CELLULAR RESPONSES TO POTENTIAL BIOMATERIALS

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Doctor of Philosophy

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Summary of Thesis

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The aim of this study was to systematically investigate the factors considered to be responsible for anchorage-dependent cell behaviour to determine which, if any, of these factors exerts greater influence. An efficient means of doing so is the *in vitro* fibroblast cell culture model. The interaction of fibroblasts with novel substrata gives information about how a biological system reacts to a foreign material. This may ultimately lead to the development of improved biomaterials. This interdisciplinary study combines the elements of surface characterisation and biological testing to determine the nature of the biomaterial/host interface. Polarity and surface charge were found to have an important influence on fibroblast adhesion to hydrogel polymers, by virtue of their water-structuring effects. These same factors were found to affect cell adhesion on undegraded PHB-HV copolymers and their blends with polysaccharides. On degraded PHB-HV copolymers, the degradation process itself played the greatest role in influencing cell response. Increasing surface charge and mechanical instability in these polymers inhibited cell adhesion. Based on the observations of hydrogels and PHB-HV copolymers a novel material, gel-spun PHB was designed for use as a wound scaffold. *In vitro* tests using human and mammalian fibroblasts accentuated the importance of polarity and surface charge in determining cellular response.

The overall view of cellular behaviour on a broad spectrum of materials highlighted the effects that polarity and surface charge have on water-structuring, and how this affects interfacial conversion. In degradable systems, mechanical stability also plays in important role in determining anchorage-dependent cell behaviour

Keywords: cell adhesion, hydrogel, polyhydroxybutyrate, wound healing.

This thesis is dedicated to the memory of my late mother without whom, so many things would not have been possible.

Gone but not forgotten

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LIST OF ABBREVIATIONS

ACM	Acrylamide
AEMA	Aminoethylmethacrylate
Ala	Alanine
Arg	Argyl
Asp	Aspartyl
BACTY	Bacteriological Grade Plastic
CHO	Chinese Hamster Ovary
COOH	Carboxyl Group
CWHRI	ConvaTec Wound Healing Research Institute
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EWC	Equilibrium Water Content
FCS	Foetal Calf Serum
FN	Fibronectin
Gly	Glycyl
HSO_3	Sulphonate Group
H_2SO_4	Sulphuric Acid
HEMA	2-hydroxyethylmethacrylate
HCD	Hydrocolloid Dressing
LMA	Lauryl Methacrylate
MBACM	Methylenebisacrylamide
MMA	Methyl Methacrylate

LIST OF ABBREVIATIONS (Continued)

Na ALG	Sodium Alginate
NNDMA	N'N'Dimethyl Acrylamide
NVP	N-Vinyl Pyrrolidone
NT	Norwegian Talc
NWM	Non-Woven Mat
OH	Hydroxyl Group
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PHB	Poly (ß-)hydroxybutyrate
HV	(ß-)hydroxyvalerate
Pro	Prolyl
RGDS	Arg-Gly-Asp-Ser
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
Ser	Serine
TC	Tissue Culture Grade Plastic
VN	Vitronectin

CHAPTER ONE

INTRODUCTION & LITERATURE REVIEW

MISSION STATEMENT

Biology and materials science would appear to have nothing in common at first sight. Yet it is the the conjunction of these two sciences that produces the new and exciting field of biomaterials research. The biological component is that of the host system; the materials science involves the manufacture of synthetic materials for use as biomaterials. The biomaterial is the interface between biology and materials science, and as such it acts as a nexus for the integration of many varied disciplines. Polymer technologists, materials scientists, biochemists and biologists are all vital to the development of a new biomaterial. This interdisciplinary approach is essential for progress in biomaterials science. It would be a mistake for materials scientists to attempt studies of biological interactions without the detailed knowledge of the underlying processes involved in biological systems, just as it would be foolish for biologists to attempt to produce materials by methods they know little of, even if they feel they can predict the properties necessary in a material. The only truly profitable approach in attempting to produce useful biomaterials is gained by the overlap of expertise contained within the relevant disciplines. It is this cross-fertilisation of ideas which produces results.

As a biologist, I see the role that I play in biomaterials science as one of extending knowledge in two areas. Firstly in the area of cell biology, I can provide further information as to the nature of *in vitro* cell culture systems. I can address the question of how anchorage dependent cell behaviour is affected by adhesion and growth on a wide variety of substrates. Secondly, by using cells as sensitive surface "probes", I can elucidate the way in which the properties of synthetic

materials can be altered to produce particular cellular responses.

The reasons for attempting this study are based on the above premises. A wide range of potential biomaterials was chosen to supplement the volume of knowledge that already exists in this area. Hydrogels are a very interesting class of polymers which have many applications, particularly as contact lenses. Much work on cellular interactions with hydrogels has been conducted in these laboratories, so this study is an extension of that research. In addition, this well characterised system is an ideal model to study because it is a stable system, and observations of these polymers may have implications for studies of cell behaviour on unstable biodegradables such as polyhydroxybutyrate. Biodegradable polymers are important in the production of biomaterials because degradation of implants may be an essential prerequisite in certain clinical situations. With this in mind, a novel process for the production of polyhydroxybutyrate has created a material which is applicable to the area of wound healing. In a purely commercial development cell biology still has an important role to play, as it provides first stage toxicity testing of products for the market place. Additionally, *in vitro* models may help to determine whether a new material possesses the properties necessary for the required biological response to that material. Thus, this study is based on the application of cell biology techniques to the field of biomaterials science. No attempt is made to relate detailed knowledge of polymer chemistry or materials science, as this lies outside the scope of the thesis. However, the ways in which the relative contributions of various surface characteristics of the materials tested influence cellular response, are discussed.

1.1 A brief history of cell culture

Since Harrison's pioneering work in the early years of this century[1], cell culture - the maintenance of cells outside the host organism from which they were derived - has advanced greatly. Considerable effort has been carried out to elucidate the correct requirements for the optimum growth of the many cell lines used in the widely varying fields of medical research.

The early problems of keeping cells alive *ex vivo* were solved by the development of balanced nutrient solutions (culture media), and by the use of scrupulous aseptic techniques to reduce the possibility of infection or contamination by pathogens such as bacteria, fungi and viruses[2]. Harrison's observations[1] and those of other researchers provided early evidence for the preference that certain cell types exhibited for growth on solid supports[3,4]. With the advent of reliable cell culture systems, a powerful new tool became available for biological research. However, routine cell culture was not without its problems. The use of glass in culture containment led to difficulties with cleaning and resterilisation. The introduction of disposable polystyrene in the 1960's solved the problem of sterility, but unfortunately, native polystyrene did not allow attachment and spreading of cell types such as fibroblasts as glassware did[5-7]. Surface treatment of polystyrene by glow discharge in oxygen plasma increased the wettability of the surface, rendering the disposable dishes suitable for cell culture[8-13]. This opened the way for the establishment of the sophisticated and reliable cell culture systems in use today.

1.2 Anchorage-dependent cells

As mentioned above, whilst some cell lines can grow in suspension others, particularly eucaryotic tissue cells, require a suitable substrate to which they can attach and spread. Stoker *et al* [14] use the term "anchorage-dependent" to describe cells which need to become attached to, and spread on a substrate, in order to undergo normal cell behaviour in cell culture.

1.3 Fibroblast cells

Fibroblast cells are a family of collagen secreting cells that are present in most tissues, and whose functions include the deposition, maintenance, degradation, and rearrangement of the extracellular matrix. Fibroblasts are anchorage-dependent and are thus useful when investigating the surface properties of potential biomaterials[15]. Fibroblasts act as a very sensitive biological probe, responding to small changes at a surface. As such, they are an important first step in determining the biocompatibility of a new material.

1.4 The application of cell culture to biomaterials research

Cell culture has useful advantages over *in vivo* models in the study of the biological effects of biomaterials. Cell culture negates the need for *in vivo* surgery and it's associated complications, such as post-experimental removal, and host trauma. Also, *in vivo* experiments are often difficult to repeat due to host characteristics, whereas *in vitro* experiments are easily repeatable under identical conditions. Such models are therefore accessible and can be used to isolate the effects of one, or a number of conditions.

1.5 Biomaterials and biocompatibility

A biomaterial may be described as a material that is used in the treatment of a patient, and which at some stage interfaces with tissue for an indeterminate length of time. For a material to be considered biocompatible it must be able to exist in a physiological environment without it adversely or significantly affecting the biological system, or the biological phase adversely affecting the biomaterial[16].

1.6 The effect of substrate on cell behaviour

In synthetic polymers, it is the surface characteristics which determine how a cell will respond. There are several physico-chemical properties that can exert an influence on cell behaviour, and these include surface chemistry[17-21], surface energy[22-24], hydrophobicity/hydrophilicity[25-28], surface morphology[29,30], and surface charge[31-33]. The relative effect each factor plays in determining biological responses is a source of great confusion in the literature. It is most likely that it is the interaction of several, if not all, of these factors which determines whether a particular surface will support cell adhesion.

1.6.1 Surface group expression

The roles of hydroxyl[34], sulphonate[31,35], and carboxyl[36] groups in cell adhesion have been well documented. The expression of specific groups at the surface is very important. A well studied example is polystyrene (PS). Native PS is non-adhesive to fibroblasts, but can be rendered adhesive by means of a surface treatment which introduces charged moieties at the surface. The work of Thomas[35] and others[17,19] have shown that acid treatment of PS produces an adhesive

surface. Thomas showed that by exposing PS to cold sulphuric acid, hydroxylation but little sulphonation of the surface occurred. These hydroxylated surfaces produced cell attachment and spreading, in accordance with the results of Curtis[10] who concluded that hydroxyl group expression at the surface was necessary for cell adhesion. Thomas also used hot sulphuric, oleum and chlorosulphonic acids to treat PS surfaces. These treatments resulted in sulphonated surfaces which led to a decreased cell attachment with no spreading. Maroudas[11] has stated that sulphonate groups promote cell adhesion, in contrast to the above findings. It may be that the sulphonate groups which are strongly negatively charged may prevent the negatively charged cell surface from attaching to the substratum due to short range electrostatic repulsion. Hydroxyl groups which are more neutral do not present the same electrostatic barrier.

1.6.2 Surface polarity

Surface energy, in particular the polar component of surface free energy, can play a crucial role in determining cell behaviour. Polar surfaces exhibiting high surface energy promote the adsorption of proteins. The surface polarity determines the relative hydrophobicity/hydrophilicity of a surface. The terms "hydrophobic" and "hydrophilic" as liberally applied in the literature to polymer surfaces are relativistic. Thus, "hydrophobic" surfaces contain some polar groups and vice-versa.

Hydrophilic surfaces have polar groups expressed at the surface which may be ionised and form dipoles. These dipole moments lead to hydrogen bonding with water. By contrast, hydrophobic surfaces have few permanent dipoles and therefore interact with water by dispersive or hydrophobic interactions.

Hydrophilic surfaces bind water strongly and orientate the water molecules around the polar groups, whereas hydrophobic surfaces structure water molecules in an ice-like formation.

Hydrophilic surfaces are more wettable i.e. water spreads more readily on a "hydrophilic" surface than on a"hydrophobic" surface. This wettability of a substratum has important implications for the biological response. Weiss[37] in 1960 was the first to report the influence of substratum wettability on cell adhesion. His findings were supported by later research[38,39,26-28]. It is believed that increased surface polarity affects the adsorption of proteins from the surrounding medium[40,41] However, it has been demonstrated that protein adsorption is greater on hydrophobic surfaces[42]. That this should be so can be explained by the fact that globular proteins in solution have charged, hydrophilic exteriors. As a result, there is likely to be greater adsorption of protein on a hydrophobic surface than on a hydrophilic surface. Indeed, Bentley and Klebe[43] have demonstrated that more fibronectin was bound to bacteriological grade plastic (amorphous polystyrene), than to tissue culture plastic (surface modified polystyrene). However, they also reported that the number of active sites exposed on the bacteriological grade plastic was less than on the tissue culture plastic. This implies that it is not purely the amount of adsorbed protein on a surface that influences cell response; it is the correct expression of those proteins that ultimately determines cell adhesion. Adhesive proteins such as fibronectin appear to adsorb in the correct conformation on more hydrophilic surfaces.

1.6.3 Surface morphology

The morphology of the substratum plays an important role in determining cellular response in both *in vivo* and *in vitro* systems. For example, the wound healing response is dependent on reepithelialisation[44,45], and the construction of new tissue along collagen pathways[46].

Recent *in vitro* studies[20,47] including the work of Thomas[35] have demonstrated the effects of surface rugosity on cell adhesion. After treating polystyrene surfaces with oleum, she found that the macroscopic surface features produced as a result, inhibited the spreading of BHK-21 fibroblasts. The degree to which the surface rugosity can influence cell behaviour can depend on the cell type. As discussed later, surface rugosity can affect the adhesion of fibroblasts such as BHK-21 and L929, whereas other cell types may not be affected to the same degree[48]. Recent work in these laboratories[49] has shown that chondrocytes can adhere to the surfaces of IPN hydrogels which exhibit large scale surface rugosity.

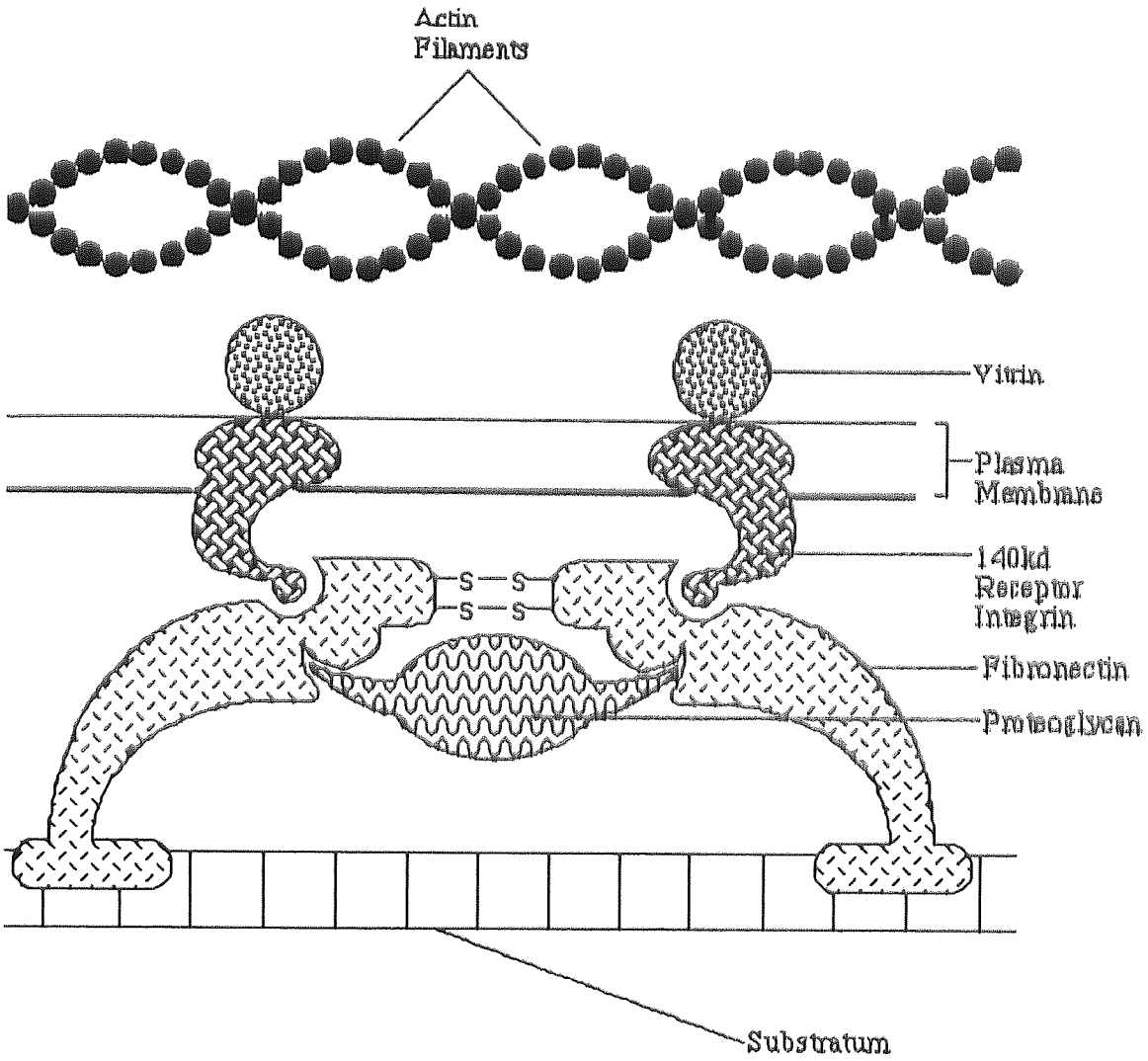
1.7 Cell adhesion

The adhesion of anchorage dependent cell types, such as fibroblasts to surfaces is a complex process which has yet to be fully elucidated. For a cell to adhere to a surface, that surface must adsorb adhesive proteins such as fibronectin and vitronectin. Cells in suspension are rounded but during adhesion, they spread out in a characteristic "stellate" shape. Cells remain in this state until mitosis, then they round up and divide; the two daughter cells spreading again. If cultured cells are allowed to reach confluence, cell division stops due to "contact inhibition".

This response is believed to maintain fully developed animal tissues in a non-dividing state[50].

When a cell comes into contact with a surface, the interaction of the cell with the potential substratum is dependent on the formation of points of attachment. The contacts that are made are influenced by cell type[51,52], substrate[5,11,53],contact time[54], motile state[55], and the presence of extracellular proteins. Cytoskeletal reorganisation results in the formation of focal adhesions. Morphologically, focal adhesions are electron dense areas found at the periphery of the spreading cell. They are very elaborate structures composed of cytoskeletal components such as actin filaments, and transmembrane protein complexes. The transmembrane proteins form a family termed integrins. Integrins act as cell surface receptors for adhesive molecules such as fibronectin and vitronectin (see later).

Figure 1.1 Idealised diagram of focal adhesion.



1.8 Surface deposition of protein

When a foreign material is exposed to a biological system, there is an immediate, active deposition from that system[56]. An adsorbed layer consisting primarily of proteins, which subsequently alters the surface characteristics of the exposed material is layed down. This interfacial conversion means that the material will take on new surface properties as a result of the adsorbed layer. There have been several major proteins so far identified that have an effect on cell behaviour notably

fibronectin, vitronectin, laminin, vinculin, and actin. Of these, the most important and widely studied is fibronectin (FN). A review of the effect of this protein on a wide range of cellular interactions and processes will be discussed here.

1.9 Fibronectin

1.9.1 Fibronectin: structure & function

Fibronectin (FN) is a large molecular weight glycoprotein present in the extracellular matrix (ECM), serum, and on the surface of fibroblasts. In structure, FN consists of 2 sub-units, each of ~250kilodaltons (kD). Each sub-unit is folded into a flexible arm ~60nm long, with the two sub-units joined at the C-terminus by a disulphide bond. Within the sub-units, quaternary globular domains exist, each with a specific binding property for different molecules[57]. These domains are linked by short protease sensitive helical regions[58] The primary biological function of FN is it's adhesiveness to a great diversity of substances such as collagen, fibrin, actin, glycosaminoglycans, factor XIII, von Willebrand factor (VWF), deoxyribonucleic acid (DNA), staphylococci, and cells[59].

1.9.2 Fibronectin: role in cell adhesion

Of the many adhesive activities, the cell-binding property of FN has elicited the most interest. From the point of view of biomaterials research, this is important because FN has been found to promote the adhesion of many cell types to artificial and natural substrata. FN is necessary for adhesion[60] and growth[61] of fibroblasts, adhesion of macrophages[62], myoblasts[63], neutrophils[64],platelets[65], and keratinocytes[66]. More recently, FN has been shown to promote the adhesion of hemopioetic cell lines[67], play a role in regulating the adhesion of lymphocytes[68]

and granulosa cells[69] ; and to play a dominant role in the attachment and spreading of mammary epithelial cells[70]. Clarke[71] and coworkers have demonstrated that FN mediates the re-epithelialisation of rat alveolar cells. Lubec[72] has shown that FN promotes attachment of glomerular cells to the basement membrane. Clearly, FN plays a major role in cell organisation. FN does not mediate only mammalian cell adhesiveness, it's effects are widespread throughout the animal kingdom from *Staphlyococcus aureus* (Bacteria:Bacillus)[4], and the slime mold *Dictyostelium discoideum* (Protozoa:Dictyostelia)[73] to *Pleurodeles waltlii* (Vertebrata: Amphibia)[74].

FN may also have an important part to play in disease. Thomas *et al* [75] have shown that FN mediates cell adherence in *Treponema pallidum*, the causative organism of syphilis, whilst Ouaissi[76], have found a similar cell adherence response in the infective stages of the parasite *Trypanosoma cruzi*, which causes the fatal Chagas' Disease of South America. Probably the most important medical aspect of the effects of FN is it's influence on the cell adhesion of tumour cell lines. M^{c}Carthy *et al*[77] have shown that FN influences the adhesion and motility of metastatic melanoma cells. Pande *et al*[78] demonstrated the importance of FN in the adhesion of a number of tumour cell lines.

1.9.3 Fibronectin: The cell binding domain

FN mediates it's cell binding activity via a cell binding domain, the active site of which has been identified and sequenced to a tetrapeptide containing the amino acid sequence arginine-glycine-aspartate-serine (RGDS)[79,80]. The Arg-Gly-Asp

residues appear to be essential for activity, whilst the serine residue can be replaced by other amino acids and yet still retain cell binding activity[81]. Piersbacher & Ruoslahti[81] postulate that the surrounding amino acids play a role in the expression of cell adhesion activity in FN. Yamada & Kennedy[79] demonstrated that the inverted form of RGDS (i.e. SDGR) is nearly as active as the forward sequence, though not if embedded in a decapeptide where this sequence exhibited minimal activity. They concluded that the adhesive recognition signal consisted of a specific arrangement of RGDS, with additional information being supplied by adjacent amino acids.

The advent of peptide synthesis has provided a useful technique with which to examine the cell recognition system of FN. Hayman *et al*[82] used synthetic peptides containing the RGD sequence to detach cells from culture substrata by inhibition of cell attachment to FN. The RGDS sequence has been shown to be important in regulating many biological systems. Synthetic RGDS peptide inhibits retinal pigment epithelial (RPE) cell attachment to FN, a process which can result in traction retinal detachment after retinal reattachment surgery. Thus RGDS may have a role to play in the management of proliferative vitreoretinopathy[83]. Ouaissi *et al*[76]found that they could use synthetic peptides with the RGDS sequence, but not other sequences of similar amino acids, to inhibit cell invasion by trypomastigotes (infective stages) of *T. cruzi*. This is because the RGDS sequence of cell surface FN is a recognition signal for the attachment of the parasite to host cells.

Poole & Thiery[84] using the synthetic peptide H-**Arg-Gly-Asp-Ser**-Pro-Ala-Ser-Lys-Pro-OH, which contains the cell recognition site of FN, showed that this peptide inhibited the migration of mesencephalic neural crest cells within the chick embryo. FN has been implicated in chemotaxis as well as cell adhesion. Long *et al*[85] have shown that the hexapeptide Gly-**Arg-Gly-Asp-Ser**-Pro affects chemotaxis of *ligamentum nuchae* fibroblasts. Lash *et al*[86] have found synthetic peptides that mimic the adhesive recognition signal of FN which have differential effects on cell-cell and cell-substratum adhesion in embryonic chick cells. With regard to tumour biology, workers have recently shown that synthetic RGDS peptides can inhibit cell adhesion in cell lines such as the PC12 pheochromocytoma line[87]. An important fact, discovered by Dedhar *et al*[88], is that some tumourous cells can overproduce FN receptor, and so resist detachment. Stimulation of these cells by synthetic peptides can selectively cause them to overproduce FN receptor. There is an accumulating amount of evidence suggesting that there may be more than one cell attachment site for FN. Schwarzbauer *et al*[89] have speculated that FN molecules with 2 distinct cell attachment sites exist, by demonstrating that, in rat FN, the expression of a second RGD sequence is controlled by alternative mRNA splicing. McCarthy *et al*[90] also found that melanoma adhesion to intact FN occurs as a result of multiple, discrete, adhesion-promoting domains, which interact with multiple receptors on the surface of the melanoma cell.

1.9.4 Fibronectin receptors

Cell adhesion and the many other processes which FN is involved in , require that there is a means by which FN can interact with the cell surface. These reactions are

mediated by cell surface receptors. The putative receptor for FN is a membrane-associated glycoprotein complex of 140kD which has been characterised and implicated in cell adhesion to extracellular FN[91]. Brown & Juliano[92] used monoclonal antibodies to this 140kD glycoprotein (gp140) to inhibit the adhesion of chinese hamster ovary cells (CHO) to FN-coated substrata. They also found that adhesion to substrata coated with other adhesive molecules such as vitronectin (VN), laminin and serum was not inhibited. Thus they concluded that gp140 is involved in the FN-mediated adhesion mechanism, but not other adhesion processes. The same workers went on to show that gp140 is expressed on the surfaces of several human cell lines, a non-adhesive human lymphoid line, but not on erythrocytes. Analysis of gp140 on sodium dodecyl gels (SDS-PAGE) revealed 2 closely migrating bands suggesting 2 closely related polypeptides[93].

The 140kD complex has reported roles in other cell processes. Duband *et al*[94], using a combined *in vitro* and *in vivo* approach have located and provided a possible role for gp140 in neural crest cell adhesion and migration in the early vertebrate embryo. They found gp140 in all 3 germ layers (Exoderm, Mesoderm, Endoderm), particularly concentrated at sites of FN enrichment, and as such was found at the base of epithelial cells. Embryonic cell migration was associated with a diffuse organisation of gp140 over the cell surface, whereas in stationary, adhered cells, gp140 was linked to the cytoskeleton at anchorage sites. During morphogenesis and cytodifferentiation of chick lung cells, Chen *et al*[95] found coupled expression and colocalisation of gp140 and FN. During the formation of airways and alveolar tissues gp140 was concentrated at basal surfaces of epithelial cells adjacent to localised concentrations of FN This colocalisation was identical in

the angiogenesis of lung mesenchyme. They conclude that gp140 is a highly active participant in cell-matrix adhesion during morphogenesis and cytodifferentiation of avian lung cells. In the Amphibia, it has been demonstrated that gp140 plays a significant part in cell adhesion and cell migration during gastrulation in *Pleurodeles waltlii*[74]. These workers also identified 3 major polypeptides of apparent molecular weights,140000, 180000,and 90000 which make up the gp140 complex. Recent evidence suggests that there is more than one receptor which mediates cell adhesion. Buck & Horwitz[96] have identified a transmembrane glycoprotein complex involved in FN-mediated adhesion structures in chick cells. Genetic analysis has shown that this receptor, termed integrin, is a member of a supergene family of cell adhesion receptors which play vital roles in cell migration, cytodifferentiation, and embryonic development, as previously discussed. Tomaselli *et al*[97] have discovered integrin-related glycoproteins which are involved in attachment and growth of a neuronal cell line in rats.

1.10 Fibroblasts and wound healing

Wound formation and repair have early origins in human history[98], and the management of these phenomena still occupy an important place in modern medicine.

The host reaction to wound formation is well established. Briefly, the damaged tissue is invaded by plasma components (e.g. fibrin) and cellular components (e.g. platelets, neutrophils) which combine to provide an effective barrier against infection. Once these barriers have been established, repair of the damaged tissue

begins with the formation of granulation tissue, the functions of which are firstly, the synthesis of new connective tissue, and secondly, the contraction of the wound site.

After the inflammatory events have occurred, the wound site is invaded by fibroblasts, which together with the ingrowth of the capilliary network complete the healing process. The origin of wound fibroblasts has yet to be demonstrated unequivocally. Silver[99] maintains that wound fibroblasts originate from surrounding tissues, whilst other researchers conclude that blood monocytes undergo metaplastic transformation and become the cells of the origin of the fibroblasts[100,101]. Regardless of their origins, wound fibroblasts proliferate and migrate during wound healing.

Collagen types I & III compose the newly synthesised connective tissue, and the importance of collagen for fibroblast motility has been demonstrated[102] by showing the chemotactic attraction of fibroblasts to types I, II & III. Collagen is essential to the wound healing process by encouraging the migration of fibroblasts into the injury site.

The contractile properties of granulation tissue can be attributed to the conversion of normal tissue fibroblasts into the contractile cells of the granulation tissue called myofibroblasts. These myofibroblasts differ from normal fibroblasts in many respects, particularly - structure, composition and function.

1.10.1 The normal fibroblast

In the normal fibroblast, the nucleus is large, containing one or more nucleoli, typical of active synthesis. Rough endoplasmic reticulum is prominent, and again indicative of active protein synthesis. The Golgi apparatus is prominent and collagen is stored here prior to secretion[103a]. Very few cytoplasmic microfilaments or intermediate filaments are seen in normal fibroblasts, and these are located close to the plasmalemma[103b]. In the normal tissues of adult humans and animals there are no cell-cell contacts, although tight junctions exist in the fibroblasts of embryonic tissues[104].

1.10.2 The cultivated fibroblast

There are great differences between the fibroblasts of normal tissue and fibroblasts that are cultured *in vitro*. In particular, differences are remarkable with regard to the content and organisation of filaments and contractile proteins. As previously mentioned, filaments are sparsely distributed, and immunoflourescent staining of normal tissue with anti-actin, anti-myosin or anti-tubulin antibodies does not show reaction of such antibodies to tissue fibroblasts[105]. In contrast, the staining of cytoskeletal proteins in cultivated fibroblasts results in a typical pattern of fixation, indicating highly organised participation of these proteins in various cellular functions[106,107,108]. The cytoplasmic representations of this protein configuration are "stress fibres", and it has been shown that the localisation of actin and myosin corresponds to these fibres[108]. The other main feature of cultured fibroblasts which are not found in normal fibroblasts are gap junctions[109] which are pathways for intercellular communication. The presence of these low resistance

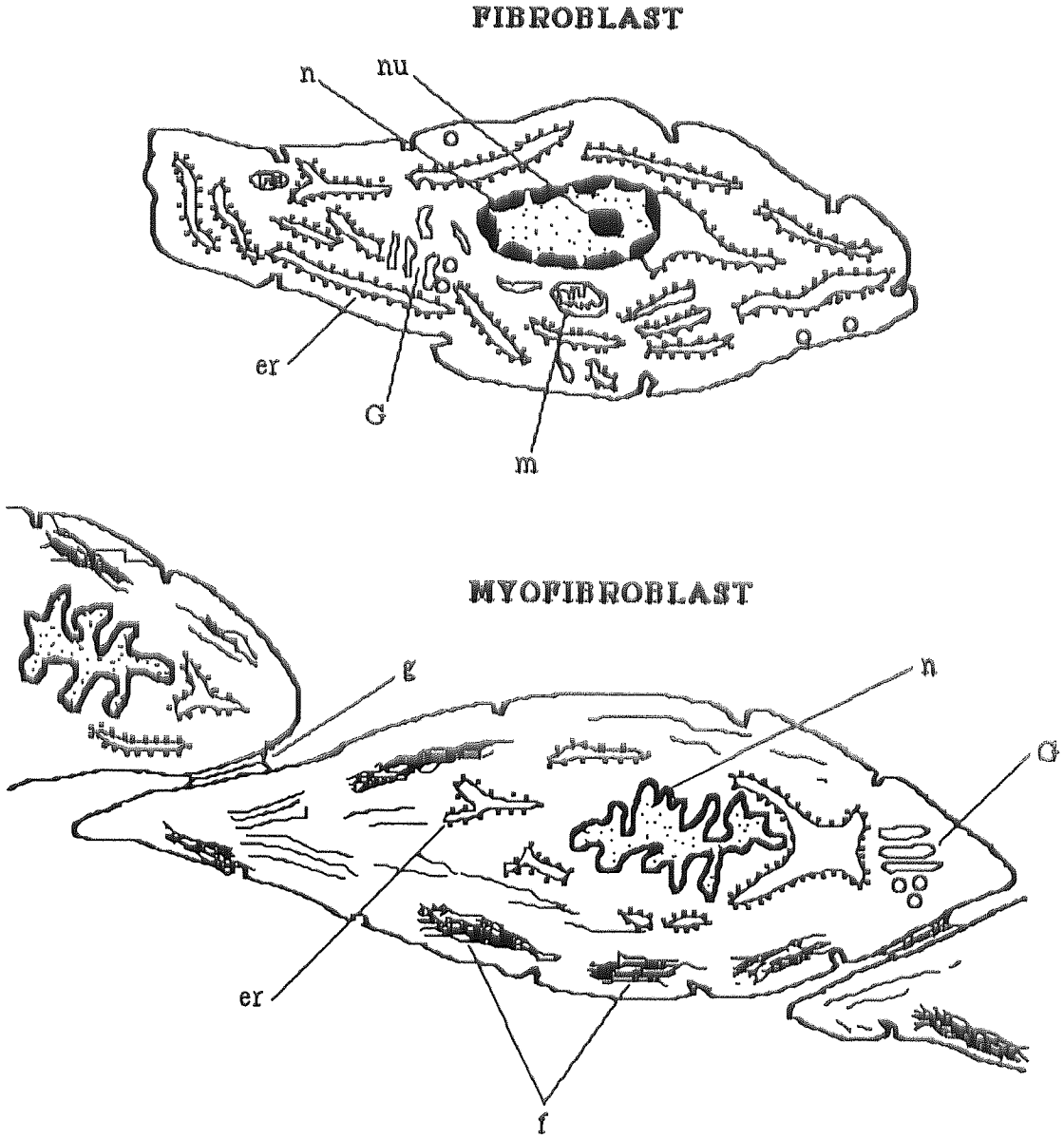
channels indicates that cultivated fibroblasts are probably metabolically linked.

1.10.3 The myofibroblast

As granulation tissue forms, so the fibroblasts acquire structural, chemical and functional differences that clearly distinguish them from normal fibroblasts. A system of fibrils develops within the cytoplasm[110] consisting of bundles of parallel fibrils resembling those of smooth muscle cells, which are oriented parallel to the long axis of the cell. The nuclei have multiple indentations, quite dissimilar to the nuclei of normal fibroblasts. There are numerous intercellular connections between myofibroblasts which take the form of gap junctions and tight junctions, with the former being far more abundant than the latter. In addition, part of the cell surface is often covered by a basal lamina, beneath which the cell often shows dense zones of fibrillar bundles reminiscent of hemidesmosomes. Granulation tissue behaves like smooth muscle when subjected to the same pharmacological stimuli which contract and relax smooth muscle[111,112]. Serotonin, angiotensin and vasopressin induce contraction, whilst paperavine and prostaglandins E_1 & E_2 induce relaxation.

These data suggest that myofibroblasts can contract either spontaneously, or in response to endogenous mediators so causing the tissue to shrink. Thus, granulation tissue is a contractile organ, which is of prime importance when closing off a wound site.

Figure 1.2 Diagram of fibroblast types (after Gabbiani[105]).



Key:

er	rough endoplasmic reticulum
f	fibrillar bundles
g	gap junction
G	Golgi apparatus
n	nucleus
nu	nucleolus
m	mitochondria

1.11 The cell as a surface "probe".

As mentioned previously, there are many physico-chemical factors which influence the biological response to a synthetic material. This has led to a multi-disciplinary approach to solving the problems encountered in biomaterials research. As a result, the overall view of biological interactions with synthetic materials is confusing.

The complete characterisation of a new material requires the integration of many techniques. Even a complete characterisation cannot predict how a potential biomaterial will perform in a host. Thus, the *in vitro* cell culture model becomes a powerful investigative tool when determining the viability of a new material for biomedical use. The integration of modern polymer chemistry and cell biology enables directed research into the fundamental questions of why certain materials are biocompatible, and others are not. An understanding of some aspects of polymer chemistry enables cell biologists to investigate a wider range of substrates with carefully controlled surface properties. An inter-, rather than multi-disciplinary approach is the way to achieve cohesive results; incorporating the chemical and structural elements of polymer science, and the subsequent interfacial phenomena of cell biology.

In the light of this method of approach, Minett[53] conducted a series of experiments to determine the response of several cell types to a unique family of polymers known as hydrogels. Hydrogels are water-swollen polymer networks produced by the polymerisation of individual monomers e.g. poly (2-hydroxyethylmethacrylate) (poly-HEMA), or from the copolymerisation of two or more comonomers as in the

case of poly (acrylamide/methacrylic acid) (poly-ACM/MAA). Since water is an essential structural element of hydrogels, they are conveniently characterised in terms of their ability to absorb water up to an equilibrium point. This property is termed the equilibrium water content (EWC).

Minett[53] studied a range of hydrogels, mostly based on poly-HEMA, with varying surface and bulk properties. He found that the EWC played an important part in determining cell behaviour. Hydrogels in the range 2-35% EWC did support high levels of cell attachment and spreading, but this level of adhesion dropped off markedly at an EWC of 35-60%. Thereafter the cell adhesion increased slowly in the 60-90% EWC zone. He thus concluded that EWC is the most important factor influencing cell adhesion on hydrogels.

However, Minett's observations[53] in the regions of high water content (his 60-90% EWC zone) were based on results obtained with a randomly chosen group of polymers not based on the poly-HEMA system he used for most of his work. Following on from Minett's research[53], Thomas[35] set out to explore the effects that extremely high water content hydrogels have on cell adhesion. She found that at these elevated levels of EWC the dominant water structuring group, which structures water at the surface, can override the effects of EWC alone. She also pointed out that Minetts results, summarised in Figure 3.25 were due to the polymers he used. The HEMA based hydrogels produced a distinctive "hema curve", where levels of cell adhesion rapidly curtailed at ~35% EWC. These hydrogels have hydroxylated surfaces, and it is the hydroxyl groups which act as

the dominant functional group. Minett's high water content results[53] were based on polymers containing different dominant functionals. Using three separate water structuring groups, Thomas[35] was able to produce a shift to the right of the hema curve for each of the three groups. These results are summarised in Figure 3.26.

The relationship between cell adhesion and surface characteristics is ideally addressed by the inter-disciplinary approach exemplified by Minett[53], and later Thomas[35]. Therefore an *in vitro* cell culture model was designed for the systematic assessment of a broad spectrum of materials including high water content hydrogel copolymers containing charged species; and a novel biosynthetic polymer poly (ß) hydroxybutyrate (PHB).

1.12 Scope of this study

1. Hydrogels are a group of polymers which are unique in that they contain a large amount of water which is bound up in the polymer matrix. From a cell biology viewpoint, these polymers are interesting because the way in which they structure water at their surfaces means that the adsorption of adhesive proteins can be promoted. This process of interface conversion greatly influences the adhesion of anchorage-dependent cells, such as the mammalian fibroblasts used in this study.

2. To observe the *in vitro* mammalian fibroblast response to a novel biosynthetic copolymer poly (ß) hydroxybutyrate-(ß) hydroxyvalerate, and its blends with polysaccharides. These thermoplastics may have uses as biodegradable surgical fixation devices, thus the elucidation of their interactions with fibroblastic cell lines is required, as cell attachment is a desirable property for this clinical situation. Degradation of these copolymers is mediated via hydrolysis and enzyme lysis. Hydrolysis is likely to be the major degradation mechanism even *in vivo*, so an *in vitro* study of the ways in which degradation of PHB-HV copolymers affects cell behaviour is essential. Water structuring at the polymer surface is therefore seen to be a major factor, which will influence cell behaviour.

3. The final section of this thesis is concerned with the application of a fibroblast cell culture model to a potential biomedical device. Using this

model, a preliminary assessment of the human and mammalian cytocompatibility of gel-spun poly (ß) hydroxybutyrate homopolymer, with a view to its use as a wound scaffold is undertaken. Studies of hydrogels and PHB-HV copolymers produced in these laboratories provide very useful information which can be applied directly to commercial products. The elucidation of the surface characteristics which influence cell behaviour in model systems can be used to tailor so called "designer" biomaterials; that is to say, materials that have particular properties which make them ideally suited to a particular clinical application.

CHAPTER TWO

MATERIALS AND EXPERIMENTAL METHODS

2.1 Polymers

The polymers used in this study were synthesised at the University of Aston[113,114,115]. They comprised a range of high water content hydrogels including N-vinylpyrrolidone (NVP) and N'N' dimethylacryamide (NNDMA) copolymerised with lauryl and methyl methacrylates (LMA & MMA); acrylamide (ACM) copolymers with aminoethylmethacrylate (AEMA) and methacrylic acid (MAA) incorporated into the polymer matrix; and also, a range of poly (ß-) hydroxybutyrate (PHB) -(ß)-hydroxyvalerate (HV) copolymers.

2.1.2 PHB-HV copolymers

Polysaccharide fillers were incorporated into the PHB-HV copolymers to produce the following compositions:

12%HV/10% Blends	**20%HV/10% Blends**
12HV/10% Amylose	20HV/10% Amylose
12HV/10% Dextran	20HV/10% Dextran
12HV/10% Dextrin	20HV/10% Dextrin
12HV/10% Sodium Alginate	20HV/10% Sodium Alginate

12%HV/30% Blends	**20%HV/30% Blends**
12HV/30% Amylose	20HV/30% Amylose
12HV/30% Dextran	20HV/30% Dextran
12HV/30% Dextrin	20HV/30% Dextrin
12HV/30% Sodium Alginate	20HV/30% Sodium Alginate

In addition, 1% Norwegian talc nucleated PHB-HV copolymers which had no polysaccharide incorporated in them were used as control substrates. These were 12%HV/1%NT and 20%HV/1%NT respectively.

2.1.3 PHB "wool"

Gel spun PHB "wool", extracted from chloroform or dichloromethane production solvents, was obtained from Marlborough Biopolymers Ltd. (courtesy of CWHRI).

2.1.3.1 Acid Washed "wool" Production

Samples of PHB "wool" were subjected to the following protocol to produce acid washed surfaces:

Treatment 1 Wool washed in 1% H_2SO_4 solution @ ambient temperature for 10 mins.

Treatment 2 Wool washed in 3% H_2SO_4 solution @ ambient temperature for 10 mins.

Treatment 3 Wool washed in 10% H_2SO_4 solution @ ambient temperature for 10 mins.

Treatment 4 .Wool washed in sterile water @ ambient temperature for 10 mins as a control.

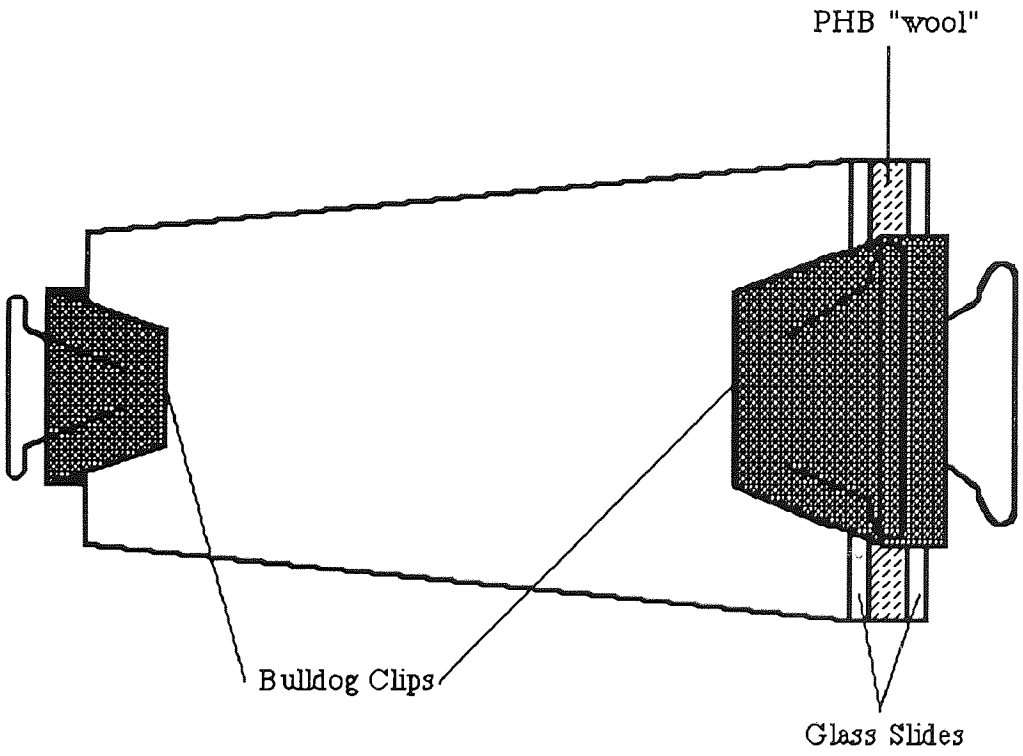
Following treatments, all samples were thoroughly washed in sterile distilled water (3x10 mins), and finally rinsed in PBS (with Ca^{2+}, Mg^{2+}) (PBS+).

2.1.4 PHB Non-woven mat production

The production techniques involved melt processing in a vacuum oven. Two layers of wool were teased apart and placed on clean glass microscope slides (76x25mm).

The 2 slides were placed together, sandwiching the 2 layers of wool between, and clamped using bulldog clips. These samples were then placed in a preheated vacuum oven (Gallenkamp) for a range of time periods. After several time periods had been tried, the best mats were produced by incubation at 180°C for 5 mins. The fibres were well bound together, producing an integral, homogeneous surface with reasonable tensile strength.

Figure 2.1: Apparatus for the production of PHB NWM's



2.1.4.1 Acid washing of PHB Non-woven mats

Acid Washing Protocol

1. Mats sterilised by autoclaving.
2. Small discs cut using aseptic technique.
3. Acid solutions sterilised by filtration (0.2µm Dynaguard filter).

4. Samples placed in sterile universals with appropriate acid treatment.
5. Universals placed in 60°C water bath for 1 hour.
6. Samples removed and thoroughly rinsed in sterile PBS(+) (3x10 mins.).

Treatments

Two types of NWM were tested. One mat was produced from PHB "wool" extracted from chloroform; and one mat was produced from PHB "wool" extracted from dichloromethane. The NWM's were then subjected to various acid wash treatments.

Treatment 1: NWM (Dichloromethane) washed in 10% H_2SO_4 @ 60°C for 60 mins.

Treatment 2: NWM (Dichloromethane) washed in 3% H_2SO_4 @ 60°C for 60 mins.

Treatment 3: NWM (Dichloromethane) washed in 1% H_2SO_4 @ 60°C for 60 mins.

Treatment 4: NWM (Chloroform) washed in 10% H_2SO_4 @ 60°C for 60 mins.

Treatment 5: NWM (Chloroform) washed in 3% H_2SO_4 @ 60°C for 60 mins.

Treatment 6: NWM (Chloroform) washed in 1% H_2SO_4 @ 60°C for 60 mins.

2.1.4.2 Alkali washing of PHB Non-woven mats

Using the alkaline buffering system of Yasin[114] it may be possible to produce a surface which will promote cell adhesion. The alkaline treatment was likely to be harsher and less controllable than the acid washes, and significant alterations to the surface morphology were expected.

The buffering system of Yasin was used in his studies of accelerated degradation of

PHB and is designed to produce pH 10.6 @ 60°C.

pH10.6 Buffering System:

The buffer consists of:

Compounds	$Na_2CO_3.10H_2O$	/	$NaHCO_3$
Ratio	9		1

Added to 1 litre of sterile distilled water and mixed thoroughly.

Alkali washing Protocol.

1. Mats sterilised by autoclaving.
2. Small discs cut using aseptic technique.
3. Alkali solutions sterilised by filtration (0.22µm Millipore filter).
4. Samples placed in sterile universals with appropriate alkali treatment.
5. Universals placed in 60°C water bath for 1 hour.
6. Samples removed and thoroughly rinsed in sterile PBS (3x10mins.).

Treatments: Once again, mats extracted from both chloroform and dichloromethane solvents were tested.

Treatment 1: NWM (Dichloromethane) washed in pH 10.6 buffer @ 60°C for 60 mins.

Treatment 2: NWM (Chloroform) washed in pH 10.6 buffer @ 60°C for 60 mins.

Treatment 3: NWM (Dichloromethane) washed in sterile water @ 60°C for 60 mins.

Treatment 4: NWM (Chloroform) washed in sterile water @ 60°C for 60 mins.

Post-treatment, samples were washed thoroughly in sterile water (3x10mins), and

finally rinsed in PBS(+).

2.2 Polymer sterilisation

It was essential that polymers being investigated were sterile, as the presence of contaminating agents such as bacteria and fungi would greatly affect the growth of all cell lines by using up metabolites, secreting toxins etc. The following protocols were used for the sterilisation of polymer samples.

2.2.1 Hydrogels

Hydrogels were sterilised by washing in a 1% Tween 20 solution for 60 minutes. Samples were then thoroughly rinsed in sterile Dulbecco's PBS (w/o Ca^{2+}, Mg^{2+}). In addition, Hydrogels were autoclaved for 15 mins @ 121°C & 3bar in a Fluidette autoclave Samples were then equilibrated overnight in Dulbecco's PBS (with Ca^{2+}, Mg^{2+}) prior to testing.

2.2.2 PHB-HV copolymer plaques

PHB plaques were subjected to the same washing regime as the hydrogel samples, but were not autoclaved, as this proved too harsh a sterilisation technique and caused the PHB to begin degrading.

2.2.3 PHB Non-woven mats

NWM's were sterilised by the autoclaving procedure outlined above.

2.3 Polymer testing

Cell adhesion assays were carried out over six hour or eighteen hour incubation periods according to the following protocols.

2.3.1 Hydrogel adhesion assays

1cm diameter discs of each hydrogel were placed in individual, discrete wells of a 24 well TC plate. Samples were then seeded with 1ml of cell suspension at a cell seeding density of $1x10^6$cells/ml. The dishes were then incubated at 37°C and 5% CO_2 for a period of 6 hours to allow cell attachment to occur. As controls, 1cm discs were cut from presterilised BACTY (Sterilin) and TC grade (Falcon) petri dishes using aseptic technique. Post incubation, samples were removed from the incubator, rinsed for 1 min. in Dulbecco's PBS(-),and then placed in fresh multiwell plates with 1ml 2.5%trypsin/EDTA solution. Once cell removal had been effected, hydrogel surfaces were checked using an inverted light microscope (Olympus) to determine whether trypsinisation was successful.

2.3.2 PHB-HV copolymer adhesion assays

Polymer squares (1x1cm) were cut from the melt processed plaques produced in these laboratories and placed in individual wells of a 24 well TC plate. They were then seeded with 1ml of cell suspension @ $1.0x10^5$ cells/ml. The plates were then placed in a CO_2 incubator for 18 hours @37°C and 5%CO_2 atmosphere. Post incubation, samples were rinsed in PBS(-), placed in fresh 24 well TC plates with 1ml of 2.5% trypsin/EDTA solution and incubated @ 37°C to effect cell removal.

2.3.3 Acid washed PHB "wool" cytocompatibility testing

Sub-confluent NOM 238 cells were harvested using 2.5% trypsin/EDTA, and inoculated onto the samples in 24 well TC plates (Costar) at a seeding density of $1x10^5$ cells/ml. The samples were incubated for 18 hrs @ 37°C and 5% CO_2

2.3.4 PHB Non-woven mat cytocompatibility testing

Cytocompatibility testing with NOM 238 and L929 cell lines was carried out in 24 well tissue culture plates (Costar). In an attempt to promote easier SEM processing, sample NWM's were placed in SEM processing "cradles" (see Chapter 2.5 later). These samples were then placed into wells and covered with 2ml of the inoculating cell suspension at a density of $1x10^5$ cells/ml. The samples were then incubated for 18hrs at 37°C in a 5% CO_2 atmosphere. Post incubation, samples were rinsed for 1 min. in PBS(-) and then prepared for SEM.

2.4 Cell counting

Cell counts were determined by using either an improved Neubauer haemocytometer or by a Coulter counter. After harvesting the cells, the polymers were removed and rinsed with PBS to a final volume of 10ml. Then, 0.5ml aliquots of this cell suspension were diluted 40-fold in Isoton II electrolyte solution and cell counts were measured twice in the Coulter Counter.

2.5 Viability tests

The Trypan Blue exclusion test was employed to determine the viability of cells immediately prior to and post testing. The basis for this test is the fact that viable

cells can exclude the dye whereas dead cells cannot therefore, their cell walls take up the dye and they stain blue. 0.1ml of a 0.4% Trypan Blue solution (Sigma, U.K.) were added to 1ml of cell suspension, cell counts taken in a haemocytometer, and the percentage of viable cells calculated.

2.6 Cell culture

Three cell lines were used in this study.

Description	Derivation
BHK-21/Clone 13	Pooled kidneys from 1 day old Syrian hamsters (Flow Laboratories, Irvine, UK)
NCTC Clone L929	Mouse areolar and adipose tissue. (Flow Laboratories, Irvine, UK)
NOM 238	Normal Oral Mucosa epithelial fibroblasts obtained from a patient in a Manchester hospital (courtesy of CWHRI).

All cell lines were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% Fungizone, and 0.5ml Gentamycin. Cells were passaged when confluent using a split ratio of 1:6 for the mammalian cell lines, and 1:3 for the human cell line. Cells were harvested using a 2.5% trypsin/EDTA solution. The action of trypsin was then arrested using a ten-fold dilution with fresh medium, and then cells were centrifuged in polystyrene centrifuge tubes (Sterilin, U.K.) @1500rpm for three minutes. The supernatent was pipetted off, and replaced with fresh medium. Cell pellets were then disaggregated using a Pasteur pipette. Fresh TC flasks

(Falcon) were seeded with cell suspension, and medium pipetted in to a final volume of 25ml per 75cm^2 flask. Cell lines were incubated @37°C in a 5% CO_2 atmosphere in a Gallenkamp Plus CO_2 incubator. All media and supplements were purchased from Gibco Life Technologies, Paisley, U.K., unless otherwise stated.

2.7 Scanning electron microscopy (SEM)

Samples were subjected to the following protocol in preparation for SEM:

1. Fixed in 2% glutaraldehyde (Sigma, U.K) in 0.1ml sodium cacodylate buffer at pH 7.4 for 30 mins.
2. Rinsed in 0.1ml sodium cacodylate buffer (Sigma, U.K.) at pH 7.4 for 30 mins.
3. Dehydration through graded ethanol/water series:
   - i. 50% EtOH/H_2O for 30 mins.
   - ii. 70% EtOH/H_2O for 30 mins.
   - iii. 90% EtOH/H_2O for 30 mins.
   - iv. 95% EtOH/H_2O for 30 mins.
   - v. 100% EtOH for 3x30 mins.
4. Critical Point Dried under liquid CO_2 in a Polaron E3100 II.
5. Samples were mounted on aluminium SEM stubs (Biorad,U.K.) using "UHU" adhesive, and left to dry overnight in a desiccator.
6. Samples were coated with gold in a Polaron sputter coating unit at 1kV and 20mA.
7. Samples were examined in either a Phillips SEM, or a Cambridge Stereoscan at accelerating voltages of 15-25kV.

In order to facilitate easier SEM processing of the samples, "cradles" were designed to hold the discs of PHB fibres (Figure 2.3.). This had the advantage that once cradled, the samples were subject to much less flexing during the rigorous SEM processing, which has caused cells to detach from the fibres[116]. The cradles were made from small screw top polypropylene vials (Sarstedt, FRG). A hole was cut in each top of the vials with a flamed size 2 cork borer. This was necessary to enable the cell suspension to flow freely through the sample. Sample fibres were placed across the open end of the vial, and the screw top put on thus securing the sample ready for incubation. The conical bottoms of the tubes were cut away, so that each cradle consisted of the screw top and the screw thread, with the sample sandwiched between. Post incubation, cradles were removed from cell suspension and placed in fresh wells and rinsed with PBS(-) to remove any remaining cell suspension. Samples were then subjected to the standard SEM preparation protocol above.

2.8 Tensile testing of PHB NWM's

The tensile properties of the manufactured mats were investigated using a Houndsfield HTi tensometer interfaced with an IBM 55SX personal computer. Dumbell shaped specimens of gauge length 8mm and width 3.3mm were cut from mats in preparation for testing. The samples were tested to break at an extension rate of 20mm/min, and the values of tensile strength and elongation to break were calculated from an average of four tests.

Table 2.1: Results of tensile testing of chloroform extracted PHB Non-woven mat.

PHB NWM	Ts/MPa	Eb%
3mins @ 180°C	0.4+/-0.2	48.8+/-3.93
5mins @ 180°C	0.7+/-0.5	39.0+/-6.72
7mins @ 180°C	14.62+/-6.68	14.92+/-3.03

Table 2.2: Results of tensile testing of dichloromethane extracted PHB Non-woven mat.

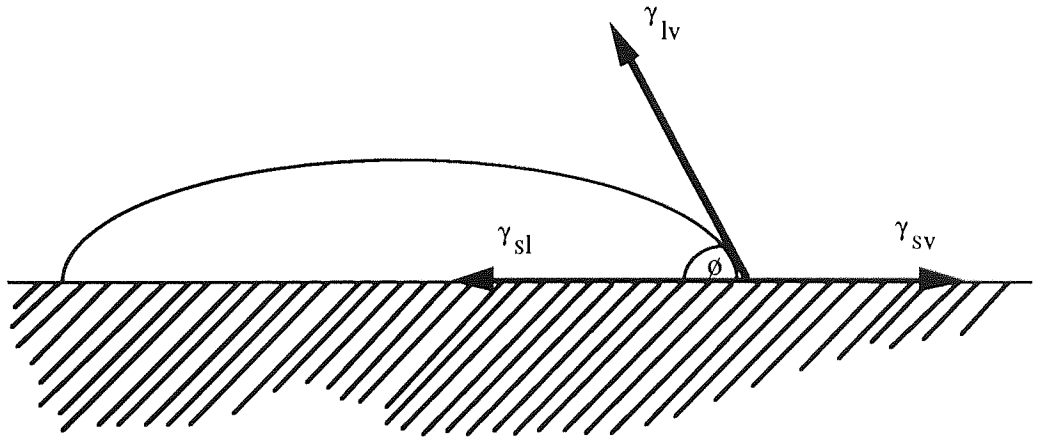
PHB NWM	Ts/MPa	Eb%
3mins @ 180°C	0.6+/-0.2	42.2+/-3.93
5mins @ 180°C	1.1+/-0.5	28.1+/-4.34
7mins @ 180°C	16.62+/-6.2	15.31+/-2.56

Looking at the above results, there is a general trend in that the tensile strength (Ts) of both chloroform & dichloromethane extracted NWM samples increases with increasing incubation times. As the mats remain in incubation for longer periods of time, so the degree of melt processing between adjacent fibres will increase, and greater fusion over the mat as a whole will occur. The sharp increase in tensile strength at the 7 min. interval is explained by the fact that after this amount of incubation, the PHB is no longer a NWM, but has completely melted to form a homogenous, transparent film. Associated with this homogeneity is a greatly increased tensile strength.

2.9 Contact angle measurements

Contact angle measurements were carried out on the polymers tested in this thesis by Baker[113], Yasin[114], and Corkhill[115] . This technique serves to evaluate the surface energy and wettability of materials[117]. The underlying principle is that most liquids will spread on contact with a solid. The extent of spreading is determined by the balance of forces at the interface between liquid, solid and air.

Figure 2.2 The forces involved in determining the contact angle of a liquid drop on a solid surface.



Owens and Wendt[118] have described the balance of forces by the following equation:

$$\text{Cos } \theta.\gamma_{lv} = \gamma_{sv} - \gamma_{sl} - \pi_e \qquad (1)$$

where γ_{lv}, γ_{sv}, and γ_{sl} are the free energies of the liquid and solid against their saturated vapour, and of the interface between solid and liquid respectively; and π_e

is the equilibrium pressure on the solid. θ is the angle of contact between a liquid droplet and a planar solid surface. When $\theta=0$, the liquid is considered to completely wet the solid; and when $\theta\neq0$, the surface is less wettable. The wettability of a material is favoured by low interfacial free energy, high solid surface free energy, and low liquid-surface free energy. Equation (1) can be further modified to calculate the value of θ as follows:

$$\text{Cos } \theta+1=2/\gamma_{lv} \{(\gamma^d_l \gamma^d_s)^{1/2} + (\gamma^p_l \gamma^p_s)^{1/2}\} \qquad (2)$$

where: γ^d_l is the dispersive component of the liquid

γ^d_s is the dispersive component of the solid

γ^p_l is the polar component of the liquid

γ^p_s is the polar component of the solid

Drops of the wetting liquids (water and methylene iodide) were placed onto separate areas of the substrate. Contact angles were measured using a calibrated eyepiece, and from the mean values of both wetting liquids, the polar and dispersive components were calculated from equation (2).

2.10 Goniophotometric analysis

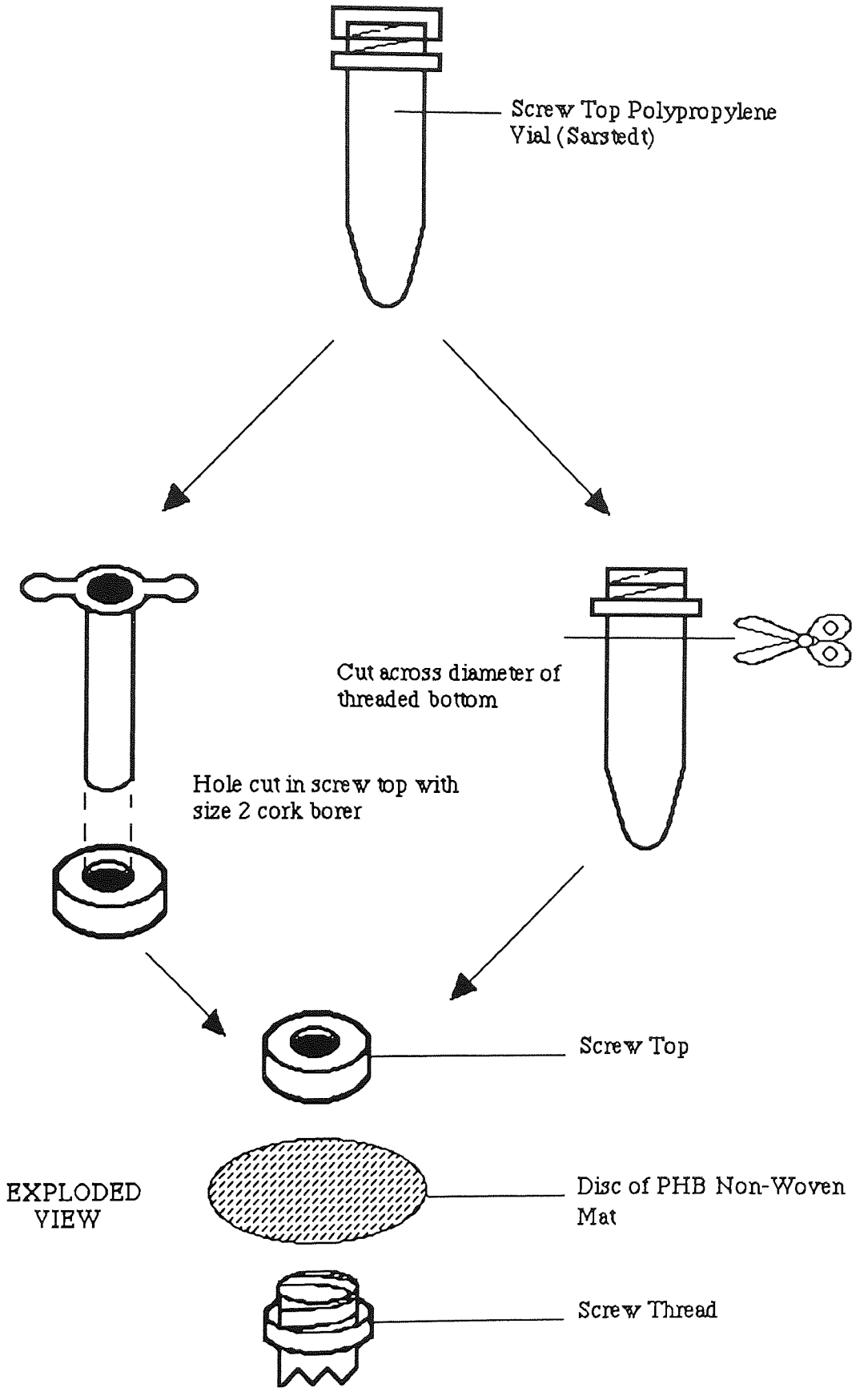
Goniophotometry is a non-destructive, comparative technique for monitoring the changes in the surface gloss of a surface. It is a quantitative technique, and is therefore a very useful way of investigating changes in the surface topography of materials. All Goniophotometric analyses of PHB-HV copolymers were carried out

by Yasin[114]. The technique involves a collimated beam of monochromatic light impinging at an angle of 45° onto the sample surface, combined with a rotating photocell which records the intensity of scattered light as a function of scattered angles.

2.11 Statistical analysis

Error bars on all graphs represent the standard errors of the means of the cell count data. Preliminary analyses of the cell count data were carried out by means of a single factor analysis-of-variance (anova), with the F statistic used as a test of significance at the 95% limit. The between cell line comparisons were carries out by means of a two-factor anova. Comparisons between treatment means were performed using Fisher's Protected Least Significant Difference method at a significance level of 95%[119].

Figure 2.3: Apparatus for the production of PHB NWM's (overleaf).


Screw Top Polypropylene Vial (Sarstedt)
Cut across diameter of threaded bottom
Hole cut in screw top with size 2 cork borer
Screw Top
EXPLODED VIEW
Disc of PHB Non-Woven Mat
Screw Thread

CHAPTER THREE

CELLULAR INTERACTIONS WITH SYNTHETIC HYDROGELS

Chapter Three: Prelude

The primary function of the biologist in biomaterials research is to elucidate the ways in which biomaterials interact with the host system. A simple, elegant means of doing this is by the use of *in vitro* cell culture techniques. Cell biology provides a powerful tool which can be used to study the interface between a synthetic material and the biological system. Cells can differentiate between small changes in surface characteristics which cannot be detected by other means. In addition, the ways in which cells respond to a surface determine what clinical situation it will be most applicable to. Single cell culture models also have the advantage that the effects of single factors can be isolated and studied far more easily than is the case with *in vivo* experimentation. The fact that the cell lines used in this study are clonal means that the biological variation experienced in complex mammalian systems is avoided.

Most cell culture systems use surface treated polystyrene as the culture substrate, as cells show very favourable attachment and spreading on this material. The surface treatment involves the oxidation of the surface so that cell adhesion is promoted because of enhanced interface conversion. This enhanced protein deposition is produced by the expression of charged groups produced at the surface as a result of the oxidation process. Polystyrene is not an ideal biomaterial for all cases however. It could not be used for contact lenses for instance because amongst other things, it is not oxygen permeable, and it does not have the requisite tensile properties for this most delicate of body sites. Hydrogels on the other hand are more mechanically suited to body sites by virtue of their relatively high water content, and are well

suited to use as contact lenses. The application of the lessons learned from surface treatment of tissue culture polystyrene to hydrogel polymers will produce a novel range of materials with exciting properties which may lead to the commercial production of new contact lenses as well as other useful biomaterials. This chapter aims to extend the knowledge of cellular interactions with hydrogels by looking at the interaction of mammalian fibroblasts with a broad spectrum of hydrogels which have very different chemical groups expressed at the surface, and which have very high water contents.

3.1 Introduction

Hydrogels are a group of water swollen polymer networks which occur both naturally, (for example cartilage), and can be produced synthetically by conventional polymerisation techniques. Modern polymer chemistry has enabled the development of a range of synthetic hydrogels with carefully designed properties for use as potential biomaterials. Synthetic hydrogels may have uses as contact lenses[120], liver support systems[121], drug delivery systems[122], replacement blood vessels[123], and as wound dressings[124].

The structural nature of of hydrogels means that they are generally well tolerated *in vivo*. Their high water content gives them a superficial resemblance to body tissues, and the water structured at the surface of the polymer transports dissolved species and also bridges any differences in surface energy between the polymer and the host system. It may be said then, that synthetic hydrogels have favourable interfacial properties which make them potentially very useful as biomaterials.

Since the water content of hydrogels is a characteristic of paramount importance, a quantitative measure of the amount of water absorbed by the hydrogel network is pertinent and necessary. A common measure of water absorption is the equilibrium water content (**EWC**). This is the ratio of the weight of water in the hydrogel to the weight of the hydrogel at equilibrium hydration expressed as a percentage[125].

$$EWC = \frac{\text{weight of water in the gel}}{\text{total weight of hydrated gel}} \times 100\%$$

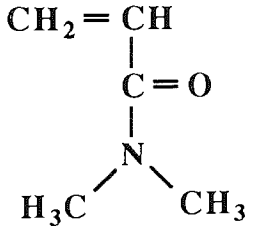
The EWC serves as an indication of the hydrophilicity and polarity of a hydrogel. As the surface polarity of a hydrogel increases, so it becomes more wettable. Non-polar polymers such as native polystyrene are not wettable but can be rendered so by the introduction of charged species[35].

Previous research in these laboratories has produced an overview of the interactions of mammalian cells with hydrogels[35,53]. The earlier work of Minett[53] indicated that the EWC was the prime factor influencing cell behaviour on hydrogels. His studies used polyHEMA in which the EWC was reduced by the introduction of hydrophobic groups such as styrene, MMA and EMA. With this range of copolymers he showed that there was a uniform zone of cell adhesion between 5-30% water content, with the rapid curtailment of cell adhesion at the water contents greater than 35%. This non-adhesive zone extended to ~60% water content, at which point cell attachment increased again, although there was no return to a fully spread morphology (Figure 3.25). However, the results obtained were based on observations made with a limited number of higher water content hydrogels available to him at the time.

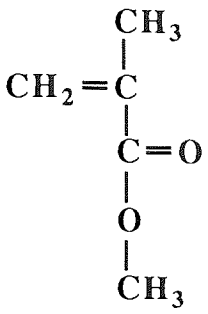
Thomas[35], using a broader range of hydrogels in her studies was able to offer a new interpretation of Minett's data. Using a range of copolymers of NVP & NNDMA with MMA & LMA, she found that these hydrogels exhibited the same "hema curve" behaviour as seen by Minett. In her results however, the curves of NVP and NNDMA copolymers were shifted to the right of Minett's polyHEMA hydrogels (Figure 3.26). Since NVP and NNDMA contain different surface

functional groups to polyHEMA, she concluded that the dominant water structuring group can override the effects of EWC and have a greater influence on the cellular response to hydrogels. In addition, using hydrogels containing the charged group methacrylic acid (MAA), she showed that the introduction of charged species to the surface can over-ride the effects of the water structuring complex. It must be emphasised here that although Minett saw adhesion in his hydrogel series rising again with increasing water content, his observations on higher water hydrogels were based on hydrogels which did not have the hydroxyl (-OH) as the dominant functional group, as polyHEMA does.

Thomas's[35] work was based on nitrogen water structuring groups at the high end of the water content scale. The aim of this study is to investigate the cellular interactions with an extended range of low and high water content hydrogels containing the same water structuring groups in an attempt to determine the relative effects of water content and functional groups on cellular response. In order to pursue these aims, a range of hydrogels synthesised in these laboratories were cell tested. The polymers were categorised into "families" on the basis of comonomer content and composition (Tables 3.1 & 3.3). N'N'Dimethylacrylamide (NNDMA) and it's copolymers with lauryl methacrylate (LMA) and methyl methacrylate (MMA) formed Families 1 and 2.

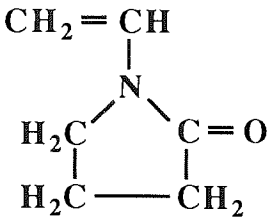


N'N' dimethylacrylamide (NNDMA)



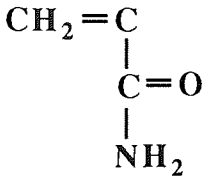
Methylmethacrylate (MMA)

N-vinyl pyrrolidone (NVP) was also copolymerised with LMA and MMA to give Families 3 and 4 respectively.

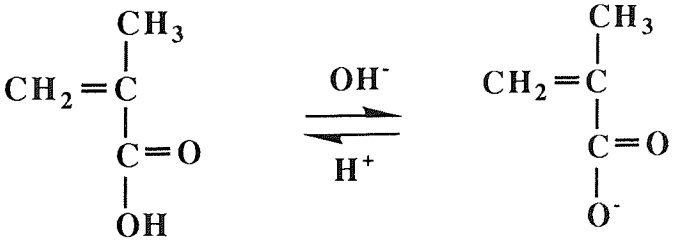


N-vinyl pyrrolidone (NVP)

A series of acrylamide (ACM) copolymers with aminoethyl methacrylate (AEMA) and methacrylic acid (MAA) formed the Families 5, 6 and 7.

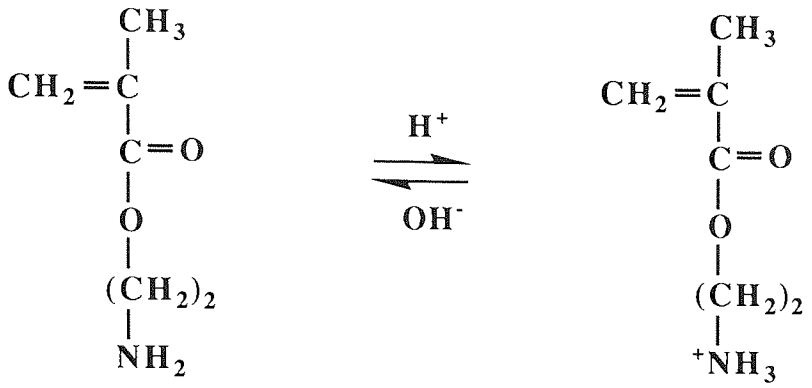


Acrylamide (ACM)



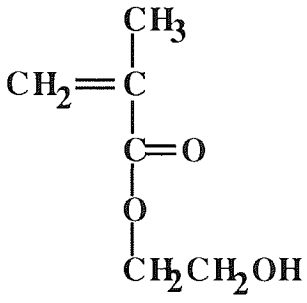
Neutral **Ionised**

Methacrylic Acid (MAA)



Neutral Ionised

2-Aminoethylmethacrylate (AEMA)



2 - hydroxyethyl methacrylate

This structure is synonymous with "hema" and polyHEMA as used in the text.

3.2 Experimental procedure

Hydrogel samples were sterilised by washing in Tween 20, rinsing thoroughly, and finally autoclaving in distilled water. After sterilisation, the samples were placed into multiwell plates under aseptic conditions, covered with cell suspension, and then placed in a CO_2 incubator for 6 hours to enable the cells to attach. Post incubation, samples were removed from the incubator, the cells were harvested using trypsin, and cell counts taken. Polymer surfaces were checked by light microscopy to ensure all cells had been detached from the polymers prior to counting.

3.3 Discussion of results

Using a categorisation scheme similar to that used by Thomas[35], the hydrogels were divided into "families" on the basis of comonomer content and composition. The members of all families were cell tested using both BHK-21 and L929 fibroblasts (Tables 3.1 - 3.4).

In general terms, all seven families supported some cell attachment and spreading, but to varying degrees (Tables 3.1-3.4). For Families 1-4, there is a correlation between functional group and cell attachment (Figures 3.1-3.8), whilst in Families 5,6 &7, the levels of cell attachment are much lower, and cell behaviour is dependent on comonomer ratio - the introduction of charged species at the surface.

The preliminary statistical analysis carried out on the cell count data for both cell lines shows a very highly significant difference ($p=0.0001$) between treatment

means for both cell lines (Appendices 1c & 1d). The conclusion, is that the treatments (i.e. changes in polymer composition) exert a significant effect on cell behaviour.

3.3.1 Cell attachment on NNDMA hydrogels.

For families 1 and 2 (F1 & F2), the levels of cell attachment follow similar trends. As the comonomer content increases, so cell attachment increases (Figures 3.1 & 3.2). Unfortunately, there is only one copolymer ratio common to each family with which to make a comparison; the 50:50 comonomer ratio. For both cell lines, there is a significant difference between the F1 and F2 values for this comonomer ratio (Appendices 3a & 3b). Comparison plots of cell count versus comonomer ratio are presented in Figures 3.17 & 3.20. There is an anomaly in these plots because the numbers of BHK-21 cells attached are greater on the MMA copolymer than the LMA copolymer, whereas the converse is true for L929 cells. The reasons for this are unclear given that the general trend is for higher levels of BHK-21 cell attachment on a surface than L929, for reasons discussed later. The cells are reacting to differences in the substrate surface. It may be that differences in surface parameters occurred during the copolymerisation process, for example, oxidation of the surface. This might lead to the creation of polar domains expressed on the copolymer surface which by chance were utilised for the L929 adhesion assays. It must be stressed however, that both BHK-21 and L929 assays were conducted on samples from the same batch, and processed under identical experimental conditions.

Also included is an 80:20 comparison for F1 and F2, although in point of fact, the F2 comonomer ratio is 85:15. These plots of cell counts were chosen because there are no other comonomer ratios common to F1 and F2 with which to draw comparison. The cell counts for both cell lines are similar (Figures 3.17 & 3.20), and are not statistically different from each other at the 95% limit (Appendices 3a & 3b). At these elevated water contents (Tables 3.1 & 3.2), these two copolymers support little cell attachment and no spreading. This is probably due to the phenomenon of substrate mobility as discussed later.

Following the results of Minett[53] and Thomas[35], plots of cell count versus EWC were produced for F1 and F2, and these are presented in figures 3.5 and 3.6. They show that curves similar to those of Thomas (Figure 3.26) are obtained. It appears that the replacement of LMA with MMA in the copolymer network produces a shift to the right in the cell attachment curve. Cell attachment curtails at slightly higher levels of EWC in MMA copolymers than with LMA copolymers.

The conclusion drawn from the comparisons within NNDMA copolymer families is that cell attachment is low at the higher water contents for F1 and F2, but that elevated levels of cell attachment and the commencement of cell spreading begin to occur as the comonomer ratio rises. In addition, the replacement of LMA with MMA significantly influences cell behaviour.

3.3.2 Cell attachment on NVP hydrogels.

The general trends seen in NNDMA copolymers seem to extend to those hydrogels containing NVP copolymerised with the same comonomers, i.e. families 3 and 4 (F3 & F4). So, as comonomer content increases, so does the level of cell attachment (Figures 3.3 & 3.4). Two comonomer ratios are common to each family; the 80:20, and 70:30 ratios. The plots for both cell lines show similar trends, with the MMA copolymer supporting slightly higher cell attachment than the corresponding LMA copolymer (Figures 3.18 & 3.21). The only statistically significant difference among the copolymers is for BHK-21 tested 70:30 copolymers (Appendix 3a). The graphs of the cell count data (Figures 3.18 & 3.21) indicate differences in cell attachment between the common MMA and LMA comonomer ratios. These differences are outside the range accountable to error (standard error), as denoted by the error bars. It is however, important to realise that the cell counts are low in percentage terms (Tables 3.1 & 3.2), and as such, it is impossible to draw significant conclusions.

Plots of cell count against EWC produce similar curves to those of the NNDMA containing copolymers (Figures 3.7 & 3.8). There are differences however between these graphs and the plots obtained for FI and F2. The diminishing of cell attachment appears to occur at slightly lower water contents in the NVP copolymers than in the NNDMA copolymers (see Figures 3.5-3.8). In the NVP copolymers, the non-adhesive trough begins at ~35% and ~65% for the LMA and MMA copolymers respectively, compared to ~40% and ~75% for the NNDMA families. Although it must be emphasised that there are differences in the comonomer ratios between families (Table 3.1), the comonomer ratios of F1 (NNDMA) and F3

(NVP) are identical, and as such serve as a useful basis for comparison.

The comparisons between the common comonomer ratios of F1 and F3 are presented in Figures 3.23 & 3.24. For both cell lines the same tendency is evident. As the LMA content increases, so cell attachment increases. For most of the copolymers, the levels of cell attachment are greater on the NNDMA copolymers than on the NVP copolymers. For the BHK-21 cell line the differences between the cell counts on F1 and F2 copolymers are statistically significant apart from the 80:20 comonomer ratio (Appendix 4a). For the L929 cell line, non-significant differences in cell counts occur for the 70:30 and 30:70 comonomer ratios.

In conclusion, the cellular responses to the NVP hydrogel families tested follow very similar trends to those exhibited by NNDMA hydrogels containing the same comonomers.

3.3.3 The effect of cell line on cell attachment

It is clear that for all hydrogel families tested, the levels of cell attachment are generally higher for BHK-21 than for L929 (Figures 3.1-3.11). The results of a two-factor anova on the cell count data shows a highly significant difference ($p=0.0001$) between the cell lines (Appendix 2a). It is a general observation that in routine culture and experimental conditions, BHK-21 culture reach confluence more quickly than L929 cultures do; they have a faster cell cycle. Consequently, over a standard test period, one would expect to see greater numbers of BHK-21 cells attached to a surface than L929 cells, all other factors being equal. Equating the

levels of cell attachment to the TC control shows a similar response to each surface by both cell lines (Tables 3.1-3.4).

3.3.4 The effect of comonomer ratio on cell attachment

For the NNDMA families, the between treatment comparisons show a significant difference between the 50:50 comonomer ratio polymer of F1 and F2 (i.e. NNDMA/LMA 50:50 and NNDMA/MMA 50:50) (Appendices 3a & 3b). Thus cell attachment is determined not only by the level of the comonomer present, but also by comonomer composition. The replacement of LMA by MMA exerts an influence on cell behaviour. The copolymer composition may explain the reason why there is a shift in the EWC curves when MMA replaces LMA in the same copolymer. Certainly the greater influence is exerted by comonomer content. As the amount of LMA or MMA increases, so cell attachment increases. Why this should be so is not immediately apparent. LMA and MMA are relatively hydrophobic, certainly far more so than either NNDMA or NVP, so one might expect a decrease in cell attachment with increasing hydrophobicity of the surface. However, NNDMA and NVP both contain nitrogen water-structuring groups which are expressed at the surface, as Thomas has stated[35]. These dominant water-structuring groups allow these polymers to structure water via hydrogen bonding. So, even though the surfaces of the polymers of families 1,2,3 and 4 become decreasingly wettable, there may still be sufficient surface polarity to promote protein adsorption from the bound water. Conversely, it may be that the surface polarity is too great when the expression of dominant functionals is high. This is an extension of Minett's "hema curve" behaviour. Thus, as the surface

polarity becomes too high, an electrostatic barrier to cell attachment is created. Another explanation for the drop off of cell attachment in these hydrogels is that at extremely elevated levels of water content, it may be impossible for a cell to become attached to the surface of the hydrogel simply because it is mechanically unstable. As mentioned in chapter one, anchorage dependent cells need a stable substratum on which to attach and spread. Fibroblasts exert a considerable tractive force during the process of cell spreading and therefore both of the cell lines used will exhibit firm attachment forces. Pratt *et al*[126] have shown that human endothelial cells can resist a shear force of 90 dynes/cm^2. Even if these hydrogel surfaces are "attractive" in terms of polarity and conditioned layer, the fibroblasts may find it impossible to spread because of the mobility of the polymer surface. Tables 3.1-3.4 show that for most of the very high water content hydrogels levels of cell attachment are very low, and there is no cell spreading.

3.4 Cell attachment on acrylamide hydrogels.

The acrylamide (ACM) hydrogels which comprise families 5, 6 and 7 are copolymers with very different properties as a consequence of the comonomers that are incorporated into the copolymer network. In contrast to the NNDMA, and NVP hydrogels which are copolymerised with hydrophobic comonomers, these ACM families are copolymerised with the charged species aminoethylmethacrylate (AEMA) and methacrylic acid (MAA).

3.4.1 The effect of comonomer ratio on cell attachment

It is immediately apparent that for both cell lines the level of cell attachment rises as the ratio of comonomer is increased (Tables 3.3 & 3.4, and Figures 3.9-3.11). There are two comonomer ratios common to F5 and F6 (99:1 and 90:10), and the cell counts recorded on these copolymers are plotted in Figures 3.19 and 3.22. The levels of cell attachment in the 99:1 copolymers are very low (~2-4% in Tables 3.3 & 3.4), and the differences are not statistically significant (Appendices 3a & 3b). The 90:10 copolymers do show statistically significant differences nevertheless between the cell attachment on AEMA and MAA containing copolymers (Appendices 3a & 3b). Thus, the copolymer composition significantly influences cell behaviour on these ACM hydrogels.

An increase in the level of the comonomer, whether it is AEMA or MAA, leads to a concurrent increase the polar component of free surface energy (γ^p) of the copolymer, as is shown by the surface polarity data obtained by Baker[113] (Figure 3.16). Both MAA and AEMA are charged species, and their surface expression will increase the wettability of that surface. As previously discussed, more wettable i.e. hydrophilic surfaces promote greater cell attachment than more hydrophobic surfaces such as native polystyrene. However, AEMA and MAA carry different charges, being positively and negatively charged respectively. What effect does this have on cell attachment?

Since the cell surface carries a net negative charge at physiological pH, one might expect electrostatic repulsion forces to prevent cell attachment on negatively charged surfaces. This is not the case, as has been demonstrated by several workers[33,36].

It is the process of interface conversion by protein deposition from the culture media which renders a surface "sticky" to fibroblasts. Charged surfaces are necessary for the deposition and more importantly, the correct expression of adhesive proteins such as fibronectin and vitronectin. Poorly wettable surfaces inhibit the adsorption of adhesive molecules in the correct conformation for cell attachment to occur. Presumably, on these surfaces, the cell binding domains are not correctly expressed, so that the cell cannot attach and spread.

In the case of Family 6 copolymers, the level of cell attachment rises with increasing MAA content (Figure 3.10). As with the hydroxyl group expression of the Minett copolymer series, so there may come a point where the MAA content increases to a level whereby cell attachment is inhibited as the negative charge at the surface becomes too great for the cell to overcome, and cell attachment will drop off (see Figure 3.25). This is obviously not the case for the copolymers of Family 6, in which the level of MAA reaches 25%.

The copolymers of Family 5 display a similar trend with respect to cell attachment as do the members of Family 6 (Figures 3.9 & 3.10). Thus as the AEMA content increases, so cell attachment increases also, due to the reasons outlined above. One point to note is that the level of cell attachment increases more rapidly at lower comonomer ratios in Family 5 than in Family 6. A possible explanation for this result lies in the difference in charge between AEMA and MAA. As AEMA is positively charged, so cell attachment is encouraged at lower levels of comonomer than the negatively charged MAA copolymers of Family 6.

Family 7 is an interesting copolymerisation of ACM, AEMA & MAA (Tables 3.3 & 3.4). It is very difficult to interpret the results of the cell attachment on these copolymers because of the uncertainty of the relative contributions of the charged species. It seems that the only clear conclusion to be drawn from adhesion assays on these copolymers is that as the level of AEMA exceeds the level of MAA, so the levels of cell attachment increase. Once again this is likely to be due to preferential cell attachment on a more positively charged surface.

All the copolymers of Families 5,6 and 7 have very high water contents (Table 3.3). There appears to be little correlation between EWC and cell attachment for these ACM hydrogels as there is for the NNDMA and NVP copolymers (Figures 3.12, 3.13 & 3.14). However, the spread of EWC's within families is small, when compared to the EWC values for the NNDMA and NVP copolymers (Table 3.1), so to what extent does the EWC influence cell behaviour? In F5, F6, and F7, the water content of the copolymer may be so high that fibroblasts experience great difficulty in attaching and spreading. The process of cellular adhesion involves quite considerable mechanical forces exerted by the cell on the substratum surface, and it may be that the flimsy nature of these hydrogels is such that they do not provide a solid enough support for anchorage-dependent cells to spread on.

3.4.2 The effect of surface polarity on cell attachment

The changes in the surface polarity, as measured by Baker[113] show that the changes in the γ^P are relatively small (Figure 3.16). One of the reasons for using the *in vitro* cell culture model is that cells such as fibroblasts serve as a very

sensitive biological "probe", and react to changes at the molecular level, as appears to be the case here.

The results of this study confirm the general trends established by Minett and Thomas. The levels of cell attachment on the NNDMA and NVP hydrogels are low relative to the tissue culture control, and also in relation to the levels of adhesion attained by the polyHEMA based hydrogels of Minett's studies (Figure 3.25). The levels of cell attachment on the acrylamide based hydrogels is also low. However, cell adhesion can be promoted by the introduction of charged species, such as AEMA and MAA at the surface.

The overall view from this study is that it is the interaction of factors such as surface charge, surface polarity and EWC which influence cell behaviour. It is difficult to determine which factor has the major influence because a change in one of these factors affects the others. Thus, increasing the surface polarity increases the wettability of a surface and its EWC. EWC is not the overriding factor influencing cell behaviour as Minett has suggested. Cell attachment and the promotion of fully spread morphologies occurs in these copolymers over a broad spectrum of water contents from ~20% - 95%. The presence of dominant functional groups which structure water and thus are able to mediate protein adsorption, influence cell behaviour, even at lower ranges of EWC as Thomas has found. As the EWC decreases due to the incorporation of less hydrophilic groups such as MMA and LMA, there is still sufficient charge expressed at the surface to bind water. The copolymer composition also influences the cellular response.

Replacing LMA with MMA produces a shift to the right in the Minett curve.

Results from the ACM hydrogel series show that even at extremely high water contents, cell attachment and spreading occurs. This is due to the introduction of the charged species, MAA and AEMA. The MAA content can be increased to 25% without any decrease in cell attachment. Much higher levels of MAA or AEMA may be warranted to see a decrease in cell adhesion. An adjunct to these observations is the fact that the cell behaviour is influenced by the sign of the charge. Many workers have reported the cellular response to negatively charged surfaces produced by hydroxylation or sulphonation. A certain amount of charge is necessary for interface conversion. However, if the concentration of negative charge at the surface becomes too great as for example, in sulphonated surfaces, the cell adhesion ceases. In Minett's polyHEMA hydrogels, the elevated levels of hydroxyl groups caused the curtailment of cell attachment, the phenomenon of "hema curve" behaviour. This is due to the electrostatic barrier established between the substratum and the negatively charged carbohydrate moieties in the cell membrane. With positively charged AEMA incorporated into the copolymer, cell attachment is encouraged at much lower comonomer ratios than for the negatively charged MAA comonomer. It is interesting to speculate what effect greatly increased positive charge would have on cell behaviour.

3.5 Conclusions

There are a range of physico-chemical factors which affect cell behaviour. In hydrogels, water, the surface charge and the group expression at the polymer surface mediate interfacial conversion, which in turn determines the cellular

response. In addition to these chemical parameters, the mechanical properties of these polymers as determined in part by the EWC can also influence cell adhesion. At extremely high water content levels, it appears that the cellular response is being affected by the mechanical instability of the polymer substrate. The next logical experimental step is to determine the ways in which unstable surfaces can influence cell behaviour. Biodegradable polymers, such as polyhydroxybutyrate (PHB) are an obvious choice for this study, and in chapter four the cellular responses to a wide range of PHB copolymers and degraded copolymers is investigated.

n = ?

Table 3.1: BHK-21 Cell adhesion on a range of high water content hydrogels.

	Polymer	Ratio of comonomers	EWC	Cell Count (cells/ml)	% Cell Attachment	
Family 1	NNDMA:LMA	80:20	69.2	4.89×10^3	4%	-
	NNDMA:LMA	70:30	63.7	4.81×10^3	4%	-
	NNDMA:LMA	50:50	56.7	1.68×10^4	15%	+
	NNDMA:LMA	40:60	45.8	3.21×10^4	28%	+
	NNDMA:LMA	30:70	37.2	4.39×10^4	38%	+
Family 2	NNDMA:MMA	99:1	87.3	2.19×10^3	3%	-
	NNDMA:MMA	95:5	86.5	3.69×10^3	4%	-
	NNDMA:MMA	85:15	85.2	5.00×10^3	6%	-
	NNDMA:MMA	50:50	63.2	2.20×10^4	26%	+
Family 3	NVP:LMA	80:20	68	3.38×10^3	3%	-
	NVP:LMA	70:30	58.4	9.69×10^3	8%	-
	NVP:LMA	50:50	36.6	2.46×10^4	21%	+
	NVP:LMA	40:60	32.2	2.93×10^4	25%	+
	NVP:LMA	30:70	26.8	3.90×10^4	34%	+
Family 4	NVP:MMA	90:10	82.3	1.25×10^3	1%	-
	NVP:MMA	80:20	76.7	4.13×10^3	4%	-
	NVP:MMA	70:30	67.8	1.24×10^4	11%	+
	NVP:MMA	60:40	55.6	1.40×10^4	12%	+
Control	BACTY	---	---	1.23×10^3	11%	-
	TC	---	---	1.15×10^5	100%	+

+ = Full spread morphology expressed

- = Full spread morphology not expressed

Table 3.2: L929 Cell adhesion on a range of high water content hydrogels.

	Polymer	Ratio of comonomers	EWC	Cell Count (cells/ml)	% Cell Attachment	
Family 1	NNDMA:LMA	80:20	69.2	5.50×10^3	6%	-
	NNDMA:LMA	70:30	63.7	4.81×10^3	6%	-
	NNDMA:LMA	50:50	56.7	2.16×10^4	25%	+
	NNDMA:LMA	40:60	45.8	2.70×10^4	31%	+
	NNDMA:LMA	30:70	37.2	3.53×10^4	41%	+
Family 2	NNDMA:MMA	99:1	87.3	1.13×10^3	1%	-
	NNDMA:MMA	95:5	86.5	3.31×10^3	4%	-
	NNDMA:MMA	85:15	85.2	4.56×10^3	5%	-
	NNDMA:MMA	50:50	63.2	1.81×10^4	21%	+
Family 3	NVP:LMA	80:20	68	2.44×10^3	3%	-
	NVP:LMA	70:30	58.4	3.50×10^3	4%	-
	NVP:LMA	50:50	36.6	1.60×10^4	19%	+
	NVP:LMA	40:60	32.2	2.98×10^4	35%	+
	NVP:LMA	30:70	26.8	3.30×10^4	39%	+
Family 4	NVP:MMA	90:10	82.3	1.06×10^3	1%	-
	NVP:MMA	80:20	76.7	3.81×10^3	5%	-
	NVP:MMA	70:30	67.8	5.10×10^4	6%	-
	NVP:MMA	60:40	55.6	1.01×10^4	12%	+
Control	BACTY	---	---	7.91×10^3	9%	-
	TC	---	---	8.50×10^4	100%	+

+ = Full spread morphology expressed

- = Full spread morphology not expressed

Table 3.3: **BHK-21 Cell adhesion on a range of high water content hydrogels.**

	Polymer	Ratio of comonomers	EWC	Cell Count (cells/ml)	% Cell Attachment	
Family 5	ACM:AEMA	99:1	96.5	4.44×10^3	4%	-
	ACM:AEMA	97:3	96.2	5.13×10^3	4%	-
	ACM:AEMA	90:10	94.7	1.55×10^4	13%	+
	ACM:AEMA	85:15	95.2	2.59×10^4	21%	+
Family 6	ACM:MAA	99:1	94.4	1.94×10^3	2%	-
	ACM:MAA	90:10	94.5	1.16×10^4	10%	+
	ACM:MAA	75:25	93.7	1.51×10^4	12%	+
Family 7	ACM:AEMA:MAA	90:2.5:7.5	95.1	2.44×10^3	2%	-
	ACM:AEMA:MAA	90:5:5	95.5	3.00×10^3	2%	-
	ACM:AEMA:MAA	90:7.5:2.5	96.5	1.38×10^4	11%	+
Control	BACTY	---	---	7.00×10^3	6%	-
	TC	---	---	1.21×10^5	100%	+

+ = Full spread morphology expressed

- = Full spread morphology not expressed

Table 3.4: L929 Cell adhesion on a range of high water content hydrogels.

	Polymer	Ratio of comonomers	EWC	Cell Count (cells/ml)	% Cell Attachment	
Family 5	ACM:AEMA	99:1	96.5	2.56×10^3	3%	-
	ACM:AEMA	97:3	96.2	6.06×10^3	6%	-
	ACM:AEMA	90:10	94.7	1.40×10^4	14%	+
	ACM:AEMA	85:15	95.2	2.01×10^4	20%	+
Family 6	ACM:MAA	99:1	94.4	1.63×10^3	2%	-
	ACM:MAA	90:10	94.5	1.04×10^4	10%	-
	ACM:MAA	75:25	93.7	2.09×10^4	20%	+
Family 7	ACM:AEMA:MAA	90:2.5:7.5	95.1	1.25×10^3	1%	-
	ACM:AEMA:MAA	90:5:5	95.5	1.19×10^3	1%	-
	ACM:AEMA:MAA	90:7.5:2.5	96.5	1.31×10^4	13%	+
Control	BACTY	---	---	4.71×10^3	5%	-
	TC	---	---	1.01×10^5	100%	+

+ = Full spread morphology expressed

- = Full spread morphology not expressed

Figure 3.1 BHK-21 & L929 cell attachment on a range of NNDMA/LMA hydrogels (Family 1).

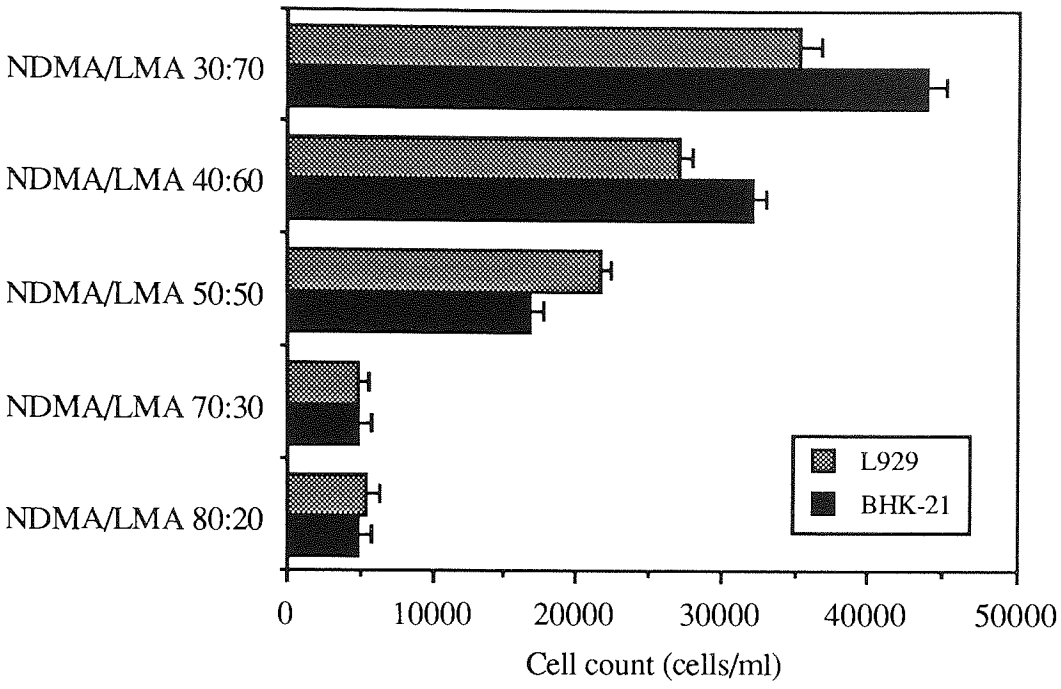


Figure 3.2 BHK-21 & L929 cell attachment on a range of NNDMA/MMA hydrogels (Family2).

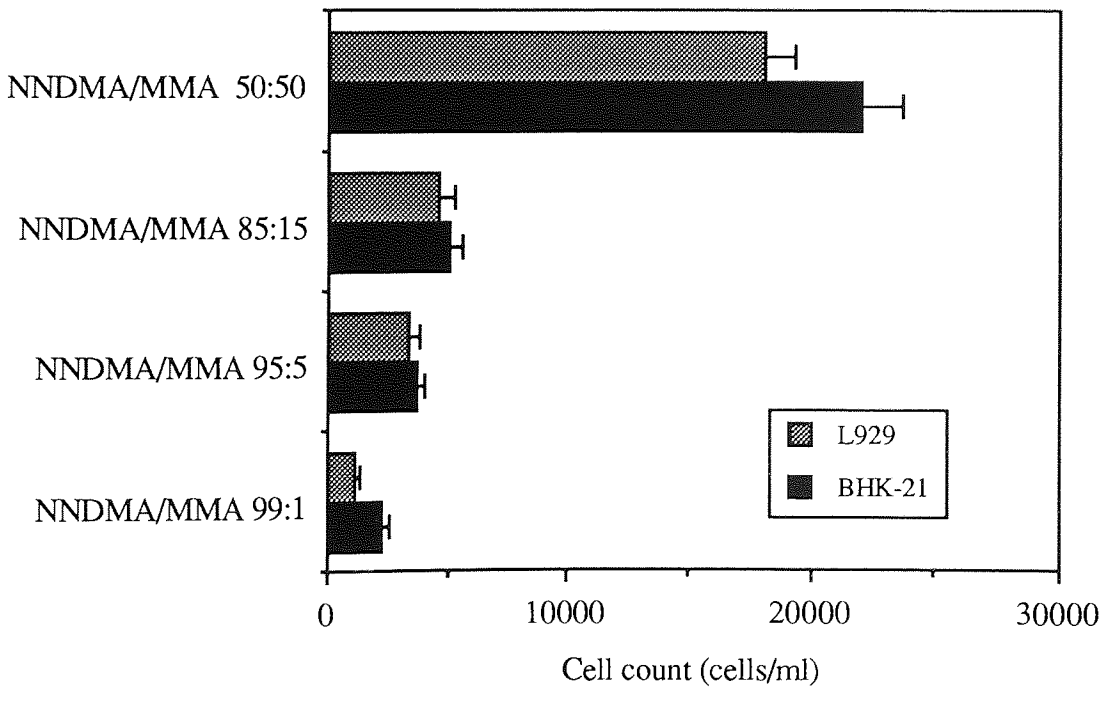


Figure 3.3 BHK-21 & L929 cell attachment on a range of NVP/LMA hydrogels (Family 3).

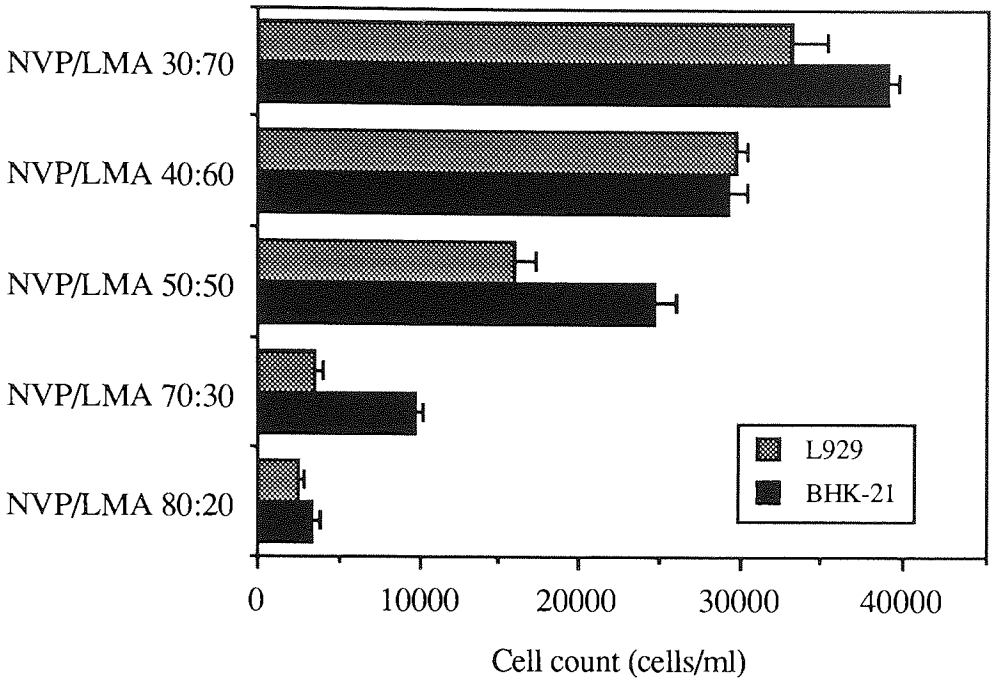


Figure 3.4 BHK-21 & L929 cell attachment on a range of NVP/MMA hydrogels (Family 4).

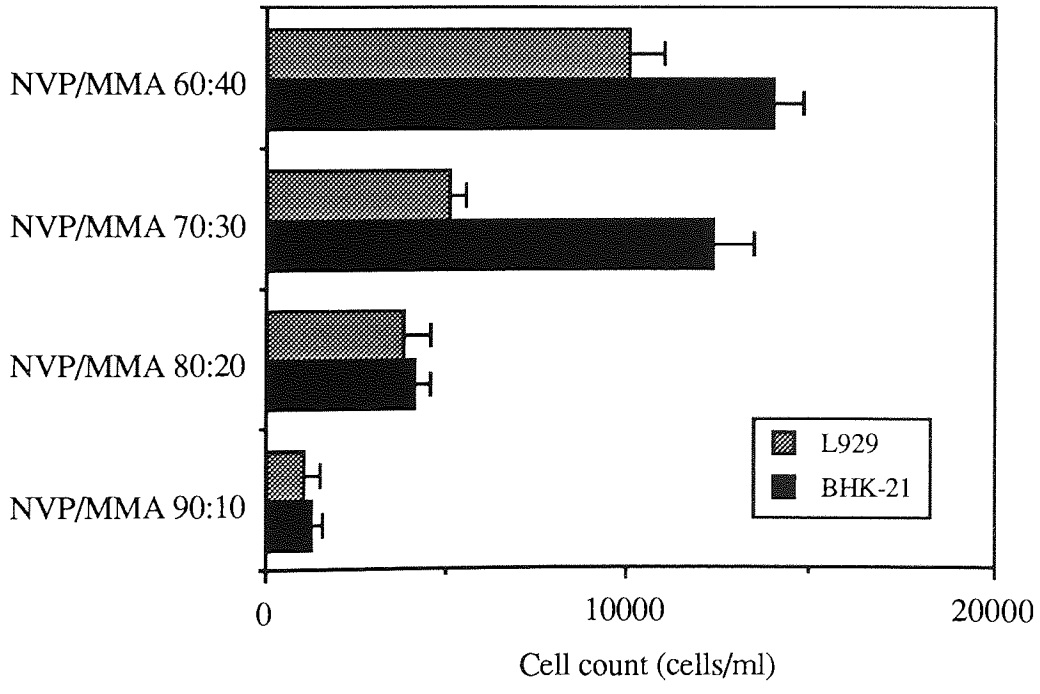


Figure 3.5 Cell count against Equilibrium Water Content (EWC) for Family 1.

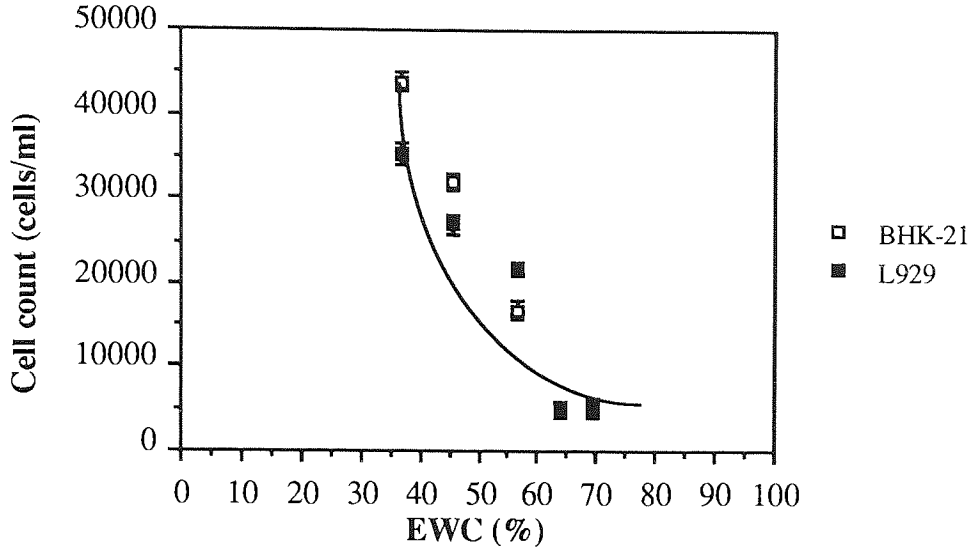


Figure 3.6 Cell count against Equilibrium Water Content (EWC) for Family 2.

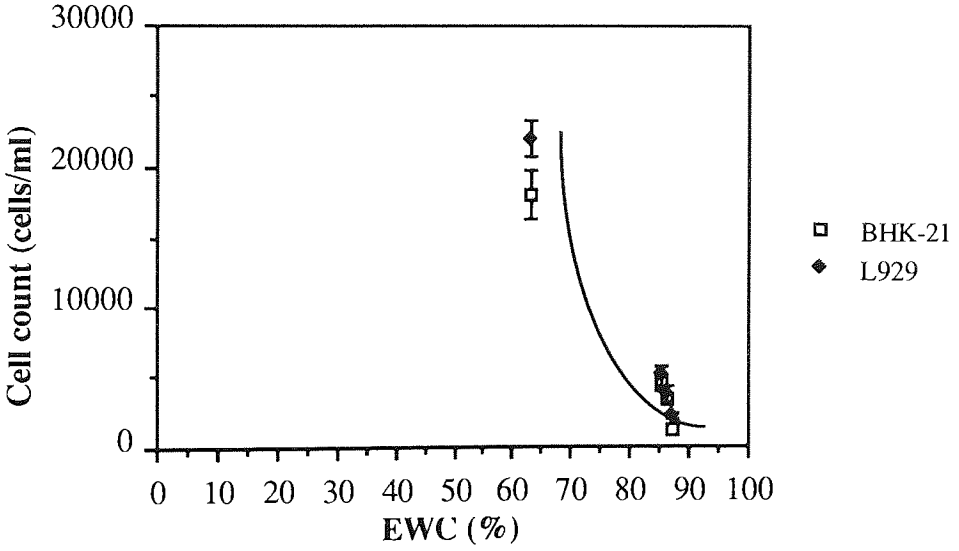


Figure 3.7 Cell count against Equilibrium Water Content (EWC) for Family 3.

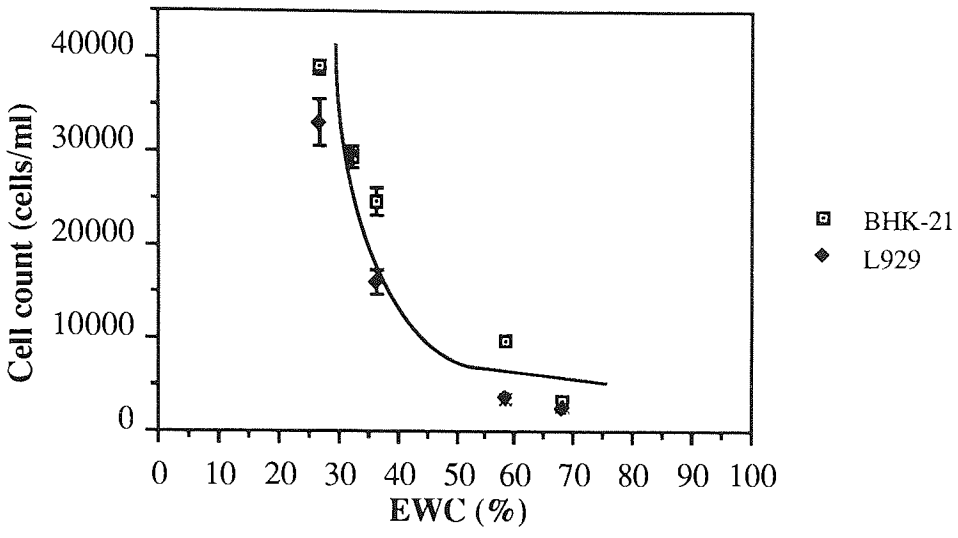


Figure 3.8 Cell count against Equilibrium Water Content (EWC) for Family 4.

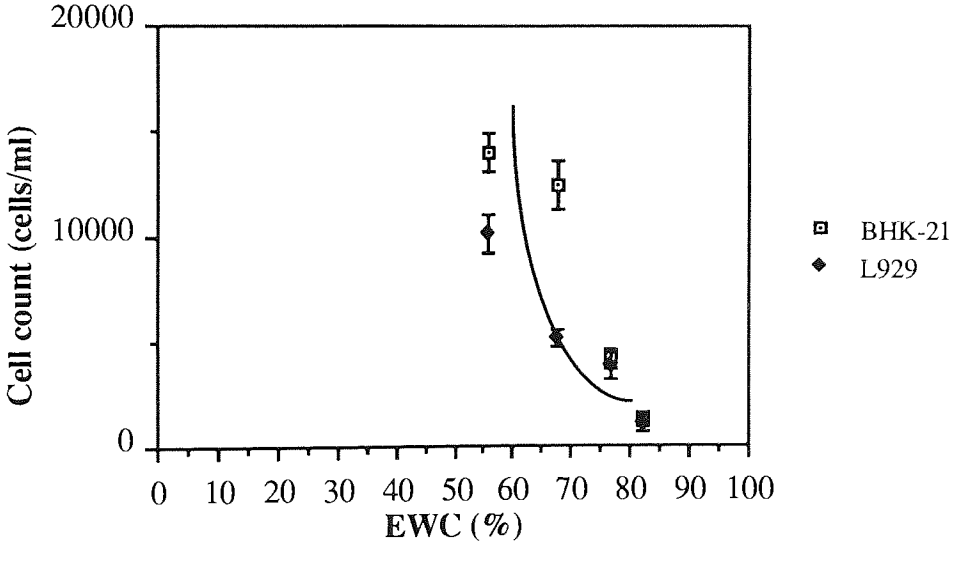


Figure 3.9 BHK-21 & L929 cell attachment on a range of ACM/AEMA hydrogels (Family 5).

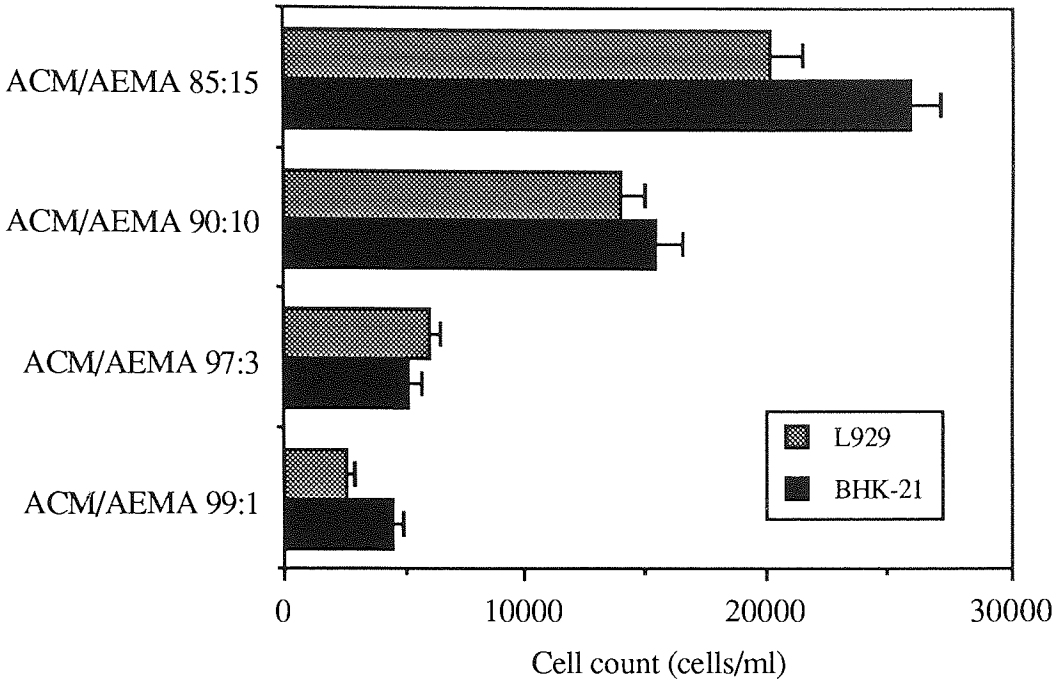


Figure 3.10 BHK-21 & L929 cell attachment on a range of ACM/MAA hydrogels (Family 6).

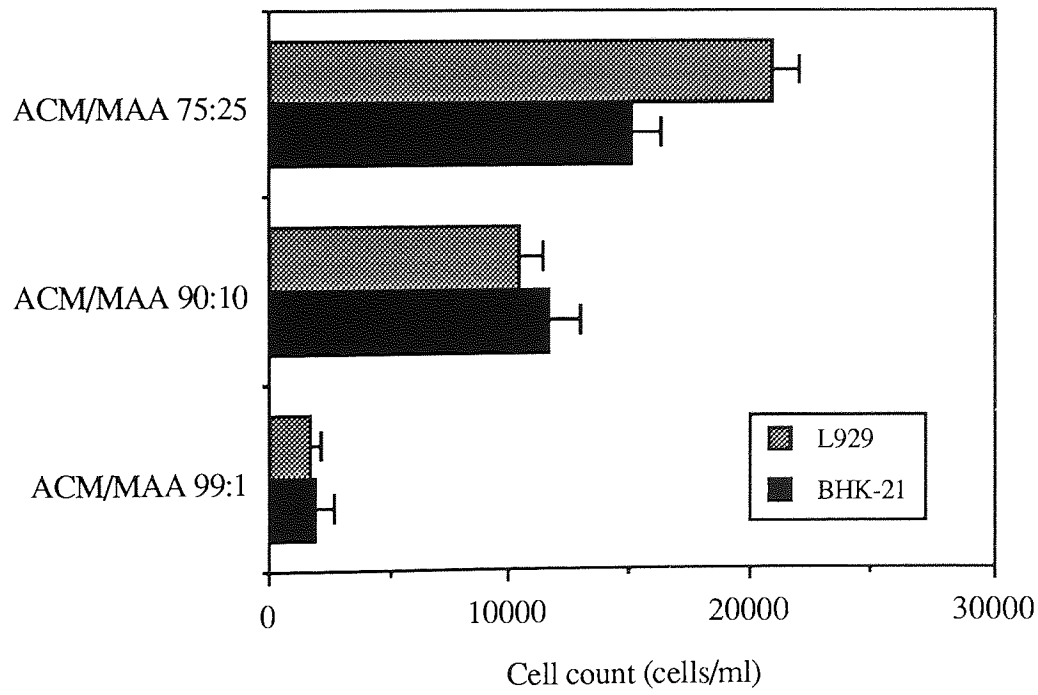


Figure 3.11 BHK-21 & L929 cell attachment on a range of ACM/MAA hydrogels (Family 7).

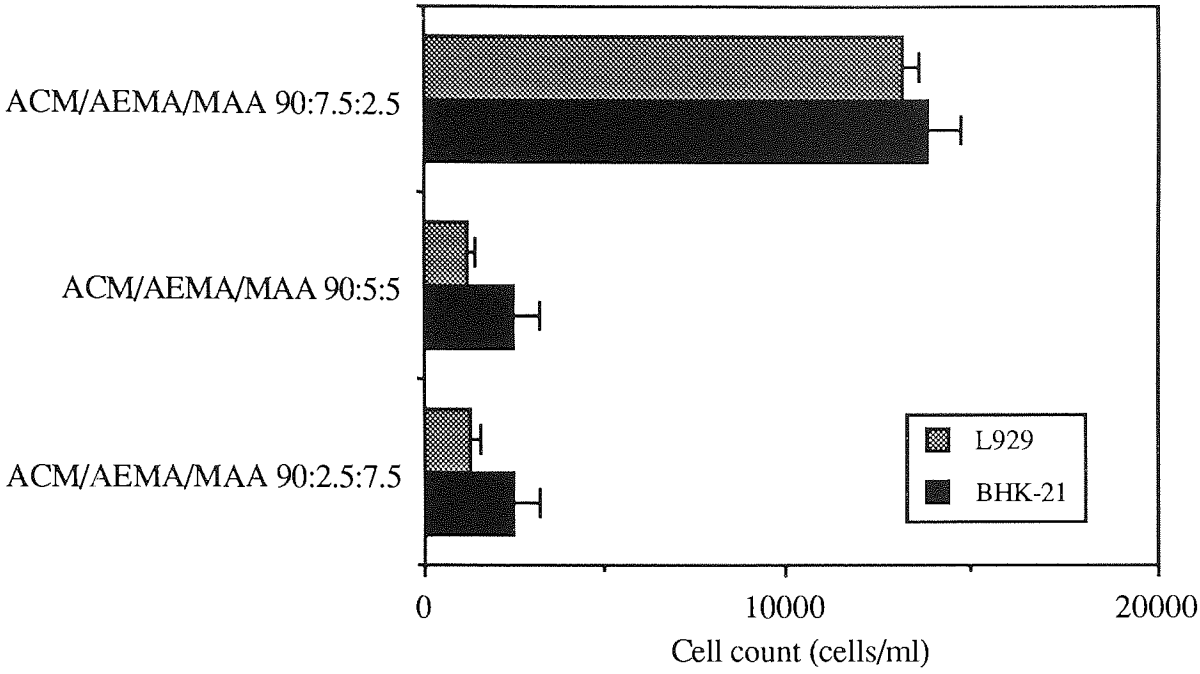


Figure 3.12 Cell count against Equilibrium Water Content (EWC) for Family 5.

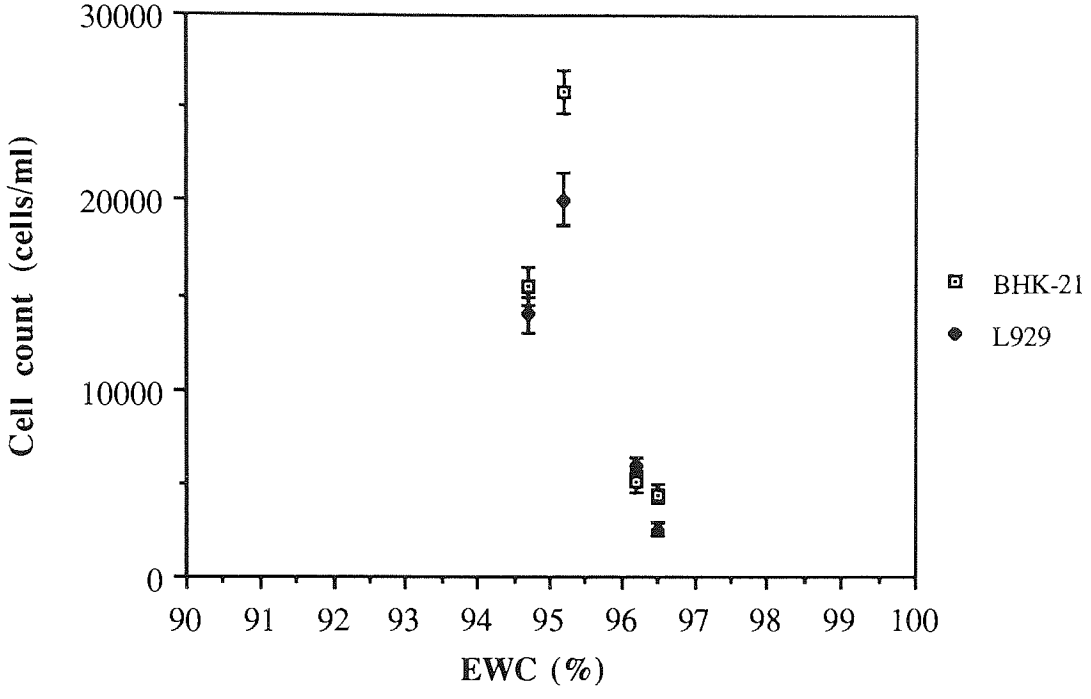


Figure 3.13 Cell count against Equilibrium Water Content (EWC) for Family 6.

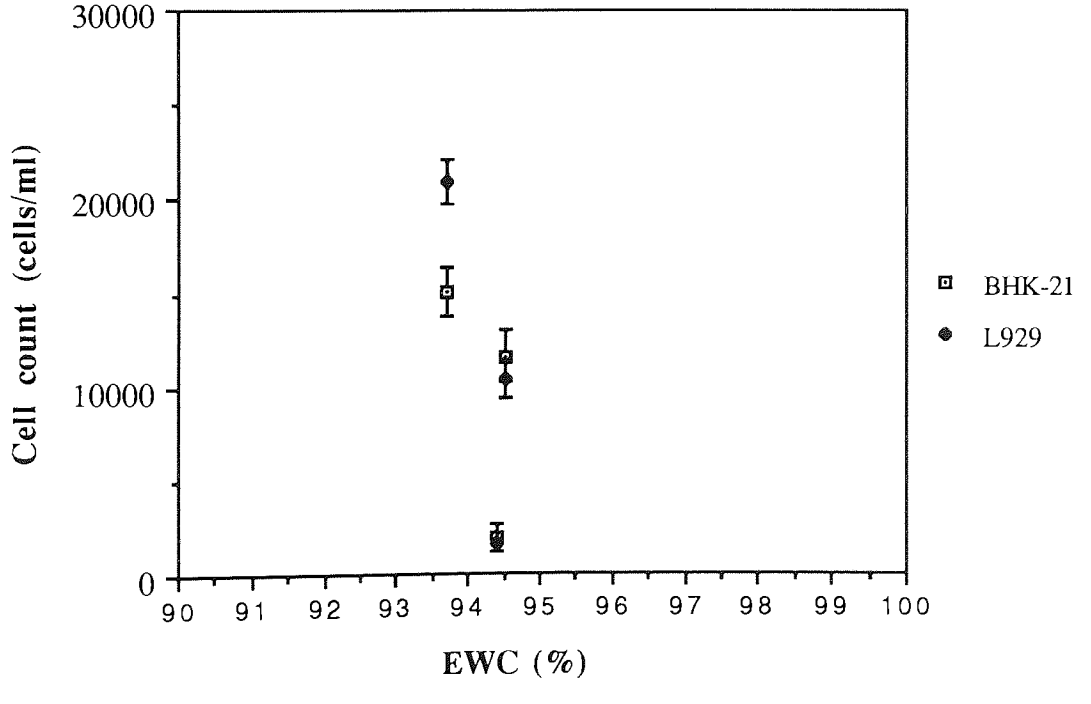


Figure 3.14 Cell count against Equilibrium Water Content (EWC) for Family 7.

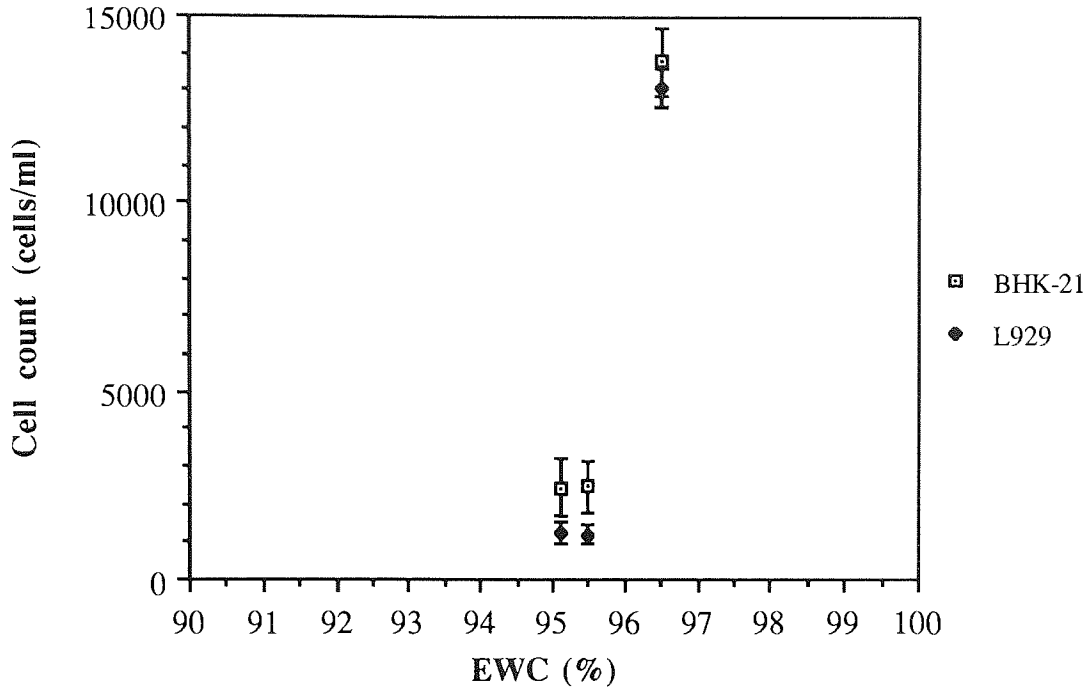


Figure 3.15 Changes in the polar component of surface energy with increasing copolymer content for NNDMA and NVP hydrogel copolymers.

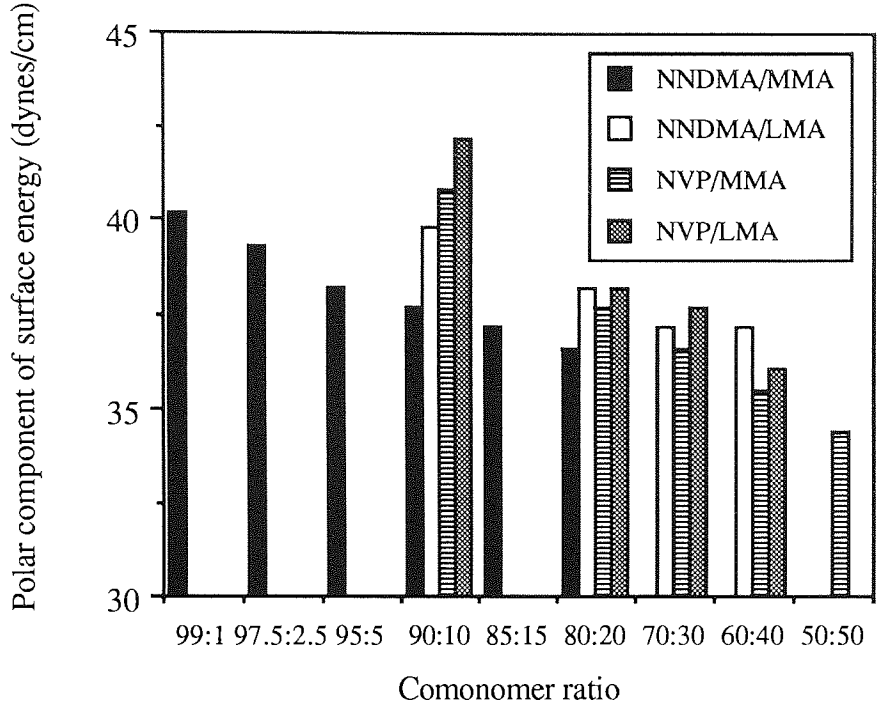


Figure 3.16 Changes in the polar component of surface energy with increasing copolymer content for ACM hydrogel copolymers.

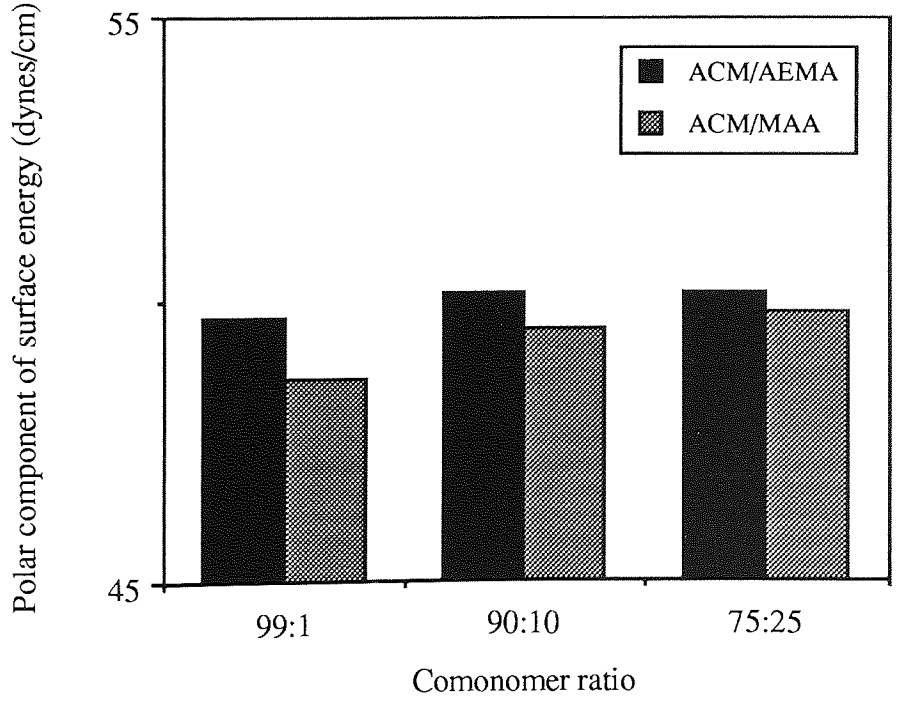


Figure 3.17 Comparisons of BHK-21 cell attachment on a range of NNDMA/LMA & NNDMA/MMA hydrogels.

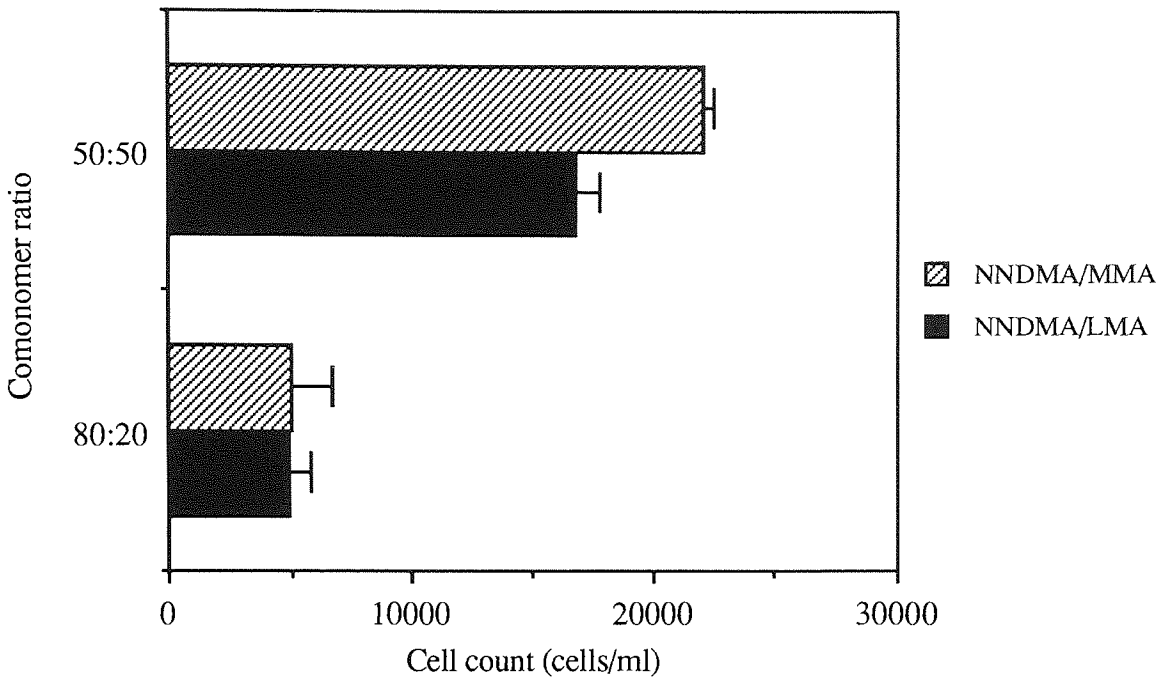


Figure 3.18 Comparisons of BHK-21 cell attachment on a range of NVP/LMA & NVP/MMA hydrogels.

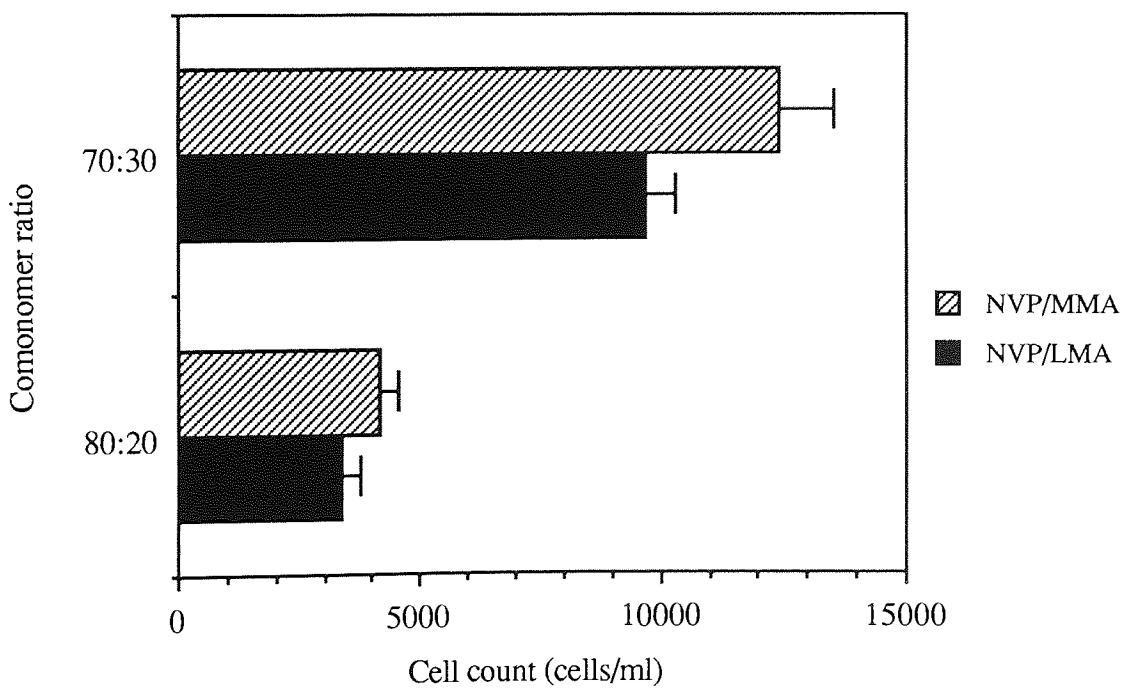


Figure 3.19 Comparisons of BHK-21 cell attachment on a range of ACM/AEMA & ACM/MAA hydrogels.

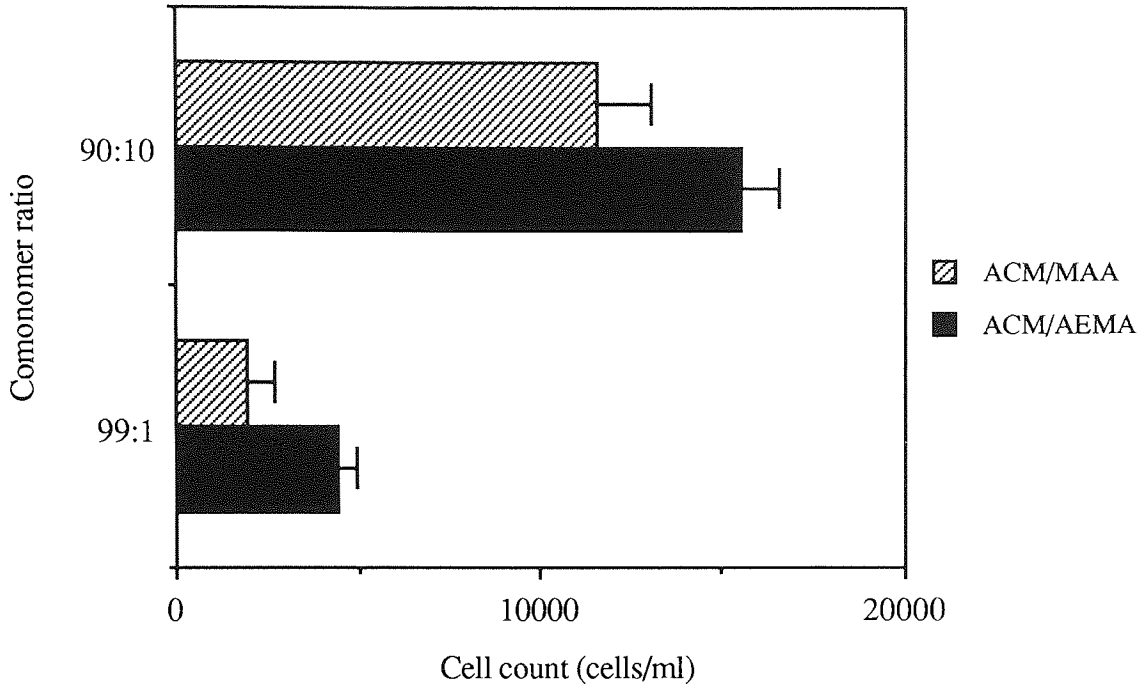


Figure 3.20 Comparisons of L929 cell attachment on a range of NNDMA/LMA & NNDMA/MMA hydrogels.

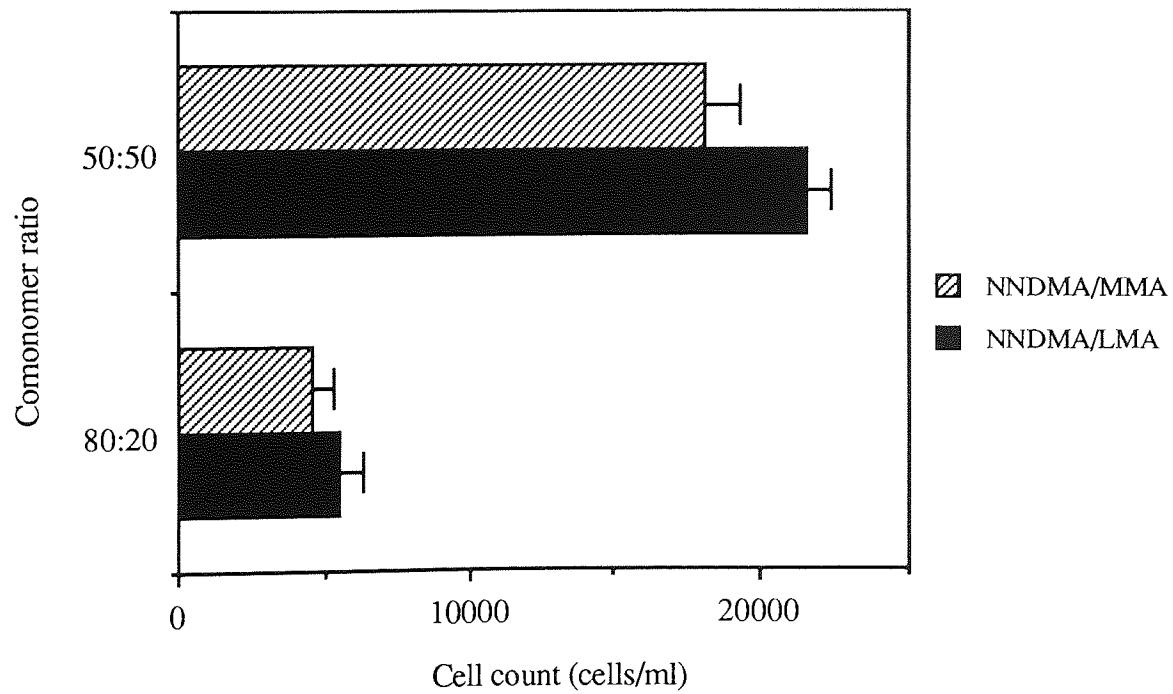


Figure 3.21 Comparisons of L929 cell attachment on a range of NVP/LMA & NVP/MMA hydrogels.

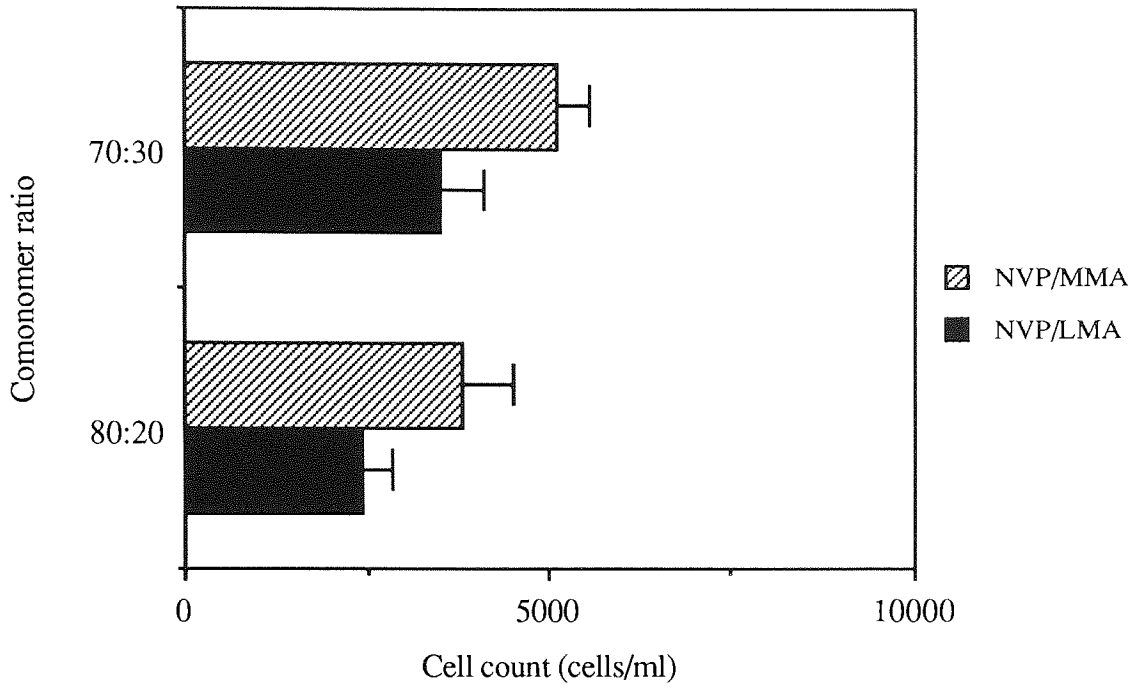


Figure 3.22 Comparisons of L929 cell attachment on a range of ACM/AEMA & ACM/MAA hydrogels.

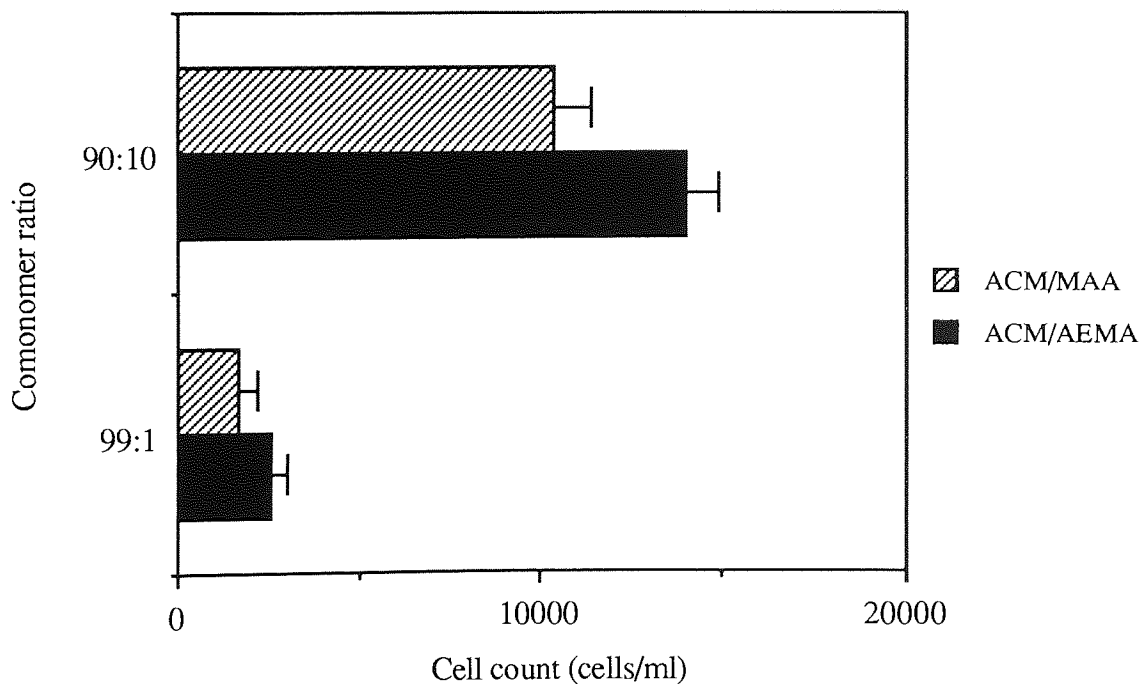


Figure 3.23 Comparisons of BHK-21 cell attachment on a range of NNDMA & NVP hydrogels.

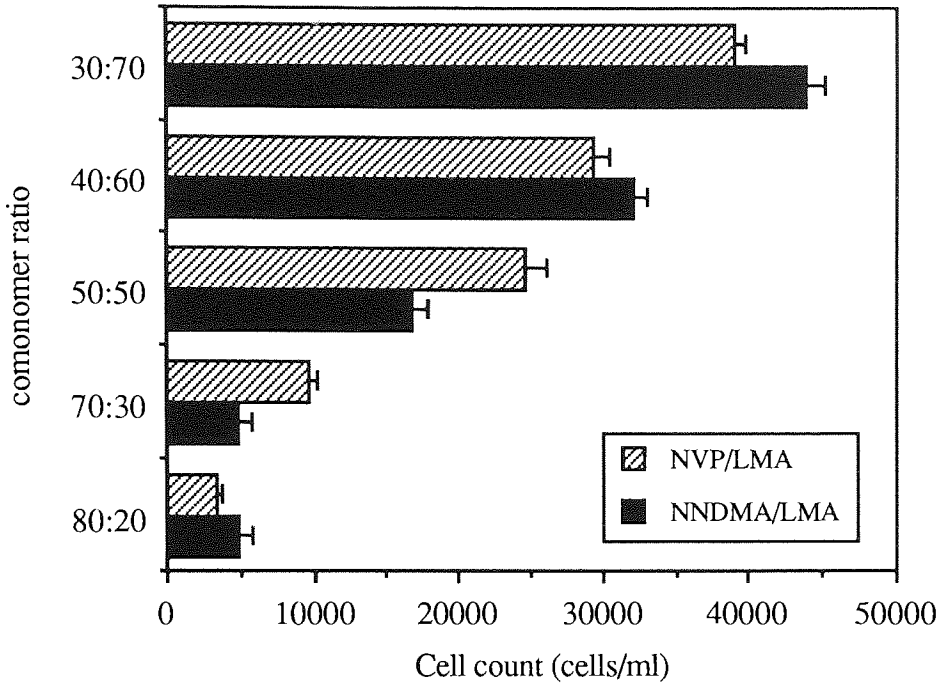


Figure 3.24 Comparisons of L929 cell attachment on a range of NNDMA & NVP hydrogels.

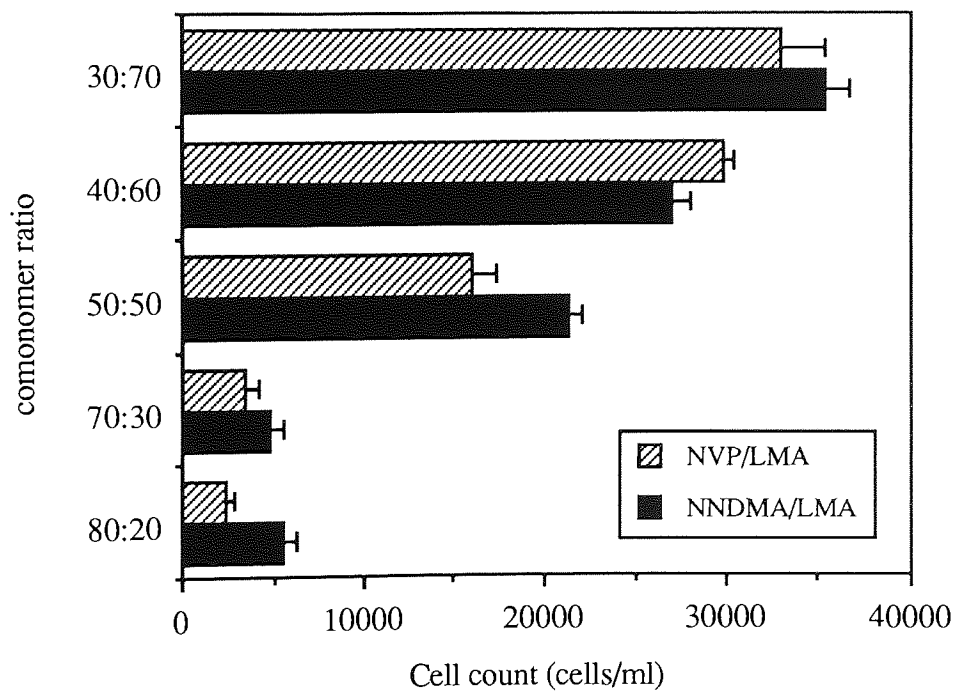


Figure 3.25 A sketch of the "Minett" curve.

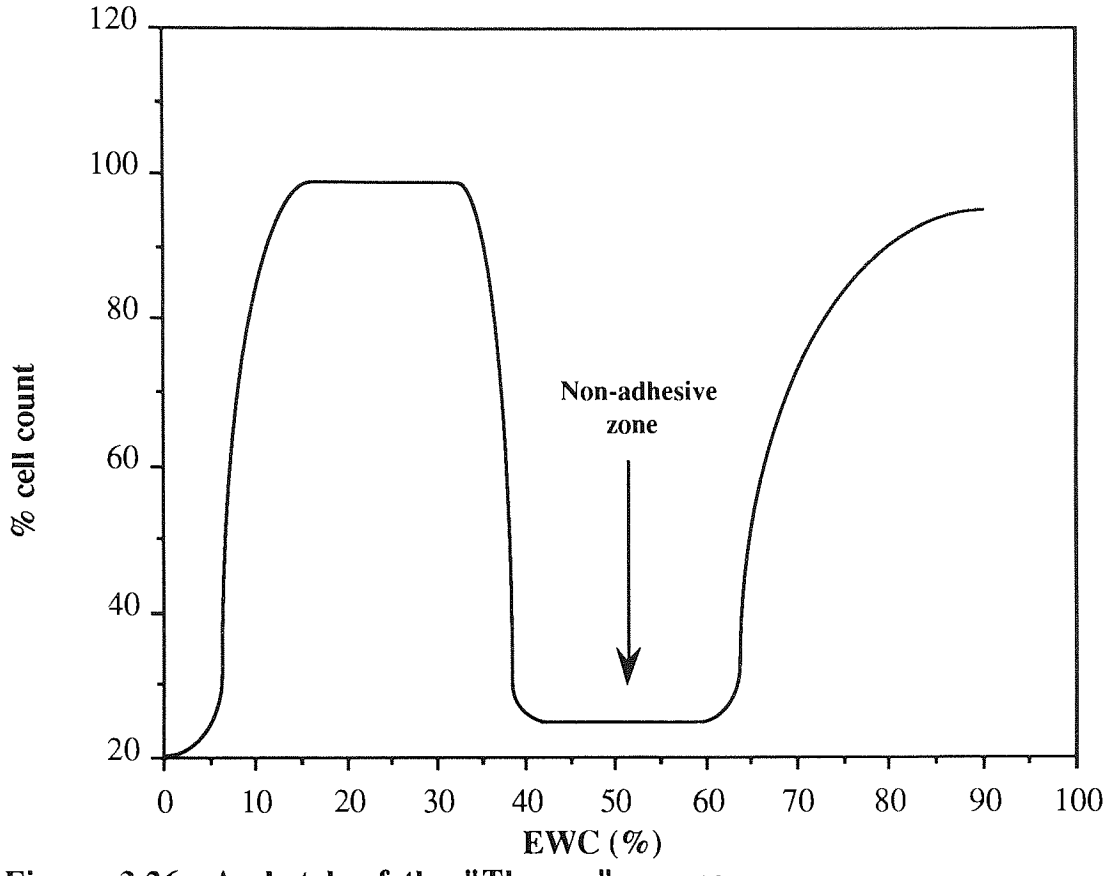
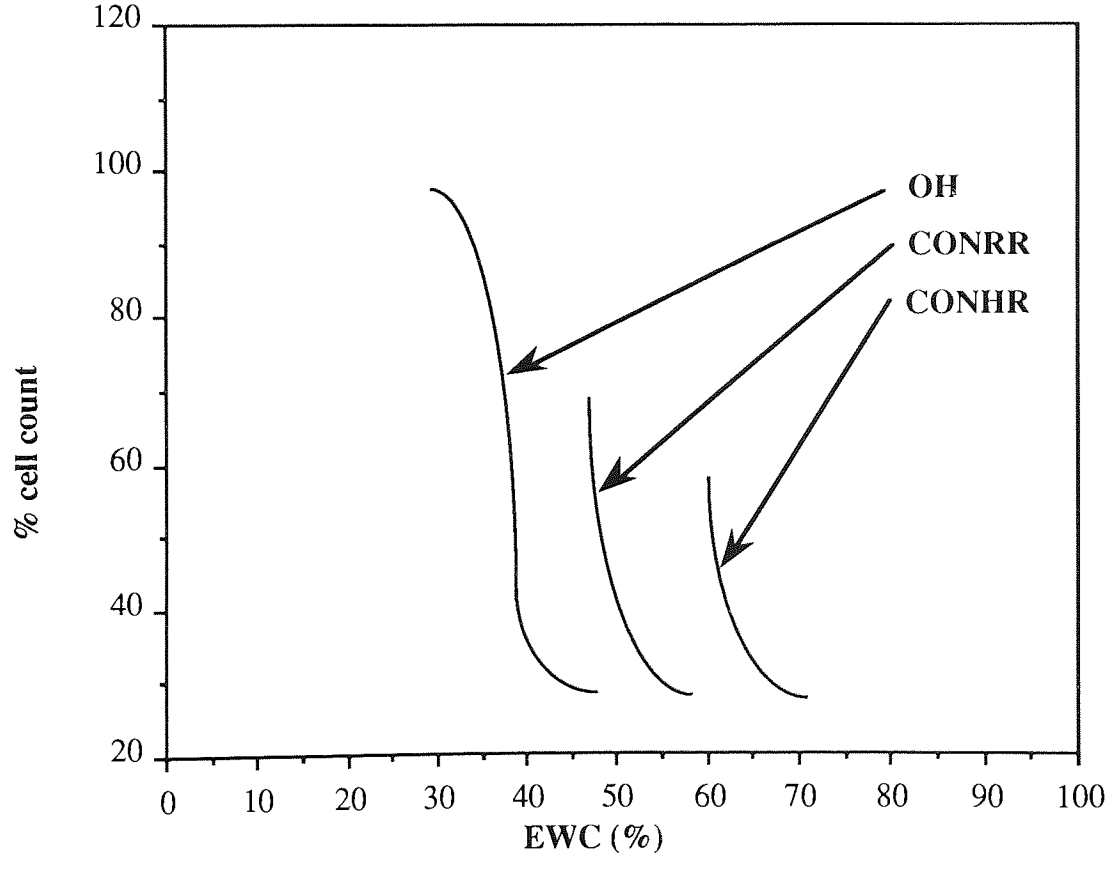


Figure 3.26 A sketch of the "Thomas" curves.



CHAPTER FOUR

CELLULAR RESPONSES TO POLYSACCHARIDE BLENDED POLY (ß-)HYDROXYBUTYRATE-(ß-)HYDROXYVALERATE COPOLYMERS

Chapter Four: Prelude

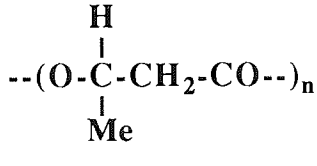
Biodegradable polymers have a very important role to play in biomaterials research. There is no universal biomaterial which is applicable to every clinical situation, and potential materials have to be tailored to meet specific needs. Some materials need to last the lifetime of the patient, for example heart valves and vascular grafts, whereas others need to be biodegradable, such as surgical fixation devices and drug release systems. In this context, polyhydroxybutyrate has a part to play as a biodegradable polymer. Biodegradable systems are very different to other biomaterials such as hydrogels because they are broken down *in situ*. They are relatively unstable systems, and as such it is important to have preliminary information from stable systems which may be applicable to cellular interactions with these polymers. The results gained from chapter three on hydrogels serve as a useful basis on which a foundation of information about the possible contributions of water and functional groups, both charged and polar, can be built up.

PHB-HV copolymers are degraded by hydrolysis mechanisms, so the surface properties of the copolymers change greatly over time. Hydrolysis will produce charged species at the surface, and these species will influence cell behaviour, as seen in chapter three. The big difference between hydrogels and PHB-HV copolymers is that the former have fixed surface characteristics determined by their production, whereas PHB-HV copolymers have constantly changing surface characteristics as a result of their degradation profile. In addition, the incorporation of various polysaccharide moieties into the copolymer matrix produces surfaces with widely differing surface polarities. The results from chapter three show that

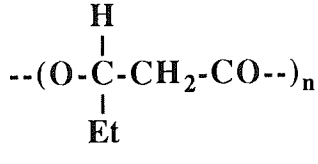
surface polarity is an important surface characteristic influencing cell behaviour, so cell adhesion assays on this range of PHB-HV copolymers are both interesting and relevant.

4.1 Polyhydroxybutyrate-hydroxyvalerate copolymers

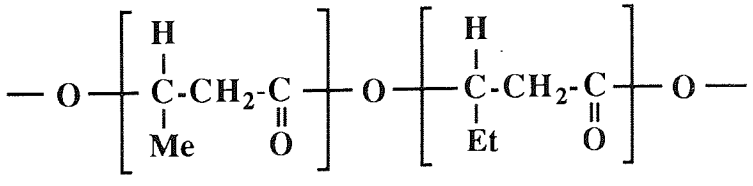
Polyhydroxybutyrate (PHB) is a naturally occurring, biodegradable polyester which is synthesised by a number of bacteria. It was first isolated from *Bacillus megaterium* by Lemoigne[127]. It forms crystalline cytoplasmic granules which have been shown to be utilised as a carbon and energy store[128]. The commercial route to production is biosynthetic, using the restricted metabolism of *Alcaligenes eutrophus*. Marlborough Biopolymers (a subsidiary of ICI, U.K.) have found that *Alcaligenes eutrophus* can accumulate up to 70% (w/w) of polyhydroxybutyrate-hydroxyvalerate (PHB-HV) copolymer using glucose and propionic acid as carbon sources. The high molecular weight fractions are commercially available as Biopol®. The copolymer has been shown to be statistically random, containing 3-hydroxybutyrate (3-HB), and 3-hydroxyvalerate (3-HV) repeat units[129]. The valerate content of the copolymer can be varied from 0-30% depending on propionic acid concentration.



Poly (ß-)hydroxybutyrate



Poly (ß-)hydroxyvalerate



Poly(ß-hydroxybutyrate)/Poly(ß-hydroxyvalerate) copolymer

4.2 Biomedical applications

PHB has many potential applications in the biomedical field, among them surgical fixation devices[130], drug delivery systems[131], and as I will discuss later, wound dressings. PHB would seem to be an ideal candidate for development as a biomaterial given it's lack of cytotoxicity[132], and the presence of it's immediate breakdown product, hydroxybutyrate in normal human serum[133]. Kennedy *et al*[134] have shown that the monomeric degradation product of PHB, hydroxybutyric acid is toxic *in vitro*, though not *in vivo*. This suggests that *in vivo*, degradation products are constantly removed from the implantation site resulting in sub-toxic concentrations. However, Miller and Williams[135] have shown that PHB may have limited uses in surgery or drug delivery due to it's slow degradation rate *in vivo*. Pouton *et al*[136] have demonstrated that, *in vitro*, thin films of PHB (85μm) have a half-life of 152 weeks.

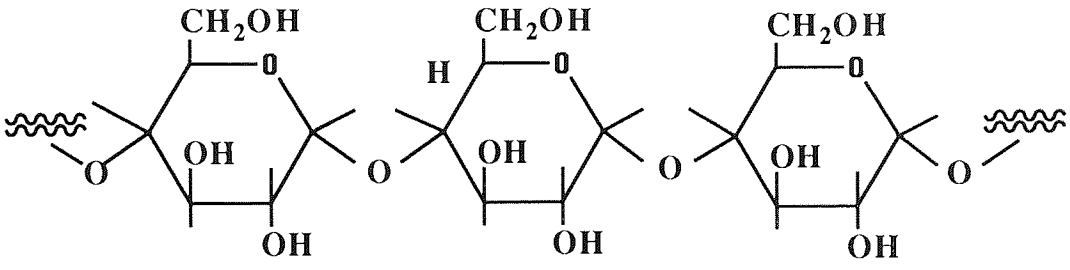
4.3 Degradation

In nature, soil fauna degrade PHB both intracellularly[137], and extracellularly[138,139] by enzymatic means, as one might expect given that PHB is an inert storage material analogous to starch. Studies in these laboratories[114] have shown that *in vitro* degradation of PHB is influenced by many variables, such as copolymer composition, molecular weight, pH and temperature. The rate of degradation is increased in alkaline conditions and at elevated temperature (pH 10.6, 70°C). In particular, the presence of increasing numbers of HV units resulted in faster degradation. The initial events of degradation occur at the surface.

4.4 Polysaccharide fillers

4.4.1 Amylose

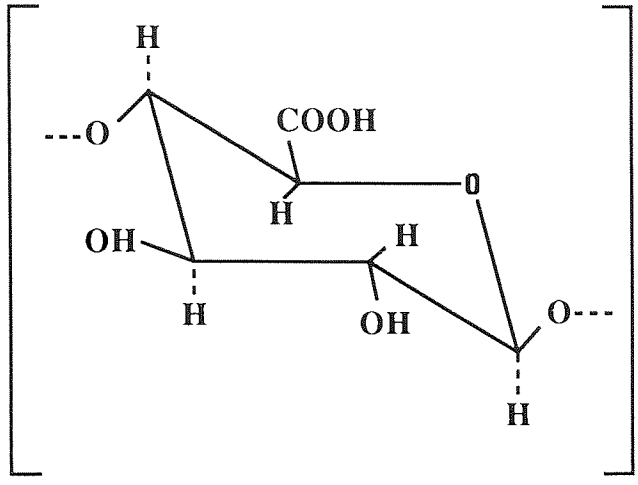
Amylose is a linear polymer composed of D-glucose sub-units, which are linked by cis α (1-4) glycosidic linkages, which results in amylose having an open helix structure (Fig. 4.1). Each sub-unit contains 2 primary, and one secondary hydroxyl group. The helical structure, and linearly arranged hydroxyl groups means that amylose molecules are attracted to each other. They bind by means of hydrogen bonds. This hydrogen bonding renders the amylose molecule very hydrophobic. Amylose is readily degradable by α & β amylase which are both found in abundance in mammalian systems.



Amylose

4.4.2 Sodium Alginate

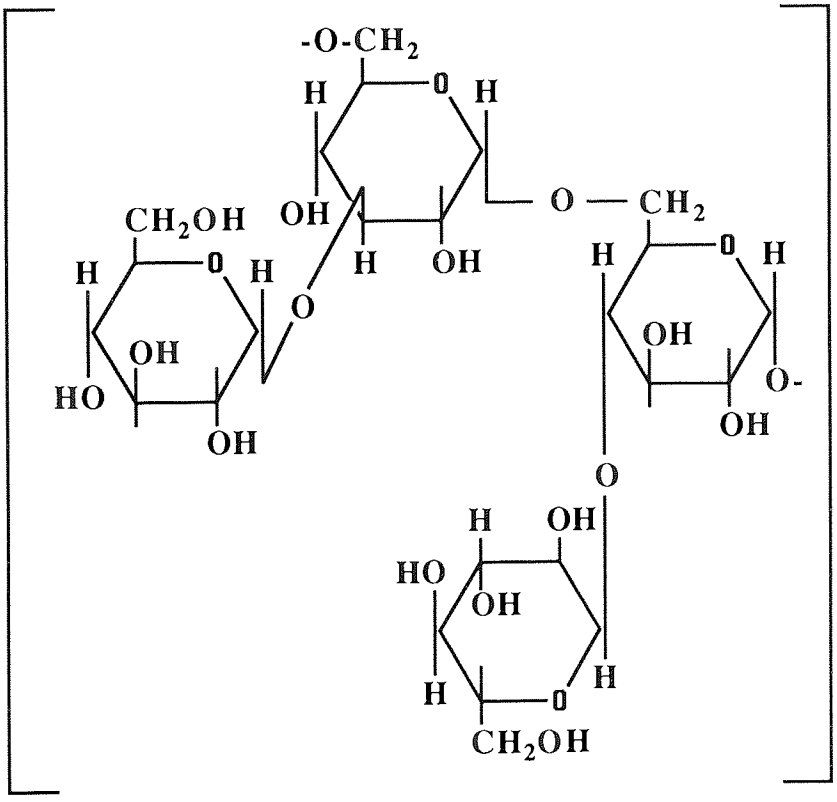
Sodium alginate is a salt of the polymeric polysaccharide alginic acid. Alginic acid is composed of β-D-(1-4) mannuronic and α-L-guluronic acid residues (Fig 4.2). The alginates (including sodium alginate) are very hydrophilic polysaccharides. Sodium alginate is gradually absorbed *in vivo* with no cytotoxic effects[140].



Alginic acid

4.4.3 Dextran

Dextrans are a class of polysaccharides synthesised from sucrose by members of the family Lactobacilli[141]. Dextran is a branched polymer consisting of D-glucose subunits linked by α (1-6) glycosidic bonds in the main chain, and by varying amounts of α (1-2), α (1-3), or α (1-4) branch linkages (Fig. 4.3). *In vivo*, the ubiquitous enzyme dextranase breaks down dextran to sugars.



Dextran

4.4.4 Dextrin

Dextrin is produced by the dextrinisation of starch, involving heating with small amounts of dilute nitric acid. This process consists of hydrolysis, transglucosidation and repolymerisation[142], resulting in a branched polymer molecule.

4.5 Degraded PHB-HV copolymers

A preliminary investigation of cell adhesion on degraded copolymers formed part of this project This is an important study because it gives initial information on how the process of degradation affects cellular response. Once a biomaterial such as PHB is placed in a host system, that system will interact with the material, and alter the properties of that material Thus degradation of PHB-HV copolymers has potentially important implications for cell adhesion. A degraded surface may enhance or inhibit fibroblast adhesion.

Two PHB-HV copolymers were available for testing the cellular response to degraded surfaces; one amylose blended copolymer, and one copolymer with no polysaccharide incorporated into the copolymer matrix.

i. 12%HV/1% apatite (nucleating agent) **UNFILLED**

ii. 12%HV/10% amylose **FILLED**

These two copolymers were subjected to the same experimental protocols as the undegraded copolymer plaques. Unfortunately, due to the availability of samples, not enough material was obtainable for SEM studies.

4.6 Experimental procedure

Injection moulded PHB-HV copolymer plaques were sterilised by washing in a 1% Tween 20 solution for one hour. PHB-HV copolymers plaques could not be autoclaved as the hydrogels were, because the extremes of temperature and pressure involved initiated degradation of the plaques. Copolymers samples were placed into individual wells of a multiwell plate and seeded with cell suspension. The plates were then placed in the CO_2 incubator for 18 hours. Post incubation, cells were removed using trypsin, and counted. Duplicate samples were processed for SEM using the protocol outlined in chapter two.

4.7 Discussion of results

The PHB-HV copolymers can be conveniently divided into two families on the basis of valerate content. Family 1 contains PHB-HV copolymers with 12%HV blended into the copolymer matrix. Family 2 contains PHB-HV copolymers with 20% HV blended into the polymer matrix. Looking at the cell count data (Tables 4.1 & 4.2) and the SEM evidence (Plates 4.1-4.36), it is clear that all of the copolymers, filled and unfilled, support cell attachment/adhesion to varying degrees.

4.8 The effect of cell line on cell adhesion

There is a significant difference ($p=0.0001$) (Appendix 2b) between the two cell lines with respect to the levels of cell attachment (Tables 4.1 & 4.2). Looking at the plots of BHK-21 against L929 (Figures 4.1 & 4.2), the levels of cell attachment for all the copolymers tested were lower for the L929 line than the BHK-21 cell line.

As stated in chapter three, it is an observation that in both routine culture, and experimental conditions, BHK-21 cultures reach confluence more rapidly than L929 cultures do because they have a faster cell cycle. Consequently, over the standard incubation period of 18 hours, one would expect to see a greater number of BHK-21 cells attached to a surface than L929 cells, assuming their responses to that surface are similar. The SEM evidence (Plates 4.1-4.36) confirms that the responses of both cell lines are very similar on all the copolymers tested. A look at the values for % cell attachment (Tables 4.1 & 4.2) also show that with respect to the TC control, the responses to each copolymer are similar for BHK-21 & L929. The difference between the levels of attachment exhibited by the two cell lines is the result of their differing growth rates.

4.9 The effect of valerate content on cell adhesion

To assess the effects of valerate content on cell behaviour, plots of 12%HV against 20%HV copolymers are reproduced in Figures 4.3-4.6. Looking at the plots of 12%HV & 20%HV blends with 10% polysaccharides (Figures 4.3 & 4.4), it is apparent that for most of the copolymers there appears to be little difference in the levels of cell attachment for either cell line. There are statistically significant differences between the 10% dextran blends, and both dextrin blends for the BHK-21 cell line (Appendix 5a). For the L929 cell line, the statistically significant differences are on the 10% dextran blends, 30% dextrin blends, and on both sodium alginate blends (Appendix 5b). There seems to be no general trend for the 10% polysaccharide blends, whereas inspection of the 30% polysaccharide blends (Figures 4.5 & 4.6) reveals a trend, with the 20%HV copolymers supporting slightly higher levels of attachment than the 12%HV copolymers. It must be

pointed out however, that levels of attachment are very close together, and when the variation about the mean is considered, this trend may mean very little. The surface energy data (Figures 4.11 & 4.12) show that the values for the polar component of surface energy are similar for all the 12% & 20%HV copolymers. One would expect this given that it is the incorporation of polysaccharides that make the greatest contribution to surface polarity. Degradation of the blends via hydrolysis increases the number of charged groups at the surface.

Relative to polysaccharide incorporation, variations in the valerate content of the copolymers have little influence on cell adhesion in this range of copolymers.

4.10 The effect of polysaccharide incorporation on cell adhesion

The results of the cell adhesion assays (Tables 4.1 & 4.2, Figures 4.1 & 4.2) show that there are great differences between the copolymers depending on which polysaccharide and how much of that polysaccharide is blended into the copolymer matrix.

4.10.1 Unblended copolymers

The unfilled (Norwegian Talc nucleated) copolymers in each of the two families give a baseline against which to judge the effects of polysaccharide incorporation into the copolymers. For both cell lines, the 12% & 20% unfilled copolymers support similar levels of cell attachment, in the range of 23-31% (Tables 4.1 & 4.2). Although this level of attachment is much less than that exhibited on the polysaccharide blends, cell spreading is expressed by both cell lines on these

copolymers (Plates 4.1, 4.2, 4.19, 4.20). The surface of the talc nucleated plaques is more hydrophobic because of the methyl and ethyl pendant groups off the copolymer backbone. As previous researchers in these laboratories[35] and elsewhere have shown, this surface hydrophobicity does not encourage cell adhesion. Lydon *et al*[23] have demonstrated that hydrophobic surfaces such as polystyrene may be rendered more wettable (i.e. more hydrophilic) by the introduction of polar species such as hydroxyl (OH) groups. This increased hydrophilicity results in an increase in cell attachment and adhesion. As a surface becomes more wettable, so more water is bound to that surface and consequently, proteins can be laid down in the process of interface conversion. It may also be that polar groups are needed for the correct expression of adhesive molecules such as fibronectin and vitronectin. It has been reported that there are greater numbers of fibronectin molecules laid down on the surface of bacteriological grade plastic, which is native polystyrene, than on the surface of tissue culture grade plastic, which is polystyrene that has been surface treated to make it more wettable[26]. The conclusion is that the fibronectin molecules are in the correct conformation for recognition by the cell surface receptors of cells in the case of tissue culture plastic, whilst they are not in the case of bacteriological grade plastic.

4.10.2 The influence of amylose on cell adhesion

The incorporation of amylose into the polymer certainly influences the cellular response. For both cell lines a copolymer content of 30% amylose keeps the levels of cell attachment much lower than those on the other copolymers (Figures 4.5 & 4.6). The micrographs for both cell lines show rounded morphologies with little or

no cell spreading (Plates 4.5, 4.6, 4.23, 4.24). Yasin[114] has determined the surface energies of a range of PHB-HV copolymers, and these show that the incorporation of amylose into the copolymer matrix will lead to an increase in surface polarity during degradation (Figures 4.11 & 4.12). For the reasons outlined above, this should lead to increased cell adhesion. The cell count data for the 10% amylose blends (Tables 4.1 & 4.2) show that the levels of cell attachment are higher than those on 30% blends, and these differences are statistically significant (Appendices 5c & 5d). An attendant fully spread cell morphology accompanies the increases in attachment levels (Plates 4.3, 4.4, 4.21, 4.22). As the amount of amylose in the polyester increases from 10-30%, cell adhesion may be curtailed because of the increase in hydroxyl groups at the surface. The cell surface is negatively charged therefore, when a negatively charged substratum is encountered, repulsive electrostatic forces will decrease cell attachment. Thus there is a balance point as far as the effects of surface negative charge are concerned. A lesser density of hydroxyl groups will lead to increased cell adhesion up to a point, beyond which the surface becomes too highly negatively charged, and then there is a decrease in cell adhesion. Investigation of the surface polarity data which is available for the 12%HV/10% amylose blend (Figure 4.11), shows that in comparison to dextran and dextrin, the surface polarity of 10% amylose blends increases rapidly with time in "physiological" conditions. This is due to hydrolysis of the amylose at the surface. As the amylose is hydrolysed, the polarity of the surface increases as more polar groups are expressed. It may be that over the period of incubation (18hours), sufficient surface hydrolysis may occur so that the surface polarity increases to such an extent that "hema curve" behaviour may have

an effect, i.e. too large a concentration of charged groups produce a surface negative charge too great for the negatively charged cell surface to overcome.

4.10.3 The influence of dextran on cell adhesion

Figure 4.11 shows the surface energy data for the 12%HV/10% dextran blends. At 0 days, the polar components of surface free energy (γ^p) for amylose, dextran and dextrin are all within 2mN/m of each other. Dextran has the lowest γ^p, and yet the 10% dextran blends produce higher levels of cell attachment than the other two polysaccharides. It is difficult to explain in terms of polarity alone why the levels of cell attachment on dextran are higher than those on amylose or dextrin. Obviously the introduction of a hydrophilic polymer such as dextran into the copolymer matrix increases the wettability of the surface by the introduction of charged groups. The increased wettability, means more water will be bound to the surface, and consequently, increased protein deposition will occur at the interface with the biological system. This in turn will increase cell attachment and promote a fully spread morphology as is evident from the SEM results (Plates 4.7-4.10, 4.25-4.28). The 30% dextran copolymers produce the highest levels of cell adhesion exhibited by any of these PHB-HV copolymers (Tables 4.1 & 4.2), approaching that of the tissue culture plastic controls. Increasing the loading of dextran in the copolymer from 10%-30% produces significant differences in the levels of cell attachment (Appendices 5c & 5d). This increase in dextran content does not have the same effect on cell behaviour that similar levels of amylose incorporation do. There is not a sufficient electrostatic barrier to overcome cell adhesion by BHK-21 and L929 fibroblasts.

4.10.4 The influence of dextrin on cell adhesion

The incorporation of dextrin into the copolymer matrix promotes similarly high levels of cell adhesion as seen in dextran (Tables 4.1 & 4.2). Dextrin blends also promote a fully spread morphology (Plates 4.11-4.14 & 4.29-4.32). The surface energy data show that dextrin has the highest γ^p of the three polysaccharides (Figure 4.11). The process of dextrinisation of starch involves hydrolysis which will produce hydroxyl groups at the surface. This is reflected in the surface energy measurements of 12%HV/10% dextrin blends. For the reasons discussed above, the cell attachment and adhesion will increase as the surface polarity increases up to a point beyond which "hema curve" behaviour may overrule the influence of hydrophilicity and reduce cell attachment, as is the case with amylose. As seen with dextran, increasing the copolymer content from 10%-30% produces a statistically significant difference in the levels of cell attachment exhibited by both copolymers (Appendices 5c & 5d). In the dextrin blends, the increase in surface polarity which results from increasing the loading level from 10%-30%, does not increase the surface negative charge to a point beyond which it is impossible for cells to overcome any repulsive electrostatic forces.

4.10.5 The influence of sodium alginate on cell adhesion

The presence of alginate in the copolymer matrix also raises the levels of cell attachment beyond those observed on the unblended polymers (Tables 4.1 & 4.2). The surface energy data (Figure 4.11) suggest that the alginate blends should produce the highest levels of cell attachment, assuming surface polarity is the major factor influencing adhesion. The alginate blends do indeed support high levels of

cell attachment (Tables 4.1 & 4.2), and a fully spread morphology in both cell lines (Plates 4.15-4.18 & 4.33-4.36). In addition, alginate blends are the fastest degrading blends tested. The rapid hydrolysis of alginate blends will produce a large concentration of charged groups at the surface, thereby rapidly increasing the surface polarity. With the 30% loading, significantly greater % cell attachment is attained (Appendices 5c & 5d), presumably due to the increase in surface polarity.

Thus there is somewhat of a paradox with alginate blends. Although they do produce high levels of attachment and spreading, these levels are not as great as those exhibited on dextran and dextrin blends. One would expect cell attachment to be greater on alginate blends, given their relatively high surface polarity. However, it may be that if the degradation of the alginate blends proceeds rapidly through the test period, the surface morphology may play a part in determining cell attachment. If the rugosity is macroscopic, the surface area available for attachment is increased. This appears to be the case from the SEM evidence presented in Plate 4.35 where cracks are appearing in the alginate blended copolymer. This fact combined with the surface polarity data lead to the conclusion that cell attachment on alginate blends should be greater than on any of the other copolymers. However, if the surface rugosity is also affected at the microscopic level, there may be fewer points of attachment for the cell, and in addition, the expression of proteins may be influenced. Another possible reason why cell adhesion is lower than predicted is that even though the surface area available for attachment has increased, the surface has become so friable that the mechanical forces involved in the cell adhesion process may break the surface up, leading to lower levels of attachment.

Supportive evidence for this theory comes from the studies of Yasin[114] who has reported that the alginate blends are the fastest to degrade of any of these polysaccharide blends.

Table 4.1: **BHK-21 Cell adhesion on filled & unfilled PHB-HV copolymers.**

	Polymer	Ratio of comonomers	Cell Count (cells/ml)	% Cell Attachment
Family 1	PHB:HV:NT	87:12:1	1.10×10^4	31%
	PHB:HV:AMYLOSE	78:12:10	1.61×10^4	45%
	PHB:HV:AMYLOSE	58:12:30	1.18×10^4	33%
	PHB:HV:DEXTRAN	78:12:10	3.10×10^4	86%
	PHB:HV:DEXTRAN	58:12:30	3.37×10^4	94%
	PHB:HV:DEXTRIN	78:12:10	2.05×10^4	57%
	PHB:HV:DEXTRIN	58:12:30	2.84×10^4	79%
	PHB:HV:Na ALG	78:12:10	2.38×10^4	66%
	PHB:HV:Na ALG	58:12:30	2.61×10^4	73%
Family 2	PHB:HV:NT	79:20:1	1.06×10^4	29%
	PHB:HV:AMYLOSE	70:20:10	1.51×10^4	42%
	PHB:HV:AMYLOSE	50:20:30	1.14×10^4	32%
	PHB:HV:DEXTRAN	70:20:10	2.86×10^4	79%
	PHB:HV:DEXTRAN	50:20:30	3.48×10^4	97%
	PHB:HV:DEXTRIN	70:20:10	2.72×10^4	76%
	PHB:HV:DEXTRIN	50:20:30	3.21×10^4	89%
	PHB:HV:Na ALG	70:20:10	2.28×10^4	63%
	PHB:HV:Na ALG	50:20:30	2.71×10^4	75%
Control	BACTY	---	3.11×10^3	9%
	TC	---	3.60×10^4	100%

Table 4.2: **L929 Cell adhesion on filled & unfilled PHB-HV copolymers.**

	Polymer	Ratio of comonomers	Cell Count (cells/ml)	% Cell Attachment
Family 1	PHB:HV:NT	87:12:1	8.44×10^3	27%
	PHB:HV:AMYLOSE	78:12:10	1.25×10^4	40%
	PHB:HV:AMYLOSE	58:12:30	8.48×10^3	27%
	PHB:HV:DEXTRAN	78:12:10	2.73×10^4	88%
	PHB:HV:DEXTRAN	58:12:30	2.96×10^4	96%
	PHB:HV:DEXTRIN	78:12:10	1.81×10^4	58%
	PHB:HV:DEXTRIN	58:12:30	2.12×10^4	69%
	PHB:HV:Na ALG	78:12:10	1.93×10^4	62%
	PHB:HV:Na ALG	58:12:30	2.08×10^4	67%
Family 2	PHB:HV:NT	79:20:1	8.13×10^3	23%
	PHB:HV:AMYLOSE	70:20:10	1.30×10^4	42%
	PHB:HV:AMYLOSE	50:20:30	8.81×10^3	28%
	PHB:HV:DEXTRAN	70:20:10	2.31×10^4	75%
	PHB:HV:DEXTRAN	50:20:30	2.98×10^4	96%
	PHB:HV:DEXTRIN	70:20:10	1.91×10^4	62%
	PHB:HV:DEXTRIN	50:20:30	2.35×10^4	76%
	PHB:HV:Na ALG	70:20:10	1.64×10^4	53%
	PHB:HV:Na ALG	50:20:30	2.43×10^4	77%
Control	BACTY	---	1.78×10^3	6%
	TC	---	3.10×10^4	100%

Figure 4.1: BHK-21 & L929 Cell attachment on 12% HV polysaccharide filled copolymers.

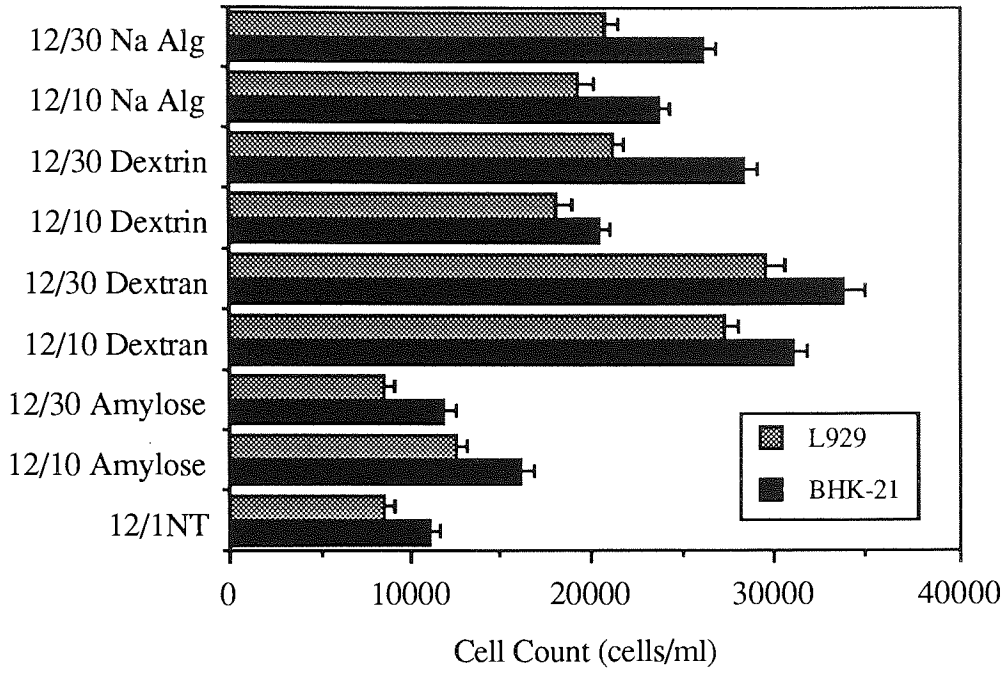


Figure 4.2: BHK-21 & L929 Cell attachment on 20% HV polysaccharide filled copolymers.

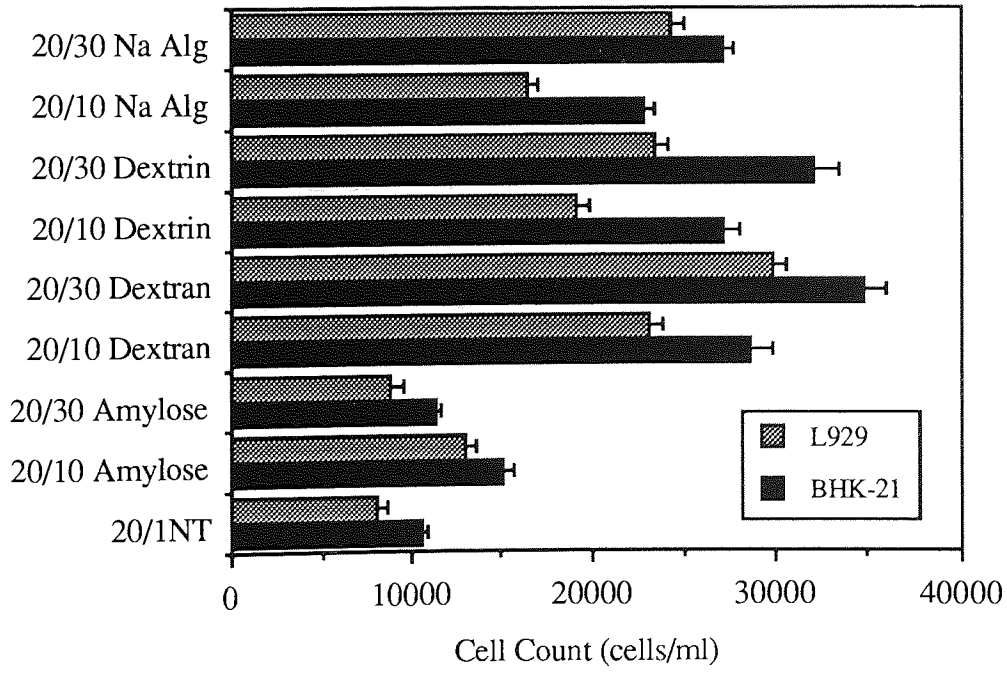


Figure 4.3: BHK-21 Cell attachment on 12% HV & 20% HV/10% polysaccharide filled copolymers.

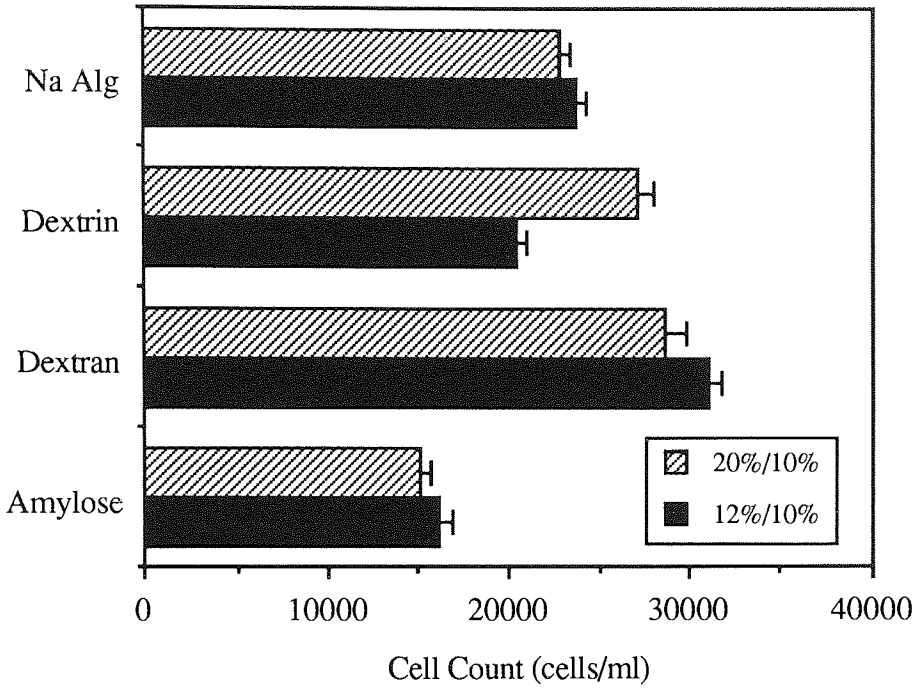


Figure 4.4: L929 Cell attachment on 12% HV & 20% HV/10% polysaccharide filled copolymers.

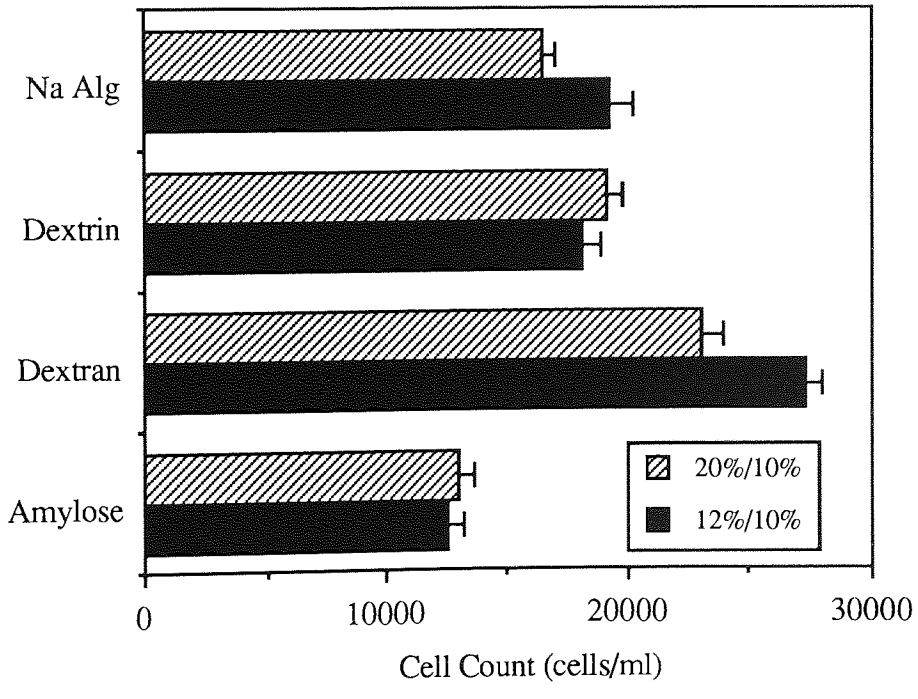


Figure 4.5: BHK-21 Cell attachment on 12% HV & 20% HV/30% polysaccharide filled copolymers.

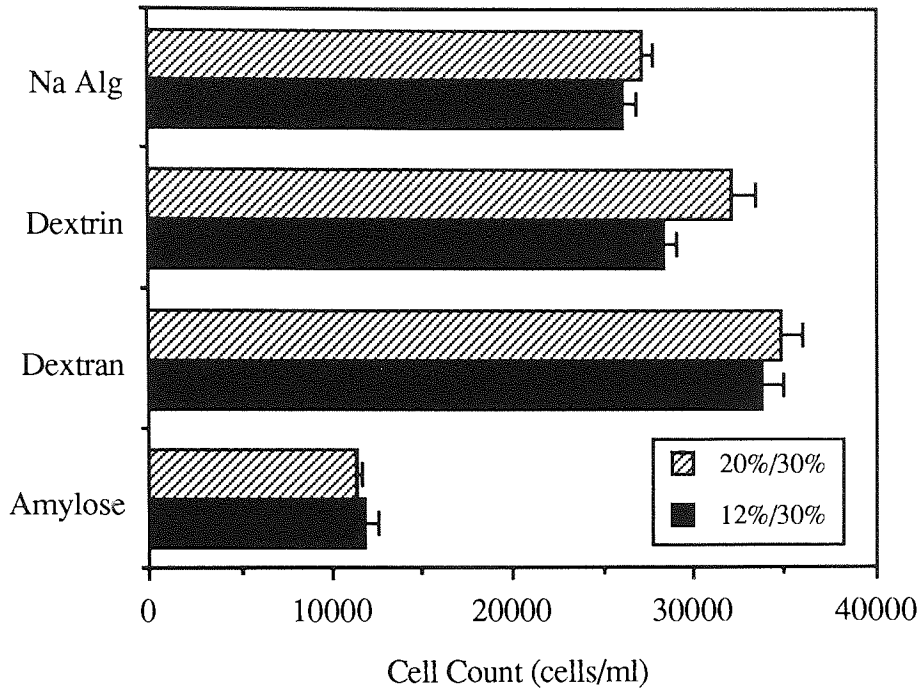


Figure 4.6: L929 Cell attachment on 12% HV & 20% HV/30% polysaccharide filled copolymers.

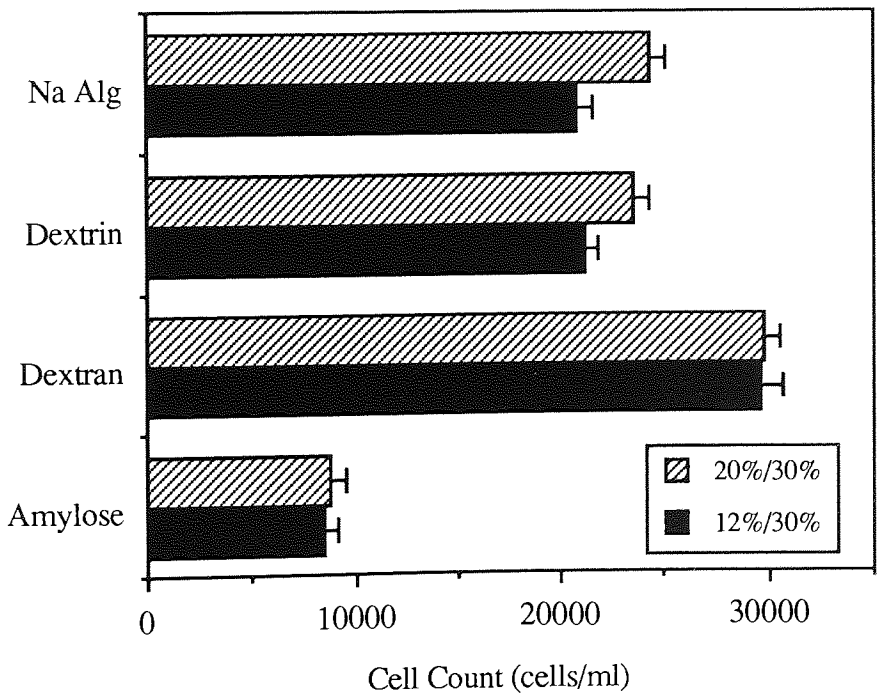


Figure 4.7: BHK-21 Cell attachment on 12% HV/10% polysaccharide & 12% HV/30% polysaccharide filled copolymers.

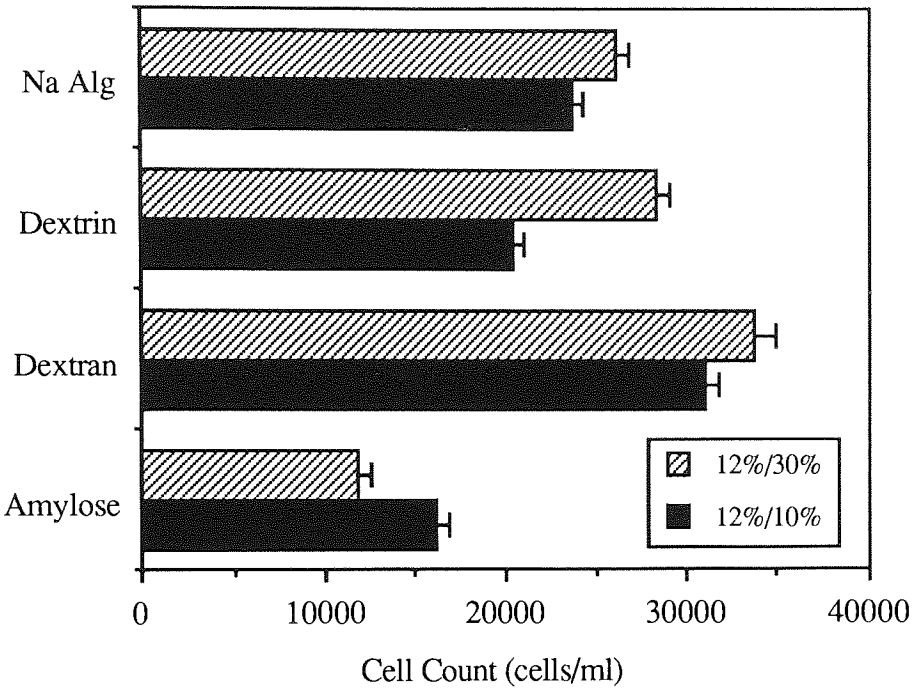


Figure 4.8: L929 Cell attachment on 12% HV/10% polysaccharide & 12% HV/30% polysaccharide filled copolymers.

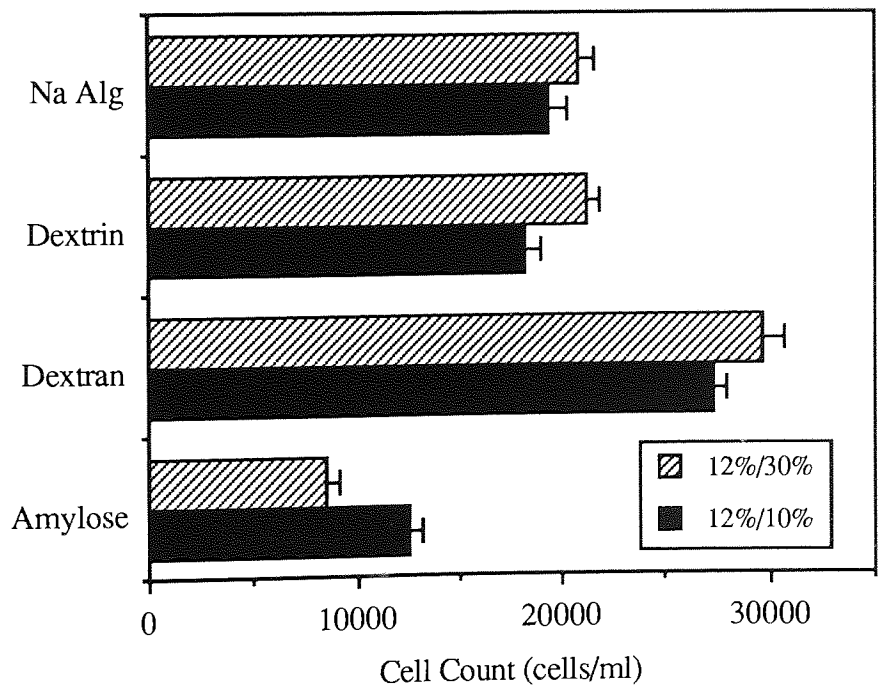


Figure 4.9: BHK-21 Cell attachment on 20% HV/10% polysaccharide & 20% HV/30% polysaccharide filled copolymers.

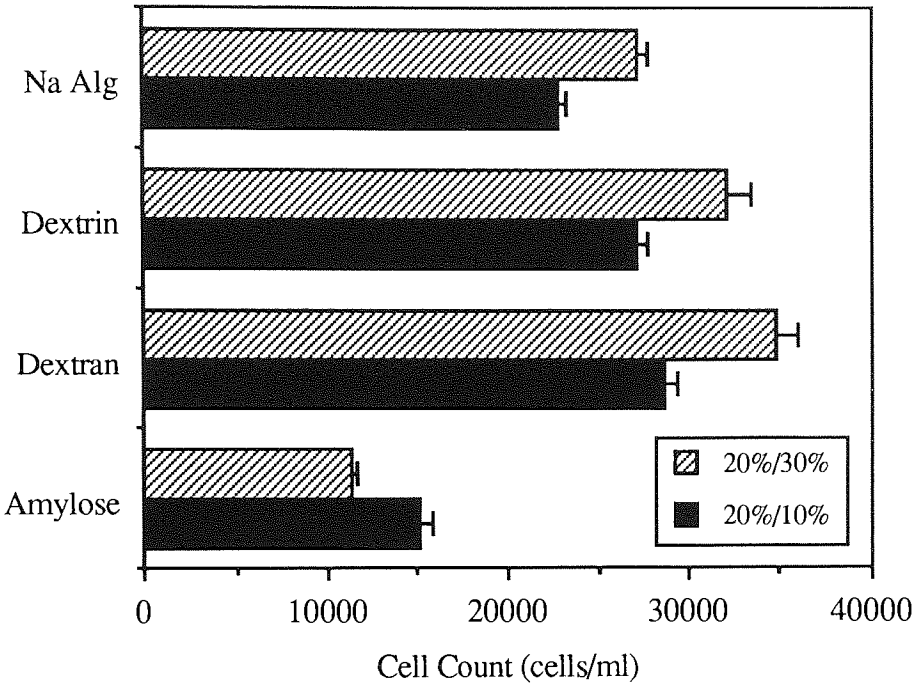


Figure 4.10: L929 Cell attachment on 20% HV/10% polysaccharide & 20% HV/30% polysaccharide filled copolymers.

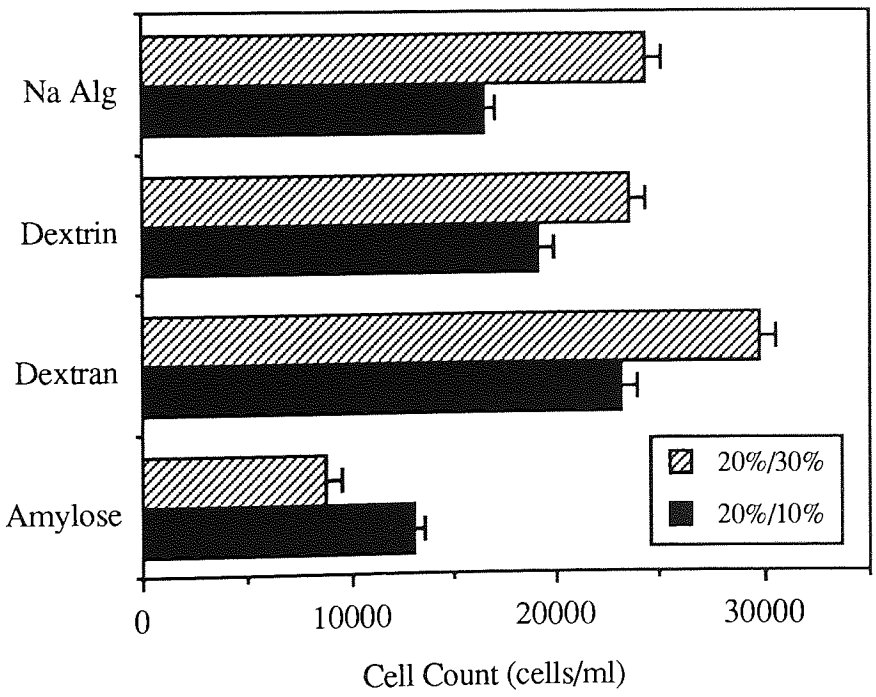


Figure 4.11: Surface energy measurements of 12%HV/10% polysaccharide blended copolymers degraded in "physiological" conditions (After Yasin[114]).

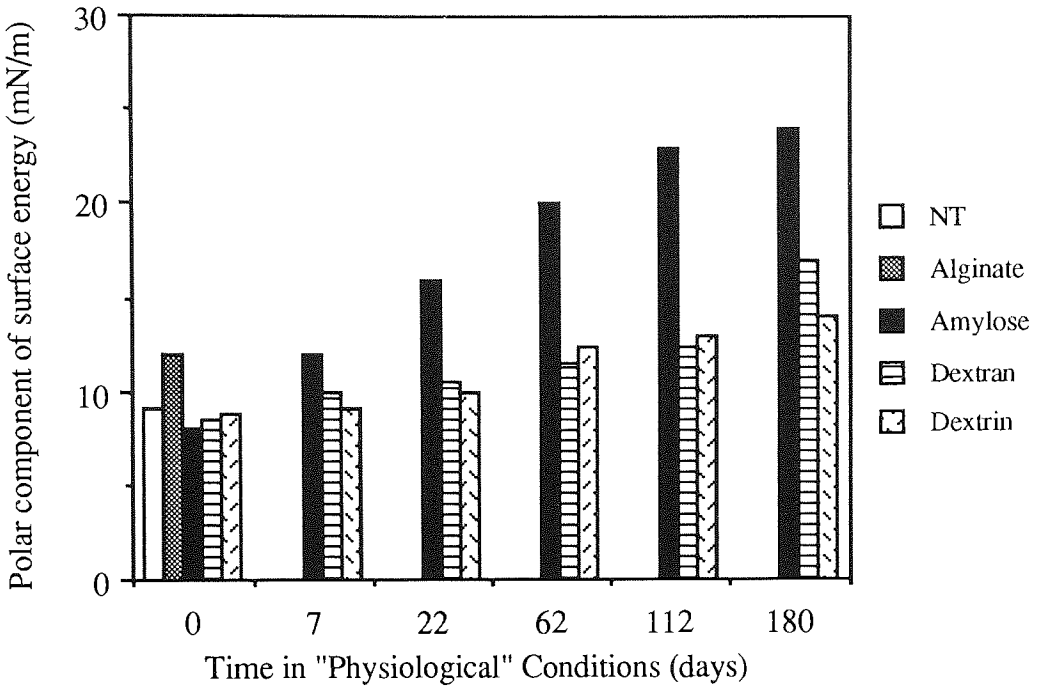
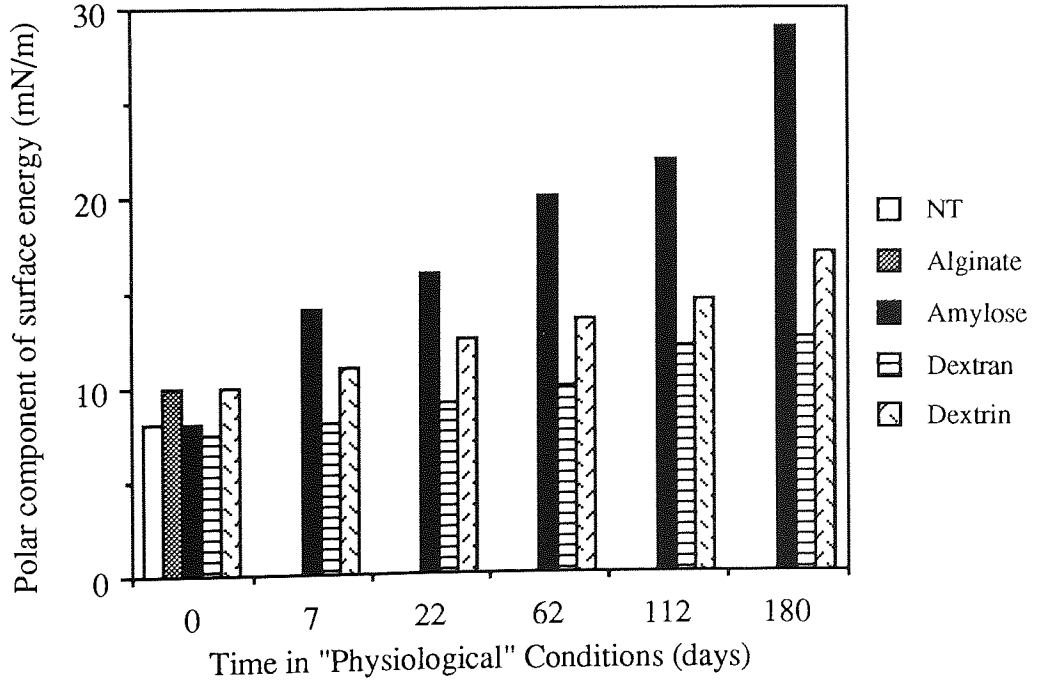


Figure 4.12: Surface energy measurements of 20%HV/10% polysaccharide blended copolymers degraded in "physiological" conditions (After Yasin[114]).



4.11 Cell adhesion on degraded PHB-HV copolymers

The results for both cell lines indicate a dichotomy between the levels of cell adhesion on the filled copolymer, and the unfilled copolymer. There is also a difference between the two cell lines as regards the levels of cell adhesion (Figures 4.17-4.22), which is accounted for by the differences in cell cycle times between the two fibroblasts. For both cell lines, the level of adhesion on the amylose filled copolymer increases rapidly from day 0 to day 7. The adhesion increases up to day 62 for both cell lines in the case of the filled copolymer, and then decreases over the remaining period up to day 250; while in the unfilled copolymer, the adhesion decreases for both cell lines after day 7 to day 250. The same general trend is observed for both cell lines. This suggests that the cell behaviour is influenced by factors other than the cell line employed.

The stepwise difference between the two copolymers with respect to levels of cell adhesion, is probably explained by increased polarity associated with extra processing. The incorporation of amylose into the polymer matrix involves a further melt-processing and milling stage, compared to the unfilled copolymer. Increasing the polarity of a surface can increase levels of cell adhesion, due to the adsorption of adhesive protein species on a charged surface. This provides the essential "conditioning layer".

A comparison of both copolymers (Figs 4.21-4.22) show that for both cell lines, there is an initial increase in the level of cell adhesion. Previous work in these laboratories has shown that the polar component of surface energy increases as

degradation proceeds[114]. It is probable that this increased polarity results in higher levels of cell adhesion, for reasons discussed earlier. Allied to this increase in cell adhesion is a small (relative to hydrogels, Chapter 3) increase in EWC. As the EWC of the copolymers increases, so "hema curve" behaviour may occur[53,35], due to the effects of the dominant water-structuring group. The increase in water-structuring groups means that more water will be bound at the surface which will lead to a concurrent increase in protein deposition from this bound water, leading to increased levels of cell adhesion.

After day 7, the levels of cell adhesion on the filled copolymer increase up to day 62, whereas the levels of cell adhesion on the unfilled copolymers decrease rapidly, more so for the L929 line than the BHK-21 line (Figures 4.21 & 4.22). Another facet to "hema curve" behaviour is the curtailment, quite rapidly, of cell adhesion to produce non-adhesive zones at higher levels of EWC. It may be that the surface charge density of hydroxyl groups has reached a critical density beyond which the negatively charged cell surface cannot overcome the short range electrostatic forces incurred by a large negative surface charge.

Surface rugosity is one of a number of factors cited as responsible for influencing cell behaviour, and in these results, may be the most important factor influencing cell adhesion on the degraded copolymer surfaces. Goniophotometric analysis carried out by Yasin[114] on the degraded surfaces of these two copolymers, show a decrease in the gloss factor for both copolymers (Figure 4.23). The gloss factor, as it's name suggests, measures the glossiness of a surface, and a decrease in the level

of this measurement indicates a surface change involving an increase in the irregularity of that surface, in other words, an increase in surface rugosity. Looking at the gloss factor (GF) data for the filled and unfilled copolymers, there is little change in the GF for the unfilled copolymer for ~20 days, followed by a decrease to day 250 (Figure 4.25). For the filled copolymer, the decrease in GF is progressive from the start. As the gloss factor decreases, so the polymer surface becomes increasingly rugose, as it degrades. Interestingly, the gloss factors converge at day 250, paralleling the convergence (and overlap in the case of L929, Figure.4.15) of levels of cell adhesion at day 250 for both cell lines on the two copolymers (Figure 4.21). At the microscopic level, as the surface becomes increasingly rugose, the surface area available for cell attachment decreases; there are less points of attachment. This may lead to decreased levels of adhesion. As the surface rugosity reaches the macroscopic level, so there will be a greater surface area available for cell attachment, and this may lead to increased levels of adhesion. Also, the rugosity may influence the expression of deposited protein species. If the proteins are in the wrong conformation, the cell-binding sites may be masked, thereby limiting cell adhesion. As the copolymer degrades, the surface may fragment due to the mechanical stresses exerted by the cell in the attachment process[126].

4.12 Conclusions

The process of degradation affects cell behaviour. If a comparison is made of the change in surface properties with the change in bulk properties of the copolymers (Fig. 4.25), the bulk properties of the copolymers, such as the crystallinity and

initial weight remain almost constant over the time period of degradation studied. It is the surface properties, the polar component of surface energy, and the surface rugosity that are changing markedly. It is the change in surface properties, rather than the bulk properties of the copolymers that influence the cell response to degraded PHB-HV copolymers.

Some of the surface characteristics which play an important part in determining the cellular response on stable systems such as the hydrogels of chapter three can also influence cell adhesion in PHB-HV copolymers. Polysaccharides which are incorporated into the copolymer matrix to accelerate degradation also greatly influence the behaviour of the fibroblastic cell lines used in this study. Hydrophilic polysaccharides increase the wettability of PHB-HV copolymers, and this increases cell adhesion. The knowledge of cellular interactions with hydrogels is applicable to these biodegradable copolymers.

Armed with the knowledge of how cells interact with the materials used in chapters three and four, the natural progression of the study was to observations of cell behaviour on a material which has been designed for a specific clinical application. This material is gel-spun PHB which has potential uses as a wound scaffold in the healing wound site.

Table 4.3: BHK-21 cell adhesion on "physiologically" degraded PHB-HV copolymers.

Polymer	Days in "Physiological" Conditions	Cell Count (cells/ml)
PHB:HV	0	1.25×10^4
(82:12)	7	1.49×10^4
	62	1.20×10^4
	180	9.81×10^3
	250	9.60×10^3
PHB:HV:AMYLOSE	0	1.30×10^4
(78:12:10)	7	1.54×10^4
	62	1.74×10^4
	180	1.31×10^4
	250	1.18×10^4
TC	---	2.12×10^4
BACTY	---	5.63×10^3

Table 4.4: L929 cell adhesion on "physiologically" degraded PHB-HV copolymers.

Polymer	Days in "Physiological" Conditions	Cell Count (cells/ml)
PHB:HV	0	1.14×10^4
(82:12)	7	1.54×10^4
	62	1.12×10^4
	180	7.10×10^3
	250	9.60×10^3
PHB:HV:AMYLOSE	0	9.92×10^3
(78:12:10)	7	1.52×10^4
	62	1.76×10^4
	180	1.21×10^4
	250	7.63×10^3
TC	---	1.72×10^4
BACTY	---	6.31×10^3

Figure 4.13: BHK-21 Adhesion on filled & unfilled PHB-HV copolymers.

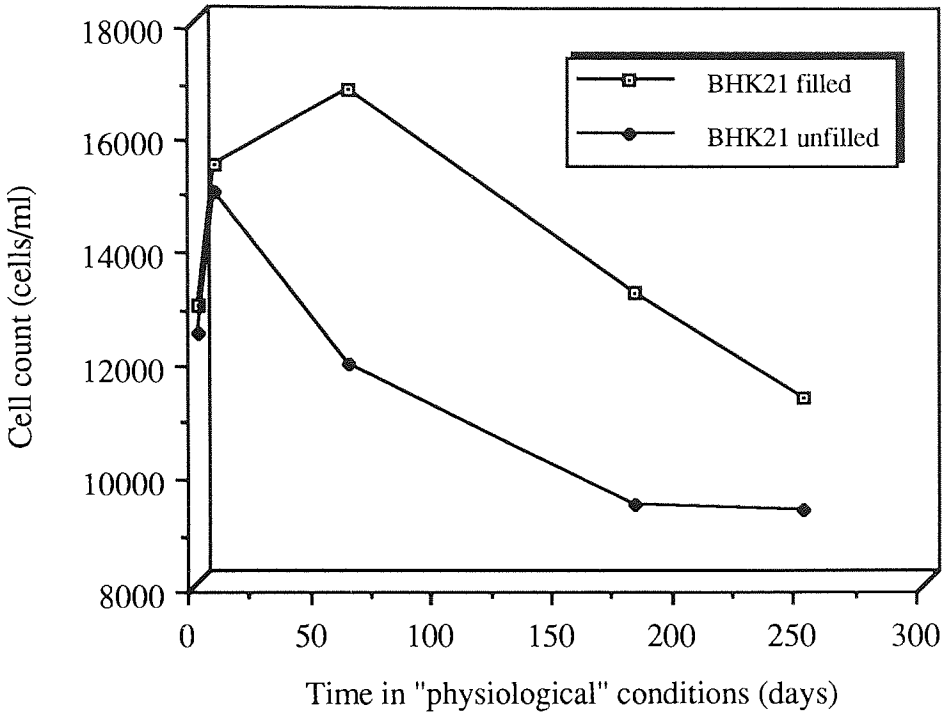


Figure 4.14: BHK-21 Adhesion on filled & unfilled PHB-HV copolymers.

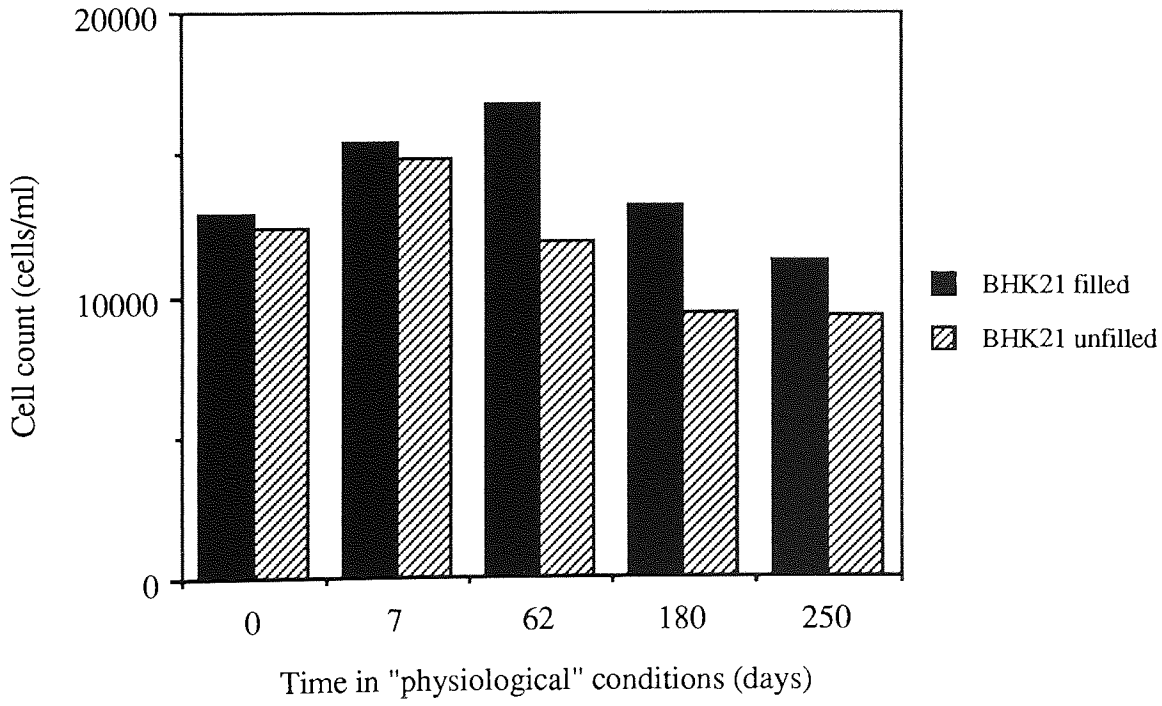


Figure 4.15: L929 Adhesion on filled & unfilled PHB-HV copolymers.

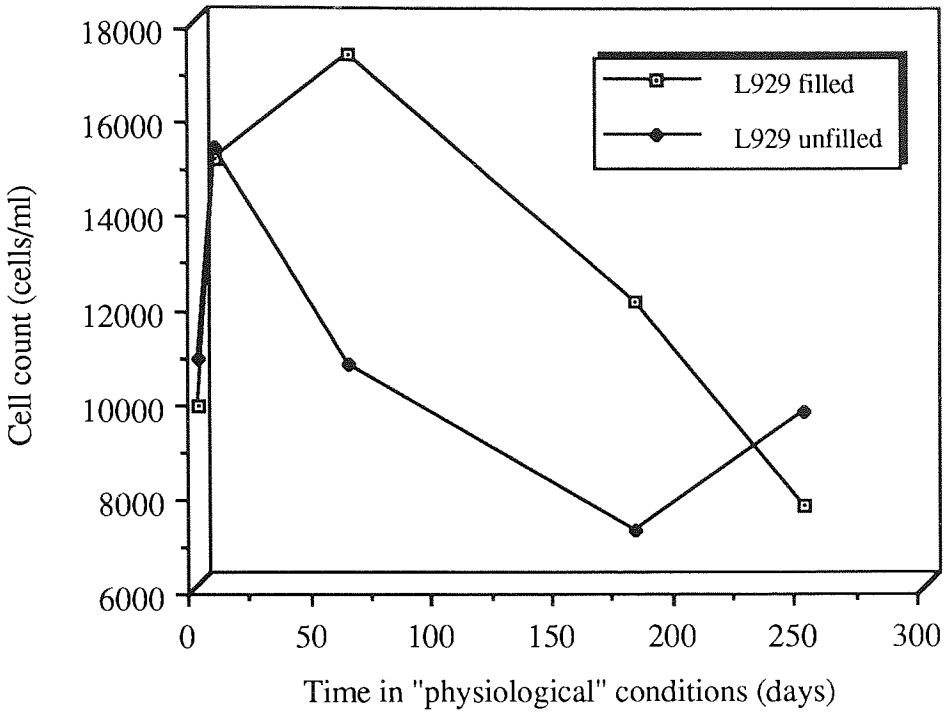


Figure 4.16: L929 Adhesion on filled & unfilled PHB-HV copolymers.

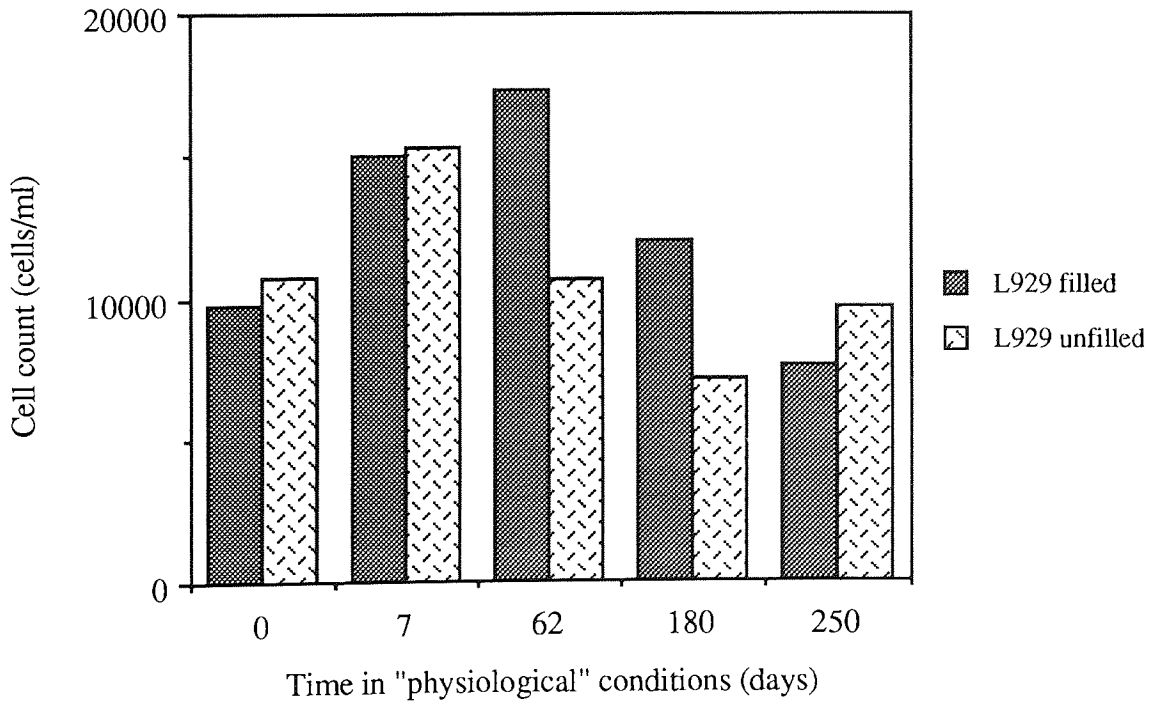


Figure 4.17: BHK-21 Vs L929 adhesion on filled PHB-HV copolymers.

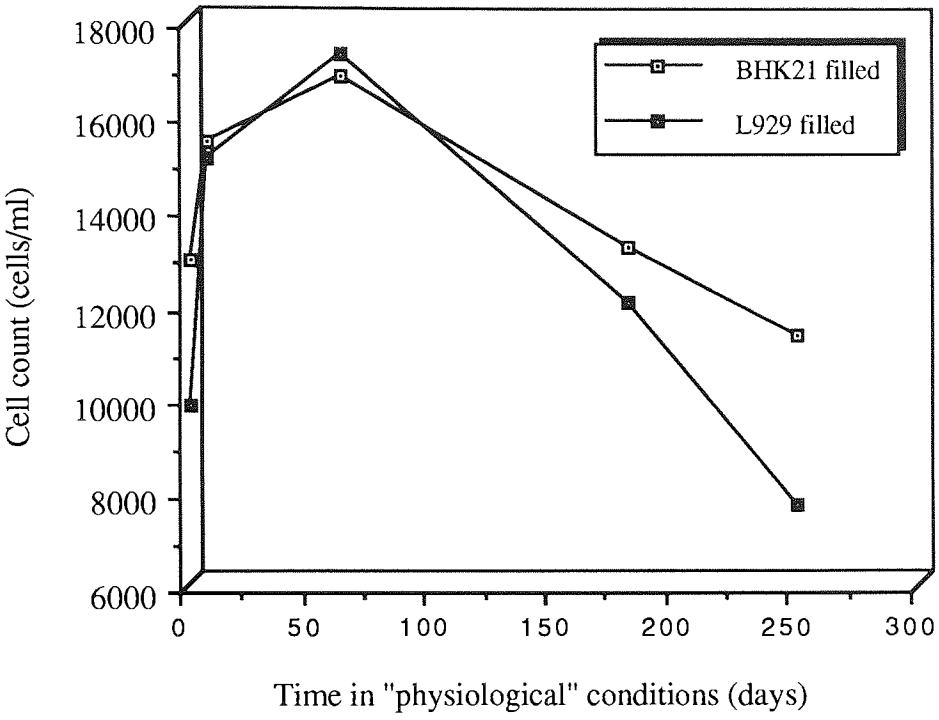


Figure 4.18: BHK-21 Vs L929 adhesion on filled PHB-HV copolymers.

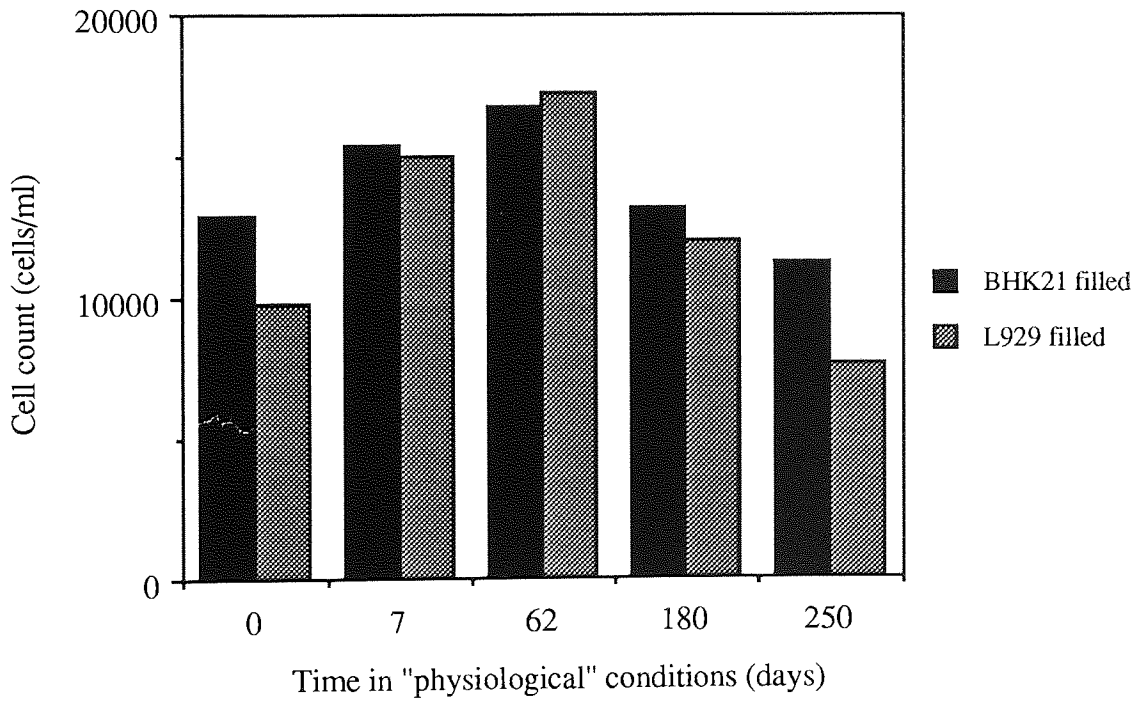


Figure 4.19: BHK-21 Vs L929 adhesion on unfilled PHB-HV copolymers.

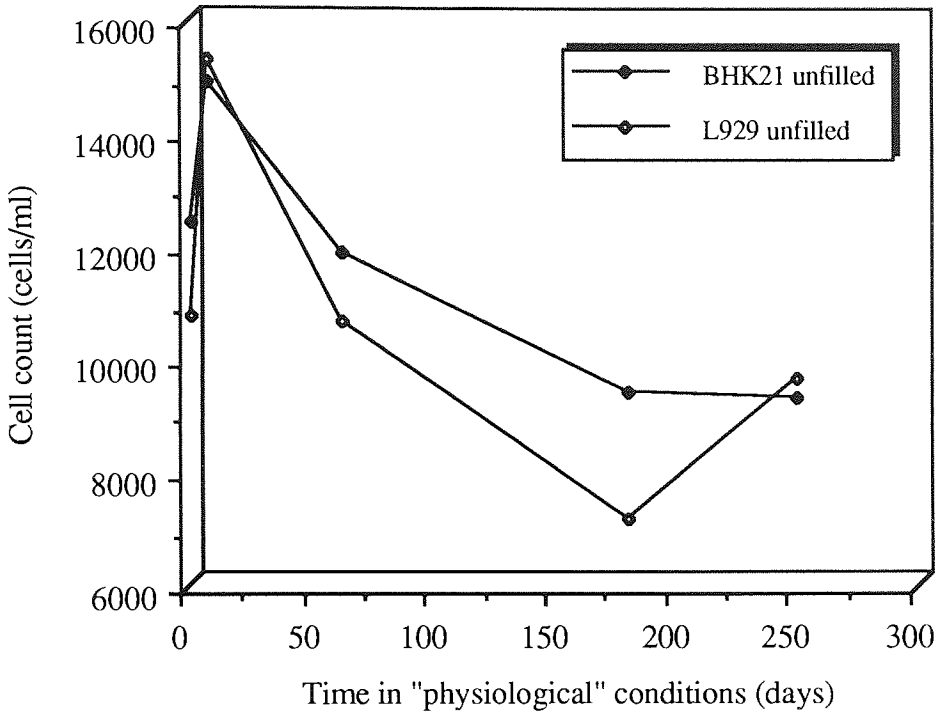


Figure 4.20: BHK-21 Vs L929 adhesion on unfilled PHB-HV copolymers.

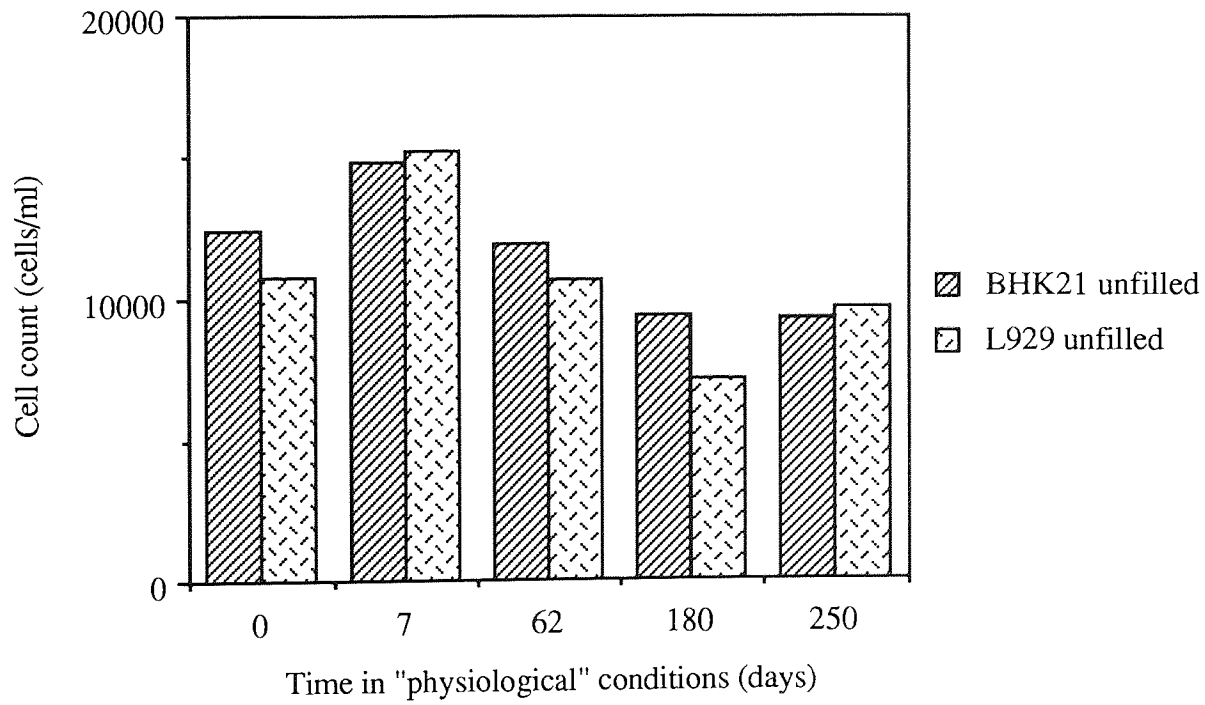


Figure 4.21: BHK-21 Vs L929 adhesion on filled & unfilled PHB-HV copolymers.

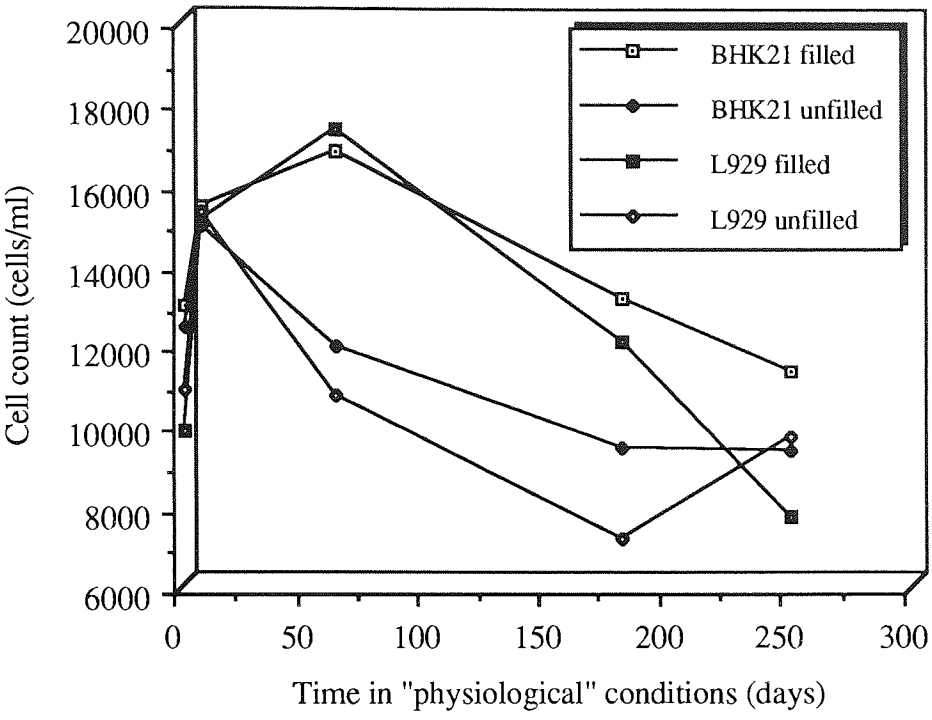


Figure 4.22: BHK-21 Vs L929 adhesion on filled & unfilled PHB-HV copolymers.

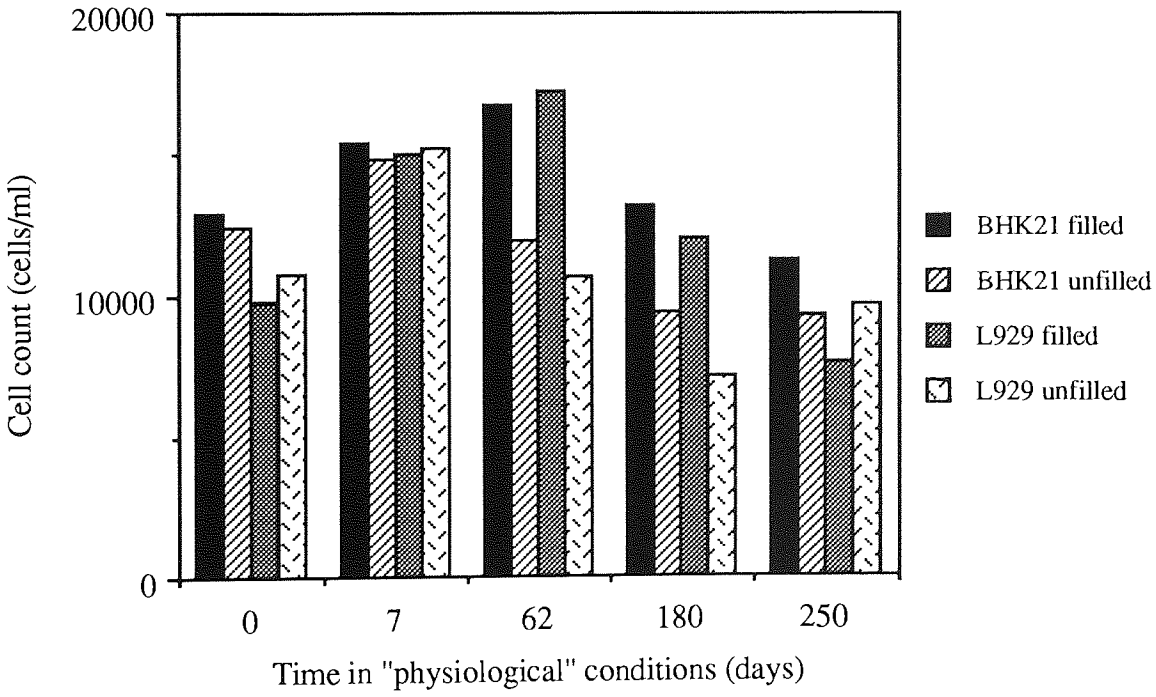


Figure 4.23: Gloss factor measurements of 12%HV/10% polysaccharide blended copolymers degraded in "physiological" conditions (After Yasin[114]).

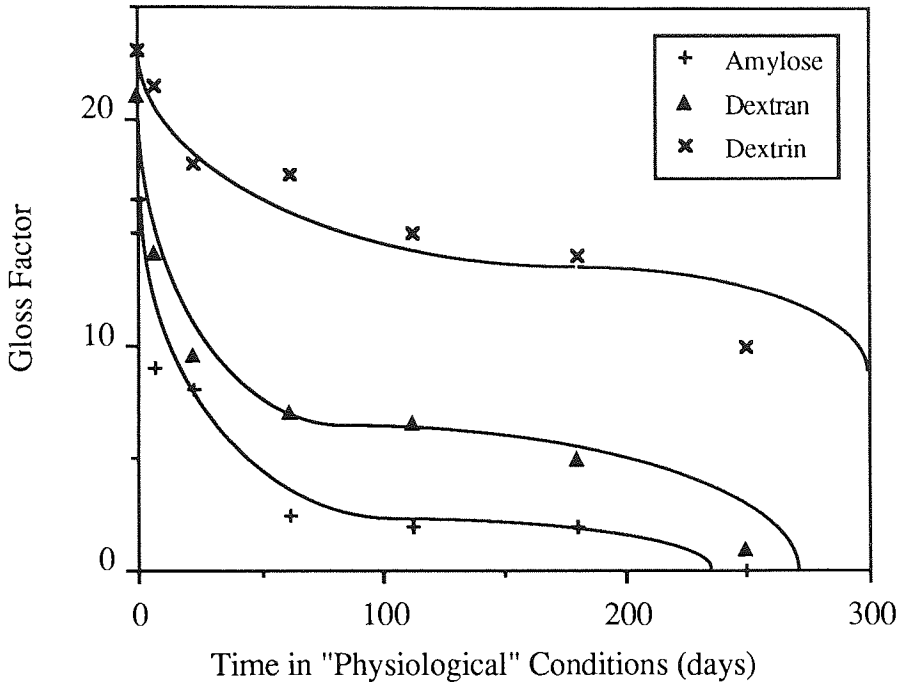


Figure 4.24: Gloss factor measurements of 20%HV/10% polysaccharide blended copolymers degraded in "physiological" conditions (After Yasin[114]).

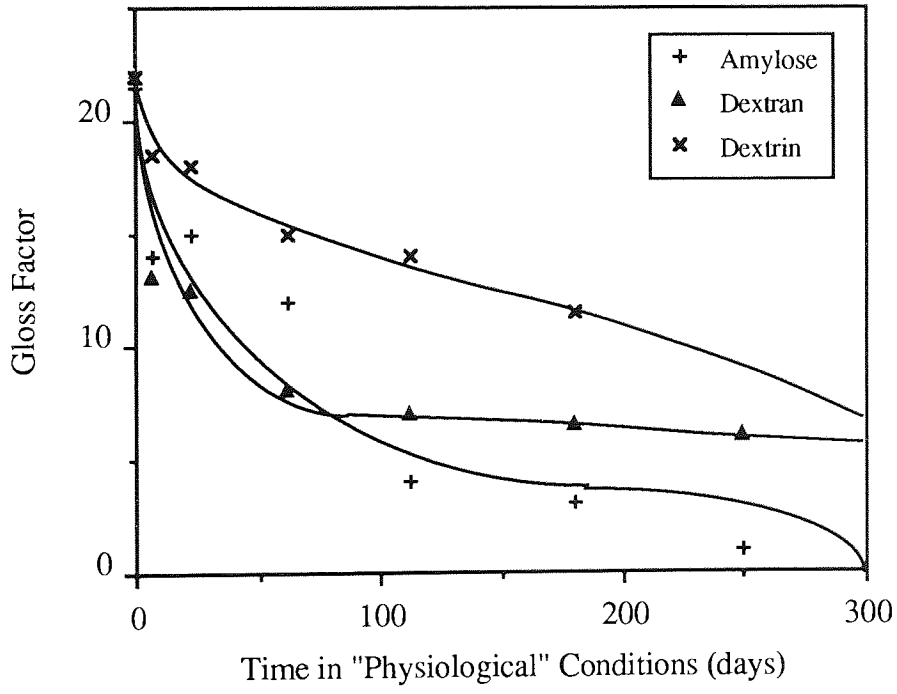


Figure 4.25: Changes in bulk and surface properties of 12%HV apatite nucleated copolymers after degradation in "physiological" conditions (After Yasin[114]).

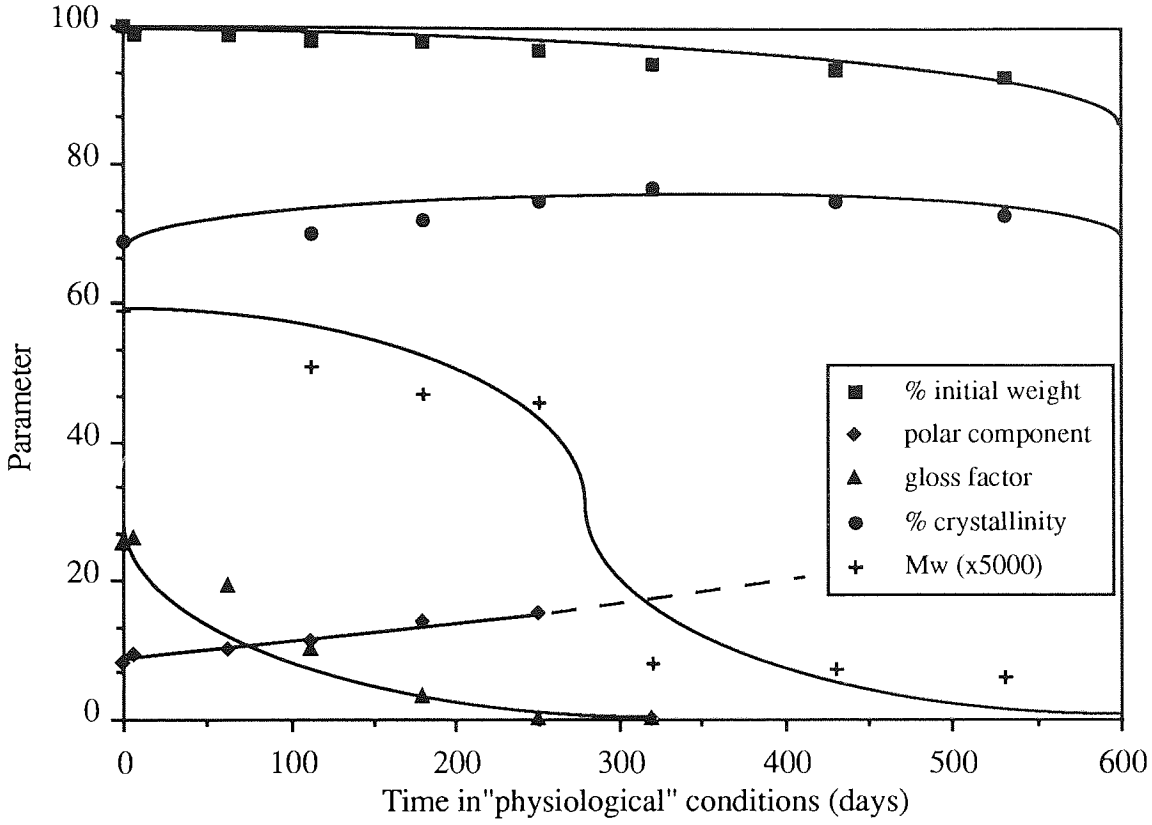


Plate 4.1: BHK-21 cell attachment on 12% HV/1% NT copolymer.

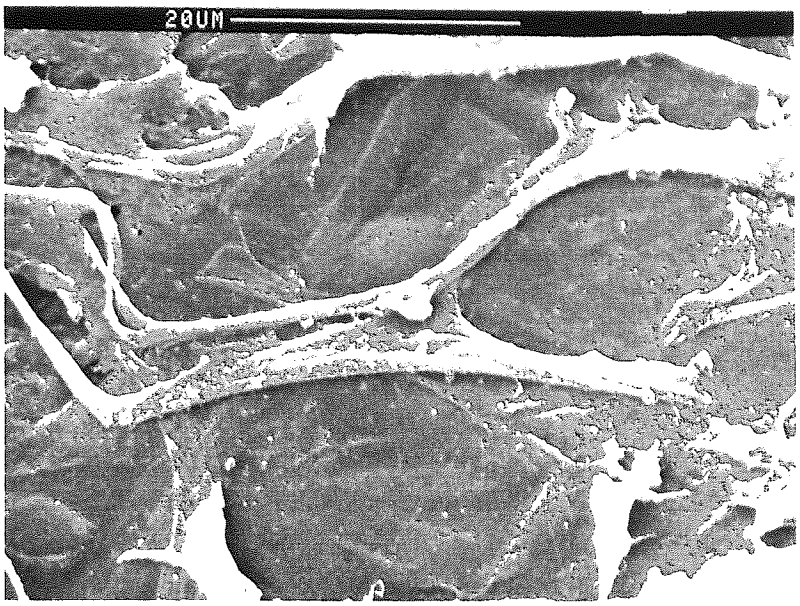


Plate 4.2: L929 cell attachment on 12% HV/1% NT copolymer.

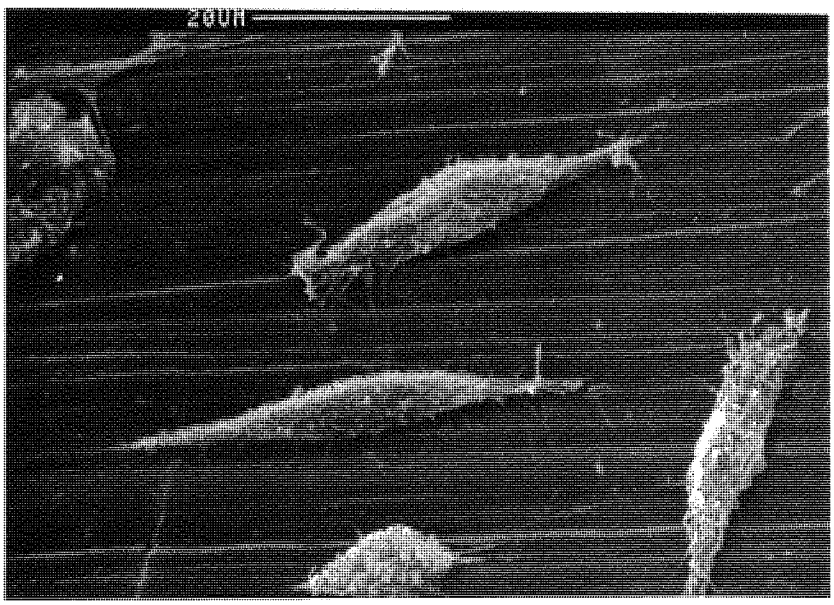
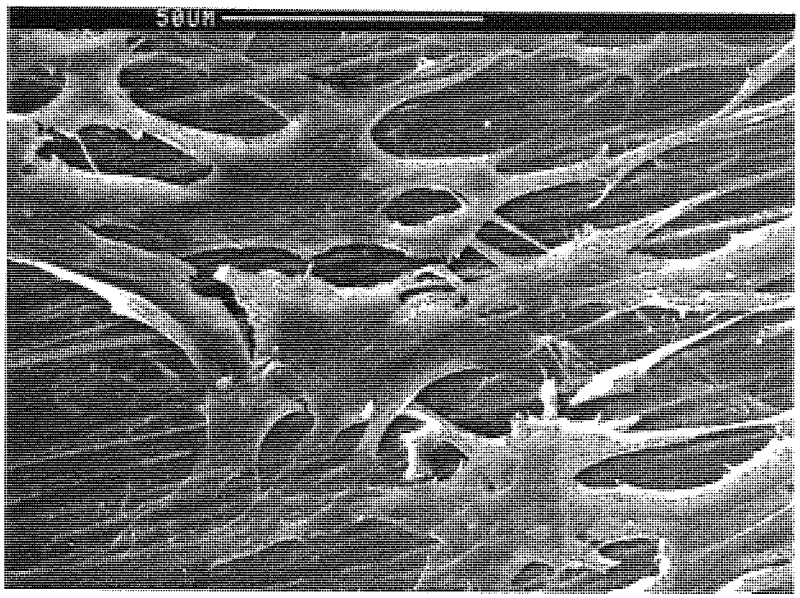


Plate 4.3: BHK-21 cell attachment on 12% HV/10% Amylose copolymer.



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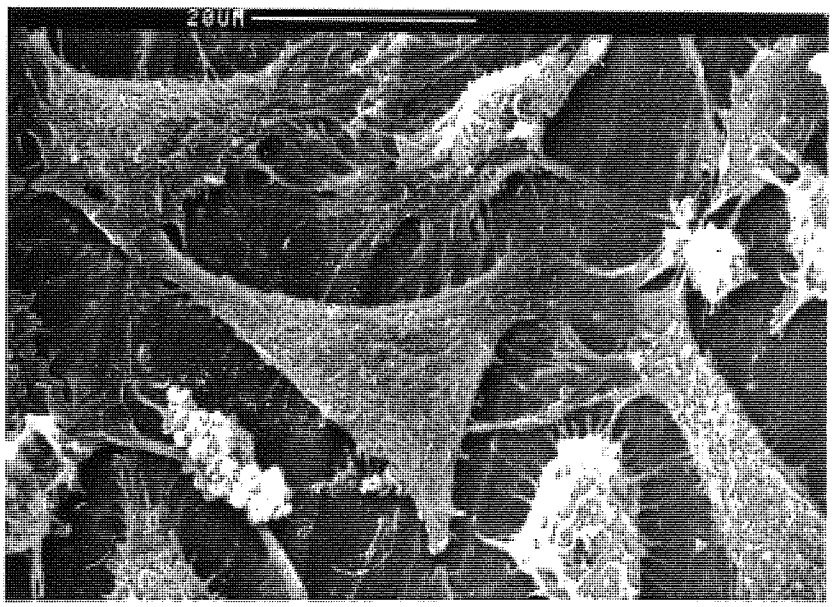
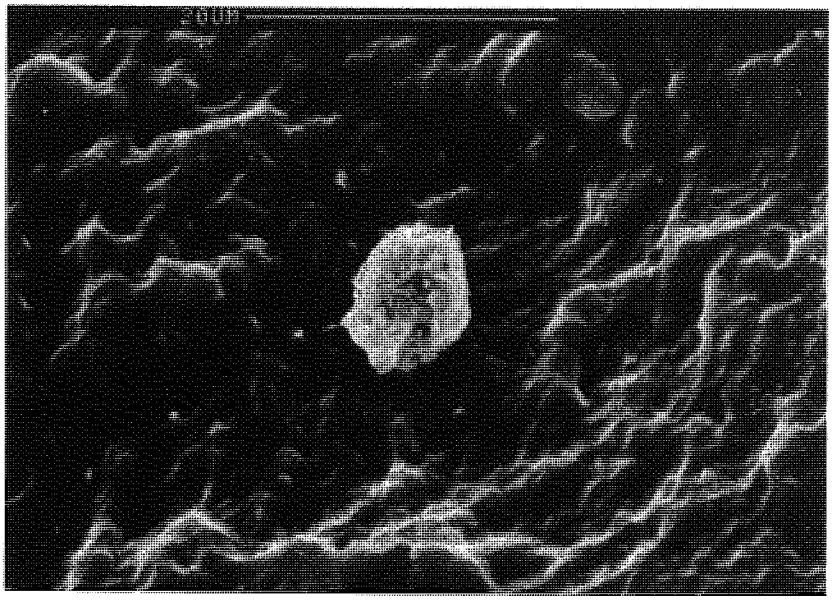


Plate 4.5: BHK-21 cell attachment on 12% HV/30% Amylose copolymer.



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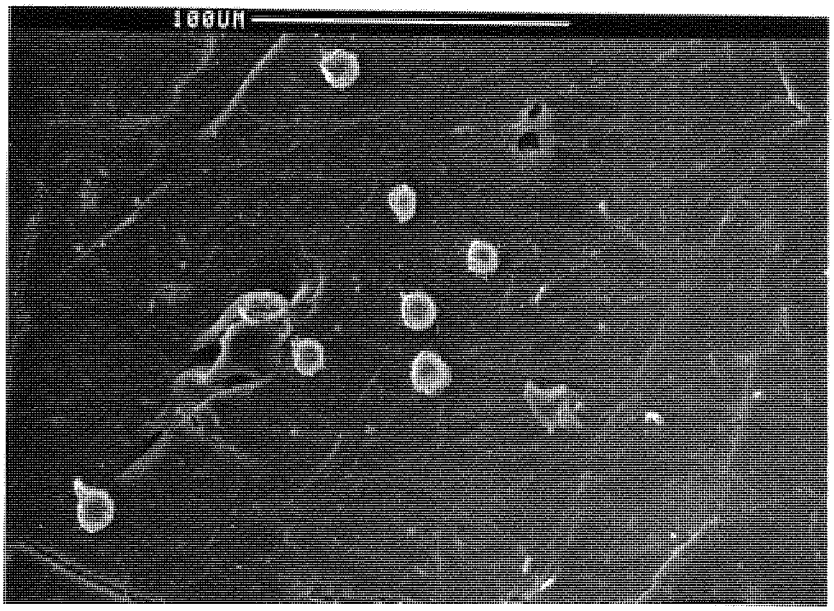
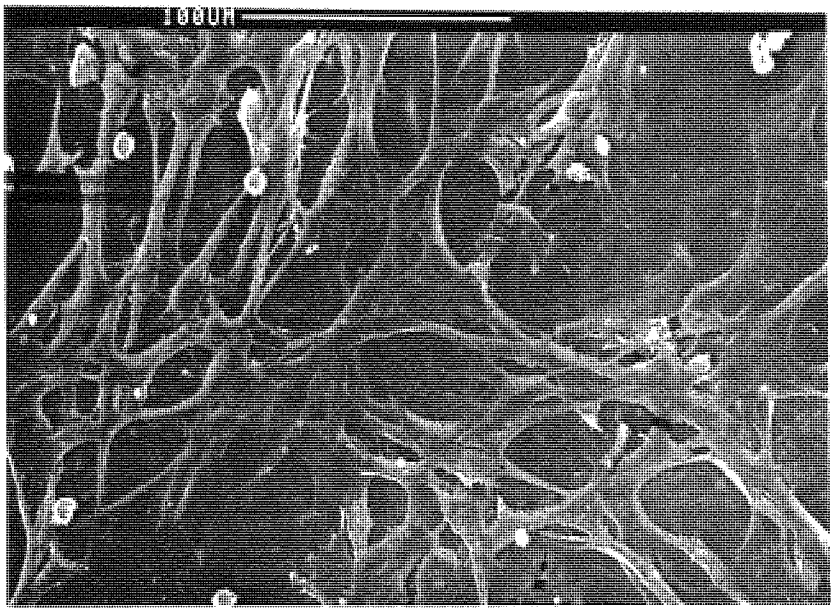


Plate 4.7: BHK-21 cell attachment on 12% HV/10% Dextran copolymer.



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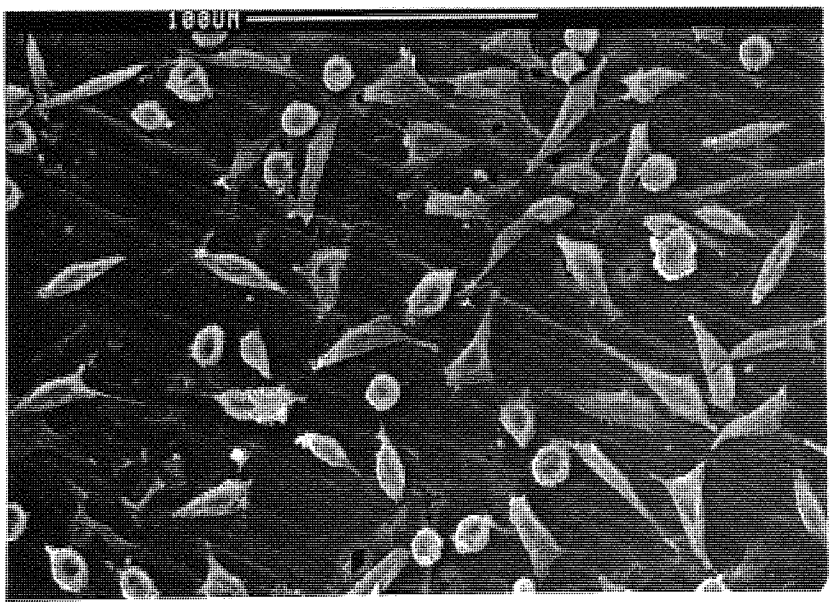


Plate 4.9: BHK-21 cell attachment on 12% HV/30% Dextran copolymer.

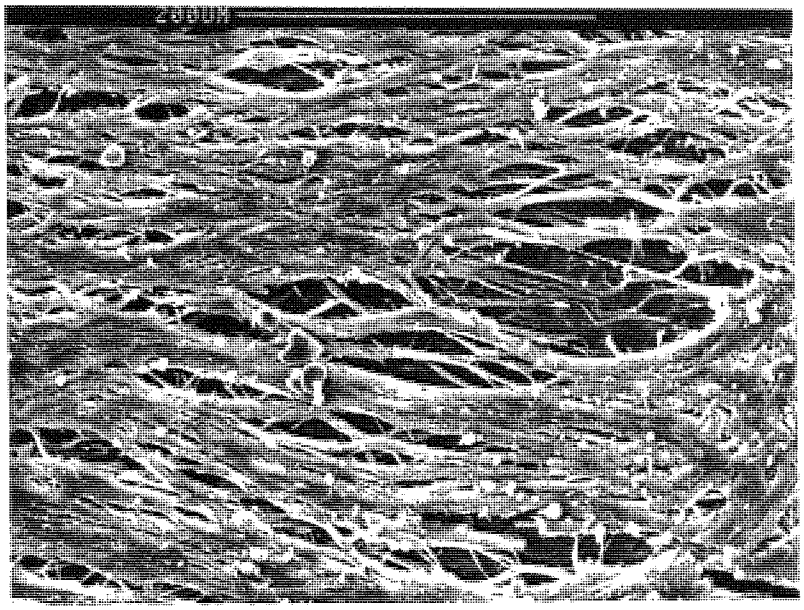


Plate 4.10: L929 cell attachment on 12% HV/30% Dextran copolymer.

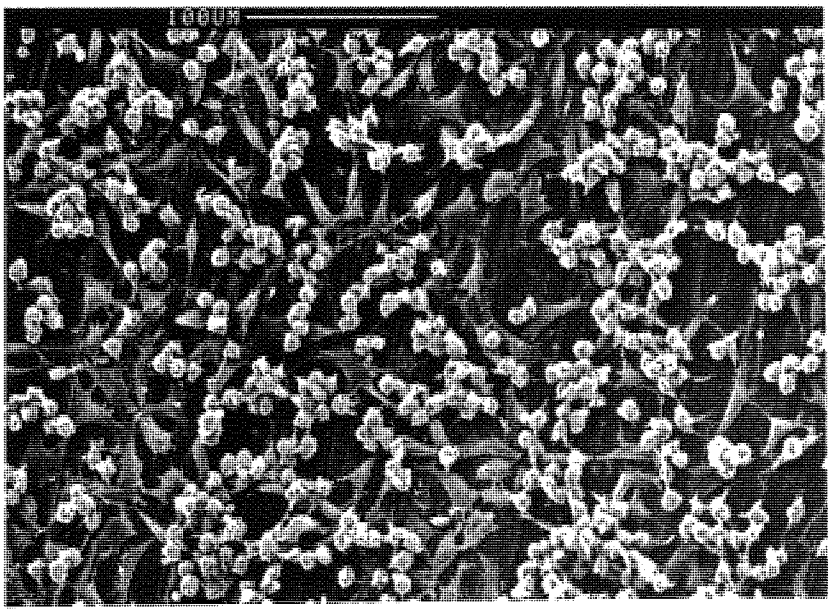


Plate 4.11: BHK-21 cell attachment on 12% HV/10% Dextrin copolymer.

Plate 4.12: L929 cell attachment on 12% HV/10% Dextrin copolymer.

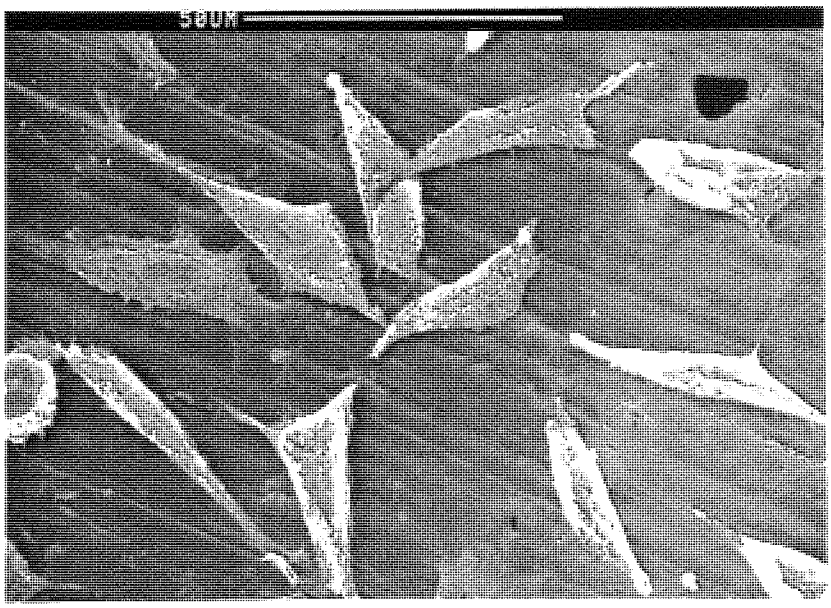


Plate 4.13: BHK-21 cell attachment on 12% HV/30% Dextrin copolymer.

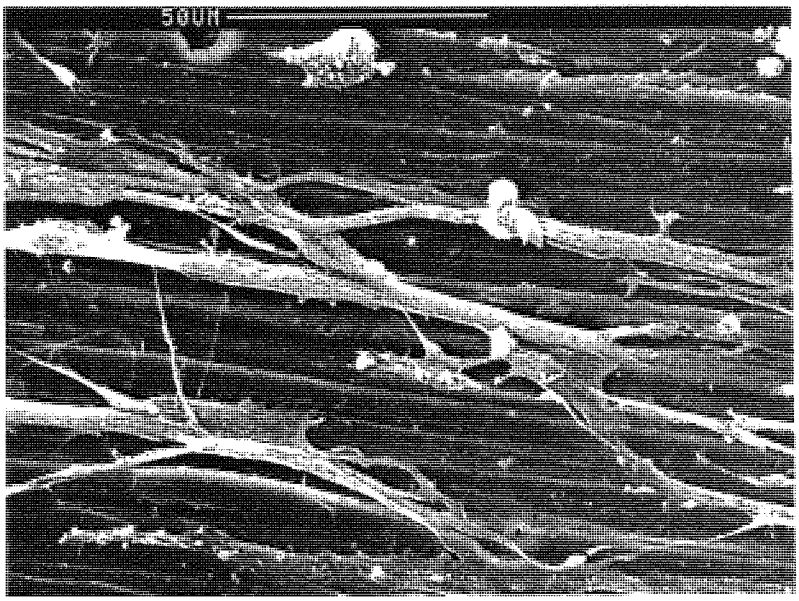


Plate 4.14: L929 cell attachment on 12% HV/30% Dextrin copolymer.

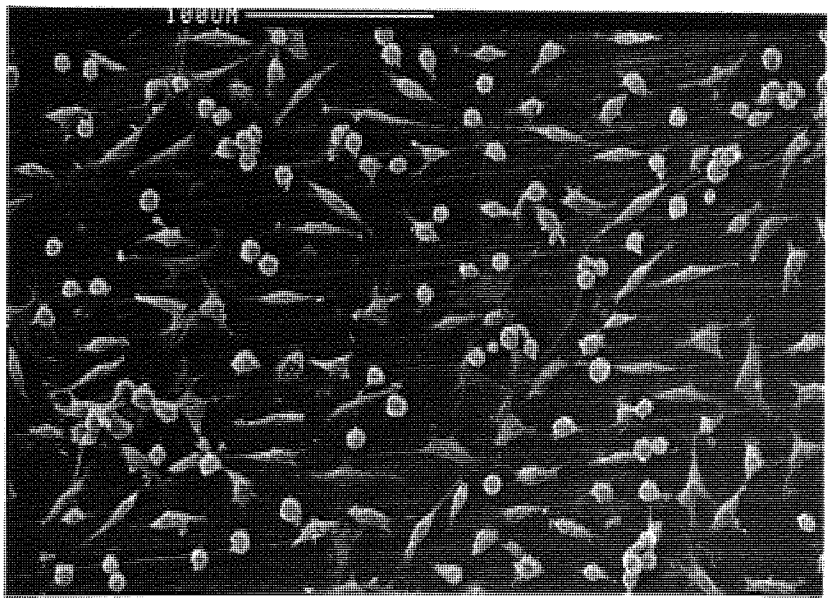


Plate 4.15: BHK-21 cell attachment on 12% HV/10% Na Alginate copolymer.

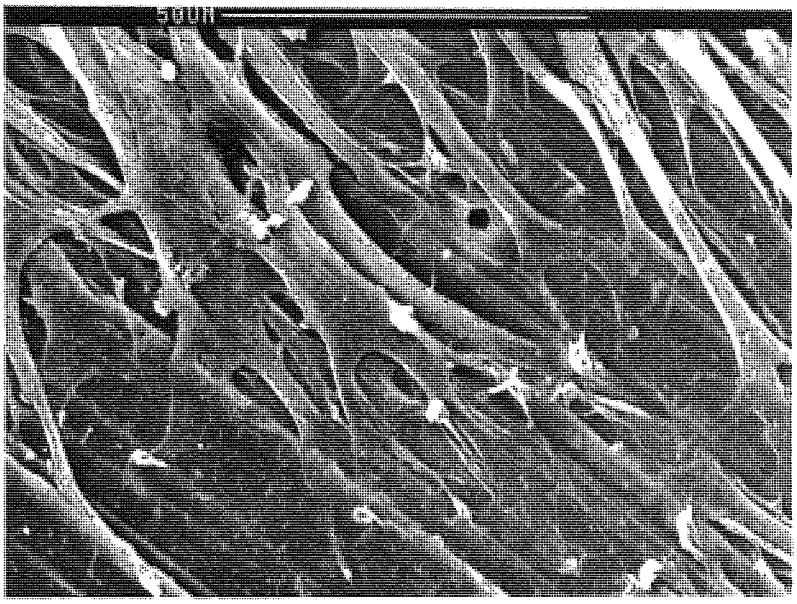


Plate 4.16: L929 cell attachment on 12% HV/10% Na Alginate copolymer.

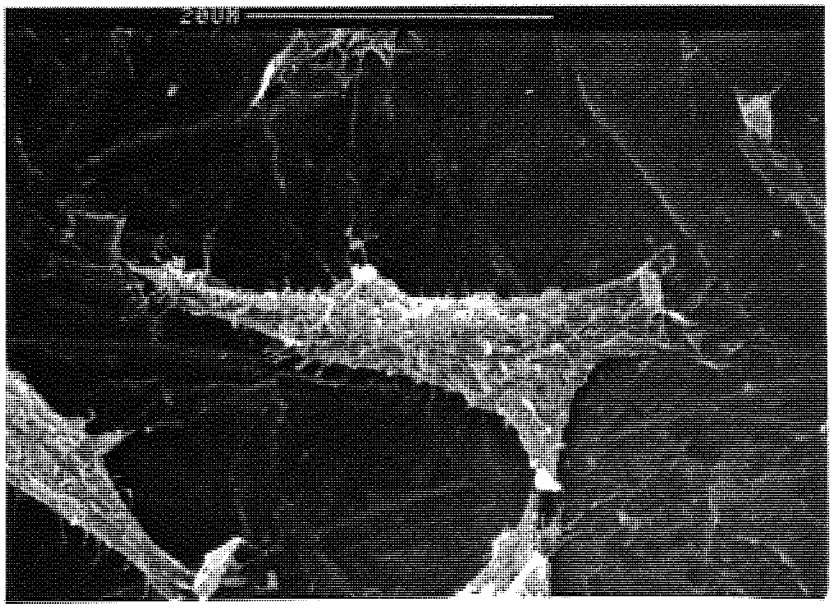


Plate 4.17: BHK-21 cell attachment on 12% HV/30% Na Alginate copolymer.

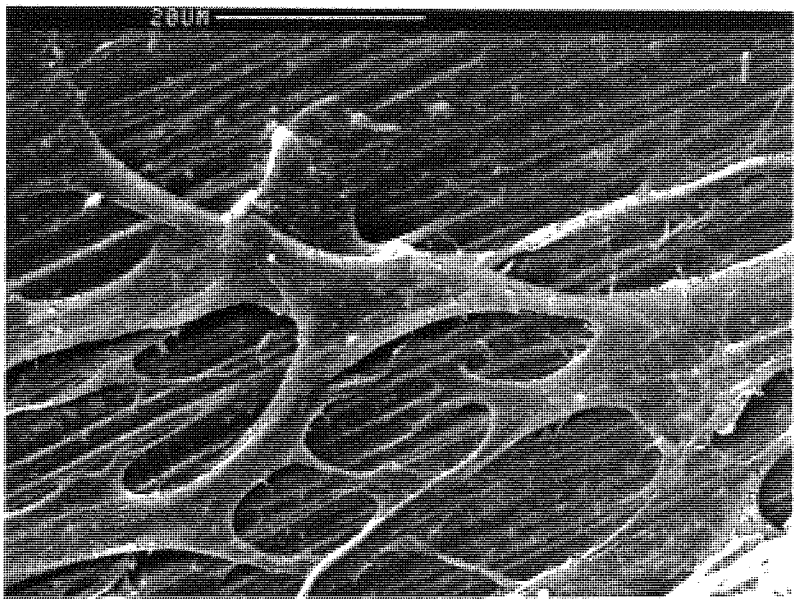


Plate 4.18: L929 cell attachment on 12% HV/30% Na Alginate copolymer.

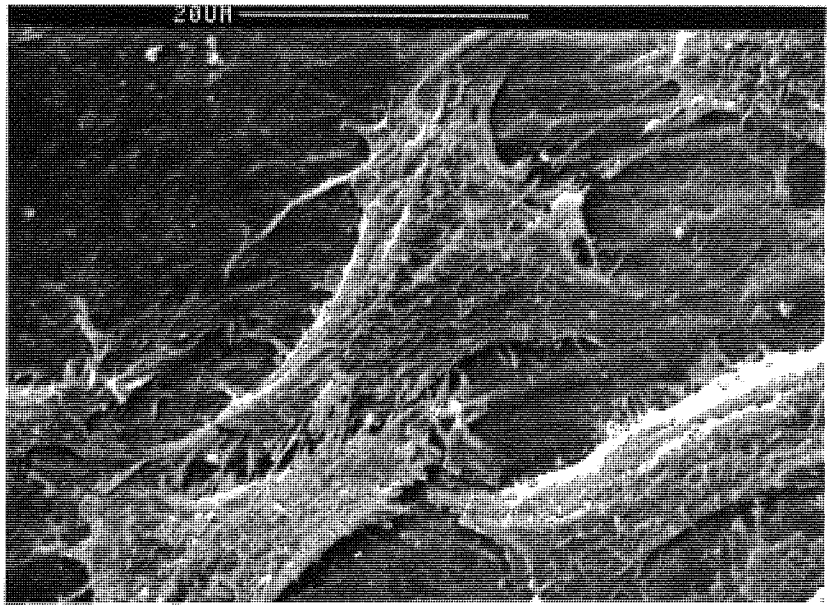


Plate 4.19: BHK-21 cell attachment on 20% HV/1% NT copolymer.

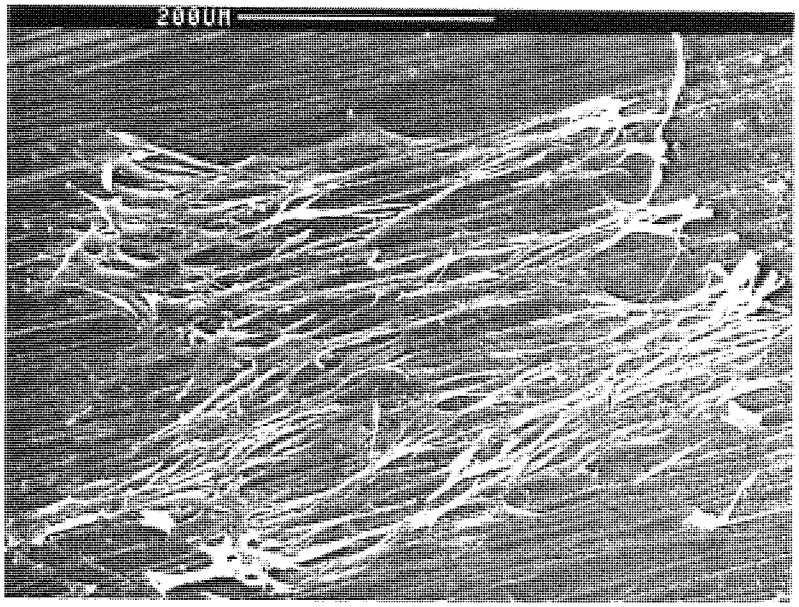


Plate 4.20: L929 cell attachment on 20% HV/1% NT copolymer.

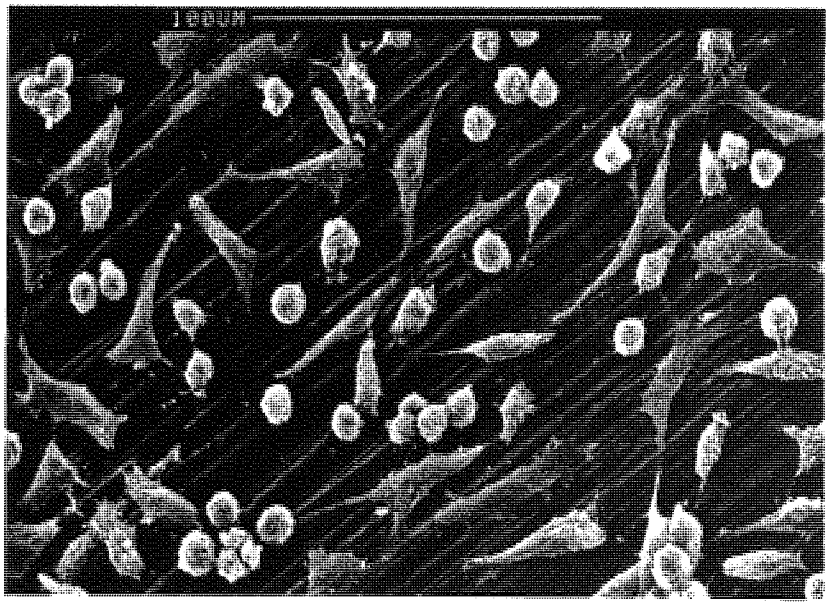


Plate 4.21: BHK-21 cell attachment on 20% HV/10% Amylose copolymer.

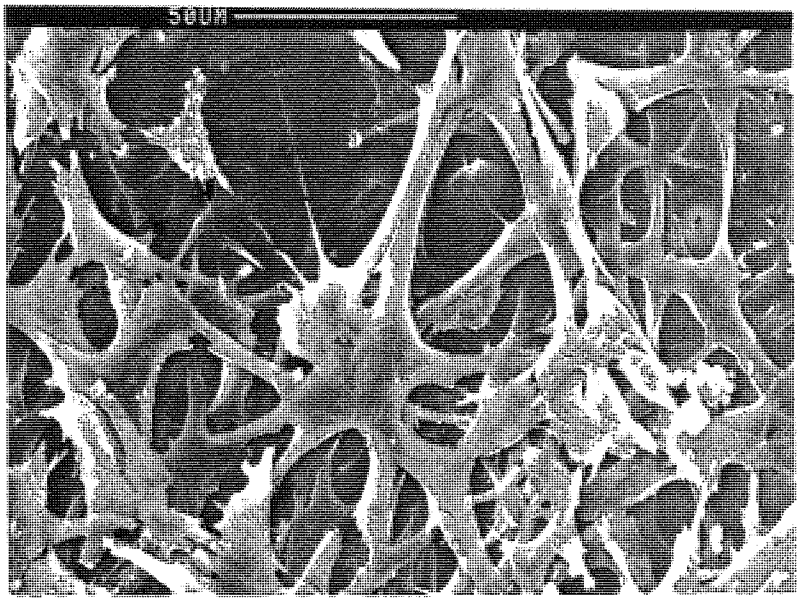


Plate 4.22: L929 cell attachment on 20% HV/10% Amylose copolymer.

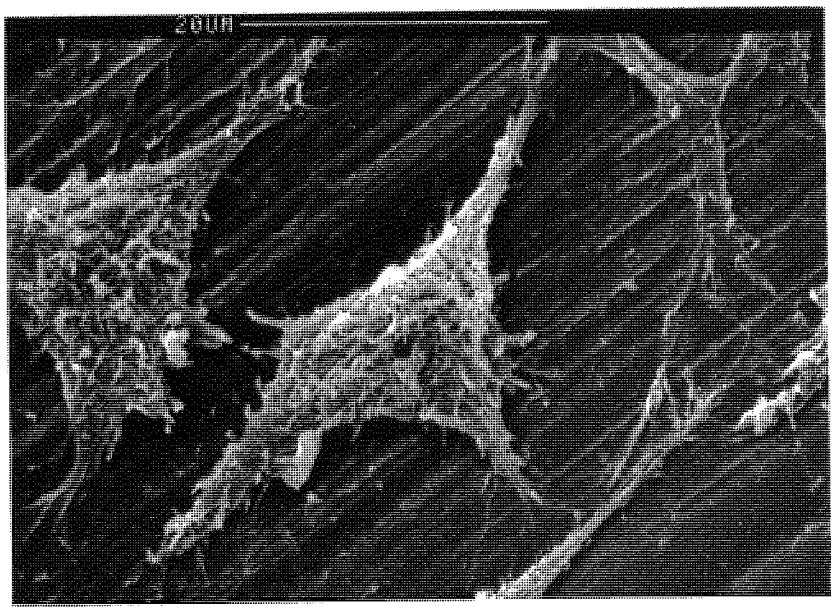


Plate 4.23: BHK-21 cell attachment on 20% HV/30% Amylose copolymer.

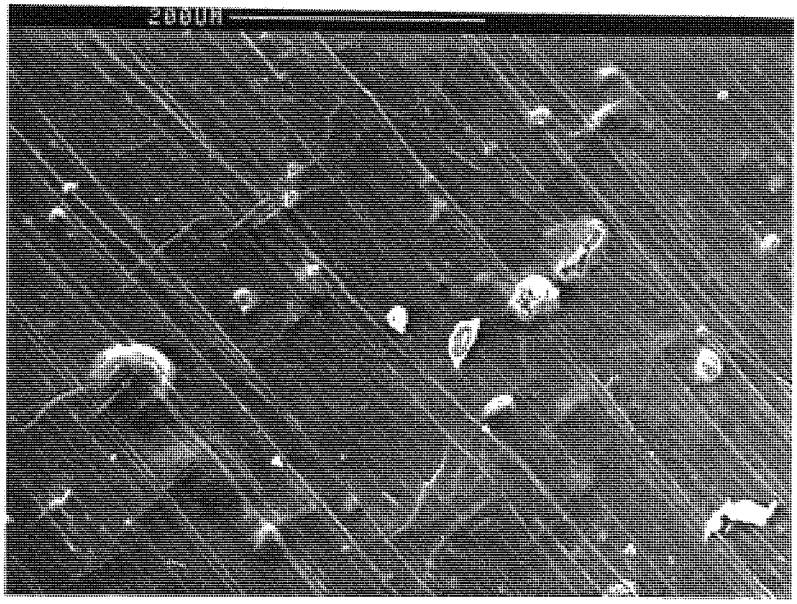


Plate 4.24: L929 cell attachment on 20% HV/30% Amylose copolymer.

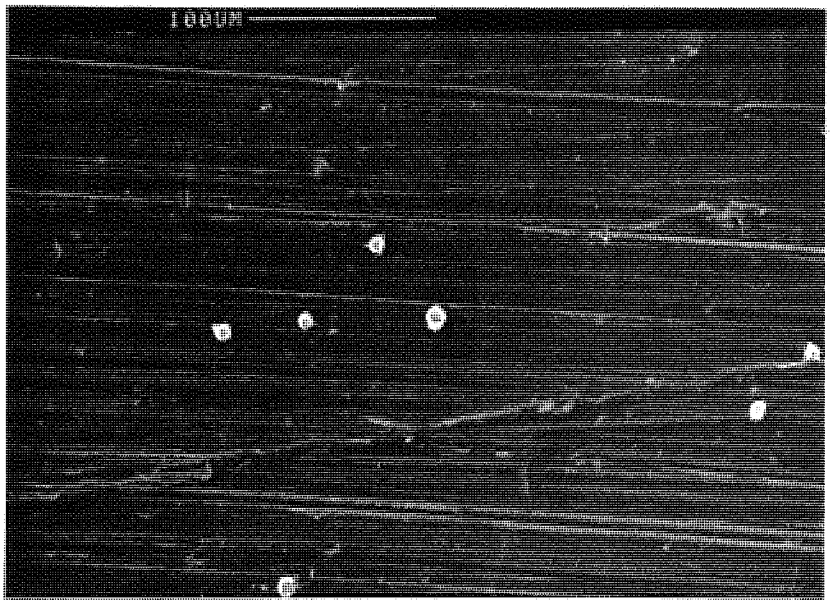


Plate 4.25: BHK-21 Cell attachment on 20% HV/10% Dextran copolymer.

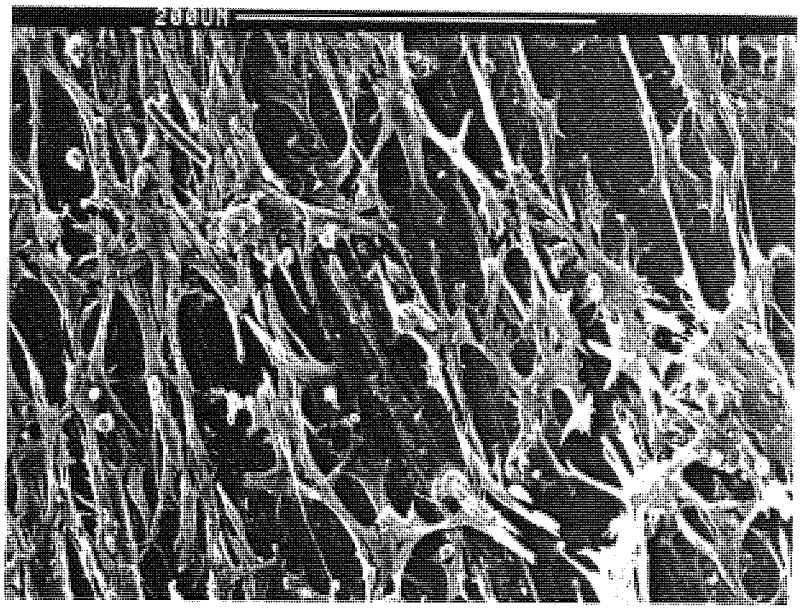


Plate 4.26: L929 cell attachment on 20% HV/10% Dextran copolymer.

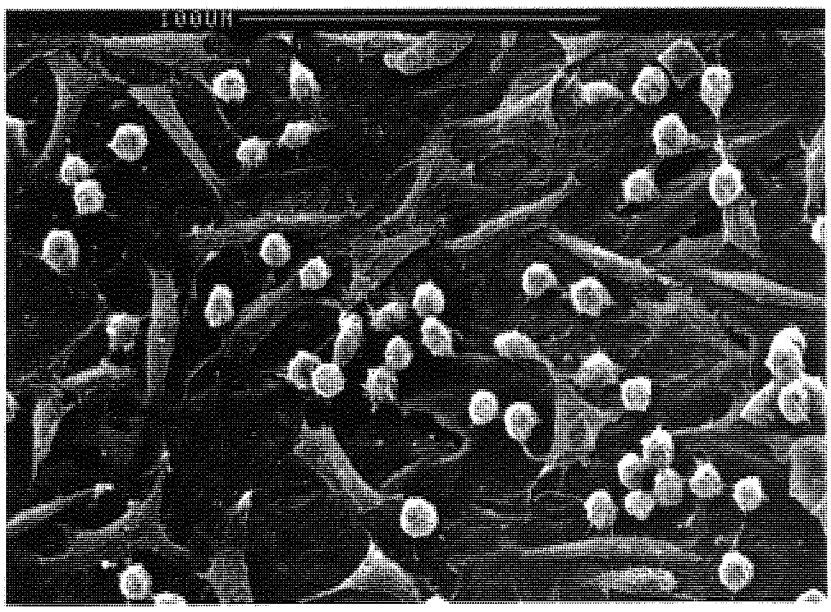


Plate 4.27: BHK-21 cell attachment on 20% HV/30% Dextran copolymer.

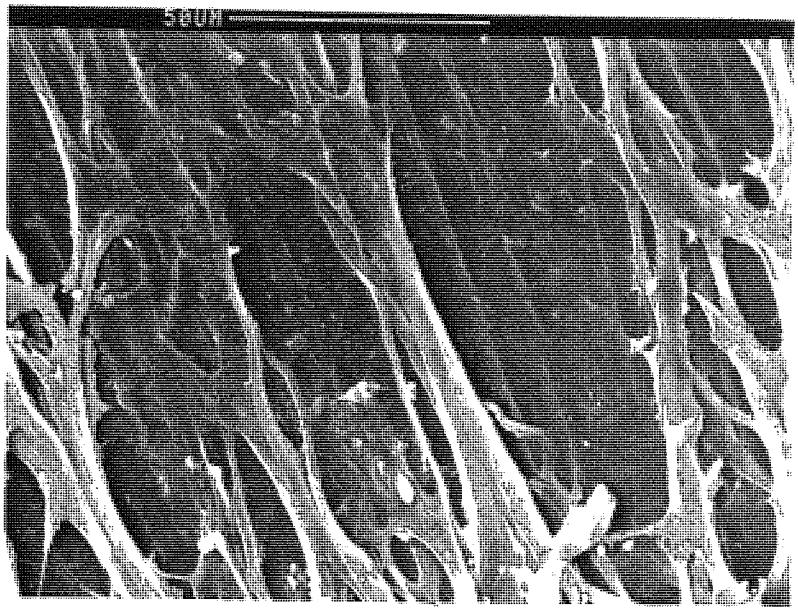


Plate 4.28: L929 cell attachment on 20% HV/30% Dextran copolymer.

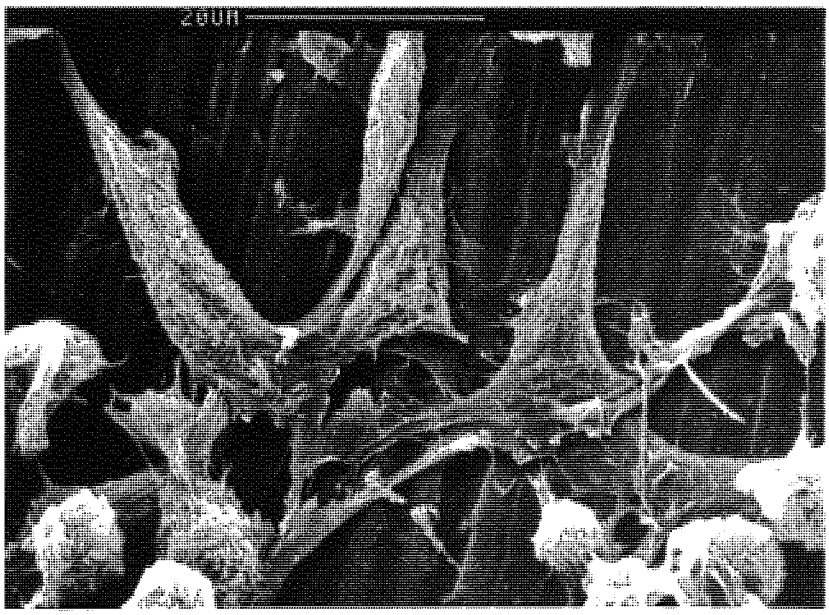


Plate 4.29: BHK-21 cell attachment on 20% HV/10% Dextrin copolymer.

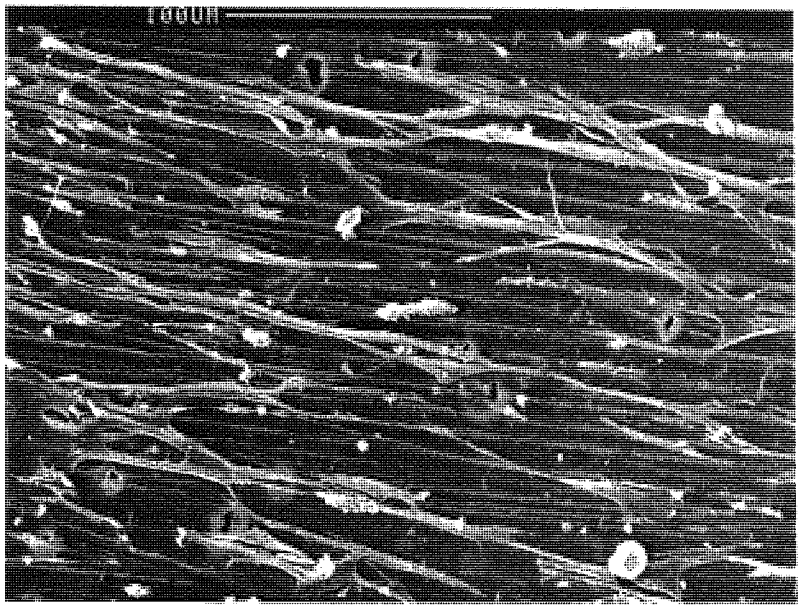


Plate 4.30: L929 cell attachment on 20% HV/10% Dextrin copolymer.

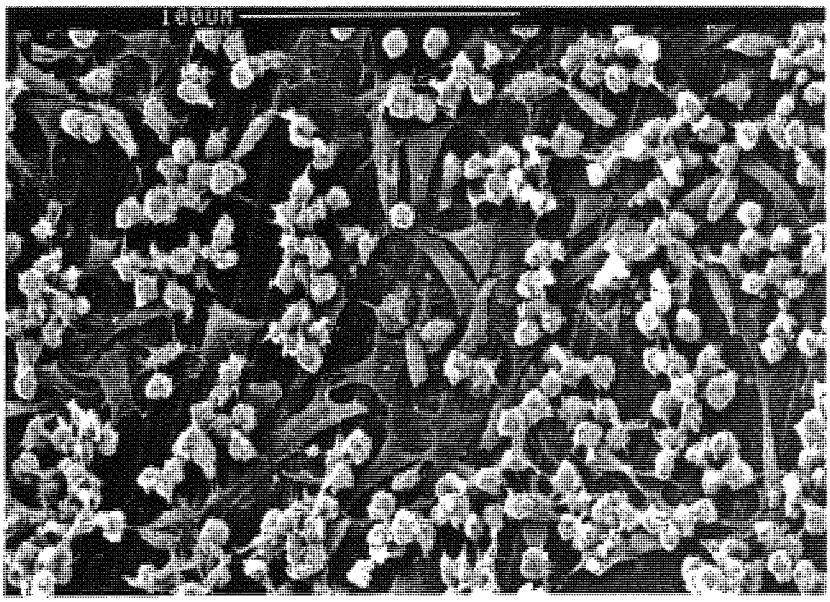


Plate 4.31: BHK-21 cell attachment on 20% HV/30% Dextrin copolymer.

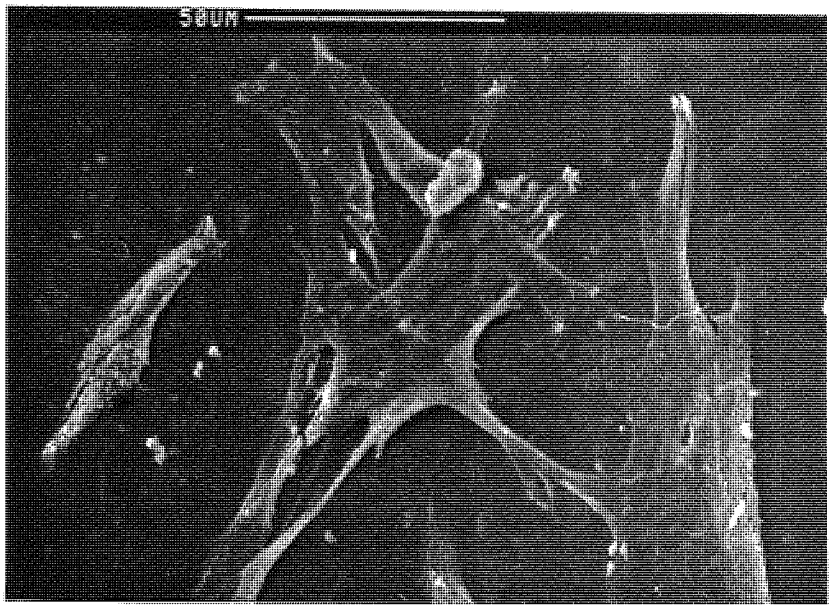


Plate 4.32: L929 cell attachment on 20% HV/30% Dextrin copolymer.

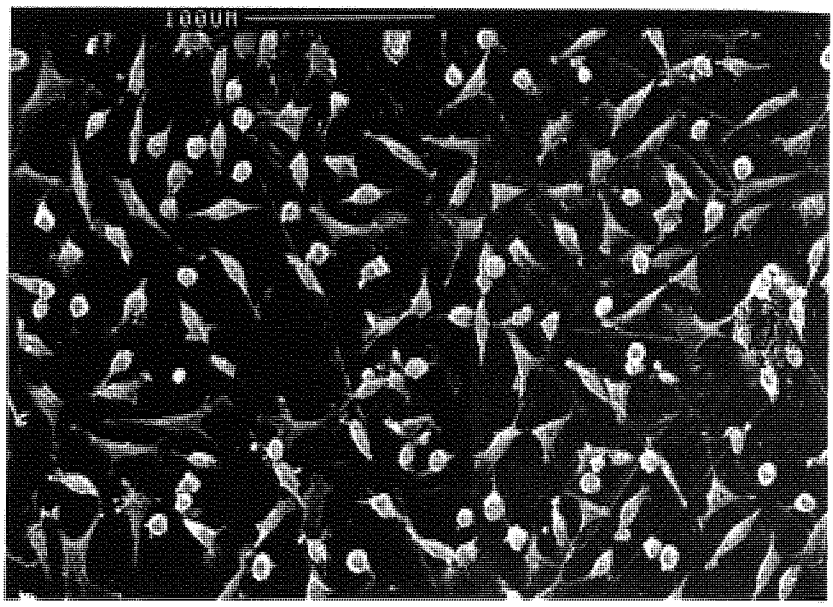


Plate 4.33: BHK-21 cell attachment on 20% HV/10% Na Alginate copolymer.

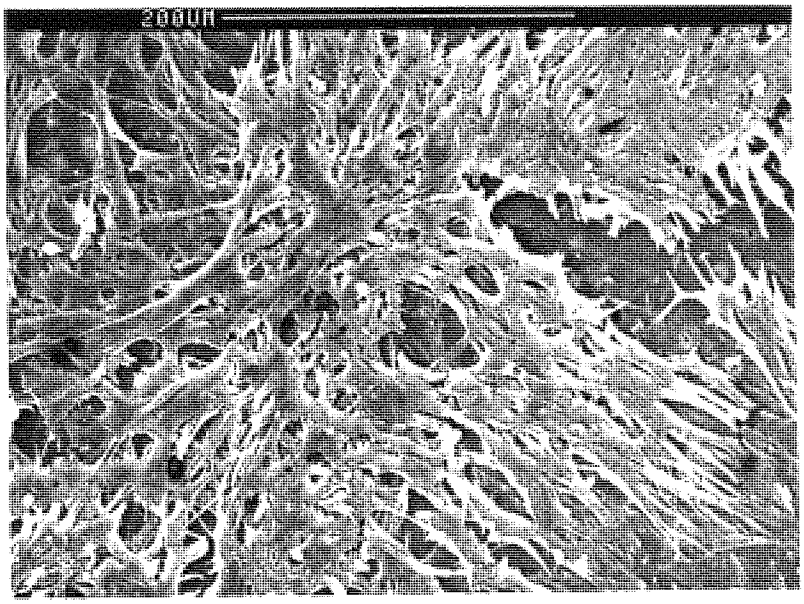


Plate 4.34: L929 cell attachment on 20% HV/10% Na Alginate copolymer.

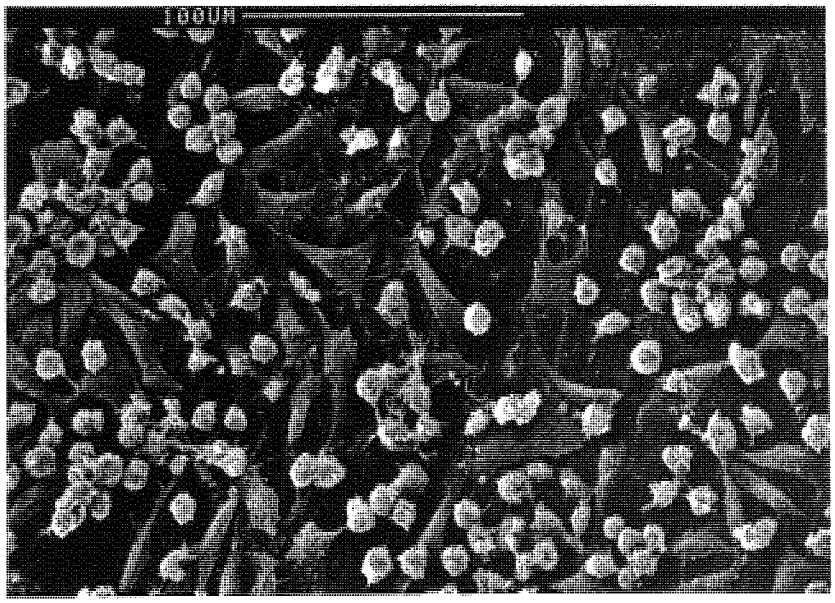


Plate 4.35: BHK-21 cell attachment on 20% HV/30% Na Alginate copolymer.

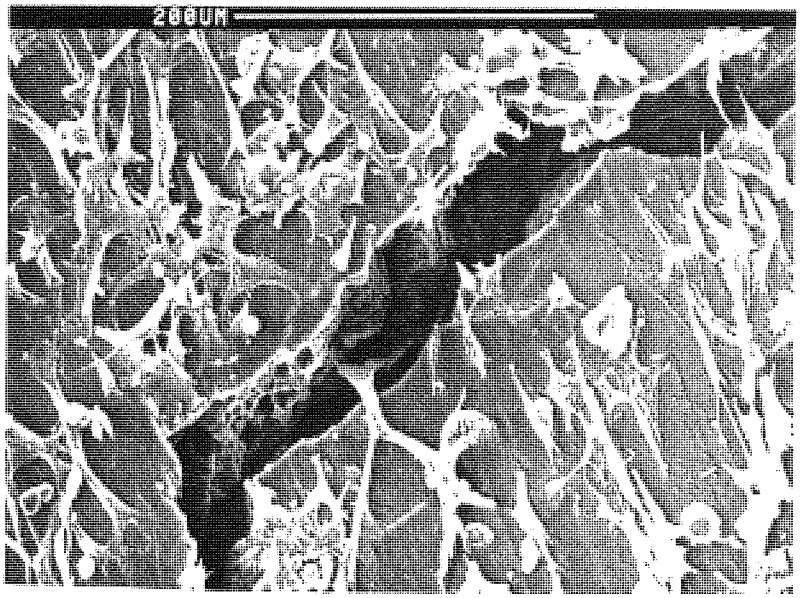
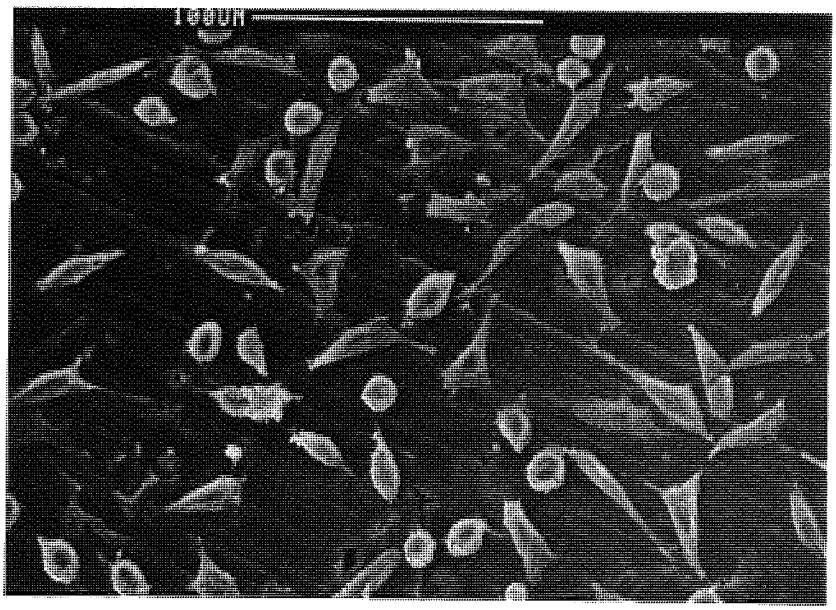


Plate 4.36: L929 cell attachment on 20% HV/30% Na Alginate copolymer.



CHAPTER FIVE

GEL SPUN POLY (ß-)HYDROXYBUTYRATE AS A POTENTIAL WOUND SCAFFOLD

Chapter Five: Prelude

Research on a wide variety of materials in chapters three and four have provided a basis upon which decisions may be made concerning the design of potential biomaterials. With the knowledge of how mammalian fibroblasts interact with hydrogels and PHB-PHV copolymers, a material was designed for specific *in vivo* use as a wound scaffold. This material is gel-spun PHB. Unlike the injection moulded plaques of chapter four, this gel-spun form is composed of filamentous PHB homopolymer: no valerate units are incorporated into the copolymer matrix. It was envisaged that this "wool" form would be useful as a wound stuffing and wound scaffold. PHB seemed to be an ideal choice for this application because of it's slow degradation rate. Any potential wound scaffold needs to last in the body sufficiently long for a replacement dermal architecture to be layed down by the collagen secreting fibroblasts. Thus, fibroblast interaction was a crucial first stage in determining PHB "wool's" potential as a wound scaffold.

The interactions of fibroblasts with hydrogels and PHB-PHV copolymers as discussed previously, highlight the importance of surface polarity and balanced surface charge expression in promoting cell adhesion. The information gained from these complementary studies enables predictions about fibroblast behaviour on gel-spun fibres to be made. The PHB homopolymer fibres may well degrade a lot slower than the PHB-HV plaques because they have no polysaccharide blended into the copolymer. However, they will still be degraded by the same hydrolysis mechanisms, so the expression of hydroxyl and carboxyl groups at the surface is to be expected.

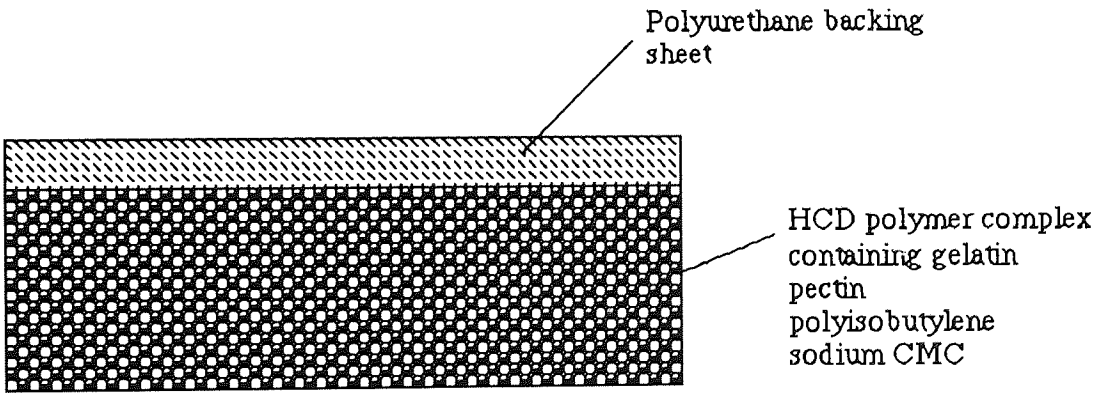
The unknown factor in predicting the cell response to gel-spun fibres is how the manufacturing process affects the fibre surface. The injection moulded plaques of chapter four allow some surface oxidation due to the melt-processing, and this promotes attachment and the expression of fully spread fibroblast morphologies on Norwegian talc nucleated PHB-HV copolymers.

Fibroblast interactions with gel-spun PHB, using human and mammalian cell lines will determine firstly, whether this material is cytotoxic, and secondly whether surface treatment is necessary to promote cell adhesion on these monofilaments.

5.1 Introduction

PHB has been manufactured by extraction from chloroform or dichloromethane using a gel-spinning process (Marlborough Biopolymers Ltd.,U.K.), to produce a "cotton wool" like form composed of fibres of varying diameter. A possible use for this form of PHB is as a potential wound stuffing material, to be used in conjunction with the existing hydrocolloid dressing (HCD) DuoDERM® (ConvaTec, Squibb, U.K.). DuoDERM® consists of an impermeable polyurethane foam backing sheet, and a hydrocolloid polymer complex composed of gelatin, pectin, polyisobutylene and sodium carboxymethylcellulose (sodium CMC).

Figure 5.1 An idealised hydrocolloid wound dressing



The HCD complex interacts and adheres to normal skin, providing an occlusive environment , impermeable to oxygen.

As long ago as 1962, Winter[143] recognised the beneficial effects of occlusive dressings on the wound healing process, reporting their ability to increase the rate of reepithelialisation. However, there were fears[144], supported by adverse

reports[145,146] that occlusion of wound sites would lead to infection, and this attitude delayed the commercial production of occlusive dressings until comparatively recently. Animal models and clinical studies of DuoDERM® applied to various wound sites have demonstrated that although bacterial populations do undergo massive increases in size, there is no concomitant increase in wound infection. In the chronic wound situation, Gilchrist & Reed[147], and Handfield-Jones *et al*[148] have reported that the levels of wound infection in leg ulcers are not increased with the use of DuoDERM®. Similar findings have been reported for donor sites[149] and burns[150]. A distinction must be made here between colonisation and infection. Given that the fears about occlusion and wound infection have largely been dispelled, occlusive dressings such as DuoDERM® have proven effective in treating many clinical situations.

5.1.1. Burn wounds

Although there are contraindications from animal models, clinical studies have shown that occlusive dressings improve the rate of reepithelialisation. In two separate studies of seventy patients with superficial and deep partial thickness burns, Hermans and Hermans[151,152] demonstrated that DuoDERM® provided faster reepithelialisation than either human allografts or silver sulfadiazene cream (SSD). Wyatt *et al*[153] in a comprehensive study using DuoDERM® in wound management provided evidence that the HCD dressing had statistically better wound healing rates than SSD and conventional dressings. There are contradictory results from pig models as demonstrated by Davis *et al*[154], who concluded that the use of DuoDERM® did not change the rate of reepithelialisation when compared with air

exposed wounds; and these observations were echoed by the results of Chvapil *et al*[155]. These findings are in stark contrast to the work of Alvarez *et al*[156] who found that DuoDERM® speeded reepithelialisation, and increased the rate of collagen synthesis. The discrepancies may be attributable to the ways in which the wounds were inflicted, and the size of the wounds. Alvarez *et al*[156] used an electrokeratome which produces a donor site type wound rather than a burn wound as those produced by the other studies. The experimental wounds inflicted by Alvarez *et al*[156] were also much smaller than those produced by Davis *et al*[154], and studies of other wound types have shown that DuoDERM® is more effective at treating smaller wounds. However, the evidence of clinical trials using DuoDERM® in burn wound management have proven it's effectiveness over conventional dressings.

5.1.2. Donor sites

Clinical studies have demonstrated the superiority of DuoDERM® over conventional dressings in the treatment of donor sites. Donati & Vigano[157] found that in comparison with a conventional dressing of paraffin gauze, DuoDERM® promoted more rapid reepithelialisation. Leicht *et al*[149] reported that DuoDERM® provided faster reepithelialisation than the semi-occlusive dressing Omiderm® (Omikron, Rehovot, Israel). Faster reepithelialisation allows the donor site to be reharvested more rapidly, a crucial factor in patients requiring multiple autografts[158]. In animal studies, Olof-Reuterving *et al*[159] showed that DuoDERM® promoted faster wound contraction than gauze dressings in rats.

5.1.3 Pressure sores

DuoDerm® has also been successfully used in the treatment of decubitus ulcers (pressure sores). Brod *et al*[160] found DuoDerm® superior to the hydrogel poly-hema in promoting faster healing rates of patients with bed sores.

5.1.4. Chronic leg ulcers

Chronic venous leg ulcers affect thousands of people in the U.K. alone[161]. It is a clinical situation which has been successfully managed by the use of DuoDERM®[162,163], and one which may be further improved by the collaborative use of DuoDERM® and gel-spun PHB as a wound scaffold.

In the chronic leg ulcer, the wound site is poorly vascularised, and open weeping sores accompanied by tissue necrosis are symptomatic. For this reason, healing may be slow or absent, creating substantial demands on resources. The ideal wound healing scenario is the reingrowth of the vascular network into the wound bed, followed by speedy reepithelialisation, and permanent wound closure. Although DuoDERM® has been demonstrated to be effective at promoting reepithelialisation, the wound site still remains fragile. There are two major reasons for this. Firstly, fibrin accumulation deters oxygen and nutrient transport across capilliaries, leading to slow healing. With respect to this problem, Mulder & Walker[164] have reported the benefits of using DuoDERM® because of it's reported fibrinolytic activity. Secondly, collagen synthesis is undergoing much more rapid turnover than is found in normal skin, resulting in a more transient dermal architecture. Supportive evidence for this fact comes from Eaglstein[165], who has

found that wound strength is related to collagen maturity not the amount of collagen synthesised.

The occlusive DuoDERM® dressing promotes rapid wound healing by preventing wound dehydration and scab formation[166]. This allows the uninterrupted migration of epithelial cells across the wound bed because the cells remain viable, and they encounter no obstructions. In wounds exposed to air, epithelial cells have to migrate beneath the scar tissue which slows down reepithelialisation[167]. In addition,under occlusive conditions, other beneficial cell types such as white blood cells also remain functional[168]. The moist environment will also assist in the maintainance of other immunological defence mechanisms such as enzyme lysis, to function.

5.2 The role of oxygen in wound healing

The oxygen tension in wound sites plays a crucial role in the wound healing process. Under occlusive dressings such as DuoDERM®, angiogenesis is promoted. Angiogenesis is the phenomenon of regrowth of the capilliary network into the wound site, and is vital for the eventual healing of the wound. Knighton *et al*[169] have demonstrated that wound angiogenesis is inversely proportional to the ambient oxygen tension. Under hypoxic conditions, macrophages migrate into the wound site and secrete angiogenic growth factors which stimulate capilliary regrowth. Varghese *et al*[170], found that reduced oxygen tension promotes *in vitro* growth of fibroblasts, and the production of angiogenic factors from tissue macrophages; and their findings have been confirmed by others[171,172]. Silver[173] has stated that a hypoxic environment encourages angiogenesis but that it reduces

fibroblast mitosis and collagen synthesis, which would seem a necessary requirement for wound healing. However, other researchers have shown that the migration and differentiation of epidermal cells occur earlier when the mitotic response is reduced[174-176].

The occlusive nature of DuoDERM® promotes the formation of granulation tissue, the healthy living tissue of a healing wound. Granulation tissue has to have a good supply of oxygen for the survival of the fibroblasts of the new dermis. The oxygen poor atmosphere beneath the dressing encourages macrophages to migrate into the wound site. It is the macrophages that secrete angiogenic growth factors which influence the regrowth of capilliaries.

5.3 Gel-spun PHB in a wound healing scenario

One possible means by which a more stable wound site could be engineered is by the *in vivo* implantation of gel-spun PHB. The PHB acts as a "scaffold", binding this fragile site over an extended period of time before being assimilated into the host. PHB is a material which is biodegradable, non-toxic, and which seems particularly suited to this application given it's reportedly slow degradation rate *in vivo*[135]. The idea is for reepithelialisation to occur over the top of the PHB implant, closing the wound, whilst the temporary scaffold provides a framework for the laying down of a permanent dermal architecture of collagen. Thus the PHB scaffold would remain *in vivo* until the rates of collagen synthesis and turnover have returned to those levels found in normal skin. Using other formulations of PHB[177], one can envisage an all-in-one "designer" wound dressing for the leg

ulcer condition based around the existing DuoDERM® format, incorporating a PHB wound scaffold, and PHB microencapsulated antibiotics and growth factors dispersed within the HCD polymer complex.

Once angiogenesis has progressed and the site is vascularised, fibroblasts will migrate into the scaffold site and start laying down the new collagen network that will become the permanent dermal architecture once the PHB is completely degraded.

5.4 Experimental technique

Samples of gel-spun PHB were sterilised by autoclaving (chapter two). These homopolymer monofilaments were found to be extremely resistant to degradation, so this sterilisation procedure could be used. Sterile mats were placed in sterile universals and acid washed in 0.2μm filtered H_2SO_4 solutions. After acid washing, the samples were thoroughly rinsed in sterile water, placed in multiwell plates, and seeded with cell suspension. The samples were then incubated at 37°C in a CO_2 incubator for 18 hours. Post incubation, samples were subjected to the SEM procedures discussed in chapter two.

5.5 Discussion of results

5.5.1 Cell adhesion on PHB "wool"

Initial experiments on native PHB "wool" which had not had any surface treatment showed that there was no cell attachment with NOM 238 cells (Plates 5.1 & 5.2). This is in contrast to the PHB-HV copolymers produced by the injection moulding process of Yasin[114]. The unblended copolymers he produced were cell adhesive to mammalian fibroblasts (see Chapter 4). As yet, no surface polarity data are available for this filamentous PHB. The mechanical production of PHB by the gel-spinning technique may prevent the possibility of oxidation of the fibre surface which would lead to the expression of charged moieties at the surface. As discussed previously, increased surface polarity is necessary for the promotion of interface conversion, which in turn governs cell adhesion.

5.5.2. Cell adhesion on acid washed PHB "wool"

Thomas[35] has shown that hydrophobic surfaces such as polystyrene can be made cell adhesive by "mild" surface treatment with sulphuric acid. She used dilute solutions of H_2SO_4 to hydroxylate the surface. The increased surface polarity associated with the introduction of charged species promoted cell adhesion. The introduction of hydroxyl and carboxyl groups at the surface of these gel-spun fibres should lead to increased cell adhesion. Acid washing the fibres will produce these two charged moieties as a result of hydrolysis of the copolymer. On the basis of this research, a regime of dilute H_2SO_4 solutions were used to wash the PHB fibres. The fibres were acid washed in 1%, 3%, and 10% H_2SO_4 at ambient temperature for ten minutes. Following acid washes, samples were cell tested with NOM 238 for 18 hours. The results of these experiments are presented in Plates 5.3-5.8.

For all of the treatments, the levels of cell attachment were low. In addition, the cells that were attached were rounded. Only in the 10% H_2SO_4 treatment did cell spreading begin to take place (Plates 5.7 & 5.8). The NOM 238 responses to chloroform and dichloromethane extracted wool samples is very similar. The method of solvent extraction plays no part in determining the cellular response to PHB wool. The solvent residues in these fibres are very low (<1%), nevertheless, both solvents are potential carcinogens, so there may be potential problems with long term usage *in vivo*.

Mild surface treatments using dilute H_2SO_4 did not increase fibroblast attachment to any great degree. PHB is chemically resistant and degrades slowly unless blended with polysaccharides[114]. The acid wash treatments employed in this initial study on PHB wool appear not to be hydrolysing the surface sufficiently to produce enough surface charge to promote cell adhesion. In addition, it may be that the filaments produced by the gel-spinning process are too small to act as a sufficient support for the anchorage-dependent fibroblasts. As discussed in Chapter 3, anchorage-dependent cells exert considerable forces on the substratum during the process of adhesion. Thus, these PHB monofilaments may be too thin to provide a solid support for NOM 238 cells. However, the gel-spun samples did show great variations in fibre diameter, and one would expect to see cell attachment on the larger fibres, if fibre diameter was a critical parameter influencing the cellular response. This is not the case. There is no cell spreading on the larger fibres as is evident from Plate 5.4, where the NOM 238 cell is attached to, but not spread on, a fibre which has a diameter greater than that of the cell itself.

5.5.3. Cell adhesion on acid washed PHB non-woven mats

Thomas[35] found by increasing reaction temperature and exposure times, that H_2SO_4 treatments promoted the expression of sulphonate groups at the surface of polystyrene, and this inhibited cell adhesion. Sulphonate groups carry a much greater negative charge, relative to hydroxyl groups, so a high surface charge density of sulphonate groups will inhibit cell adhesion due to electrostatic repulsion. Since the "mild" H_2SO_4 treatments did not succeed in producing an adhesive surface on the PHB wool, increasing the severity of the treatments was attempted in

order to hydrolyse the fibre surface to a greater degree. The same acid wash concentrations were used, but the exposure time was increased to 60 minutes, and the reaction temperature was increased to 60°C. In addition, mammalian L929 fibroblasts were used to test the fibres.

A concomitant change in the physical appearance of the "wool" also accompanied these new treatment regimes. In the clinical situation, it has been found that PHB "wool" can be difficult to work with[178]. It does not tease apart as readily as cotton wool, and is also very "clingy", possibly due to the build up of electrostatic charge. A more manageable form of PHB is desirable, and it was thought that a mat-like form would provide a more stable basis than "wool". However, woven mats would be expensive, as they could only be produced using sophisticated tooling procedures. So it was decided to opt for an initial study involving non-woven mats (NWM's). NWM's have the advantage that they are a quick and cheap means of manufacture (Chapter 2). Another important reason why NWM's might prove valuable is biological. It has been noted that cells find it difficult to "bridge" the gaps between adjacent fibres in the very open structure of PHB "wool", as seen by the existence of broken filopodia[116]. It is difficult to interpret whether this filopodial breakage is due to the mechanics of cell growth, or the rigours of SEM processing. Nevertheless, the fact that cells have to "stretch" over greater distances means they will be much more susceptible to external mechanical forces, such as wound contraction imposed on the "wool". In a NWM, adjacent fibres are much closer together, so the bridging distances will be much less. Hopefully, this will encourage fully spread cell morphology, and better migration of fibroblasts into the "wool" matrix.

Micrographs of the two mat types show that they are structurally similar (Plates 5.9 & 5.10). The dichloromethane extracted mats generally have a greater number of large diameter fibres. The results of cell adhesion assays on acid washed NWM's are presented in Plates 5.11-5.22 The levels of cell attachment on these mats are much higher than seen in PHB wool. The NOM 238 cells are well spread on all the treatments, with the greatest degree of attachment and spreading on the 10% H_2SO_4 treated mats. The L929 cell line does not show the same degree of spreading or attachment levels as the NOM 238 line, although the 10% treated mats exhibit some cell spreading (Plates 5.20 & 5.22). The overall picture of fibroblast adhesion on acid treated NWM's is encouraging. Levels of cell attachment and spreading are much higher than those on the wool samples.

The promotion of cell adhesion on these PHB monofilaments is due to a change in the surface characteristics of these fibres. This change may be due to two reasons. Firstly, the severe acid wash treatments may have hydrolysed the fibre surface producing hydroxyl groups. These hydroxyl groups will structure water and mediate protein adsorption. Adhesive proteins such as fibronectin are correctly expressed, thus cell adhesion is encouraged. Secondly, the extra melt processing associated with mat production (Chapter 2) may lead to an increase in surface polarity due to oxidation. As discussed earlier, surface polarity can play a crucial role in determining cellular responses. A combination of both factors provide the most likely reason for the increase in cell adhesion. To determine which, if either, factor was most influential in determining cell adhesion, control mats which had not been subjected to acid washing were cell tested. These untreated controls do not

support cell spreading in either cell line (Plates 5.27-5.30). It is the acid washing not any changes associated with melt processing which promote cell adhesion on NWM's.

5.5.4. Cell adhesion on alkali washed non-woven mats

As an adjunct to the acid wash treatments, alkali washed mats were tested to determine the effects of this form of surface modification on cellular response. The alkali washes were based on the pH10.6 buffering system used by Yasin[114] in his degradation studies of PHB-HV copolymers. This method is harsher and less controllable than the acid washes. The results for the two cell lines are quite different. The NOM 238 exhibit fully spread morphologies (Plates 5.23 & 5.25) and high levels of attachment, whereas the L929 lines supports lower attachment and spreading (Plates 5.24 & 5.26). In earlier chapters, L929 cells generally exhibited lower levels of attachment than BHK-21 cells for reasons of cell cycle times. In routine culture L929 cells reach confluence quicker than NOM 238, so the differences between the two cell lines with respect to cell attachment cannot be explained merely in terms of growth rates. The alkali wash system used for these experiments produces considerably more surface degradation than the acid wash treatments, as exemplified by Plate 5.26. The two cell types may respond to surface rugosity differently. The NOM 238 epithelially derived cells may be more able to bridge small scale surface features and form sufficient points of attachment than L929 cells.

The use of alkaline washing systems will lead to the production of carboxyl groups at the surface as well as hydroxyl groups. Carboxyl groups are more strongly negatively charged than hydroxyl groups. There may be a balance point as far as the relative effects of each of these groups is concerned; and the balance point may be different for each cell line. If the L929 cells carry a greater negative charge at their cell surface due to negatively charged carbohydrates for instance, then the electrostatic force of repulsion will be greater against L929 than NOM 238 cells. The L929 cells cannot tolerate the surface charge density of carboxyl groups. It may be that for the L929 line, the critical density of carboxyl groups has been reached. Less harsh alkaline treatments may promote cell attachment of L929 cells.

5.6 Conclusions

Gel-spun PHB homopolymer in its native state does not promote cell adhesion. This is in contrast to the results presented in Chapter 4 for PHB-HV copolymers which support high levels of adhesion. Using acid washes alters the surface characteristics of these fibres by introducing charged species, which encourages cell adhesion on PHB "wool". The manufacture of non-woven mats to produce a closely knit fibre matrix, combined with the increased severity of acid washes enhances mammalian and human fibroblast adhesion. Alkaline washes can also promote cell adhesion, but the process is less controllable and the balance of surface charge expression affects cell behaviour. PHB is chemically resistant to attack, and the simple hydrolysis mechanisms used in this study will differ from the conditions experienced *in vivo*. In the host system, it is likely that enzyme lysis in combination with hydrolysis will degrade the PHB faster. This has implications for

cell adhesion. Enzyme mediated reactions will create an entirely different surface to that produced by hydrolysis alone. The new surface that is created by the degradation process will have very different surface characteristics to the undegraded surface. This new substratum may enhance cell adhesion or inhibit it.

Plate 5.1: NOM 238 cell attachment on untreated chloroform extracted PHB "wool".

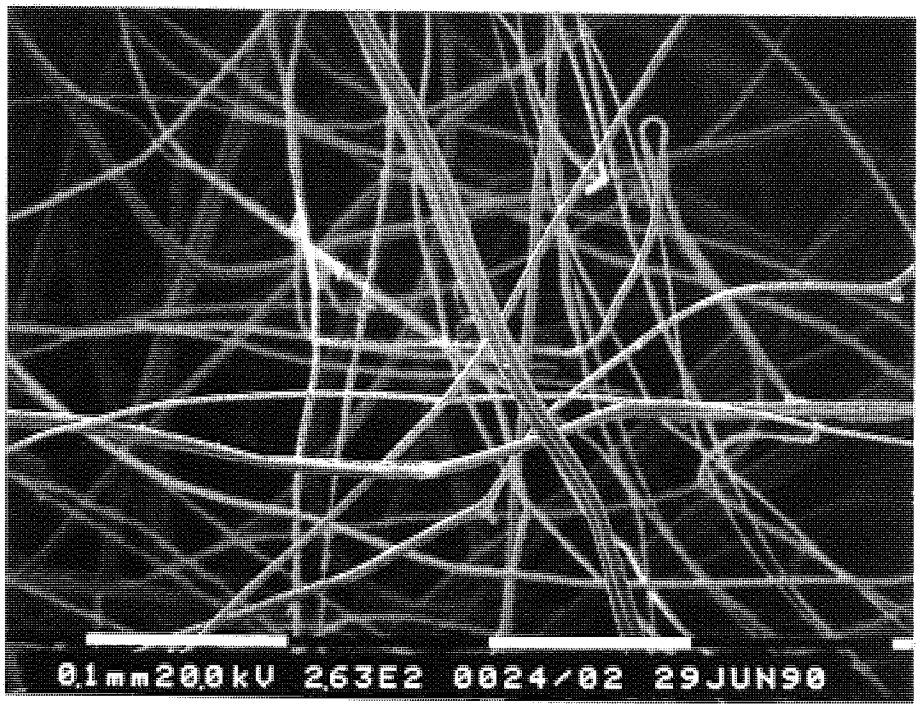


Plate 5.2: NOM 238 cell attachment on dichloromethane extracted PHB "wool".

Plate 5.3: NOM 238 cell attachment on 1% H_2SO_4 treated chloroform extracted PHB "wool".

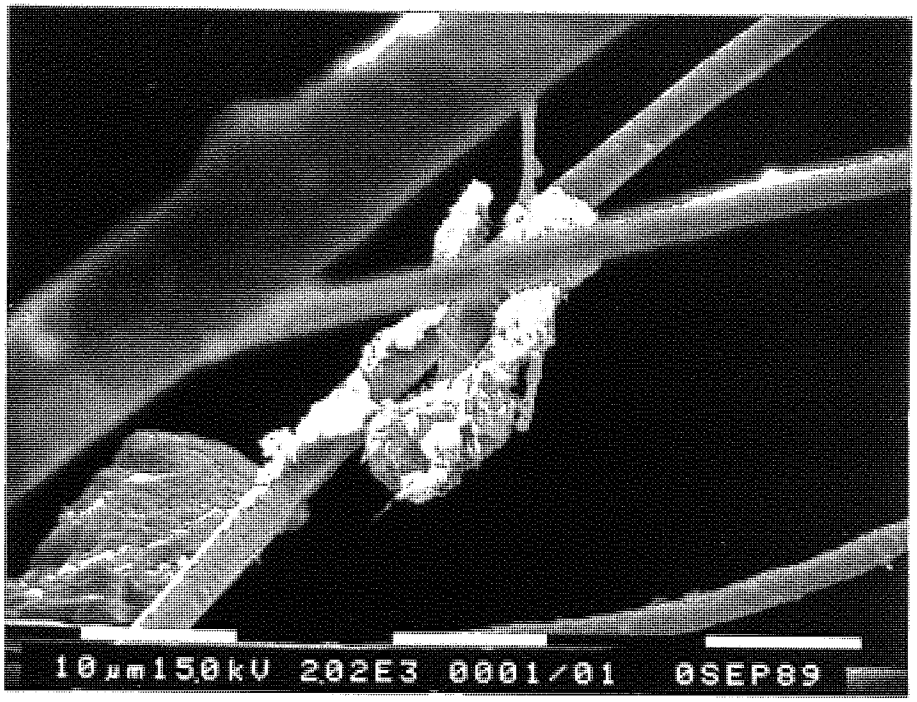


Plate 5.4: NOM 238 Cell attachment on 1% H_2SO_4 treated dichloromethane extracted PHB "wool".

Plate 5.5: NOM 238 Cell attachment on 3% H_2SO_4 treated chloroform extracted PHB "wool".

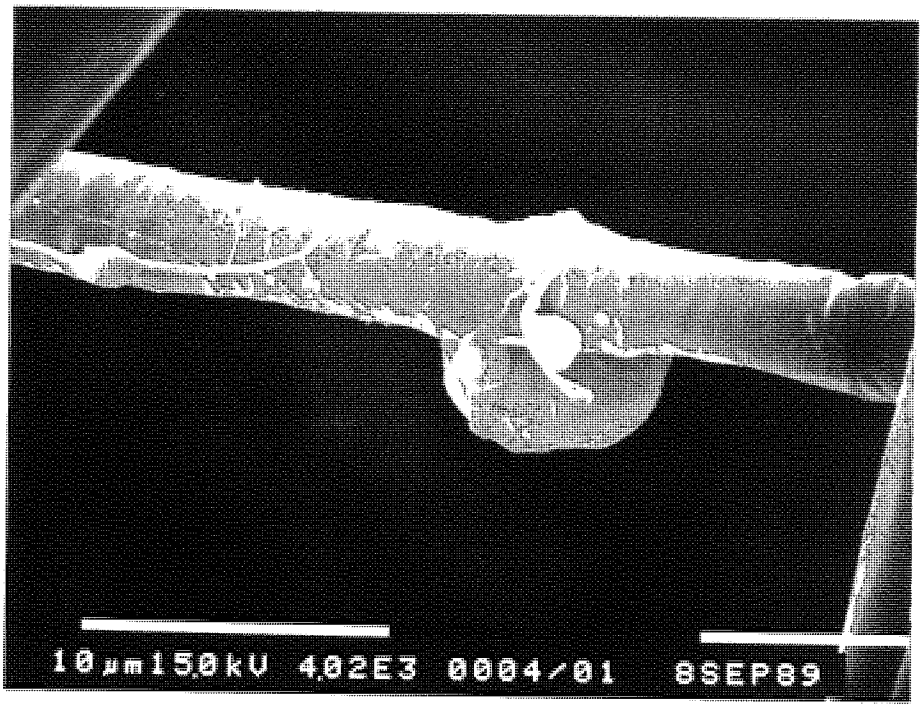


Plate 5.6: NOM 238 cell attachment on 3% H_2SO_4 treated dichloromethane extracted PHB "wool".

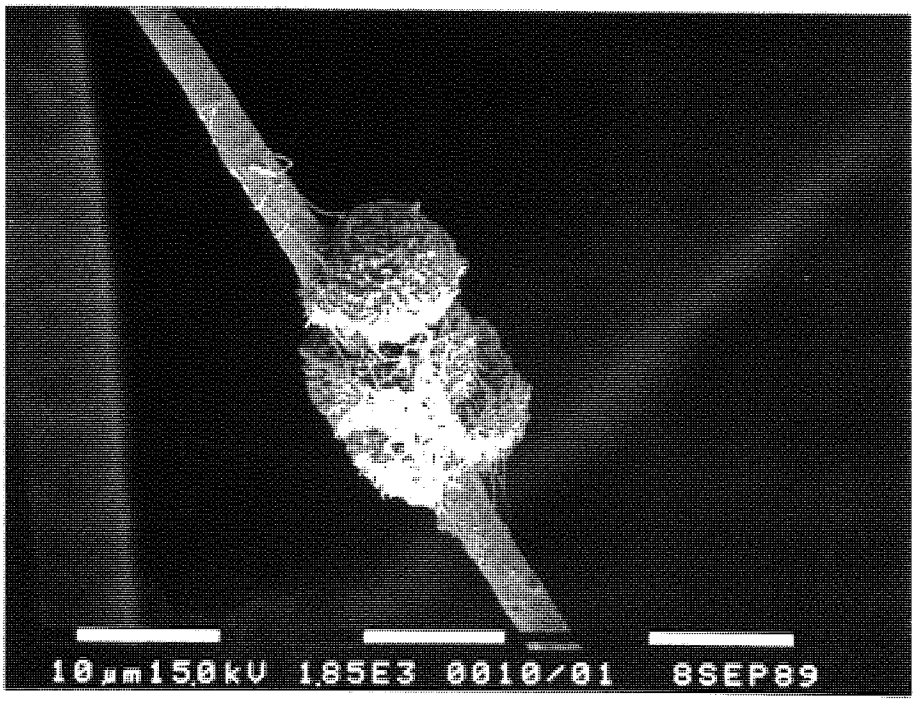


Plate 5.7: NOM 238 cell attachment on 10% H_2SO_4 treated chloroform extracted PHB "wool".

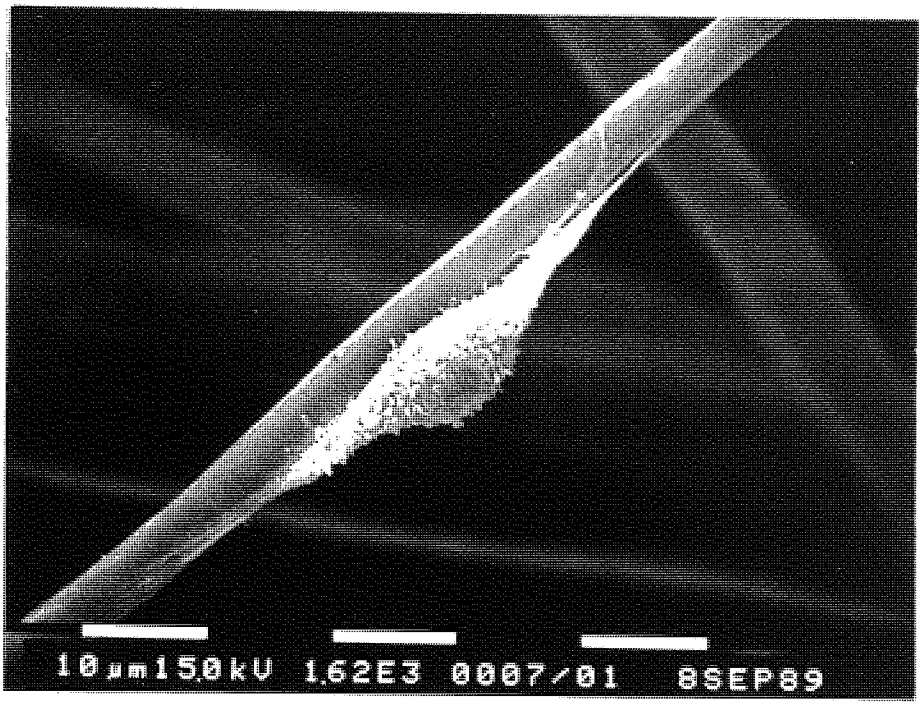


Plate 5.8: NOM 238 cell attachment on 10% H_2SO_4 treated dichloromethane extracted PHB "wool".

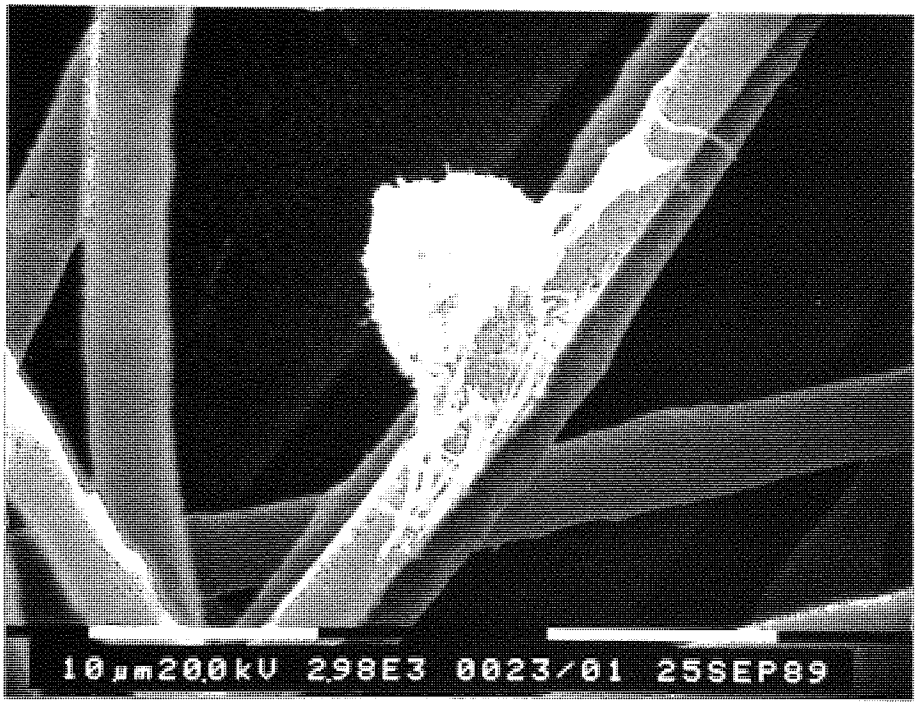


Plate 5.9: Chloroform extracted PHB NWM.

Plate 5.10: Dichloromethane extracted PHB NWM.

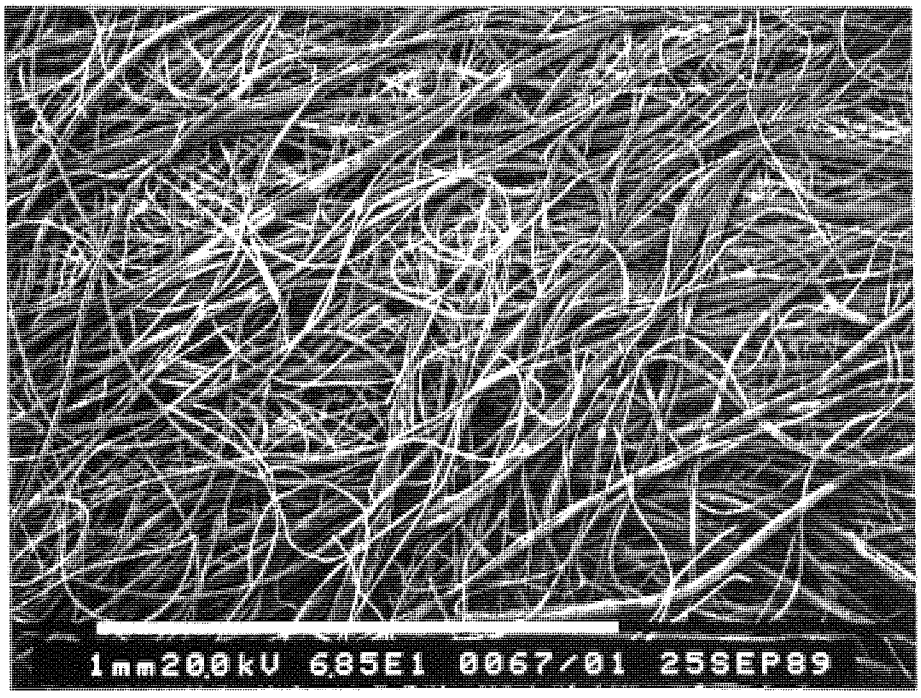


Plate 5.11: NOM 238 cell attachment on 1% H_2SO_4 treated chloroform extracted PHB NWM.

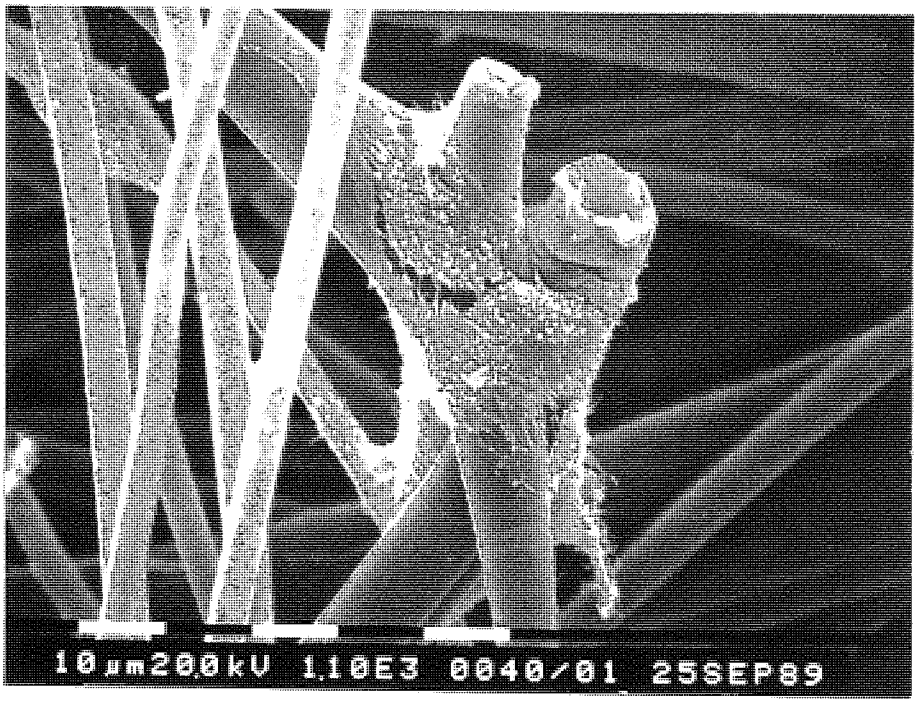


Plate 5.12: L929 cell attachment on 1% H_2SO_4 treated chloroform extracted PHB NWM.

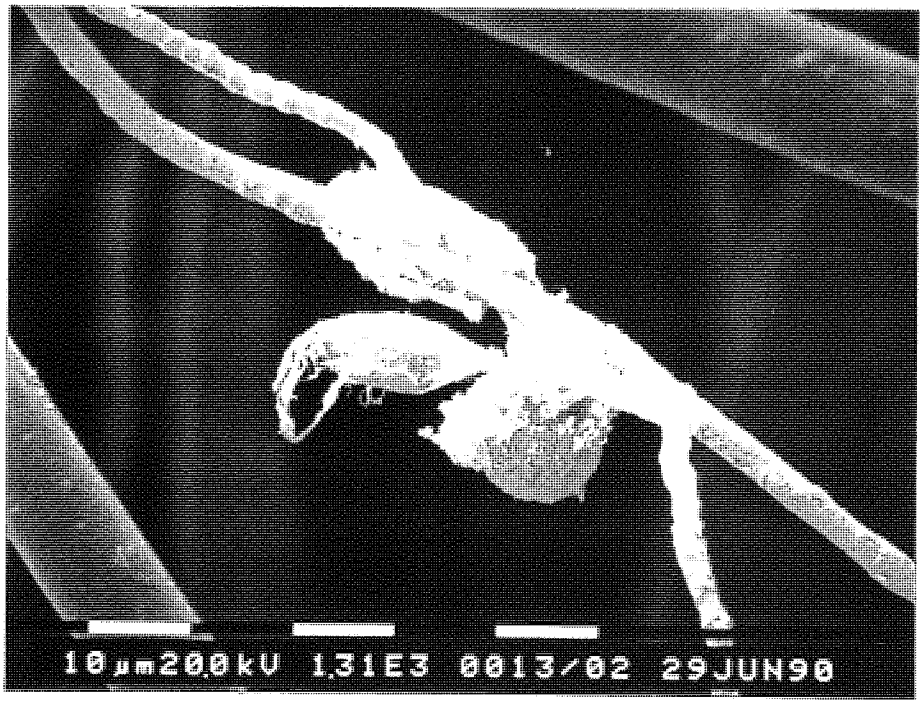


Plate 5.13: NOM 238 cell attachment on 1% H_2SO_4 treated dichloromethane extracted PHB NWM.

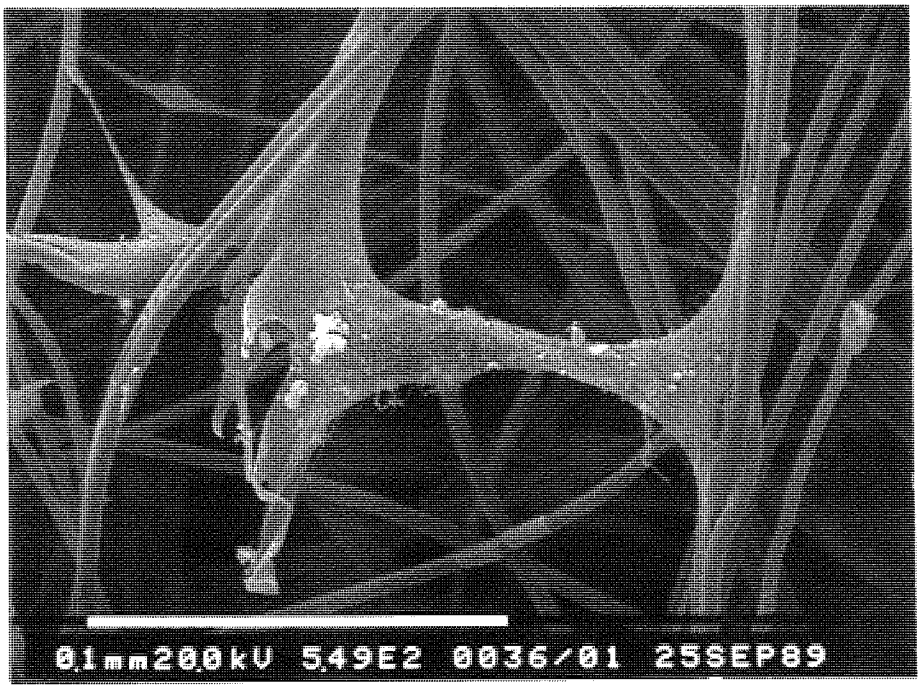


Plate 5.14: L929 cell attachment on 1% H_2SO_4 treated dichloromethane extracted PHB NWM.

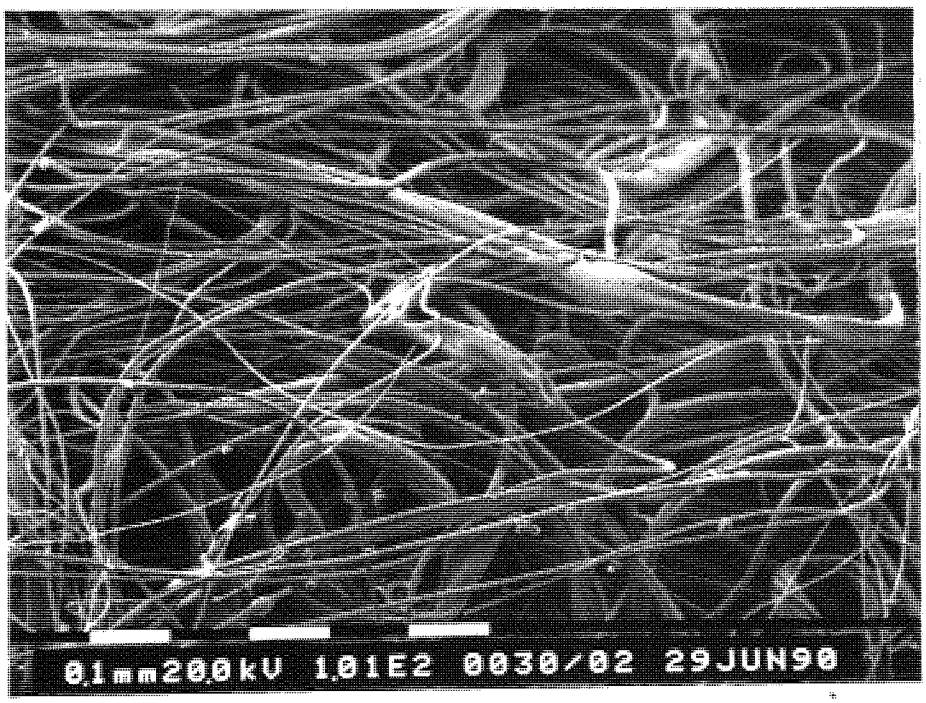


Plate 5.15: NOM 238 cell attachment on 3% H_2SO_4 treated chloroform extracted PHB NWM.

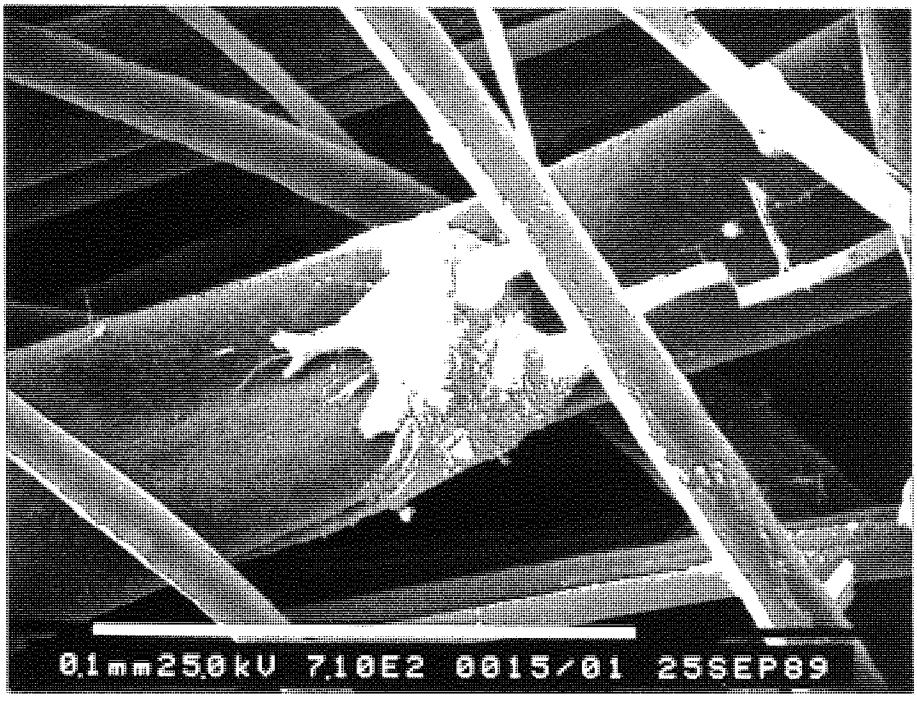


Plate 5.16: L929 cell attachment on 3% H_2SO_4 treated chloroform extracted PHB NWM.

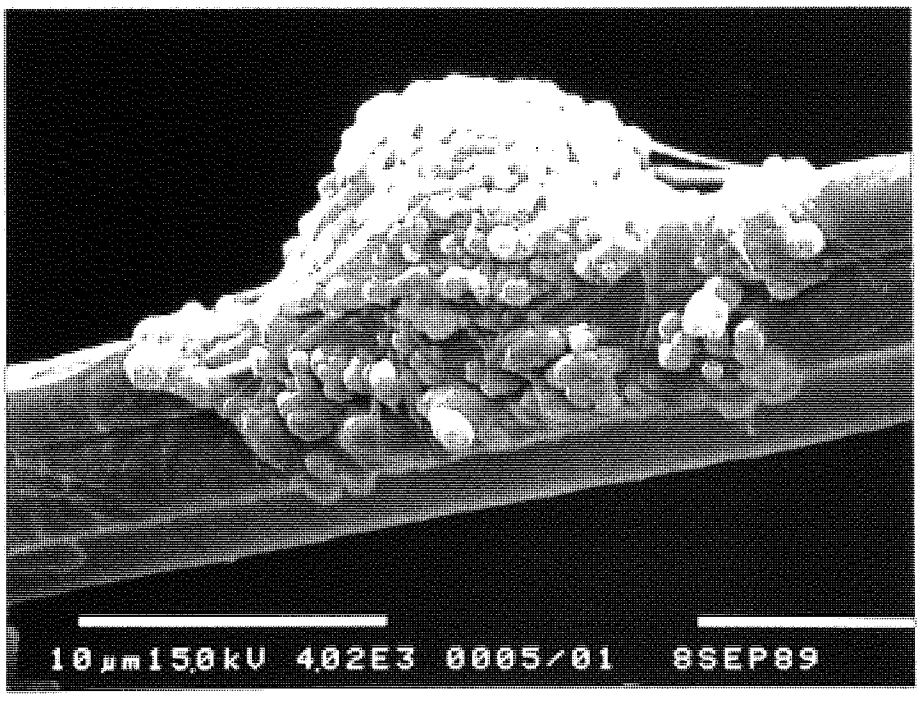


Plate 5.17: NOM 238 cell attachment on 3% H_2SO_4 treated dichloromethane extracted PHB NWM.

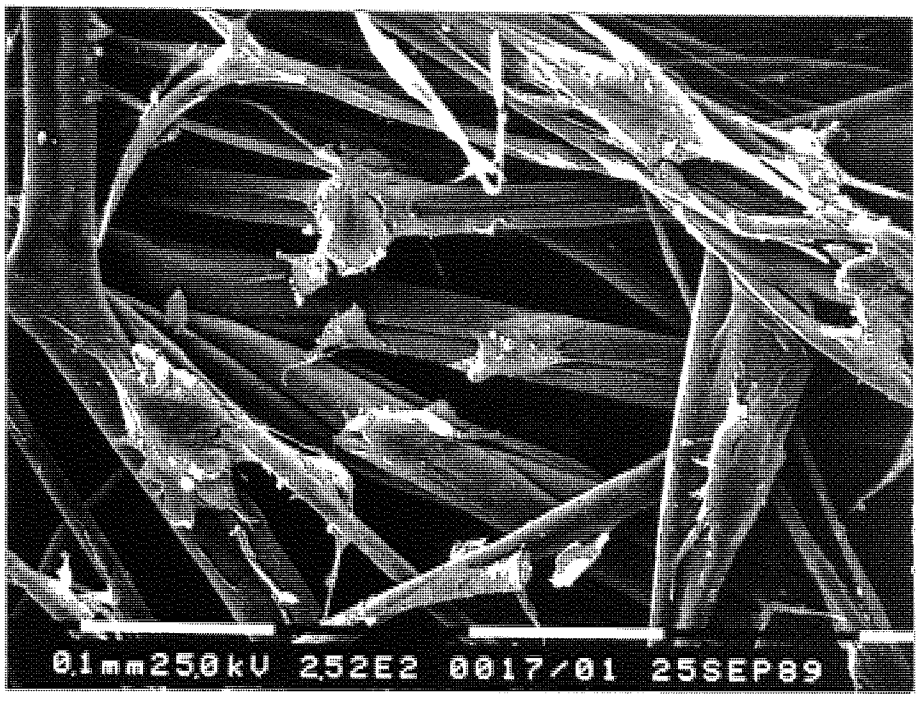


Plate 5.18: L929 cell attachment on 3% H_2SO_4 treated dichloromethane extracted PHB NWM.

Plate 5.19: NOM 238 cell attachment on 10% H_2SO_4 treated chloroform extracted PHB NWM.

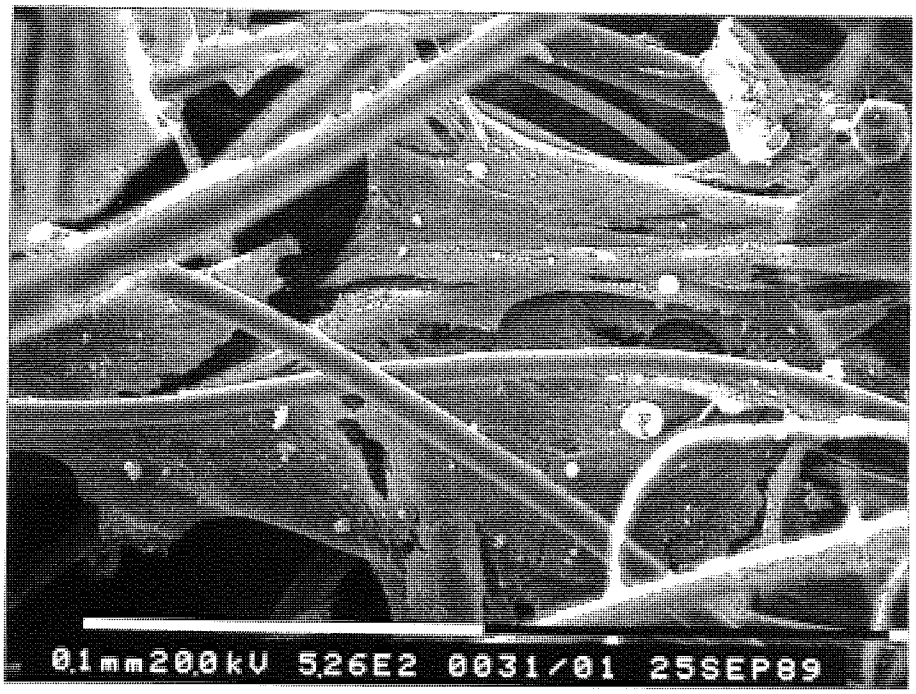


Plate 5.20: L929 cell attachment on 10% H_2SO_4 treated chloroform extracted PHB NWM.

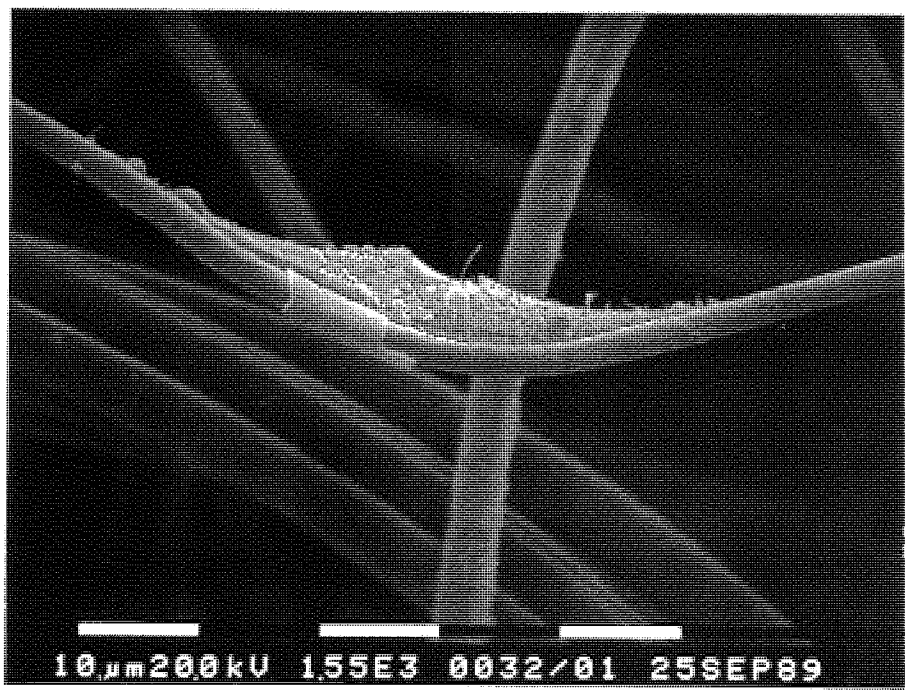


Plate 5.21: NOM 238 cell attachment on 10% H_2SO_4 treated dichloromethane extracted PHB NWM.

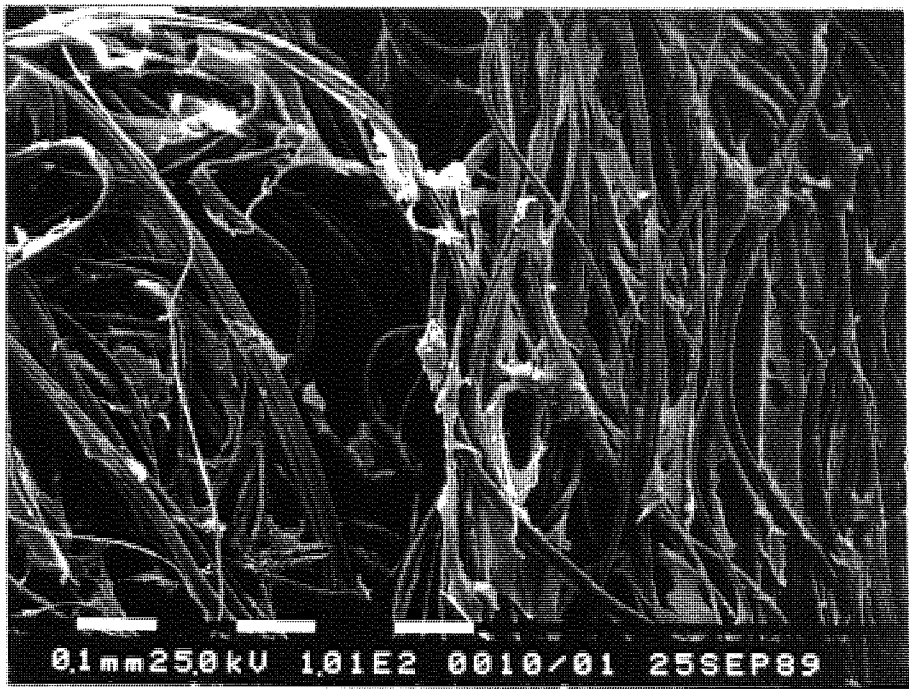


Plate 5.22: L929 cell attachment on 10% H_2SO_4 treated dichloromethane extracted PHB NWM.

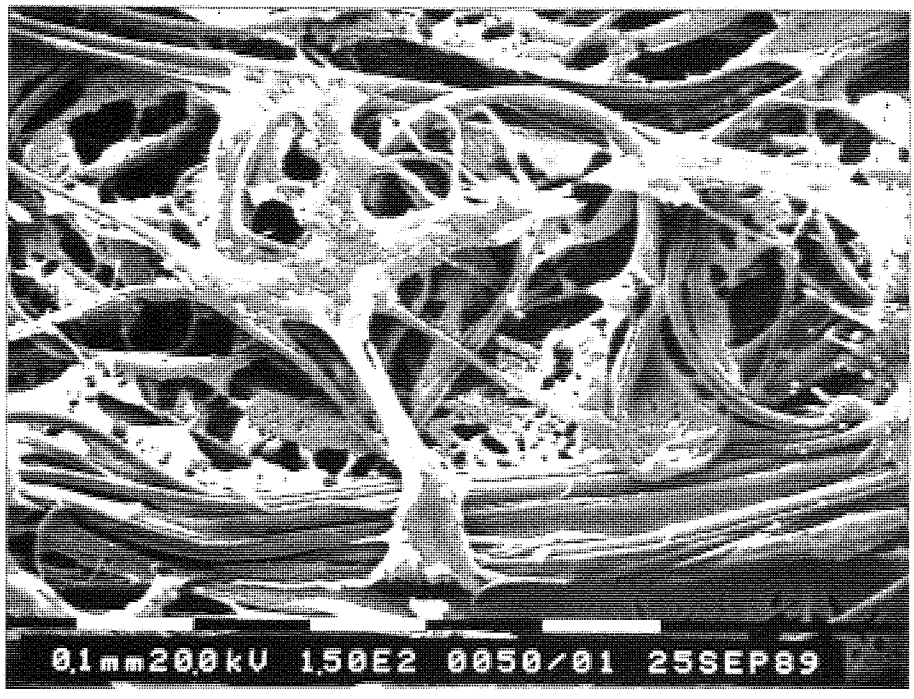


Plate 5.23: NOM 238 cell attachment on alkali treated chloroform extracted PHB NWM.

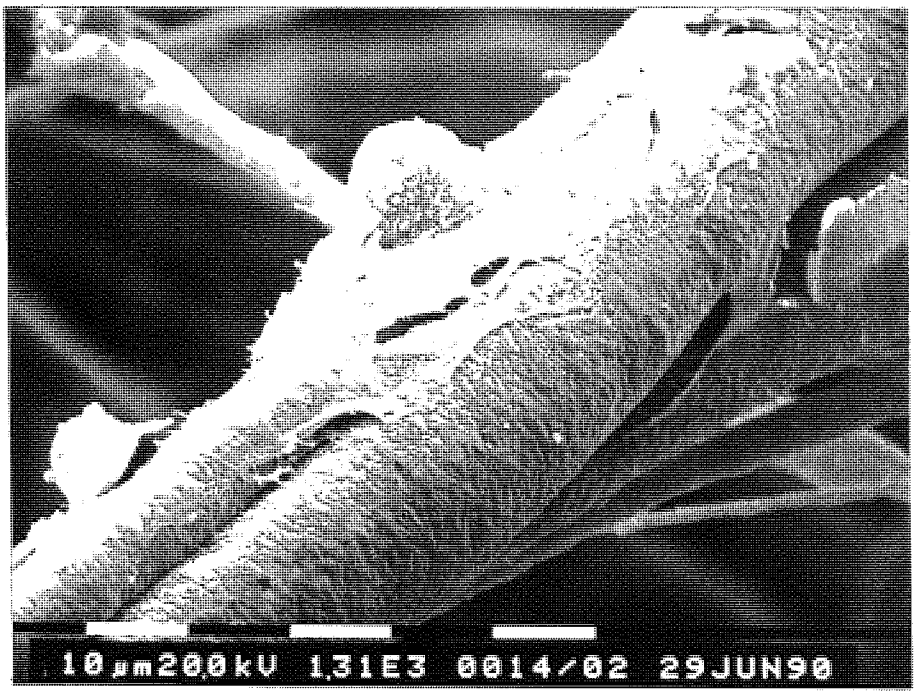


Plate 5.24: L929 cell attachment on alkali treated chloroform extracted PHB NWM.

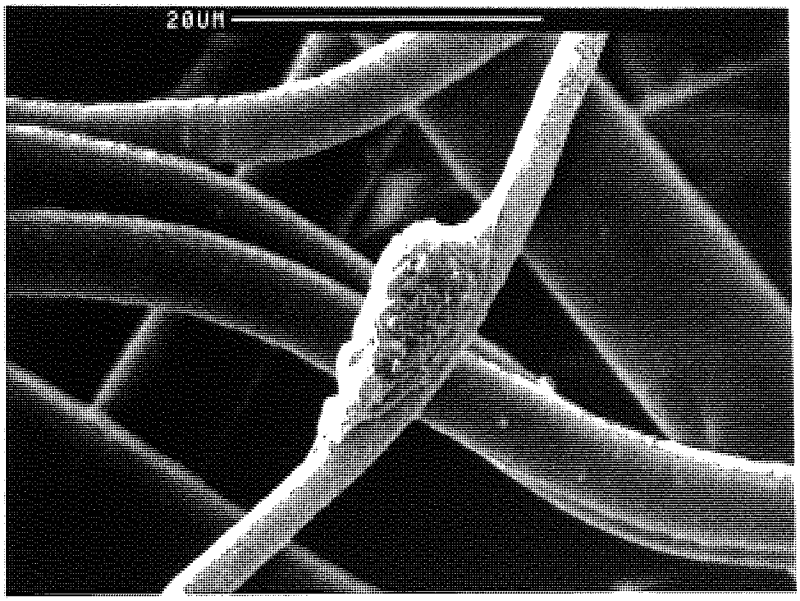


Plate 5.25: NOM 238 cell attachment on alkali treated dichloromethane extracted PHB NWM.

Plate 5.26: L929 cell attachment on alkali treated dichloromethane extracted PHB NWM.

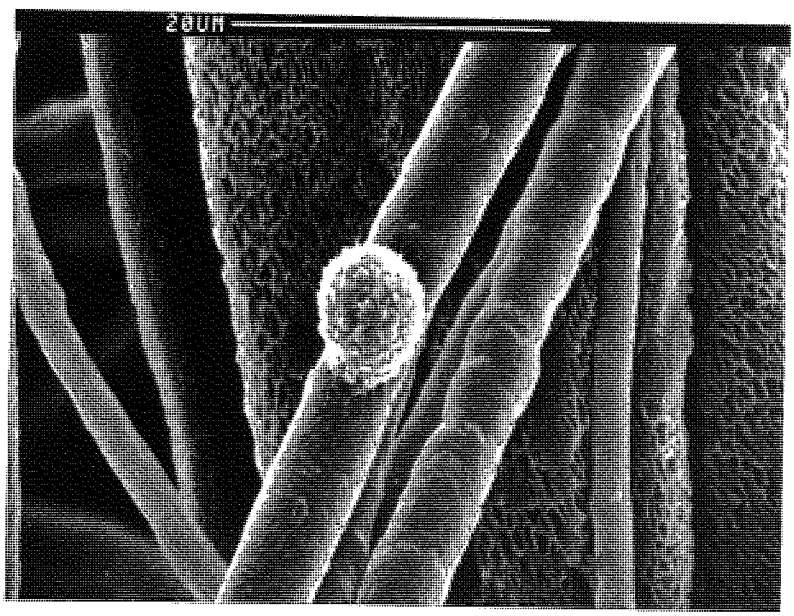


Plate 5.27: NOM 238 cell attachment on untreated chloroform extracted PHB NWM.

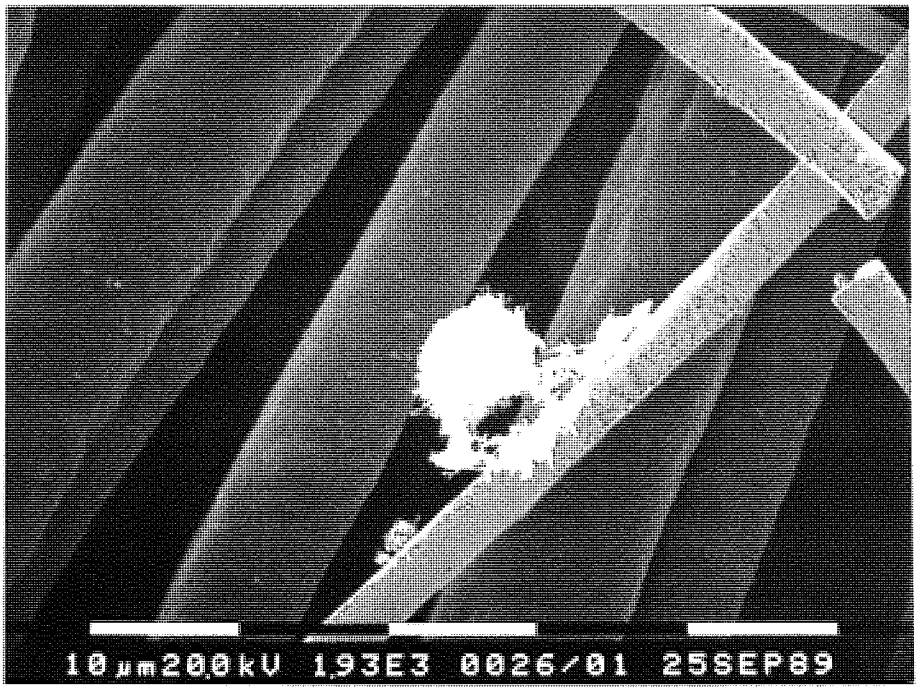


Plate 5.28: L929 cell attachment on untreated chloroform extracted PHB NWM.

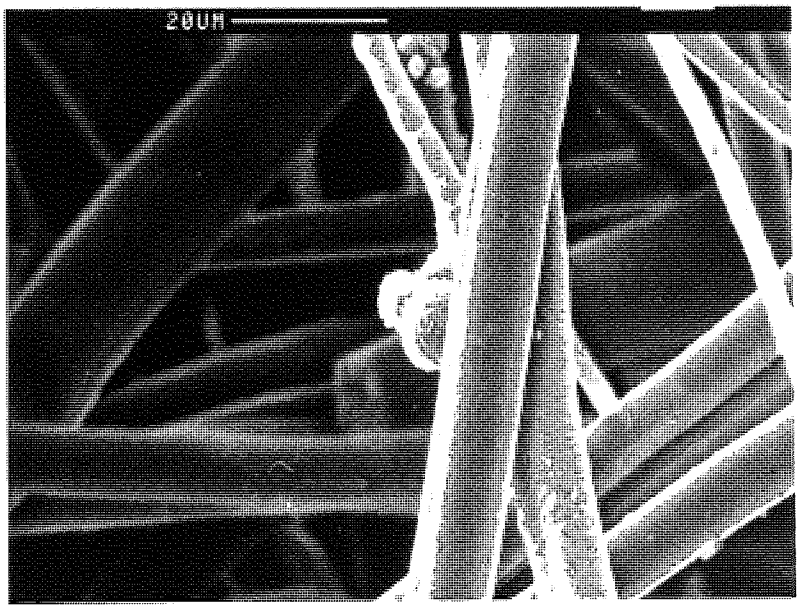


Plate 5.29: NOM 238 cell attachment on untreated dichloromethane extracted PHB NWM.

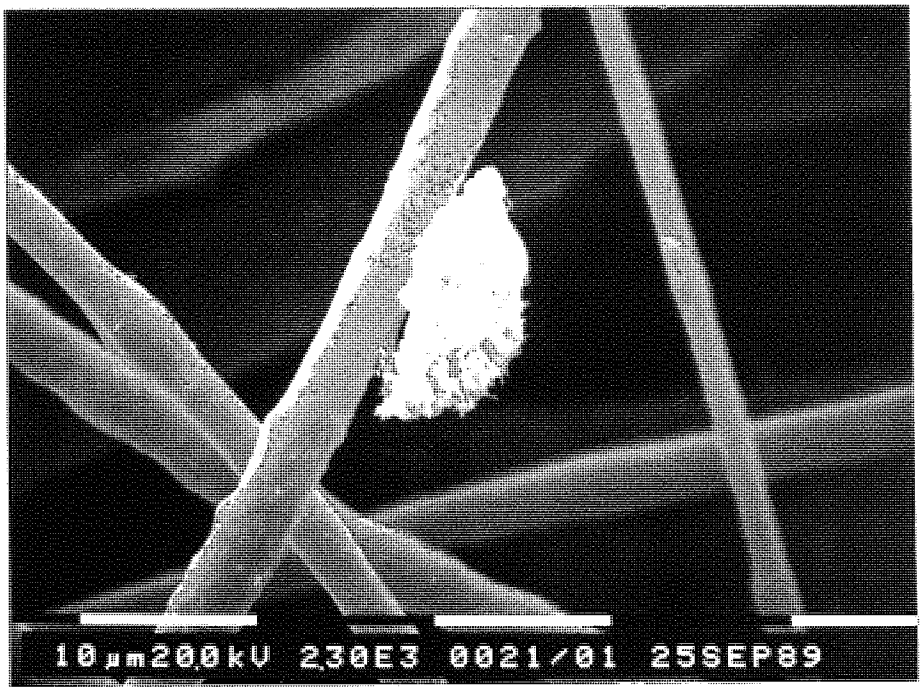
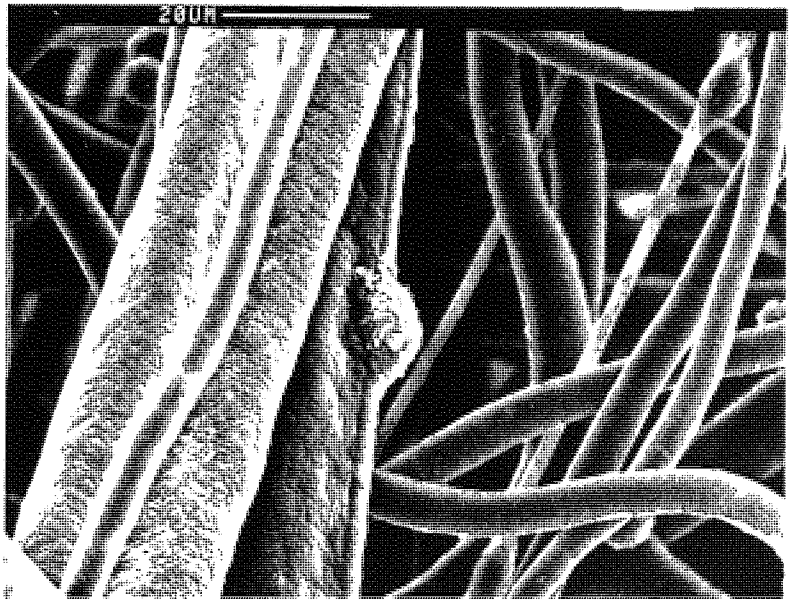


Plate 5.30: L929 cell attachment on untreated dichloromethane extracted PHB NWM.



CHAPTER SIX

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Concluding discussion

The results of this study have shown that the interactions of anchorage-dependent cells with novel substrata provide a plethora of information which the biomaterials scientist can utilise in the formulation of new materials.

Hydrogels are a unique group of polymers in that they contain water. The water is bound throughout the backbone and at the surface, and this characteristic water-structuring ability plays an important role in determining how cells interact with a particular hydrogel. The hydrogels used in this study have a high polar component of surface energy (Figures 3.15 & 3.16), and this increases the wettability of the surface. Wettability is a measure of the hydrophilicity of a surface. Earlier reports indicate that relatively hydrophilic surfaces can promote cell adhesion. The presence of polar groups and charged species at the surface mediate the interface conversion, the processes that determine the adhesiveness of a surface.

Research on the interactions of cells with hydrogels has confirmed the importance of the volume fraction of water in the gel, and by implication, at the surface. Protein deposition from this bound water converts the polymer surface and this may encourage cellular adhesion. The presence of dominant functional groups such as the nitrogen containing NNDMA and NVP polymers raises the polarity of the surface, and this in turn appears to promote the correct conformational deposition of adhesive proteins. It is this process of interface conversion which determines whether a cell will spread on a surface. Thus, surfaces that are considered "sticky" to fibroblasts are those which promote the deposition of adhesive proteins such as

fibronectin and vitronectin.

Earlier work from this group has shown that if the volume fraction of water in a hydrogel of given chemical structure is too high, cell attachment and spreading will not take place. In the extreme case, a layer of liquid water is clearly unable to support cell growth. The work in this thesis has helped to rationalise the importance of the ability of the chemical groups in the polymer to "organise", "structure", or "bind" the water. We have shown that different chemical groups have different water binding abilities, as indicated by the cellular response.

In NNDMA and NVP based hydrogels, the introduction of the hydrophobic groups MMA and LMA decreases the hydrophilicity of the copolymer, and as a result, the EWC drops. The nitrogen containing groups of the NNDMA and NVP have the ability to structure water as Thomas has stated. This means they can mediate protein adsorption and thus influence cell adhesion. Yet, the introduction of the hydrophobic comonomers MMA and LMA increases cell adhesion. In this case, the amount of mobile water at the surface may be too great for cell adhesion to take place; the observations recorded here fall on the right hand side of the "hema curve". As the mobile water is reduced by the presence of MMA or LMA, so the conditions for adsorption of conformationally correct proteins are restored, and cell adhesion can commence Increasing the comonomer content to 50% or more leads to even greater cell adhesion.

With respect to the plots of EWC against cell counts (Figures 3.5-3.8), two trends are apparent. Firstly, the NNDMA copolymers are able to "tolerate" more water than the corresponding NVP copolymers. This is because the nitrogen water-structuring groups in the NNDMA copolymers are more accessible than those in the closed ring of the NVP copolymers (see structures, chapter three). Therefore, the NNDMA hydrogels can bind more water at the surface than can the NVP series. This produces a shift to the right in the Minett curve.

An additional factor to take into consideration is the effect of the comonomer. Replacing LMA with MMA produces a further curve shift to the right in both NNDMA and NVP copolymers (Figures 3.5-3.8). This is because the methyl group in MMA sterically hinders the underlying water-structuring groups less than does the lauryl group in LMA. LMA is a long hydrophobic chain of 11 repeat units, which is flexible and may "bend over" the more polar oxygen and nitrogen containing functional groups, and thus exclude water.

An alternative explanation for the trend of cell adhesion on these copolymers is that as the EWC decreases with increasing comonomer content, so the mechanical stability of the surface increases. High water content hydrogels are by nature flimsy, and it is possible that at these elevated water contents, the polymer surface is too mobile to provide the necessary support for the tractive process of cell adhesion.

The incorporation of charged species can override the effects of the dominant functional group. ACM polymers are weakly basic so they exert their own water structuring effect, but this is overrun by the effects of the charged comonomers MAA and AEMA. Increasing the amounts of these comonomers raises the levels of cell adhesion in these hydrogels. It appears that in addition to polarity, surface charge plays an important role in determining the biological response to synthetic polymers. The most fascinating aspect of this study on hydrogels is the observation that not only does the presence of charged groups at the surface influence cell behaviour, but so does the nature of that charge. The positively charged AEMA comonomer encouraged cell adhesion at lower concentration levels than the corresponding negatively charged MAA. As discussed previously, many workers have reported on the effects of negatively charged species, such as, COO^-, and HSO_3^- on cell response. Indeed, the manufacture of tissue culture plastic utilises treatments which oxidise the surface of native polystyrene to render it cell adhesive. Minett and Thomas showed that in hydrogels, the effect of increasing this negative charge produced a cut-off point in cell adhesion on these surfaces. Once the electronegative charge at the surface becomes too great, the negatively charged cell surface has difficulty overcoming this electrostatic barrier, even if the charge is masked by adsorbed proteins. There is no such cut-off point in the ACM/AEMA copolymers, as indeed there is not for the MAA containing copolymers. This is in part due to the low molecular concentration of charged groups. Another facet to cell response on these extremely high water content polymers is structural mobility of the surface. Fibroblasts exert high tractive forces during the process of adhesion, and it may be that the surfaces of those copolymers with small amounts of comonomer in them are too fluid to support fibroblast

adhesion, as has already been suggested. The incorporation of charged comonomers does decrease the EWC of the surface, and this may make them more stable. However, these changes in EWC are very small, and the water contents remain well over 90%. It is the charged groups which have the greatest influence on cell behaviour on ACM copolymers. It would be interesting to see how the presence of dramatically increasing positive charge affects cell behaviour.

The overall picture of cellular adhesion on hydrogels highlights a number of physical and chemical factors which are responsible for influencing the cellular response. Altering any one of these factors affects the others. It is a combination of factors which determine fibroblast adhesion on these polymers.

The results gained from the work on hydrogels can be applied to biodegradable systems such as PHB-HV copolymers. Hydrogels and PHB polymers represent two systems; stable and unstable. In the case of hydrogels, the surface properties are determined by molecular design parameters such as the amount and type of comonomer. Thus, the surface properties are fixed in a way that is not possible in a biodegradable system. In PHB for example, one can determine the initial surface characteristics of a copolymer by processing, but predicting the way in which the degradation process affects the surface is guesswork at best. Degradation therefore is an important factor affecting the cellular response. In hydrogel systems, the amount of surface charge is a fixed quantity, and as we have seen, too much surface charge inhibits cell adhesion. When we come to look at PHB, the amount of charge that is present at the surface is a function of the extent of hydrolysis.

Random chain scission means that a hydroxyl and carboxyl group are created at every scission event, which will contribute to the surface charge effect, because of the ionisation of COOH to COO^-. It is to be expected that this rapidly increasing surface charge will eventually inhibit the attachment of fibroblasts due to the presence of an electrostatic barrier. In addition, the increased rate of hydrolysis experienced as a result of the incorporation of polysaccharides means that the surface becomes unstable. Gloss factor measurements (Figures 4.23-4.25) show that as degradation proceeds, the surface rugosity increases, and this becomes the most important factor influencing cell response on PHB-HV copolymers. Small scale rugosity (less that the cell diameter) may decrease cell attachment because there are less points of attachment for fibroblasts. As the scale of the rugosity becomes greater than the diameter of the cell, one would expect to see higher levels of attachment because of the increased surface area. That this is not the case is due to two factors. Firstly, it is likely that by this stage in the degradation of the copolymer, the concentration of electronegative carboxyl groups will be high, and will thus inhibit cell adhesion. In addition, as degradation proceeds, the surface becomes friable. The physical instability of the surface is not conducive to the attachment of fibroblasts due to the considerable forces that they exert on the substratum during adhesion. The incorporation of polysaccharides into undegraded PHB-HV copolymer matrices exerts a great influence on cellular behaviour. All of the polysaccharides used in this study are hydrophilic, therefore blending them with PHB-HV increases the wettability of the surface. This relationship between polymer, water and hydrolysis is very important. Blending polysaccharides increases the bound water at the surface. This will increase the deposition of

protein over the surface, which in turn influences the cell adhesion. The presence of polysaccharides may act as a double edged sword however. Since they make the system more susceptible to hydrolysis during degradation, surface charge will accumulate more rapidly than on unblended PHB copolymers which degrade slowly.

Increasing the polysaccharide content from 10 to 30% increases adhesion in all the polymers except amylose. Yasin's data (Figure 4.11 & 4.12) show that the surface polarity of the amylose blends increases much more rapidly than that of the other comonomers, with the possible exception of alginate blends for which no data are available. Increasing the amylose loading from 10-30% may mean that over the course of incubation, enough hydrolysis takes place to decrease cell adhesion due to the concentration of carboxyl groups at the surface.

The results gained from work on hydrogels and PHB-HV copolymers enabled the modification of PHB in a gel-spun form for use as a wound scaffold. The human and mammalian cell culture models used in this study have provided valuable information about the cytotoxicity of this material, as well as the fibroblast interaction.

Native PHB "wool" is non-adhesive for both cell lines. Presumably, the gel-spinning process does not allow sufficient oxidisation of the surface to take place, so the polarity, and hence wettability of the fibres is low. Unfortunately, no data exist as yet to confirm or deny this. The observations on hydrogels and PHB-HV

copolymers, as well as the work of other researchers enabled prediction of the type of surface treatment which would enhance cell adhesion. The initial surface treatments with cold sulphuric acid over short exposure times did not appreciably increase cell adhesion, and cell spreading only commenced with a 10% H_2SO_4 treatment. Insufficient hydrolysis of the surface does not produce enough charged groups to promote adhesion. The more severe treatment regime of elevated temperature and exposure produced surfaces which were very adhesive to both cell lines. Many more cells were attached, and cell spreading was evident over the whole range of acid wash concentrations. These harsher treatments increased the rate of hydrolysis and produced charged COO^- groups at the surface with every chain scission event. The fibres are better able to structure water at the surface and so promote protein deposition. The alkali treatments were found to be much harsher than the acid washes, and they promote rapid degradation of the fibres, as evidenced by the surface morphology of the larger fibres. The L929 mammalian fibroblasts were unable to tolerate these alkali washed surfaces, in comparison to the well spread NOM 238 line. This may be due in part to the surface morphology. Small scale features may disrupt cellular adhesion because of the reduced number of attachment points. The concentration of surface charge due to rapid hydrolysis may also be too great for the L929 cells to overcome.

In conclusion, the overview gained from this thesis highlights the contributions of surface polarity, surface charge and mechanical stability in determining cellular response. Polar surfaces are more wettable and more able to structure water at the surface. Protein deposition from this bound water "converts" the surface and consequently, cell adhesion is encouraged. The concentration of surface charge

also plays an important role in cell adhesion. A much greater concentration of surface charge in high water content hydrogels can be tolerated by cells because of the amount of water in these polymers. Since the EWC of the ACM copolymers is well in excess of 90%, the charge is diluted, so that even high comonomer ratios do not inhibit cell adhesion. This is not the case in the PHB polymers which contain much less water. In these polymers, the surface charge is a more important factor because its effects can either promote or inhibit cell adhesion. If the density of negatively charged species, particularly the strongly charged COOH becomes too great, "hema curve" behaviour occurs, and cell adhesion is curtailed.

In biodegradable systems, the process of degradation itself greatly affects the cellular response. The surface rugosity increases, and this may lead to decreased levels of attachment for the reasons outlined above. Eventually, the surface of the copolymer becomes too unstable to support anchorage-dependent cell growth, and this obviously decreases the cell adhesion.

Previously, biomaterials were utilised before the fundamental reasons for their applicability had been elucidated. The results presented here and elsewhere in the field of biomaterials science enable predictions about the biological performance of novel materials to be made. It is to be hoped that this will enable the production of tailor made biomaterials which will be well suited to their particular application.

6.2 Suggestions for further work

On the basis of the results, it appears that adhesive proteins such as Fn and Vn which mediate the adhesion of cultured fibroblasts adsorb as well, if not better, to positively charged surfaces than negatively charged ones. FTIR microscopy and photoacoustic spectroscopy (PAS) are novel techniques which hopefully, in future, will allow the determination of the nature of bound protein species. It is the process of interfacial conversion, the initial moments of host/biomaterial interaction which will determine whether a material is suited to a particular application.

A more comprehensive series of degraded PHB-HV copolymers and polysaccharide blends need to be cell tested to extend the fuller picture of the effect that degradation has on cell behaviour. In addition, cell assays on a wider range of time points over the degradation period is advisable. In the copolymers used in this study, general trends were established for two cell lines. However these results may be masking underlying trends because of the availability of samples along the degradation curve, on which adhesion assays could be carried out (i.e. 0,7,62,180,250 days).

The interaction of gel-spun PHB fibres with fibroblasts has provided useful information on its cytocompatibility with both human and mammalian cell lines. However, there are many other cell types which are involved in the wound healing process, and an assessment of the interaction of PHB "wool" with these cell types would be very useful. In particular, the response of macrophages to these fibres would be pertinent. In leg ulcers, it is these cells which initially migrate into the

hypoxic environment of the wound site, and secrete angiogenic growth factors. These growth factors encourage the regrowth of the capilliary network, oxygenating the wound and promoting the influx of ECM secreting cells such as fibroblasts. In addition, information on the surface characteristics, such as the polarity of these fibres would be of great benefit.

APPENDICES

Appendix 1a: Results of a single-factor anova on the BHK-21 treatment means of PHB-HV copolymers.

One Factor ANOVA X 1 : Polymer Type Y 1 : Cell Count:BHK-21

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	17	9362453125	550732536.765	115.72
Within groups	126	599656250	4759176.587	p = .0001
Total	143	9962109375		

Model II estimate of between component variance = 68246670.022

Appendix 1b: Results of a single-factor anova on the L929 treatment means of PHB-HV copolymers.

One Factor ANOVA X 1 : Polymer Type Y 1 : Cell Count:L929

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	17	8367578125	492210477.941	114.683
Within groups	126	540781250	4291914.683	p = .0001
Total	143	8908359375		

Model II estimate of between component variance = 60989820.407

Appendix 1c: Results of a single-factor anova on the BHK-21 treatment means of all hydrogel copolymer "families".

One Factor ANOVA X 1 : Polymer type Y 1 : Cell count BHK-21

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	27	3.081E10	1.141E9	160.554
Within groups	196	1392937500	7106823.98	p = .0001
Total	223	3.220E10		

Model II estimate of between component variance = 141740387.909

Appendix 1d: Results of a single-factor anova on the L929 treatment means of all hydrogel copolymer "families".

One Factor ANOVA X 1 : Polymer type Y 1 : Cell count L929

Analysis of Variance Table

Source:	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	27	2.423E10	897357928.241	136.379
Within groups	196	1289656250	6579878.827	p = .0001
Total	223	2.552E10		

Model II estimate of between component variance = 111347256.177

Appendix 2a: Results of a two-factor anova on the treatment means of all hydrogel copolymer "families".

Anova table for a 2-factor Analysis of Variance on Y_1 : Cell count

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Polymer type (A)	27	5.373E10	1.99E9	290.812	.0001
Cell line (B)	1	425295200.893	425295200.893	62.147	.0001
AB	27	1.303E9	48258163.856	7.052	.0001
Error	392	2682593750	6843351.403		

Appendix 2a: Results of a two-factor anova on the treatment means of PHB-HV copolymers.

Anova table for a 2-factor Analysis of Variance on Y_1 : Cell Count

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Polymer Type (A)	17	1.739E10	1.023E9	226.015	.0001
cell line (B)	1	1512500000	1512500000	334.214	.0001
AB	17	341750000	20102941.176	4.442	.0001
Error	252	1140437500	4525545.635		

Appendix 3a: Results of a single-factor anova on the treatment means of BHK-21 tested hydrogel copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count BHK-21

Comparison	Mean diff:	Fisher PLSD:
NNDMA/LMA 80:20 Vs NNDMA/MMA 85:15	-125	2628.988
NNDMA/LMA 50:50 Vs NNDMA/MMA 50:50	-5187.5	2628.988*
NVP/LMA 80:20 Vs NVP/MMA 80:20	-850	2628.988
NVP/LMA 70:30 Vs NVP/MMA 70:30	-2750	2628.988*
ACM/AEMA 99:1 Vs ACM/MAA 99:1	2500	2628.988
ACM/AEMA 90:10 Vs ACM/MAA 90:10	-3900	2628.988*

* Significant at 95%

Appendix 3b: Results of a single-factor anova on the treatment means of L929 tested hydrogel copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count L929

Comparison	Mean Diff:	Fisher PLSD
NNDMA/LMA 80:20 Vs NNDMA/MMA 85:15	937.5	2529.646
NNDMA/LMA 50:50 Vs NNDMA/MMA 50:50	3500	2529.646*
NVP/LMA 80:20 Vs NVP/MMA 80:20	-1375	2529.646
NVP/LMA 70:30 Vs NVP/MMA 70:30	-1625	2529.646
ACM/AEMA 99:1 Vs ACM/MAA 99:1	937.5	2529.646
ACM/AEMA 90:10 Vs ACM/MAA 90:1	-4375	2529.646*

* Significant at 95%

Appendix 4a: Results of a single-factor anova on the treatment means of BHK-21 tested hydrogel copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count BHK-21

Comparison	Mean diff:	Fisher PLSD:
NNDMA/LMA 80:20 Vs NVP/LMA 80:20	1500	2628.988
NNDMA/LMA 70:30 Vs NVP/LMA 70:30	-4875	2628.988*
NNDMA/LMA 50:50 Vs NVP/LMA 50:50	-7750	2628.988*
NNDMA/LMA 40:60 Vs NVP/LMA 40:60	2875	2628.988*
NNDMA/LMA 30:70 Vs NVP/LMA 30:70	4937.5	2628.988*

* Significant at 95%

Appendix 4b: Results of a single-factor anova on the treatment means of L929 tested hydrogel copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count L929

Comparison	Mean diff:	Fisher PLSD:
NNDMA/LMA 80:20 Vs NVP/LMA 80:20	3062.5	2529.646*
NNDMA/LMA 70:30 Vs NVP/LMA 70:30	1312.5	2529.646
NNDMA/LMA 50:50 Vs NVP/LMA 50:50	5625	2529.646*
NNDMA/LMA 40:60 Vs NVP/LMA 40:60	-2750	2529.646*
NNDMA/LMA 30:70 Vs NVP/LMA 30:70	2312.5	2529.646

* Significant at 95%

Appendix 5a: Results of a single factor anova on the BHK-21 treatment means of 10% & 30% polysaccharide blended PHB-HV copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count BHK-21

Comparison	Mean diff:	Fisher PLSD:
12%PHV/1% NT Vs 20%PHV/1% NT	375	2158.829
12%PHV/10% AMYLOSE Vs 20%PHV/10% AMYLOSE	937.5	2158.829
12%PHV/30% AMYLOSE Vs 20%PHV/30% AMYLOSE	437.5	2158.829
12%PHV/10% DEXTRAN Vs 20%PHV/10% DEXTRAN	2437.5	2158.829*
12%PHV/30% DEXTRAN Vs 20%PHV/30% DEXTRAN	-1062.5	2158.829
12%PHV/10% DEXTRIN Vs 20%PHV/10% DEXTRIN	-3937.5	2158.829*
12%PHV/30% DEXTRIN Vs 20%PHV/30% DEXTRIN	-3687.5	2158.829*
12%PHV/10% Na ALG Vs 20%PHV/10% Na ALG	1000	2158.829
12%PHV/30% Na ALG Vs 20%PHV/30% Na ALG	-937.5	2158.829

* Significant at 95%

Appendix 5b: Results of a single factor anova on the L929 treatment means of 10% & 30% polysaccharide blended PHB-HV copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count L929

Comparison	Mean diff:	Fisher PLSD:
12%PHV/1% NT Vs 20%PHV/1% NT	312.5	2050.113
12%PHV/10% AMYLOSE Vs 20%PHV/10% AMYLOSE	-500	2050.113
12%PHV/30% AMYLOSE Vs 20%PHV/30% AMYLOSE	-337.5	2050.113
12%PHV/10% DEXTRAN Vs 20%PHV/10% DEXTRAN	4200	2050.113*
12%PHV/30% DEXTRAN Vs 20%PHV/30% DEXTRAN	-250	2050.113
12%PHV/10% DEXTRIN Vs 20%PHV/10% DEXTRIN	-1000	2050.113
12%PHV/30% DEXTRIN Vs 20%PHV/30% DEXTRIN	-2250	2050.113*
12%PHV/10% Na ALG Vs 20%PHV/10% Na ALG	2875	2050.113*
12%PHV/30% Na ALG Vs 20%PHV/30% Na ALG	-3500	2050.113*

* Significant at 95%

Appendix 5c: Results of a single factor anova on the BHK-21 treatment means of 10% & 30% polysaccharide blended PHB-HV copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count BHK-21

Comparison	Mean diff:	Fisher PLSD:
12%PHV/10% AMYLOSE Vs 12%PHV/30% AMYLOSE	4250	2158.829*
12%PHV/10% DEXTRAN Vs 12%PHV/30% DEXTRAN	-2687.5	2158.829*
12%PHV/10% DEXTRIN Vs 12%PHV/30% DEXTRIN	-7950	2158.829*
12%PHV/10% Na ALG Vs 12%PHV/30% Na ALG	-2375	2158.829*
20%PHV/10% AMYLOSE Vs 20%PHV/30% AMYLOSE	3750	2158.829*
20%PHV/10% DEXTRAN Vs 20%PHV/30% DEXTRAN	-6187.5	2158.829*
20%PHV/10% DEXTRIN Vs 20%PHV/30% DEXTRIN	-4875	2158.829*
20%PHV/10% Na ALG Vs 20%PHV/30% Na ALG	-4312.5	2158.829*

* Significant at 95%

Appendix 5d: Results of a single factor anova on the L929 treatment means of 10% & 30% polysaccharide blended PHB-HV copolymers.

One Factor ANOVA X1: Polymer type Y1: Cell count L929

Comparison	Mean diff:	Fisher PLSD:
12%PHV/10% AMYLOSE Vs 12%PHV/30% AMYLOSE	4187.5	2050.113*
12%PHV/10% DEXTRAN Vs 12%PHV/30% DEXTRAN	-2300	2050.113*
12%PHV/10% DEXTRIN Vs 12%PHV/30% DEXTRIN	-3125	2050.113*
12%PHV/10% Na ALG Vs 12%PHV/30% Na ALG	-1562.5	2050.113
20%PHV/10% AMYLOSE Vs 20%PHV/30% AMYLOSE	4187.5	2050.113*
20%PHV/10% DEXTRAN Vs 20%PHV/30% DEXTRAN	-6750	2050.113*
20%PHV/10% DEXTRIN Vs 20%PHV/30% DEXTRIN	-4375	2050.113*
20%PHV/10% Na ALG Vs 20%PHV/30% Na ALG	-8000	2050.113*

* Significant at 95%

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