characteristics and appearance of the material.

Before qualitative analysis of cells in culture can be made it is important to describe the phases of the cell cycle and the appearance of cells at the various stages of their growth. The growth cycle is divided into three parts: the lag phase, log phase and the plateau phase. The lag phase follows subculture and re-seeding onto a new surface. There is very little increase in cell number at this stage and the cell is essentially replacing elements lost during trypsinisation. The cells attach to the substrate and spread out with this lag phase lasting up to 24-48 hours depending on the cell type. The log phase follows the lag phase and is dependent on the initial cell seeding density. The log phase is characterised by an exponential increase in cell number and stops when confluence is reached. Sampling should take place during the log phase as the cells are at their most uniform and cell viability is high. The plateau phase takes place when the cells have reached confluence and growth has decreased to 0-10%. The cells at this phase are less motile and ruffling of the plasma membrane is reduced so the cells occupy less surface area. Some cells such as fibroblasts and osteoblasts may align themselves in the same direction forming swirls as they start to produce extracellular matrix.

Most *in-vitro* testing has involved fibroblast (Al Nazhan, 1990), osteoblast (Elgendy *et al* 1993; Puleo *et al* 1993), monocyte/macrophage (Miller *et al* 1989) and hepatocyte (Cima

et al 1991) growth on the test material where the cell type is chosen with respect to the final site of the implantation. Bone cells and fibroblasts of human or mouse origin have been used for *in-vitro* testing of resorbable materials and for the testing of materials intended for orthopaedic use which are discussed further in this chapter. Other cell types, usually of rat origin, have been used to carry out biocompatibility screening of a range of biomaterials. Puleo *et al* (1991) used neonatal rat calvarial osteoblasts for testing various orthopaedic implant materials. The materials tested were stainless steel, titanium alloy, cobalt chrome, HA and borosilicate glass, all of which are non-degradable. The results were compared to tissue culture plastic, which favours cell attachment and is often used as a control. The proliferation was measured by performing cell counts after day 1, 4 and 7 using a Coulter counter. They concluded that *in-vitro* methods were suitable for examining several aspects of cell interactions with orthopaedic biomaterials.

Discrepancies have been reported with *in-vitro* work that has been carried out by other workers, as a comparison is made difficult by the differences in methods used for isolating cells, the type of cells used and the numbers used to seed on to biomaterial surfaces. Before direct comparison of tests and data can be made, test methods need to be standardised to allow for comparison. Frequent problems include the differences in shape, size and surface features of materials. This is an important factor as it plays a major role in the tissue or cell response in terms of cell adherence and spreading on the surface. In the main basic cell culture methods are usually similar with only slight modifications in the growth media, but there are differences in the cell type and the cell seeding density used. Begley *et al* (1993) used human osteoblasts to test bone graft substitutes and found that a higher cell seeding density led to more of the materials giving positive results. An important recurrent factor in many different studies published is that the cell seeding density for the experiments *in-vitro* is different. This means that the results from one experiment which may use the same cell type and the same biomaterial cannot be compared to other experiments which may have used different seeding densities.

The attachment of cells to biomaterials has been extensively studied however very few of these papers include degradable polymers. Boyan *et al* (1996) demonstrated the changes in the response of bone and cartilage cells to changes in material surfaces. Malik *et al* (1992) for example studied the morphology of neonatal rat calvarial osteoblasts on the surfaces of HA, alumina and bone *in-vitro* for the first 2 hours of attachment. They found that the morphology of the cells was different on the different surfaces. Anchorage dependent cells such as fibroblasts and osteoblasts need to attach before translation and transcription of cellular events can begin (Ben-Ze'ev *et al* 1980).

The aim of this study was to assess cell attachment and proliferation on the various polymer surfaces over time. The effect of cell seeding density was also investigated. The

effect of the polymer surface on cell morphology and cell proliferation was investigated using light microscopy, scanning microscopy and transmission electron microscopy. The cells were seeded onto the polymer surfaces and cultured for up to 24 days for the long term experiments. The effect of degradation products from PGA on the cells in culture was assessed qualitatively. PGA degrades rapidly and the degradation products released from the polymer were assessed using studying cell morphology and the gross appearance of the cell by SEM. The choice of a cell type for general *in-vitro* testing is important as a cell type may be more susceptible or resistant to injury by a particular test agent. This is particularly so if there are subtle differences in the materials and biocompatibility of the material can be influenced by small changes. Al Nazhan and Spangberg (1990) found differences in human periodontal fibroblasts and L929 cells in their resistance to damage by endodontic materials. So, in devising tests for biocompatibility, we need to determine not only the type of test to be carried out but also the number of different cell types that should be used.

MATERIALS AND METHODS

HOS cell culture

A human osteosarcoma (HOS) cell line TE85 (ECACC No 87070202), was used unless stated otherwise. The HOS cell line was obtained from the European Collection of Animal Cell Cultures (ECACC) and was used between passages 10 and 15 for all experiments. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% FCS, Foetal Calf Serum, 0.02M HEPES, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2mM L-Glutamine, 1% Penicillin/streptomycin (all Life technologies) and 150µg/ml Ascorbate. Cells were grown to confluence at 37°C with 5% CO^2 in a humidified atmosphere and were trypsinised using Trypsin (Sigma) 0.02% in Phosphate Buffered Saline (PBS), (Sigma) with HEPES (Life technologies). The cells were resuspended in complete medium and after a viability count with trypan blue, seeded onto the polymer surfaces at the stated concentrations. Thermanox disks (15mm) in diameter was used as the control surface (Life technologies). The Thermanox coverslips and the polymer disks cut 15mm in diameter were placed into 24 well tissue culture plates (Beckton Dickinson). At each time point the disks were removed and processed for light, scanning and transmission electron microscopy.

Light Microscopy

The specimens for light microscopy after tissue culture were rinsed in 0.1M sodium cacodylate buffer and fixed in 4% formaldehyde for 30 minutes then rinsed again in 0.1M sodium cacodylate buffer. They were then stained with Harris's haematoxylin for 4 minutes and rinsed in water; they were then differentiated in acid alcohol for 5 seconds and "blued" in Scotts water. The films were placed into 1% eosin for 30 seconds and rinsed in water before dehydration through a series of alcohols from 70-100%. These were then air dried and mounted with Aqua mount (BDH). The specimens were viewed using a light microscope and photographed.

SEM Processing

Cells on the polymer surfaces could not be critical point dried as this required acetone which affected all the polymers by dissolving them completely or distorting some of the surface structure. The initial methods employed to study cell attachment to the polymers involved fixing in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 minutes at 4°C. The cells were then dehydrated through a graded series of ethanols (20-100%) before being air dried and viewed. The results obtained from these were poor and modifications to the method were made. The cells were fixed in 2.5% glutaraldehyde in

0.1M sodium cacodylate buffer for 30 minutes at 4°C. The films were then rinsed in 0.1 M sodium cacodylate buffer and placed into 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour. These were rinsed in 0.1 M sodium cacodylate buffer and placed in 1% tannic acid for 30 minutes. After a further rinse they were dehydrated through a series of ethanols (20-70%) for 2 x 5 minutes. The specimens were placed in 2% uranyl acetate made up in 70% alcohol for 30 minutes and then washed in 70% alcohol. The dehydration was completed by following through to 100% (Wollweber *et al* 1980). After the 100% then the specimens were placed in HMDS (Sigma,), for 2 x 5 minutes, air dried and mounted onto SEM stubs. These were sputter coated with gold for 5 minutes and viewed under a Joel 35C SEM. The method for SEM using HMDS as described by Nation (1983) gave superior results.

TEM Processing

Cells on the surfaces were fixed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 30 minutes. The specimens were placed in secondary fixative 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for one hour. They were then dehydrated through a graded series of ethanols (70-100%) and propylene oxide and then vacuum infiltrated for 2 hours and embedded in Spurr's resin (Spurr, 1969). Ultra thin sections (60-100 nm) were cut and collected on pioloform coated 200 mesh nickel grids which were stained with saturated aqueous uranyl acetate and lead citrate (Reynolds, 1963) for 10 minutes each. Specimens were viewed by a Philips CM12 transmission electron microscope.

RESULTS

Figures 3.1 to 3.4 show the results in the form of scanning electron micrographs carried out in order to determine the attachment and proliferation of HOS cells seeded onto various polymers over a 120 hour period. The cells were seeded onto the surfaces at 4 x 10^4 cells/cm^2. After each time point the films were removed and processed for SEM. Although the individual morphology of the cells was difficult to see, the total cell coverage of the polymer surface gave a good indication of the extent of proliferation that had occurred in that time period.

In the first 24 hours the cells adhered to the polymers in varying degrees with greater cell adherence seen on to Thermanox (figure 3.1a) and PLA (figure 3.1b). Fewer cells adhered to PCL (figure 3.1c) followed by PHB (figure 3.1e) and PHB-PHV (figure 3.1f) and the individual morphology of the cells on PHB was more rounded with very little spreading occurring on the surface. Cells on PHB-PHV were also less spread out than on PLA, PCL and Thermanox. The individual cells on the PCL surface appeared to be more

flattened and had more processes than the cells on the other surfaces as can be seen from the higher magnification (figure 3.1d).

After 48 hours the cells on Thermanox (figure 3.2a and figure 3.2b) were confluent as were cells on PLA (figure 3.2c). Interestingly the cells on PCL (figure 3.2d) followed the pattern of the spherulites and did not grow as well in between the spherulites. The cells on PHB (figure 3.2e) and PHB-PHV (figure 3.2f) were not as rounded in appearance as they had been after 24 hours. The cells on PHB formed colonies which then joined up forming a network giving it a "lacy" appearance.

After 72 hours the cells on Thermanox (figure 3.3a), PCL (figure 3.3b) and PLA (figure 3.3c) were confluent. Cells on PCL were also confluent and, while cells on PHB (figure 3.3d) and PHB-PHV (figure 3.3e) had increased in number, they were not confluent. In addition cells on PHB-PHV did not appear to have many processes compared to the cells on PCL, Thermanox and PLA as seen from the higher magnification (figure 3.3f) of the cells on the polymer surface.

By 120 hours cells on Thermanox (figure 3.4a), PLA (figure 3.4b), PCL (figure 3.4c) and PHB-PHV (figure 3.4e) were confluent with the individual morphology of the cells on the polymer surfaces becoming difficult to distinguish. Cells on PHB (figure 3.4d), however, were not confluent. On the PHB surface, where the cells had initially adhered and started to proliferate, cells were densley populated but in the areas between the colonies cell density was low. It was interesting to note that, although the cell density when adhering to PHB-PHV surface were lower than the other polymers, the same degree of confluence was achieved by 120 hours as the other polymers. The cells on Thermanox had started to form multilayers; with cells stacking on top of each other and, as a result, in some areas cells had begun to detach from the polymer surface. This detachment may have been due to a processing artifact or due to a shortage of nutrients for the cell layer closest to the Thermanox surface. Evidence of the multi cell layer can be seen in the transmission electron micrographs (figure 3.7).

Figure 3.1a: HOS cells on Thermanox after 24 hours in culture were well spread out and had already covered a large area of the polymer surface.

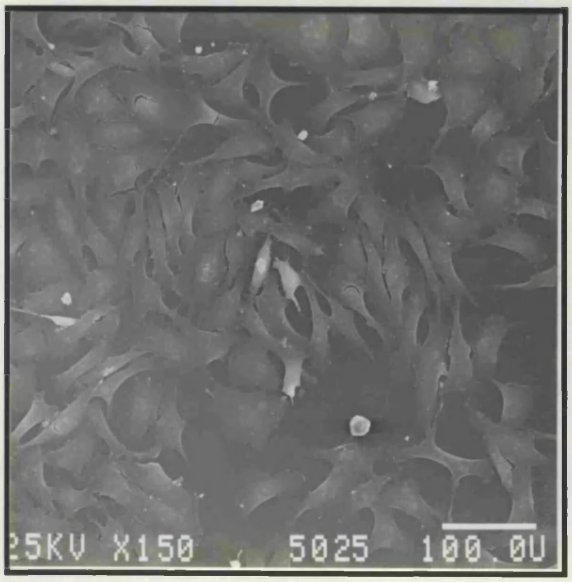
Figure 3.1b: HOS cells on PLA after 24 hours in culture were well spread out and there appeared to be more cells attaching to the polymer surface. Rounded cells were present which may be cells undergoing division.

Figure 3.1c: HOS cells on PCL after 24 hours in culture were well spread but fewer cells were attaching to the polymer surface. Note the round structures which are the spherulites (s).

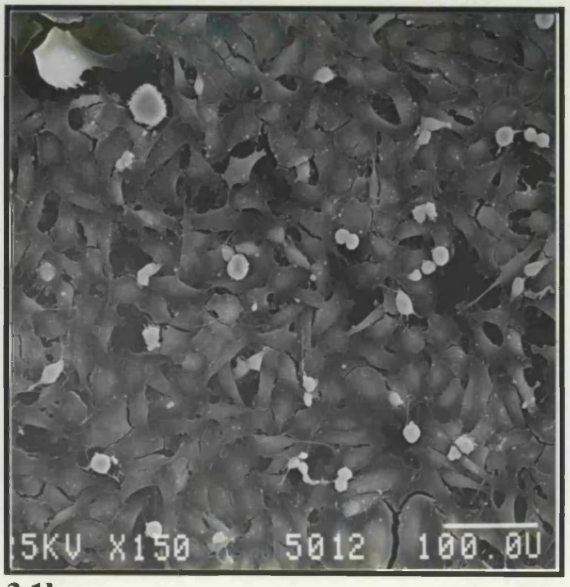
Figure 3.1d: HOS cells on PCL after 24 hours in culture at a higher magnification showed that the cells had many processes and were spreading across the edges of the spherulites.

Figure 3.1e: HOS cells on PHB after 24 hours in culture were different in morphology to PLA, PCL and Thermanox surfaces. There were fewer cells attaching and these were more rounded with some cylindrical shaped cells.

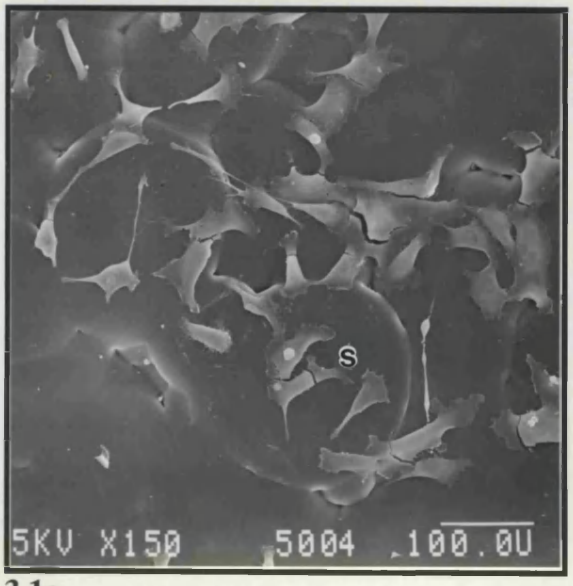
Figure 3.1f: HOS cells on PHB-PHV after 24 hours in culture were similar in morphology to cells on PHB with fewer cells attaching to the surface compared to Thermanox, PCL and PHB.



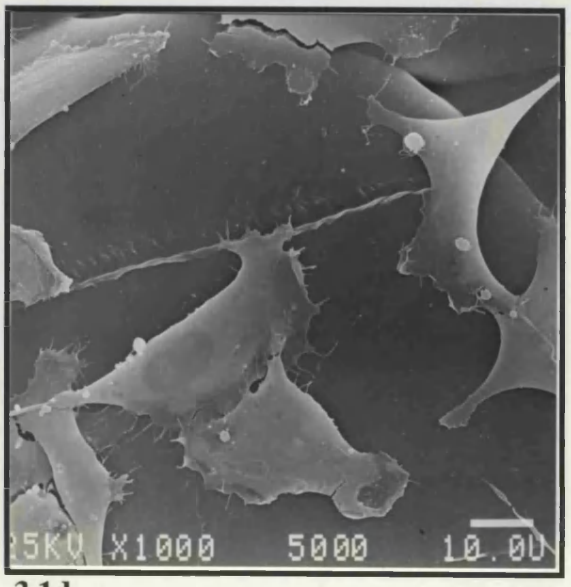
3.1a



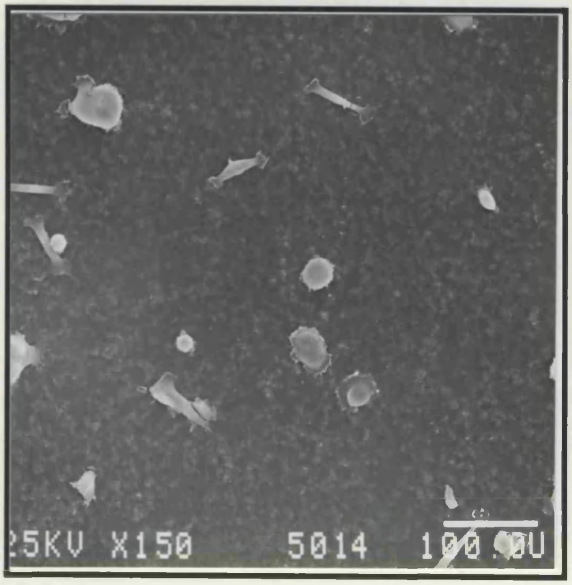
3.1b



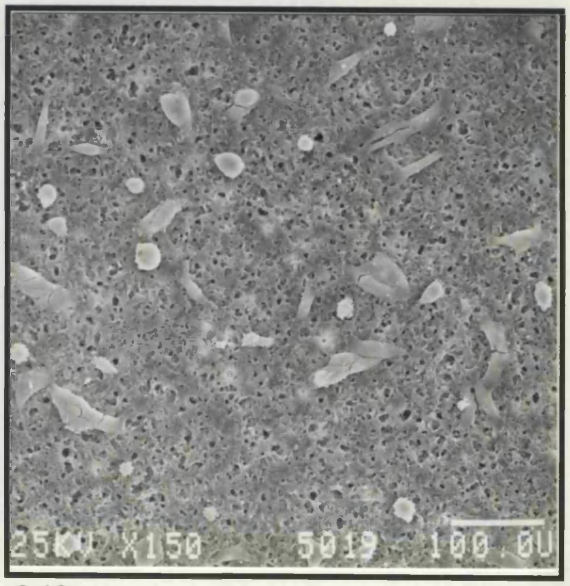
3.1c



3.1d



3.1e



3.1f

Figure 3.2a: HOS cells on Thermanox after 48 hours in culture had the same morphology as cells after 24 hours but had a larger coverage of the surface with a few rounded cells observed indicating cells undergoing division.

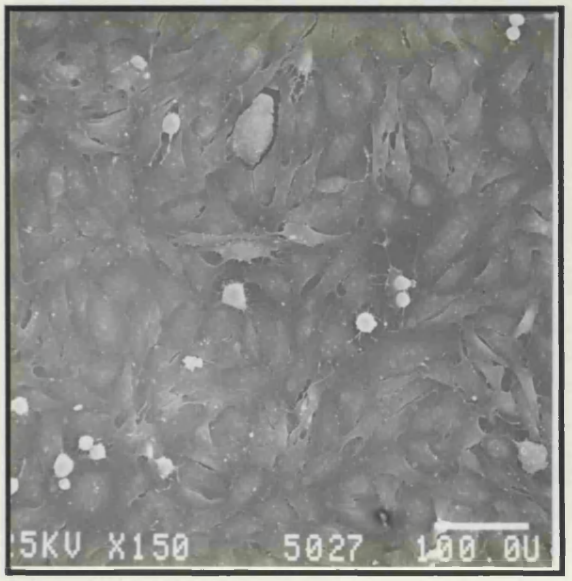
Figure 3.2b: HOS cells on Thermanox after 48 hours in culture; a higher magnification shows that the cells were well spread with some rounded cells. The rounded cells had more processes compared to the flattened cells.

Figure 3.2c: HOS cells on PLA after 48 hours in culture had covered more of the polymer surface with many rounded cells present and very little of the PLA surface visible under the cell layer.

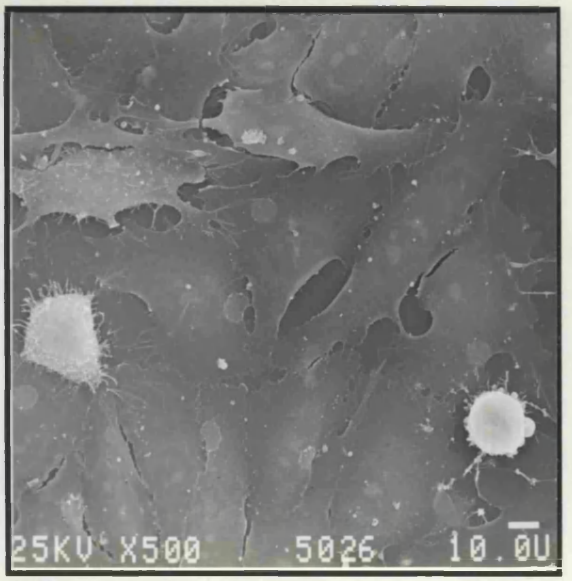
Figure 3.2d: HOS cells on PCL after 48 hours in culture were flat and the number of cells had increased with the cells mainly adhering on the spherulites.

Figure 3.2e: HOS cells on PHB after 48 hours in culture still had an elongated morphology with an increase in cell number. The cells were forming colonies where the cells had initially adhered.

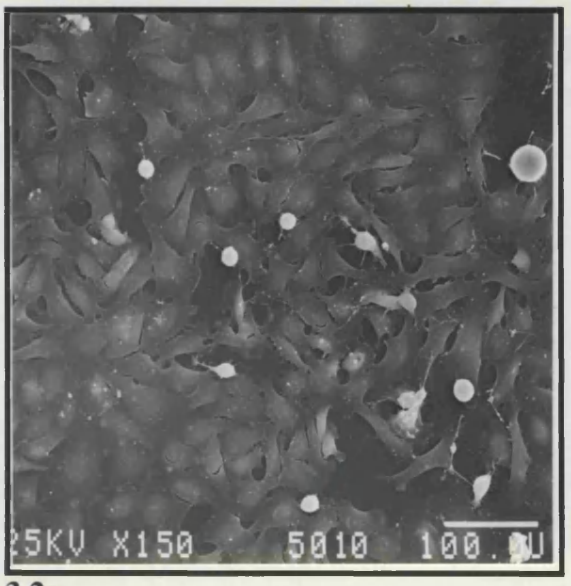
Figure 3.2f: HOS cells on PHV after 48 hours in culture; note the change in morphology with the cells appearing more elongated.



3.2a



3.2b



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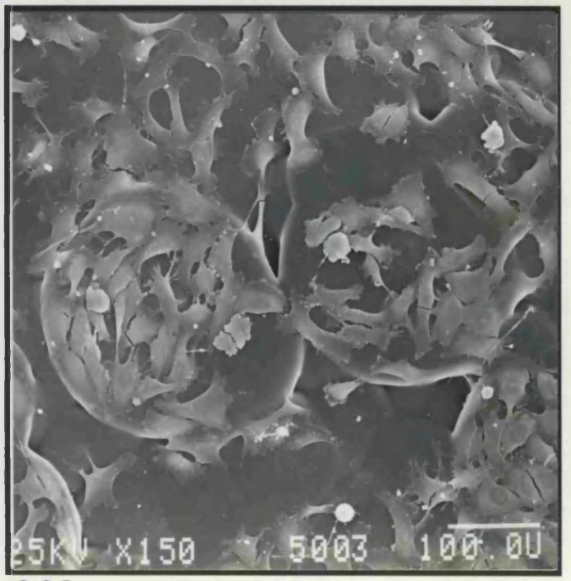
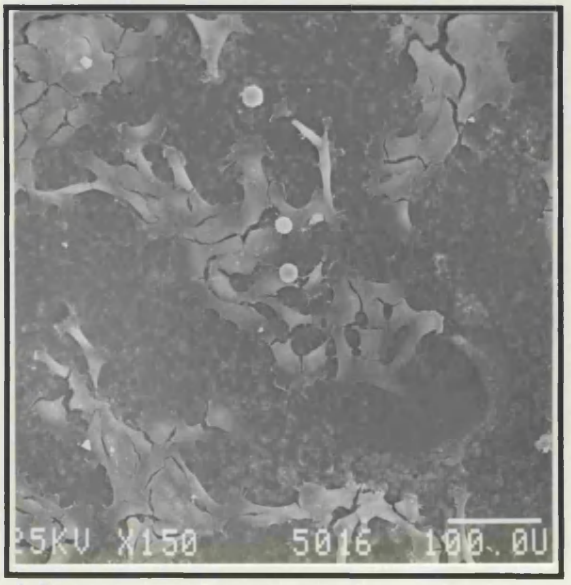


3.2d



3.2e

3.2f

Figure 3.3a: HOS cells on Thermanox after 72 hours in culture were confluent with individual cells difficult to distinguish. Note the presence of rounded cells undergoing division.

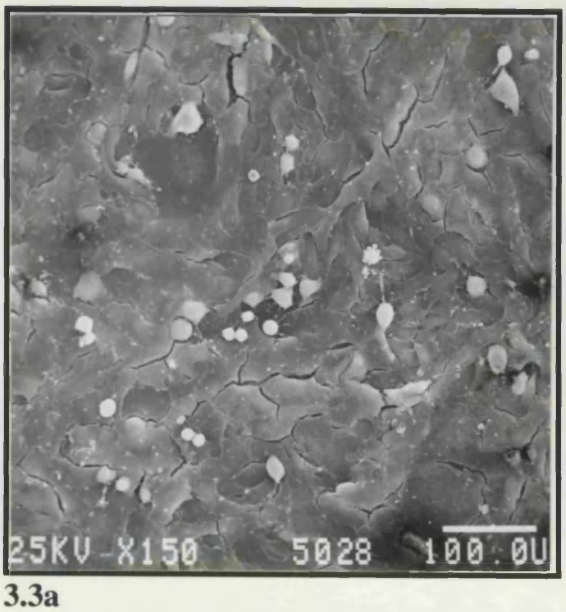
Figure 3.3b: HOS cells on PCL after 72 hours in culture were similar in appearance to figure 3.3a with rounded cells also present.

Figure 3.3c: HOS cells on PLA after 72 hours in culture at a higher magnification showed a very flattened morphology with many cellular processes. Some rounded cells were present and the cells were similar in the degree of confluence to figure 3.3a.

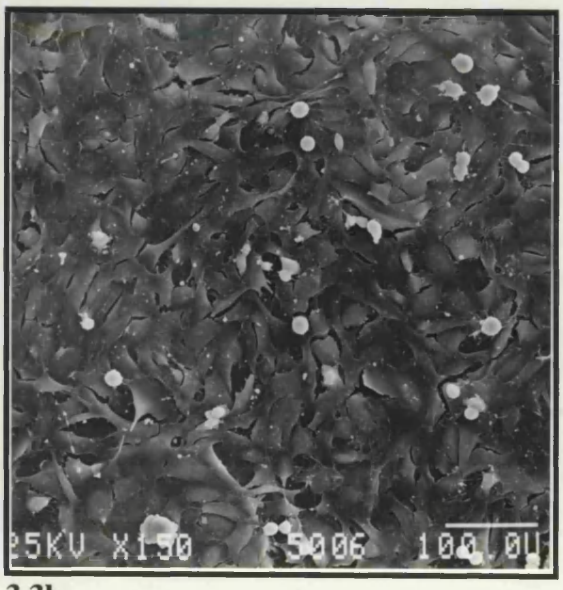
Figure 3.3d: HOS cells on PHB after 72 hours in culture had not reached confluence and the morphology was different to Thermanox, PLA and PCL. The cells were elongated with few processes and the cells were forming clumps.

Figure 3.3e: The morphology of HOS cells on PHB-PHV after 72 hours in culture was different to the morphology at 24 and 48 hours. The cells now had a flatter morphology with more cell to cell contact occurring. However there were fewer cells as compared to Thermanox, PLA and PCL.

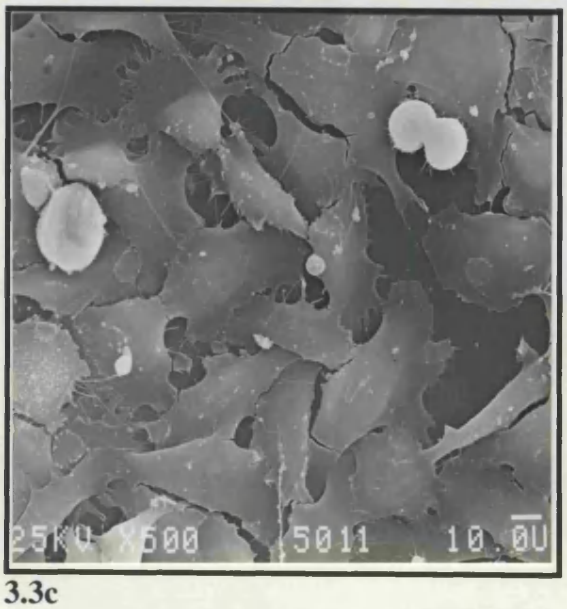
Figure 3.3f: HOS cells on PHB-PHV after 72 hours in culture at a higher magnification showed the cells to have a few processes which were invading into the polymer surface.



3.3a



3.3b



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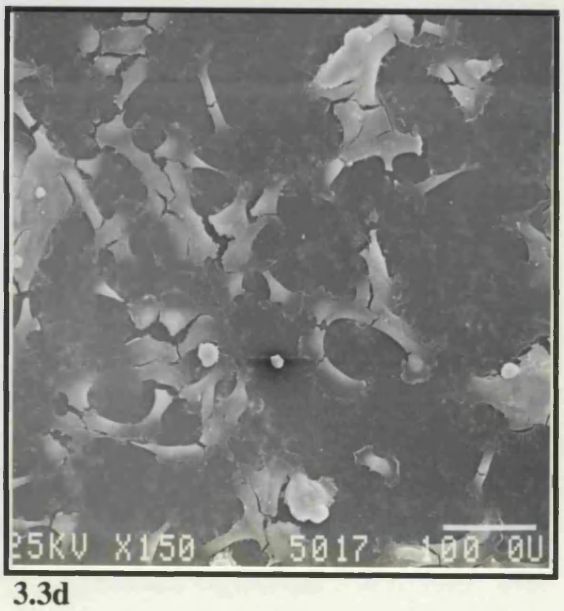

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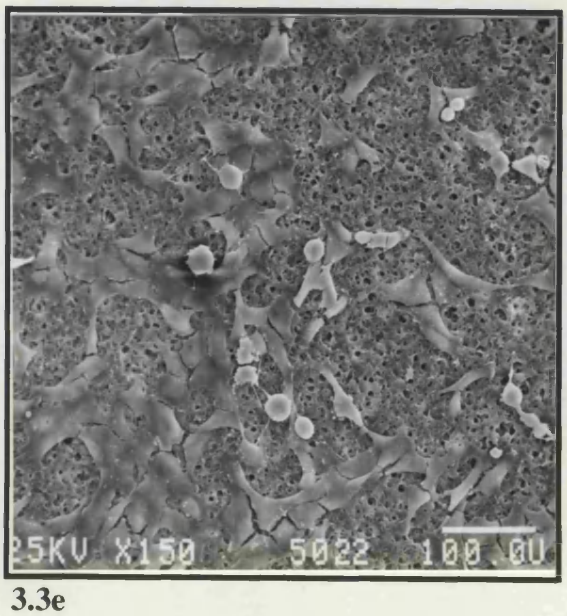
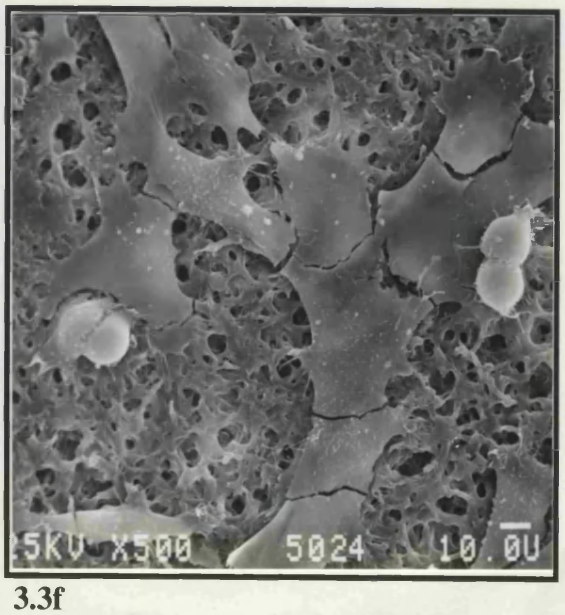


3.3e



3.3f

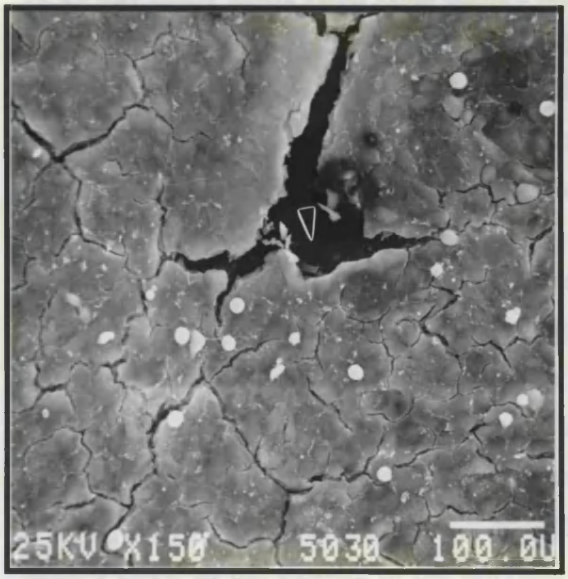
Figure 3.4a: HOS cells on Thermanox after 120 hours in culture were confluent and the cell layer had detached from the surface (arrowhead). However there were rounded cells present on the top layer indicating cell division may still have been taking place.

Figure 3.4b: HOS cells on PLA after 120 hours in culture had reached confluence and were similar in appearance to cells on Thermanox.

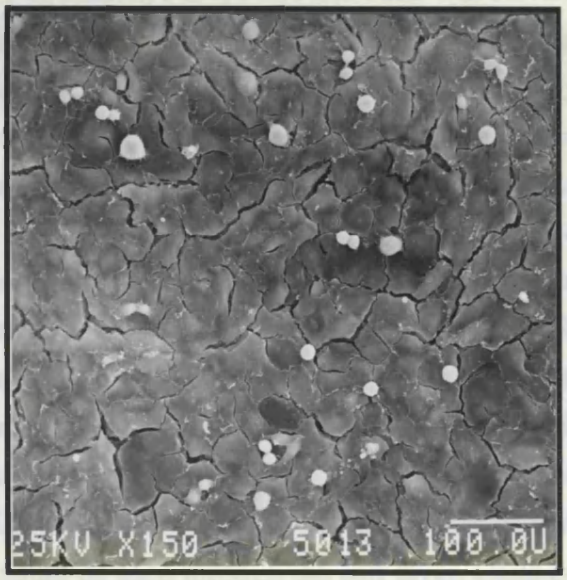
Figure 3.4c: HOS cells on PCL after 120 hours in culture had reached confluence and individual spherulites were covered in cells with cells spreading across the spherulites.

Figure 3.4d: HOS cells on PHB after 120 hours in culture were not confluent but the areas of initial colonization were heavily populated. The cellular morphology at the edge of the clumps remained elongated.

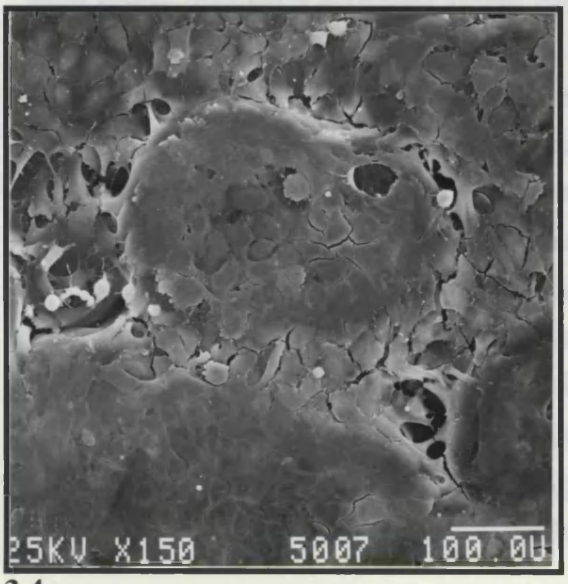
Figure 3.4e: HOS cells on PHB-PHV after 120 hours in culture were confluent. A small area of polymer surface was still visible (arrowhead) but the majority of the surface was covered in a monolayer of cells.



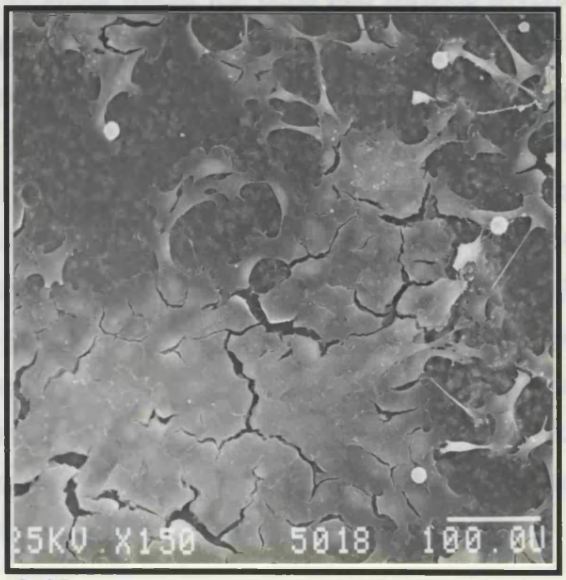
3.4a



3.4b



3.4c



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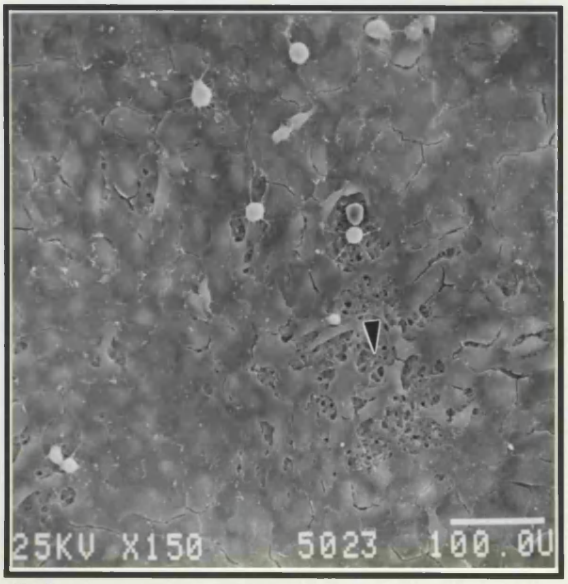


3.4e

A 24 day study using HOS cells seeded on Thermanox, PLA, PGA and PHB was carried out to determine the effect of the polymer surface and degradation products on the morphology and viability of the cells. Cells were seeded onto the surfaces at a seeding density of 4 x 10^4/ cm^2. Cells on Thermanox were confluent after 48 hours (not shown) and by day 7 (figure 3.5a) the individual morphology of the cells was difficult to distinguish. However the extent of cell coverage is emphasised by the unocvered Thermanox surface in the corner of the micrograph. Cells on PLA after 48 hours (not shown) and 7 days (figure 3.5b) were similar in appearance to cells on Thermanox. Individual cells, however, could be seen adhering to the edge of the polymer surface which showed a normal polygonal cell morphology with many processes. Cells on PHB after 7 days (figure 3.5c) were confluent and individual cells were difficult to outline. The number of cells adhering on the PHB surface was lower than on Thermanox and PLA after 7 days, as the surface of the polymer could be seen through the cell layer. Cells on Thermanox, PLA and PHB were further cultured until day 24 and no dramatic differences were noted. The cells appeared viable but in areas where the cells had formed several layers, there was some detachment from the polymer surface. Cells on PGA after 48 hours (figure 3.5d) were confluent but some areas of the polymer can be seen between the cells. Cells on PGA after 7 days (figure 3.5e) appeared intact but cracks had appeared on the polymer surface and the cell layer had also cracked as a result. The cells away from the cracks still appeared intact. Cells on PGA after 12 days (figure 3.5f) appeared damaged with the cytoplasm becoming "lacy" and the multi cell layer no longer present. The culture medium, which contained a phenol red indicator, turned bright yellow indicating its acidic nature. After 24 days the cells on PGA (not shown) retained a similar morphology to that at 12 hours although there were fewer cells present with more cracks appearing on the polymer surface. The polymer had not completely disintegrated but had become difficult to handle without pieces breaking off during processing.

Figure 3.5a: SEM of HOS cells cultured on Thermanox for 7 days the cells were several layers thick. By day 12 however the cell layer had started to break up at the surface (micrograph not shown).

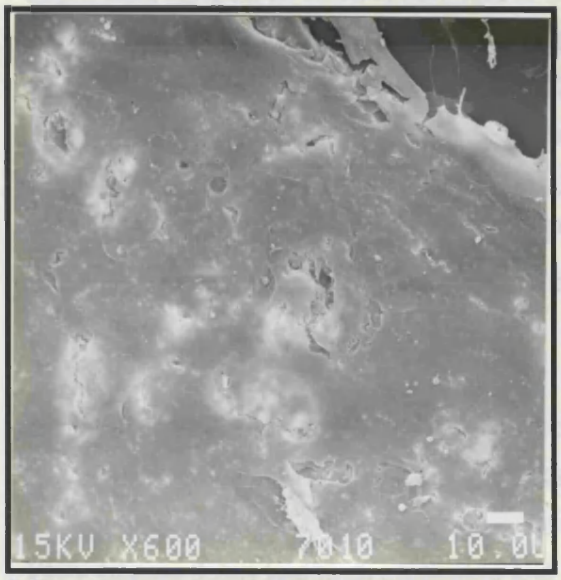
Figure 3.5b: SEM of HOS cells cultured on PLA for 7 days; the cells were confluent and individual cells can be seen "climbing" across the polymer width (arrowhead).

Figure 3.5c: SEM of HOS cells cultured on PHB for 7 days; the cells had reached confluence and were several layers thick. Individual cells could not be distinguished from each other and the polymer surface was not visible.

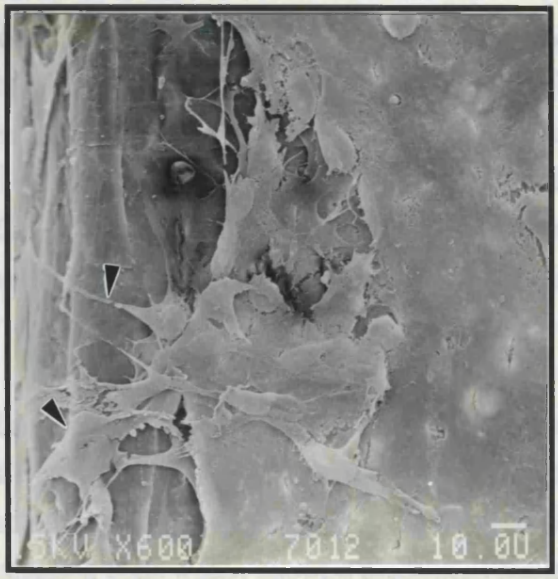
Figure 3.5d: SEM of HOS cells cultured on PGA for 48 hours shows the cells were confluent with some areas of the polymer still visible (arrowhead).

Figure 3.5e: SEM of HOS cells cultured on PGA for 7 days; the cells appeared different compared to cells on PLA (figure 3.5b), Thermanox (figure 3.5a) and PHB (figure 3.5c) on day 7. The polymer had started to break up with cracks appearing on the polymer surface (arrowhead).

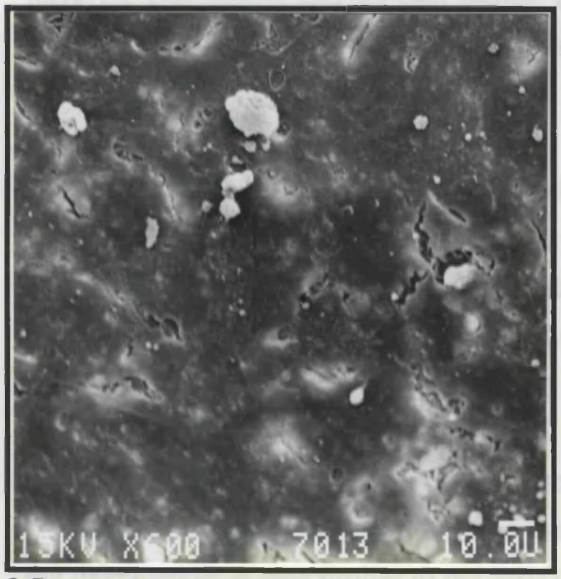
Figure 3.5f: SEM of HOS cells cultured on PGA for 12 days shows that, as well as the polymer having undergone degradation, the cells appeared damaged. The cytoplasm appeared perforated and many cells had detached from the surface.



3.5a



3.5b



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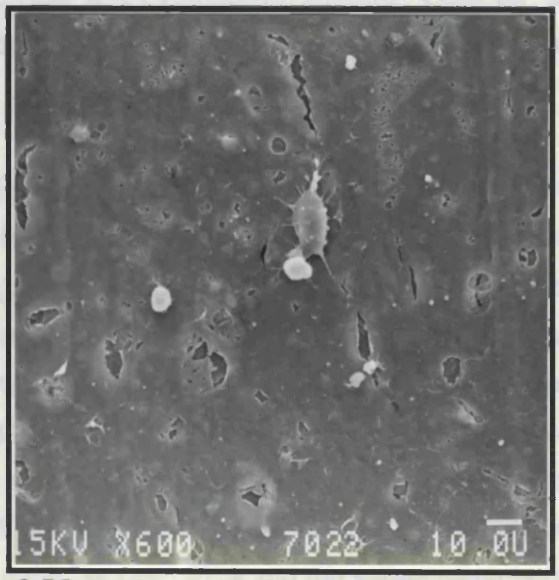
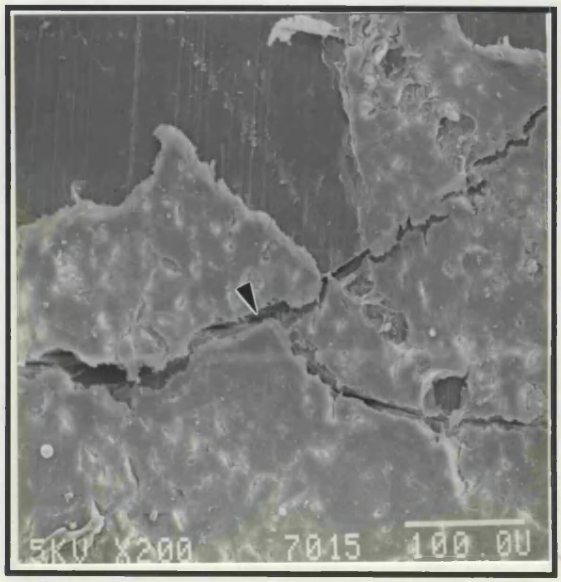
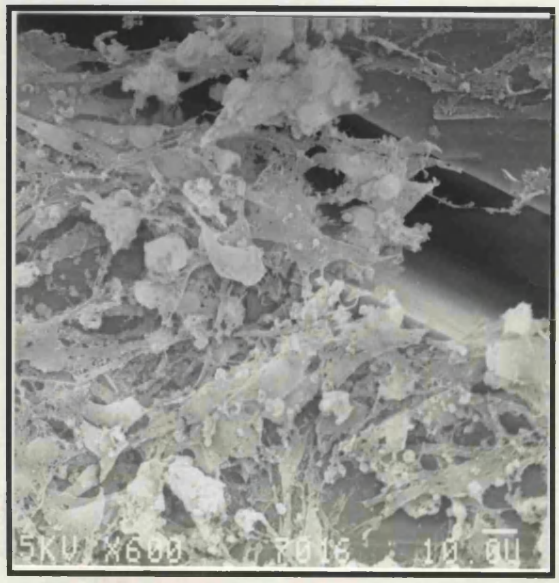


3.5d



3.5e



3.5f

Light micrographs of HOS cells (figures 3.6a-h) were used to determine the morphology of the individual cells on the polymer surfaces. The nuclei were also strongly stained and hence clearly visible in the micrographs. The cells on Thermanox (figure 3.6a) were well spread out with prominent nuclei. There were many nucleoli present and the size of the nuclei varied from cell to cell. The overall morphology of the cells was cuboidal with some cells appearing elongated. Cells on PCL (figure 3.6b) appeared different to cells on Thermanox. The cells were well spread out with fewer elongated cells and rounder in appearance with prominent nuclei.

Cells on PHB (figure 3.6c) were forming clumps with individual morphology difficult to interpret. There were some cells that had adhered individually showing spindle like morphology which was very similar to the scanning electron micrographs. Cells on PHB-PHV (figure 3.6d) had both elongated and rounded cells which showed prominent nuclei with many cells adhering. HOS cells on glass (figure 3.6e) were similar in appearance to those on Thermanox and showed similar nuclear and cytoplasmic staining. Cells on "as cast" PLA (figure 3.6f) also appeared normal with good cell to polymer contact. Few cells however had adhered to the surface.

Cells on gamma-irradiated PLA (figure 3.6g) appeared similar in appearance to cells on the "as cast" PLA althought there were a few cracks on the polymer surface. Cells on degraded PLA showed a similar morphology to cells on the other PLA. As the polymer was difficult to handle the photograph had to be taken under a phase contrast microscope. Quantitative tests could not be carried out with this material due to the variability of the films. The results were interesting in that, even though the PLA had degraded substantially, cells were still able to adhere and proliferate on this polymer. The polymer did not cause a significant drop in the pH of the medium. The morphology of the cells on the degraded PLA was similar to the cells on the "as cast" PLA and the gamma-irradiated PLA.

Transmission electron micrographs of HOS cells on the polymer surfaces can be seen in figures 3.7a-3.7d. Cells on Thermanox (figure 3.7a) appear very active with many processes extending out from the surface of the cells, at least four layers of cells were seen. Good contact was observed between the polymer and the cellular processes and healthy mitochondria and endoplasmic reiticilum were seen.

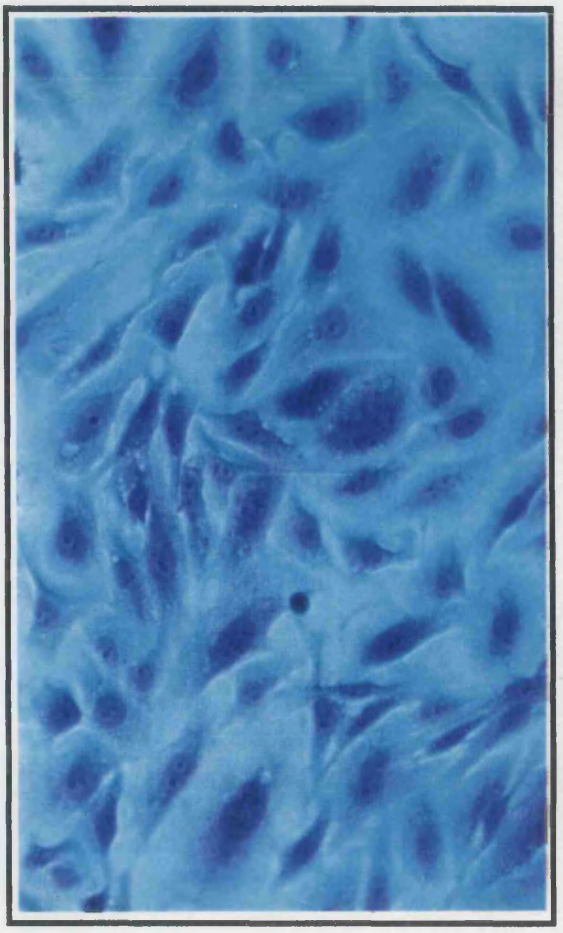
Cells on PCL (figure 3.7b) appeared very active with 6 to 7 layers of cells adhering to the surface. There was close contact with the polymer and the mitochondria and endoplasmic riticilum appeared healthy. Fewer processes emerging form the cells as compared to the cells on Thermanox were observed. Although the cells adhering to PHB (figure 3.7c) appeared healthy there were very few cells present on the surface. There were only two layers of cells and the cells were not in close contact with the polymer surface. Generally the cells did not appear to adhere and proliferate well on this polymer. Cells on PHV (figure 3.7d) were many layers thick with healthy mitochondria present within the cells. There was good contact between the cells and the polymer although areas of cell spread were very sparse with clumps of cells appearing scattered on the surface.

Figure 3.6a: Light micrographs of HOS cells on Thermanox for 48 hours showed cells to be well spread with prominent nuclei. There were many nucleoli present and the size of the nuclei varied from cell to cell (Magnification = 330x).

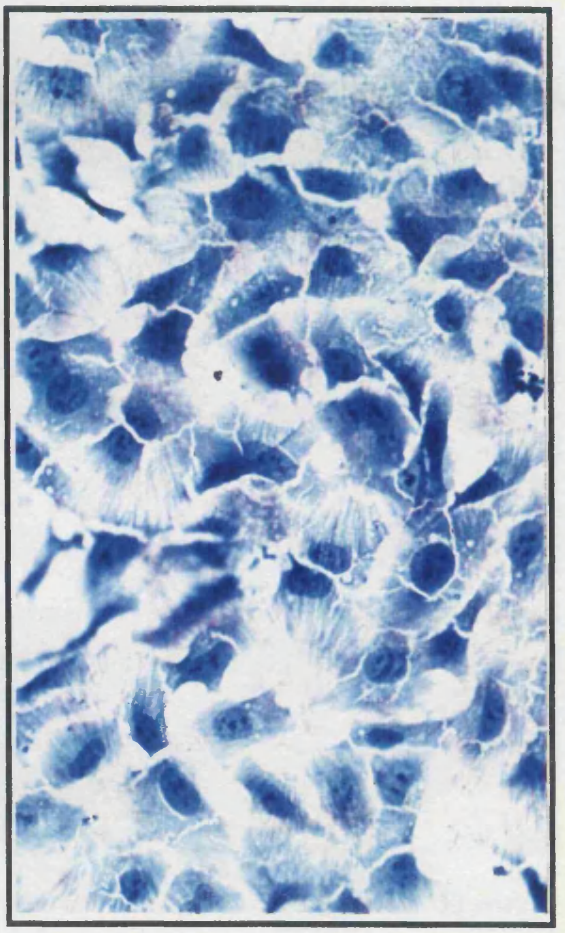
Figure 3.6b: Light micrographs of HOS cells on PCL affor 48 hours showed a different morphology to cells on Thermanox. The cells were more spread out with fewer elongated cells and had prominent nuclei (Magnification = 330x).

Figure 3.6c: Light micrographs of HOS cells on PHB after 48 hours showed the cells were forming clumps with some individual cells scattered. Individual nuclei could not be distinguished and few processes were observed (Magnification = 330x).

Figure 3.6d: Light micrographs of HOS cells on PHB-PHV after 48 hours showed the cells with flattened morphology with a few elongated cells. The cytoplasm appeared feathery and the nuclei were prominent (Magnification = 330x).



3.6a



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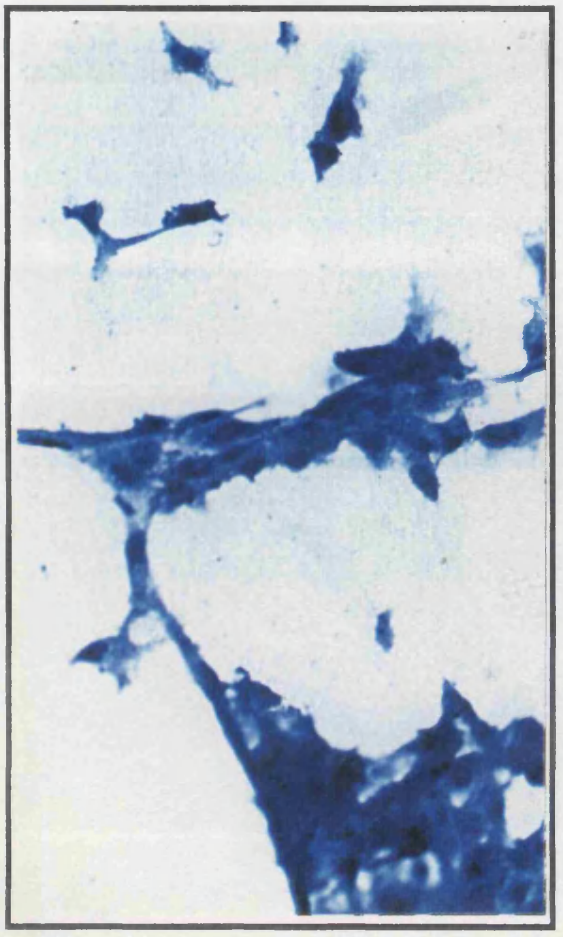

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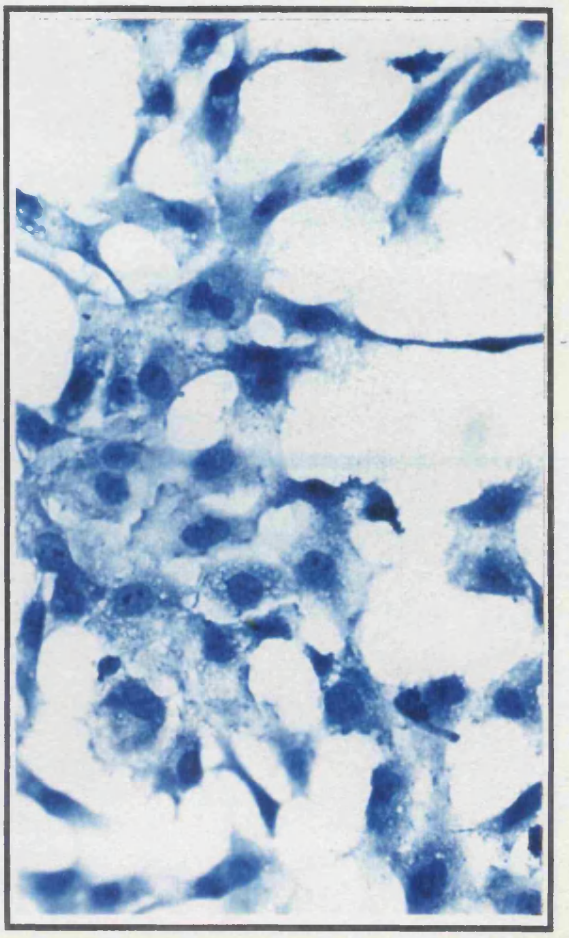


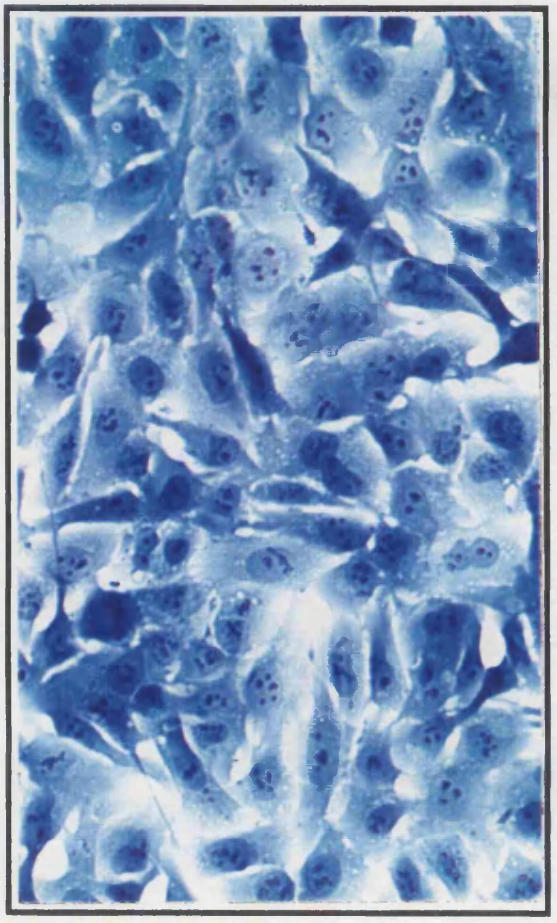
3.6d

Figure 3.6e: Light micrographs of HOS cells on glass for 48 hours showed a similar morphology to cells on Thermanox. The number of cells attaching to the glass surface was also similar to Thermanox (Magnification = 330x).

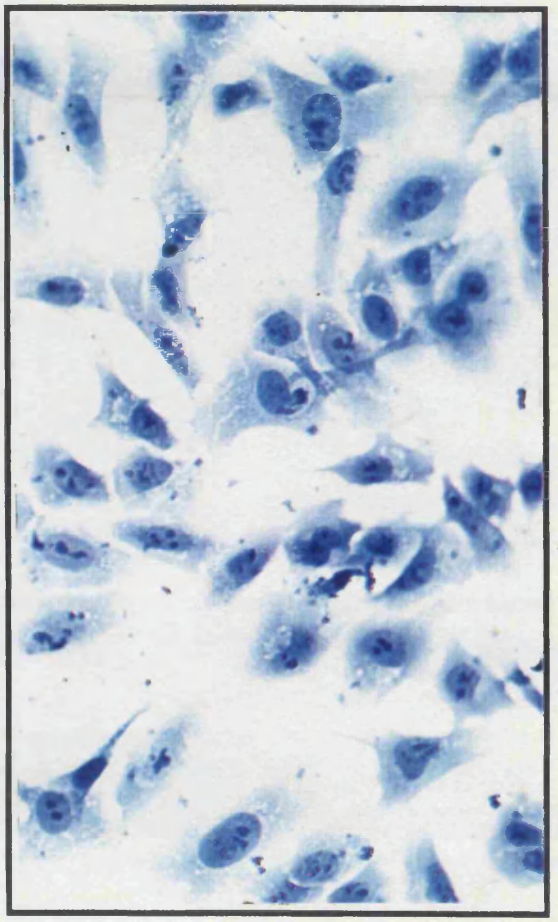
Figure 3.6f: Light micrographs of HOS cells on PLA after 48 hours were well spread but fewer cells were attaching as compared to glass and Thermanox (Magnification = 330x).

Figure 3.6g: Light micrographs of HOS cells on gamma-irradiated PLA after 48 hours showed a similar morphology to cells on "as cast" PLA (figure 3.6f) (Magnification = 330x).

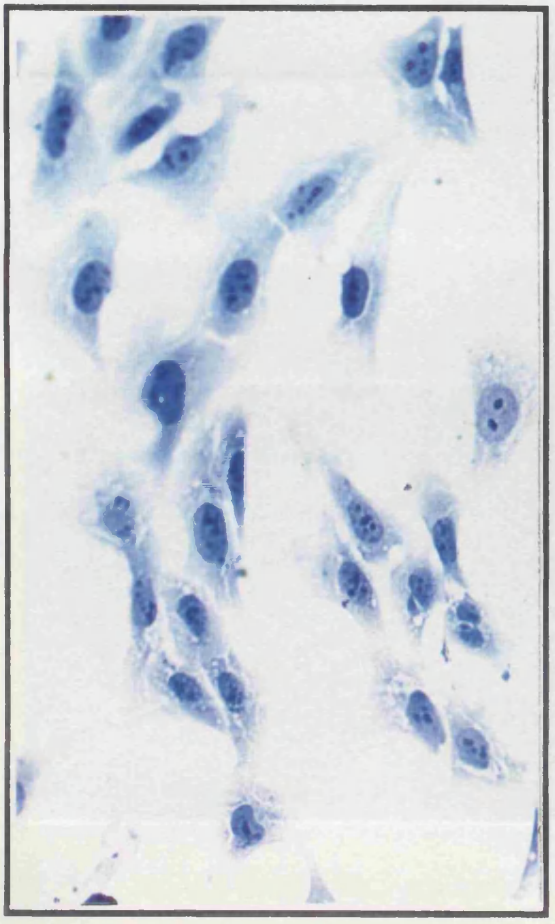
Figure 3.6h: Light micrographs of HOS cells on degraded PLA after 48 hours showed a similar morphology to the other PLA films. The cells were adhering to the PLA spherulites as well as the glass surface the polymer had been cast on (Magnification = 330x).



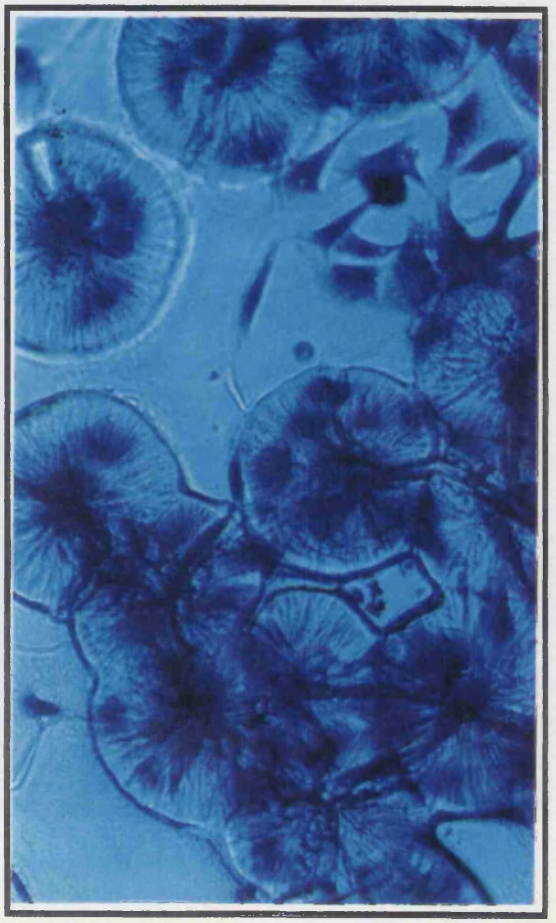
3.6e



3.6f



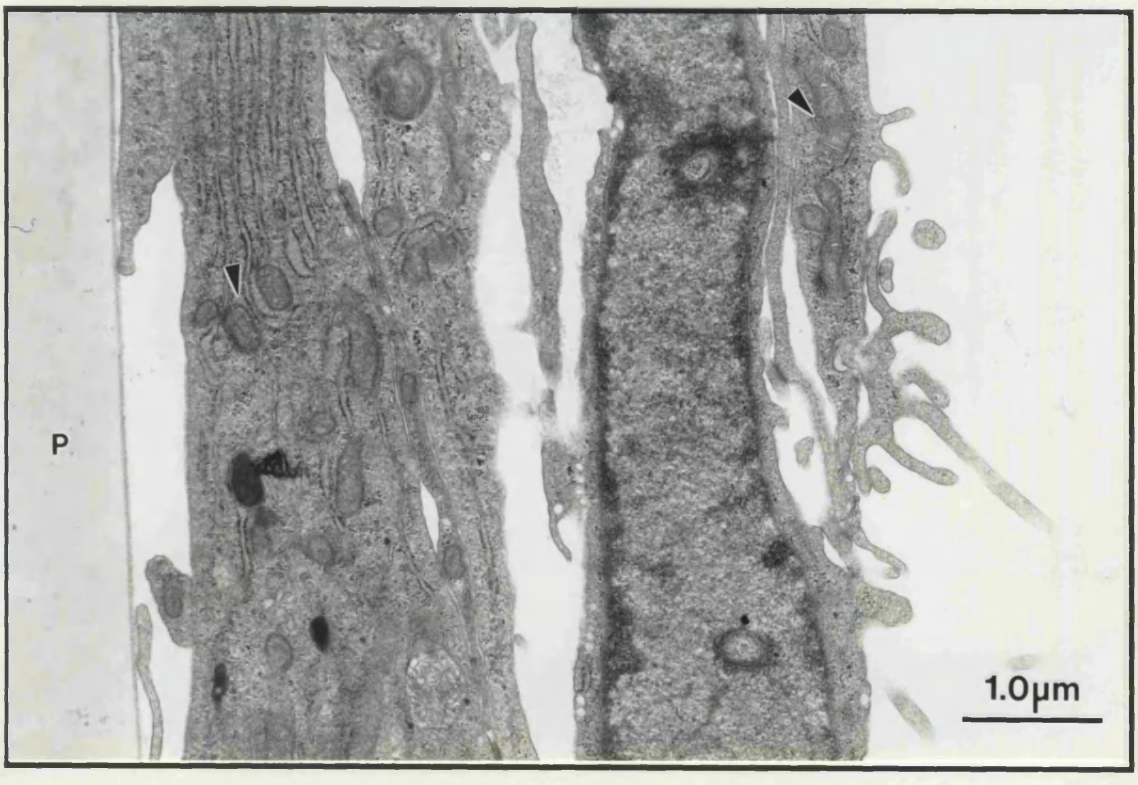
3.6g



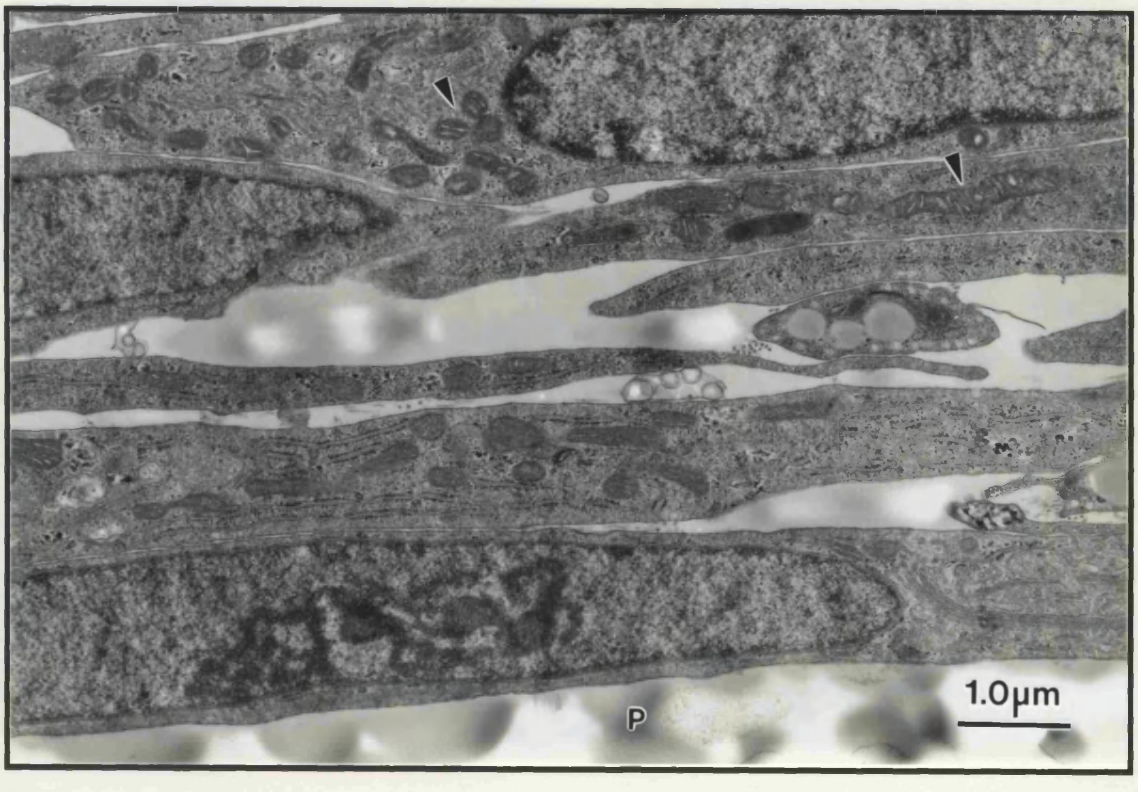
3.6h

Figure 3.7a: TEM of HOS cells on Thermanox which appeared active with many processes extending out from the cell surfaces. There was good cell to polymer (P) contact and healthy mitochondria (arrowhead) and endoplasmic reticulum was seen.

Figure 3.7b: TEM of HOS cells on PCL showed the cells to be active with healthy mitochondria present (arrowhead) and endoplasmic reticulum present. There were cells six to seven layers thick in areas with close contact occurring between polymer (P) and cell.



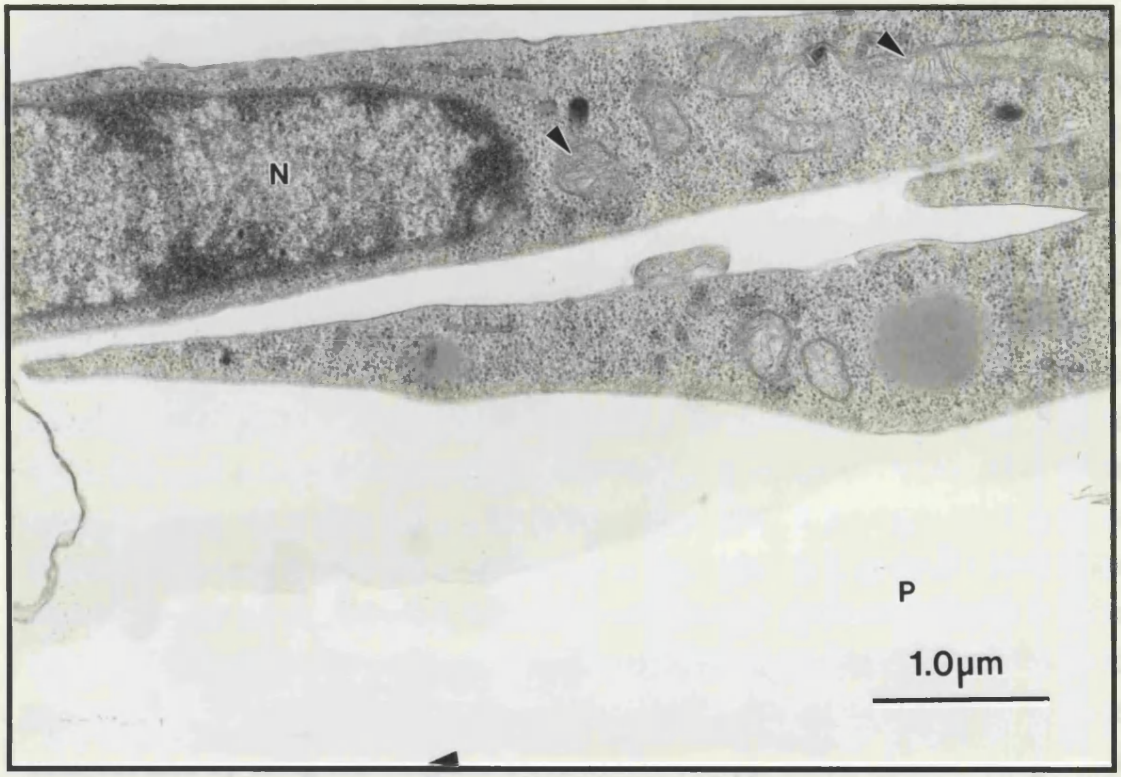
3.7a



3.7b

Figure 3.7c: TEM of HOS cells on PHB showed the cells appeared healthy with few cells present on the surface. Only two cell layers were observed and there was reduced contact between the cell and the polymer (P).

Figure 3.7d: TEM of HOS cells on PHB-PHV which appeared healthy with mitochondria present (arrowhead). There was good cell to cell contact but cell to polymer contact was not visible as the polymer (P) was easily burnt by the electron beam.



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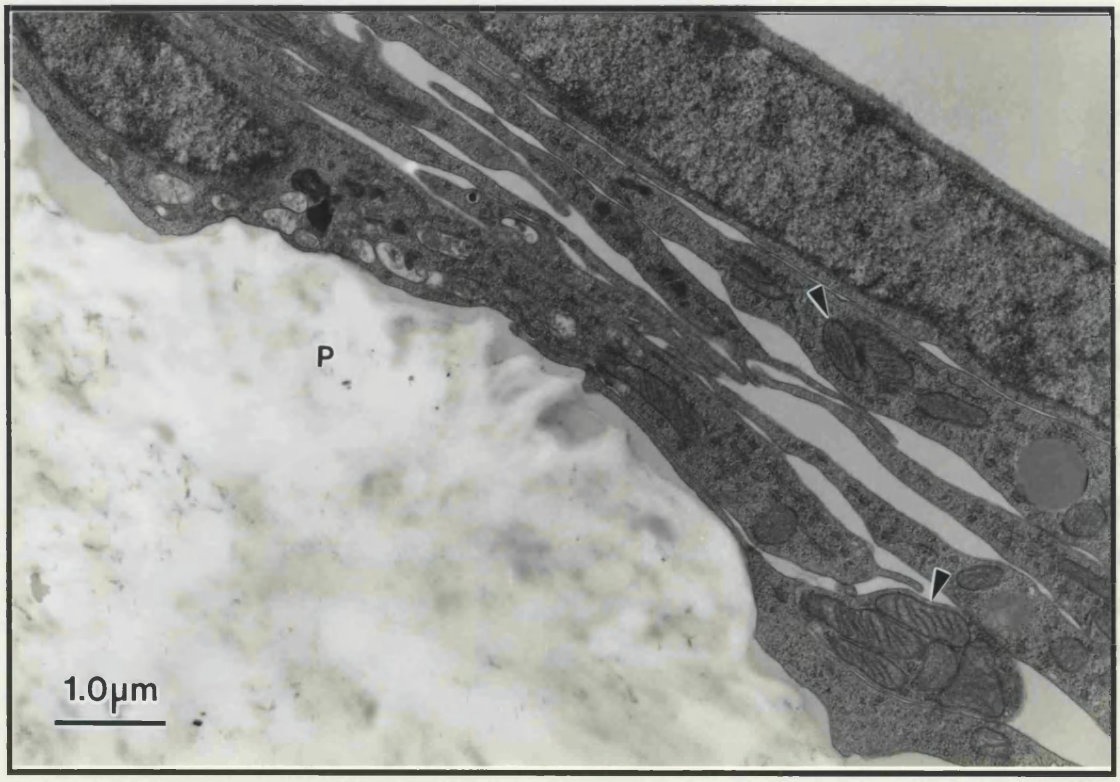


3.7d

Discussion

Toxicity of a material or degradation products from the material can lead to one or more of the following events occuring. There may be cell death which can be observed directly by a light microscope during cell culture with the cells rounding up and detaching from the surface they were adhering to. Nuclear shrinkage and fragmentation of the cytoplasm may occurr and there may be reduced cell adhesion which may in some cases be wrongly interpreted as cell death. Reduced cell adhesion is not necessarily due to a toxic effect (Kirkpatrick and Mittermayer, 1990). As well as cell death, reduced cell adhesion cells on biomaterial surfaces may alter their morphology in response to the polymer surface. Assessment of cellular morphology on the surfaces of biomaterials is the simplest method that can be used to identify cells and determine their morphological integrity. Changes in morphology may be due to reduced or increased spreading of the cells, an increase or decrease in the number of adhesion plaques present per cell or changes in the intracellular organisation of the cells. There may be an increase in vacuolation in the cytoplasm with a reduction in the amount of cellular proteins present or changes to the Golgi apparatus and mitochondria. in this chapter changes to mitochondria, nuclei and cellular processes were demonstrated by using scanning and electron microscopy. Other cellular changes that may occur in response to toxicity are reduced cell proliferationa and reduced biosynthetic activity and these are further discussed in detail in chapter four.

Changes in cellular response are not always due to the biomaterial directly but may be due to the composition of the medium and the environment which can alter the morphology of the cell (Freshney, 1983). Therefore when carrying out *in-vitro* tests and comparing cells on various surfaces, it is crucial to have the same methodology for all the polymers to be tested and even differences in the culture plastic can have a significant effect.

Results from the scanning electron micrographs have indicated that cell morphology was highly dependent on the polymer surface. The degree of total surface area covered by the cells on the polymer surfaces varied with time; with PLA and Thermanox showing the highest cell adherence and attachment, initially. Generally individual cells on these surfaces appeared larger and consequently covered a larger surface area compared to the cells on the PHB and the PHB-PHV copolymer. The cells on the PCL had a similar morphology to the cells on the PLA and Thermanox, but the processes appeared longer when making cell-cell contact. This could be due to there being fewer cells present on the surface thus the cells had to extend further to make cell to cell contact.

The cells on PHB and PHB-PHV after 24 hours appeared rounded and, if this had been a short term biocompatibility study, this could have been interpreted as an unfavourable

polymer surface for HOS cell attachment and proliferation. This stresses the importance of carrying out "long term" biocompatibility tests. There were also fewer cells attaching to the PHB and PHV surfaces indicating that cells did not attach as readily to these. Changes were observed after 48 hours and, although the individual morphologies of the cells on Thermanox, PLA and PCL remained similar to that at 24 hours, the morphology of cells on the PHB and PHV had changed significantly. The cells on PHB and PHV at 48 hours had become more well spread and appeared to be greater in number with a definite increase in cell density occurring in the original "colonies". This suggested that initially the cells did not favour the polymer surfaces for attachment but the few that did adhere were able to proliferate and by day 120 had reached confluence. The reason for this increase in proliferation after 48 hours could be due to the adsorption of protein onto the polymer surfaces. The culture media was supplemented with foetal calf serum which contains many different proteins and it is known that protein adherence to surfaces influences the behaviour of cells *in-vivo* and *in-vitro*. There are many different proteins present in serum which makes up 10% of the growth media and this could cause surface changes. Hydrophilicity of the polymers may have an effect on cell adherence and proliferation caused by a reorganisation of the cytoskeleton.

There are two types of cell adhesion: cell-cell and cell-matrix adhesion (Ruoslahti and Obrink, 1996). Many adhesion molecules have been discovered and characterised the majority of which also serve as signalling molecules. Cell adhesion has a role in proliferation, motility, cellular trafficking, differentiation, apoptosis and tissue architecture. The adhesion of cells in suspension on to a biomaterial surface occurs in a series of steps. Firstly, there is adsorption of serum proteins onto the polymer surface (Matsuda *et al* 1987) followed by contact of the cells with the material or the proteins adhered to the material surface (Davies *et al* 1988). Once contact has been made the cells can then attach to the surface via cellular processes and then spread on the surface (Ben-Ze'ev *et al* 1980) and the degree of spreading is dependent on the surface roughness and size of the particles. Brunette (1988) for example studied the effect of surface topography of implants on the attachment and migratory behaviour of cells and found that small grooves ≈ 0.5μm in depth caused the alignment and directed the migration of both fibroblasts and epithelial cells *in-vitro*. He concluded that the surface topography of the biomaterial affected cell attachment and influenced cell migration at implant surfaces. Harmands(1991) studies also concluded that the surface of a biomaterial plays an important part in cell adherence and cytocompatibility.

Zislis *et al* (1989) carried out SEM studies of *in-vivo* implanted polymers for cell attachment 7, 14 and 21 days post implantation. They found that degradation may inhibit cell attachment by the formation of a collagenous matrix around the degrading implant. Degradation was also thought to be due to the fluid environment rather than the cells.

The importance of the morphology of cells on the overall biocompatibility of materials is still unknown but it appears to play a major role as cell shape is known to influence and regulate proliferation and differentiated gene expression in a variety of cell types. Newman and Watt (1988) found cytochalasin-D$^{35}SO_4$ prevented articular chondrocytes from rounding up and spread cells were encouraged to round up with an increase in proteoglycan production also observed.

This study has demonstrated that there are changes in cellular morphology of osteoblast-like cells *in-vitro* when cultured on different polymer surfaces. The results have shown that qualitative evaluations using microscopy can be used as an effective tool in the biocompatibility testing of biodegradable biomaterials. Problems caused by conventional processing techniques were overcome using new methods which gave good cellular resolution, so cellular details could be visualised more easily. Although qualitative methods cannot give results which can be compared directly to other results they are a good method for visualising cells on polymer surfaces (Gurav and Downes 1994). The results have to be used in conjunction with quantitative data as they can be misleading if used on their own. Microscopy does provide information on the events occuring at the very early stages following cell seeding on the polymer surfaces. This type of approach allows the determination of the polymers more favourable for cell attachment and subsequent proliferation.

CHAPTER IV

Quantitative Evaluation of Cells on Materials

Introduction

There has been limited use of primary osteoblasts and osteoblast-like cell lines in biocompatibility testing to date. The majority of the work has been done using *in-vivo* experiments involving various animal models. Some of the studies described in the literature have used MC3T3-E1 cells (an osteoblast-like cell line) seeded onto various biomaterial surfaces, for example, Itakura *et al* (1989) used these cells to test the osteocompatibility of platinum-plated titanium. They found that the deposition of calcium was affected more than alkaline phosphatase activity. Elgendy *et al* (1993) also used these cells; they seeded MC3T3-E1 cells on to various polymer surfaces and assessed cell proliferation by cell counts and cellular morphology by SEM. Total DNA and alkaline phosphatase activity were also determined. The highest cell attachment was observed on P(LA-GA) surfaces which was comparable to the tissue culture plastic controls. Cell attachment on HA was, however, significantly lower than the other polymers. Attawia *et al* (1996) also used MC3T3-E1 cells to test the biocompatibility of poly(anhydride-co-imides) containing pyromellithylimido-alanine. They measured cell number and cell viability using cell counts by dye exclusion and the morphology of the cells on the polymers was examined using environmental SEM. The production of osteocalcin, an osteoblastic marker protein, was also measured over a 21 day period and the materials were found to maintain the osteoblastic phenotype over the study period. Macnair *et al* (1997) also tested primary rat and human osteoblast response to various materials and found the osteoblasts to be a useful *in-vitro* model for the biocompatibility screening orthopaedic biomaterials

Other cell types have also been used to test degradable materials and they have been able to support hepatocyte (Cima *et al* 1991), chondrocyte (Vacanti *et al* 1991) and fibroblast (Hansborough *et al* 1992) growth on the surfaces. Mikos *et al* (1993), used hepatocytes on PGA bonded fibre structures and found that, although initial cell attachment was sparse, after seven days the cells were forming clumps and cell interaction was observed. Other types of degradable materials, such as collagen sponges, based on type I and III bovine collagen, glycosaminoglycans (GAG) and chitosan have been used to measure collagen synthesis by fibroblasts. These were found to be biocompatible with fibroblasts and produced twice as much collagen in the sponge as opposed to a mono-layer culture (Berthod *et al* 1993).

This chapter deals with the quantitative evaluation of cells on materials. What is a quantitative test? Quantitative tests enable direct comparisons to be made between two or more sets of data. These may include tests such as the measurement of proliferation rates, cell numbers, total DNA, protein and the incorporation of radio-labelled nucleotides (Wieslander *et al* 1993). Chapter three dealt with qualitative observations of the

appearance of the cells on the different polymer surfaces. Qualitative observations are very much subject to the individual observer and definite conclusions cannot be drawn from these data. However qualitative observations can be quantified allowing for statistically significant comparisons to be made. An example of this would be the cell size on a particular polymer surface. A qualitative observation would simply suggest that the size of a particular cell on a material was larger than on another surface. If, however, the size was measured (using image analysis systems) the qualitative data could become quantitative and direct comparison of the size of cells on the surfaces could be made. In this chapter the response of the cells to the polymers and the effect of the polymers on the cells was assessed using a range of quantitative methods.

Although *in-vivo* tests have been carried out extensively they can be difficult to quantify due to limited numbers thus *in-vitro* tests provide a good tool for quantifying responses with statical significance. Work by Johnson *et al* (1985), showed that their *in-vitro* assays were more sensitive to material composition when compared to a 90 day subcutaneous implantation. However the studies also showed that *in-vitro* assays using cell lines were more reproducible than *in-vitro* assays using primary cells. The use of cell lines to test one agent or one response allows the quantification of the results in real terms and comparison with other test agents. Species differences can be eliminated as well as the individual variations which occur with *in-vivo* models. Very little data, however, is available on *in-vitro* tests of degradable materials and this may be due to some of the problems encountered with quantification of *in-vitro* tests, in particular those involving biomaterials. These include interference from the materials being tested or the effect of any leachables or degradation products of the polymer. There can be problems in processing of the polymers in that the polymers can be difficult to cast or shape into a precise shape, thus the starting material is different to begin with. For example, in this study, the polymers were cast into films and cut out into the appropriate shapes but, due to the low weight of the polymer films, they tended to float when placed into tissue culture dishes with medium. This made cell seeding very difficult and cells seeded on to the polymer films tended to fall on and adhere to the tissue culture plastic rather than the polymer surface.

Another problem encountered with polymers was reproducibility and differences in surface structure caused by changes in crystallinity as a result of casting conditions. The effect of crystallinity on cell adherence and proliferation was not measured but changes occurring to the polymer surfaces were visualised using light microscopy. With cell culture systems there are problems associated with reproducibility of plates in addition to plate drift, cell seeding densities and variability of cells at the different passages, all of which can also have a significant bearing on the final outcome. Although cells obtained

from passages 10-15 were consistently used for all cell experiments, differences even within these passages may have occurred.

Choosing the appropriate test for the quantitative analysis is important and is dependent upon the information required and the final use of the material in question. In this study ^{3}H-thymidine incorporation into cellular DNA was used to measure the proliferation of the cells in this chapter. The total DNA was also measured and proliferation was expressed as the amount of ^{3}H-thymidine incorporation per microgram of DNA. The incorporation of ^{3}H-thymidine into DNA as a measure of cell proliferation has pitfalls as reviewed in a paper by Maurer (1981). One of the pitfalls may be the binding of ^{3}H-thymidine decomposition products to macromolecules other than DNA and RNA. It is also assumed that ^{3}H-thymidine does not affect normal function but studies have shown that functional changes to the cells and mutations, DNA breakages and chromosomal aberrations, as well as growth retardation, occur. The effect of pH and temperature on the stability of tritium when incorporated into nucleotides also causes instability (Evans *et al* 1969). The results obtained from the ^{3}H-thymidine incorporation studies provide useful data on cell proliferation which is otherwise difficult to obtain, but these results have to be interpreted with care if they are to be used for routine biocompatibility testing of materials. Unfortunately, this is due to the limited methods available for determining cell proliferation to date. However, despite these shortcomings, studies in the literature have utilised ^{3}H-thymidine incorporation as a means of studying cell proliferation on both degradable and non-degradable materials (Chu *et al* 1995).

Hunter *et al* (1992), used a human fibroblast cell line (HF-19) on a range of non-degradable materials using tissue culture plastic as control. They measured adhesion using immunofluorescence labelling of vinculin and proliferation by ^{3}H-thymidine incorporation of the cells over 24 hours. They found significant differences in the proliferation of the cells on the materials and differences in the number of adhesion plaques which could be used to quantify cell adhesion to different materials. Hunter *et al* (1995), used fibroblast (HF-19) and osteoblast (UMR 106.1) cell lines to test attachment and proliferation on various non-degradable biomaterials. They found differences in the response of osteoblasts and fibroblasts, again stressing the drawbacks in comparing two different cell types to test the same material.

In the cell culture studies PGA could not be used as it was insoluble in the solvents tested and thus films could not be cast. Results from chapter three however, showed the effect of the polymer during degradation on cells in culture. Over the relatively short term study of 24 days PGA bars had started to degrade by day 7 and by day 12 cell damage had occurred. The methods used in this chapter to artificially degrade were enzymes and other aqueous solutions. High temperature was not used although it was used in chapter two.

Other studies have utilised high temperature to obtain rapidly degraded polymers (Buchholz 1993; Gogolewski and Mainil-Varlet 1996; 1997). Studies have been described in the literature which have used PLA and PLA:PGA copolymers for biocompatibility testing *in-vitro*. Ishaug *et al* (1994) seeded rat osteoblasts onto films of PLLA, PLGA (75:25), PLGA (50:50) and PGA over a 14 day period. The ALP activity was measured and was compared to a TCP control. The ALP for cells on 75:25 PLGA increased from day 7 to day 14, which was comparable to TCP, but did not increase for the other polymers. Collagen synthesis did not vary for the polymers compared to TCP and they concluded that the morphology of the osteoblasts was not affected by degradation of the polymer surfaces. This was in contrast to the results obtained in chapter III where degradation products from PGA did affect cell morphology after day 7.

The polymer studies for quantitative analysis of the cells in culture were performed on films cast onto glass slides. Variability in the surface of films was visible with the PCL films. The micrographs (figure 4.4a-d) show the different surfaces formed after casting. The films were cast in similar conditions at room temperature, and dried under a glass petri dish. The differences in the surfaces may have been due to the drying temperature, the temperature of the environment or variability in the glass surface used for casting. Differences in the other polymer surfaces cast may also have occurred but these were difficult to visualise as they were smoother and did not have prominent features which could be used as markers. There are other methods available, such as goniophometry, which can quantify the surface properties (Knowles and Hastings, 1991; 1992). Goniophotometry is the measurement of reflected light with respect to viewing angle. Measurement of surface roughness and gloss factor can be achieved by analysing the reflectance pattern from a beam of light.

Although there are limitations with all the methods described, if these are taken into consideration when assessing biocompatibility and statistical significant test numbers are used, it is possible to obtain a quantifiable measure or assessment of biocompatibility. No one test can give an accurate assessment thus a number of tests measuring different parameters need to be used.

MATERIALS AND METHODS

Polymer Formulation

Polymer films were cast onto glass slides as described in chapter II and cut into 15 mm disks using a cork borer. The films were rinsed in 70% ethanol followed by 100% ethanol before being air dried and placed into 24 well plates. For the first few experiments the films were not hydrated prior to cell seeding but later the films were hydrated for 24 hours with complete medium prior to cell seeding in order to remove residual chloroform.

Cell culture

For all experiments a cell seeding density of 4×10^4 cells/cm^2 was used unless stated otherwise. The cells were cultured on the films for the appropriate time period at 37°C with 5% CO_2 in a humidified atmosphere. For biochemical analysis after cell culture the films with the cells on them were removed and rinsed in PBS before being transferred to a fresh plate with 1ml of distilled water. This was placed at 37°C for 30 minutes then at -70°C for 30 minutes and the cycle repeated three times to rupture the cells. The cell digest was then used for the DNA assay and the ^{3}H-thymidine counts.

DNA assay

The amount of total DNA present in the samples which had been freeze-thawed to rupture the cells was measured by a method modified by Kapuscinski and Skoczylas (1977). DNA obtained from calf thymus (Sigma) was used to obtain a standard curve from 0-20μg/ml. The DNA in the unknown samples was detected by the binding of the DNA with DAPI (4',6-Diamidino-2-Phenylindole, Sigma) to form a fluorescent complex. This fluorescence was measured at 460 nm on a Perkin-Elmer fluorometer and read off the standard curve to give the amount of DNA present in the samples.

^{3}H-thymidine labelling

^{3}H-thymidine labelling is used to obtain information on the rate of proliferation of cells expressed as a percentage of the total DNA. Cells in the S phase take up ^{3}H-thymidine for incorporation into new DNA. If the thymidine is labelled with tritium the amount of newly synthesised DNA can be measured. Cells were cultured on the polymer to be tested or on tissue culture plastic as control and cultured for 24 hours. The ^{3}H-thymidine was added to the growing cells at 1 μCi/ml in complete supplemented medium. The cells were further incubated for the desired time period, usually 24 hours, before removal of the media. The cells were rinsed with PBS at 37°C and stored in 1ml of deionised water. They were

freeze-thawed at -70°C and 37° C three times to ensure the cells had ruptured. The cell lysate was used for the various assays. For counting the ^{3}H-thymidine incorporation 100 µl of the digest was placed into 96 well filtration plates 0.45µm pore size (Millipore) with 20% TCA at -4°C for 30 minutes to precipitate the DNA. The precipitate was rinsed in 10% cold TCA to remove any unbound tritium using a vacuum filter system. The plate was dried at 37°C and the filters punched into scintillation vials. 0.01M potassium hydroxide was used to dissolve the precipitate and the sample mixed for 30 minutes. 4 mls of Emulsifier Scintillator-Plus fluid (Canberra-Packard) was added to each vial and mixed for a further 30 minutes before reading on the scintillation counter Minaxi Tri-carb 4,000 series, (Canberra Packard) for 5 minutes each. Results were expressed as counts per minute (CPM).

Statistics

The results obtained were analysed using appropriate statistical tests. The data sets were tested for normality using the Shapiro-Wilk W test. If p values obtained were less than 0.05 the data was not normal. If the data was normal (parametric) an analysis of variance was used, and if the data was not normal (non-parametric) the Mann-Whitney test was used. Depending on the results from these tests a multiple comparison test was carried out, the Tukey Kramer-Honestly Significant Difference test (TK-HSD). All tests were carried out using alpha values of 0.05 and 0.01 and significance was denoted * for significance at $p<0.05$ and ** for significance at $p<0.01$. Where multiple comparison was not required and comparisons could be made to a control the Dunnett's test was carried out at alpha values of 0.05 and 0.01 and significance was denoted as above. Some data sets could not be analysed due to small sample sizes.

RESULTS

Figures 4.1-4.3 show the total DNA and ^{3}H-thymidine incorporation into HOS cells seeded at increasing cell densities on tissue culture plastic over a period of 0-96 hours. The total DNA measured at the lower seeding densities was very low for the first 72 hours but it increased thereafter up to 96 hours. The other seeding densities also showed low DNA concentrations during the period of 0-24 hours but increased steadily up to 96 hours. Although an increase in DNA was observed for all the seeding densities, a plateau was not reached during the time period studied. Figure 4.2 shows the total ^{3}H-thymidine incorporation per test sample. The DNA content increased for all seeding densities with time. Increase in cells at the two lowest seeding densities was slow for the first 72 hours but by 96 hours the cells increased dramatically in number. However, this was not the case for the highest seeding density which increased steadily in cell number. ^{3}H-thymidine incorporation (figure 4.2) was seen for all the seeding densities over time with the exception of the highest seeding density. (For this sample seeding density incorporation) was measured, but did not increase with time. The greatest proliferation (figure 4.3) was observed with the lowest seeding density at 0-24 hours although, the degree of error was higher for these values compared to the other seeding densities when expressed per µg of DNA.

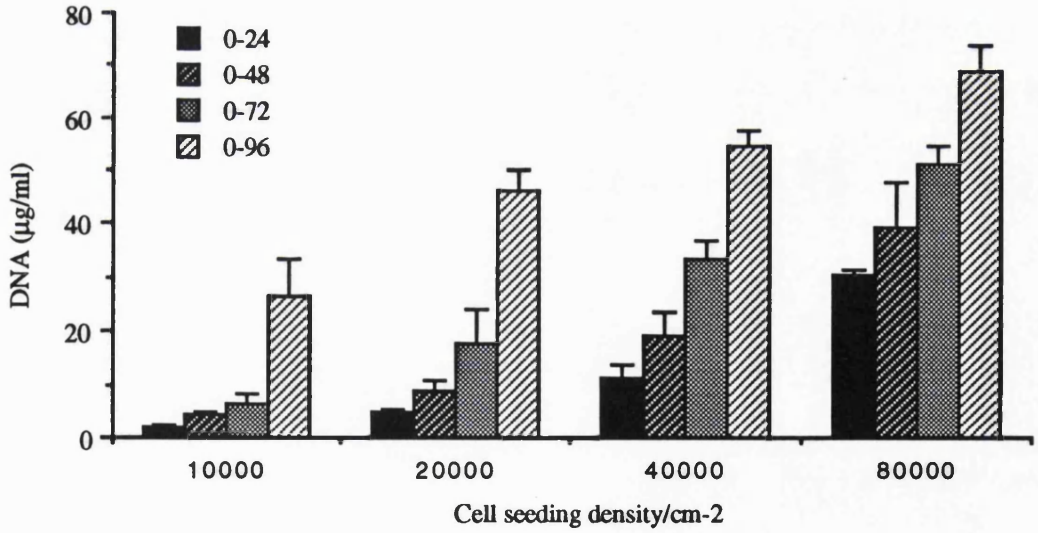


Figure 4.1 shows the total DNA concentration of HOS cells seeded at different initial seeding densities on tissue culture plastic at 24 hour intervals for 96 hours. Each point represents six replicates with the error bars being the percent standard error of the means. An increase in DNA was observed for all the seeding densities with time (TK HSD at $p<0.05$).

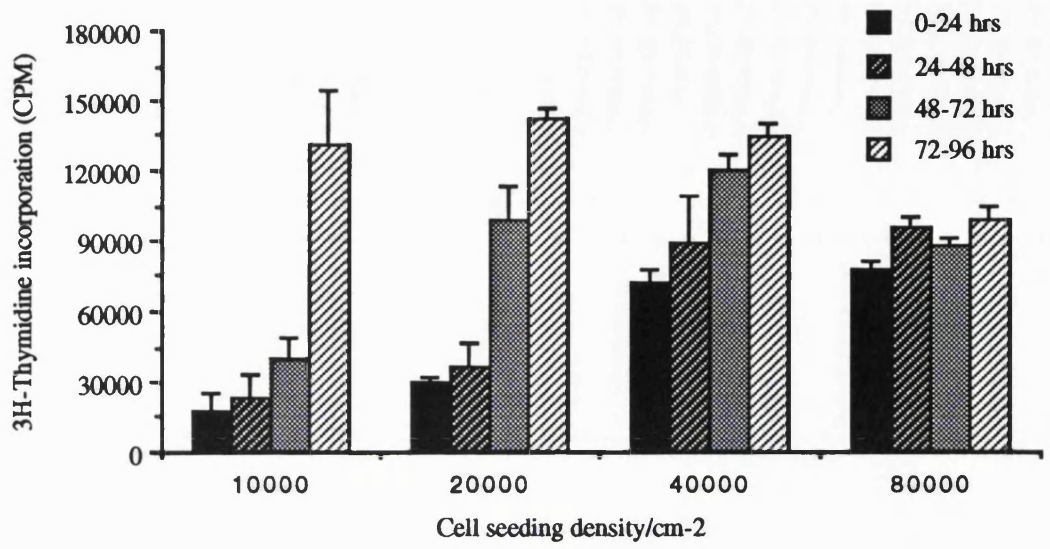


Figure 4.2 shows the ^{3}H-thymidine incorporation into HOS cells at the different cell seeding densities. Each point represents six replicates with the error bars being the percent standard error of the means. An increase in the ^{3}H-thymidine incorporation was observed for all the seeding densities with time with the exception of the highest seeding density. This seeding density showed no significant increase or decrease with time TK-HSD ($p<0.05$).

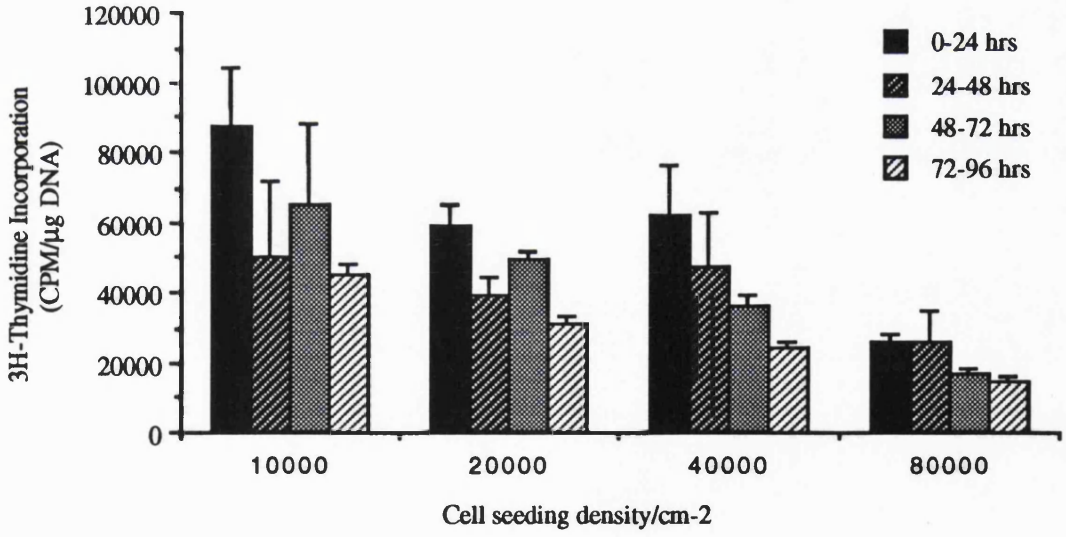
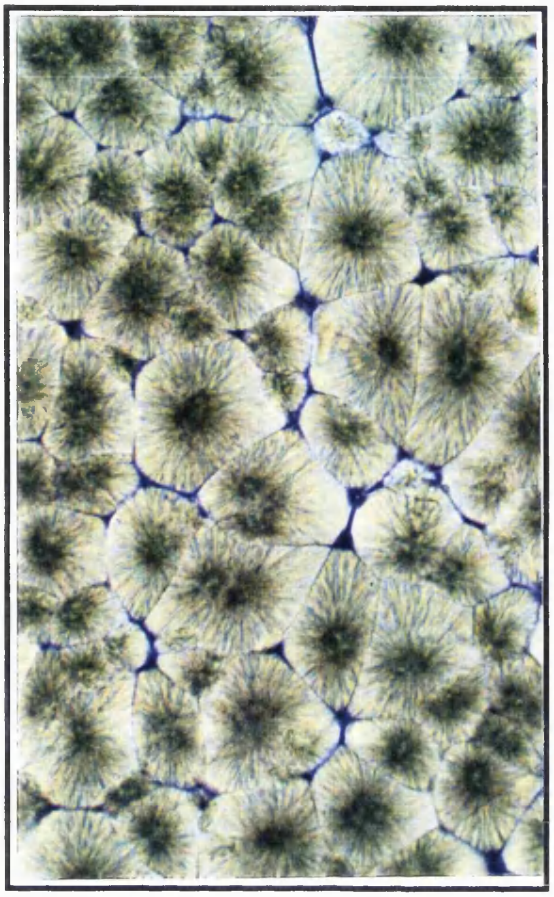
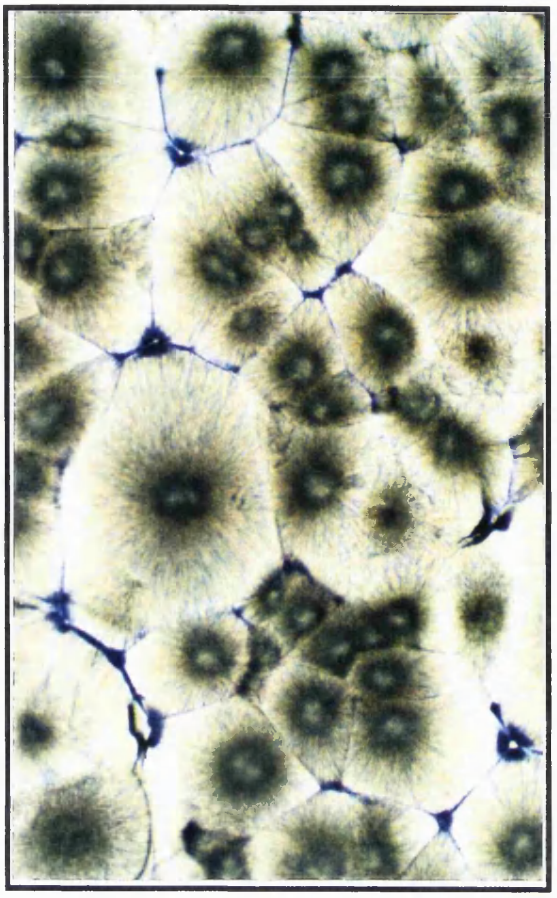


Figure 4.3 shows the ^{3}H-thymidine incorporated per µg of DNA after each time interval. Each point represents six replicates with the error bars being the percent standard error of the means. The ^{3}H-thymidine incorporation per µg of DNA decreased with increasing seeding density and with time.

Figures 4.4a-4.4d shows the variations in the surfaces PCL visualised by light microscopy. The spherulites varied in size as well as shape and surface structure (Magnification = 330x).



4.4a



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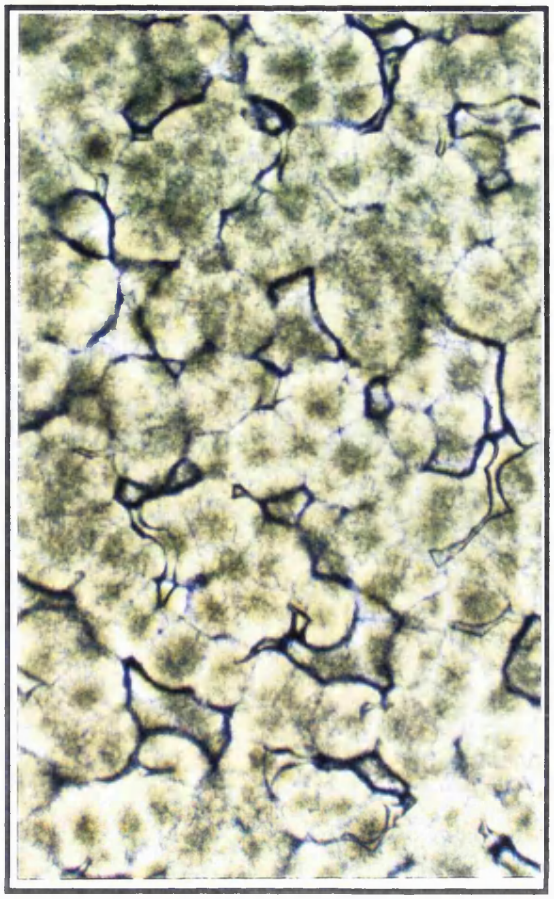
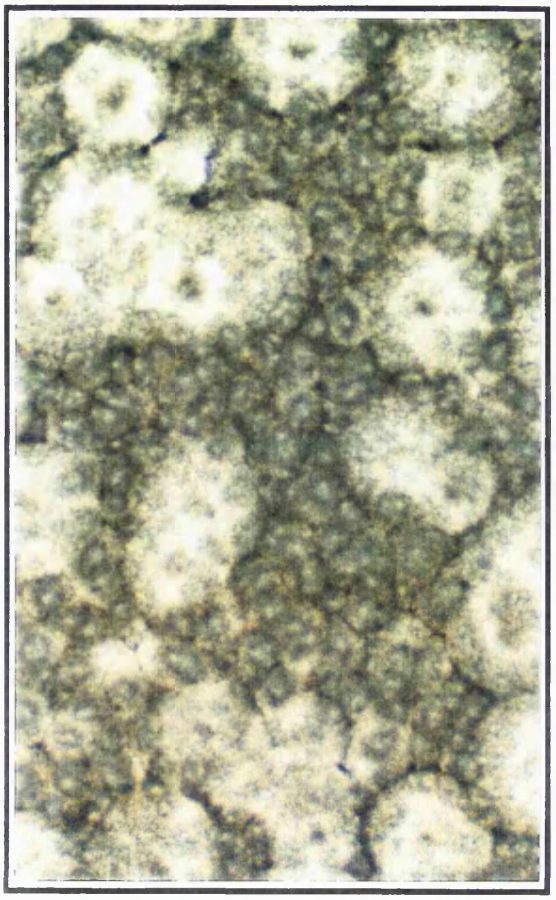


4.4c



4.4d

Figures 4.5-4.7 show the total DNA and ^{3}H-thymidine incorporation over a 0-72 hour period. After 24 hours (figure 4.5) the DNA on PCL and PLA was significantly lower than that on PHB and PHB-PHV. After 48 hours and 72 hours no significant differences were observed. The total DNA for the polymers increased on all the polymers at 48 hours and after 72 hours, except for PLA and PHB-PHV, where it decreased. The reason for this is not known and may have been due to polymer variability as the results were always variable.

^{3}H-thymidine incorporation into HOS cells cultured on the polymers is shown in figure 4.6. The graph shows the increase in ^{3}H-thymidine incorporation into cells cultured on all the polymer surfaces with time it increased up to 72 hours with the exception of PHB-PHV where a small decrease was observed which was not significant. PCL was significantly lower that PHB and PHB-PHV. No significant differences were observed between PHB and PHB-PHV, and PLA was significantly lower than PHB-PHV only (TK-HSD $p<0.05$). PCL was significantly lower than PLA at 0-24 hours and at 48-72 hours. No other significant differences were observed. The CPM/μg of DNA is significantly less on PCL as compared to Thermanox and PLA.

The time points on the DNA graphs are expressed differently to the ^{3}H-thymidine incorporation graphs due to the nature of the assays. The DNA measured after 48 hours was the total DNA from time 0 while the ^{3}H-thymidine incorporation was measured only for the 24 hour window stated. This was one of the drawbacks of the method as ^{3}H-thymidine incorporation for 24-48 hours was being expressed for DNA measured from 0-48 hours. The increase in DNA from 24-48 hours could not be separated thus it was important to include the ^{3}H-thymidine graph not expressed as μg/DNA. This gave a relative measurement of the total activity regardless of the cell number.

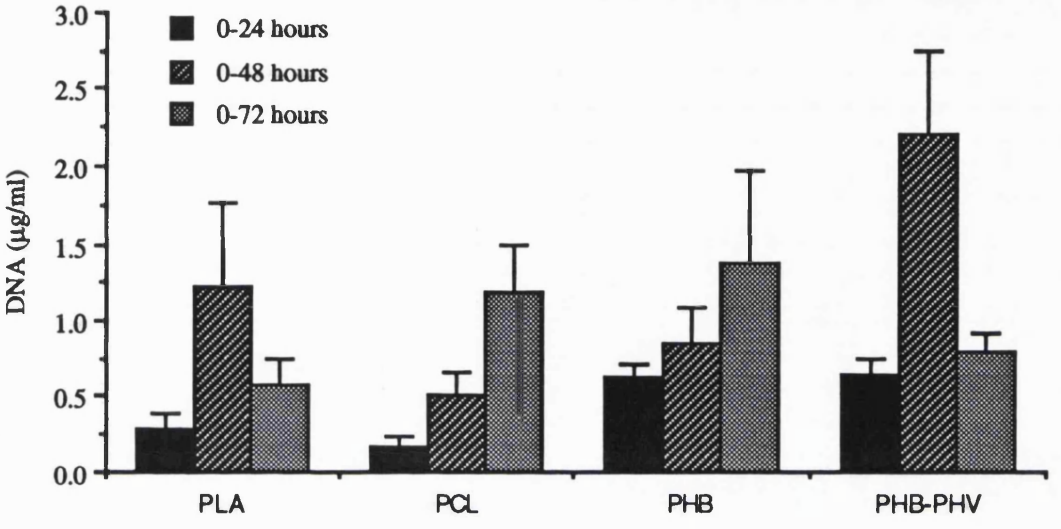


Figure 4.5 shows the DNA content of cells grown on the four polymer surfaces over a 0-72 hour period. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

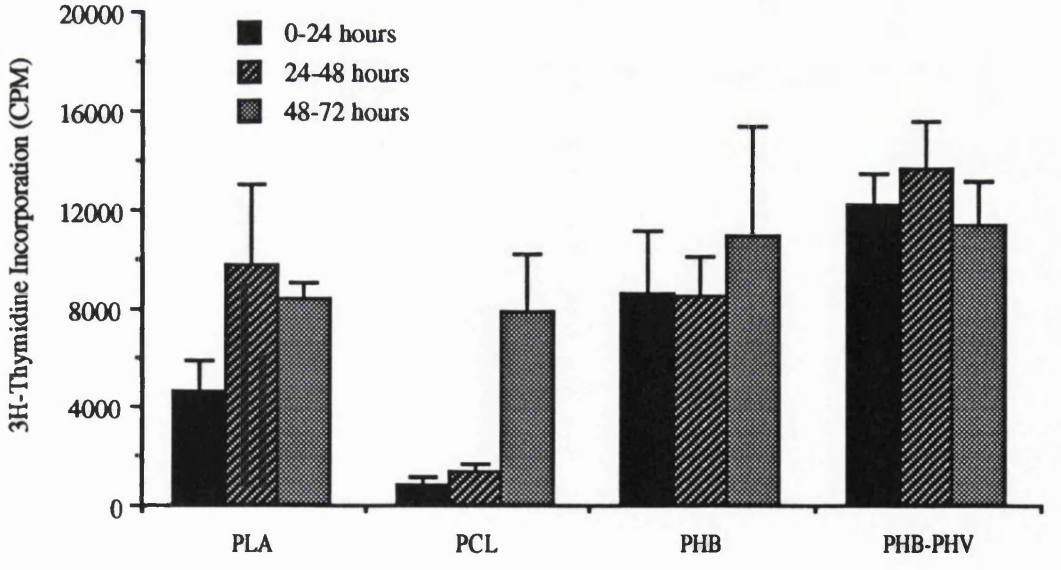


Figure 4.6 shows the total ^{3}H-thymidine incorporation into HOS cells as they were proliferating on the polymer surfaces. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

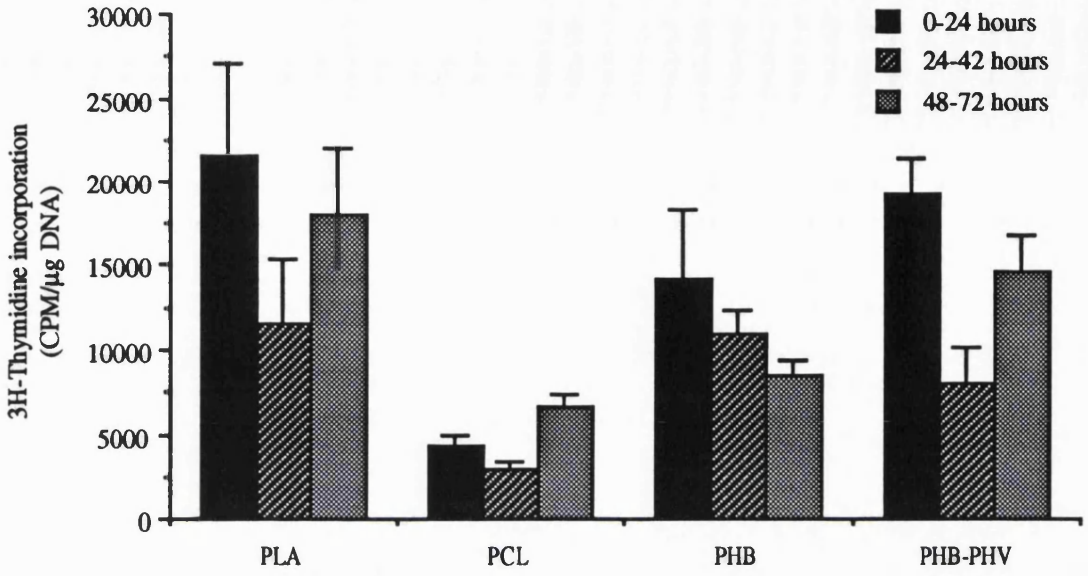


Figure 4.7 shows the ^{3}H-thymidine incorporation into HOS cells expressed as CPM/ µg of DNA. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

Figures 4.8-4.10 show the culture of HOS cells on the polymers using Thermanox as a control. Thermanox is a polymer treated specifically for tissue culture use and cells adhere and proliferate well on this material. What is noticeable is the variability in the DNA, ^{3}H-thymidine incorporation in this set of results and those shown in figures 4.5-4.7. This illustrates the problems associated with biocompatibility testing *in-vitro* and the need to have controls each time a test is carried out. The differences may be due to the polymers themselves or the cell culture conditions. The cell seeding densities used were the same and the size of the polymers was also the same. Figure 4.8 shows the DNA content of cells on the polymer surfaces over 0-120 hours. The DNA did not increase significantly for the polymers from 0-24 hours to 0-48 hours. There was a significant difference, however, from 48 hours to 120 hours. The highest levels of DNA were observed on Thermanox, followed by PLA and PCL. The lowest DNA levels were found on PHB with PHB-PHV being marginally higher. The greatest increase in DNA was between 48-120 hours which is confirmed in figure 4.9 where most ^{3}H-thymidine incorporation is seen at 72-120 hours with the exception of PHB-PHV which shows a reduction in ^{3}H-thymidine incorporation. Figure 4.10 shows the ^{3}H-thymidine incorporation/µg of DNA of cells on the polymer surfaces. Most incorporation was seen at 24-48 hours and was highest for PLA. This is in contradiction to the DNA results (figure 4.8) where the least DNA was seen for PLA at 0-48 hours. The reason for this discrepency is not known and could be due to polymer surface or assay sensitibity.

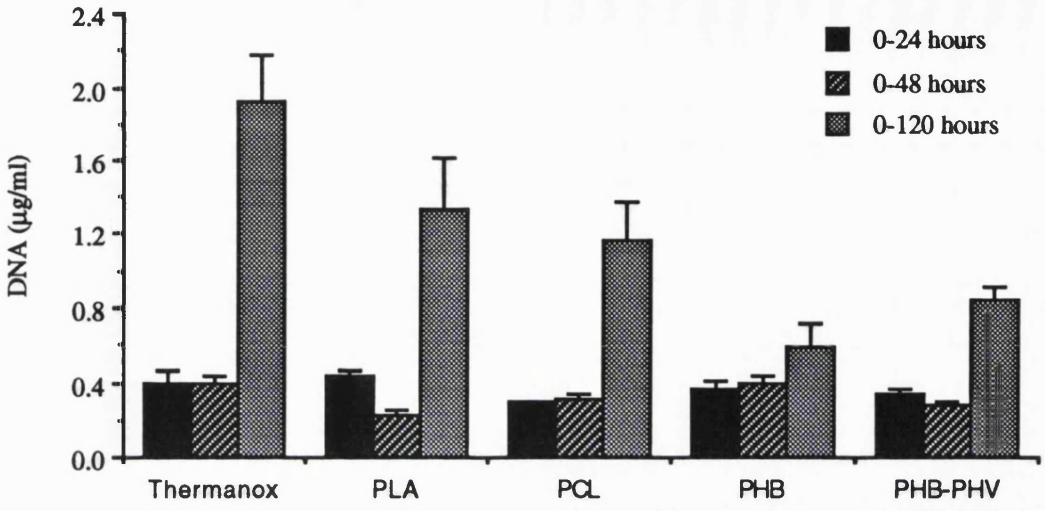


Figure 4.8 Total DNA of HOS cells cultured on polymer surfaces over three different time periods. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

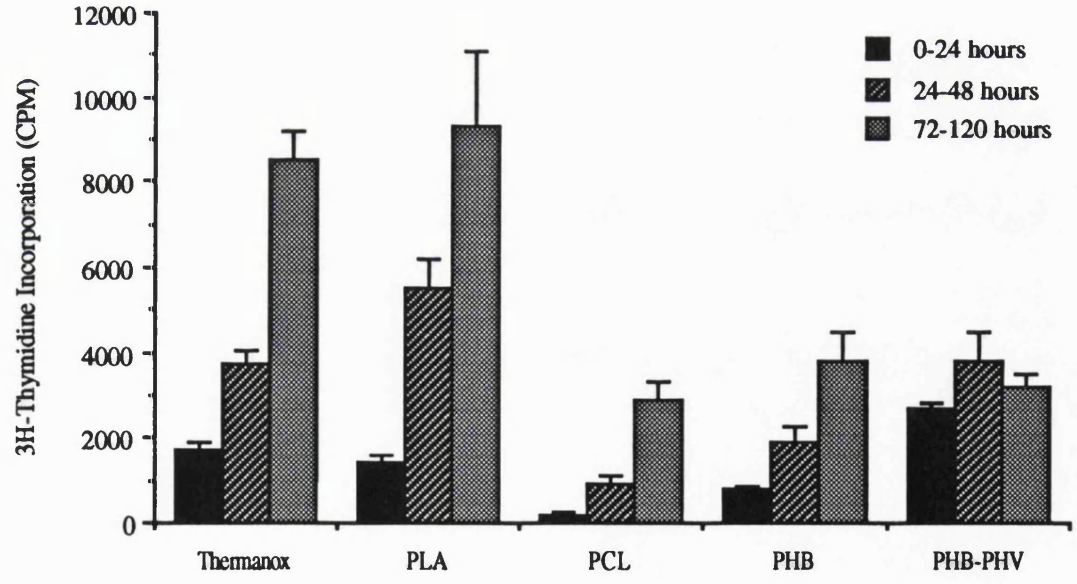


Figure 4.9 Graph of total thymidine incorporation into HOS cells cultured on polymer surfaces over three time periods. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

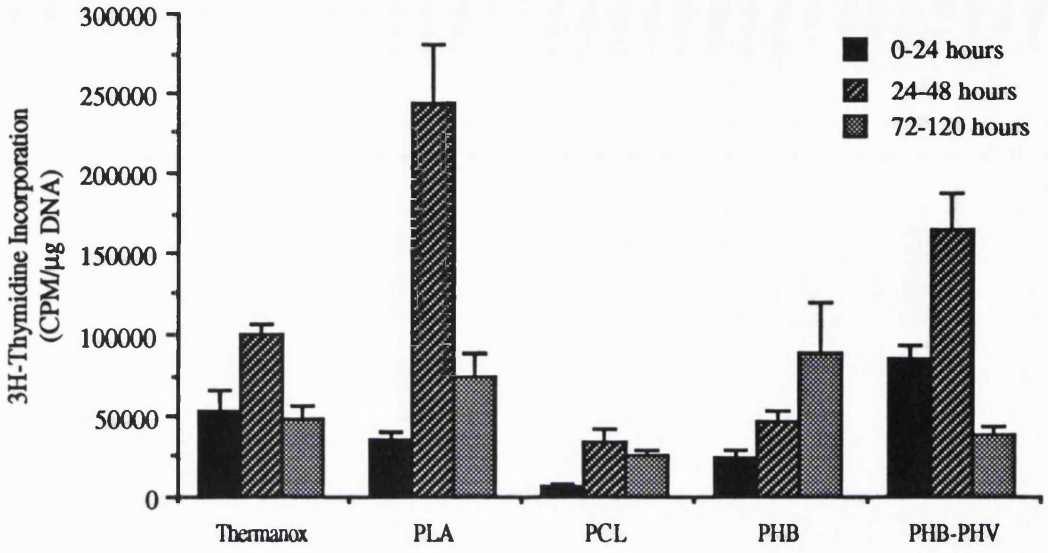


Figure 4.10 Graph of ^{3}H-thymidine incorporation/µg of DNA when cultured on polymer surfaces over a 120 hour period. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

Figures 4.11 to 4.15 show a series of results of HOS cell seeding on polymers for 48 hours (with ^{3}H-thymidine incorporation measured over the 24-48 hour period) that had been degraded in various solutions. Figure 4.11 is the "as cast" control and shows the data expressed as a percent of the TCP. There were no significant differences in the total DNA on the polymers however PCL showed significantly less ^{3}H-thymidine incorporation compared to the other polymers and TCP thus the ^{3}H-thymidine incorporation/µg of DNA was low suggesting that cells on PCL were not dividing. Cells on PLA showed high DNA and ^{3}H-thymidine incorporation hence the highest ^{3}H-thymidine incorporation/µg DNA.

Figure 4.12 shows the DNA of cells on polymers following storge in enzymes expressed as a percent of TCP. Cells on all the polymers were lower than the TCP. DNA on PHB was higher on the "as cast" and enzyme stored polymers. PHV in papain was not shown due to the polymer undergoing breakage thus reproducible discs could not be obtained. Storage of PCL with trypsin and papain increased the DNA compared to the "as cast" polymer whereas for PHB-PHV it decreased following incubation in trypsin. DNA on PHB stored in all the solutions was higher compared to PCL with the exception of storage in papain where DNA on PCL was significantly higher.

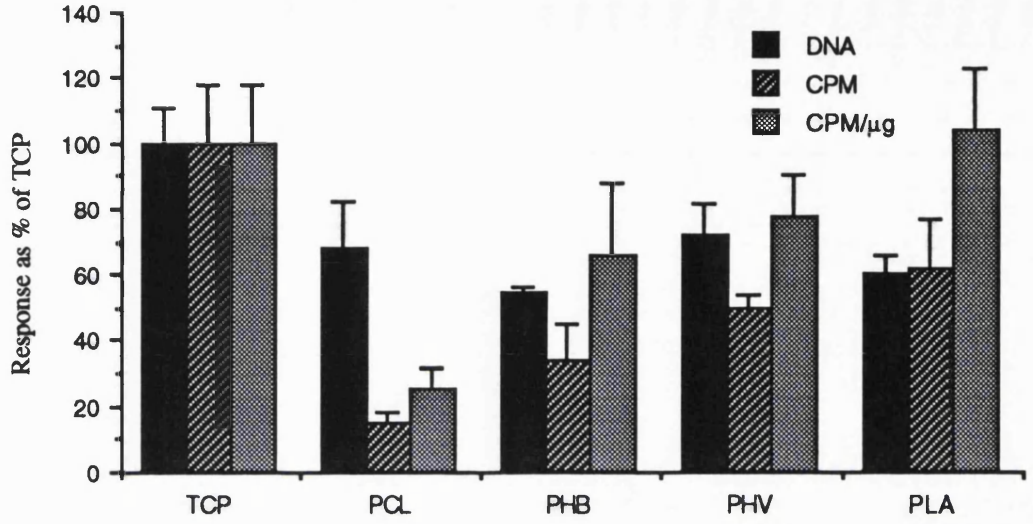


Figure 4.11 shows the DNA, total ^{3}H-thymidine incorporation and ^{3}H-thymidine incorporation/µg DNA into HOS cells on polymer surfaces expressed as a percent of TCP control. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

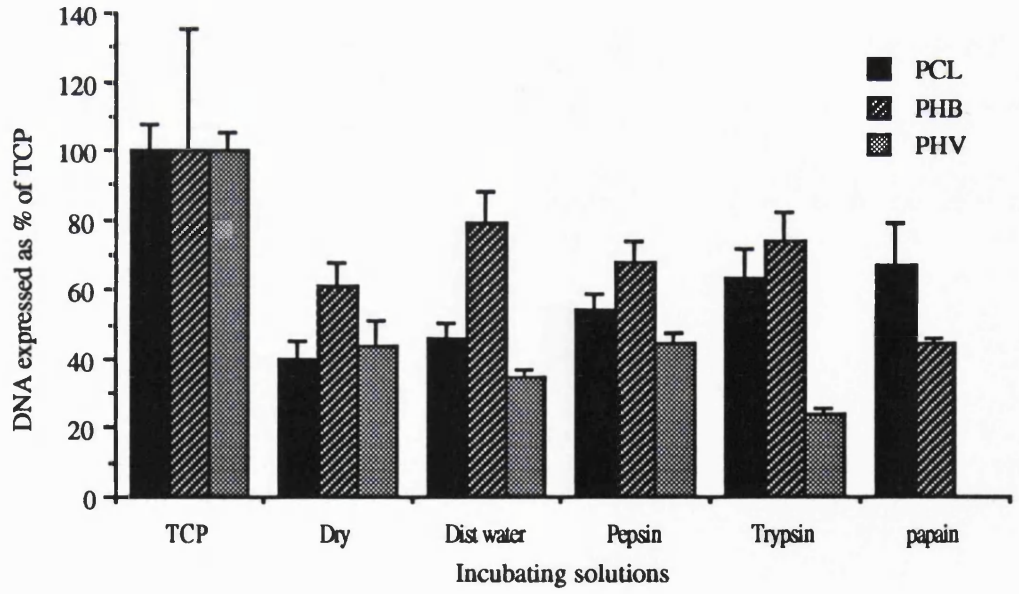


Figure 4.12 Shows the total DNA of cells cultured on polymers which had undergone treatment in various solutions for 5 weeks. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

Figure 4.13 shows the DNA, ^{3}H-thymidine incorporation into PCL following storage in all the solutions compared to TCP. The measured response of the cells on the polymers was lower compared to TCP. No significant differences were observed between the different treatments except for DNA being significantly higher on papain and trypsin treated polymers.

Figure 4.14 shows results obtained with PHB following storage in the differnt solutions for 5 weeks. No significant differences were observed between the different treatments with the exception of storage in papain. Storage in papain resulted in lower DNA and lower ^{3}H-thymidine incorporation. There was no significant difference compared to the TCP control for DNA and ^{3}H-thymidine incorporation.

Figure 4.15 shows the results obtained with PHB-PHV following storage in the different solutions. The response of the DNA and ^{3}H-thymidine incorporation values on all the polymers was lower than TCP. The proliferation was also lower on all the polymers with the exception of the dry "as cast" polymer which was significantly higher than all the polymers and TCP.

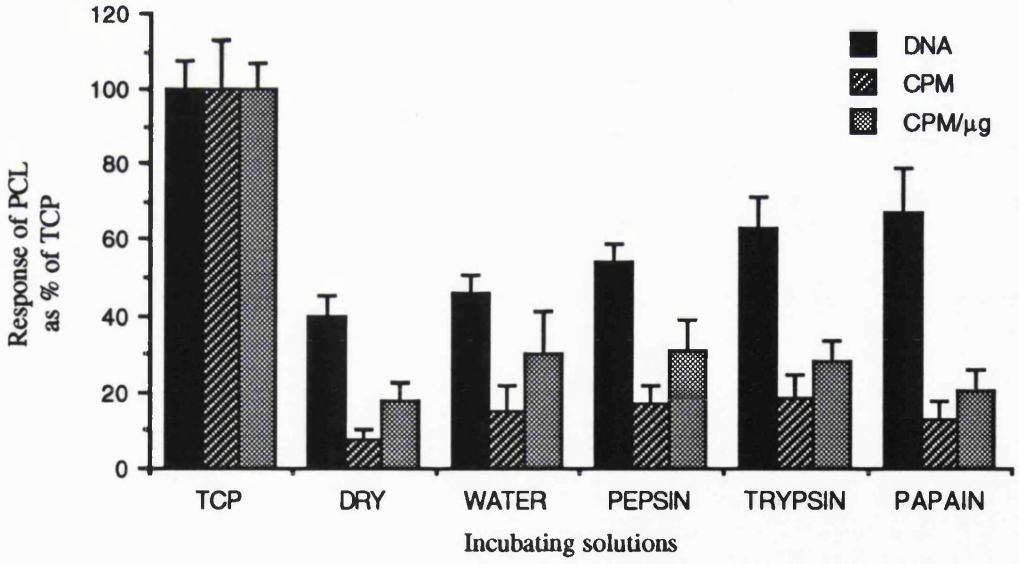


Figure 4.13 shows the DNA, total ^{3}H-thymidine incorporation and ^{3}H-thymidine incorporation/µg DNA into HOS cells on PCL surfaces incubated in various solutions for five weeks expressed as a percent of TCP control. Cells were cultured for 48 hours and ^{3}H-thymidine incorporation measured between 24-48 hours. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

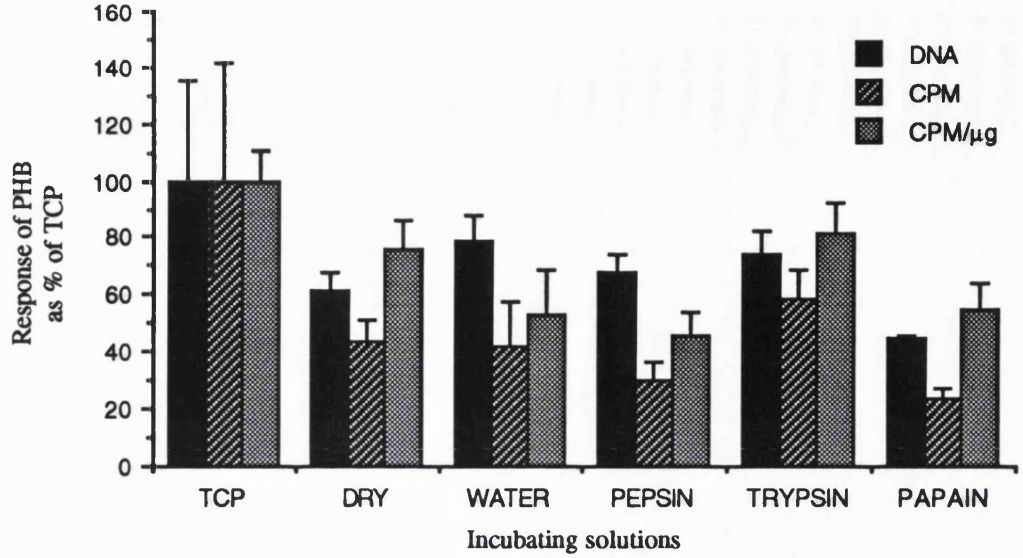


Figure 4.14 shows the DNA, total ^{3}H-thymidine incorporation and ^{3}H-thymidine incorporation/µg DNA into HOS cells on PHB surfaces incubated in various solutions for five weeks expressed as a percent of TCP control. Cells were cultured for 48 hours and ^{3}H-thymidine incorporation measured between 24-48 hours. Each point on the graph is a mean of four samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

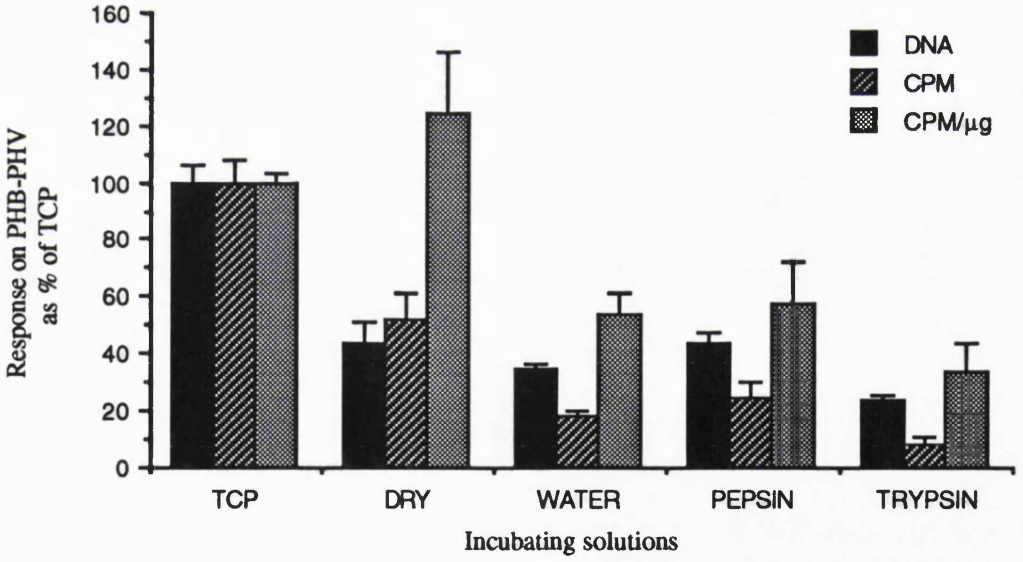


Figure 4.15 shows the DNA, total ^{3}H-thymidine incorporation and ^{3}H-thymidine incorporation/µg DNA into HOS cells on PHB-PHV surfaces incubated in various solutions for five weeks expressed as a percent of TCP control. Cells were cultured for 48 hours and ^{3}H-thymidine incorporation measured between 24-48 hours. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

An experiment carried out to determine if differences in the "as cast" polymers was due to residual chloroform or surface roughness was carried out using washed and stored polymers. Figure 4.16 shows the DNA on the polymers. The DNA was not significantly affected for PCL, PHB or PHB-PHV for any of the treatments however, storage of PCL in PBS resulted in higher errors.

Figure 4.17 shows the ^{3}H-thymidine incorporation into the polymers and no significant differences were observed for the polymers however the response of the cells on the polymers was significantly lower than the Thermanox control.

Figure 4.18 shows the ^{3}H-thymidine incorporation/μg of DNA on PHB and PHB-PHV was significantly higher than Thermanox however no significant differences were obtained between the different treatments. The proliferation of cells on PCL following storage in PBS for 5 weeks resulted in a higher proliferation.

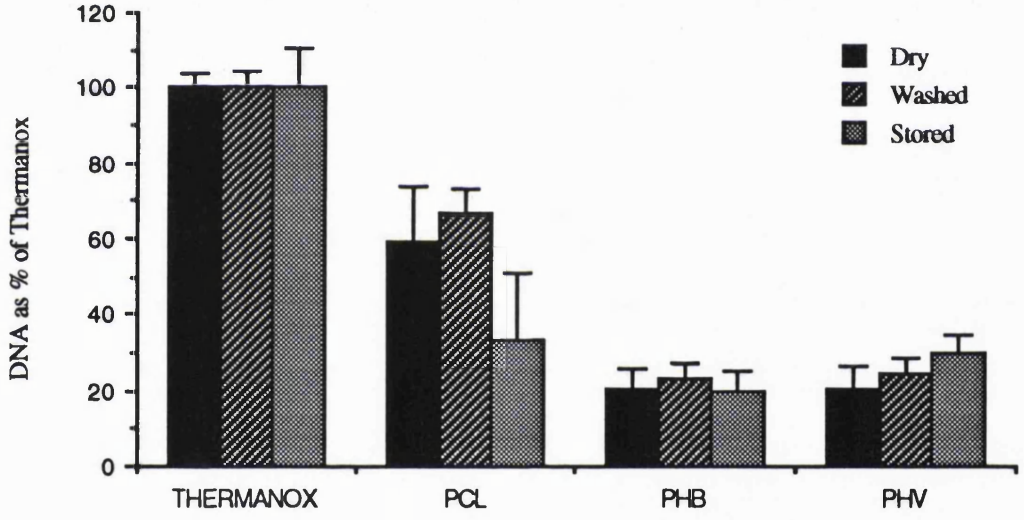


Figure 4.16 Total DNA content of cells cultured on dry, washed and stored PCL, PHB and PHB-PHV over 48 hours. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

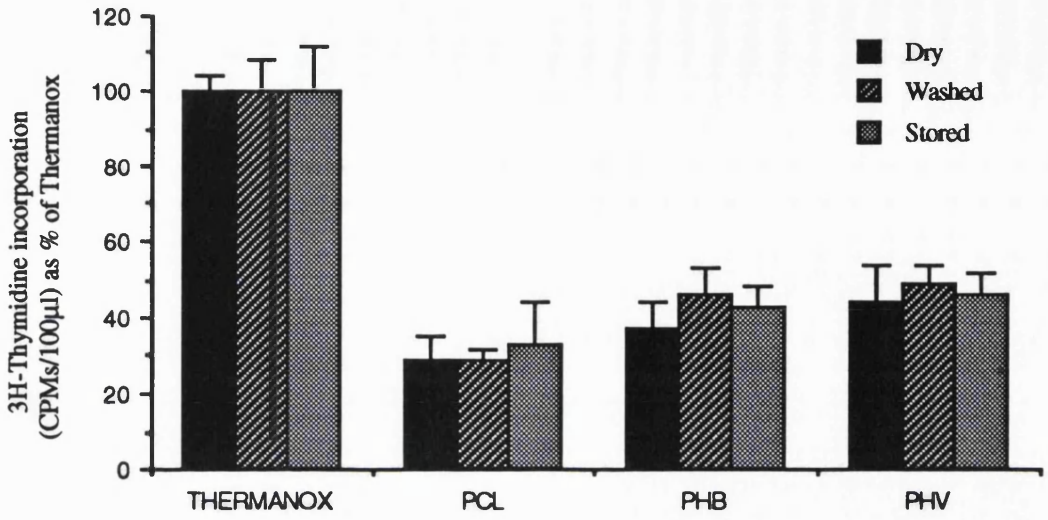


Figure 4.17 Total ^{3}H-thymidine incorporation into cells cultured on dry, washed and stored PCL, PHB and PHB-PHV over 48 hours. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

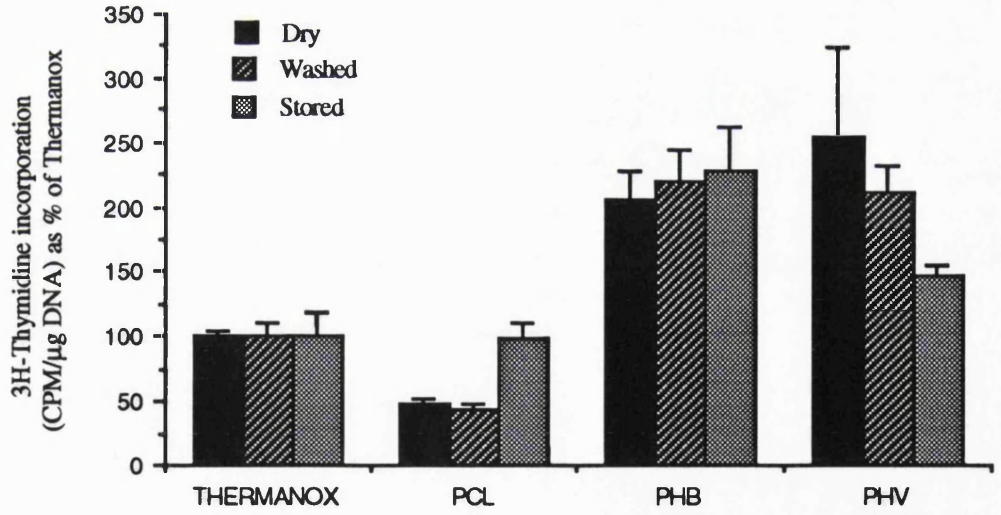


Figure 4.18 ^{3}H-thymidine incorporation, expressed as counts/µg DNA for cells cultured on dry, washed and stored PCL, PHB and PHB-PHV over 48 hours. Each point on the graph is a mean of four different samples assayed in duplicate with, the error bars as the percent standard error of the mean. The TK-HSD test was carried out and all significance is at $p<0.05$.

DISCUSSION

The results obtained from the biochemical assessment have been difficult to interpret due to the variability of the polymers and the sensitivity of the assays employed. For quantitative tests to be confirmatory there are many parameters which must first be standardised. Firstly the material being has to be standardised, including the size, shape, surface finish, crystallinity and molecular weight of the material to name but a few. Chapter two discussed the differences which can result in the polymer samples by varying the temperature and concentration. This study endeavoured to use materials that were of the same size and surface finish, using the same concentrations for each of the polymer solutions and drying them in a constant environment to get reproducible surface finishes. The films were cast on glass slides in similar conditions at room temperature, and dried under a glass petri dish. Figures 4.4a-d, however, show that surface reproducibility was not always achieved and different surfaces were formed after casting of PCL. Surface differences and changes in the crystallinity occurred and all these factors affect the overall behaviour of the polymers particularly in such a sensitive system involving cells. The differences in the surfaces may have been due to the drying temperature, the temperature of the environment or variability in the glass surface used for casting. Differences in the other polymer surfaces cast may also have occurred but these were difficult to visualise as they were smoother and did not have prominent features which could be used as markers. There are methods available, such as goniophotometry, which can quantify the surface properties. Tissue culture systems are already subject to between culture dish, plate and across plate variations without the involvement of polymers and, with these additional variables, the reproducibility can be affected dramatically.

The DNA assay and tritium incorporation results were variable. The scintillation counter was sensitive at the lower range and is able to measure small amounts of radioactivity. The DNA assay, however, proved to be less sensitive and, with the low cell numbers, the amount of DNA was usually too low thus making it difficult to distinguish from the lowest detection limit of the assay. Therefore the values for CPM/μg DNA for some experiments were unrealistically high due to the relatively small amount of DNA present in the sample. A more sensitive method for DNA measurement needs to be developed, to be used specifically for biocompatibility testing of polymers as its end use, as this usually has to take into consideration low cell numbers when performing such tests. For *in-vitro* testing to be standardised such tests need to be modified so these tests can be performed routinely and reproducibly. Beumer *et al* (1993) used *in-vitro* predegraded particles of PLLA to test the biocompatibility with fibroblasts and keratinocytes. The accelerated degradation of the polymers using high temperature proved to be effective in obtaining aged polymers and differences were observed in the response of the cells.

Increasing the initial seeding density would increase the amounts of DNA available for detection but this would also reduce the time taken for the cells to reach confluence on a polymer surface reducing the exposure time. Furthermore figures 4.1-4.3 show that the rates of proliferation and thymidine incorporation slow down at higher seeding densities. Higher cell seeding densities also make it difficult to determine individual cellular morphology on the polymer surfaces.

Total protein was also measured, but due to the high degree of variability the results were not used. This was probably due to the non-specificity of the method in measuring the protein. The method measured the total amount of protein present which could not be distinguished from the protein adsorbed on to the polymer surfaces from the serum contained in the medium. This method only allows the detection of a protein to be measured, it does not distinguish its specificity. Methods which measured specific proteins, such as gel electrophoresis or assays measuring specific proteins which would be more useful.

Binding of proteins to polymer surfaces, can influence cellular adhesion dramatically (Anderson *et al* 1990). Adherence of cells to the polymer surfaces is the first stage in the cell-polymer interaction. Therefore one needs to determine whether the differences observed in the numbers of cells adhering to the polymers are due to the type of proteins adsorbed onto the polymer surface, or whether it is purely a polymer surface-cell based interaction. Various methods can be used to analyse biomaterial surfaces and interaction of polymers and include Electron Spectroscopy for Chemical Analysis (ESCA), Secondary Ion Mass Spectroscopy (SIMS) and Surface-Enhanced Raman Spectroscopy (SERS).

Whilst results from this chapter proved to be variable when evaluating the adherence and proliferation of HOS cells on the polymer surfaces, the methods used have, however resulted in the quantification of biocompatibility of cells on the materials. The differences observed in the results were due to the polymer surfaces, in terms of changes in surface structures, and possibly due to crystallinity, caused by the uptake of fluid from the medium. Before reproducible tests can be developed the polymer formulation and variability caused by casting and processing need to be controlled. Films may not be the best form of the material for testing and larger pieces, more representative of the devices that would be used for clinical use, should be used. The polymer films were easy to handle; however the surfaces of cast polymer films are different to moulded polymer devices so the results may be different as surface structure appears to be important in determining cellular morphology and attachment. Limitations of sample size may have been the cause of some of the variability and better quality control methods used to

produce the samples would have decreased the errors and in addition be more representive of the actual sample.

With larger devices and better knowledge of the buffering capacity of the acidic environment caused *in-vivo*, tests can be devised which would mimic the long term degradation of polymers. The effect of surface changes on cell adherence, proliferation and expression of appropriate markers for that cell type could then be measured with better reproducibility and accuracy. The method for removing cells from the polymer surfaces for the assays is important. A freeze thawing was used in this study although other methods were tested which were not as effective for this system. Trypsinisation of cells from the surface could be used but there were problems associated with this resulting in loss of cells during the procedure. The presence of trypsin would also have interfered with the protein assay so that the cells would have to be spun down and resuspended in water or buffer leading to further loss of cells. Detergents were tested for lysing the cells and proved to be very effective; however this too interfered with the protein and DNA assays.

In-vitro cell culture systems are sensitive to differences in temperature, humidity and the immediate environment around the cells in culture. Plate drift is a major problem with cells and it was found that cells cultured in 48 or 96 well plates were subject to variations due to their position in these plates. Work by Clifford and Downes (1996) shows the results from various assays and measured plate drift. The use of different cells lines and the sensitivity of methods used, can give rise to misleading results. In addition material production and batch variation can give rise to variable results. In order to obain meaningful data a standardisation of methods and materials is necessary.

CHAPTER V

The Effect of Monomers on Cellular Activity

INTRODUCTION

As polymer degradation proceeds the main polymer chain breaks up to form oligomers which further break down into monomers, the basic polymer unit. Monomers are the final degradation products of degradable polymers; they are released during bulk hydrolysis and surface degradation of polymers, and play an important role in determining the overall biocompatibility of that polymer. Monomers can be acidic in nature, and at high concentrations can have a deleterious effect on cellular viability and activity when implanted into a site. The problems associated with monomers or degradation products from resorbable polymers have to date not been described and therefore this is an area which requires investigation.

The effect of monomers on cells *in-vitro* can be investigated using various viability and colorimetric assays, but there can be problems associated with these tests, when making direct comparisons of the *in-vitro* results with those obtained *in-vivo*. In an *in-vivo* situation there is the advantage with buffering of the environment occurring around the implant, however the extent of this buffering is unclear. HEPES has been used for buffering of the culture in cell systems but this remains effective over a small pH range only. The pH changes that occur by the addition of high concentrations of LA and GA may be due to inadequate buffering in the *in-vitro* situation. *In-vitro* it would be possible to minimise dramatic fluctuations in the pH of the system by frequent changes of the growth medium, but this would not be satisfactory as it would be time consuming and expensive. Additionally the frequent changing of the medium would lead to the removal of growth factors produced by cells which may retard cellular growth. An alternative would be to determine the pH of the environment surrounding the implant as it is degrading and devise *in-vitro* experiments using the pH range obtained *in-vivo*.

Most cells have an optimal pH of 7.4 and the pH of a fully supplemented buffered medium with serum is approximately 7.3-7.5. Phenol red is used as an indicator which is red at pH 7.4, orange at pH 7.0, yellow at pH 6.5, bluish red at pH 7.6 and purple at pH 7.8. It is usually advisable to use phenol red in the medium during culture of cells as differences in pH caused by a decrease or increase in CO_2 levels in the incubator can be readily detected. However, in some assays, particularly colorimetric assays, phenol red interferes and has to be omitted from the medium.

In addition to causing changes in pH, the addition of acids or bases causes changes in osmolality. Further neutralisation of the solutions by sodium hydroxide also affects the osmolality. Osmolality is defined as the number of solute molecules present in a solution independent of their size and structure. Osmolality is measured by freezing point depression or elevation of vapour pressure and it is important to monitor for changes that

may occur as a result of additives in the medium. Most cells have the ability to tolerate osmolality changes in the range of 260-320 mosm/kg (Freshney, 1983) but, when adding large quantities of monomers or salts, the osmolality changes can be large enough to cause cell damage or death.

In recent years there has been great interest in apoptosis, a process of programmed cell death. It is relevant in this study to distinguish those cells undergoing apoptosis from those dying as a result of necrosis. Apoptosis and necrosis are two very different types of cell death; although the end result may be the same the processes involved are different and, when investigating cellular viability, the results can be misleading as cells undergoing apoptosis may appear healthy (Evan *et al* 1995). As briefly mentioned in the general introduction, necrotic cell death usually occurs as a result of injury and the presence of excessive toxins while apoptosis is a mechanism for the natural elimination of cells from the body and is an important mechanism for tissue homeostasis (Kerr *et al* 1971; 1972). During apoptosis various morphological and ultrastructural changes occur in the cell that can be detected microscopically. (The most important feature of apoptotic death in this study was cell organelles such as mitochondria remained viable late into the process). This has implications for the MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay which has been used to determine changes in cell viability caused by monomers which is discussed in greater detail later in the chapter.

There are many methods available for detecting apoptosis, although these are still in their infancy and quantitative methods are few. Apoptosis can be detected ultrastructurally using transmission microscopy, or by using various DNA fluorescent stains and by gel electrophoresis. The quickest method is to use fluorescent dyes which bind to the damaged DNA which fluoresces under the microscope. In the body apoptosis affects single scattered cells which rapidly condense their nuclei and cytoplasm with abundant protrusions emerging from the cytoplasm surface. The nucleus then fragments and disperses through the cytoplasm and organelles in the cytoplasm become crowded but remain intact (Searle *et al* 1982).

Currently there are several methods available for measuring and counting proliferating cells some of which include radioactive labelling and inclusion and exclusion of various dyes. Most of these methods, however, are time consuming and require special apparatus such as the radioactive assays. The tetrazolium-based assay (MTT) was first introduced by Mosmann in 1983 as an assay for cellular growth and survival as it detected living but not dead cells. In the assay the tetrazolium salt MTT is cleaved to give a blue coloured product (formazan) by the enzyme succinate dehydrogenase which is present in mitochondria. The MTT assay was further modified by Denziot in 1986 to give a more sensitive and reliable procedure which could replace the ^{3}H-thymidine incorporation

assays used for cytotoxicity testing. The MTT assay has also been used by Ciapetti *et al* (1994) to determine the biocompatibility of various biomaterials. It was found to have good correlation with other *in-vitro* proliferation assays such as ^{3}H-thymidine incorporation. The main advantage of the assay is the speed at which large number of samples can be analysed and also the reproducibility of the results. Plumb *et al* (1989) found that pH had an effect on the sensitivity of the assay and for this reason in this chapter the monomers were removed before the addition of MTT. Sgouras and Duncan (1990) also used the MTT assay to determine biocompatibility of various polymers. They investigated the cytotoxicity of various polymers and found the MTT assay to be a useful technique for the primary and rapid evaluation of the cytotoxicity of soluble polymers. Sladowski *et al* (1993) also used the assay to test the effect of IL-2 concentration on different cell types in a a 96 well plate and found good reprodcibility.

The aim of this chapter was to measure the effect of increasing monomer concentrations on cell activity by using assays which monitor different aspects of cellular activity. The assays used were the MTT assay and ^{3}H-thymidine incorporation. The results from these two assays were compared for differences as both assays measure different aspects of cellular activity. The effect on the morphology and ultrastructure of HOS cells in the presence of both neutralised and non-neutralised monomers was investigated by SEM and TEM. Evidence of apoptosis was investigated by fluorescent microscopy using a dye which binds to the DNA of apoptotic cells. The results from this chapter have shown that monomers have an effect on cellular viability and proliferation. These tests have shown that *in-vitro* long term biocompatibility of polymers is greatly influenced by the presence of monomers released from degrading polymers. However before assays involving biomaterials can be used routinely, optimization of existing assays and elimination of interfering factors need to be carried out.

MATERIALS AND METHODS

Monomer preparation

A stock solution of the three monomers LA, GA and D3-HB was made up in supplemented DMEM and an appropriate volume neutralised with concentrated sodium hydroxide to make the neutralised monomer solutions. Non-neutralised monomers are those that were not neutralised with sodium hydroxide and are abbreviated as NN. The solutions were made up in µg/ml but converted into moles/ml for graphical purposes and to allow direct comparisons to be made between the monomers. The osmolality of the different monomers in medium was measured in duplicate using an osmometer (Camlab). Tables 5.1 and 5.2 show the concentrations in µg/ml and the corresponding values in moles/L for LA, GA and D3-HB.

Table 5.1

Lactic acid (µg/L)	Lactic acid (moles/L)	Glycolic acid (µg/L)	Glycolic acid (moles/L)
1,000	0.011	1,000	0.013
2,000	0.022	2,000	0.026
3,000	0.033	3,000	0.039
4,000	0.044	4,000	0.053
5,000	0.056	5,000	0.066
6,000	0.067	6,000	0.079
7,000	0.078	7,000	0.092
8,000	0.089	8,000	0.105
9,000	0.100	9,000	0.118
10,000	0.111	10,000	0.132
12,000	0.133	12,000	0.158
14,000	0.155	14,000	0.184
16,000	0.178	16,000	0.210
18,000	0.200	18,000	0.237
20,000	0.222	20,000	0.263

Table 5.2

D3-hydroxybutyric acid (μg/ml)	D3-hydroxybutyric acid (moles/ L)
3,000	0.029
6,000	0.058
9,000	0.087
12,000	0.115
15,000	0.144
18,000	0.173
21,000	0.202
24,000	0.231
27,000	0.259
30,000	0.288

MTT assay

100 μl of 8 x 10^4 cells/ml were seeded into 96 well plates and incubated for 24 hours at 37°C. The medium was then removed and 100μl of monomer incorporated media at various concentrations was added. The plates were incubated for a further 24 hours. After this time the plate was inverted and the solution removed by tapping gently onto some tissue paper. 100μl of complete medium without ascorbate was then added to the cells with 10μl of MTT at a stock concentration of 5μg/ml. The ascorbate was removed from the medium because it interfered with the assay. The plates were incubated for 4 hours and the contents removed by inverting the plate. The crystals were solubilised with 100μl of DMSO (Merck) and shaken gently for 5 minutes till all the crystals had dissolved. The plate was read at 540 nm on a Biorad plate reader. The blank was a column of wells which had undergone all the procedures but had no cells. All the sample readings were expressed as a% of the control which was set to 100%.

Detection of apoptosis via fluorescence light microscopy

Signs of apoptosis were investigated in HOS cells exposed to neutralised and non-neutralised monomers. The cells were cultured normally for 24 hours in 24-well plates on glass cover slips. These were then exposed to various concentrations of monomers for 4 hours. After this time the monomer media were removed and 5μg/ml of Hoechst dye-HO 33342, (Sigma) in supplemented media was added to the cells. These were incubated for 30 minutes at 37°C. Following this the cells on the coverslip were rinsed in PBS, fixed in 1:1 methanol/acetone fixative for five minutes, then rinsed in PBS, mounted with DABCO and viewed under a UV microscope (Olympus).

SEM processing

Samples for SEM evaluation were cultured on Thermanox for 24 hours at a seeding density of $4x10^4$/ml in 24 well plates. They were then exposed to 1 ml of monomer incorporated medium that was neutralised or non neutralised. After 24 hours or 48 hours the discs were rinsed in PBS and fixed in 1.5% gluteraldehyde in 0.1M sodium cacodylate buffer for at least 30 minutes. These were then osmicated with 2% osmium tetroxide for 1 hour, rinsed in buffer, placed in 1% tannic acid and dehydrated through a series of alcohols to 70%; they were then placed in 2% uranyl acetate made up in 70% alcohol for 30 minutes and finally dehydrated to 100% alcohol. The films were then placed into HMDS for 5x2 minutes and allowed to air dry. They were then coated with gold palladium and viewed under a Joel 35C SEM.

Statistics

The results obtained were analysed using appropriate statistical tests. The data sets were tested for normality using the Shapiro-Wilk W test; if the p values obtained were less than 0.05 the data was not normal. If the data was normal (parametric) an analysis of variance was used, and if the data was not normal (non-parametric) the Mann-Whitney test was used. Depending on the results from these tests a multiple comparison test was carried out the Tukey Kramer-Honestly Significant Difference test (TK-HSD). All tests were carried out using alpha values of 0.05 and 0.01 and significance was denoted * for significance at $p<0.05$ and ** for significance at $p<0.01$. Where multiple comparison was not required and comparisons could be made to a control the Dunnett's test was carried out at alpha values of 0.05 and 0.01 and significance was denoted as above. Some data sets could not be analysed due to small sample sizes.

RESULTS

Figure 5.1 shows the change in pH occurring with the addition of monomers to PBS with increasing concentration. The concentration scale is in moles/L although the actual solutions were made up in µg/ml (table 5.1). There was a rapid decrease in pH of the buffer to approximately 3.5 at a concentration of 0.01 moles/L of both LA and GA. Above this concentration the pH continued to decrease but at a slower rate reaching a pH of 2.5 with 0.06 moles/L of the GA monomer.

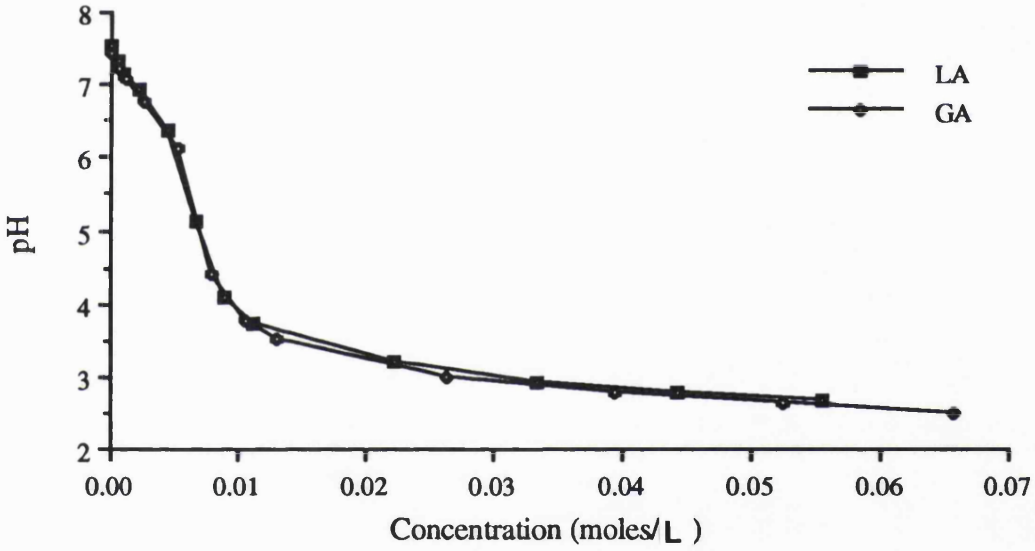


Figure 5.1 Shows the effect of increasing non-neutralised monomer concentration on the pH of PBS.

Figures 5.2-5.6 show the cellular activity of HOS cells exposed to increasing concentrations of monomers measured using the MTT and ^{3}H-thymidine assay. Figure 5.2 shows that, with increasing NLA concentration there was a decrease in cell viability. There were, however, significant differences between the two assays. At the lower concentrations ^{3}H-thymidine incorporation into the cells was higher than mitochondrial activity. At 8,000µg/ml (0.089moles/L) there was no difference and there was a crossover after which mitochondrial activity was higher than ^{3}H-thymidine incorporation. For NGA (figure 5.3) the graph was different with the crossover occurring earlier and there was no significant difference between the two assays at the lower concentrations. At a concentration above 6,000µg/ml (0.079moles/L) ^{3}H-thymidine incorporation was significantly lower than mitochondrial activity.

With NNLA (figure 5.4) and NNGA (figure 5.5) there were no significant differences between the two assays. The concentration required for HOS cells exposed to NNLA and NNGA to reach zero activity was 0.039-0.044moles/L; this, for the NNLA, is equivalent to 4,000µg/ml, and for the NNGA 3,000µg/ml.

Figure 5.6 shows the activity of HOS cells exposed to D3-HB up to a concentration of 30,000µg/ml (0.288moles/L). There was no significant difference between the two assays at the lower concentrations but, after 12,000µg/ml (0.115moles/L), the ^{3}H-thymidine incorporation was significantly lower than mitochondrial activity. However, at a concentration of 27,000µg/ml (0.288moles/L), viability was virtually zero for both the assays.

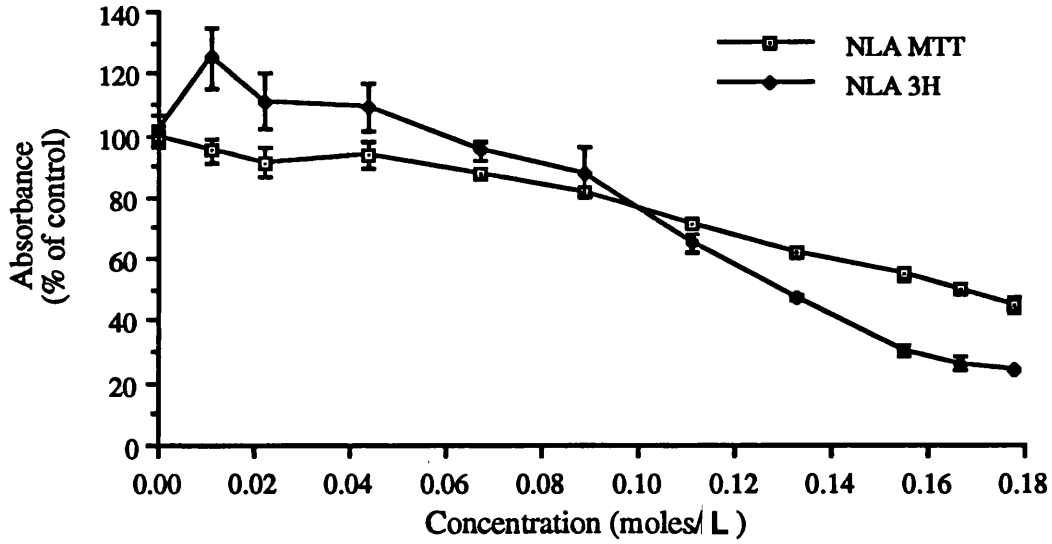


Figure 5.2 Cellular activity following exposure to increasing concentrations of NLA using the ^{3}H-thymidine incorporation and MTT assay (expressed as a percent of the control). Each point is the mean of six replicates with the error bars being the percent standard error of the mean.

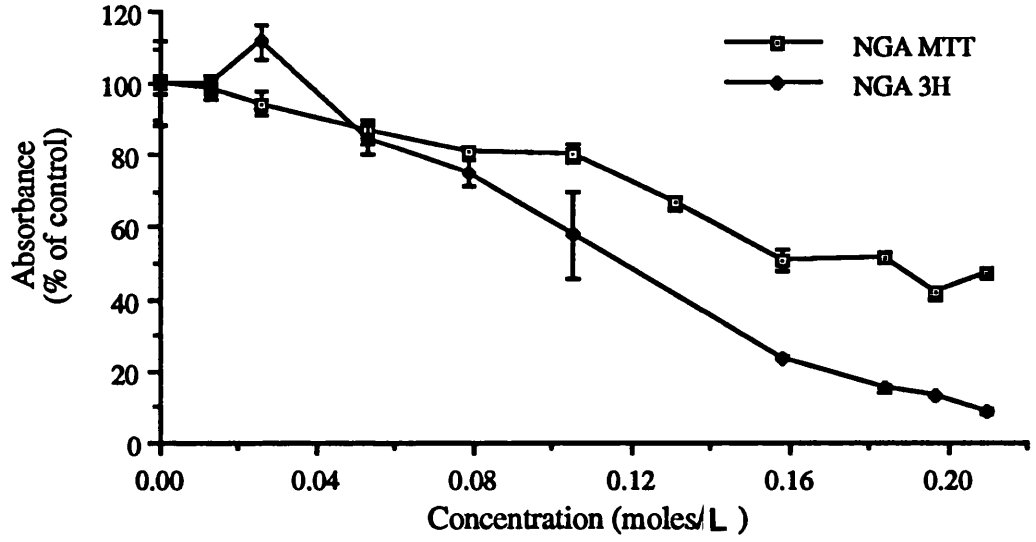


Figure 5.3 Cellular activity following exposure to increasing concentrations of NGA using the ^{3}H-thymidine incorporation and MTT assay (expressed as a percent of the control). Each point is the mean of six replicates with the error bars being the percent standard error of the mean.

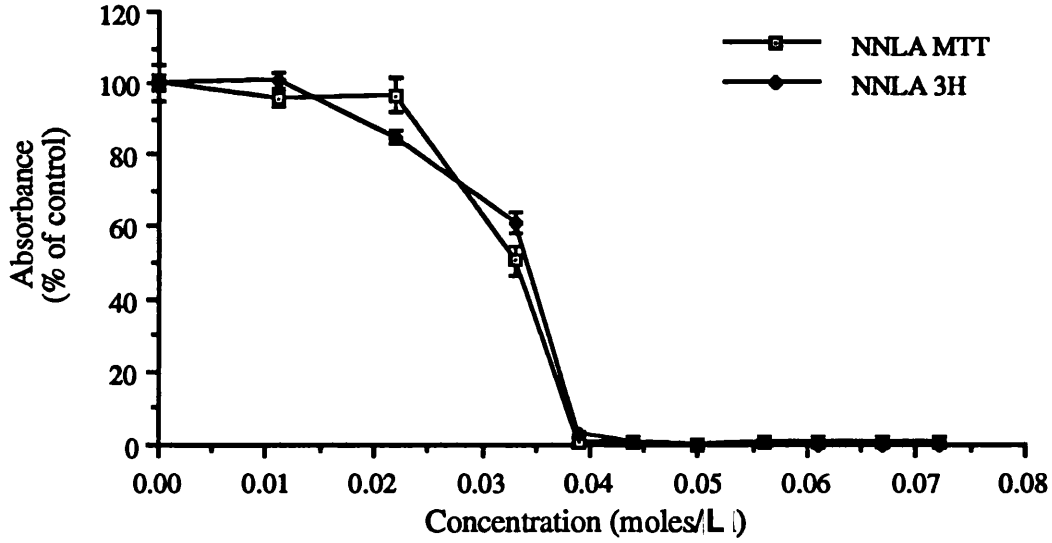


Figure 5.4 Cellular activity following exposure to increasing concentrations of NNLA using the ^{3}H-thymidine incorporation and MTT assay (expressed as a percent of the control). Each point is the mean of six replicates with the error bars being the percent standard error of the mean.

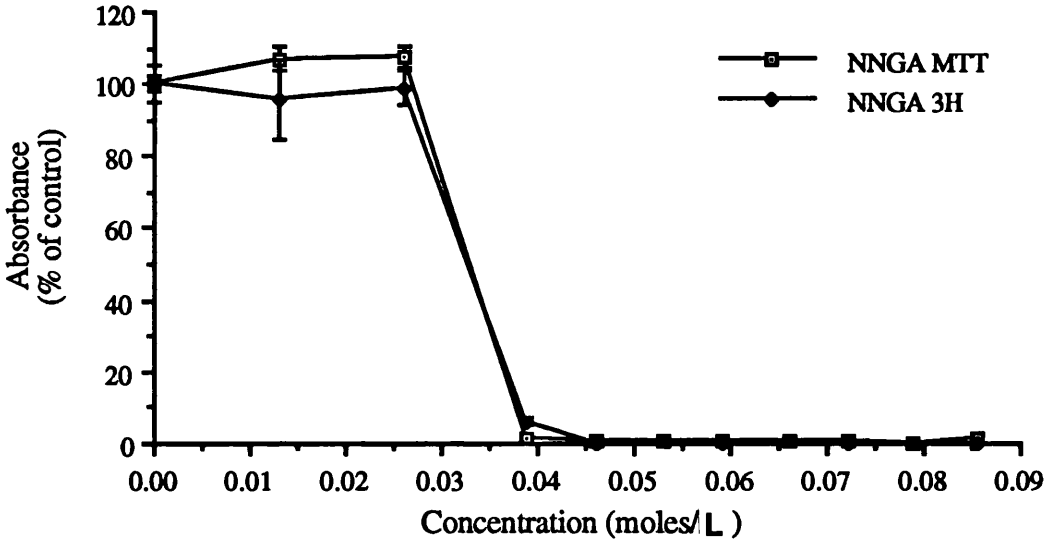


Figure 5.5 Cellular activity following exposure to increasing concentrations of NNGA using the ^{3}H-thymidine incorporation and MTT assay (expressed as a percent of the control). Each point is the mean of six replicates with the error bars being the percent standard error of the mean.

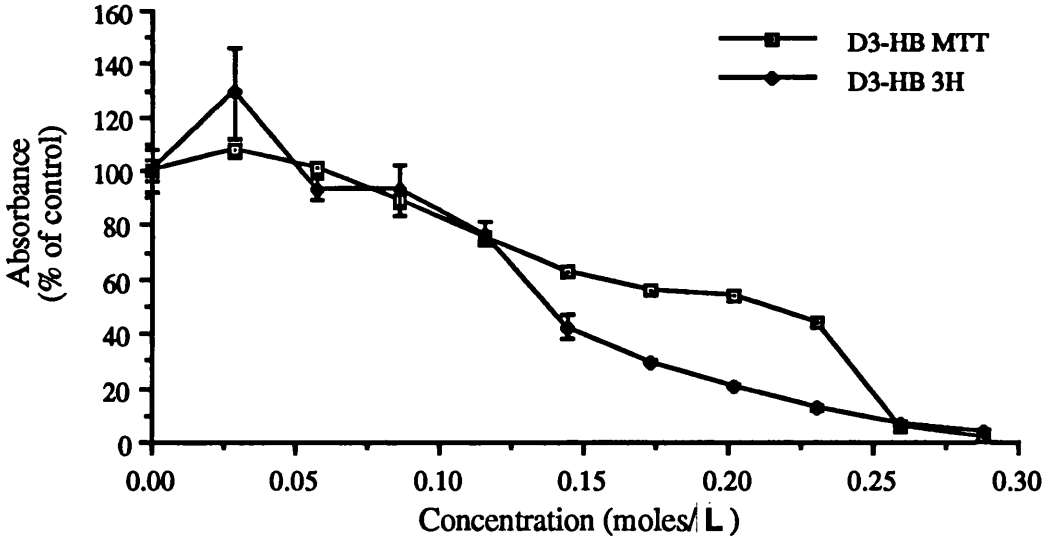


Figure 5.6 Cellular activity following exposure to increasing concentrations of D3-HB using the ^{3}H-thymidine incorporation and MTT assay (expressed as a percent of the control). Each point is the mean of six replicates with the error bars being the percent standard error of the mean.

Cells seeded on Thermanox were cultured with neutralised and non-neutralised monomers of LA, GA and D3-HB (Figures 5.7-5.11). The samples were in duplicate and the cells were exposed to the monomers for 24 hours. Cells on Thermanox without the presence of monomer were used as the control.

HOS cells cultured in the presence of NLA (figure 5.7) did not appear to be affected at the lower concentrations and, at the higher concentration, a slight reduction in cells adhered to the Thermanox was observed. The overall structure and morphology of the cells remained similar to the control. This was not observed for the NNLA exposed cells (figure 5.8). At the lower concentration of 1,000μg/ml (figure 5.8b) the cells appeared normal with mitotic figures being present. At 2,000μg/ml (figure 5.8c) the cells had still retained their normal morphology but there were fewer mitotic figures observed on the surface. At 3,000μg/ml (figure 5.8d) there was a reduction in cell numbers on the polymer but the overall morphology appeared normal. At a concentration of 4,000μg/ml (figure 5.8e) there were very few cells adhering to the surface of the Thermanox and the morphology of the cells that remained was dissimilar to the control. The cells were broken in places and had shrunk to form a rounded mass rather than being flattened out over the surface.

The cells exposed to NGA (figure 5.9) behaved very differently to the cells exposed to NLA (figure 5.7). At 4,000 μg/ml (figure 5.9b) the cells appeared normal with good cell-to-cell contact, the integrity of the cytoplasm was retained and the cells had processes extending out from the surfaces which were undamaged. At 8,000μg/ml, (figure 5.9c) however, the cells did not appear to be in close contact with the adjacent cells in certain areas although the overall cellular morphology appeared normal with some mitotic figures visible. At 12,000μg/ml (figure 5.9d) a marked reduction in the number of adherent cells can be seen; the cellular morphology appeared different to the control but not abnormal. There was a noticeable increase in the number of processes emerging from the cells. At 16,000μg/ml (figure 5.9e) the number of adherent cells was further reduced and only a few cells appeared on the surface. These were still quite well spread out and the morphology remained fairly healthy but the remains of cellular processes and cellular debris could be seen on the surface of the Thermanox in the background.

With NNGA (figure 5.10) the cells appeared normal and retained normal morphology at a 1,000 (figure 5.10b) and 2,000 μg/ml (figure 5.10c) . However, at 3,000 μg/ml (figure 5.10d), the cells appeared very different. There were fewer cells still adherent to the surface of the Thermanox and the cells remaining were different in morphology. There was a mixture of rounded cells and flat cells. The flat cells did not appear similar to the control or the cells exposed to lower concentration. The cells had thinner cytoplasm which appeared to be breaking up. At the concentration of 4,000μg/ml (figure 5.10e) the

cells were mostly flat with thin cytoplasm and the area around the nuclei was starting to break up with holes appearing within the cytoplasm.

The cells exposed to D3-HB (figure 5.11) monomer retained a normal morphology and good cell density at 5,000μg/ml (figure 5.11b) and at 10,000μg/ml (figure 5.11c). At 15,000μg/ml (figure 5.11d), however, the cell number dropped dramatically although the cell morphology appeared normal as seen from the micrograph at the higher magnification. The cells, at 20,000μg/ml (figure 5.11e) appeared damaged with the cytoplasm separating from the nuclei and the cells taking on a more elongated morphology as well as fewer cells appearing on the surface of the Thermanox.

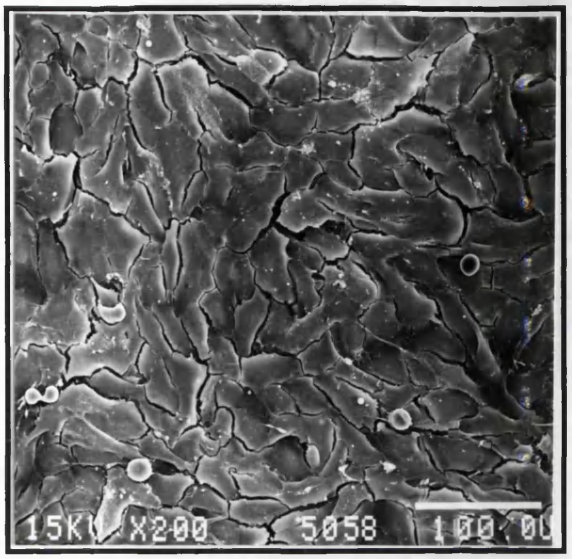
Figure 5.7a Cells cultured on Thermanox as a control showing normal morphology with a confluent layer of cells.

Figure 5.7b Cells on Thermanox with NLA at a concentration of 4,000µg/ml appeared normal and there were many cells adhering to the surface of the Thermanox.

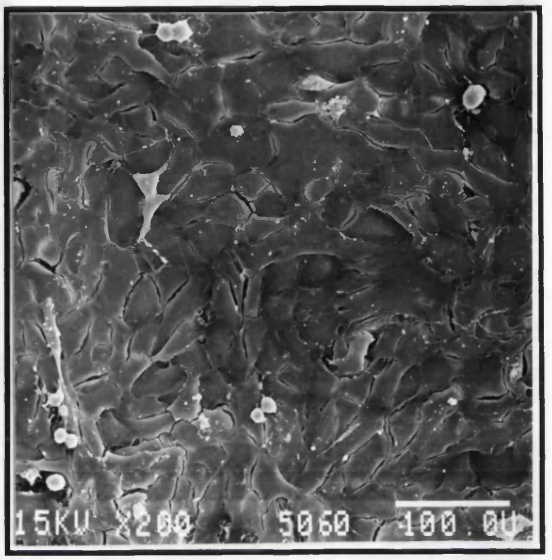
Figure 5.7c Cells on Thermanox with NLA at a concentration of 8,000µg/ml showed very little change in the overall cellular morphology but there were fewer cells on the surface.

Figure 5.7d Shows cells on Thermanox with NLA at a concentration of 12,000µg/ml with a similar cellular morphology to the control and a further decrease in cells on the surface.

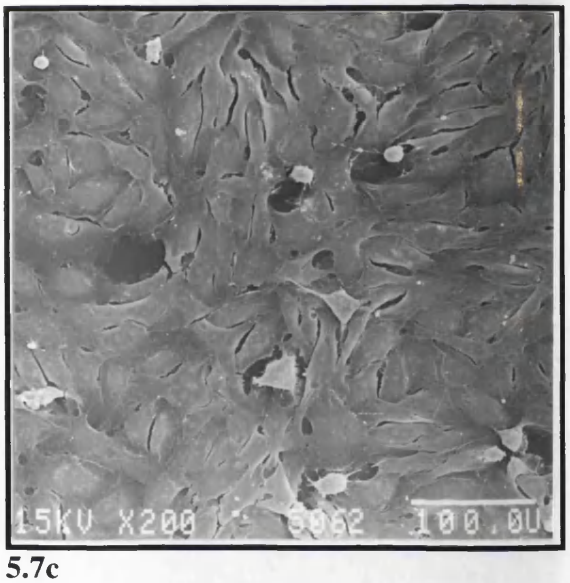
Figure 5.7e Shows cells on Thermanox with NLA at a concentration of 16,000µg/ml there was very little cell damage with the exception of cracks appearing around the nuclei and there was a further decrease in the number of cells on the polymer surface.



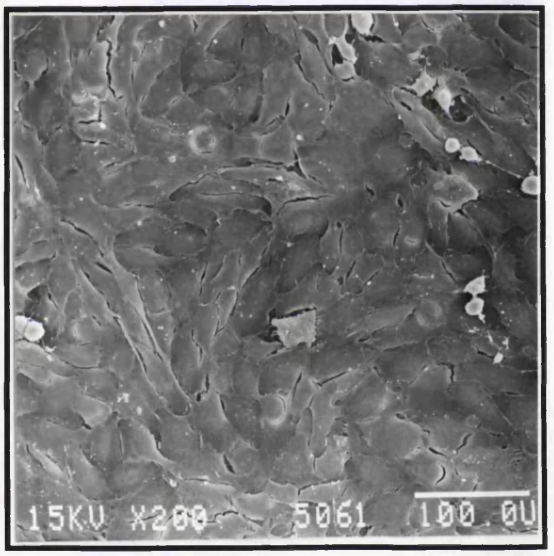
5.7a



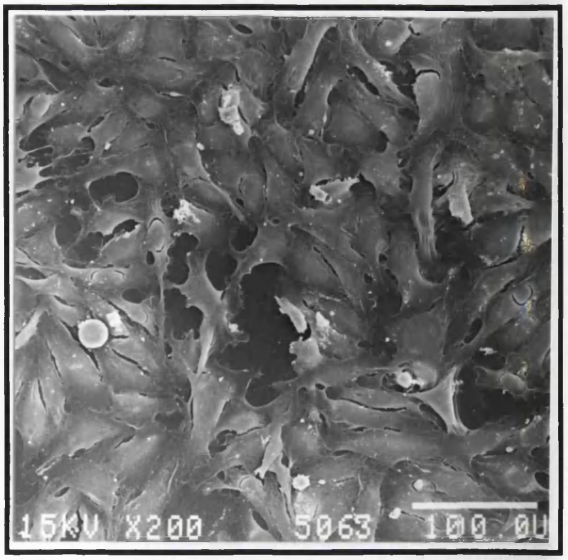
5.7b



5.7c



5.7d



5.7e

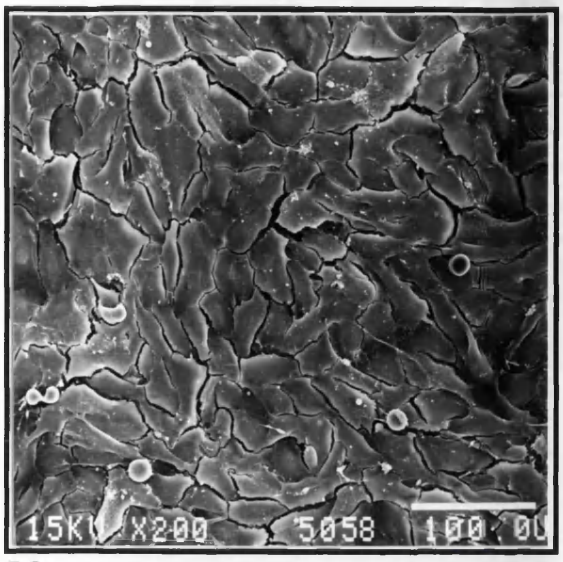
Figure 5.8a: Cells cultured on Thermanox as a control showing normal morphology with a confluent layer of cells.

Figure 5.8b: Cells cultured on Thermanox with 1,000μg/ml of NNLA showed normal cellular morphology and a confluent layer of cells.

Figure 5.8c: Cells cultured on Thermanox with 2,000μg/ml of NNLA showed normal cellular morphology with the individual cells becoming more defined.

Figure 5.8d: Cells cultured on Thermanox with 3,000μg/ml of NNLA showed normal cellular morphology but there were fewer cells present on the polymer surface.

Figure 5.8e: Cells cultured on Thermanox with 4,000μg/ml of NNLA appeared damaged. There were very few cells present on the polymer surface and the cellular morphology was more rounded.



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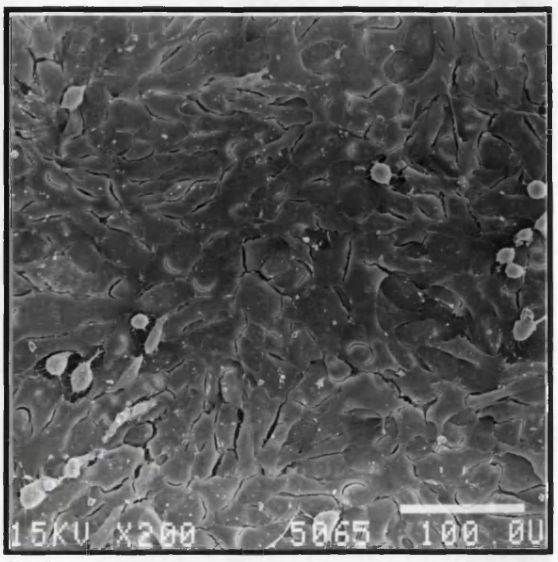
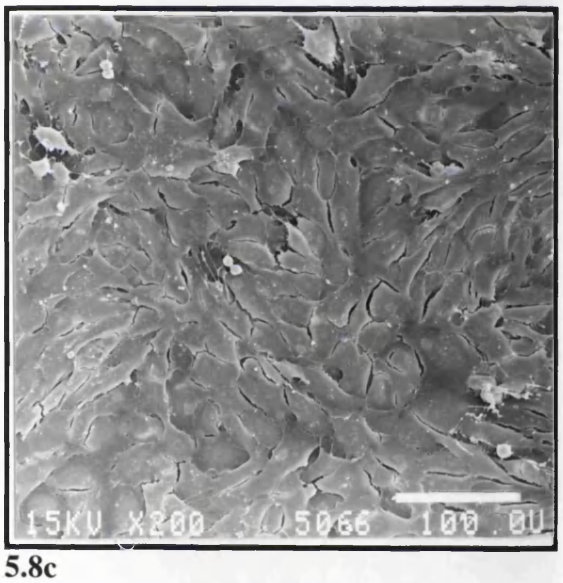


5.8b



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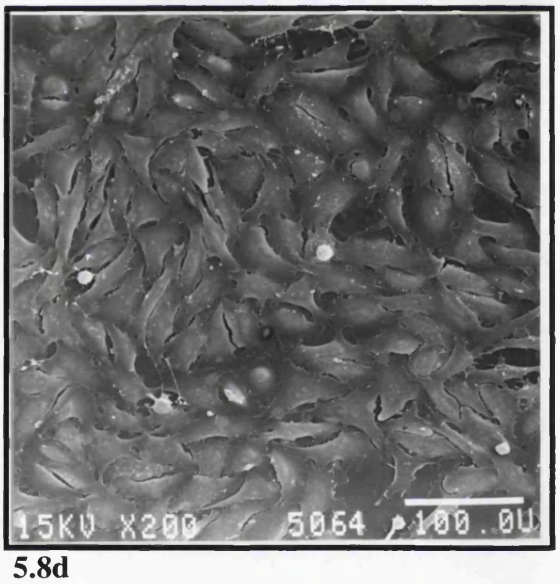

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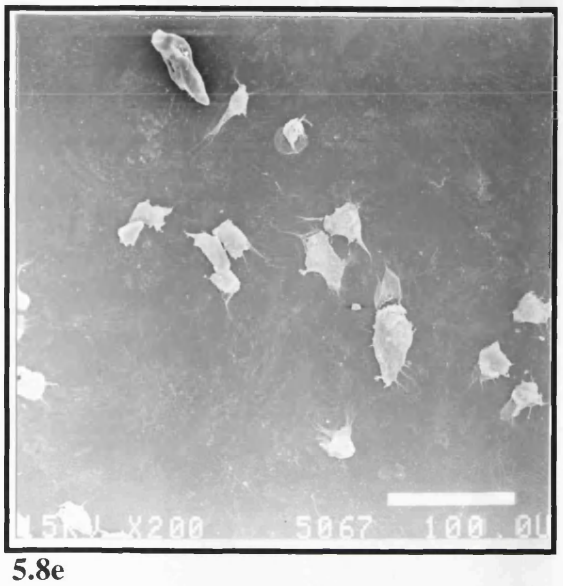


5.8e

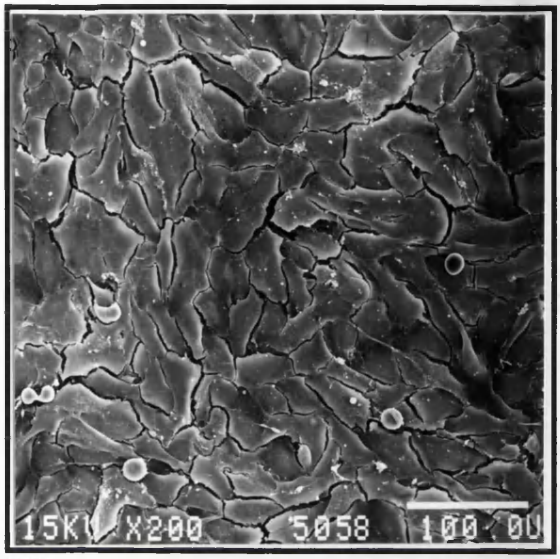
Figure 5.9a: Cells cultured on Thermanox as a control showing normal morphology with a confluent layer of cells.

Figure 5.9b: HOS cells cultured with 4,000μg/ml of NGA have good cell to cell contact with healthy cytoplasm and nuclei with many processes extending from the cells.

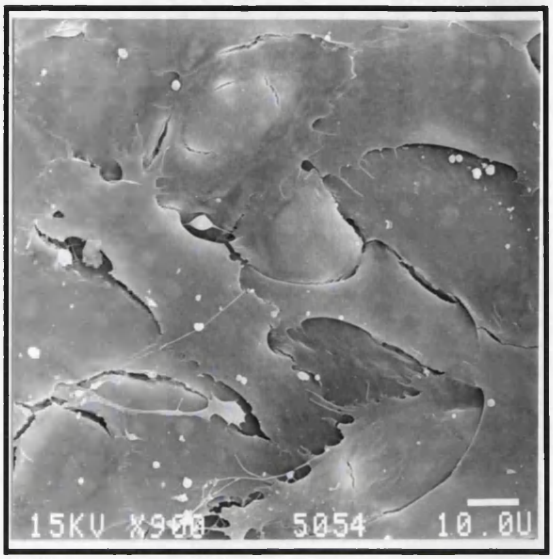
Figure 5.9c: Cells cultured with 8,000μg/ml of NGA show a reduction in the number of cells on the Thermanox with the cells appearing lacy at the edges and in the cytoplasm.

Figure 5.9d: The cells cultured with 12,000μg/ml of NGA show a marked reduction in cells compared to the control and the cells appear damaged although they still adhered to the surface.

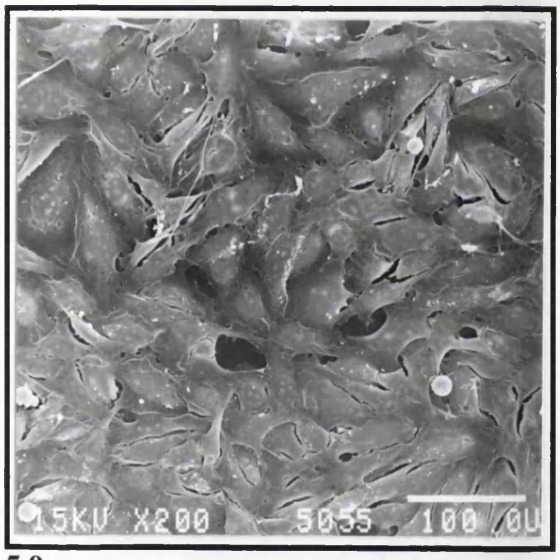
Figure 5.9e: The cells cultured with 16,000μg/ml of NGA show very few cells remained attached to the surface which had contracted.



5.9a



5.9b



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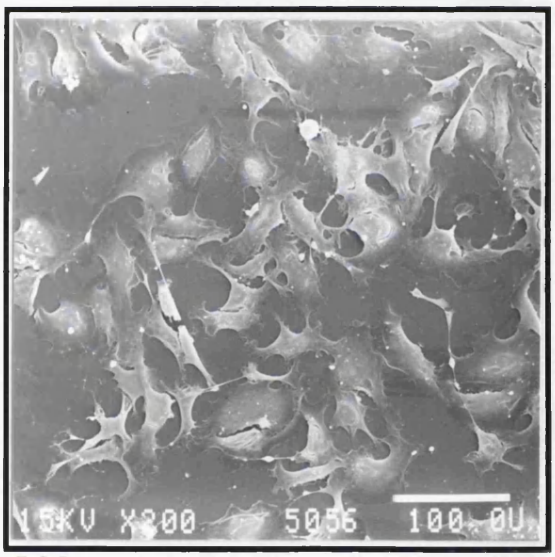

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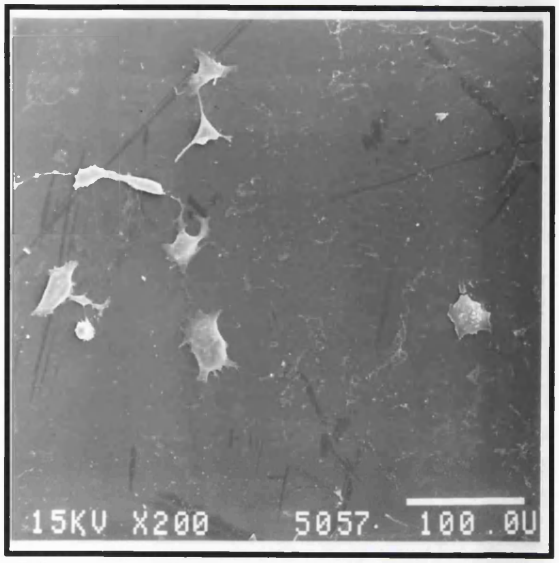


5.9e

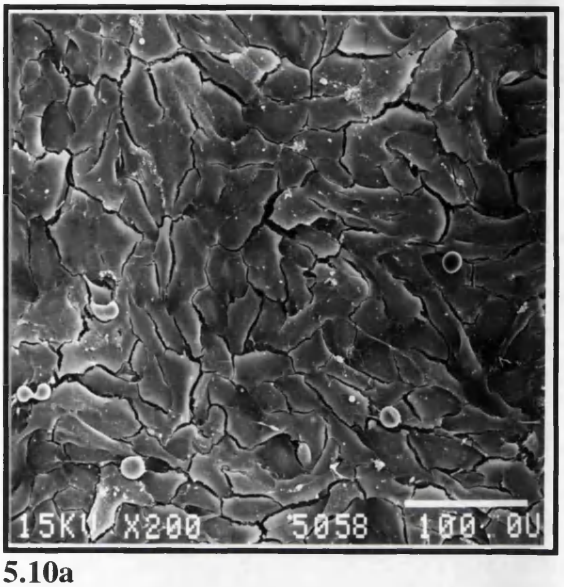
Figure 5.10a: Cells cultured on Thermanox as a control showing normal morphology with a confluent layer of cells.

Figure 5.10b: Cells cultured with 1,000μg/ml NNGA show a normal morphology with a confluent monolayer of cells.

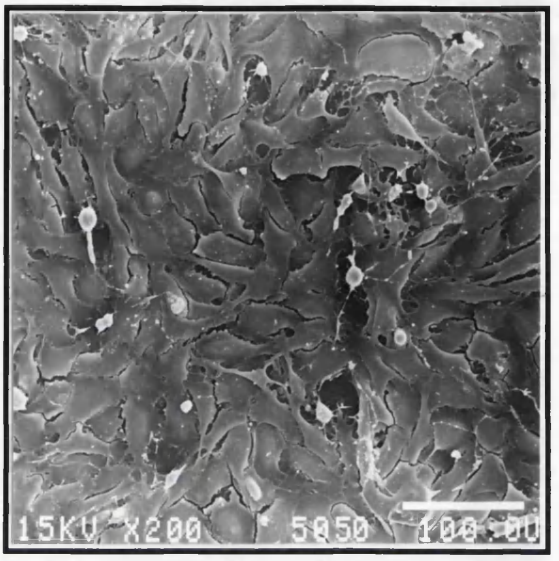
Figure 5.10c: Cells cultured with 2,000μg/ml NNGA showed normal morphology but there was a reduction in the number of cells on the surface.

Figure 5.10d: Cells cultured with 3,000μg/ml NNGA showed a marked decrease in the numbers adhering to the surface with the cells that remained appearing damaged. The cytoplasm was lacy and cracks were visible around the nuclei.

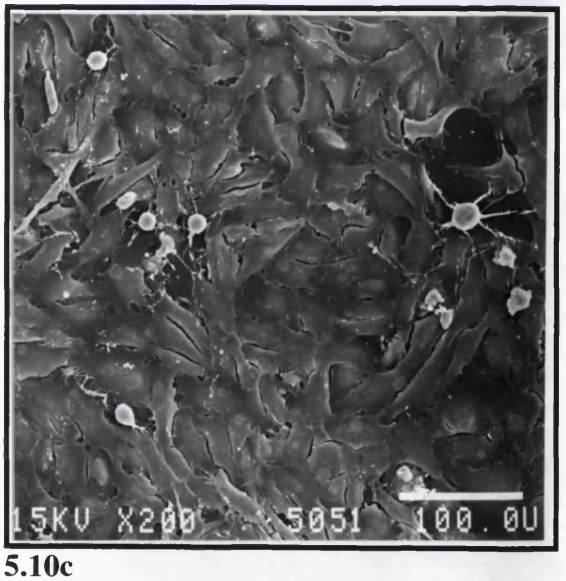
Figure 5.10e: Cells cultured with 4,000μg/ml of NNGA showed an increase in the numbers adhering to the surface compared to figure 5.10d. However the cells were damaged with lacy cytoplasm and cracks around the nuclei.



5.10a



5.10b



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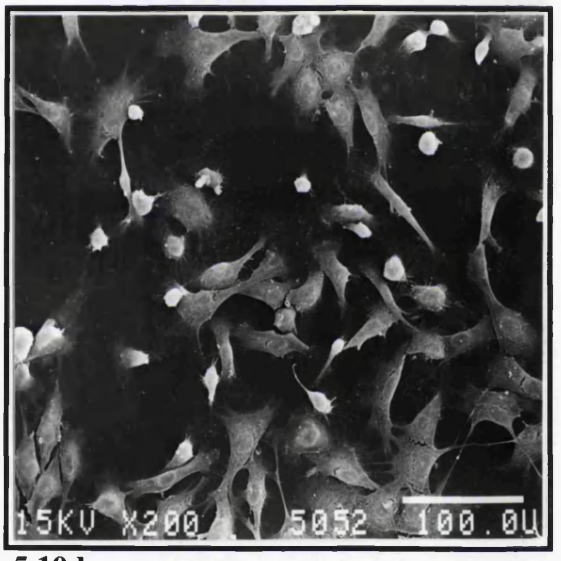
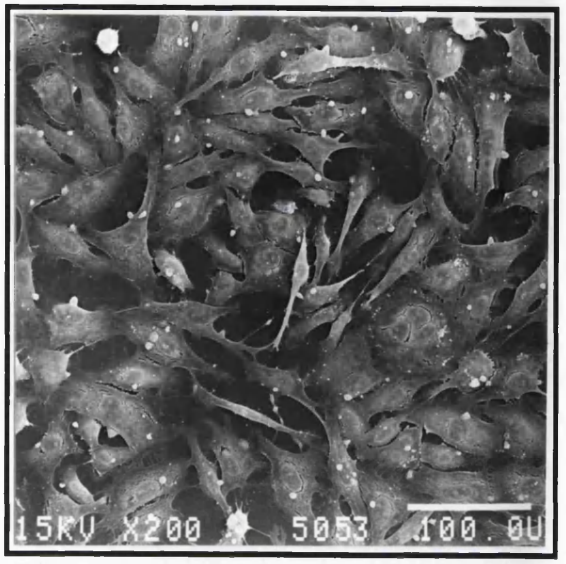


5.10d



5.10e

Figure 5.11a: Cells cultured on Thermanox as a control showing normal morphology with a confluent layer of cells.

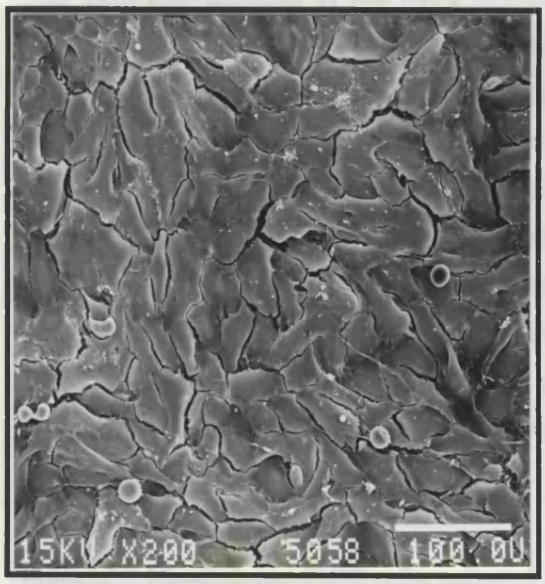
Figure 5.11b: Cells cultured with 5,000μg/ml of D3-HB monomer showed a normal morphology with a confluent layer of cells.

Figure 5.11c: Cells cultured with 10,000μg/ml of D3-HB monomer showed a normal morphology but there appeared to be fewer cells in some areas.

Figure 5.11d: Cells cultured with 15,000μg/ml of D3-HB monomer showed a marked reduction in the number present on the surface with the attached cells appearing normal.

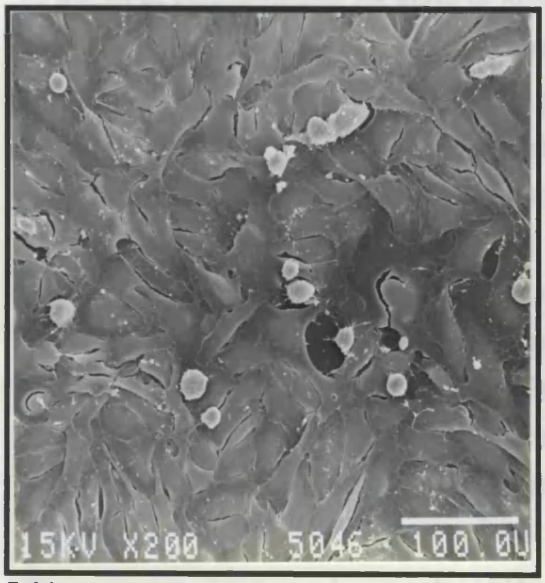
Figure 5.11e: Cells cultured with 15,000μg/ml of D3-HB monomer at a higher magnification; the cell remained intact with no damage occurring to the cytoplasm or the nuclei.

Figure 5.11f: Cells cultured with 20,000μg/ml of D3-HB monomer show a reduction in the number present on the surface; the cells remaining appeared damaged around the nuclei and the cytoplasm.

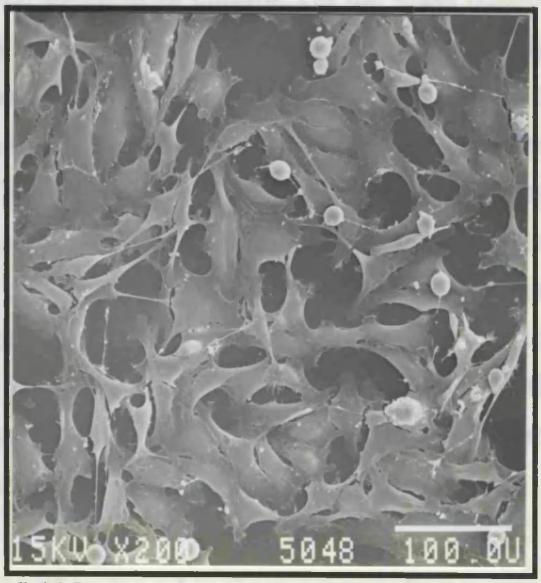


5.11a

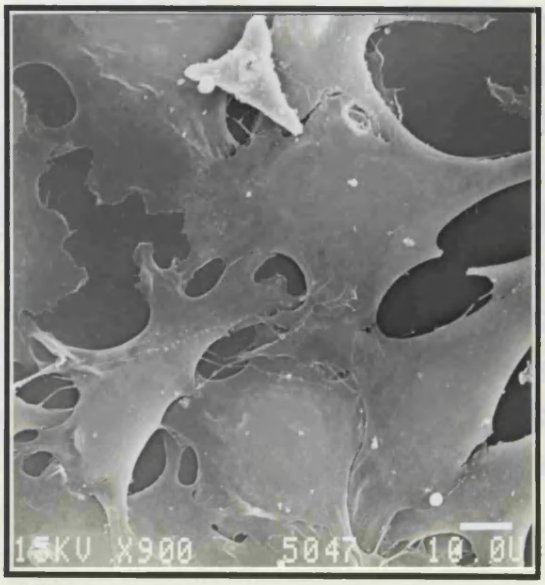
5.11b



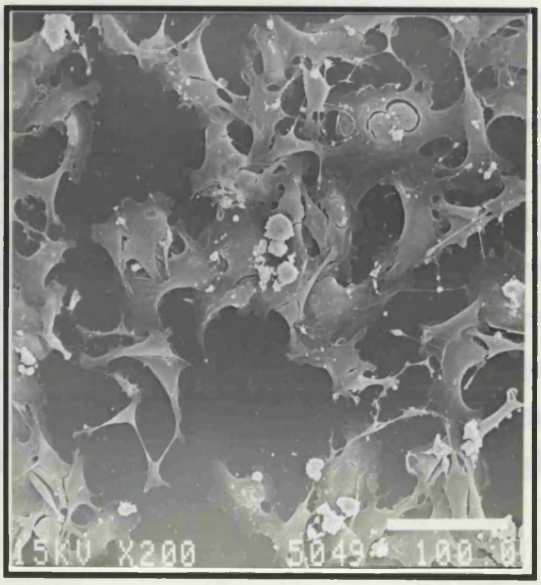
5.11c



5.11d



5.11e



5.11f

The light micrographs of the MTT crystals shows the effect of the monomers on the individual cells as compared to the control. Cells cultured in normal conditions (figure 5.12a) showed good crystal formation and crystal growth had progressed so that they were large and clearly visible. No intact cells were visible on the Thermanox surface after exposure to MTT. In figure 5.12b the cells were exposed to 5,000µg/ml NLA for 24 hours and show that, although some crystals appear similar to the crystals in figure 5.12a the majority remained small and were at the early stages of formation. These tended to be more concentrated around the cell membrane. With 7,500µg/ml of NGA there were very few cells and crystals remaining but the cells that remained still showed good crystal formation. Cells cultured with 20,000µg/ml of D3-HB showed good crystal formation with no intact cells visible.

Figures 5.13a-d shows HOS cells cultured on glass for 24 hours followed by exposure to monomers for 24 hours and stained for cells undergoing apoptosis these were compared to the HOS cells cultured on glass in normal medium as control. On the glass (figure 5.13a) a few cells could be seen which were positive for apoptosis although the majority of the cells were negatively stained. Some positive apoptotic cells are usually present in a normal population of cells. Figure 5.13b shows a positive control of HOS cells on glass which had been exposed to 5% ethanol for 3 hours to initiate apoptosis. Although many cells had detached from the surface the remaining cells were strongly positive for apoptosis. Figure 5.13c shows HOS cells with 2,500 µg/ml of NGA with many cells positively stained for apoptosis. A large number of the cells, however, were not stained positively. Figure 5.13d shows HOS cells cultured with 20,000µg/ml of D3-HB with many cells positive for apoptosis but also many not positive for apoptosis. NLA was also tested but at the low concentrations tested the cells did not stain positively for apoptosis.

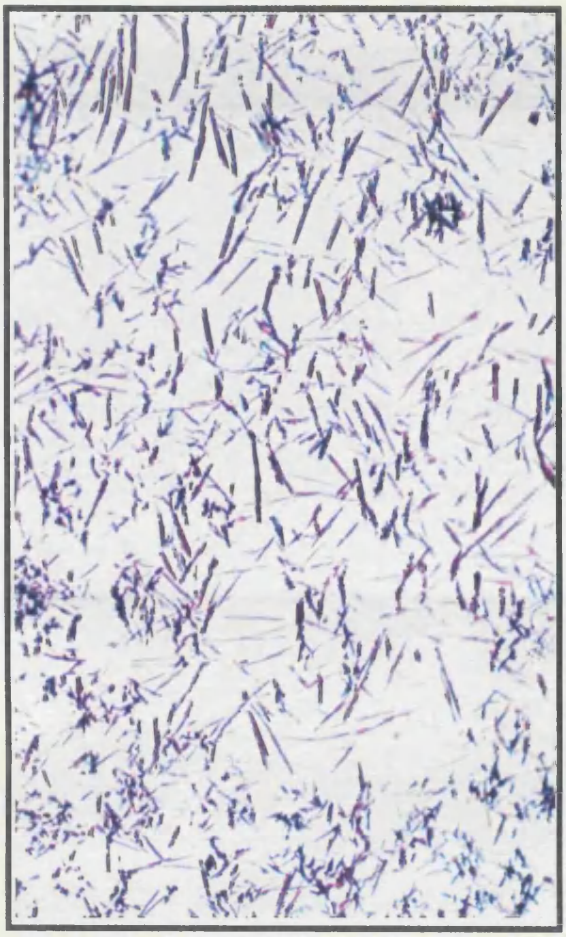
Transmission electron micrographs of HOS cells cultured in the presence of monomers are shown in figures 5.14a-5.14f. Figure 5.14a shows HOS cells on Thermanox with healthy mitochondria and intact nuclei. Figure 5.14b shows cells in the presence of 20,000µg/ml of NGA with no organised structure and perforations in the cell membrane with loss of material occurring. Figure 5.14c shows HOS cells cultured in the presence of 5,000µg/ml of NLA. There was a lack of cellular protein compared to figure 5.14a but cellular membrane integrity was retained and mitochondria were present. HOS cells cultured in the presence of 20,000µg/ml of NLA (figure 5.14d) showed cells with an intact cell membrane but damaged mitochondria with no cristae. Figure 5.14e shows cells cultured in the presence of 1,000µg/ml NNLA which had healthy mitochondria but lacked cellular protein. Figure 5.14f shows cells cultured in the presence of 4,000µg/ml of NNLA. The cells were dead with loss of cell membrane integrity occurring although some cellular material still remained.

Figure 5.12a: MTT crystals growing out of HOS cells cultured on Thermanox. The non uniformity in the size of the crystals, which were at different stages in their "growth" could be seen (Magnification = 330x).

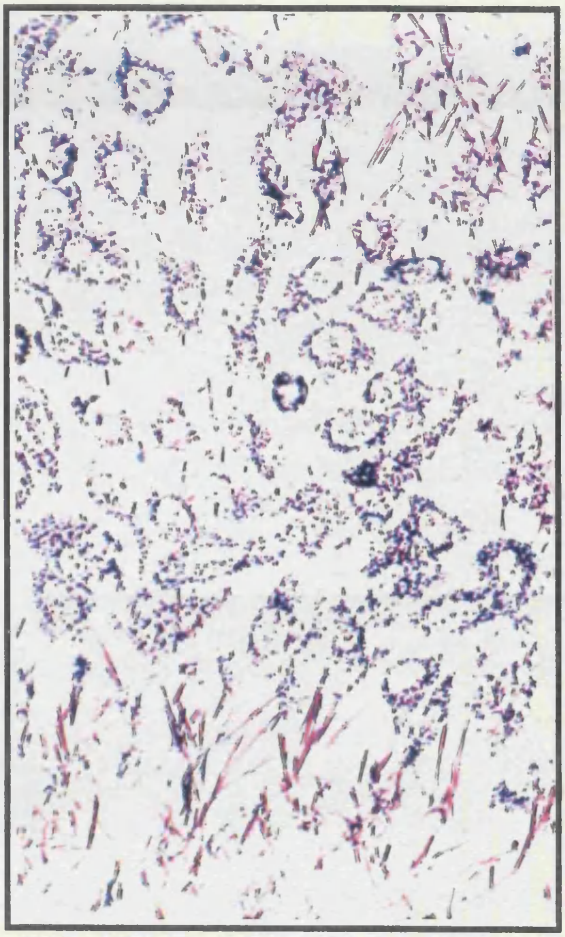
Figure 5.12b: MTT crystals growing out of HOS cells cultured on Thermanox in the presence of NLA at 5,000µg/ml. The crystals were not as developed as the ones in figure 5.12a and they were most concentrated at the edge of the cell (Magnification = 330x).

Figure 5.12c: MTT crystals growing out of the remaining viable HOS cells cultured on Thermanox in the presence of NNGA at 7500µg/ml. There were fewer cells remaining on the surface compared to figures 5.12a and 5.12b, but, these showed good crystal formation (Magnification = 330x).

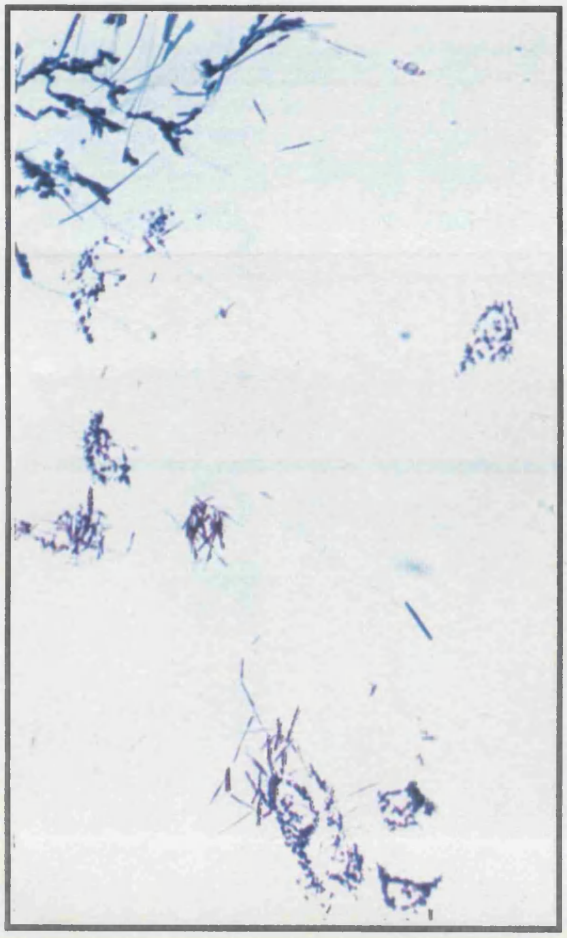
Figure 5.12d: MTT crystals at high magnification cultured on Thermanox in the presence of 20,000µg/ml of D3-hydroxybutyric acid. There were fewer cells that had remained adhered to the surface compared to figures 5.12a and 5.12b, but, these showed good crystal formations (Magnification = 600x).



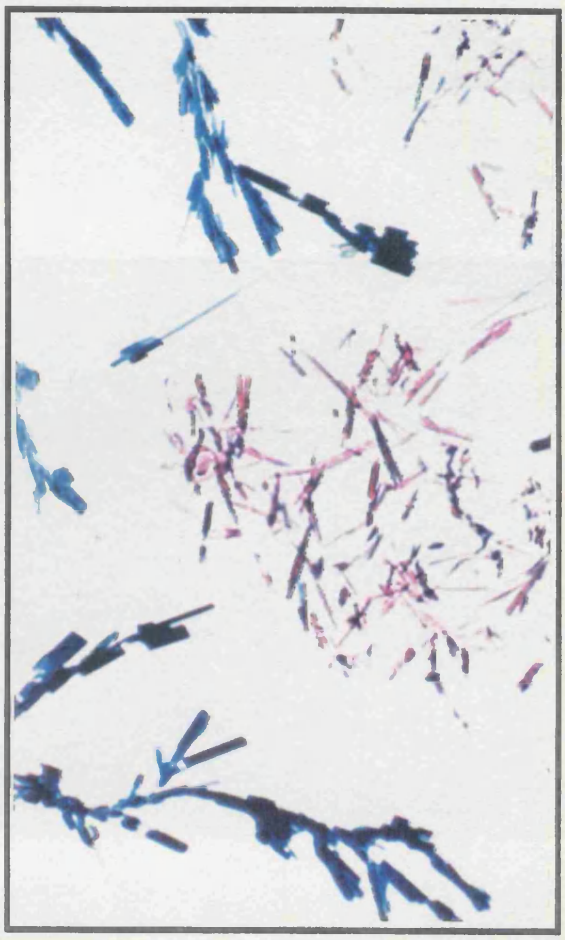
5.12a



5.12b



5.12c



5.12d

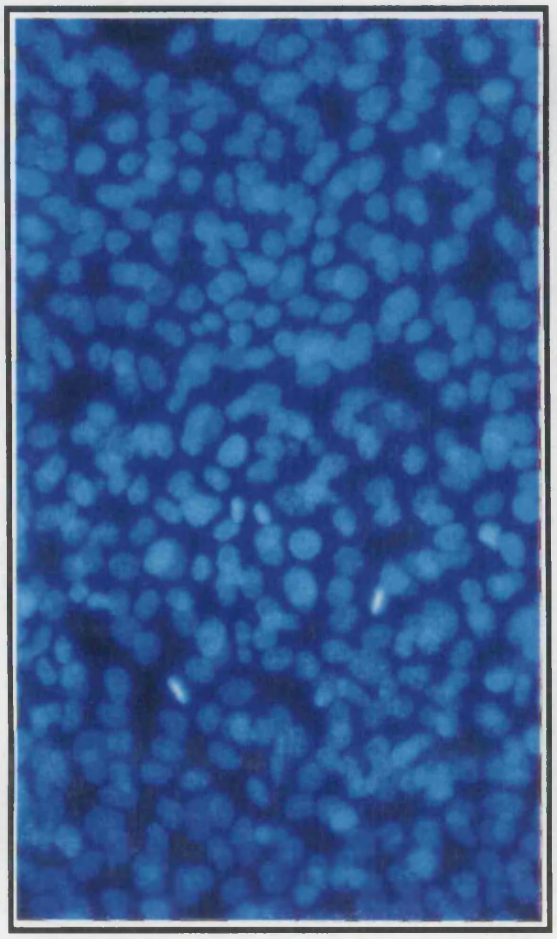
Cells stained with a fluorescent stain Hoescht-HO 33342, (Sigma), for the detection of apoptotic cells.

Figure 5.13a: HOS cells on glass cultured in normal medium for 48 hours; most cells stained negative for apoptosis but a few were positive (Magnification = 330x).

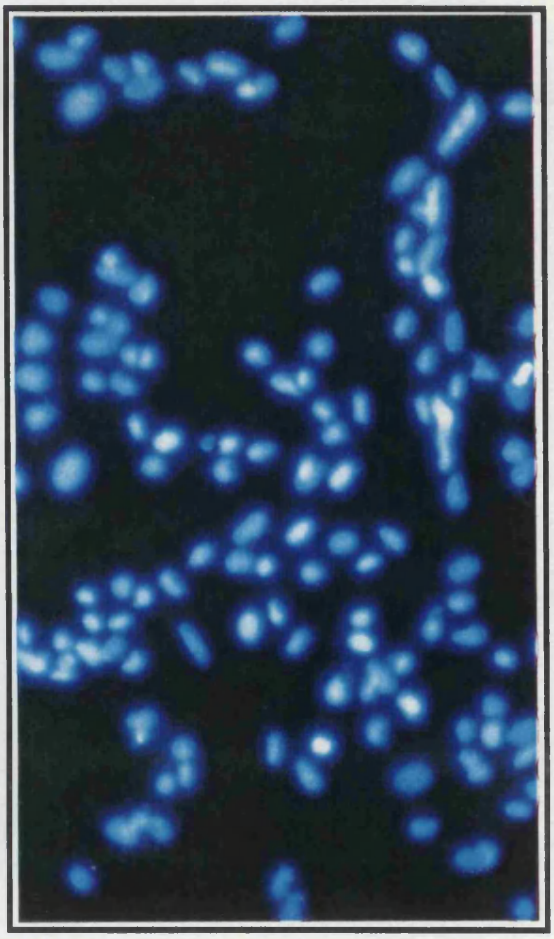
Figure 5.13b: HOS cells on glass cultured with 5% ethanol in normal medium for 3 hours; the remaining adherent cells were strongly positive for apoptosis (Magnification = 330x).

Figure 5.13c: HOS cells cultured on glass with 2500µg/ml of NGA showed a large number of cells positively stained for apoptosis (Magnification = 330x)

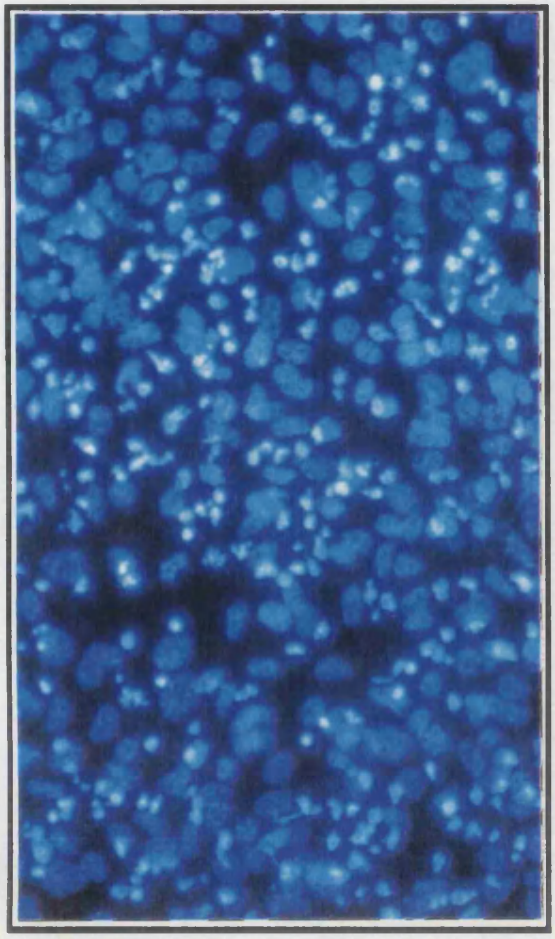
Figure 5.13d: HOS cells cultured in the presence of D3-HB at a concentration of 20,000 µg/ml showed a large number of cells positively stained for apoptosis (Magnification = 330x).



5.13a



5.13b



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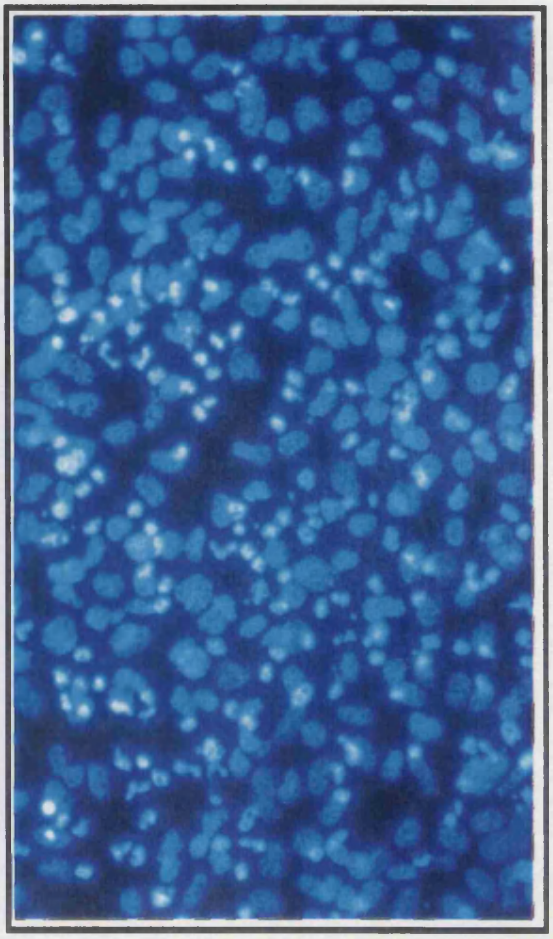
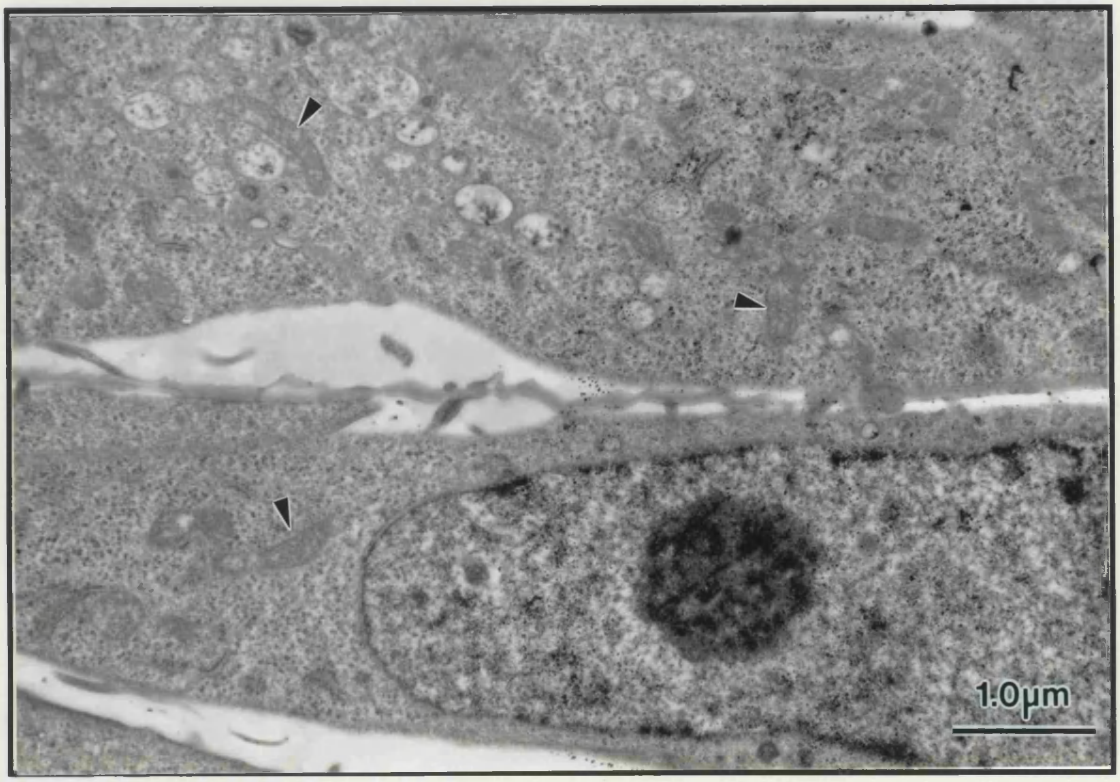


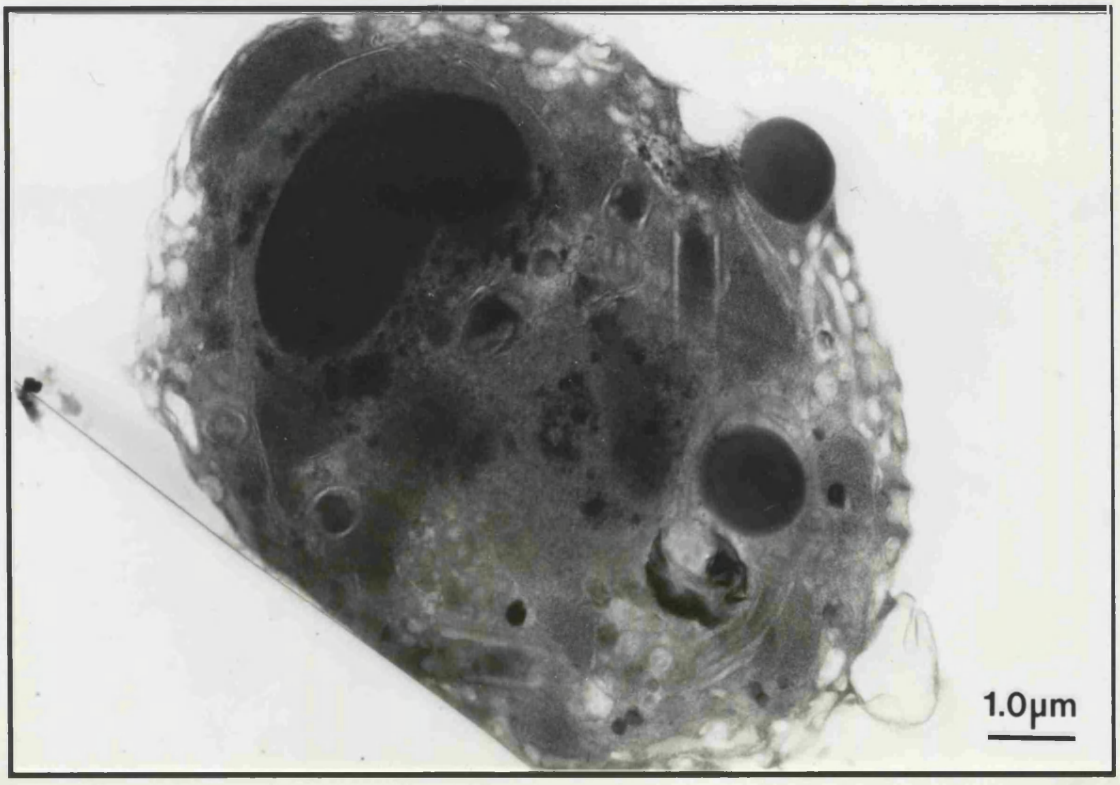
5.13d

Figure 5.14a: TEM of HOS cells cultured on Thermanox for 48 hours showing healthy mitochondria (arrowhead) and an intact nucleus with many cell layers visible.

Figure 5.14b: TEM of HOS cells cultured on Thermanox for 48 hours in the presence of 20,000 µg/ml NGA, the few remaining cells were rounded with no organised structure present and many lipid droplets. There were perforations in the cell membrane with loss of cellular material occurring.



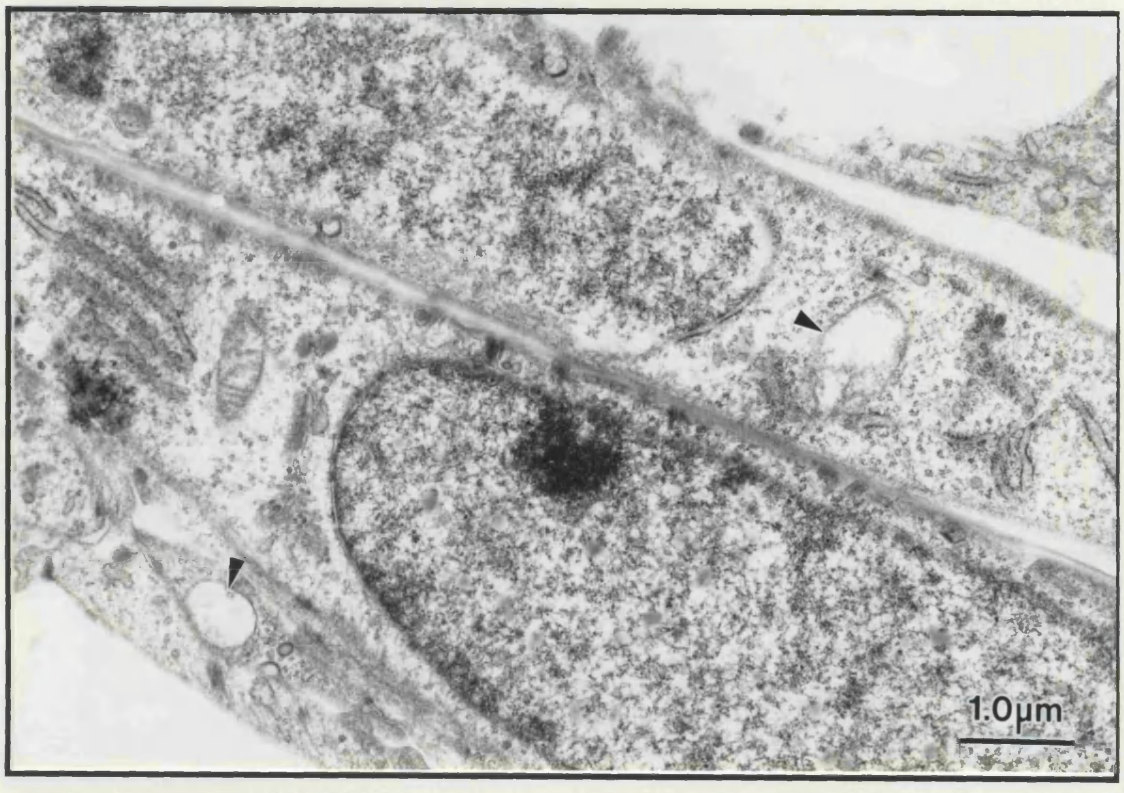
5.14a



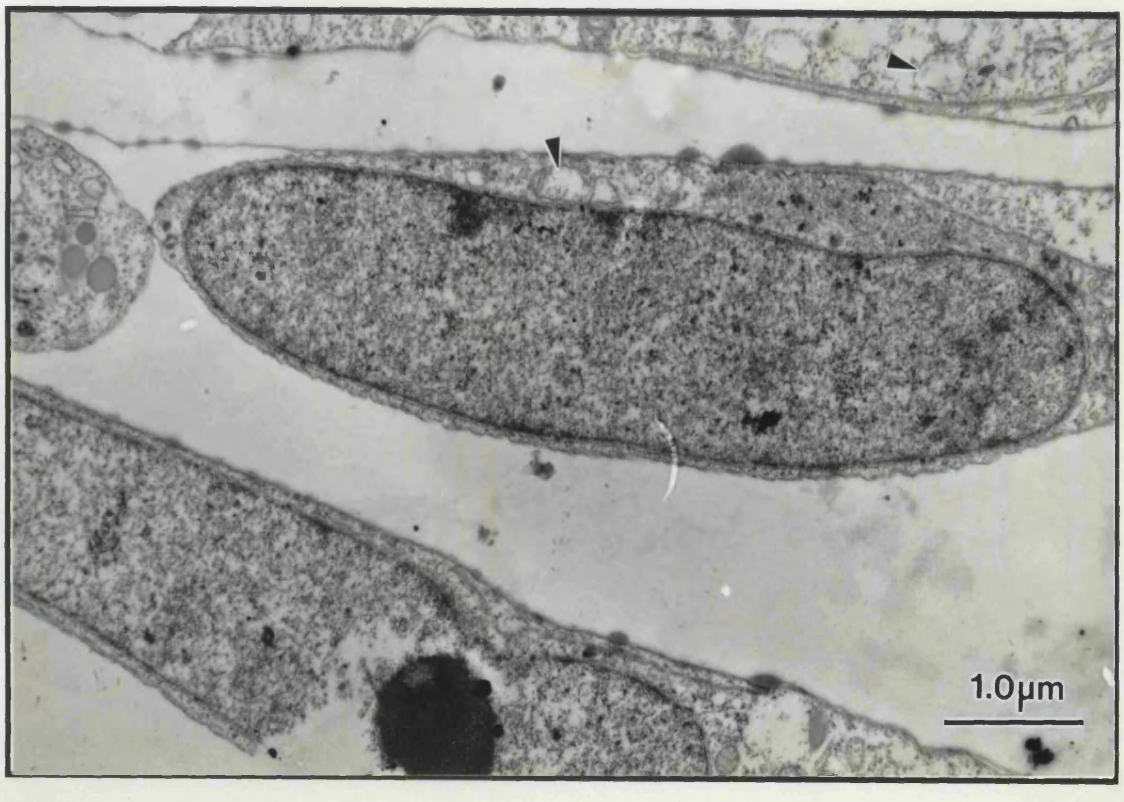
5.14b

Figure 5.14c: TEM of HOS cells cultured on Thermanox for 48 hours in the presence of 5,000μg/ml NLA; the cells showed a lack of cellular protein compared to figure 5.14a. However the cellular membrane integrity was retained and mitochondria were present (arrowhead) but these lacked cristae and cellular processes.

Figure 5.14d: TEM of HOS cells cultured on Thermanox for 48 hours in the presence of 20,000 μg/ml NLA showed cells with an intact cell membrane but a damaged appearance. Mitochondria were present but these were damaged with no cristae present within (arrowhead).



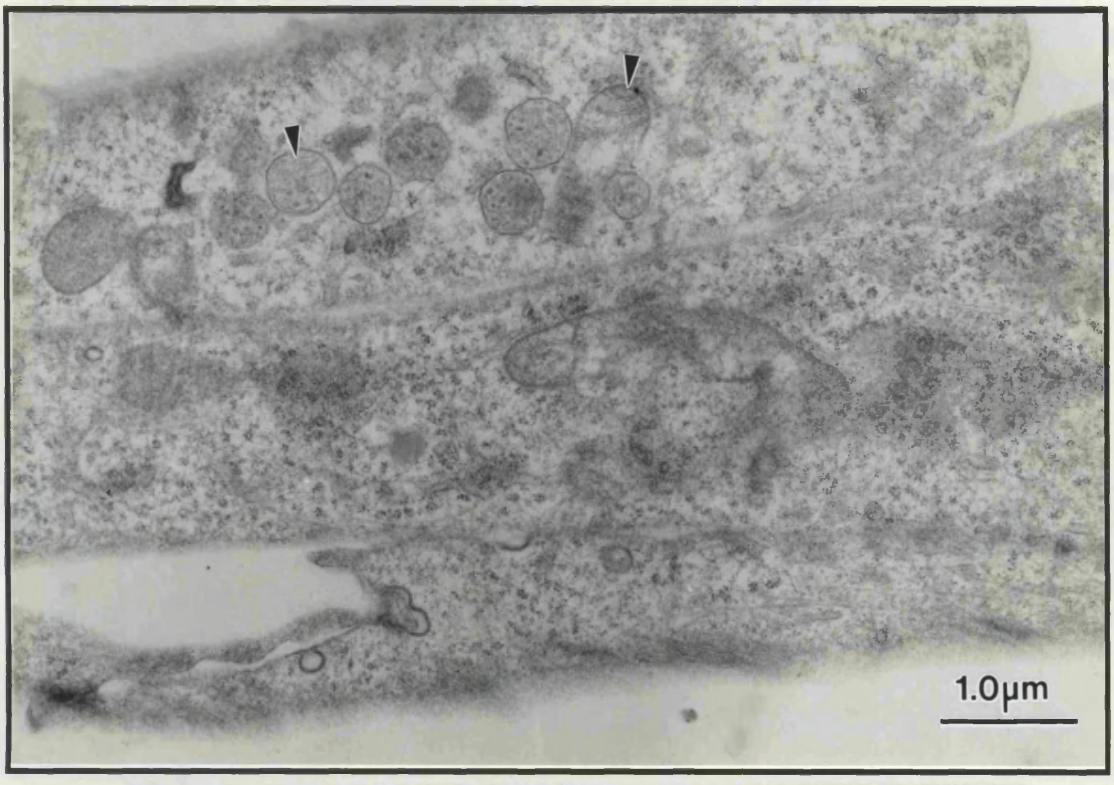
5.14c



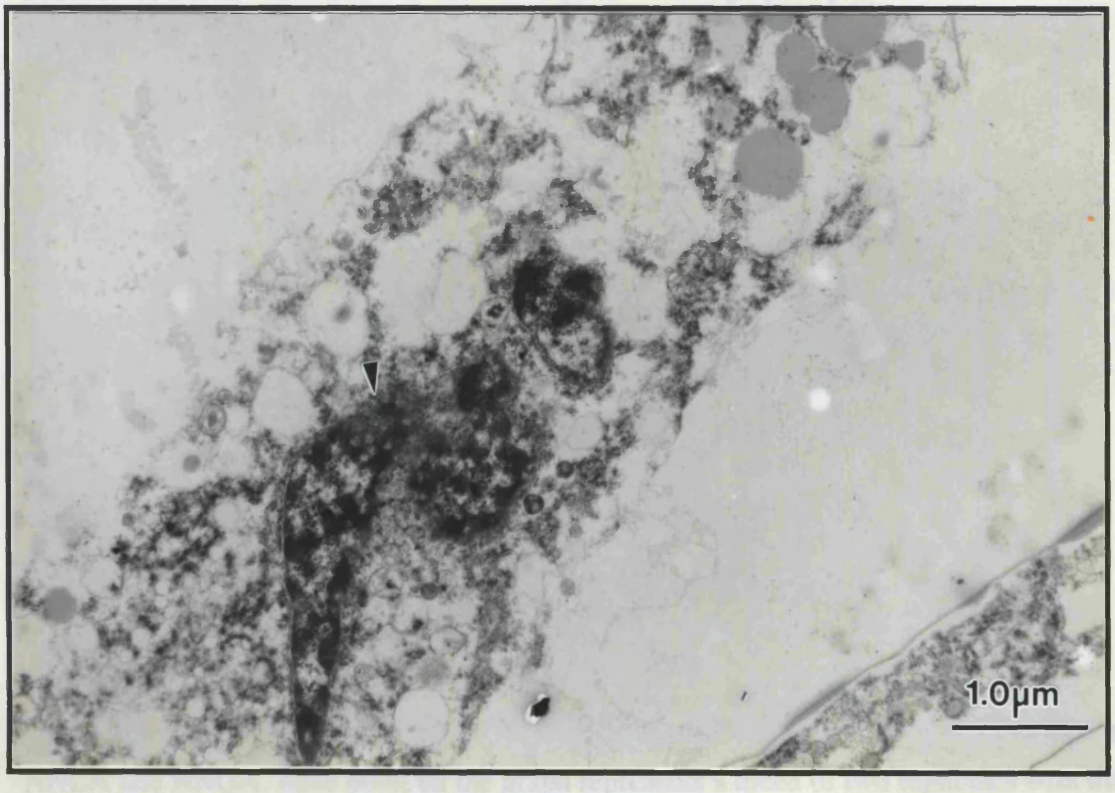
5.14d

Figure 5.14e: TEM of HOS cells cultured on Thermanox for 48 hours in the presence of 1,000μg/ml NNLA. The cells appeared normal with healthy intact mitochondria (arrowhead); however the cells lacked cellular protein.

Figure 5.14f: TEM of HOS cells cultured on Thermanox for 48 hours in the presence of 4,000 μg/ml NNLA. The cells were dead although complete loss of cellular membrane and cellular material had not occurred. Possible remains of nuclear protein were still visible (arrowhead).



5.14e



5.14f

Figures 5.15 and 5.16 show the osmolality of neutralised and non-neutralised medium with the addition of LA, GA and D3-HB monomers. Fully supplemented DMEM with 10% FCS, had an osmolality of 300mosm which increased for all three neutralised monomers due to the addition of sodium hydroxide. With NNLA and NNGA there was no significant increase in the osmolality of the medium; this suggests that the monomer was not the cause of the increase in osmolality but it was caused by the sodium hydroxide used for neutralising the monomers.

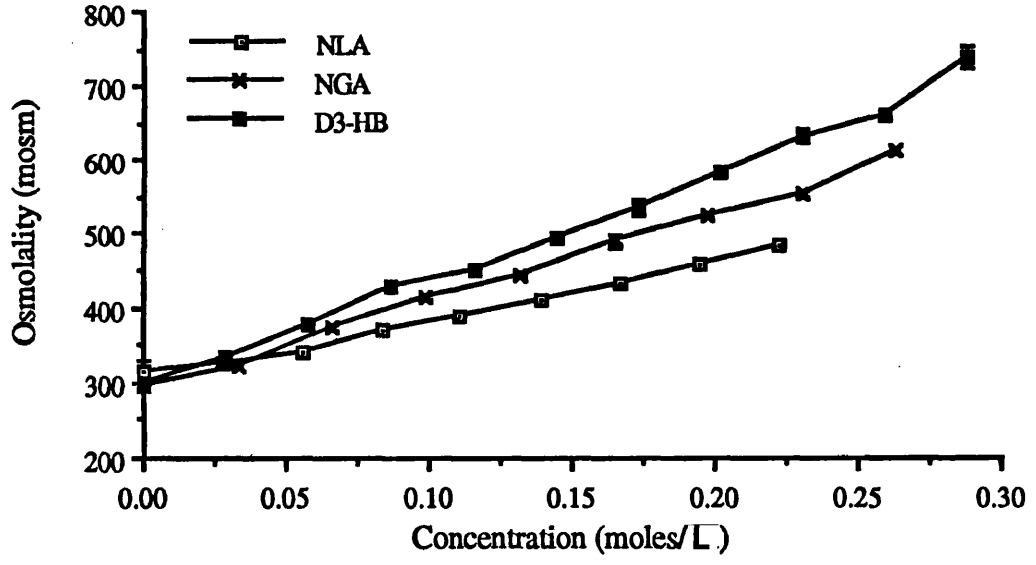


Figure 5.15 shows the osmolality changes occurring to the medium following the addition of NLA, NGA and D3-HB. Each point on the graph represents a mean of two replicates with the error bars as the percent standard error of the mean.

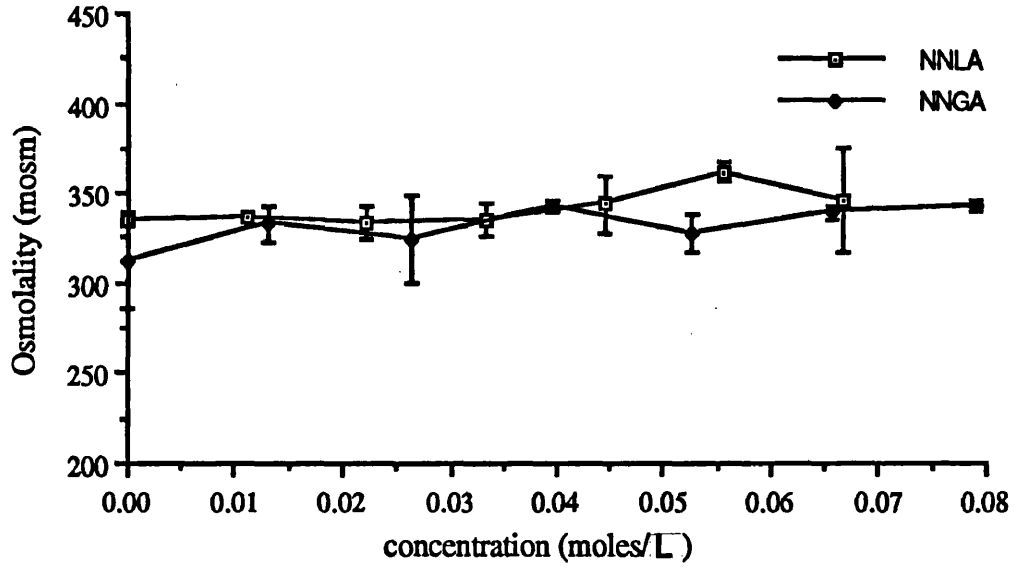


Figure 5.16 shows osmolality changes occurring to the medium with the addition NNLA and NNGA. Each point on the graph represents a mean of two replicates with the error bars as the percent standard error of the mean.

Figures 5.17-5.20 show the results of experiments carried out on HOS cells seeded into 96 well plates and cultured for 24 hours. The normal medium was replaced with monomer loaded medium and the cells exposed to increasing concentrations for 24 hours. Each set was carried out on a separate 96 well plate. After 24 hours one of the plates was assayed and the other plate was rinsed with fresh medium and incubated in complete medium for a further 24 hours. The second plate was then taken through the MTT assay. All points on the graph are replicates of six wells with the error bars being the percent standard error of the mean.

The MTT results from HOS cells cultured in the presence of NLA (figure 5.17) for 24 hours and then revived with complete medium for another 24 hours showed a decrease in cell viability for both treatments. The cells with NLA exposure at the lower concentrations showed a lower cell viability but, following the removal of the monomer, the cells were able to show increased mitochondrial activity as a percent of the control. At the higher concentrations there was no difference between the revived and un-revived values and the mitochondrial activity for both sets was 70%.

With NGA (figure 5.18) a similar pattern was observed but at the higher concentrations viability was down to 60% whereas for LA the viability had not decreased as much. At lower concentrations there was no significant difference between the revived and un-revived treatments.

With NNLA and NNGA (figure 5.19)there was no difference in the values between revived and un-revived. There were also no differences between the NNLA and NNGA monomers. There was a rapid decrease in mitochondrial activity which reached zero at 0.039moles/L.

With D3-HB (figure 5.20) a similar pattern to figure 5.17 was observed. Viability for the revived cells at the lower concentrations was higher than the un-revived cells. After a crossover point of 0.16moles/L the viability however was below the un-revived cells which decreased further with increasing concentrations of the monomer.

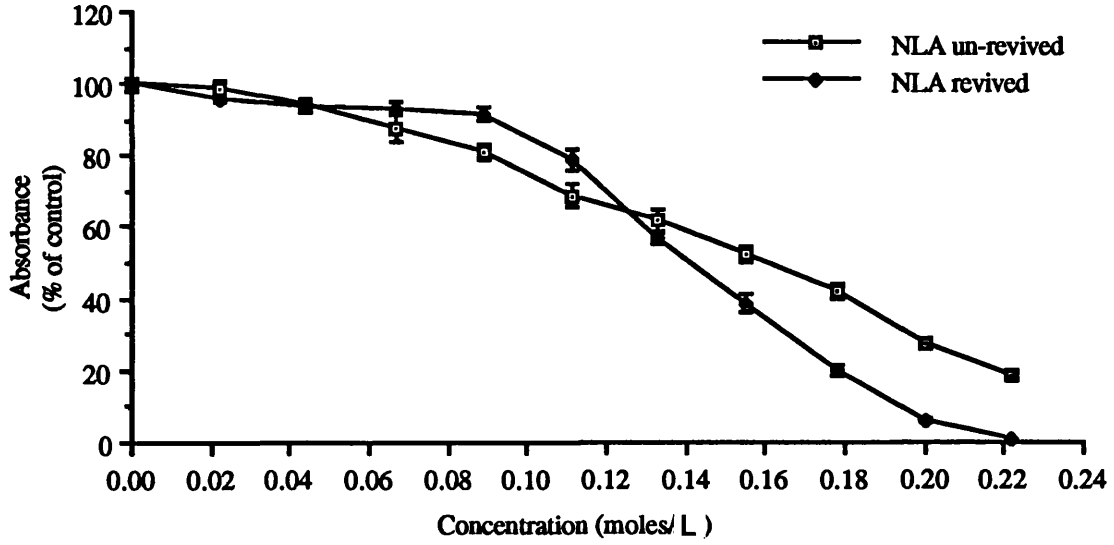


Figure 5.17 shows the mitochondrial activity of HOS cells cultured in the presence of NLA for 24 hours and then "revived" for 24 hours with complete medium.

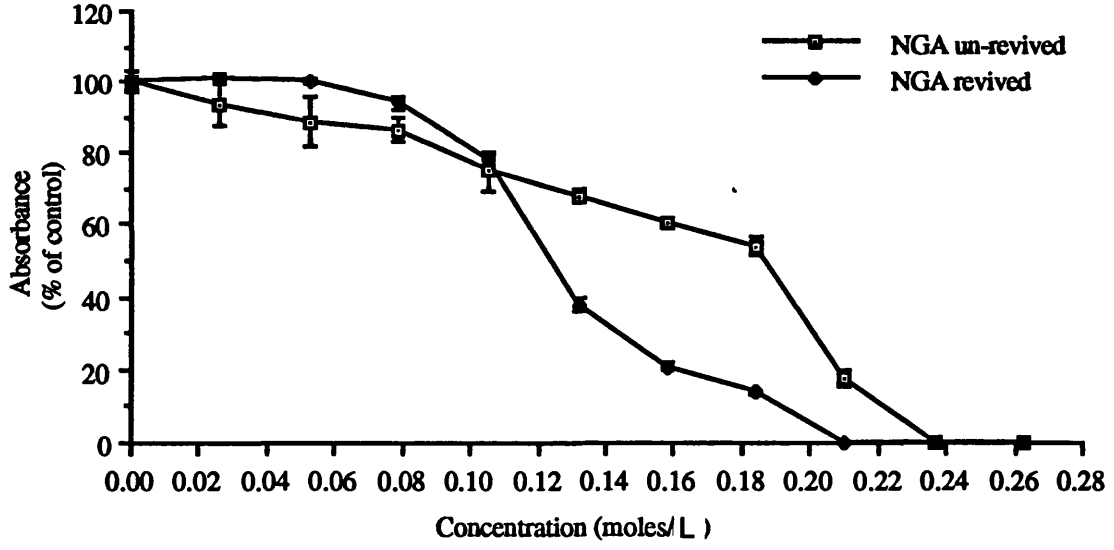


Figure 5.18 shows the mitochondrial activity of HOS cells cultured in the presence of NGA for 24 hours and then "revived" for 24 hours with complete medium.

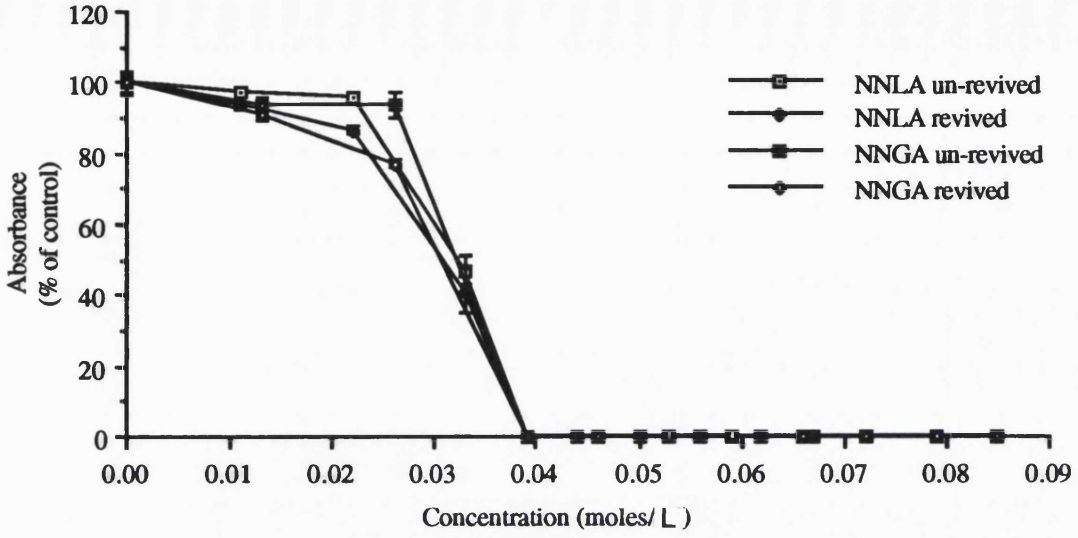


Figure 5.19 shows the mitochondrial activity of HOS cells cultured in the presence of NNLA and NNGA for 24 hours and then "revived" for 24 hours with complete medium.

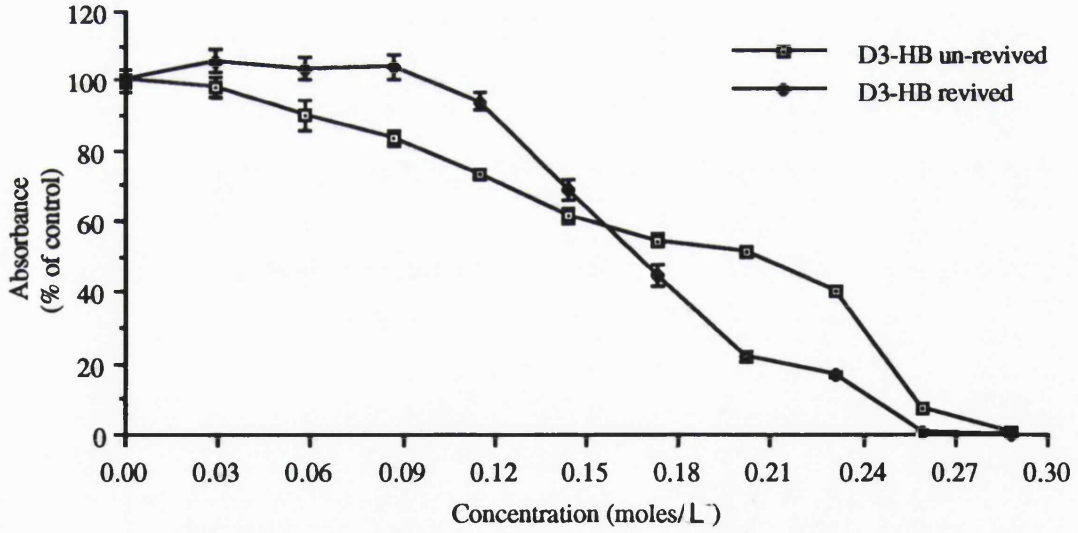


Figure 5.20 shows the mitochondrial activity of HOS cells cultured in the presence of D3-HB for 24 hours and then "revived" for 24 hours with complete medium.

DISCUSSION

Figure 5.1 shows the change in pH of PBS with increasing concentrations of LA and GA. The graph showed a rapid decrease in pH to 4 with a concentration of 0.01 moles/l L (1,000μg/ml) for both LA and GA. This suggests that the buffering ability of PBS will be effective only at low concentrations. Thus the amounts released from the polymers into the incubating solutions will be quite low. Diffusion of bigger molecules such as enzymes into polymers is more difficult than water. An increase in temperature sometimes improves the quality of the solvent leading to a higher swelling but also shrinkage is possible so temperature could promote hydrolysis and also diffusion of water.

In this study the effect of three different monomers were tested on the activity of HOS cells. All the monomers were found to affect the cells and although D3-HB addition did not cause a pH drop the monomer did cause a decrease in mitochondrial activity with increase in concentrations. Other work in this field is sparse but work by Taylor *et al* (1994) tested the degradation products from PGA, PLA and PCL and poly (ortho ester), (POE) on a bioluminescent bacteria acute toxicity system. The response was variable with the degradation products of PLA being the least toxic followed by PCL, PGA and with POE being the most toxic. Although PCL and POE was not tested in my study the same results were obtained for PLA and PGA where PGA was more toxic that PLA.

There have been very few studies on the effect of monomers on osteoblast cells *in-vitro*. However, Sliedregt (1992; 1993; 1995) used fibroblasts, epithelial cells and osteosarcoma cells to investigate the effect of Polylactic acid films, monomers and degradation products which had been artificially aged. Fibroblasts were seeded at a seeding density of $1x10^5$ cells/ 35 cm dish that is $1.04\ x10^4$ cells /cm^2. L and D monomers of Lactic acid were added to fibroblasts and epithelial cells at a concentration of 1, 10, 1,000, 10,000 μg/ml where the pH was adjusted by sodium hydroxide. There was some decrease in the number of cells on the films but no differences in the molecular weights. The degradation products from the artificially aged media were not different to the control. There were no differences between the L and D monomers and only cells cultured with 10,000 μg/ml of lactic acid showed a significant decrease in cell number. It is difficult to make direct comparisons of the results of this study with results discussed in this chapter as the cell types used were different and the culture conditions were different. They also concluded that osmolarity as well as the concentration and nature of the material influenced cell morphology, proliferation and activity.

Otto *et al* (1996) measured the effect of PLLA on the proliferation of primary bone cells. The results were an upregulation of ALP synthesis but no increase in DNA. They found the monomer increased DNA content at lower concentrations but not higher which is in

agreement with the results in this study where an increase in activity was observed for lower LA concentrations. They concluded that this supported *in-vivo* data where new bone formed around PLA implants.

The LDH assay which is a measure of cell death has been used by other groups and could be used (Bordenave *et al* 1993). Martin*et al* (1996) measured the acidity near eroding polylactide-polyglycolide *in-vitro* and *in-vivo* in rabbit tibial bone chambers and found that the acidity did not inhibit angiogenesis or bone defect healing although the drop in pH was significant. The sample size however was small and these were in film form the dimensions were 100 μm thick, had an outer diameter of 1.6 mm and an inner diameter of 0.8 mm.

Comparison of the MTT and ^{3}H-thymidine incorporation showed that there was a significant difference in the two sets of data. The difference in that could be due to apoptotic cell death where the cells were no longer able to divide due to the damage to DNA but not as much to mitochondria. Apoptotic process takes about 30-60 minutes. Other assays which measure cell death or cell viability should be used for further studies to confirm these results. Ciapetti (1993) found good correlation between the MTT assay and ^{3}H-incorporation assay but this was different to our study. There was significant difference between the MTT assay and ^{3}H-thymidine incorporation when exposed to monomers from resorbable polymers.

Figure 5.17-5.20 shows the effect of monomers on cells following exposure for 24 hours to determine whether the cells had the capacity to recover. The activity of the cells decreased following exposure to all the monomers and was most rapid for the non-neutralised monomers. The effect of reviving was only noticable on the neutralised monomers at higher concentrations and at the lower concentrations the cells showed no difference in activity. At the higher concentrations however, the cell activity dropped significantly after a "crossover" point. This suggested that at higher concentrations the cells were continuing to die after the removal of the monomer; it is possible that this death was by apoptosis rather than necrosis.

This study has demonstrated the variability in sensitivity of assays which measure different aspects of cell behaviour or activity. The values obtained for the two differnt assays that is, ^{3}H-thymidine incorporation and MTT gave varying results on the effect of the monomers on HOS cells. This variability in assay sensitivity has also been demonstrated by Clifford and Downes (1996) who tested other assays such as the neutral red and the MTS assay and found these to be different. Therefore during biocompatibility or cytotoxicity testing it is important to have at least two different reproducible assays which measure various aspects of cellular activity.

CHAPTER VI

The Monocyte/Macrophage Response to Degradable Polymers and their Monomers

The immune system consists of two divisions, the innate and the adaptive immune system. The innate system is the first line of defence and, if this fails, the adaptive immune system comes into play. The immune system has various lymphoid cells and molecules associated with it of which the leucocytes, are the most important. These fall into two categories phagocytes and lymphocytes. The Phagocytes from the myeloid lineage and include the neutrophils, monocytes, macrophages, basophils, eosinophils, mast cells, platelets and polymorphonuclear granulocytes. The lymphocytes are derived from the lymphoid lineage which include the T and B cells. Macrophages are derived from bone marrow promonocytes which differentiate into blood monocytes and then into macrophages in tissues where they make up the mononuclear phagocyte system. Macrophages are end of line cells that have rough surfaced endoplasmic reticilum and mitochondria and can live for many days. Monocytes/macrophages adhere to glass and plastic surfaces and phagocytose organisms and tumour cells *in-vitro* .

The immune response to an implanted polymer is determined by various factors such as the size, shape, toxicity and leachables released from the polymer in addition to the site of implantation and the surface properties of the polymer. Sliedregt *et al* (1992) however stated that the molecular weight did not have an effect. When phagocytosis occurs there is an increase in the oxygen consumption due to the generation on NADPH. Oxygen is converted into superoxide anion, hydrogen peroxide and hydroxyl and oxygen radicals which have also been implicated in the degradation of polymers. These cells also contain peroxidase which inactivate peroxide ions generated during killing of the ingested microorganisms and produce complement components, such as prostaglandins, interferon monokines, interleukin-1 and TNF.

Much work has been done on the effect of degradable polymer fragments on the morphology and viability of cells. Lam *et al* (1993) used predegraded PLLA and non treated PLLA particles both with diameters of less than 38μm injected intraperitoneally using an 0.3% ethanol/0.9% saline solution into mice. The degraded particles were obtained by gamma irradiation at 25KGy with polytetrafluoroethylene (PTFE) used as the non-degradable control. After various time points the cells were harvested from the abdominal cavity. TEM analysis showed that macrophages from the peritoneal cavity with the pre degraded particles had phagocytosed particles and showed signs of cell damage, cell death and cell lysis. However, the non-treated PLLA and PTFE were not significantly different from those cells exposed to just the solution. The highest amount of necrotic death occurred at 2 days and it was concluded that the high amount of cell damage was caused by phagocytosed Poly(L-lactic) particles. This is highly suggestive that there should be concern on the effect of degradation products on the viability of macrophages.

Initial interest in degradable polymers concentrated around their use as suture materials as these are the most common "implants" used in humans. Of the absorbable are catgut and collagen, usually obtained from ox or sheep which is absorbable within 90 days. The new breed of materials are the synthetic polymers of which the most common is glycolide supplied under the trade name Dexon which is also absorbed in 90 days. There are also copolymers of glycolide with lactide(trade name Vicryl)with a glycolic acid to lactic acid ratio of 90:10 which is absorbable within 90 days. There is also interest in some other polymers;Polydioxanone (Ray *et al* 1981) and polyhydroxyvalerate which are absorbable in approximately 180 days. There are also non degradable sutures which are made form silk, linen, cotton, polyester, polyamide, polypropylene, and steel. They are implanted in most parts of the body and therefore, come into contact with most cell types present in the body. There are absorbable sutures and non absorbable. The cellular response can be assessed in three ways *in-vivo*, *in-vitro* and in situ (Capperauld *et al* 1986). Matlaga *et al* (1976) demonstrated that shape and size of the suture influenced the tissue reaction where the round shape caused the least tissue reaction and braided shapes caused more of a reaction. It is not only the shape but also the site of implantation that determines the overall immune response stressing the importance of chosing implantation sites carefully for biocompatibility testing (Bakker *et al* 1988)

Macrophages have a major function in the body, they influence many aspects of the immune and inflammatory response and secrete a wide range of substances which vary in molecular mass. They release polypeptide hormones, complement components, coagulation factors, enzymes and acids which have an effect on almost all the processes that happen in the body. TNF-α and IL-1 are known to be released from macrophages when they are activated and these cytokines have an effect on cells in culture. They have been documented to decrease bone synthesis by affecting osteoblast proliferation but in comparison increase the proliferation of fibroblasts. This has a direct effect on the overall biocompatibility of a material that is being used for osteointegration. The cytokines released from the macrophages will therefore have an effect on the direct function that the material is trying to promote(Nathan 1987).

Degradation, and the effect of degradation products from polyesterurethane foam on macrophages and osteoblasts have been investigated by Saad *et al* (1996). A macrophage cell line, a osteoblast cell line and primary osteoblasts were used for the study. There was no macrophage activation or a cytotoxic effect observed on the osteoblasts. The degradation of the polymer released D3-hydroxybutyric acid and a further study showed that the macrophages were able to phagocytose particles of PHB-P. At low concentrations no adverse effects were noted but at high concentrations, higher than 400pg PHB-P/cell, macrophages were affected and to a lesser extent osteoblasts.

Macrophage/biomaterial interactions have been investigated as modulators of endothelial cell proliferation. Rabbit peritoneal macrophages were harvested and seeded into culture flasks with DMEM. PG910 particles were found to be present in the cytoplasm of the macrophages, but Dacron was not phagocytosed. Medium collected at 5-10 weeks from these were added to lung epithelial cells. The Dacron group showed no significant difference from the control group but the PG910 group showed increased mitogenic activity from weeks 6 to 10, in addition to an increase of 620% in DNA synthesis. It was concluded that macrophage activation by bioresorbable prosthesesyielded growth factors which increased endothelial cell proliferation (Greisler, 1989).

Macrophages when releasing growth factors and cytokines can affect cells in various ways. As well as being able to enhance endothelial cell proliferation they have been documented to decrease bone production(Anderson and Miller 1984; Remes and Williams 1992). In the design of various prosthes and devices it is very important to consider the site of implantation and the effect the degradation products are going to have on all the cell types it will come in contact with.

Biomaterials upon implantation ilicit an inflammatory response that will affect the healing of the implant site. Various models have been developed to measure the effects of the polymer on the inflammatory cells immediately adjacent to the polymer such as subcutaneous pouches and tissue cages. Freyria *et al* (1991) used pieces of materials and implanted them into the peritoneal cavity and measured a graded response related to the different materials. Krause *et al* (1993) developed an air pouch model into which biomaterials could be implanted and removed for analysis. They quantified the production of intracellular hydrogen peroxide by inflammatory cells at 2 and 7 days afer implantation and found significant differences.

Polymers and macrophages

In another study a fluorescent labelled PHB segment synthesised and precipitated into crystalline particles was cultured with a macrophage cell line. Light and fluorescent microscopy was used for the analysis. After one hour fluorescent particles were found to be internalised in the phagosomes. The number of phagocytosed particles however, had decreased after 8 days incubation. HPLC analysis of cell supernatant extracts showed degradation products of PHB after 8 days suggesting that macrophages were able to degrade low molecular weight PHB (Ciardelli *et al* 1995).

A major problem with *in-vitro* systems in the lack of information currently on their relevance to what occurs in the *in-vivo*. Miller *et al* (1989) compared *in-vitro* and *in-vivo* models for the production of IL-1 like activity on response to different polymer implants

and concluded that biomaterials could affect the activation of macrophages and thus affecting the production of growth factor mediators such as IL-1. *In-vivo* and *in-vitro* methods could be combined to develop a "powerful tool" which could be used to better understand the biocompatibility response

Ali *et al* (1994) have looked at interactions between polymers and inflammatory cells and their relative biocompatibility *in-vivo*. Inflammatory cells were found to be involved in the biodegradation of PCL and PDLA by the release of highly reactive free radicals into the area surrounding the implant. Thus, hydroxyl radicals may be one of the main causes of polymer degradation.

Bergsma *et al* (1993) reported long term results of fixing displaced zygomatic fractures using PLLA plates and screws. Three years post-operatively four patients returned and the remaining six were recalled. Remnants of degraded PLLA were found surrounded by a dense fibrous capsule. The swelling observed in these was considered to be a foreign body reaction to the degraded PLLA and crystalline PLLA was found internalised in the cytoplasm of cells.

The aim of the work described in this chapter was to determine the monocyte/macrophage response to the various degradable materials. Methods were used to quantify the response of the THP-1 cell line to the various polymer surfaces and the monomers that would be released from the degradable polymers. The MTS assay was used as opposed to the MTT assay as it is more sensitive to non adherent cells and as the THP-1 cell line is predominantly non adherent this was found to be the best method.

Cells cultured on the polymer surfaces were also stained for non specific esterase after five days in culture to determine their morphology and expression of phenotype. The morphology of the cells on the polymer surfaces was also investigated using scanning electron microscopy after 48 hours. The cells were also cultured with neutralised and non-neutralised monomers to determine their effect with increasing concentrations and how these compared to the osteoblast-like cells.

MATERIALS AND METHODS

Human monocytes (THP-1 cells) able to differentiate into macrophages, ECACC number 88081201 were used for all *in-vitro* studies. The cells are phagocytic and possess Fc and C3b receptors but lack surface and cytoplasmic immunoglobulins. They were derived from the peripheral blood of a one year old boy with acute monocytic leukaemia. The cells were cultured in RPMI 1640 (Sigma) supplemented with 10% FCS (Sigma) and $2x10^{-5}$M, 2-Mercaptoethanol (Sigma) and maintained at a concentration of $9x10^5$ cells/ml with 5% CO_2 in a humidified atmosphere at 37°C. A cell suspension of $2.5x10^5$ cells/ml in supplemented RPMI was made and the cell suspension was seeded onto to the polymer films in 24 well plates. The films were prepared using the method described in chapter II. The cells were incubated for the times stated and removed for sampling at the various stages.

THP-1 cells on polymer surfaces
α-Naphthyl acetate esterase staining (Non specific esterase)

THP-1 cells were cultured on the polymer surfaces for the time points stated and viewed by SEM and Light microscopy. A α-naphthyl acetate esterase kit, procedure number 91, (Sigma) was used to test the expression of phenotype of the monocyte/macrophages on the various polymer surfaces. The purpose of this study was to determine if such stains could be used for visualising the cells on the polymer surfaces and if these could be used to identify monocytes/macrophages from a mixed population of cells. The kit utilised provides a method for distinguishing granulocytes from monocytes. The cells are incubated with α-naphthyl acetate in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds which couple with the diazonium salt, forming coloured deposits at the site of enzyme activity.

MTS assay of THP-1 cells with monomers

Monomer preparation

Concentrations of monomers were made up in complete medium and neutralised with NaOH for the neutralised monomers. The concentrations were made up in μg/ml but for data analysis were converted to moles/ml. The table in chapter five (table 5.1) shows the concentrations in μg/ml and the equivalent value in moles/L..

This is a method for determining the proliferation of cells using CellTiter 96™ Aqueous Non-radioactive Cell Proliferation Assay (Promega Cat no G5421). A tetrazolium compound MTS is reduced by viable cells in a an electron coupled reaction utilising a

electron coupling reagent PMS. A coloured formazan product is formed which can be measured at 490 nm. The THP-1 cells were cultured in phenol red free RPMI in 96 well plates. The monomers were added for 24 hours in various concentrations. 100 µl of PMS is added to 2 ml of MTS of which 20 µl is added to each well containing 100 µl of the media with cells. The plate was incubated for 2-4 hours at 37°C after which the absorbance was read at 490 nm on an ELISA plate reader.

Statistics

The results obtained were analysed using appropriate statistical tests. The data sets were tested for normality using the Shapiro-Wilk W test. If p values obtained were less than 0.05 the data was not normal. If the data was normal (parametric) an analysis of variance was used and if the data was not normal (non-parametric) the Mann-Whitney test was used. Depending on the results from these tests a multiple comparison test was carried out; the Tukey Kramer-Honestly Significant Difference test (TK-HSD). All tests were carried out using alpha values of 0.05 and 0.01 and significance was denoted * for significance at $p<0.05$ and ** for significance at $p<0.01$. Where multiple comparison was not required and comparisons could be made to a control the Dunnett's test was carried out at alpha values of 0.05 and 0.01 and significance was denoted as above. Some data sets could not be analysed due to small sample sizes.

Results

The results from the MTS assay show the differences obtained when the cells were cultured on the polymer surfaces over a 72 hour period. Cells on Thermanox were used as a control and for the three time periods Thermanox values were set at 100%. PCL showed the highest increase after culture on the polymer for 72 hours with PHB-PHV showing little increase. There were no significant differences observed in cell proliferation on the material surfaces at the three time points tested with the exception of PCL which was significantly higher than PHB at 24 hours (Fig 6.1).

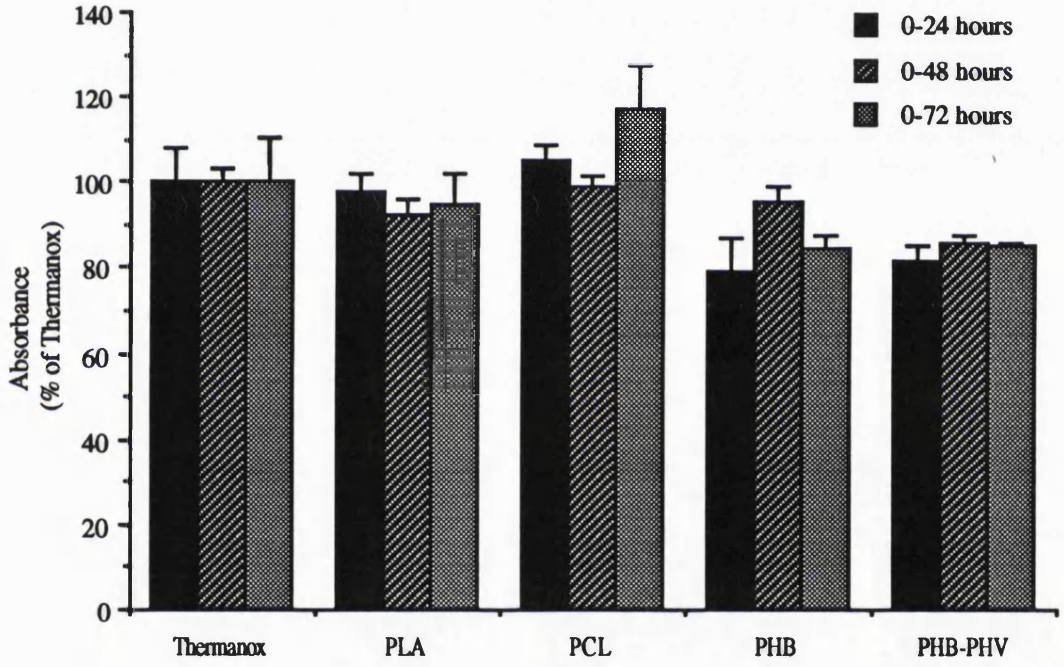


Figure 6.1: MTS conversion by THP-1 cells cultured on various polymer surfaces. Cells cultured on Thermanox were used as a control and significance was denoted by an astrix (*) at ($p>0.05$) using the Dunnetts test. The TK-HSD test was carried out to determine differences between the polymers. At 0-24 hours PCL was found to be significantly higher than PHB. At 0-48 and at 0-72 hours no significant differences were observed ($p>0.05$).

THP-1 monocyte/macrophage cell lines were used to study the immune response and the effect of polymer surfaces on the morphology of these cells. Following cell seeding, cells were cultured for 48 hours on the various polymer surfaces and viewed by SEM. The cells had very different morphologies on the polymers. When monocytes become activated they become macrophage like and take on the characteristic morphology which is very flattened with many processes. Two magnifications were used for each polymer and the lower magnification shows the extent of cells adhering to the polymer surface and the higher magnification shows a more detailed view of the cells and individual morphologies of the cells.

Cells on glass (figure 6.2) were different to all the materials with some of the cells having a rounded morphology while others were more spread out with many processes. Cells on PLA both on the "as cast" (figure 6.3) and the gamma irradiated polymer (figure 6.4) appeared to be similar in morphology to each other and the cells on glass. Cells were ruffled (this was were the cytoplasm had folded several times upon itself) and were often observed in clumps on the gamma irradiated PLA. Cells on PCL (figure 6.5) had the most varied morphology of all the polymers tested. They had a flattened shape with very few processes and in some areas the cells appeared to be fusing together to form one giant mass. Cells on PHB (figure 6.6) were sparse with the cells that were present being rounded. There were more cells present on the PHB-PHV (figure 6.7) surface and the individual cells had few processes and the surfaces were quite smooth as compared to the PLA and glass.

No text for figs 6.8 – 6.12.

Figure 6.2a: Scanning electron micrographs of THP-1 cells cultured on glass for 48 hours showing many cells adhering to the polymer surface but not appearing activated with the exception of one or two.

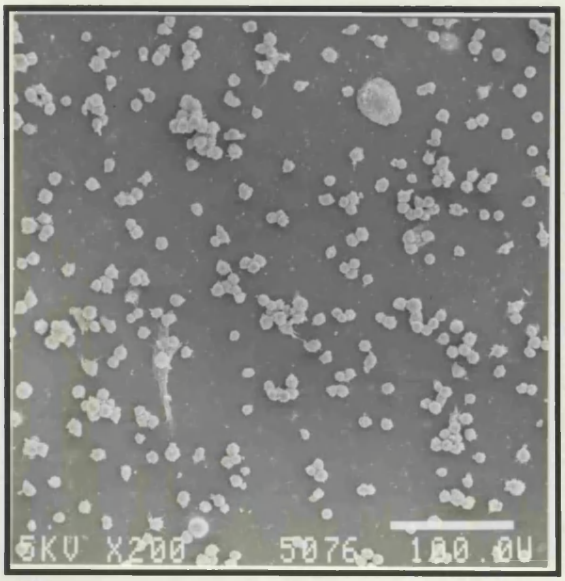
Figure 6.2b: Scanning electron micrographs of THP-1 cells cultured on glass for 48 hours showed one activated (arrowhead) cell and the other cells still rounded but with many processes.

Figure 6.3a: Scanning electron micrographs of THP-1 cells cultured on PLA for 48 hours showed cells similar in appearance to cells on glass, but cell clumping was observed.

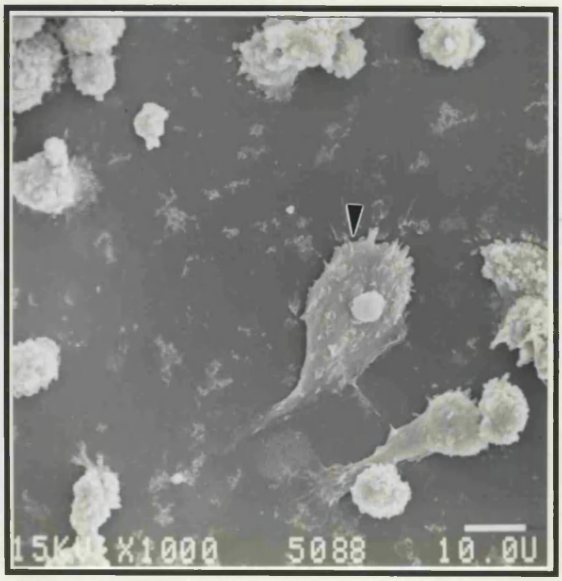
Figure 6.3b: Scanning electron micrographs of THP-1 cells cultured on PLA for 48 hours showed the cells were forming clumps and although they had fewer processes ruffling of the cytoplasm was occurring.

Figure 6.4a: Scanning electron micrographs of THP-1 cells cultured on gamma irradiated PLA showed a greater number of cell clumps were present with most of the cells appearing rounded.

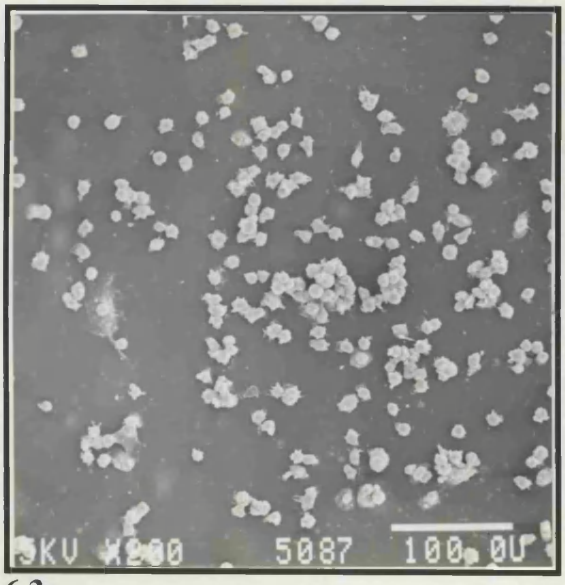
Figure 6.4b: Scanning electron micrographs of THP-1 cells cultured on gamma irradiated PLA showed cells were rounded and had very few processes and a reduced ruffling of the cytoplasm compared to figure 6.3b.



6.2a



6.2b



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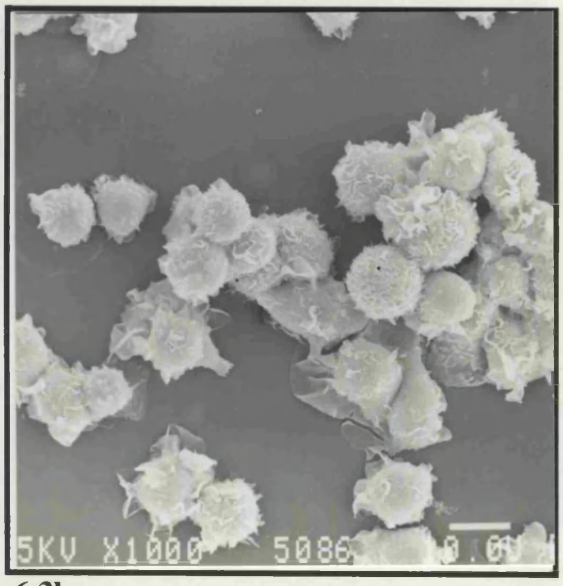
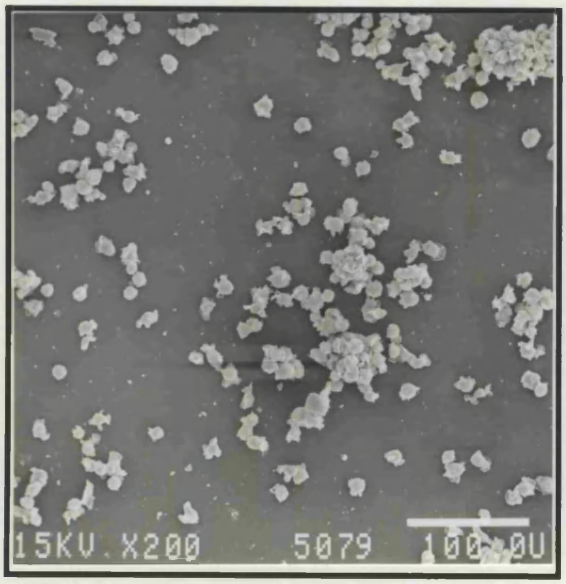


6.3b



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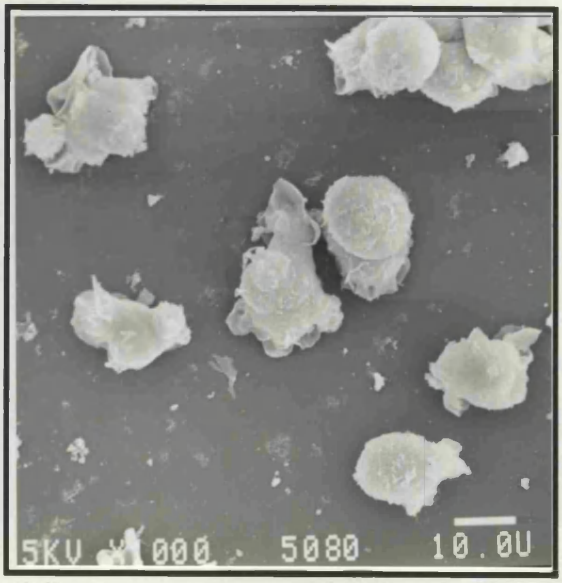


6.4b

Figure 6.5a: Scanning electron micrographs of THP-1 cells cultured on PCL for 48 hours showed the cellular morphology to be very different compared to all the other polymers. Cells were forming clumps (arrowhead) and flattened.

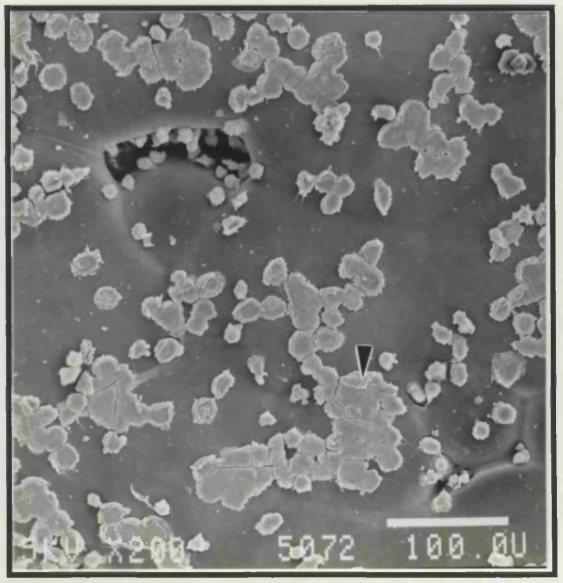
Figure 6.5b: Scanning electron micrographs of THP-1 cells cultured on PCL for 48 hours showed that the cells were flat with few processes.

Figure 6.6a: Scanning electron micrographs of THP-1 cells cultured on PHB for 48 hours showed very few cells adhering with some forming clumps.

Figure 6.6b: Scanning electron micrographs of THP-1 cells cultured on PHB for 48 hours shows the individual cells to be very rounded and with very few processes.

Figure 6.7a: Scanning electron micrographs of THP-1 cells cultured on PHB-PHV for 48 hours showed many cells adhering to the polymer surface with some clumping occurring.

Figure 6.7b: Scanning electron micrographs of THP-1 cells cultured on PHB-PHV for 48 hours shows the individual morphology of the cells to be both flat and rounded with very few processes.



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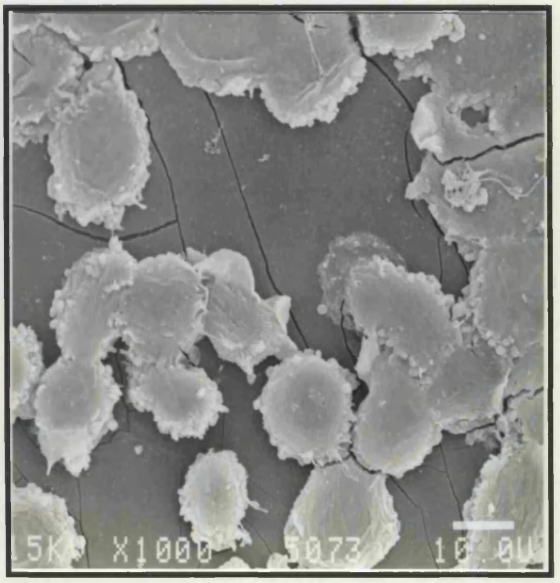

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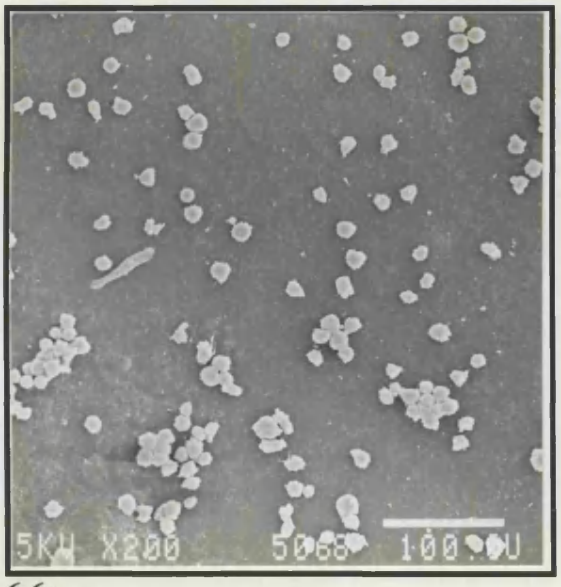

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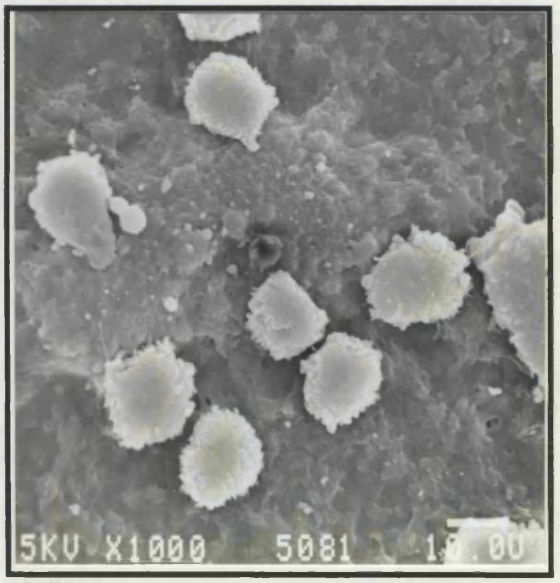

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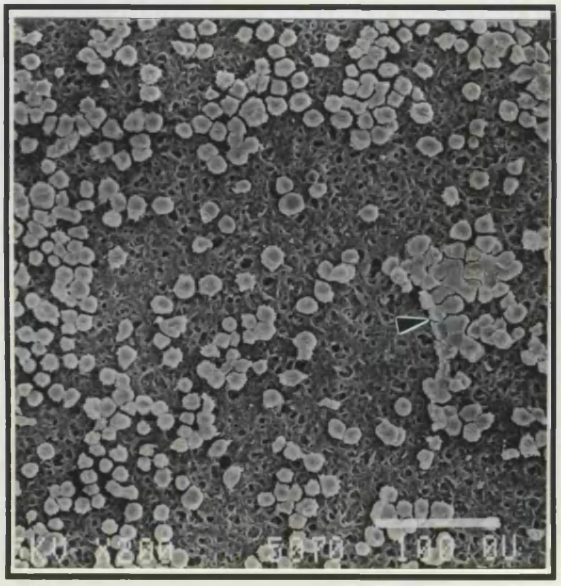

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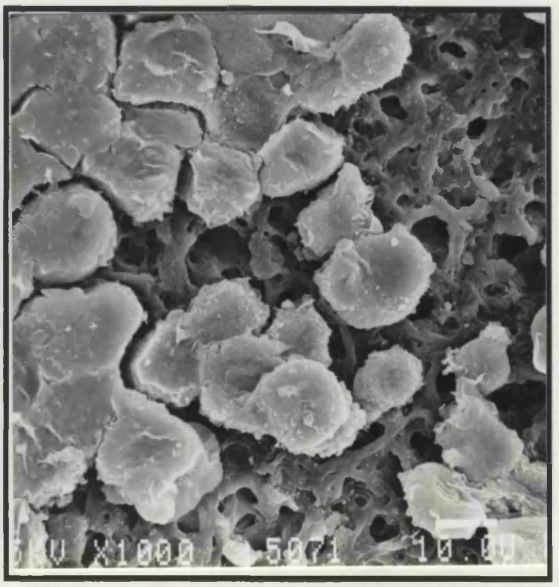
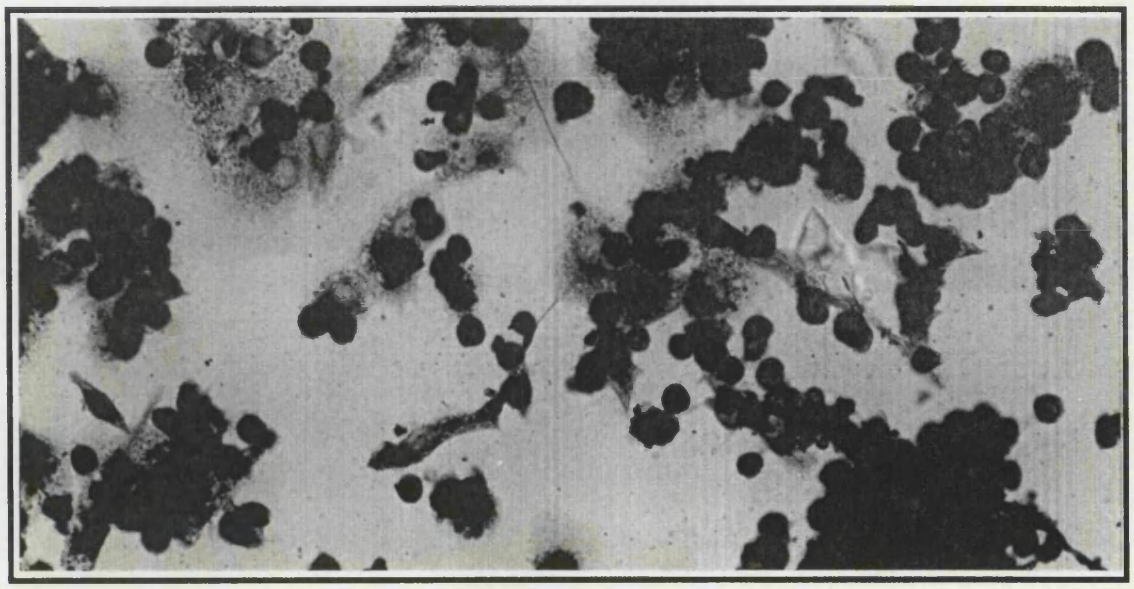


6.7b

Figure 6.8: THP-1 cells on Thermanox after 5 days in culture stained with non specific esterase staining. Monocytes and macrophages take up the stain and appear black. The cells on Thermanox appeared activated as they were very spread out with many processes (Magnification = 330x).

Figure 6.9: THP-1 cells on PLA after 5 days in culture stained with non specific esterase staining showed fewer cells adhering to the polymer surface compared to Thermanox but the morphology was similar (Magnification = 330x).

Figure 6.10: THP-1 cells on PCL after 5 days in culture stained with non specific esterase staining were difficult to visualise due to the uneven polymer surface but the cells were positively stained and were rounded (Magnification = 330x).



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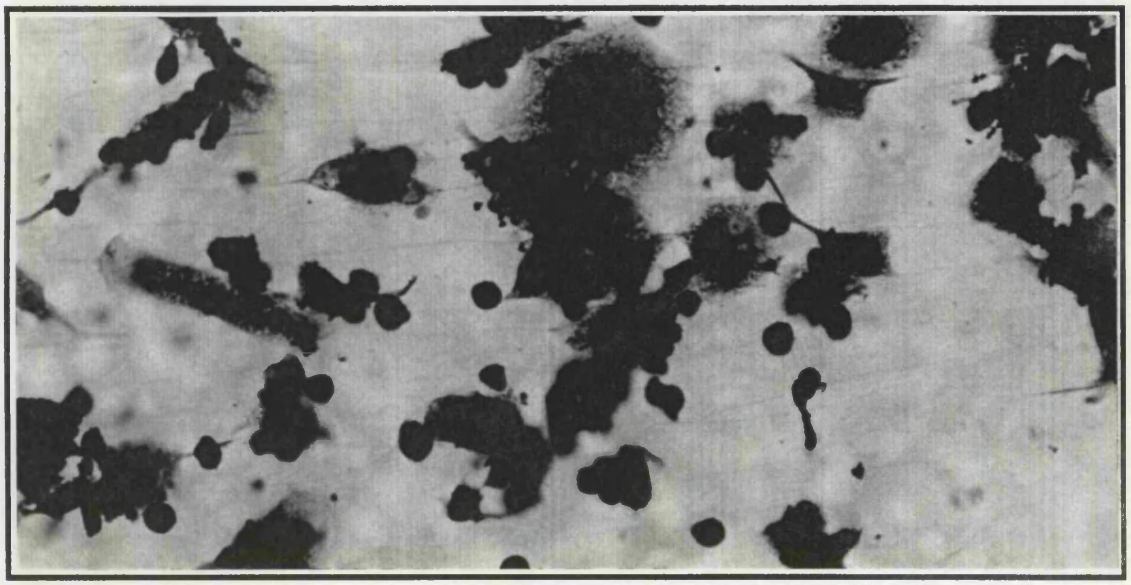

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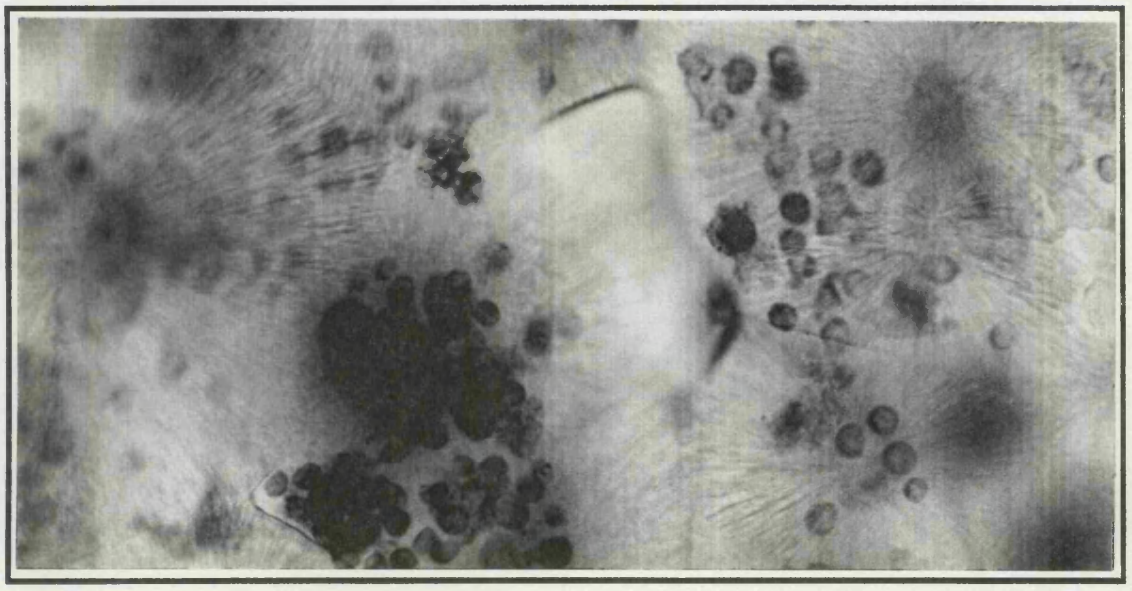
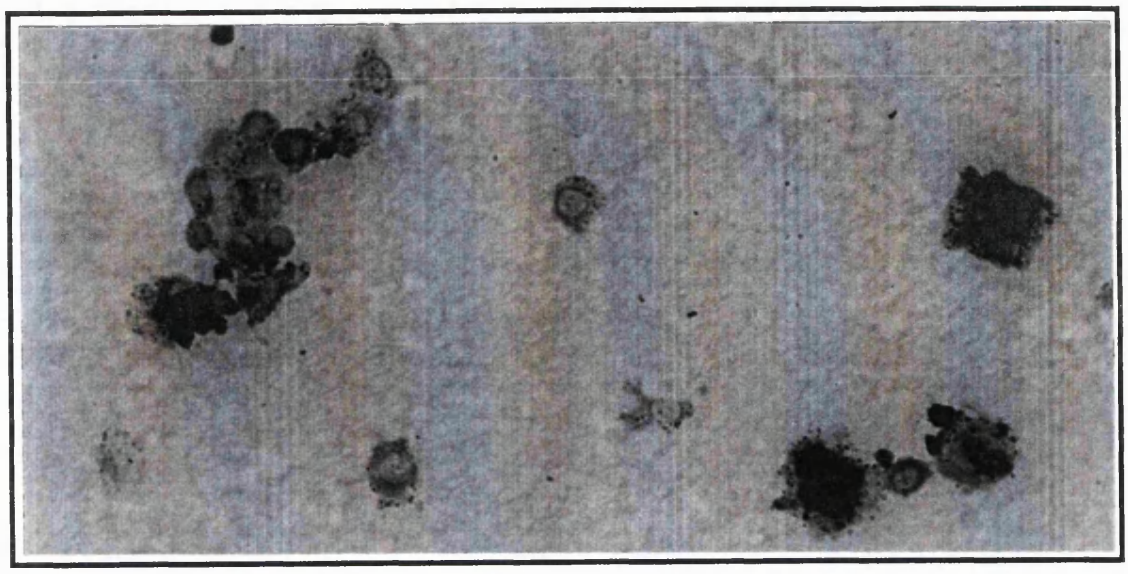


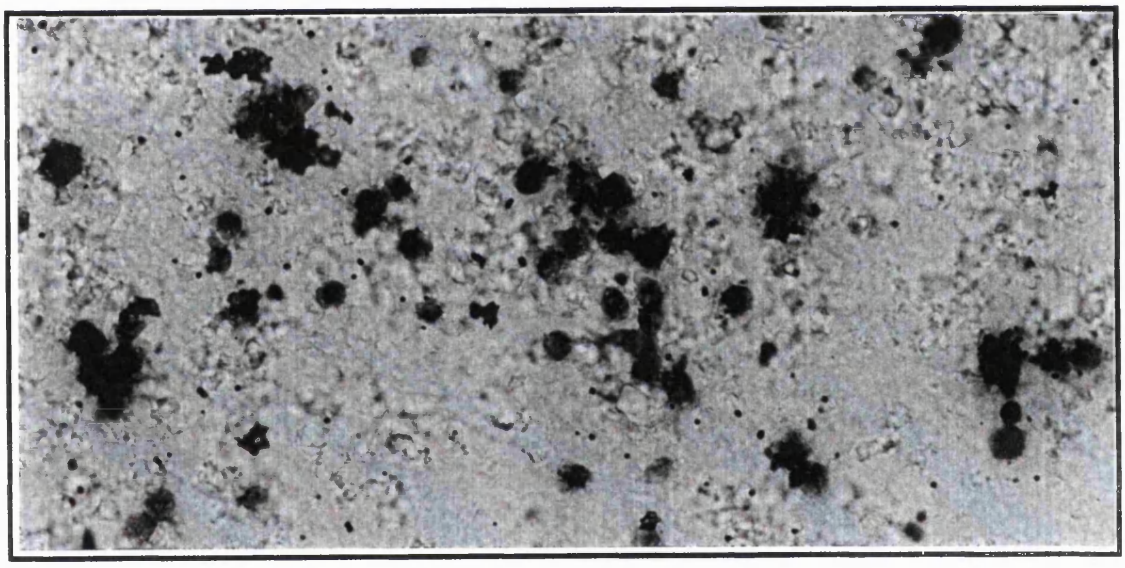
6.10

Figure 6.11: THP-1 cells on PHB after 5 days in culture stained with non specific esterase staining were rounded and some cells had clumped together although there were fewer cells adhering as compared to Thermanox (Magnification = 330x).

Figure 6.12: THP-1 cells on PHB-PHV after 5 days in culture stained with non specific esterase staining appeared small and irregular with very few cells adhering but the degree of staining was intense (Magnification = 330x).



6.11



6.12

Figures 6.13- 6.17 show the effect of increasing monomer concentrations on the activity of THP-1 cells measured using the MTS assay. By increasing the concentrations of NLA a decrease in the absorbance of the media for both time points was observed. Low NLA (figure 6.13) concentrations an increase in the absorbance can be seen at both time points, this, however stops at 0.111moles/L ., (10,000µg/ml) after which the cells show a decrease in absorbance as compared to the control. NNLA (figure 6.14) caused a dramatic decrease in the absorbance at both time points however at 0.011moles/L ., (1,000µg/ml) there was some recovery of the cells over the period of 0-48 hours. NGA (figure 6.15) as compared to NLA did not increase cell proliferation at low concentrations. A decrease in absorbance with increasing concentrations of the monomer at both time points was observed. At a concentration of 0.053moles/L , (4,000 µg/ml) there was a significant difference in the absorbance of the two time points and this may be due to the buffering ability of the medium and the ability of the cells to recover. NNGA (figure 6.16) at both time points killed the cells rapidly and above a concentration of 0.039moles/L ., (3,000 µg/ml) the cells were no longer viable. Cells cultured with D3-HB (figure 6.17) were able to survive much higher concentrations and cells were viable over both time points up to the final concentration of 0.288moles/L ., (30,000µg/ml) studied.

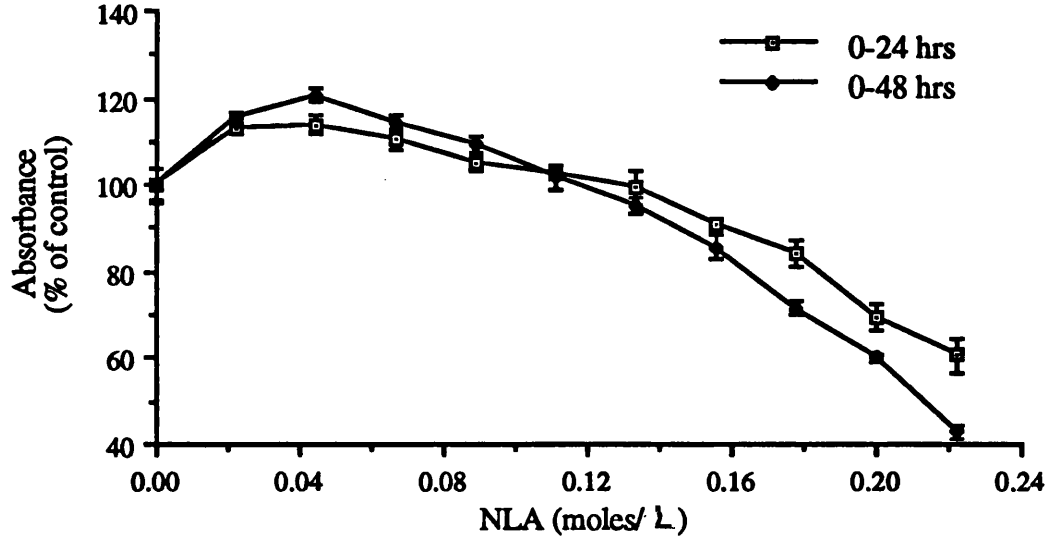


Figure 6.13: MTS conversion by THP-1 cells cultured with NLA. There was an initial increase in cellular activity at the lower concentrations with decrease in activity occurring with the addition of 0.111moles/L (10,000 µg/ml) of the neutralised monomer or above. The decrease in activity was greater if the cells had been exposed to the monomer for a longer time period. There were significant differences between the 0-24 hour and 0-48 hour exposure to the monomers ($p<0.05$).

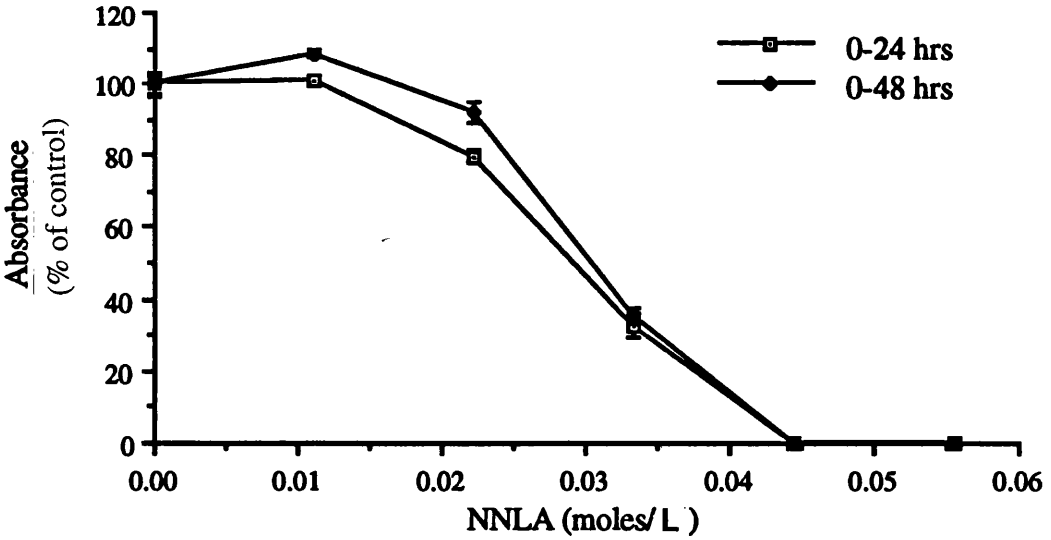


Figure 6.14: MTS conversion by THP-1 cells cultured with NNLA. There was a very slight increase in activity at the lowest concentration which decreased rapidly at the other three concentrations and cells were non viable at a concentration of 0.044 moles/L (4,000 µg/ml). No significant differences were observed between the 0-24 hour and 0-48 hour exposure to the monomer ($p<0.05$).

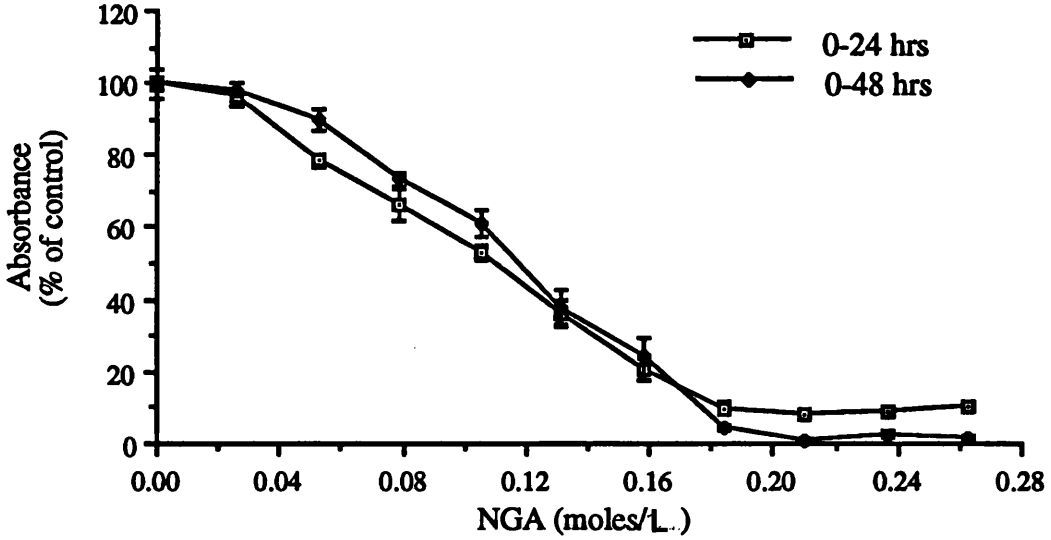


Figure 6.15: MTS conversion by THP-1 cells cultured with NGA. There was no increase in cellular activity at the lower concentrations and cellular activity continued to decrease with increasing monomer concentrations. At a concentration of 0.158 moles/L (12,000 µg/ml) the cells showed 0-10% activity compared to the control. No significant differences were observed between the 0-24 hour and 0-48 hour exposure ($p<0.05$).

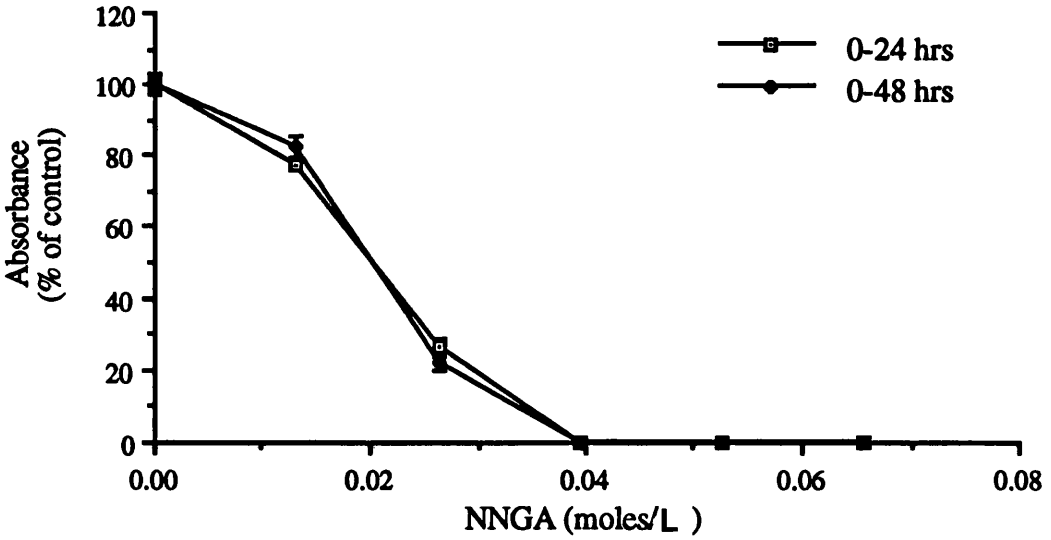


Figure 6.16: MTS conversion by THP-1 cells cultured with NNGA showed an immediate drop in cellular activity and cells were at 0% of the control at 0.039moles/L (3,000 µg/ml). No significant differences were observed at the 0-24 and 0-48 hour time points ($p<0.05$).

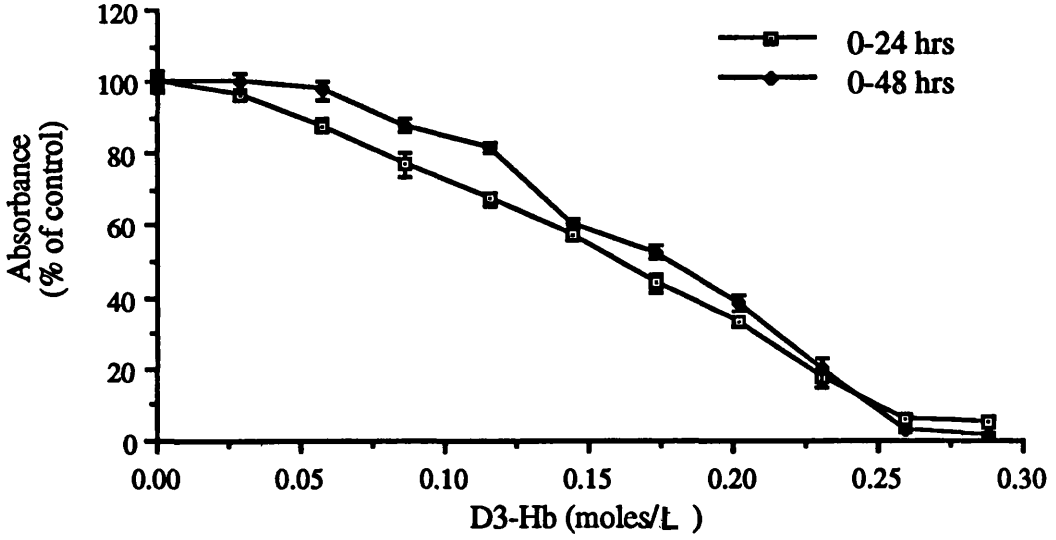


Figure 6.17: MTS conversion by THP-1 cells cultured with D3-hydroxybutyric acid. There was a decrease in cellular activity with increasing concentrations of the monomer and cells were 0-10% active of the control at 0.259 moles/L (27,000 µg/ml). There were significant differences observed between the 0-24 hour and 0-48 hour exposures at the lower concentrations with the 0-48 hour being more active at the lower concentrations ($p<0.05$).

Discussion

The first step for a successful host-foreign body interaction is dependent on good cell adherence and proliferation on the material surface (Gristina, 1987). Cytokines such as IL-1 produced in the early stages may influence tissue integration of the biomaterial and are known to affect biocompatibility. Soluble IL-1 may influence recruitment of other cells in the repair process, and membrane associated interleukin 1 (mIL-1) produced on the biomaterial may influence adherence and proliferation of cells adjacent to the biomaterial surface (Gristina, 1987). Krause *et al* (1990) measured the production of mIL-1 a cytokine found on the surface of macrophages activated by biomaterials. This cytokine may also act as a local stimulant to other cells adherent to the surface of the biomaterial. The production of mIL-1 was dependent on the structure and composition of the materials. The structure of cells was compared to determine the extent of activation. SEM of cells was used to examine morphologic alterations by cells. More mIL-1 was measured from activated macrophages and were characterised by cells having numerous pseudopodia and extensive cytoplasmic spreading. They concluded that the correlation of the structure of adherent cells with their functional activity may be useful in establishing the host response to specific biomaterials. Miller and Anderson (1988) also used *in-vitro* techniques to show that macrophages could be activated to produce significantly different quantities of soluble IL-1 which could be quantified in the supernatant. Kurt-Jones *et al* (1984) measured the production of mIL-1 on adherence of rodent peritoneal macrophages when activated after adherence to polystyrene.

Following implantation into a site there an immediate adsorption of blood proteins onto the biomaterial surface, followed by cellular interactions which lead to cell adhesion, proliferation and activation (Baier, 1969). Anderson *et al* (1990) investigated the effect of protein coated biomedical polymers on cytokine release from human monocytes and macrophages *in-vitro*. They concluded that the adsorption of proteins on biomedical polymers can significantly alter the activation of macrophages and the synthesis of IL-1 resulting in an altered biocompatibility of the polymer. As protein adsorption is a major factor in determining the biocompatibility of a material more tests need to be incorporated into the protocol which deal with this. This is particularly important as the surface of a biomaterial changes dramatically with adsorption of proteins so the start material in no longer the same after implantion or when placed into an *in-vitro* tissue culture system.

It is difficult to conclude from figure 6.1 whether the polymers were activating the cells as the assay cannot distinguish between those cells adhering to the polymer and those still in suspension. To determine if there was an increase in cell number on the polymer surfaces cell counts would be needed corroborated by visual data such as scanning or light micrographs. The MTS assay was used for testing the mitochondrial activity of

THP-1 cells on the polymers. This assay was used as it is more sensitive to suspension cells and differs from the MTT assay in that the formazan product is soluble. Work by Clifford and Downes (1996) showed the variation in the different assays tested and found the MTS assay to have poor reproducibility. More meaningful assays that could be used for macrophages for activation and proliferation would be direct cell counts, and assays that measured IL-1, PGE2, hydrogen peroxide and glucuronidase release. However the assay was used for testing the effect of monomers on the cells and proved to be very reproducible and this was possibly due to the fact that the effect of the monomers on the cells was more dramatic. There were significant differences between increasing concentrations and different monomers.

With colorimetric assays it is difficult to compare the results from two different experiments due to inter-assay differences observed in blank values and the control values which can sometimes be quite large. The assay is, however, useful in measuring the effect of degradation products and monomers on the viability of suspension cells. In this study THP-1 cells were used for all our activation studies as a monocyte/macrophage cell line. Although there are limitations with this cell line it has been shown to be a suitable model to study morphological and proliferation. These limitations are the inability to produce hydrogen peroxide and nitric oxide which can be used as a markers of activation.

The scanning electron micrographs show the difference in morphologies of the cells on the polymers with fusion of the cells occuring on some surfaces. Foreign body giant cell (FBGC) formation mechanisms are not clearly understood but work in the literature shows that they can be formed from macrophage fusion (Sutton and Weiss, 1966; Abe *et al* 1987). Kao *et al* (1994) state that macrophage adhesion, activation and FBGC formation and other stages of the inflammatory response could be influenced by the physical and chemical properties of the polymers they were in contact with. The extent of macrophage and multinucleated giant cell involvement in tissue surrounding the implant is dependent on the number and size of particles present and on the rate and clerance of these particles (Howie *et al* 1993).

Other markers of activation and inflammation can also be measured such as nitric oxide (NO) which is a free radical gas, and in small concentrations acts as a physiological and pathophysiological mediator (Moilanen and Vapaatalo, 1995). TNF and IL-1 have also been demonstrated to be released by human monocytes in response to biomaterials (Cardona *et al* 1992). NO has various roles in the inflammatory and immune responses. It has a cytotoxic action against microorganisms and when produced in "large" amounts can suppress lymphocyte proliferation (Moncada *et al* 1991). NO production in this study was measured but was not detected and this was due to either small amounts of NO

produced which were undetectable or THP-1 cells were not able to produce NO. Other parameters can also be measured for example Lundberg *et al* (1995) measured the relese of leukotriene B_4 (LTB_4) in response to expanded ePTFE and polyglactin 910. This was measured to analyse the chemoattractant ability of LTB_4 on leucocytes 24 hour and 6 day post surgery. Leucocyte numbers increased for both materials with time whereas LTB_4 content decreased with time. The numbers of leucocytes were significantly higher but the LTB_4 significantly lower with PG 910 compared with ePTFE. It was concluded that both non-degradable and degradable polymers were able to elicit activation of inflammatory cells following implantation. Other factors can also be measured for example, Horowitz and Gonzales (1997) investigated the effect of polyethylene on macrophages *in-vitro* and found that exposure led to the release of TNF-α and prostaglandin E_2.

As well as NO, superoxide release from neutrophils has been measured as a marker of cell activation (Kaplan *et al* 1992). In a further study Kaplan *et al* (1994) stated that activation of cells in direct contact with a biomaterial was different to cells in suspension thus the same conclusions about the activation mechanisms could not be drawn for different test conditions.

The THP-1 cell line used in this study has been used by other groups for example as a model to measure the response to cobalt-chromium corrosion products *in-vitro* (Lee *et al* (1997); but for our studies production of cytokines and activation products could not be detected. Further study should therefore utilise other monocyte/macrophage cell lines as well as other cell types involved in the immune response such as neutrophils to measure the response to biomaterials. For example Mora *et al* (1990) measured the ability of several prosthetic materials for their ability to activate human neutrophils. Differences in the different materials were found where the materials had a direct inhibitory effect on the neutrophils.

There is a large amount of work published on non-degradable materials and the immune response due to their role in aseptic loosening. There is however relatively small amount work published with bioresorbable materials and cells of the immune system *in-vivo* and *in-vitro*. Some work that is available is discussed in context to the work carried out in this chapter. The effect of the monomers on the cell type was dramatic however it is unclear if this is due to the pH alone or the monomers in combination with the pH. Work by Dawes and Rushton (1994) investigated the effect of LA on PGE_2 production, a bone resorbing mediator, by macrophages and fibroblasts. Cell viability, number and LDH assays were used as a measure of the cellular response. It was concluded that the presence of the lactate molecule and not just the osmolality change caused by the Sodium Hydroxide used to neutralise the monomers had an effect on cell viability which was in agreement with the results in this study.

CHAPTER VII

General Discussion and Future Work

Bioresorbable materials are becoming increasingly important in many areas of medical and scientific research, with new applications including reconstructive surgery, drug delivery (Miyamoto *et al* 1990), nerve regeneration (Pergo *et al* 1995), embolic materials to stop bleeding post-operatively (Grandfils *et al* 1992) and as intravascular stents (Agrawal *et al* 1992). This thesis has investigated the biocompatibility of various resorbable biomaterials some of which have not yet been approved for clinical use. The assessment of biocompatibility and biodegradation of a range of degradable polymers has been performed using both osteoblasts and macrophages. A variety of *in-vitro* techniques have been applied with the aim of developing methods to predict the long-term biocompatibility of the materials. The problems encountered in comparing two different cell types to test the same material have been illustrated by several groups (Jansen *et al* 1991; Hunter *et al* 1995 and Al Nazhan *et al* 1990). In this study, macrophage cell cultures were used to evaluate the behaviour of the phagocytic cells of the immune system with the materials. Whereas, osteoblasts were used to predict the bone conducive ability of materials.

Imaging cells on polymers can be very useful in biocompatibility screening but there are various problems encountered with the processing techniques involved. For example the instability of the polymers in the various solvents used in "normal" processing were overcome using newly available techniques. The polymers could not undergo critical point drying for SEM, and therefore air drying was used, this resulted in poor cell preservation, HMDS was therefore used which gave results comparable to CP drying. Another problem encountered was during tissue culture when the polymer films tended to float. As no glue could be used due to additional factors influencing biocompatibility the polymers were first hydrated for 24 hours and thus they were able to stay at the bottom of the 24 well plates. Crystallinity of the polymer films gave rise to various surface structures which in turn influenced the cell adherence and proliferation.

The major problem associated with biocompatibility testing of polymers is the long time periods associated with their degradation. As a result of this the *in-vivo* results are slow to emerge and incorrect conclusions are drawn regarding the biocompatibility of the polymers. For example, Cordewener *et al* (1996) used Poly(L-Lactide) implants for the repair of human orbital floor defects where six patients were followed for up to 3.5 to 6.5 years. The implants used were block polymerised PLLA, 30 mm in diameter and 0.4mm in thickness with a Mv of 9.5×10^5. The follow-up examinations consisted of an interview and visual inspection of the patients. None of the patients reported any problems associated with infection, migrations or extrusion of the implants or pain. The patients showed no symptoms of abnormal or increased tissue reaction and concluded that the implants were biocompatible but the slow degradation rate of PLLA was unfavourable. Further studies were conducted to modify and increase the degradation rates. However

this does not tackle the problem caused by the rapid degradation of the implant leading to severe inflammatory responses which are listed and discussed in the table in the appendix. To tackle the problems with long term degradation more studies are being carried out which use polymers or polymer degradation products that have being aged *in-vitro* prior to implanation into animal models (Gibbons et al 1994; Mainil-Varlet *et al* 1997).

GPC was used to assess the molecular weight changes of polymers following exposure to cell cultures. However, due to the long degradation periods of these polymers long term degradation studies involving cells could not be carried out extensively. It would be desirable to have polymers that could be degraded to various molecular weights, cast into films and then exposed to *in-vitro* tests for up to 4 weeks. PLA which had been degraded to a nominal Mw of 5,000 was used for *in-vitro* tests and cast into films. The film however, was very crystalline and tended to break up and disks could not be cut succesfully. The solution was therefore cast directly onto Thermanox and placed into tissue culture after careful washing in alcohol. The cells grew on the polymers surfaces that were forming spherulites and the polymers appeared to be supporting cell growth very well. Future work should determine whether the cells would be biocompatible if the polymer started releasing monomer and large quantities of degradation products. It is possible that with small films the amounts of degradation products and monomers released from the films could be buffered by the cell culture medium.

From the *in-vitro* experiments, culturing cells directly on polymer films, it was clear that all the polymers were biocompatible. This was observed for all the polymer films although the amount of cell adherence and proliferation varied for each. Due to the length of time required to optimise the GPC equipment insufficient time remained for biocompatibility testing of pre-degraded polymers. Some tests were however carried out which gave some prediction in the long term biocompatibility of some of the polymers tested. PGA for example was cultured *in-vitro* with HOS cells for a period of 21 days and in this time the material started to degrade and release acidic degradation products. This was visible by eye as the indicator in the culture media turned yellow. The cells on the surface were examined by SEM at 48 hours, 7, 12 and 21 days but after 7 days the materials had started to crack. As GPC analysis of PGA was not possible due to its insolubility in any of the solvents tested it is not possible to comment on the Mw changes occurring with this polymer. The pH drop caused by the release of the monomers was sufficient however, even after 12 days, to start causing cell death. Cell death may also be attributed to the physical changes occurring to the polymer itself during degradation.

The shape and size of degrading surfaces as well as the degradation products being released may influence the adherence and proliferation of cells on the surfaces. The

degradation of the polymers tested and release of degradation products in the different solutions was variable. The surfaces obtained upon incubation with the solutions also varied and this would influence the adherence and proliferation of cells at any stage during degradation. Anderson *et al* (1990) concluded that for blood contact materials the material surface was important in determining the biocompatibility. Thus it is very likely that cells would change their behaviour in terms of morphology during polymer degradation.

In this study the effect of free monomers on cell behaviour was extensively investigated. The concentrations of neutralised and non-neutralised monomers added to cells in culture caused a decrease in the viability of both HOS and THP-1 cells. Whether this was at a range of concentrations encountered *in-vivo* is not known as there are at present no indication on concentrations that may be encountered *in-vivo*. If the monomer concentrations occurring around implants could be determined tests could be devised which would deal with the correct pH range and concentrations. Changes caused to the environment both *in-vitro* and *in-vivo* surrounding degradable polymers were investigated by Martin *et al* (1996). They used a micro pH device which was able to measure subtle changes in pH around degrading films and found no damage was caused to the cells around the site although the drop in pH was significant. This pH drop however, was quite small and in this study it was found that a dramatic drop in pH occurs even with relatively small concentrations of monomer. The drawbacks however were that the polymer films used were too small to represent changes in pH that would be occurring with larger devices. With certain modifications however it would be possible to make an electrode to measure changes in pH around larger samples or devices. This would enable tests to be devised which would be more representative of the *in-vivo* situation.

Several conclusions have been drawn from this study. The major disadvantage of current resorbable materials is that they either degrade too quickly or too slowly. This limits their use for various applications, thus various copolymers and blends of certain polymers have future promise once optimised. Many degradable polymers hydrolyse to acidic degradation products. This has caused many problems in the *in-vitro* and *in-vivo* biocompatibility testing of these polymers. A literature review is listed in the appendix summarising the *in-vivo* response and the results from clinical implants. In the majority of cases degradable polymers have caused an inflammatory reaction in the site of implantation and further biocompatibility testing is required prior to clinical applications. The results from chapter two have shown that it is possible to achieve controllable methods resulting in meaningful data to address the problem of long term degradation. With a combination of accelerated degradation by temperature or enzymes quantitative *in-vitro* tests can be developed which will give useful information of the biocompatibiliy of a polymer as it is degrading.

Ashammakhi and Rokkanen (1997) in a review stated that PGA had been routinely used in their hospital for the internal fixation of bone fractures. It had proved to be biocompatible in 1.7 percent of human cases and although sinus formation had developed in some of the cases it did not disturb the healing. They also concluded that PGA/PLA *in-vitro* as compared to other polymers used by Elgendy *et al* (1993)were found to have the highest cell adhesion and attachment thus confirming the biocompatibility of these polymers *in-vitro*. The study did yield good information on the short term biocompatibility of the cells, however, the experimental period was seven days and it is unlikely that the polymer would have undergone substantial degradation but this is difficult to determine as the molecular weight of the polymer was not stated. They found PLA-PGA copolymer to have the highest cells attachment which is in agreement with results discussed in chapters three and four; cells on PLA and PGA adhered and proliferated very well over a short time period. The PGA started to degrade after 7 days therefore it was easy to determine the effect of the degradation products on the cells. It is hypothesised that as PLA is similar to polyglycolic acid as observed from the cytotoxicity testing of the monomers it is likely that once PLA starts to degrade the degradation products would have an detrimental effect on cells cultured on the surface or cells that may be present in contact with the degradation products. The effect may not be as drastic as the degradation products of PGA but this would depend on the buffering ability of the medium or the surrounding environment.

Protein adsorption plays a major role in cellular adhesion and activation of cells on polymers surfaces. The type of proteins that may adhere to a surface are determined by the physical and chemical properties of a polymers surface. In this study cells were cultured in medium supplemented with foetal calf serum, which contains numerous proteins, some as yet, unidentified. Further work has been carried out on protein pre-coating and its effect on cell adhesion and spreading, this has concentrated on non-degradable materials (Schakenraad *et al* 1989; Truskey and Proulx 1993). Future work involving degradable polymers should include protein adsorption studies and their effect on biocompatibility. Recent work has shown the pH has an effect on protein adsorption (Sharpe et al 1997); thus further work should include the effect of the changing pH caused by the degrading imlant on protein adsorption also.

Superoxide degradation of polymers has been extensively investigated and has been implicated in the degradation of various polymers. Superoxides are considered as one of the causes of material degradation because they are released from cells involved in the immune response after implantation. Macrophages and neutrophils when activated and phagocytic, release many enzymes as well as hydrogen peroxide, NO and hydroxyl radicals. The release of NO was also briefly discussed in chapter six. As NO is a free

radical it has been documented to cause damage to cartilage (Murrell *et al* 1996) during infection and inflammation and this same mechanism could cause polymer degradation during activation of macrophages. Thus it is important to investigate NO and hydrogen peroxide release and their role in accelerated degradation of polymers and to determine the extent of their effect.

It has been demonstrated in this study that methods can be used to obtain polymers that have been artificially aged and methods have been developed which can measure the extent of degradation. This ageing process affects the polymer surfaces as well as the bulk polymer and results in the accelerated release of degradation products Grizzi *et al* (1995). The methods included GPC, SEM and ELISA for the measurement of monomers. Cell growth which occurred on all the polymer surfaces was illustrated by using SEM and TEM. The biochemical results proved the importance of having reproducible polymer samples; that is with the same surface structure, molecular weight, surface finish, crystallinity, size and shape. The influence of surface structure in the cell response was demonstrated in a study by (Gomi and Davies (1993). A reproducible surface was difficult to obtain from casting the films in a laboratory although the conditions were attempted to be kept constant. This difference in polymer batches may have led to the variations seen the DNA, ^{3}H-thymidine incorporation and proliferation.

The release of monomers from the polymers during degradation led to an acidic environment which caused cell death in both HOS and THP-1 cells. The method of cell death was investigated for both apoptosis or necrosis. Cells were found to undergo apoptotic cell death in the presence of neutralised monomers of NGA and D3-HB at the concentrations tested. It is important to determine whether this occurs in the *in-vivo* situation as the two mechanisms are very different and the process of apoptotic cell death may be preventable using survival factors. It also suggests that the effect of the neutralised monomers on the cells was a slow process rather than the sudden process which occurred with the non-neutralised monomers where the cells showed no signs of apoptosis.

The choice of material will determine the rigidity, degradation and biocompatibility *in-vivo* and *in-vitro*. When a particular material is "developed" for a specific application firstly its intended use needs to be determined followed by the production of reproducible and representitive samples of the final device. Primary cells or cell lines then need to be selected preferably of human origin which will be in contact with the material in the *in-vivo* situation. Then appropriate tests need to be determined and optimised. For example if macrophages are to be used it is necessary to determine if they produce markers of activation or release the factor that is going to be measured without external stimuli. For osteoblasts it is important to determine that they release osteopontin, osteonectin and

alkaline phosphatase as markers of bone cell phenotype if the material being developed is to be used for bone induction.

The biocompatibility of materials in the long term needs to be determined and *in-vitro* tests need to be accurate if they are to replace *in-vivo* tests. Currently, there is too much uncertainty about the degradation products, the buffering capacity of the area around the implant site and whether cells *in-vitro* possess the same levels of resistance as cells *in-vivo*. To avoid these discrepencies in results we need to develop methods which will measure the pH around a degrading implant *in-vivo* and the concentrations of degradation products that are released. The time taken for such levels to accumulate when they start to be released and how quickly they are cleared needs to be determined in order for comparable tests to be designed. In addition standardisation of methods needs to occur such as cell numbers, seeding densities and the initial characteristics of the test materials needs to be clearly evaluated.

For biocompatibility testing of materials cell culture systems need to be developed and optimised prior to clinical use. Mainil-Varlet *et al* (1997) studied the long-term *in-vivo* degradation of various polylactides over a period of one year. They found the polymers to be biocompatible however, this is where the problem arises with most tests in the literature carried out so far. The work in this thesis has demonstrated that the polymers are slow to degrade and are biocompatible *in-vitro,* in the early stages therefore, for accurate results to be obtained testing needs to be carried out at the later stages of degradation. In this area *in-vitro* tests can prove very usefull as polymers can be artificially degraded before cell culture or implantation into an animal model. In this study the use of simple, reliable quantitative methods employing cell culture and other *in-vitro* techniques were developed which would allow the screening for cytotoxicity of materials as well as their degradation products. This type of approach allows the study of the biological response and mechanism of action of various materials during tissue material interaction as well as cytotoxicity to be evaluated.

Biomaterial-cell interactions are influenced by the geometry and surfaces charges of the biomaterials as well as cell surface receptors and membrane molecules on the cells. Modifying biomaterial surfaces at the developmental stage will lead to a better understanding of cell-material interactions and lead to the development of specific polymers designed for a particular use. Degradable materials have a lot more to offer and by using qualitative and quantitative methods which have been selected and validated in a controlled manner more information can be obtained on currently available polymers and their suitability for specific applications.

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APPENDIX

Appendix 1

AUTHOR	MATERIAL/ DEVICE	TEST PERFORMED	CONCLUSIONS
Majola *et al* (1989)	SR-PLLA and PDLLA	Implanted into cancellous bone of 56 rabbits and followed up for 48 weeks.	Adsorption of PDLLA/PLLA faster than PLLA but implants were still visible after 48 weeks. Concluded that there was sufficient mechanical strength for the fixation of osteotomies.
Santavirta et al (1990)	PGA implants	Immune response to PGA implants measured	PGA found to be immunologically inert implant materials although it did induce slight non-specific lympocyte activation. However this study was only carried out over 5 days.
Vasenius *et al* (1990)	PGA rods	Degradation *in-vivo* and *in-vitro* measured.	*In-vivo* reduction of strength was faster than *in-vitro*.
Bostman *et al* (1991)	PGA rods	67 patients treated with ankle fractures and followed up for 2-4 months after implantation.	17 patients had sudden painful swelling around implant site and discharged fluid which had remnants of degrading implant. The duration of the discharge was 4 weeks. There was a non specific foreign body reaction and osteolytic areas were seen upon degradation of the implant. Concluded that currently degradable implants could not replace conventional implants.
Bostman *et al* (1991)	PGA Screws	Implanted into the cancellous bone of rabbits for 36 weeks.	The tissue response to the implant and replacement of the implant with bone was variable. Occasionally large defects were observed.
Matsusue *et al* (1991)	PLLA compared to stainless steel	Implanted into 25 rabbits and followed up for 16 weeks.	Concluded PLLA could possibly be used for clinical treatment of human fractures.
Suuronen (1991)	SR-PLLA screws	Fixation of 9 osteotomies in sheep carried out and compared to metallic screws. follow up of 6-24 weeks.	Consolidation faster in PLA group that in the metallic group.
Bos *et al* (1992)	PLLA with Mv of ($9x10^5$) used.	Implanted subcuatneously into rats.	Within 3 months there was a rapid decrease in Mw but even after 143 weeks PLLA had not resorbed completely.
Bostman *et al* (1992)	PGA screws	24 out of 216 patients were observed 3 months after malleolar fractures due to the development of a non-specific foreign-body reaction.	Absorbable implants cannot be used as an established method of internal fixation. The immune response found is unique to degradable polyester implants although the failures due to bacterial infection and other common problems seem to be the same for metal and absorbable implants.

Eppley (1992)	PLA/PGA rods	Craniofacial fixation of 14 rabbits	Initial study suggests that large plate size and slow resorption have similar restriction in bone growth as metal fixation. Whether thinner plates or rapidly degrading polymers will allow normal growth is being investigated.
Kumta *et al* (1992)	PGA rods and kirschner wires	Intramedullary and subcutaneous.implantation into rabbits	After six months no difference in the two groups noted.
Laitinen *et al* (1992)	PLLA fibres	Immersed in PBS at 37°C and implanted subcutaneously into rabbits	PLLA degradation was found to be faster *in-vivo* than *in-vitro*
Majola *et al* (1992)	SR-PLLA and PDLLA	Implanted subcuatneously into rabbits and followed up for 36 weeks.	PDLA implants lost their bending strength faster than PLLA.
Makela *et al* (1992)	SR-PGA pins	Used for the fixation of distal humerus fractures in children. Mean follow up time of 17.2 months with the longest of 26 months.	Results were satisfactory and work concluded that biodegradable implants had established themselves as a procedure of choice for simple physeal fracture fixation.
Manninen *et al* (1992)	SR-PGA and SR-PLLA rods	Implanted into the distal femur of 42 rabbits and followed up of for 6-12 weeks.	No definite conclusions drawn except sight foreign body reaction and infiltration of macrophages observed.
Matsusue *et al* (1992)	PLLA	Degradation measured *in-vivo* and *in-vitro* with a follow up time of 78 weeks.	No inflammation was observed and no differences in vivo and *in-vitro* were observed.
Suuronen *et al* (1992)	SR-PLLA screws	For the fixation of sagittal split osteotomies in 6 sheep, followed up for 16 weeks.	Concluded that healing of osteotomies was good and PLLA would be suitable for fracture fixation in humans.
Suuronen *et al* (1992)	SR-PLLA	18 transverse osteotomies in sheep carried out. 9 with PLLA and 9 with metallic with 24 week follow up.	With both boney union occurred with callus formation.
Suuronen *et al* (1992)	SR-PLLA	PLLA studied for tensile strengths *in-vivo* and *in-vitro.*	Loss of strength faster *in-vivo* than *in-vitro.*
Partio *et al* (1992)	SR-PGA and SR-PLLA screws.	11 patients with 12 ankle joint fixations. Follow up time of 7-22 months.	Union occurred in 11 out of 12 cases and the fusion was fibrous. Generally concluded that the main benefit was the length of leg could be maintained and re-operation was avoided.
Bergsma *et al* (1993)	PLA bone plates and screws	Fixing of unstable zygomatic fractures in 10 human patients. The test was followed up 3 years postoperatively.	After 3 years 4 patients returned with swelling at the site of implantation. The remaining were recalled and re-operated on to investigate the nature of swelling. Intracellular and extracellular degradation slow. Crystal like PLLA internalised by various cells. There was a foreign body reaction around site. "Because of the clinical manifestation of the foreign body reaction " PLLA must be examined in more detail before it can be used.

Bostman *et al* (1993)	SR-PGA pins	Internal fixation of fractures using PGA pins 1.5-2.0 mm in diameter.	Mechanical reliability of PGA pins was satisfactory in all fractures except in supracondylar fractures of the humerus. Greater no of treatments will determine if the incidence of a non specific inflammatory reactions occurring in two patients is a problem which will occur frequently.
Gerlach (1993)	PLLA of different molecular weights	Used for the fixation of maxillofacial defects in animal model and as clinical pilot study.	Found good biocompatibility with slight tissue response. 2 out of the 15 patients had a late foreign body reaction and concluded that long term follow up was needed. The swelling around implant after 30 months had PLA remnants with Mw which ranged from 500-10,000.
Gogolewski *et al* (1993)	PLA, PHB, PHB/PHV	Implanted into mice subcutaniously and follow up time of 1, 3, 6 months.	Tolerated well by tissue. Number of inflammatory cells increased with increasing content of PHV. Variability in degradation rate maybe due to variable crystalline and amorphous regions within the sample.
Hirvensalo *et al* (1993)	SR-PGA pins	SR-PGA pins used for the fixation of the humeral capitellum.	The follow up was one year and out of 8 patients one developed a sterile synovitis which was drained without further complication.
Laitinen *et al* (1993)	Braided PLLA	Tested in sheep for anterior cruciate ligament (ACL) reconstruction and followed up for 48 weeks.	Degradation was not complete by 48 weeks but concluded that PLLA was a suitable material for ACL reconstruction.
Lam *et al* (1993)	PLA particles		Cell damage sometimes leading to cell death by phagocytosed PLA particles. Phagocytosis of PLA fragments on cellular morphology and viability. Injected in mice and found that cells in the peritoneal cavity showed signs of cell lysis, damage and death. This could be due to phagocytosis of large amounts of PLA particles.
Mannienen and Pohjonen (1993)	SR-PLLA rods	Used for fixation of cortical bone osteotomies and followed up for 24 weeks.	Concluded that PLLA rods were strong enough to be used in intramedullary nailing.
Niskenen *et al* (1993)	SR-PLLA rods or Kirschner wires	39 patients for fixation of the metatarso-phalangeal joint in rheumatoid arthritis.	The degradable implants performed similarly to kirschner wires.
Suganuma & Alexander (1993)	PLA Intramedullary bone implantation	Tested effect of PLA crystals on biocompatibility.	Large PLA particles did not influence the response of bone to PLA but small particles < 2μm particles induced a foreign body reaction and bone resorption. pH decrease also influenced cell kinetics.

Vasenius *et al* (1993)	SR-PLLA pins	Used to fix subcapital femoral osteotomies in nine sheep.	At 6 weeks 1 out of 3 had failed but by 12 weeks all the osteotomies had healed and it was concluded that the pins could be used to fix this type of osteotomy.
Verheyen *et al* (1993)	PLLA and PLLA composites as plugs	Transcortical implantation of the polymers into the femora of one goat.	Concluded that crystalline degradation products of the polymer could limit its application.
Winet and Hollinger (1993)	PLA-PGA copolymer	Implanted into a cortical defect into rabbit tibias.	Concluded that copolymer synthesis had to result in a predictable structure before its incorporability in a given reconstruction or fixation site could be predicted.
Bucholz *et al* (1994)	PLA and metallic screws compared	Fixation of displaced malleolar fractures in 155 patients. Follow up of 37 months.	PLA screws were found to be safe and effective although the study did not say if the implants had completely degraded in the study period.
Lautiainen *et al* (1994)	SR-PGA implant (1.1 mm diam and 35 mm length)	Implanted into a transphyseal channel drilled up to the diaphysis of the right femur in 29 rats.	Implants were broken at the plane of the growth plate due to the growth pressure exceeding the tensile load carrying capacity of the implant. Concluded that small-calliper SR-PGA rods were suitable mainly in the fixation of rapidly healing fractures due to the rapid loss in mechanical strength of PGA.
Pelto *et al* (1994)	PGA pins used for radial head fixation.	Follow up on 38 patients of 27 months. Results comparable to metallic devices.	Use of PGA appears feasible and devices do not need to be removed.
Philajamaki *et al* (1994)	PLLA expansion plug.	PLLA expansion plug for the fixation of recurrent anterior dislocation of the gleno-humeral joint. Mean follow up was 18 months.	No inflammatory reactions found and the degradable expansion plug promising for this type of fixation as compared to metal screws.
Rozema *et al* (1994)	Predegraded PLLA particles (<500μm) and implanted into rats subcutaniously	High Mw PLLA used for orbital floor reconstruction in animals and humans	GPC showed particle Mn to be 5500. Fibrous capsule formed around PLLA particles after 2 weeks
Suuronen *et al* (1994)	SR-PGA screws	Shortest follow up of 15 months and longest 23 months.	No clinical complication were note thus far.
Ashammakhi *et al* (1995)	SR-PGA	Tested the polymer *in-vivo* and *in-vitro* for strength retention.	This was thought to be due to the formation of a biomembrane around the implant.

Bergsma *et al* (1995)	PLA particles	*in-vivo* degradation of predegraded PLA particles.	Conclude that PLLA resorption is slow, approximately 5.6 years. The predegraded PLLA induced a mild foreign body reaction and copolymerised PLLA with D-Lactide was better.
Bergsma *et al* 1995)	Predegraded and non predegraded PLA	Implanted polymers after *in-vitro* degradation to measure response.	In vitro in combination with *in-vivo* is a good method to simulate long term degradation.
Hanafusa *et al* (1995)	PLA	Plate fixation, 8, 25, 40 weeks test period. A thin layer of fibrous tissue observed at the interface between bone and plate.	There have been no reports of successful use of PLLA for fixation of long bone fracture under load bearing conditions. Need a greater initial mechanical strength and surface coating which will prevent infiltration of fluids and thus cracking of the implant.
Juutilanen *et al* (1995)	SR-PLLA , SR-PGA screws and wires compared to metal implants.	Olecranon and patella fractures fixed. Follow up time from 1.4 to 1.8 years.	No differences were found between the degradable and the metallic groups. Concluded that biodegradable implants were suitable for treating these fractures as rather than metal wires.
Kankare *et al* (1995)	SR-PGA rods	Treatment of alcoholics with PGA rods as compared with metallic implants.	Significantly high failure rate in the PGA group occurred and this was put down as a failure of the patients to co-operate leading to the implants breaking.
Van der Elst *et al* (1995)	PLLA versus stainless steel nails	Interlocking nails used for fracture fixation in the femoral bone of young pigs and followed up for 3 months.	Concluded that no differences in the tissue reaction were observed between the two materials although mechanical properties deteriorated during implantation and chemical properties changed significantly.
Vasenius *et al* (1995)	SR-PGA pin or Herbert screw	Internal fixation carried out in 34 patients. Average follow up of 68 months in PGA group and 58 months in Herbert group.	A non specific tissue reaction occurred in 25 % of the PGA group. PGA group unsatisfactory thus use in delayed union and ununited scaphoid fractures not recommended.
Bergsma *et al* (1996)	PLLA and PDLA copolymer	Pre-degraded polymers implanted into rabbit tibia and followed up for 26 weeks.	High rate of fragmentation and internalisation of the particles by macrophages.
Cordewener *et al* (1996)	PLLA implants	Used for the treatment of orbital floor fractures. Patients followed up over a period of 3.5 - 6.5 years.	No problems were reported by patients and MRIs showed no abnormalities in the orbital region. However due to the slow degradation of PLLA it was unsure if late degradation resulting in highly crystalline PLLA particles would effect the surrounding area. Thus studies were being conducting towards the possibility of accelerating the degradation rates of PLLA.
Heino *et al* (1996)	PLLA thread	Used for the fascial closures of rats and followed up for 52 weeks.	PLA thread was found to be suitable for wounds requiring healing times of less than 28 weeks.

Rokkanen *et al* (1996)	Review of cases from 2500 patients	Fixation of bone or ligament using degradable polymer devices. Course uneventful in 90 % of the cases. In 3.6 % bacterial wound infection seen and in 3.7 % failure of fixation.	Non specific foreign body reaction observed in 2.3% of patients with PGA but not PLA implants. 1000 reoperations avoided
Sinisaari *et al* (1996)	Review of patients following fixation with metal and resorbable implants.	Infection rates similar for both groups with 2073 metal, and 1012 with absorbable implants.	Concluded that implant material did not have an affect on infection rates.
Van Der Elst *et al* (1996)	PLLA powder	Powder placed into medullary cavity of rabbit femur.	No clinical inflammation could be detected during the 8 week follow-up period although histological analysis showed a mild inflammatory tissue response.
Sahli *et al* (1997)	PLA and PLA-PEO nano particles	Interaction of the polymers with plasma factors of the coagulation system measured.	PLA particles interact with thrombin, factor V, calcium ions however PLA-PEO were inert.

Journal of Materials Science: Materials in Medicine

CHAPMAN & HALL

JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 784–787

A qualitative *in vitro* evaluation of the degradable materials poly(caprolactone), poly(hydroxybutyrate) and a poly(hydroxybutyrate)-(hydroxyvalerate) copolymer

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A qualitative *in vitro* evaluation of poly(caprolactone) (PCL), poly(hydroxybutyrate) (PHB) and a poly(hydroxybutyrate)-(hydroxyvalerate) (PHB-PHV) copolymer was carried out using primary human osteoblasts (HOB) and a human osteosarcoma (HOS) cell line. The cells were grown on films of these polymers and cultured for 2 and 4 days with cells grown on Thermanox as a control. The cells on each of the polymers exhibited different cellular morphologies with different rates of cell proliferation. Results from a preliminary degradation study demonstrated that biodegradable materials can be partially degraded using enzymes such as papain and trypsin. Of the solutions tested, papain caused the greatest degradation, with phosphate-buffered saline (PBS) a physiological buffer having very little effect over a six week period. The bone cells were grown on partially degraded polymers and no differences in the performance of HOS and HOB cells on the materials were observed.

1. Introduction

There has been increasing interest in degradable polymer systems for use in biomedical applications such as drug delivery [1–6], fracture repair [7–10], bone and cartilage remodelling [11–14] and soft tissue implants [15]. Degradable materials have certain advantages that make them desirable for orthopaedic use. Their degradation rates and tensile strengths can be controlled by varying their molecular weights [16–18] and, for copolymers, varying the ratio of the components can also dramatically affect their degradation rates [16, 18, 19].

These materials have been poorly characterized using *in vitro* methods which have simply involved the assessment of fibroblast and some osteoblast and hepatocyte growth on them [20–25]. There has been considerable work done with degradable polymers *in vivo* [7–14] but the mechanisms of cell attachment and proliferation on these polymers has not been investigated. Moreover, the effect of the cellular activity on the degradation of the polymer and the effect of the degradation products on the cells are not well understood.

The effect of degradative enzymes on PHB and PCL was studied to gain information on the degradation characteristics of the materials in order to develop methods to artificially degrade the polymers prior to cell culture. The cellular response of primary human osteoblasts and a human osteosarcoma cell line cultured on the polymers was investigated to produce a system where a polymer can be partially degraded in order to study the effect of the degradation products on the cells and the effect of surface changes caused by the degrading polymer on cell adherence and proliferation.

2. Materials and methods

2.1. Polymer formulation

A 3% solution of PHB (ICI) and PHB–PHV (ICI, PHV content 7%) was made in chloroform and dissolved by refluxing at 70 °C for 4 h. A 7% solution of polycaprolactone (Aldrich) was dissolved in chloroform at 37 °C and did not require refluxing. The solutions were cast on to glass slides and dried under glass petri dishes overnight. These were further allowed to stand in an open container for 7–10 days to allow the chloroform to evaporate.

2.2. Degradation studies

The dried polymers were cut into 1 cm × 1 cm squares and incubated in the following solutions at 37 °C: PBS (PBS, Oxoid); trypsin (Sigma, 0.2% solution in PBS) and papain (Sigma, 0.2% in PBS). The films were exposed to constant roller mixing at 37 °C and at various time points the films were removed, washed in distilled water and allowed to dry in air. These were

0957–4530

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Journal of Materials Science: Materials in Medicine is published bi-monthly by Chapman & Hall, 2-6 Boundary Row, London SE1 8HN. It may be purchased separately or as a combined subscription with *Journal of Materials Science.* Subscription details are available from: Subscriptions Department, International Thomson Publishing Services Ltd., Cheriton House, North Way, Andover, Hampshire SP10 5BE, UK. Telephone: 0264 332424; Fax: 0264 333471.

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sputter coated with gold and viewed under a scanning electron microscope (SEM). The partially degraded polymers were compared to the "as cast" films.

2.3. Cell culture methods

Both the primary human osteoblasts (HOB) and a commercial human osteosarcoma cell line (HOS), TE-85, ECACC No (87070202) were used at passages 10–15 for the experiments in order to compare the performance of the two cell types on the various materials. The cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FCS (Gibco), 0.02 M HEPES (Gibco), 2 mM L-Glutamine (Gibco), 150 μg/ml Ascorbate and 1% Penicillin/Streptomycin (Gibco). The HOBS were isolated from femoral heads obtained from patients undergoing surgery for total joint replacement. Trabecular bone fragments were removed, washed in PBS and incubated in supplemented DMEM as above for a period of 4–5 days. The fragments were then digested using collagenase (1000 u/ml in PBS) and trypsin (0.02% in PBS) for 20 min after which the solution was centrifuged and the cell pellet washed twice in complete medium, resuspended and seeded out at an appropriate density. Both HOS and HOB cells were grown to confluency at 37 °C with 5% CO_2 and then removed from the tissue plastic surface using trypsin (0.02% in PBS and HEPES). Both were resuspended in

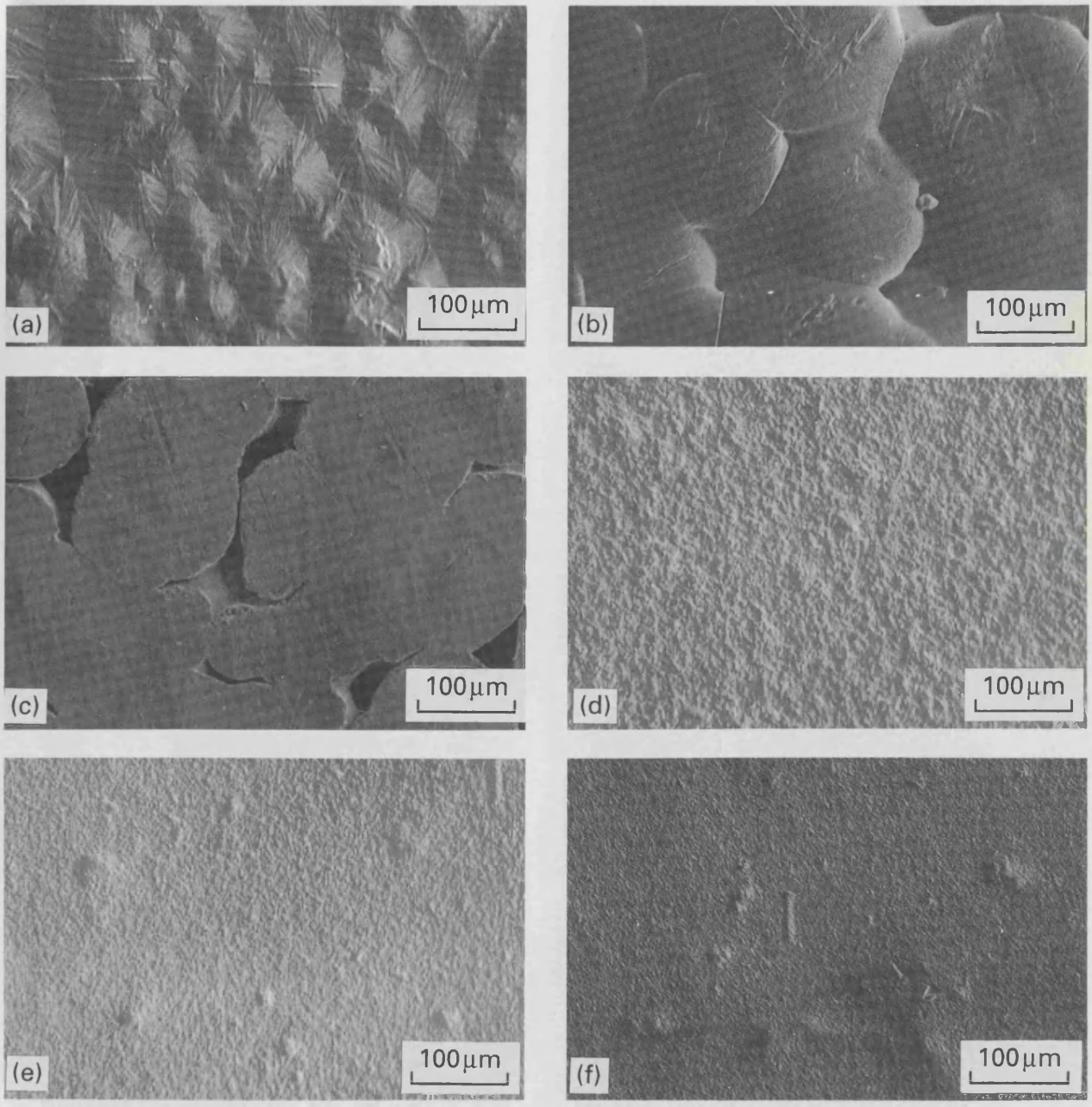

Figure 1 Scanning electron micrographs of the untreated surface of PHB and PCL and surfaces of the polymers after incubation in trypsin and papain solutions over a six week period. (a) PCL untreated; the surface of the polymer is irregular due to the spherulites which extend outwards and vary in size. (b) PCL in trypsin; the spherulites have become enlarged and smooth with small gaps appearing in between the enlarged structures. (c) PCL in papain; the spherulites have been degraded down to a smooth flat surface with large gaps appearing in between the flat surfaces. (d) PHB untreated; the structure is granular with the presence of tiny nodules. (e) PHB in trypsin; the overall granular structure remains, with fewer nodules being present. (f) PHB in papain; the surface appears "smoother" as compared to the untreated PHB but the overall structure remains intact.

cell types to give a final cell concentration of 8 $\times 10^4$/ml.

3. Results

The dry surface of PCL consisted of spherulites which ranged in size from 50–100 μm in diameter (Fig. 1a). Upon incubation with trypsin the spherulites became enlarged, possibly due to water absorption, leading to a smoother surface with the presence of small holes in between the spherulites (Fig. 1b). The effect of papain was even more marked with the spherulites appearing flattened and large holes occurring in between the flat surfaces (Fig. 1c). The effect on PHB by trypsin and papain was less dramatic with very few surface structure changes observed. Both trypsin (Fig. 1e) and papain (Fig. 1f) did not appear to affect the surface of PHB and at most there was a slight smoothing of the granular surface of PHB. HOBs grown on Thermanox after 2 days in culture appeared spread out and exhibited normal cellular morphology (Fig. 2a). At day 4 (Fig. 2b) the cells were nearing confluence due to cell proliferation. On PHB at day 2 (Fig. 2c) and at day 4 (Fig. 2d) the cells were less prevalent compared to the Thermanox control and appeared to aggregate

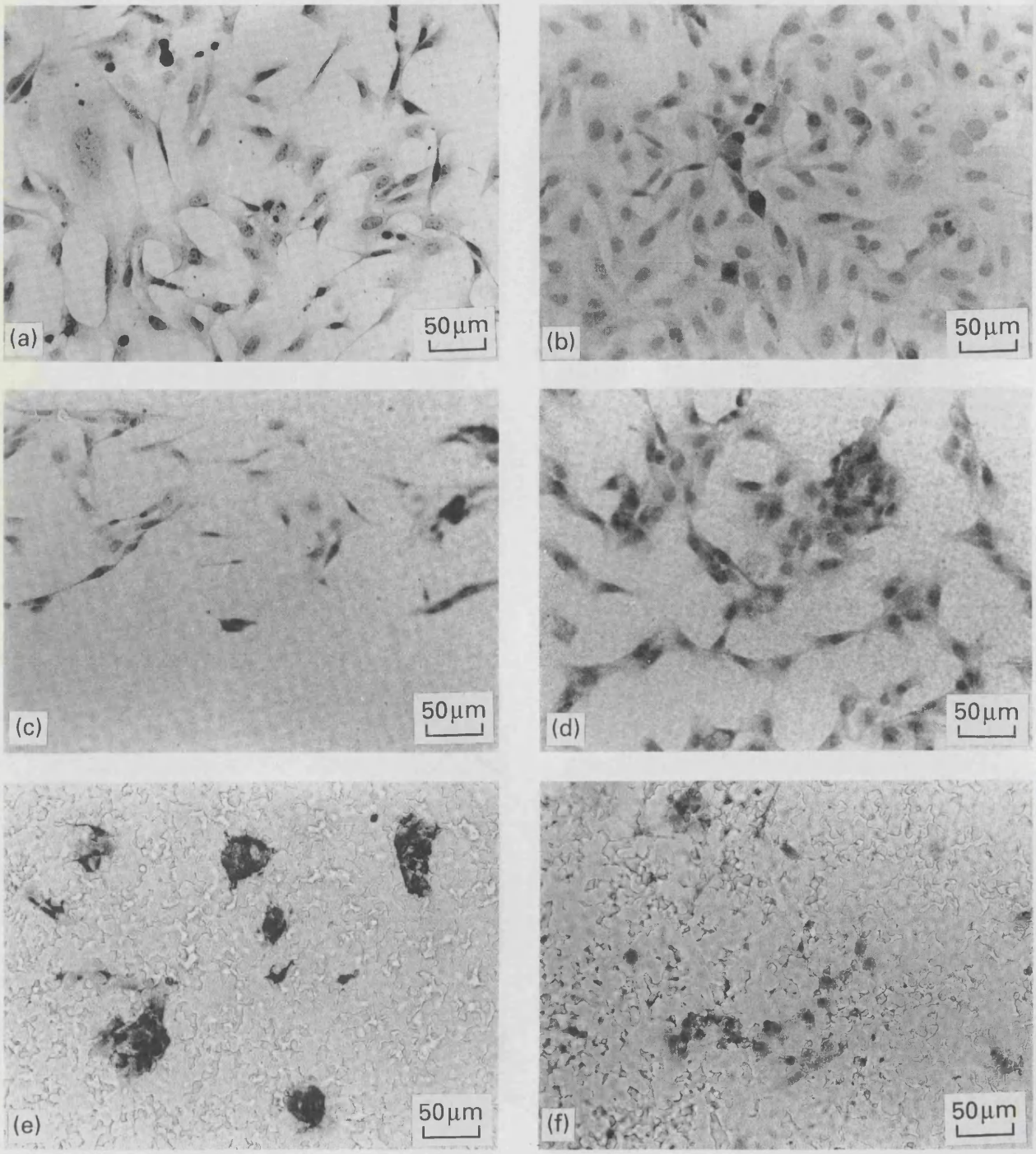

Figure 2 Light micrographs of HOBs on the surfaces of Thermanox, PHB and PHB-PHV copolymer at day 2 and day 4 in culture. (a) On Thermanox at day 2; the cells are viable and subconfluent. (b) On Thermanox at day 4; the cells are almost confluent and retain a normal morphology. (c) On PHB at day 2; the HOBs on the PHB are sparse with very little of the surface area covered. (d) On PHB at day 4; the cells have divided and a larger surface area has been covered by the cells. (e) On PHB–PHV at day 2; the cells are clumped and very few have adhered to the polymer. (f) On PHB–PHV at day 4; the cell clumps have become disaggregated and appear to be invading into the gaps on the polymer surface.

in one area first and then spread outward. The individual cellular morphology appeared normal although the proliferation rate did not appear to be as rapid as that for the control. Cells on PHB-PHV at day 2 (Fig. 2e) were present in clumps which by day 4 (Fig. 2f) had disaggregated but there did not seem to be an increase in cell number. However on PHB-PHV the cells appeared to be invading the holes present on the polymer surface: this will be further investigated using transmission electron microscopy.

4. Discussion

Degradation of polymers is affected by their molecular weights [18, 19, 26], copolymer ratios [18, 25–27], methods of sterilization and formulation [26], crystallinity [28], porosity [29] and their environment [30–32]. In the assessment of biocompatibility of degradable materials it is necessary to examine cellular performance on both the "as cast" surfaces of the materials and partially degraded materials because, during degradation, structural and chemical changes occur within the materials. The effects of these changes on cellular behaviour are not well understood. Williams has demonstrated the effects of hydrolytic enzyme activity on groups of degradable polyesters [33, 34] and more stable polymers [35, 36]. Our results show that there was a significant change in the surface properties of PCL after enzymatic degradation while the only effect of the enzyme solutions on PHB was a slight smoothing of the surfaces. This result supports previous studies which have shown PHB degrades slowly *in vivo* [37].

The results from the cell culture studies show that, although cell attachment and proliferation on the polymers does occur, the extent varies for each polymer type. On Thermanox controls the cells were seen to be more widespread and covered a larger surface area by day 4 compared to cells on either the PHB or PHB-PHV. The morphology of the cells was also different on each of the polymers, with cells on the PHB being more spread out than cells on PHB-PHV which formed clumps. These differences could possibly be explained by the difference in the surface properties of the polymers. Further work involving growth of cells on these polymers which are at different stages of degradation will be carried out and these results should provide information about cell material interactions during polymer degradation.

Acknowledgements

This work is part of the MTS programme on the measurement methods for the characterization and performance of advanced materials funded by the Department of Trade and Industry.

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described above. The gelatin was prepared as described, and each of the following cross-linking agents were tested: glycidyl acrylate 4% (Aldrich, Gillingham, Dorset, UK), acyl azide (Sigma) and carbodiimide (Sigma) using concentrations of 0.5 and 1 mg/ml, respectively. In each case the respective cross-linking agent was added to the gelatin and mixed thoroughly at room temperature for 2 h with gentle heating until the gelatin was dissolved. The different mixtures were then divided into two aliquots, and GH was added to one half of each gelatin batch containing the different cross-linker. The microspheres were then made by the method described above.

Elution of growth hormone from the spheres

The release of GH was monitored in PBS (Oxoid, Unipath, Basingstoke, UK) and horse serum (Gibco, Paisley, UK), by adding 2.5 ml of either solution to each 0.1 g of microspheres. The release of GH was carried out at 37°C on a continuous rotating mixer. The PBS and horse serum was removed and replaced with 2.5 ml of fresh PBS or horse serum after 1 h, and then daily. The elution fluid was frozen at −20°C for GH assay.

Effect of ultrasonication

The microspheres were divided into two groups, one in PBS and one in horse serum. These groups were then subdivided into a control group and an ultrasonication group. Those microspheres that were ultrasonicated were exposed for 2 min at an ultrasonic frequency of 40 kHz (using a DAWE 6441 ultrasonication bath) prior to sampling of the PBS or horse serum. The temperature of the microspheres was monitored and not allowed to exceed 37°C, the PBS and horse serum were replaced as above at each time point. The elution media was retained and frozen for GH assay.

Effect of pH

The effect of changing the pH of the dissolution medium was tested for both plain and GH-loaded microspheres. This was done by examining the swelling kinetics of the microspheres in PBS at pH 2.4, pH 7.2 and pH 10.5. The microspheres were weighed dry, and 0.1 g of gelatin microspheres (mean = 28 microspheres) were placed in 2.5 ml of PBS at the different pHs. At different time points, the microspheres were weighed and the change in weight recorded for their respective media. The swelling ratios were calculated by comparing the weights of the swollen microspheres to the dry weight of the microspheres with time.

Enzymatic degradation

The effect of pepsin was investigated on both plain and GH-loaded microspheres. In order to establish the optimal concentration of pepsin, a range from 20-660 IU/ml was tested. A final concentration of 660 IU/ml of pepsin was used to test for degradation. Prior to the start of the experiment, the microspheres were weighed and a dry weight of 0.1 g of beads was used. PBS (2.5 ml) was added to each set of microspheres containing varying concentrations of pepsin. These were incubated at 37°C and, at hourly intervals, the change in weight for each test sample was recorded.

Growth hormone assay

The assay used for GH was an in-house, enzyme-linked, immunoadsorbent assay (ELISA). This assay uses two polyclonal antibodies, the coating antibody is a guinea-pig immunoglobulin G fraction and the conjugate, a peroxidase-labelled Fab'-fragment of guinea-pig anti-human GH with 3,3'-5,5'-tetramethylbenzidine (TMB) as the substrate for the enzymatic reaction. The optical density was measured at 490 nm with a reference wavelength of 650 nm. The standard used was 22K-recombinant human GH (antibodies and standard GH were supplied by Novo Nordisk A/S, Gentofte, Denmark).

Assay validation

Accurate dilutions of the GH standard were performed in both PBS and horse serum (Gibco) to ensure that adequate recovery was observed in parallel dilution, and so confirm the absence of cross-reactivity within the GH assay. Further GH recovery studies were performed in 'binding protein-free' horse serum (briefly, removal of binding proteins was achieved by using an acid/ethanol extraction procedure), to determine whether the presence of binding proteins affected the amount of measurable GH.

Biocompatibility

The gelatin microspheres were washed several times in complete Dulbecco's Minimum Essential Medium (10% fetal calf serum, supplemented with 1% L-glutamine, 1% non-essential amino acids, 2% HEPES and streptomycin, from Gibco, UK). In order to observe their biocompatibility primary human osteoblasts were then seeded onto the gelatin microspheres and left in culture in DMEM at 37°C, in a humidified atmosphere of 5% CO_2. The gelatin microspheres were examined using SEM (Joel JSM 35) at 5 d post-seeding.

RESULTS

Gelatin spheres

The method described produced homogeneous and transparent microspheres with a diameter between 0.3 and 0.5 mm. The spheres possessed a uniform, spherical shape with a smooth surface. Although various methods were tested in order to achieve suitably cross-linked microspheres, the addition of cross-linkers such as glycidyl acrylate, acyl azide and carbodiimide proved unsuccessful with complete degradation of the microspheres at 37°C within a few hours of commencing the elution study. The optimal cross-linking for this system was achieved in the presence of 25% glutaraldehyde vapour. Complete cross-linking of the microspheres occurred within 48 h, with a colour change from pale yellow to deep yellow. Drying of the microspheres in a stream of cool air in a fume cupboard, allowed evaporation of any residual chloro-

Biodegradable microspheres: a new delivery system for growth hormone

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A drug delivery system for biologically active agents targeted to specific cells could be used to improve tissue repair in orthopaedics. The system should be controllable and capable of drug release over an extended period of time. Biodegradable, membrane-moderated, monolithic microspheres for the controlled release of growth hormone (GH) were developed and the release of GH was monitored *in vitro*. Cross-linked gelatin microspheres were used as the vehicle, with the drug dispersed within the gelatin. The amount of GH released from the microspheres was increased following ultrasonication. The release of growth hormone was monitored in phosphate buffered saline and horse serum. Interestingly, a higher level of GH was detected in the phosphate buffered saline than in serum. In addition, both pH and enzyme-induced degradation had an effect on the swelling kinetics of the gelatin microspheres. The release of GH from the microspheres was diffusion controlled, during the time period studied. *Biomaterials* (1994) **15**, (11) 931–936

Keywords: Drug delivery, microspheres, biodegradation, growth hormone, osteoblasts

Received 25 October 1993; accepted 11 November 1993

Controlled drug delivery has caused considerable interest in recent years, particularly in the development of biodegradable controlled-release systems. A problem common to most systems is that they display either a constant release rate or the release is dependent upon degradation of the system with time. Methods of drug delivery described in the literature include encapsulation[1], membrane-enclosed 'reservoir' devices[2], and monolithic systems[3–5]. There have, however, been few reports on methods for modulating the release rate with time. The approach used was to develop a monolithic microspherical drug delivery system in which growth hormone (GH) is dispersed within a biodegradable, biocompatible matrix. The delivery of GH from biomaterials can be an important therapeutic factor in the healing process of many tissues. It is known that GH can have both direct and indirect effects on osteoblast proliferation and differentiation and it also plays a role in the regulation of bone remodelling[6–10]. It has been shown previously that GH can be delivered from bone cement[11,12], ceramics[13,14] and polymer systems[15]. These biomaterials were selected primarily for their function in orthopaedics, and were modified further to allow drug delivery as a secondary function. In this work, we have specifically designed a release system for GH and other growth factors. Gelatin, a naturally occurring non-toxic polymer, was used to deliver the GH in a controllable manner, with the further advantage of being able to alter the amount of drug released on demand.

Correspondence to Dr S. Downes.

MATERIALS AND METHODS

Preparation of the spheres

A 20% gelatin solution was prepared using 300 Bloom gelatin (Swine skin type 1, Sigma, Poole, Dorset, UK) dissolved in sterile water. This was mixed, and the gelatin left to dissolve at 40°C. The solution was then divided into two aliquots, one of which was left plain as a control. Growth hormone solution (Novo Nordisk, Gentofte, Denmark) was added to the second aliquot at a concentration of 8 IU in 10 ml of sterile water. The microspheres were formed by forcing the gelatin mixture through a pre-heated syringe with a 21G needle into chilled paraffin oil in a 30 cm cooled column. The microspheres solidified as they passed through the column and were collected at the bottom. The paraffin oil was removed, and the microspheres were collected and washed three times in chloroform to remove all traces of the oil. They were cross-linked by placing them on a platform, in a dessicating chamber with 25 ml of 25% glutaraldehyde solution in the base, for 48 h. After cross-linking, the microspheres became a deep yellow colour. They were then washed several times in 200 ml of phosphate buffered saline (PBS), and dried in a stream of cool air overnight. Although several other methods of cross-linking were tested, this was found to be the optimal method.

Optimization of microsphere stabilization

Various methods for cross-linking the microspheres were investigated prior to choosing the method

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during the first 3 h of the experiment, and a rapid increase was seen in all cases. This was followed, however, by a marked fall in the swelling of the microspheres in pH 2.4 and 10.5. At pH 7.2, a very slow fall was observed in the swelling kinetics with a much lower diffusivity of GH. These findings were the same for both GH-loaded and plain microspheres.

Enzymatic degradation

Pepsin had a marked effect on the swelling kinetics of both plain and GH loaded microspheres. The plain microspheres in PBS showed a swelling ratio of approximately 7.9 after 7 h compared with 6.6 in the presence of pepsin (*Figure 5*). In contrast, a swelling ratio of 5.7 was observed in the absence of pepsin compared with 3.9 for the GH-loaded microspheres (*Figure 6*). This experiment indicates that the extent of cross-linking is important, and that the degradation of the microspheres by a proteolytic enzyme is dependent on this.

Biocompatibility

The microspheres showed no evidence of cytotoxicity. Scanning electron microscopy showed that viable cells were able to grow on the gelatin microspheres, indicating that they were biocompatible and not toxic to the osteoblasts (*Figure 7*). Good cell adhesion was observed *in vitro*, with penetration and invasion of the cells within the gelatin microspheres (*Figure 8*). Although some degree of degradation was visible, the microspheres were still not fully degraded even after 2 wks in culture in the presence of cells (*Figure 9*).

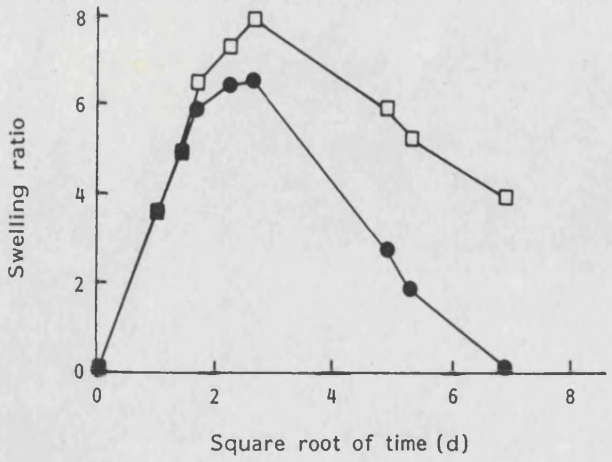

Figure 5 Swelling kinetics of plain microspheres in the presence, ●, and absence, □, of pepsin.

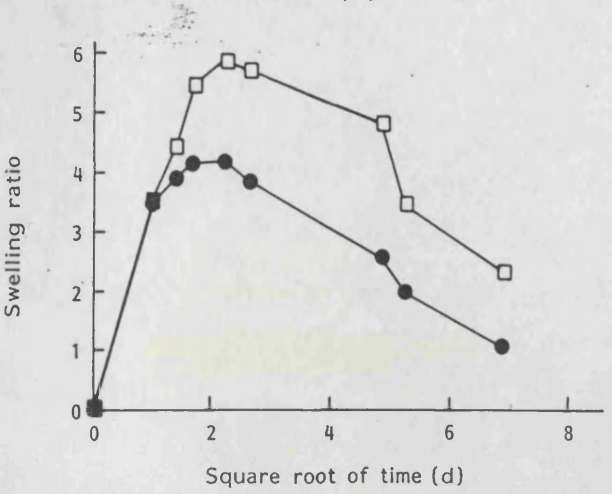

Figure 6 Swelling kinetics of growth hormone-loaded microspheres in the presence ●, and absence, □, of pepsin.

DISCUSSION

The concept of sustained release of therapeutic agents has been in existence for many years and many systems have been described, including encapsulated beads or pellets, coatings and porous materials containing the dispersed drug[16–18]. Many of the swellable systems are dependent on the polymer swelling and releasing its contents into the surrounding fluid, this can, however, result in problems with solute stability.

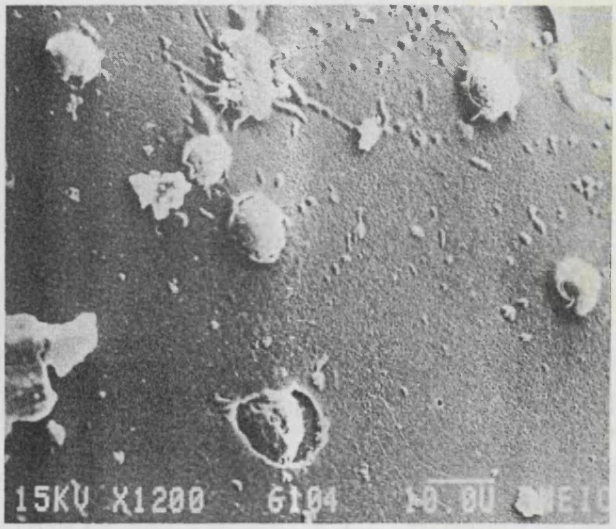

Figure 7 Scanning electron micrograph of a gelatin microsphere showing the adherence of numerous human osteoblasts 5 d post seeding.

Figure 8 Scanning electron micrograph of a human osteoblast cell on a gelatin microsphere, penetration of the cells within the gelatin matrix was visible 5 d post-seeding.

form or glutaraldehyde. This method provided microspheres that lasted for over 2 months *in vitro* in the absence of cells and over 2 wks in the presence of cells in culture.

Growth hormone release

The microspheres released high concentrations of GH into both PBS and horse serum. The results indicated that the release of the GH from the microspheres was diffusion controlled. A plot of the rate of release versus the square root of time was linear during the early stages of GH release for the period studied, in both PBS and horse serum (*Figures 1* and *2*). It was noted that the plot line did not go through the origin, which suggests that release of GH also occurs from the surface. Ultrasonication of the microspheres in PBS resulted in a release of approximately 23% of the total GH incorporated, compared with 10% in those microspheres which were not exposed to ultrasonication. However, only 15% of the total GH incorporated was released from the ultrasonicated microspheres in horse serum compared with 8% from the control group. Although a difference was observed between the release of GH in PBS and the horse serum in the control samples, it was not significant. There was, however, significantly more GH detected in the PBS than in the horse serum ($P = 0.017$, Student's t-test) indicating that ultrasonication of the microspheres had increased the amount of GH released.

Parallelism was observed in both the PBS and the horse serum standard curve indicating that there was no cross-reactivity. Studies using 'neat' horse serum and 'GH-binding-protein free' horse serum spiked with GH gave recoveries of 73% and 88%, respectively. These figures were not significant, thus indicating that the difference in the amount of GH detected was not due to the presence of GH-binding proteins.

Effect of pH

These results indicate that the swelling of the microspheres is pH sensitive for both plain and GH-loaded microspheres (*Figures 3* and *4*). The pH of the media (2.4, 7.2, 10.5) did not affect the swelling ratio

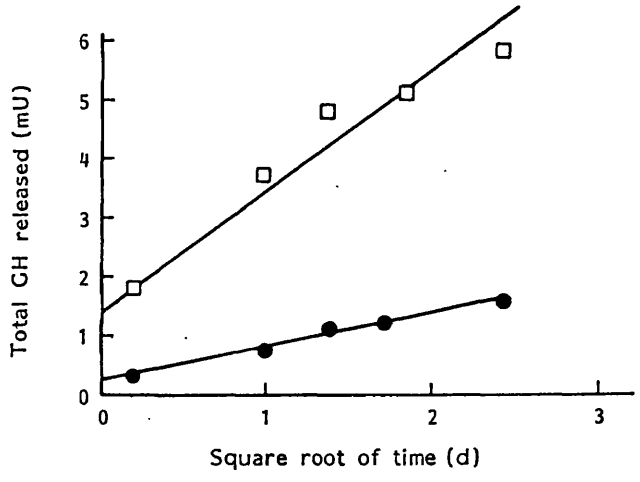

Figure 1 Total amount of growth hormone released in phosphate buffered saline for control microspheres and for microspheres subjected to ultrasonication (data are mean for $n = 4$): □, ultrasonication; ●, no ultrasonication.

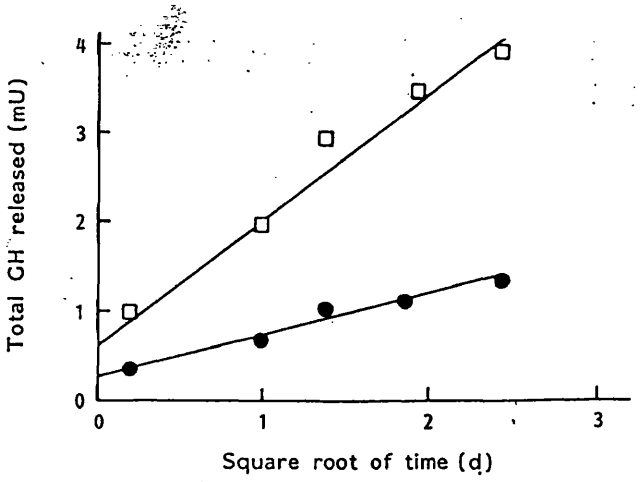

Figure 2 Total amount of growth hormone released in horse serum for control microspheres and for microspheres subjected to ultrasonication (data are mean for $n = 4$): □, ultrasonication; ●, no ultrasonication.

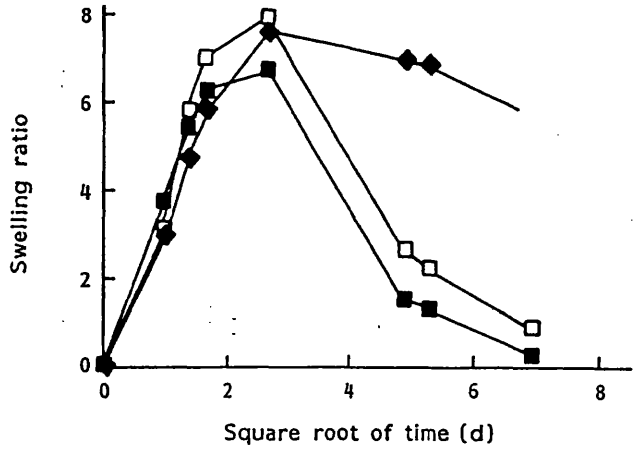

Figure 3 The effect of pH on the swelling kinetics of plain gelatin microspheres. The swelling ratio was calculated by comparing the weights of the swollen microspheres with their dry weight: □, pH 2.4; ◆, pH 7.2; ■, pH 10.5.

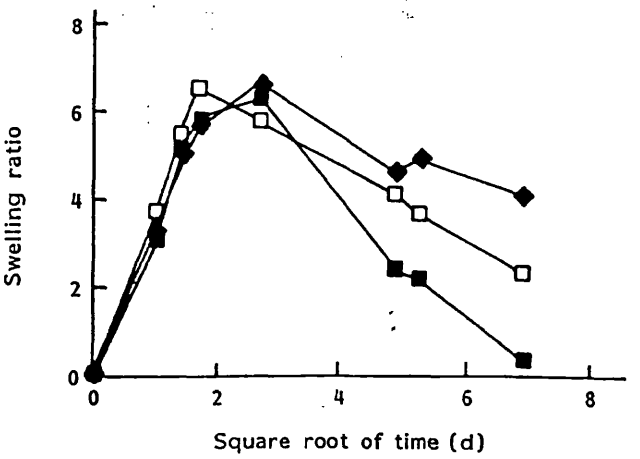

Figure 4 The effect of different pH on the swelling of growth hormone-loaded microspheres was studied by examining the swelling kinetics of the dried microspheres: □, pH 2.4; ◆, pH 7.2; ■, pH 10.5.

enzymes in the horse serum causing a more rapid degradation of the GH released.

Kost *et al.*[26,27] have shown that it is possible to increase significantly the degradation rate of polymers and the release rate of incorporated agents using ultrasound. Ultrasonication of the microspheres was used in our system to increase the amount of GH released. The structure of the gelatin microspheres was probably broken down by the ultrasonication. This in turn causes a change in the kinetics resulting in further degradation, with an increase in the amount of water penetrating into the matrix, causing an increase in the diffusion coefficient and in the release of incorporated GH. Modulation of drug delivery by an external means may improve the release pattern and the efficacy of peptides such as GH which is usually released in a pulsatile manner. Such release systems are potentially useful in a variety of clinical applications.

CONCLUSIONS

The monolithic gelatin microspheres in our system provide a simple and effective drug delivery system. The advantages of the system are that the matrix used is a natural polymer, which is both biodegradable and biocompatible, and its preparation does not involve the addition of any toxic component. It can respond to an external stimulus, and the amount of drug released can be increased when required.

ACKNOWLEDGEMENTS

This work was funded by the Scientific Engineering Research Council for the IRC in Biomedical Materials.

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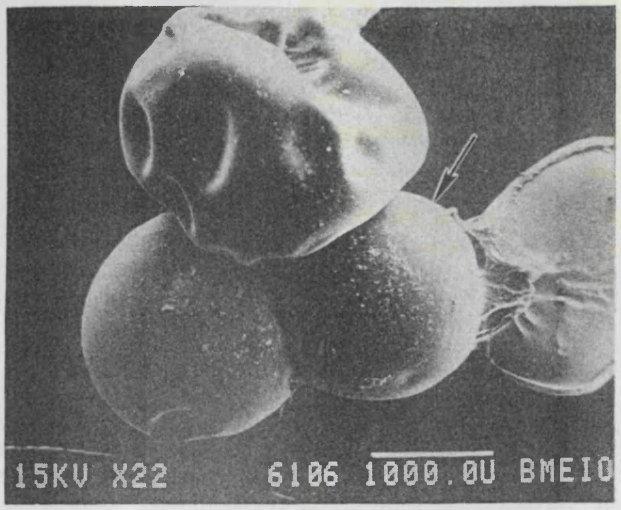

Figure 9 Scanning electron micrograph of gelatin microspheres following 5 d in culture with human osteoblasts. Cells are visible on the microspheres and some degree of degradation was noticeable. The microsphere examined in *Figures 7* and *8* is indicated by

The release of agents from monolithic systems usually has zero-order kinetics, with release rates diminishing with time[19]. Mathematical models for sustained release of water soluble drugs have been described, which indicate that the release is dependent upon the amount of drug loaded and upon dissolution and diffusion. In part these can be ascribed to Fick's Law where the diffusion coefficient is constant with time, and the dimensions of the system remain the same[20–23].

In this study, we have shown that microspheres can be used as a delivery system for GH. The drug was dispersed uniformly within the gelatin matrix. The release of GH from the microspheres is partially due to diffusion. If the release process is diffusion controlled, then the theory for diffusion from a sphere should apply[24]:

$$M_t/M_\infty = C_o\,(Dt/a^2)^{1/2}$$

$$\left\{1/\Pi^{1/2} + 2\sum_{n=1}^{\infty} \text{ierfc}\,{}^{na}/\sqrt{Dt}\right\} - 3Dt/a^2 \qquad (1)$$

In which M_t = total amount of drug released at time t; M_∞ = total amount released at infinite time; C_o = initial drug loading; t = time; a = radius of microsphere; D = diffusion coefficient; and ierfc = the integrated error function.

However, as the data presented represent a loss of approximately 22% of the total drug incorporated (the remainder retained probably as a result of cross-linking), and in view of the fact that the plots in *Figures 1* and *2* are linear, it is reasonable to assume that

$$\text{ierfc}\,{}^{na}/\sqrt{Dt} \text{ is } < 1/\Pi^{1/2}$$

Equation (1) then reduces to:

$$M_t/M_\infty = 6\,(Dt/\Pi a^2)^{1/2} - 3Dt/a^2 \qquad (2)$$

This equation is a general quadratic, but because of the linearity of the $t^{1/2}$ plot, it is further assumed that the second term is much less than the first. This transpires to be the case. For the highest diffusion coefficient taken and for a time of 4 d:

$$3Dt/a^2 = 0.6, \text{ and } 6\,(Dt/\Pi a^2)^{1/2} = 1.51$$

Also noting that:

$$M_\infty = \frac{4}{3}\,\Pi a^3\,C_o$$

in which C_o is the concentration of the drug incorporated.

Equation (2) then reduces to:

$$M_t = 8\,C_o\,a^2\,(\Pi Dt)^{1/2} \qquad (3)$$

However, a number of microspheres are used experimentally, hence if there are n_i microspheres of radius a_i, then *Equation (3)* becomes:

$$M_t = 8\,C_o\,(\Pi Dt)^{1/2} \sum_i n\,a_i^2 \qquad (4)$$

Because the range of microsphere sizes is 0.3–0.5 mm, *Equation (4)* can be rewritten as;

$$M_t = 8\,C_o\,(\Pi Dt)^{1/2}\,na^2 \qquad (5)$$

in which n = total number of microspheres; and a = mean radius (mm).

The slope (s) of the $t^{1/2}$ plot is:

$$s = 8\,C_o\,(\Pi D)^{1/2}\,na^2$$

$$D = \frac{s^2}{64\,C_o^2\,\Pi\,n^2\,a^4} \qquad (6)$$

The outward diffusion of the drug is dependent upon inward diffusion of water, so the process is more complex than the above. The diffusion of the water into the gelatin microsphere will be concentration dependent and possibly time dependent as well. In view of the linear $t^{1/2}$ plots for the release of the GH, this was not time dependent over the period studied, but may have been concentration dependent.

More GH was detected in PBS than in serum indicating that the media into which the microspheres were placed had an effect on both the rate and the amount of drug released. There are several possible explanations for this observation. Proteins in the horse serum could have been adsorbed onto the gelatin microspheres resulting in a protective proteinaceous coating around them. It has been shown that coatings on drug delivery vehicles can affect the release rates and dissolution of the incorporated drugs[25]. This seems unlikely because different diffusion coefficients would be expected, depending upon the medium, and this was not the case. Another possibility could be that GH was released in the same quantities in both cases, but in the horse serum the GH may have formed a complex with the GH-binding proteins, thereby preventing all the immunoreactive GH from being detected. Recovery studies, however, showed this not to be the case. The most probable explanation for the difference observed was due to the presence of