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Cells, Surfaces and Adhesion

Janet Helen Fitton

Doctor of Philosophy

The University of Aston in Birmingham

1993

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Summary of thesis

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This thesis concerns cell adhesion to polymer surfaces with an experimental emphasis on hydrogels. The thesis begins with a review of the literature and a synthesis of recent evidence to describe the process of cell adhesion in a given situation. The importance of understanding integrin-adhesion protein interactions and adhesion protein-surface interactions is emphasised.

The experimental chapters describe three areas of investigation. Firstly, *in vitro* cell culture techniques are used to explore a variety of surfaces including polyethylene glycol methacrylate (PEGMA) substituted hydrogels, sequence distribution modified hydrogels and worn contact lenses. Cell adhesion to PEGMA substituted gels is found to decrease with increases in polyethylene oxide chain length and correlations are made between sequence distribution and adhesion. Worn contact lenses are investigated for their cell adhesion properties in the presence of antibodies to specific adhesion proteins, demonstrating the presence of vitronectin and fibronectin on the lenses. The second experimental chapter addresses divalent cation regulation of integrin mediated cell adhesion. Several cell types and various cations are used. Zinc, previously not regarded as an important cation in the process, is found to inhibit 3T3 cell adhesion to vitronectin that is promoted by other divalent cations. The final experimental chapter concerns cell adhesion and growth on macroporous hydrogels. A variety of freeze-thaw formed porous gels are investigated and found generally to promote cell growth rate. Interpenetrating network based gels (IPN) are made porous by elution of dextrin particles of varying size and loading density. These materials provide the basis for synthetic cartilage. Cartilage cells (chondrocytes) plated onto the surface of the porous IPN materials maintain a rounded shape and hence phenotypic function when a critical pore size and density is achieved. In this way, a prospective implant, made porous at the perpendicular edges contacting natural cartilage can be both mechanically stabilised and encourage the maintenance of normal matrix production at the tissue interface.

The experimental work in this thesis has demonstrated the use of cell adhesion studies to characterise and probe surfaces and to directly aid in the design of an implant material prior to animal studies. The mechanism of cell adhesion was studied with reference to the divalent cation requirement leading to recognition of a new role for zinc.

KEYWORDS: cell adhesion, vitronectin, polyethylene oxide, divalent cations, zinc, macroporous hydrogels, synthetic cartilage.

Acknowledgements

Many thanks to Dr. David Brown (Ighite) for advice and helpful criticisms over the last three years. Within the group at Aston, I am indebted to Dr. Paul Corbett, Dr. Helen Doherty, Naveed Ashraf and Mark Smith for providing polymer samples, and to Lyndee Jones for providing electron images. For use of the excellent SEM and X-ray facilities, I am indebted to Roger Howell. Steve Turchi provided some much appreciated feedback on matters physiological and chemical.

I am grateful to Professor Charles Archer for use of microscope facilities at Cardiff and for expert technical assistance on the artificial cartilage project.

Patrick Lawton, former secretary from Dr. Adams, gave much of his time to argue the case, provided moral and academic support on a daily basis and made a major contribution to my understanding of many things. I would not have done half so far without him.

In fond memory of my late parents, Sydney and Enid Fitton, and to my dear aunt and guardian, Margaret Rastrick.

Many thanks to Ray Holman, Janet Johnson, and Pam Baggie for providing and growing the cartilage samples during the last few months.

Financial assistance was kindly provided by SERC and Kodak under the Cardiff contract scheme.

Finally I wish to acknowledge the late Professor Mike Platt who provided me with a letter of introduction to Aston. His criticism and enthusiasm provided the inspiration for this particular research. I will always be grateful for the lively conversation and advice that he gave me.

Acknowledgements

Many thanks to Professor Brian Tighe for advice and helpful criticism over the last three years. Within the group at Aston, I am indebted to Dr Phil Corkhill, Dr Helen Oxley, Naweed Ashraf and Mark Smith for providing polymer samples, and to Lyndon Jones for providing contact lenses. For use of the excellent SEM and darkroom facilities, I am indebted to Roger Howell. Steve Tonge provided some much appreciated feedback on matters physiological and chemical.

I am grateful to Professor Charlie Archer for use of microscope facilities at Cardiff and for expert feedback on the artificial cartilage project.

Patrick Lawlor, famous scotsman from St Albans, gave much of his time to argue the facts, provided moral and academic support on a daily basis and made a major contribution to my understanding of integrins. I would not have come half so far without him.




I would also like to thank Dr John Edwards at Glasgow and Dr Steve Clarke at Kodak for helpful conversations about divalent cation regulation of cellular adhesion.


Many thanks to Kay Hobson, Laura Hobson, and Paul Briggs for proofreading and good company and beer during the last few months.


Financial assistance was kindly provided by SERC and Kodak under the CASE award scheme.

Finally I wish to acknowledge the late Professor Mike Flint who provided me with a job in Auckland in 1989. His brilliance and enthusiasm provided the inspiration for me to pursue this research. I will always be grateful for the lively conversation and advice that he gave so freely.


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
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
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
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1.0 General Introduction

This thesis endeavours firstly to outline the present understanding of cellular responses to materials with particular attention to recent integrin studies, and secondly, to utilise current technology to develop new cell based screening techniques for materials.

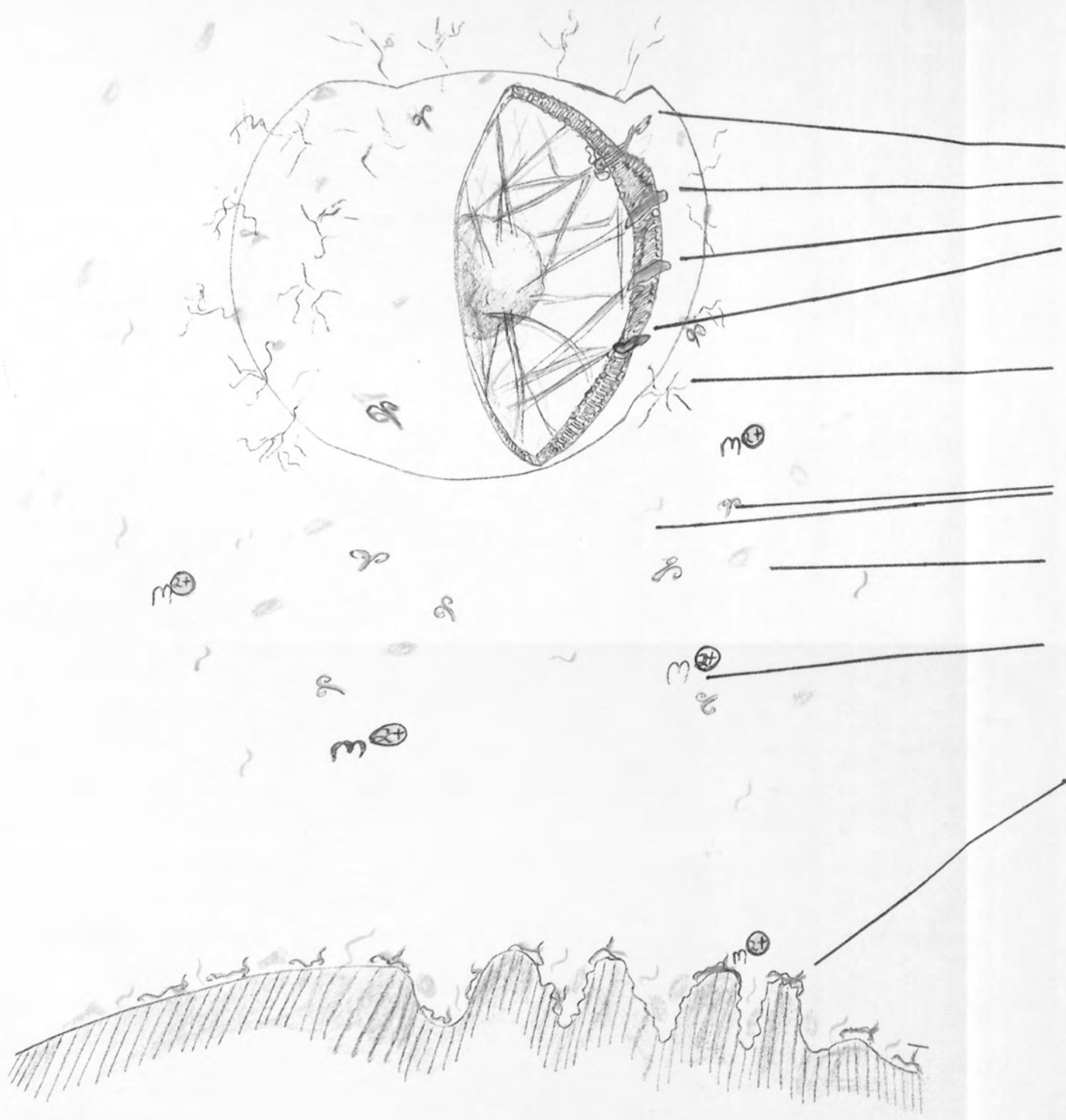
When designing a material for use in biological situations, an understanding of the relationship between the material and its immediate environment is vital. Polymers presently used for devices may not be best suited for their function. The recent controversy over the failures of silicone breast implants and subsequent autoimmune responses (1,2) emphasises the importance of continuing research into suitable materials.

Consideration of the materials' bulk and surface properties, including chemical, physical and biological aspects is the starting point in any device design procedure. Biological devices often require materials resistant to protein fouling and cellular or bacterial adhesion, so control of the surface properties is especially important. For example, catheters and sensor materials need to be totally non-fouling whilst materials used for vascular grafts should promote endothelialisation and discourage protein deposition likely to lead to thrombosis. The latter material needs to be selectively adhesive for endothelialisation. A good biomaterial is one which performs its function over the required periods of time with minimal effects on the rest of the system.

There are many methods described in the literature for physical and chemical characterisation of a surface. These include contact angle analysis for surface energy estimations, X-ray photoelectron spectroscopy (ESCA) to determine surface chemistry and scanning electron microscopy (SEM) to examine morphology. Biological characterisation can be achieved by a diverse set of *in vivo* and *in vitro* methods including protein binding and activation experiments, tissue response studies and cell adhesion or growth assessment.

The importance of understanding the cell-polymer surface system as a whole is stressed. In recent years some of the mechanisms of cell/adhesion protein interactions and adhesion protein/surface interactions have been unravelled. This insight has provided ground for innovation in biomaterials design and a reinterpretation of known cell/polymer interactions. The experimental part of this thesis is concerned with the interactions between mammalian cells and polymer surfaces *in vitro* under static conditions, in an attempt to further understand the mechanisms of mammalian cell adhesion, and to demonstrate the use of adhesion assays as a tool in the design process.

An overview of all mechanisms involved in the adhesion process is necessary for design of cell culture based experiments and interpretation of results. This literature review is divided into three areas which consider a) the cell, b) the surface and c) the medium. Each section ends with a list of keypoints. A synthesis of the views expressed in this introduction is provided by Figure 1. The reader is advised to refer to this before proceeding with the text.



Cell Adhesion to a Surface

This diagram shows a cell with a cutaway section revealing the nucleus and stylised cytoskeleton. The cell is suspended in a protein containing solution above a surface where proteins are adsorbed.

The cell expresses four major categories of adhesion receptor

Integrins-major cell-adhesion protein interactions

cadherins-self associating cell-cell interactions

immunoglobulins-self associating (cell-cell) and integrin interacting receptors

selectins-cell-cell interactions via carbohydrate binding

These receptors span the cell membrane and interact with the cytoskeleton and various regulatory proteins (see figure 3)

The receptor interactions are modified by other features of the surface such as glycosylated residues.

The immediate environment contains adhesion proteins such as **fibronectin and vitronectin**, which can associate with the integrins in solution. This binding is not as strong as binding to the immobilised adhesion protein.

Other proteins, e.g. albumin compete with adhesion proteins as they adsorb to the surface. These proteins are nonadhesive to the cell.

Binding may also be modified by serum components such as proteoglycans.

Divalent cations are essential for integrin-adhesion protein interaction. Interaction is centered on the completion of coordination of the ions within a binding site on the integrin by the peptide sequence on the adhesion protein.

Adsorbed proteins have altered conformation. This may expose specific peptide sequences that interact with integrin receptors.

The surface properties that affect adsorption include;

Chemical groups expressed at the surface- Hydroxyl, sulphonate and amide groups tend to increase adsorption of adhesion proteins in active conformation

Hydrophilicity/hydrophobicity- hydrophilic surfaces tend to adsorb more adhesion proteins relative to their bulk concentration. This does not hold true for some hydrogels. Hydrophobic surfaces tend to be dominated by albumins.

Surface energy-related to the above properties. Surfaces with high surface energy tend to be cell adhesive

Equilibrium water content- high water content polymers tend to be less adhesive to cells.

Topology- roughness on a nm scale may enhance adhesion. Roughness on larger scales may inhibit adhesion due to cytoskeletal constraints in some cells.

Figure 1

1.1 The Cell

1.1.0 Introduction

Cells are the basic unit of all organisms (other than viruses and prions). Although this thesis concerns adhesion in mammalian cell types, recent evidence shows that slime moulds, bacteria and plants (3,4,5) all rely on similar adhesion strategies; the binding properties and core peptide sequences of adhesion proteins, such as vitronectin (Vn) are highly conserved among these organisms (6). Many pathogens either mimic adhesion proteins or have receptors for host produced adhesion proteins which enable them to invade other organisms. In higher organisms, adhesion strategies are vital to cells for the organisation, synthesis and maintenance of various tissues according to the position and function that are assigned during the development of the embryo (7). A histological view of cells in mammalian systems might categorise about 200 separate cell types. There are of course many more, including cells at different stages of maturity or responding to various stimuli. Generally, cells can be loosely categorised as belonging to one of two groups. Fully differentiated committed cells, such as liver cells, divide to produce two daughter cells of the same type. Other cells are renewed from an undifferentiated stem cell. Good examples of the latter group are intestinal epithelial cells and skin cells. Haemopoetic stem cells are pluripotent and can give rise to differing cell types including a number of specialised immune cells and monocytes. Some monocytes fuse to become osteoclasts (large multinucleate, bone resorbing cells). Most organs (areas of tissue with a specialised function) are bounded by a layer of epithelial cells and basement membrane. No cells other than specialised immune cells and macrophages can cross this boundary, except when cells become cancerous and lose their territorial stability. It is easy to see from this brief description that cells in the body require a diverse and variable set of adhesion parameters according to their function.

1.1.1 Cells *in vitro*

The experimental work on cell adhesion described in this thesis deals mainly with the basic differentiated connective tissue cell type called a fibroblast. Monocytes and primary chondrocytes are also used. The cells are grown in an adhesion dependent culture system in specialised nutrient media and retain the essential characteristics of cells in the tissue from which they were first isolated. There are two classes of cell used for *in vitro* work: cell lines and primary cells (8). Cell lines are immortalised cells having characteristics of a particular cell type. They can be derived from naturally occurring tumours or, alternatively, generated from normal cells by either transfection or transfection with an immortalised cell. Cell lines can be passaged many times without loss of phenotype. Primary cells are those cells isolated from a normal tissue without further treatment. They can be cultured for relatively few passages after

which they begin to lose the phenotype of the original tissue. Whilst cultured cells can give useful indications of the behaviour of a particular cell type, the *in vivo* situation can never be replaced as a final test situation for materials.

1.1.2 Adhesion receptors

What determines the adhesive qualities of a cell type? This question is highly pertinent to many disciplines from embryology and immunology to biomaterials design. Common themes began to emerge in research and now four major classes of adhesion receptors on the cell surface have been identified. These are:

- a) Cadherins, which are calcium dependent, homotypic cell-cell adhesion molecules ⁽⁹⁾
- b) Immunoglobulin superfamily receptors (Ig), a very large group of receptors, encompassing divalent cation dependent receptors which bind to integrin type receptors on other cells and divalent cation independent homophilic receptors ⁽¹⁰⁾
- c) Selectins, calcium dependent receptors that bind to carbohydrate moieties ⁽¹¹⁾
- d) Integrins, heterodimeric receptors with ligand specificities controlled by divalent cations, binding to extracellular matrix proteins and Ig and some integrin receptors on other cells ^(12,13).

1.1.3 Integrins

It is the last group of cell surface receptors, the integrins, which are of particular interest as they mediate adhesion to proteins such as fibronectin (Fn), vitronectin (Vn) and laminin which can be adsorbed to material surfaces from tissue fluids and serum. The integrins are a large family of membrane spanning cell surface glycoproteins which also mediate a number of cell-cell adhesion interactions and serve as receptors for immunoglobulin complexes which participate in immune responses. Structurally integrins consist of heterodimer complexes of noncovalently bound α and β subunits ⁽¹⁴⁾. There has been rapid and significant growth in understanding of this receptor group since the first identification of the 'classical' Fn receptor, $\alpha 5 \beta 1$. At least twenty different integrin heterodimers are identified to date, consisting of combinations of 14 different α units and 8 different β units (see figure 2, overleaf). Most integrins seem to recognise short peptide active sites such as the RGD and LDV sequences in specific adhesion protein ligands. The variation in type of α unit associated with an β subunit alters the ligand specificity of the integrin. For example the Fn receptor, $\alpha 5 \beta 1$ binds to Fn, whereas $\alpha 2 \beta 1$ binds to both collagen and laminin. However, receptors are relatively promiscuous; recent research suggests that the Vn receptor $\alpha 5 \beta 3$ also binds Fn specifically and probably acts in concert with $\alpha 5 \beta 1$ to promote cell surface Fn binding in normal cells ^(15,16). This ability may also explain the ability of malignant melanoma cells to adhere to extracellular matrices in the apparent absence of Fn receptors. Humphries has already shown that administration of adhesion protein-like peptides in an embryo can



Aston University

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Figure 2 The known associations of various integrin subunits and their ligands

(after Ruoslahti 1991)

Key

Fn	Fibronectin
Vn	Vitronectin
Coll	Collagen
Fb	Fibrinogen
VWF	von Willebrands Factor
lam	laminin
PPA	Peyers patch addressin
RGD	classical adhesion tripeptide
C3bi	part of complement pathway
?	unknown

Integrin adhesion receptors isolated from cell membrane preparations have been shown to consist of two subunits, designated α and β . There are several identifiable types of α and β subunits which may combine to form an integrin with defined ligand specificity, as shown in the diagram above.

dramatically affect the translocation of cells (17). There is much interest in exploiting adhesion peptide therapy for various forms of metastasis through control of integrin function (18). The $\beta 1$ or "VLA" group of integrins are associated with most cell types, whereas the $\beta 2$ group are generally restricted to cells of the immune system. Nomenclature may be variable in the literature due to different modes of discovery. A list of equivalent terms can be found in the appendix.

Expression of an integrin type may be limited to a particular cell type. For example, gpIIB/IIIa ($\beta 3 \alpha IIIa$) is expressed solely by megakaryocytes and platelets (19) and $\alpha 6 \beta 4$ seems to be solely expressed by epithelia (20). Some human cell lines are now available with a limited and defined integrin expression, but few generalised fibroblasts and primary derived cells have been classified in this way. It can be assumed that connective tissue cells such as fibroblasts express a variety of receptors for basement membrane components including those for Fn and Vn, and probably collagen and laminin.

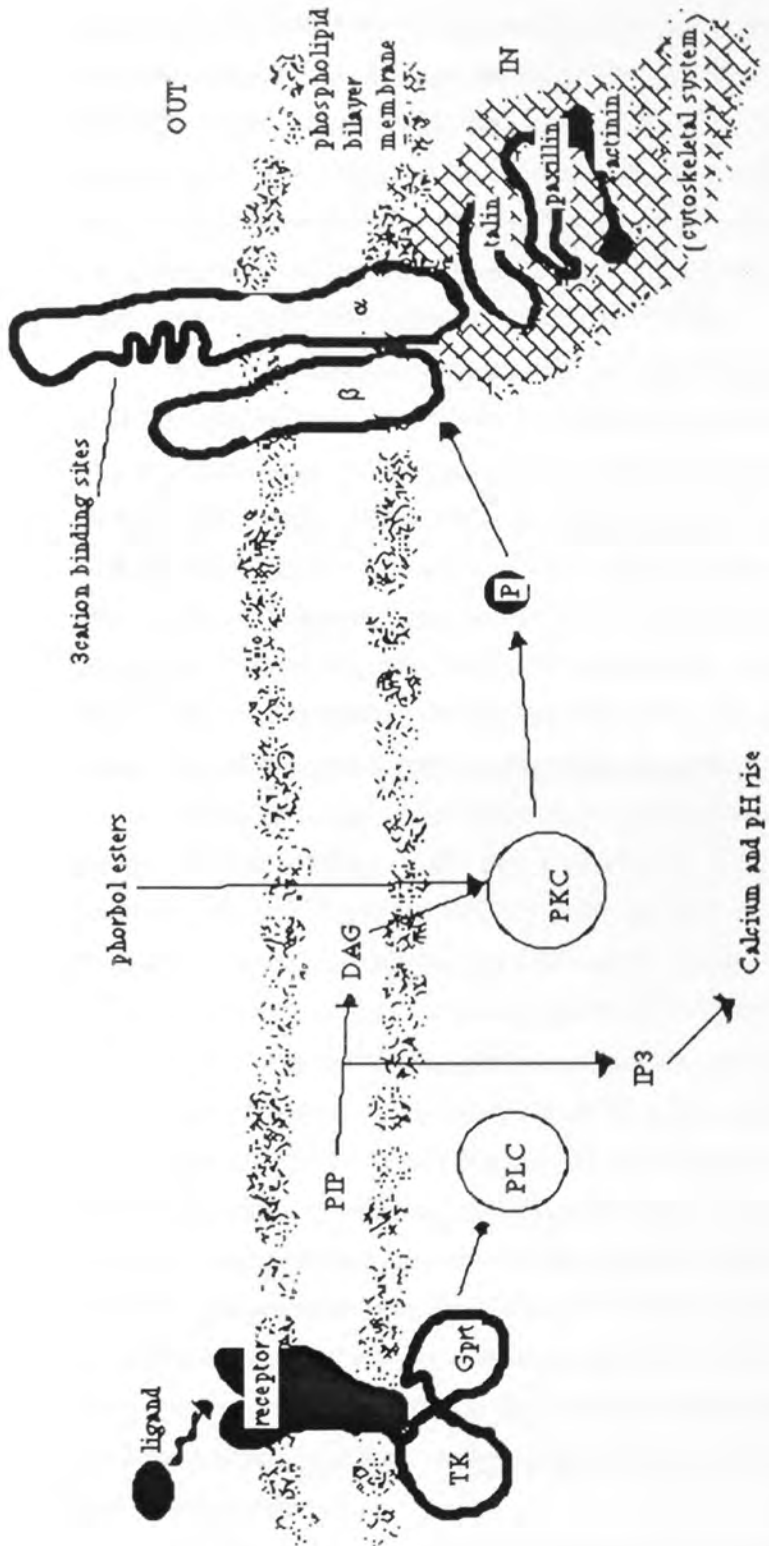
1.1.4 Connections to the cytoskeleton

Integrins span the membrane of the cell, interacting with adhesion proteins on the outside of the cell and cytoskeletal elements within the cell (see figure 3 overleaf). The cytoskeletal connections were first observed using fluorescent antibodies to demonstrate that actin (one of the principal components of the cytoskeleton) codistributes with the adhesive plaques on cells adherent to Fn. Antibody probes were then developed to show the clustered transmembrane linking entity now known as an integrin (21). After the initial adhesive contact there is a reorganisation of the cytoskeleton and formation of focal adhesions. The cell then begins to spread as more focal adhesions form peripherally. The focal adhesions represent a clustering of receptors. This spatial organisation of receptors may be important in signalling within the cell and in subsequent cell behaviour (see section 1.1.5).

A complex set of proteins including paxillin (22) talin, α actinin, fimbrin and tensin are located at the junction between cytoskeleton and integrin (23). A number of regulatory proteins are also present such as the calcium dependent protease calpain II and protein kinase C. It is likely that phosphorylation of paxillin by protein kinase C, and perhaps other proteins at the location causes changes in the organisation of the focal adhesion proteins. A recent study by Edwards et al (24) has shown that vanadate -a substance known to block tyrosine phosphorylation in cells- can inhibit spreading of BHK fibroblasts on Fn but not induce rounding in previously spread cells. It seems that tyrosyl phosphatase may have been involved in this mechanism.

1.1.5 Integrin activation

Expression of an integrin does not necessarily correlate with binding function. It has become clear within the past few years that many receptors require activation. Secondary



Ligand binding to a receptor, possibly another integrin, results in the activation of tyrosine kinase (TK) or a G protein (Gprot). This in turn, activates phospholipase C (PLC) mediated hydrolysis of phosphoinositol phosphates (PIP) in the cell membrane, producing diacylglycerol (DAG) and inositol phosphates (IP3). Inositol phosphates participate in the secondary messenger system of the cell leading to rises in intracellular calcium and pH. DAG or externally applied phorbol esters may activate protein kinaseC (PKC) which phosphorylates(P) the integrin leading to a conformational change. This changes the affinity of the integrin for adhesion molecules such as vitronectin. Binding of such an adhesion molecule leads to cytoskeletal changes e.g. the polymerisation of actin and further signalling via the pathway just described. This may also be the signal for receptor clustering which leads to focal adhesions commonly seen in adherent cells.

Figure 3. The activation of integrin receptors

messenger systems within the cell may play an important part in the activation process, possibly by phosphorylation of specific cytoskeletal elements associated with the cytoplasmic tail of the integrin, but the precise mechanisms are still obscure. A recent investigation of intracellular mechanisms in response to adhesion to extracellular adhesion proteins suggests that protein kinase C and other regulatory elements are involved (24,25,26). These internal changes in the cell could be initiated by binding of unrelated or related receptors in an "adhesion cascade", a cellular response to cytokines, or by other changes in the immediate environment-pH for example. Integrin activation from within the cell is known as "inside-out signalling" (25,26). Figure 3 shows a speculative mechanism based on recent evidence. A classic example of integrin activation is the platelet integrin glycoprotein IIb/IIIa which requires activation before binding to fibrinogen, but binds to smaller adhesion peptides and snake venom peptides without activation (27,28,29).

The investigation of such a mechanism is far from trivial in the consideration of cellular response to biomaterials. Imagine that a small quantity of endotoxin (lipopolysaccharide produced by gram negative bacteria) is present in the bloodstream of a patient. This might trigger a rise in interleukin levels leading to activation of receptors on various cell types or platelets, or directly activate the cell, for example via a CD14 receptor (30,31). Any foreign material present in the body would be susceptible to changes in adhesion processes. A relatively adhesion resistant material prior to rise in endotoxin levels could now be subject to an immune cell response or connective tissue adhesion, even though the site of endotoxin production is remote from the material.

"Outside-in signaling" is the term used to refer to the signal transduction effected by the integrin ligand binding (32,33). Many examples of this have been extensively researched-for example, the binding of monocytes to Fn via a $\beta 1$ receptor activates CR1 and MAC1 ($\beta 2$ type receptors) to bind and internalise C3b and iC3b (part of the complement pathway, see later)(34). Lymphocyte stimulation via the complement receptor CR3 activates $\beta 1$ receptors for Fn and laminin on T cells which has obvious implications for immune response (binding T cells to tissue sites proximal to infection)(35). In this way, material adsorbed proteins can make a substantial difference to subsequent cellular response. As the material surface governs the initial adsorption of adhesion proteins and other components, it can indirectly affect cellular function. For example, monocytes can be stimulated by adherence to various polymers to produce growth factors and cytokines (36). Polystyrene has been shown to effect a decreased neutrophil respiratory burst which compromises the ability to respond to infection *in vivo* (37). This may be due to the reduced binding of complement and increased binding of complement inhibitors associated with hydrophobic surfaces, but these highly complex mechanisms are still poorly understood.

Finally, there is substantial evidence to suggest that Fn binding to its receptor may merely activate other adhesion mechanisms within the cell, rather than causing adhesion directly. This adds weight to the evidence for signalling and raises new questions about the way in which cells bind to surfaces of all types (38,39).

1.1.6 Divalent cation requirement

Cell substrate adhesion mediated by integrins in the presence of adhesion proteins requires the presence of a divalent cation. Integrin α subunits contain three or four cation binding sites which bear close homology to EF hand, a 13 residue cation binding site found in calmodulin and parvalbumin. The α subunit sequences only differ from those in calmodulin by the absence of an aspartate residue (D) normally found at position 12 in EF hand (40,41). The presence of this residue in nearly all of the critical binding sites on the corresponding adhesion peptides has led to the proposal that D provides the final co-ordinating position for the cation resulting in binding between ligand and receptor. There are 3-4 binding regions on each α subunit and it is not yet clear how occupancy of one or all these sites affects binding. There is however, substantial evidence to suggest that cation binding activates integrins as well as catalysing the ligand receptor binding (42, 43). Perhaps occupancy of one site alters conformation to produce activity and the further sites are responsible for the binding. Most research interest to date centres upon the intracellular and extracellular controls acting on the α subunit, but β subunits also play a regulatory part in binding (23).

Magnesium correlates with the active form of many integrins whilst calcium tends to modulate the effects of magnesium. Manganese activates very many integrins at very low concentrations, whilst results obtained for this thesis suggests that zinc may play an important role in inhibition of Vn receptors. This subject will be discussed in greater detail in chapter 4.

1.1.7 Other factors which modulate adhesion in cells

It should be noted that carbohydrates, such as the glycosylated parts of adhesion proteins (44), and some lipids, also have a role to play in adhesion. The adhesive role of proteoglycans centres on the binding of the protein core to the relevant integrin, but the glycosylation pattern endows regulatory specificity. Other 'nonadhesive' proteoglycans can regulate the adhesion process. For example, dermatan sulphate derived from cartilage was found to reduce the number of focal adhesions in cells adhered to Fn (45). Membrane lipids may play a part in altering the conformation of integrins, and the lipid composition of the cell membrane plays a regulatory role in the migration and assembly of receptors to produce adhesion plaques after initial binding (46).

Finally, the cell type on which the integrin is expressed can affect the function of that integrin. A good example of cell specific integrin function is the $\alpha 2\beta 1$ integrin of platelets, which only binds to collagen when expressed on platelets, but can also bind to Fn and laminin when expressed on other cell types (47). This reflects the point made earlier, that conditions prevailing within the cell affect the activation and selectivity of the integrin.

1.1.8 What happens in a protein free situation?

If cells are introduced to a surface in a simple ionic medium such as buffered saline, adhesion is governed by purely physicochemical interactions between the glycosylated surfaces of the cell and the substrate; those forces which usually determine the adsorption of proteins from solution in advance of the cells. It is easy to distinguish between integrin modulated adhesion and purely physicochemical interactions, as the former requires cells to be maintained at 37°C to support the metabolically active process (48a). However, some selectin mediated adhesion, such as lymphocyte adhesion to endothelia, can occur at 7°C. At this temperature, cells must still retain some metabolic activity, since only viable cells, and not dead cells will adhere. The mechanism behind this adhesion phenomenon is not yet understood (48b).

What are the physical forces involved in adhesion of cells to surfaces in serum free media? Attractive forces include the relatively long range Van der Waals force, whilst shorter range forces include the attraction between hydrophobic molecules in an aqueous situation. Most of the forces are repulsive, such as the electrical repulsion between like charged surfaces and steric hindrance.

In the absence of serum, cells adhere strongly and very rapidly to hydrophobic surfaces such as polystyrene, slightly less to the less hydrophobic tissue culture plastic, and also to the hydrophilic HEMA surfaces as shown in the results section. However, providing the cells are metabolically active, they begin to produce protein which competes effectively for surface adsorption and cells gradually desorb. (It is entirely possible that measuring the profile of cell desorption after adding various proteins could provide a method for measuring protein adsorption, given a known set of parameters). In the presence of serum, the effect of adsorption and desorption of other proteins usually overrides the initial direct interaction of cell and substrate so that cell adhesion occurs slowly over a period of an hour or so to adsorbed adhesion proteins from the serum. However, cell adhesive coatings such as the highly positively charged polylysine and Celltak work on the principle of charge interactions even in the presence of competing proteins. Celltak is especially interesting as it is derived from a protein produced by mussels in order to adhere to rocks. It appears that this protein, known as "byssus protein", adsorbs to a surface via decapeptide regions that include L-DOPA residues. Its cell adhesive activity seems likely to be due to the high proportion of lysine residues in the remainder of the protein (49).

Cells carry different surface glycosylation patterns and hence different charge densities and hydrophobicity. They can therefore be separated on the basis of physicochemical interactions with various surfaces. A good example of cell separation on the basis of differential charge density, is the modified polymer surface which provided the basis for a rapid separation of B and T lymphocytes at low temperatures (50) later shown to be a result of charge interactions (51). This was a real improvement on previous methods which had relied on integrin mediated adhesion to serum coated nylon (52).

Red blood cell adhesion has been extensively studied, and it has been found that these cells can adhere to surfaces which seem to repel the body of the cell. Adhesion occurs by means of small tethers from the cell surface and is based on the adsorption of a cell surface associated protein (53). This extension of membrane projections is common to many cells. Cells adhered to apparently separate microspikes were able to extend cellular projections to join their nearest neighbours and eventually spread on a self secreted matrix (54). It is important to remember that cells produce adhesion proteins which remain associated with their surfaces. Nonstandardised trypsinisation procedures prior to experimentation can therefore lead to variable cell surface adhesion measurements. Adhesion measured over an extended period or in serum free medium must take into account adhesion proteins secreted by the plated cells.

1.1.9 Cell shape

Cell shape appears to be an important regulator of cell function. Binding to an adhesive ligand causes actin polymerisation and changes the architecture of the cell cytoskeleton, which leads to changes in cell behaviour (55,56,57,58). Most connective tissue cells must spread in order to proceed through the cell cycle, which requires the presence of adhesive glycoproteins such as Fn or laminin. pHEMA has often been used as a nonadhesive coating in experimental systems to control cell shape. A variety of incomplete pHEMA coatings were first used to control cell shape by Folkman and Moscona (57). These incomplete coatings effectively limited the area of cell adhesion to the underlying substrate. Adhesive islands of palladium were evaporated onto complete pHEMA background substrates (56) in experiments to determine the effects of cell shape on growth and the cell cycle. Alteration of cell shape can affect cell phenotype. For example, chondrocytes, which are rounded in intact cartilage, will assume a spread cell shape *in vitro* if they can attach to a solid substrate. This change in cell shape is accompanied by a switch from synthesis of cartilage matrix components to synthesis of a fibroblastic type matrix. The spread area of the cell, and hence matrix synthesis, can be controlled by the area available for adhesion on the substrate (59). Dedifferentiated chondrocytes can also be returned to the undifferentiated state if grown under suitable conditions and so provide an excellent model for cell shape studies (see chapter 5).



Key points

- 1) All cells express *adhesion receptors*. Common mechanisms exist amongst diverse organisms.
- 2) Mammalian cells express a number of adhesion receptors including cadherins, selectins, immunoglobulins and *integrins*.
- 3) Integrins are membrane spanning heterodimeric receptors for adhesion proteins such as fibronectin and vitronectin.
- 4) Expression of integrin does not necessarily correlate with adhesion. Activation may be required. The requirement for activation may depend on the nature of the ligand; activation may simply alter ligand specificity.
- 5) "*Inside-out signalling*" Activation seems to involve phosphorylation of specific cytoskeletal elements via secondary messenger systems.
- 6) "*Outside-in signalling*" Receptor-ligand binding may activate other cell responses.
- 7) *Divalent cations are essential* for integrin receptor-ligand binding. Binding affinity varies according to cation. Cations may promote or counteract the effects of other cations, playing a crucial role in adhesion modulation.
- 8) The state of glycosylation, presence of lipids and proteoglycans may also affect the affinity of receptor for ligand.
- 9) In the absence of serum, adhesion occurs by physicochemical forces between cell and substrate surfaces. This type of adhesion occurs in the absence of cell metabolism i.e. at $<37^{\circ}\text{C}$ and can be used as a basis for rapid cell separation.

1.2 The Surface

1.2.0 Introduction

Which surface properties have effects on protein adsorption and subsequent cell adhesion? There is a degree of conflict in the literature reflecting a rather reductionist approach to this question. Measurable qualities fall broadly into the following categories: chemical groups expressed at surface, surface energy and fractional polarity, hydrophobicity and hydrophilicity, equilibrium water content and rugosity. It is important to realise that no single parameter can be divorced from the others. Some properties correlate with each other. For example, an increase in hydroxylation increases the polar component of surface energy and usually the hydrophilicity of the surface. Water structuring effects are affected by all of these parameters and may be one of the major considerations in adsorption of proteins and cell adhesion. It is emphasised that in serum containing medium, cell surface adhesion is largely governed by the amount of adhesion proteins adsorbed in a conformationally active state as a proportion of the total adsorbed protein. In protein free medium, it is the physicochemical interactions between the surfaces of the cell and the substrate that govern cell adhesion.

1.2.1 Surface chemical groups

The pendant sidegroups exposed to the medium on a polymer surface depend on the nature of the aqueous environment and on the rotational and lateral mobility of the surface polymer chains. Generally, polar groups turn towards the aqueous environment. The presence and density of various chemical groups at the polymer surface affect the adsorption and subsequent conformation of specific adhesion proteins (and hence cell adhesion) in serum containing systems. In protein free systems, the overall physicochemical nature of the surface presented to the cell varies according to the various groups and their distribution. Effects of surface groups cannot be divorced from other surface properties. Generally, the introduction of charged groups leads to greater cell adhesive properties. In serum containing medium, this may be due to either, or both, of two effects: the direct interaction of positive charge with the cell membrane, and the increase in adsorption of adhesion proteins from the serum.

Polystyrene (PS) is used extensively in culture systems as it is cheap and therefore disposable. Untreated PS is uncharged, and not generally cell adhesive in the presence of serum although it does adsorb proteins. Precoating with a layer of adhesion proteins like Fn or charged polylysine molecules makes PS adhesive. Tissue culture plastic (TCP) is glow discharge treated or plasma treated PS. This oxidised, slightly charged, and more wettable PS surface is very adhesive to cells. It should perhaps be noted more widely that some of the oxidised species formed by plasma and glow discharge methods are water soluble. About half are washed away by washing in water, and presumably, immersion in

culture medium ⁽⁶⁰⁾. Therefore, quantification of surface groups by X-ray photoelectron spectroscopy or other methods prior to water washing is unlikely to lead to true correlations with cell culture experiments. Early research into the cell adhesion phenomenon suggested that plasma generated hydroxyl groups (OH) ^(61, 62) were a major factor controlling cell adhesion to plasma treated polystyrene surfaces. Acid treatment also renders PS adhesive, which led to arguments for the role of sulphonate, amine (NH₂) and carboxyl groups (COOH) ^(63, 64). Investigations of acid treatment in these laboratories showed ⁽⁶⁵⁾ that mild treatment induced backbone hydroxylation and resulted in surfaces that were able to support cell adhesion. More extensive treatment with hot sulphuric or chlorosulphonic acid produced substantial surface sulphonation. The resulting surface supported little cell attachment but not spreading.

Research on covalently derivatised substrata has concluded that NH₂ and OH surface groups are most likely to lead to adsorption of adhesion proteins such as fibronectin (Fn) and vitronectin (Vn) ⁽⁶⁶⁾. Steele et al examined nitrogen (N₂) and oxygen (O₂) plasma modified fluoropolymer surfaces. It was found that whilst Vn adsorption on O₂ containing films was a controlling factor for endothelial cell adhesion in the presence of whole serum, both Fn and Vn were cooperative in the adhesion response on N₂ modified surfaces. Whether these findings can be extended to other materials or cell types remains to be seen ⁽⁶⁷⁾. Carbonyl groups also increase endothelial cell adhesion and growth, although high densities of surface carbonyl groups did not support any attachment ⁽⁶⁸⁾. A retrospective view of the literature with the recently gained knowledge about integrin mediated adhesion endorses these conclusions. Pendant groups substituted onto hydrogel polymers also influence cell adhesion, although the equilibrium water content of these materials also seems to be an important regulatory factor ⁽⁶⁹⁾.

Experimental details of reports should always be examined, as it is not always clear whether adhesion is mediated by adhesion proteins. For example, leukocytes were found to preferentially adhere to NH₂ or COOH modified polyurethanes in comparison to the unmodified polymer ⁽⁷⁰⁾. Thus, these modified materials were suggested as leukocyte strippers for whole blood. The experiments were, however, carried out in the absence of any other blood components which may well completely alter the results. *In vivo*, or on surfaces contacting complex biological fluids such as blood, cell adhesion may be quite different from that seen *in vitro*. For example, OH, CO, NH and (CH₃)₂N groups on the surface of a subcutaneous implant increased macrophage spreading, and SO₃H and COOH groups inhibited fusion and spreading ⁽⁷¹⁾. These results contrasted with the *in vitro* investigations of the same study, and the *in vitro* experiments of Lentz and Horbett ⁽⁷²⁾. This anomaly may have been due to cellular enzyme modification of the surface, or perhaps the adsorption of tissue fluid components that were not mimicked *in vitro*.

It is obvious that OH groups cannot be the sole factor in adhesion as the polymer hydrogel poly (2-hydroxyethyl methacrylate) (polyHEMA) with low interfacial energy and rich in surface OH groups is definitely not cell adhesive. Poly (methyl

methacrylate) (polyMMA) and poly (ethyl methacrylate) (poly EMA) are cell adhesive in the absence of OH groups. Investigations of a range of copolymers of HEMA and EMA to determine the relative contributions of the OH group concentration and the overall physicochemical nature of the substrate showed no linear correlation between cell spreading and OH content ⁽⁶⁹⁾. McAuslan and Johnson ⁽⁷³⁾ found that brief hydrolytic etching of nonadhesive HEMA with sulphuric acid introduced COOH groups and rendered it cell adhesive (nearly to the extent of tissue culture plastic). Etching with HCl or HF was not effective, although this also introduced COOH groups. A recent paper by Ratner's group suggests that the analysis was incomplete and may have overlooked the possibilities of transesterification reactions ⁽⁶⁸⁾, yet subtle differences in the arrangement, density and orientation of the groups may have occurred in the context of different polymer structures and different treatments. Further to this, the rugosity of a surface makes a large difference to cell adhesion. The HF and HCl may have etched the surface to a greater extent than the sulphuric acid, making it too rough for adhesion (see section 1.2.6).

Positively charged polymer surfaces bind tightly to the cell membrane, increasing cell adhesion in culture systems ⁽⁷⁴⁾ and other researchers have noted the prevention of lateral diffusion of Concanavalin A receptors ⁽⁷⁵⁾ and inhibition of spreading of HeLa cells on a polyethyleneimine surface ⁽⁷⁶⁾. *In vivo*, the differential exposure of charged amino groups on collagen fibres in different tensional states has been implicated in the control of cellular morphology and synthetic activity ⁽⁷⁷⁾. Tendon cells grown on electrically positively charged carbon surfaces as a model of tensioned collagen, became trypsin resistant after 24 hours of continuous stimulation regardless of a final two hours of charge reversal. SEM revealed that although the cells became rounded, they remained attached by many residual cytoplasmic adhesion sites onto the carbon surface. This indicated a very close association between the surface and the plasma membrane, effected by a persistent cell mediated response, although the mechanism for this was not elucidated ⁽⁷⁸⁾. Whether charged surfaces *in vitro* interact directly with the cell membrane or via adsorbed adhesion proteins is unclear. It is likely that both mechanisms contribute to the increased cell adhesion response. Very highly positively charged surfaces are toxic to cells ⁽⁷⁴⁾. This may be attributable to disruption of the cell membrane.

Tissue reactions to materials *in vivo* are not only dependent on direct cell adhesion and behaviour, but also on the response of the immune system. Complement, a system of proteins that participate in the humoral immune response can be activated by material surfaces ^(79,80). Vn is involved in the regulation of this response, although it is not clear how its involvement ultimately affects cell adhesion. Oxidised PS (OH groups) or sulphonated PS (SO₃ groups) activate complement by the alternative pathway, but surfaces bearing an equal balance of OH and SO₃ groups inactivate the pathway ⁽⁸¹⁾. Complement activation is a very complex mechanism, and its inactivation at any stage is not easily explained. The presence of these groups in balanced quantities may mimic the properties of heparin which can also passivate a surface ⁽⁸²⁾.

Finally, on HEMA rich hydrogels ⁽⁸³⁾ and cuprophane ⁽⁸⁴⁾, there is a lack of the fibrinogen peak that characterises the Vroman response to materials in plasma. This effect is well defined on EMA rich copolymers ⁽⁸⁴⁾, and has puzzled researchers for some time. Recent evidence suggests that the surface adsorption of complement C3 ⁽⁸⁴⁾ may prevent the Vroman effect; cuprophane and HEMA copolymers are known to activate C3 ⁽⁸⁵⁾. This interference with the deposition and exchange of proteins presumably extends to adhesion proteins and could be another route to consider in the development of non adhesive hydrogels.

1.2.2 Hydrogels and equilibrium water content.

Hydrogels are water swollen polymers based on various hydrophilic monomers. Poly 2-hydroxyethyl methacrylate (poly HEMA) is probably the most common polymer hydrogel. When other monomers, such as N-vinylpyrrolidone (NVP), or other methacrylates, are introduced into the polymer, a large variety of water contents and surface properties can be achieved. The water content of the swollen polymer seems to exist in at least two states: 'bound' water, associated with the polymer chains that does not freeze or efficiently solvate ions, and 'free' water which can move within the polymer and solvate ions and molecules. The equilibrium water content is the maximum water content achieved by the hydrogel. It can be determined experimentally by measuring the dry and wet weights of the gel. Equilibrium water content is equivalent to $\{(\text{wet weight} - \text{dry weight}) / (\text{wet weight})\} \times 100$.

Hydrogels can provide a model substrate for cell growth that could be said to mimic extracellular matrix. Manipulation of such hydrogels may lead to further understanding of functional groups within normal extracellular matrix, as well as providing necessary information for the development of biomaterials. Work from these laboratories has demonstrated direct relationships between equilibrium water content (EWC) and cell adhesion in hydrogels containing similar water structuring groups ^(65,69). Fibroblasts will adhere and spread onto homogeneous HEMA copolymers within a certain EWC "window" of 5 - 35 % water whilst similar higher water content hydrogels (40-60%) do not support adhesion. A further investigation of the phenomenon showed that the 'nonadhesive zone' for cells with respect to the EWC of the gel could be shifted upwards if OH groups in the backbone of the polymer were substituted with other groups. Functional groups effects followed the trend OH (HEMA) < CONRR (NVP) < CONHR < CONH₂ (acrylamide), which broadly corresponds with the trends defined by other researchers on alternative polymers ⁽⁶⁶⁾. Bergethon discussed cell adhesion to HEMA gels modified with collagen and various ionisable functional groups as cell culture substrates ⁽⁸⁶⁾. He reported some adhesion without spreading to homogeneous HEMA gels indicating the presence of impurities or surface oxidation in the polymer systems. The results however, were broadly in line with those discussed above, showing that the presence of ionisable surface groups influences cell spreading and growth.

What effects do surface chemical functionalities have that results in differences in cell adhesion on hydrogels? The presence of chemical groups not only affects the amount

and conformation of adsorbed adhesion molecules but gives rise to a number of physicochemical effects such as reorganisation of water and distribution of divalent cations at the interface. The introduction of charged groups into polyHEMA gels for example, increases the water structuring effects at the surface. Polar groups such as COOH, NH₂ give rise to sheaths of irrotationally bound water. However, introduction of uncharged MMA to polyHEMA gels also produces an adhesive surface although no polar pendant groups are present. This is because MMA and other non polar groups tend to decrease the water content of the hydrogel, bringing the water content back into the 'adhesive' zone.

In chapter 5, semi- interpenetrating network hydrogels (sIPNs) will be investigated in the context of synthetic cartilage replacement materials. Thomas ⁽⁶⁵⁾ found little correlation between water content and cell adhesion in IPN materials and attributed this lack of correlation to surface domain formation. An alternative explanation may be that IPNs offer a greater density of adhesion promoting surface groups as compared to a simple hydrogel of equal water content; the interpenetrant component of the IPN may dominate the surface region of the polymer.

To summarise, alterations in physico chemical surface properties caused by various pendant groups substituted onto the backbone of the polymer, indirectly affect the adsorption of adhesion proteins. Adhesion proteins also interact directly with these charged groups. This may explain the increase in adhesion seen on various copolymer surfaces. The density of the chemical functionalities at the surface is obviously a factor in the subsequent adhesion molecule adsorption. The density of charge, for example is related to the size of the polar pendant group and its distribution within the polymer. The sequence distribution of monomers within copolymers is one of the effects examined in Chapter 3 of this thesis.

1.2.3 Surface energy

Surface energy and fractional polarity are potential indicators of the adhesive potential of a surface. These parameters are easily measured by the use of contact angle measurement methods of Owen and Wendt ⁽⁸⁷⁾. Adhesion protein adsorption is generally considered to be more likely on a surface exhibiting a moderate or high surface energy, whereas surfaces of low energy are less adhesive. The polar component of the surface energy is a result of hydrogen bonding and dipole interactions, hence the introduction of hydroxyl groups tends to increase it. However, surfaces with very low fractional polarity and low EWC do not all offer the same degree of adhesiveness. For example amorphous polystyrene is nonadhesive, whereas crystalline PTFE, polypropylene and polyethylene are cell adhesive. This may reflect other differences in surface properties ⁽⁶⁵⁾.

1.2.4 Hydrophobicity and hydrophilicity

Hydrophobicity and hydrophilicity are related to the surface energy and chemistry of a surface. Hydrogels like HEMA copolymers tend to be very hydrophilic whereas styrene or dimethylsiloxane (both commonly used as copolymers in biomedical materials) tend to be hydrophobic in nature. Hydrophilic surfaces have polar forces at the surface which hydrogen bond with water. The water molecules orient around the polar groups. Hydrophobic surfaces tend to cause an 'ice-like' configuration of water molecules at the surface. These water structuring effects may, of course, be mixed on a surface and the complex water structures produced may then have effects on adsorption characteristics of a surface (differential solubility of ions in regions of organised water molecules, for example, may have implications for the divalent cation requirement in cell adhesion). Differential water structuring across a surface may be a method by which certain block copolymers exert their nonadhesive effects.

There is a general consensus that the more hydrophobic surfaces cause greater denaturation of adsorbed proteins which do not desorb as easily as from hydrophilic surfaces, due to greater conformational changes in adsorbed proteins. These conformational changes may expose critical binding sequences in adsorbed proteins. Hydrophobic surfaces adsorb less C3, a central component of the complement system, and cause conformational changes in adsorbed C3 that increase the binding of inhibitory factor H⁽⁸⁸⁾. Hydrophilic surfaces offer a variety of protein adhesion profiles but these adsorbed proteins are more easily desorbed. This may well explain the nonadhesive qualities of some hydrogels, as specific adhesion proteins such as Vn and Fn must undergo some conformational changes for maximum cell adhesive activity. Rapoza and Horbett⁽⁸⁹⁾ recently investigated the adsorption of fibrinogen (an important adhesion protein in the process of blood clotting) onto a series of hydrophobic and hydrophilic surfaces. They found that glassy, rigid, polymers were highly adsorptive whether or not they were hydrophobic, but that the fibrinogen was more loosely bound to hydrophilic and oxygen containing polymers. This conclusion correlates with the results of Lu and Park⁽⁹⁰⁾ who showed that fibrinogen adsorbs more strongly to hydrophobic surfaces than to hydrophilic surfaces and that this is accompanied by the greatest conformational changes in the secondary structure of the protein.

1.2.5 Sequence distribution and domain morphology

Many copolymers undergo phase separation and the resulting surface consists of microdomains of hydrophilic areas within hydrophobic matrices or vice versa. The relative proportions of monomer lead to different patterns of hydrophilic - hydrophobic islands on a μM scale⁽⁹¹⁾. Manipulation of domain size and structure with block and graft copolymers and interpenetrating networks provides a novel means of controlling protein adsorption and cell adhesion as the organisation of the polymer surface seems to have a great effect on

subsequent protein adsorption. Blocky distribution of monomers in a polymer leads to tiny domain structures in the surface. This may result in increased protein adsorption, whereas "dispersed monomer" polymers may exhibit more resistant properties. This situation is addressed further in chapter 3.

Other research groups have designed surfaces to exhibit variable domain size and structure for use in cell separation. It has previously been emphasised in this thesis that cell adhesion to surfaces in protein containing solution is very different to that in protein free solution. Cell separation can be achieved under both conditions. For example, in a protein free system, island domains of polyamino groups in a polyHEMA gel caused preferential adsorption of B lymphocytes from a mixture of blood cells (50). The amino-HEMA copolymer was used to coat glass beads offering a simple and rapid alternative to the usually more lengthy integrin based method of separation for these cells (serum coated nylon fibres). Muramatsu et al (51) later demonstrated that this was due to differential electrostatic interactions between the cells and the polymer. In serum containing systems, block copolypeptides tended to inhibit cell attachment whilst their counterpart random copolymers supported cell attachment (92). *In vivo*, Okano et al found that HEMA : styrene and HEMA : dimethylsiloxane (DMS) block copolymers were relatively resistant to thrombogenesis as compared to polyHEMA, polystyrene or polyDMS alone. They followed this work up with an investigation of HEMA : polyethylene oxide(PEO) block copolymers (93). PEO containing polymers are recognised for their potential resistance to protein deposition, a subject that is discussed further in chapter 3. Generally, greater percentages of PEO in a copolymer would be expected to decrease protein adsorption. The results of Okano et al are relevant in the context of microdomain structure as they found that 38% mole fraction of PEO in the block copolymer was more effective than higher mole fraction compositions in reducing platelet adhesion. This effect was attributed to the microdomain structure of the PEO in the resulting copolymer surface.

More recently, Lai et al have investigated the adsorption of adhesion proteins and platelets onto copolymer surfaces using immunotechnology (94). They have shown that preferential adsorption of proteins occurs even on the nanometre scale of the surface distribution of groups. Using immunolabelling, they have shown that the surface microdomain patterns may be critical in determining adsorption. Cells, depending on type, form adhesive focal and/or point contacts with the surface, and require a minimum space in which to adhere permanently. If the area containing adsorbed adhesion protein is too small or too widely spaced from the next, the cell will be unable to spread, and may not be able to achieve stable adhesion at all. Protein adsorption requires areas of surface chemistry sufficiently large to achieve stable physico chemical interactions between protein and material surface.

The surface domains mentioned in the works above are generally of diameter ~100-1000nm. The effect of domain size on cell adhesion, investigated by O'Neill et al (56), used larger "macrodomains" etched into a nonadhesive surface. They found that the total adhesive area available to cells applied to a lithographically etched polyHEMA gel was proportional to

the growth rate of the cells and that the proliferation reached a maximum on islands of $\sim 5000\mu\text{m}^2$. Linear islands were more effective than triangular or circular islands at inducing proliferation. These effects are induced by large areas, but undoubtedly are mirrored in cell adhesion to some phase separated polymer surfaces where domain size can reach μm size.

1.2.6 Surface topology

How does surface structure affect cell adhesion? This question can be explored from two viewpoints. Firstly, the limits determined by the cell cytoskeleton and secondly, the effects on adhesion protein adsorption. Topology is addressed further in chapter 5 which explores cell adhesion and growth on macroporous hydrogel membranes.

Structural organisation of tissues plays a major part in deciding the degree and direction of cell growth. Extracellular matrix in basement membranes serves as a guide for cells during growth and development⁽⁹⁵⁾ and the reorganisation of tissues after wound healing is dependent on cell response to aligned collagen⁽⁹⁶⁾. Abercrombie, in 1953, was the first to observe cytoskeletal constraints on the morphology of the cell in culture⁽⁹⁷⁾. Since then, many studies have found inhibition of cell movement across grossly roughened substrata and increases in cell adhesion to mildly roughened as compared to smooth substrata. Cells react differently to variations in surface rugosity; neutrophils are not inhibited by the same grade of roughness as BHK cells for example⁽⁹⁸⁾, and macrophages accumulate preferentially on roughened and hydrophobic surfaces in direct contrast to fibroblasts⁽⁹⁹⁾. Recent work on microelectronically fabricated surfaces⁽¹⁰⁰⁾ also revealed differences between fibroblast cell types in culture, with reference to the geometry of the adhesive pattern. More importantly, this work showed that cells were able to bridge limited nonadhesive zones to contact neighbouring cells and migrate. This ability was dramatically demonstrated by Rovinsky et al⁽⁵⁴⁾ who showed the connections between cells cultured on the tips of microspikes.

Whilst investigating osteoclasts, giant multinucleate bone resorbing cells, the author of this thesis noted that osteoclasts, but not other cells within a heterogeneous bone cell preparation, would adhere to rough substrates⁽¹⁰¹⁾. Osteoclasts and giant cells always stick to surfaces more quickly than fibroblastic cells. These differential adhesion effects may be due in some way to the size of the cell. More of the cell comes into contact with a surface more quickly. The larger cell can afford to span a gap as it only has to dedicate a relatively small part of its structure to the enterprise. Recent investigations into osteoclast behaviour show that increased rugosity of synthetic calcium hydroxyapatite surfaces (the calcium salt that constitutes bone mineral) leads to increase in fusion of osteoclast precursors⁽¹⁰²⁾.

In vivo, surface texture has pronounced effect on the reactive cell population around an implant. Roughened polymer surfaces may attract more macrophages and foreign body giant cells for a longer period of time leading to greater inflammation and subsequent fibrous encapsulation. However, movement of the implant also tends to lead to fibrosis, so that in some cases, the stability afforded by a roughened surface may reduce fibrous encapsulation.

Superior compatibility is often associated with smoother surfaces with no sharp edges (103, 104, 105). Materials may break down in the body and when particles reach a critical size they may be subject to an immune response. This is particularly well illustrated by the case of wear particles from carbon fibre materials used as ligament prostheses or cartilage support implants. Macrophages and other phagocytic cell types can ingest particles <20µm in diameter and transport them to sites remote from the implant e.g. the lymph nodes. This response has been noted *in vivo* and investigated *in vitro* (106).

Topology of a surface also effects the adsorption of proteins over time. This was well demonstrated by Vroman et al with blood proteins, but could equally occur with tissue fluids or cell culture medium. Generally the exchange of the first adsorbed protein (usually the one with the highest bulk concentration such as albumin) for another of higher affinity is slowed, but still occurs, in narrow spaces due to steric hindrance and limited diffusion. Fn, however, was shown to be exceptional (107). On flat surfaces, Fn is usually displaced and not adsorbed at significant concentrations under cell culture conditions or in whole serum. However, in narrow spaces, Fn deposits and is not displaced, despite high concentrations of competing proteins (Vn has not been investigated in this way). Could this effect explain the increased adhesiveness of slightly rough surfaces to many cells?

How can roughness be quantified? Fractal descriptors of surface structure (108) are used in metallurgy and particle science, and fractal analysis of cell morphology is recognised as a tool in cellular pathology (109, 110). Quantitative assessment of structure on stated scales is achieved, and this technology may be an ideal aid to assessing cell behaviour on rough surfaces. Refer to the appendix for an explanation of the techniques involved in fractal quantitation of cell shape and material surfaces.



Key points

- 1) There are several measurable physical and chemical parameters of a surface. No single parameter can be correlated with a cell adhesion response without reference to the others.
- 2) The adsorption of a conformationally active adhesion protein is the critical factor in determining whether a surface is cell adhesive. This may be overridden by direct surface-cell interactions where a surface is very positively charged.
- 3) High water content simple hydrogels may be nonadhesive to cells.
- 4) Charged groups such as NH_2 and COOH expressed at a material surface increase the adsorption of adhesion proteins and hence cell adhesion. Introduction of these groups into a polymer may decrease the nonadhesive nature of a high water content hydrogel.
- 5) The spatial distribution of surface chemistries influences protein adsorption and cell adhesive properties of the surface.
- 6) Surface topology affects cell adhesion from two standpoints; the limitations of the cell cytoskeleton and the adsorption of specific adhesion proteins.

1.3 The Medium

1.3.0 Introduction

The composition of the immediate environment in contact with the cell and the material surface is the third major factor determining adhesion.

In classical cell culture conditions, the medium is a salt solution with a serum supplement. *In vivo*, the environment could be blood, tissue fluids, saliva or tears. The following descriptions refer mainly to serum containing cell culture conditions but this is not necessarily an intelligent choice of medium for assessing cell response to a material. Tissue fluids adjacent to a subcutaneous implant for example, would have a very different composition from 10% serum. Very few publications address this anomaly, yet *in vivo* results are known to differ from *in vitro* results for many materials. What are the major adhesion promoting proteins in serum? How do they adsorb to a surface? What other factors modulate this process?

To answer these questions it is important to understand why any protein in solution adsorbs to a surface. All the direct physicochemical interactions between cells and surfaces mentioned in sections 1.18 also apply to proteins. Most globular proteins such as albumin and fibrinogen, have charged hydrophilic exteriors and hydrophobic interiors. Unfolding on a suitable surface allows further water exclusion from the hydrophobic domains, which lowers the Gibbs free energy of the system and favours adsorption. The words "suitable surface" are part of the key to the situation; some surfaces discourage any adsorption, for example, the polyethylene oxide modified surfaces discussed in chapter 3. Norde and Lyklema (111) consider the thermodynamics of protein adsorption and summarise that proteins in general are more likely to adsorb to surfaces if: 1) the substrate is more hydrophobic, 2) the protein exterior is more hydrophobic, 3) the protein is not rigid, 4) the electrostatic potentials of the substrate and protein have opposite values, 5) very few ions are in the contact region and 6) the ions have a high valency and lower Gibbs energy of hydration.

In complex solutions, the adsorption of proteins depends on their bulk concentration, their affinity for the surface and conditions such as pH and temperature. Vroman's studies with blood proteins led to a greatly increased understanding of these processes (112). The protein with the highest bulk concentration tends to adsorb first, and is displaced over time by proteins of higher affinity. This process occurs over several minutes or hours until an equilibrium is reached. Fn is usually displaced by fibrinogen in normal serum, whereas vitronectin (Vn) seems to adsorb very highly relative to its bulk concentration on many surfaces (113, 114, 115).

Once adsorbed, the cell adhesive nature of the specific adhesion proteins is governed by their conformation and density relative to the nonadhesive adsorbed proteins. Coadsorption with albumin may increase the proportion of adhesion protein adsorbed in active

conformation. Hydrophobic surfaces tend to cause massive conformational changes i.e. denature proteins. This can expose binding sites on adhesion proteins, but nonadhesive hydrophobic core proteins such as albumin and globulins tend to dominate hydrophobic surfaces. Relatively hydrophilic surfaces tend to be cell adhesive. Globular proteins adhere less strongly to hydrophilic surfaces and offer less competition to the adhesion proteins which then occupy a higher proportion of the surface. Very hydrophilic surfaces, such as polyethylene oxide modified surfaces, do not support adsorption of proteins of any kind (although smaller proteins may diffuse into the matrix of hydrophilic gels). In the case of polyethylene oxide modified surfaces, adsorption is inhibited by steric occlusion effects of the long PEO chains (see chapter 3).

1.3.1 Adhesion Proteins

Essentially, any protein that the cell has a receptor for and adsorbs to surfaces is an adhesion protein. The serum glycoproteins Fibronectin (Fn) and Vitronectin (Vn) are perhaps the best recognised as adhesion proteins in culture systems but this group also includes proteins like von Willebrand factor, fibrinogen, thrombospondin, cytotactin (tenascin), osteonectin (SPARC protein) and collagen (note that many of these are components of the basement membrane). Adhesion proteins have domains that bind to cells and also domains that bind to other proteins. The cell adhesive sequences of many adhesion proteins centre around a short peptide sequence such the classical Fn sequence 'RGDS'. Small variations in this sequence, or its position in the protein alter its integrin specificity and the binding strength. Tenascin, osteonectin and thrombospondin came to be named 'antiadhesive proteins'⁽¹¹⁶⁾ yet their cell adhesiveness is actually variable. The adhesive nature is governed by other conditions; either the presence of other cooperative proteins, conformational state or perhaps cation binding. This is well illustrated by the J1 group of tenascin related neuronal adhesion proteins in which zinc and calcium binding turn on and off the adhesive qualities of the protein ⁽¹¹⁷⁾. Thrombospondin is more active if calcium is chelated, or the protein is reduced with Clelands reagent ⁽¹¹⁸⁾.

How do cell adhesion sequences on adhesion proteins interact with cell surface integrins? Figure 4 shows a selection of binding interactions between cell receptors and adhesion protein sequences. This table is not comprehensive as other binding sequences exist in these proteins, and the sequences that bind to other proteins are not listed. It is important to remember that glycosylation of adhesion proteins plays a major part in their affinity ⁽⁴⁴⁾. These sequences are present on relatively mobile parts of the adhesion molecule and current theory proposes that the aspartate (D) residues serve to complete the co-ordination of divalent cations together with the cell surface integrin ^(41,119). Studies of calcium binding proteins have led to consensus sequences for the metal binding area, known as EF hand. Integrins α subunits have very high sequence homology with this sequence. The EF hand motif has 13

Adhesion Protein	Recognised sequences	Receptor examples	Possible cell types
Fibronectin	RGDS	GPIIbIIIa $\alpha 5\beta 1$	Platelets fibroblasts
	LDV(CSI sequence)	$\alpha 4\beta 1$	lymphocytes
	REDV type III repeat, synergistic site	$\alpha 4\beta 1$	melanoma most cells
Vitronectin	RGDV	$\alpha v\beta 5$	most cells
Laminin	RGDN YIGSR LRE PDSGR IKVAV	$\alpha 3\beta 1, \alpha 2\beta 1, \alpha 6\beta 1$	epithelial cells
Collagen I	RGDTP DGEA	$\alpha 2\beta 1$	fibroblasts platelets
Collagen IV	IV-HI seq	$\alpha 2\beta 1$	melanoma
Thrombospondin	RGDA VTXG	$\alpha IIb\beta 3$ (GPIIbIIIa)	platelets
Von Willebrand Factor	RGDS	$\alpha IIb\beta 3$ (GPIIbIIIa)	platelets.
Fibrinogen	RGDS HHLGGAKQAGD V	$\alpha v\beta 3, \alpha m\beta 2$	platelets

Figure 4 Integrin receptors of various cell types and their recognition sequences

residues, but the integrins without exception, lack a critical coordinating residue at position 12. Instead of aspartate or glutamate that is usually seen in EF hand, they have a hydrophobic residue. The partial coordination of cations by the integrin is completed by the interaction of specific D residues presented by the corresponding adhesion protein.

An excess of small peptides containing the RGD motif or similar sequences introduced into the culture medium, can prevent cell binding to immobilised adhesion peptides (120,121) or interfere with *in vivo* embryonic cell translocation processes (17). Because of this, targeted peptide therapy holds potential for therapeutic treatment of metastasis and possibly for immune therapies (viruses such as HIV enter cells via integrin binding as they have RGD sequences in their coat proteins).

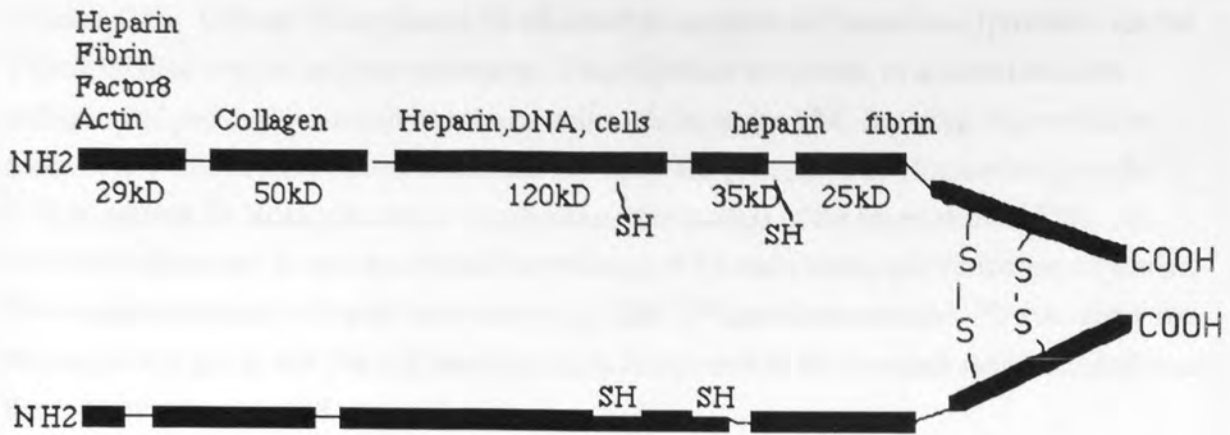
Inclusion of adhesive peptide sequences has already found biomaterials application. This is very well illustrated the recent work by Hubbell et al (122). Incorporation of a short peptide REDV into a polyethylene oxide modified surface resulted in a biomaterial that was highly specific for endothelial cells. Using this technique, they hoped to produce a non thrombogenic surface by supporting only endothelial growth.

1.3.2 Fibronectin and vitronectin

These two well conserved adhesion proteins have received most attention as they constitute the major adhesive proteins adsorbed from serum (see figure 5 overleaf). Definite correlations have been observed between the amount of Fn adsorbed and cell spreading by many researchers (123) yet Fn adsorption is strongly inhibited by other serum proteins where the serum concentration is above 0.1% (113,114,115,124). There is now a strong consensus that Vn is the major adhesive protein for most cells under standard culture conditions. Fn is adhesive to cells when coated as a single protein and induces more cell spreading than Vn.

Fn is a 500kD dimer glycoprotein (see diagram), first discovered in 1972 (125). It has since been shown to be a highly versatile and important protein. It has a role in the development of the embryo, in basement membranes, in wound healing and in the adhesion of cells to surfaces. It is a relatively minor component of the normal basement membrane but is especially abundant in extracellular matrix during wound healing, for example in the eye. A thin Fn and fibrinogen film forms across the corneal wound and the epithelial cells migrate onto the area. This response is speeded by the addition of extra Fn and this is now used clinically as treatment for corneal ulcer repairs. The presence of Fn is crucial to the contraction of wounds (126) as fibroblasts draw together the collagen fibrils using Fn as an anchorage point.

Several forms of Fn exist *in vivo* as a result of differential splicing of mRNA. Cold insoluble globulin (plasma Fn) is a slightly smaller protein than cellular Fn as it lacks the type III homology repeats that have since proved to be synergistic in adhesion effects. Like many proteins, plasma Fn undergoes a conformational change upon adsorption that yields it adhesive to the cell. Adsorbed Fn has 50 times the cell binding affinity of plasma Fn in



The diagram above shows the commonly accepted primary structure for the 440kD fibronectin dimer, joined by disulphide bonds at the carboxy termini of the monomers. The primary structure consists of three types of short homologous repeat sequences. There are three regions of alternative splicing (EDI, EDII, IIICS) which result in 5 different isoforms in humans. Two major forms are the soluble dimeric fibronectin in plasma which is produced by liver and endothelial cells, and the multimeric cell surface generated fibronectin. The diagram below shows the Homandberg model for the tertiary folding pattern of the dimer. The cell binding region is tucked into a cleft at the top of the dimer. Adsorption of the dimer to a surface may expose this sequence so that coordination of a cation in the integrin binding site can be completed.



The diagram below shows the structure of vitronectin. Although smaller than Fn, it is similarly structurally arranged into distinct binding regions for cells and a variety of other proteins.

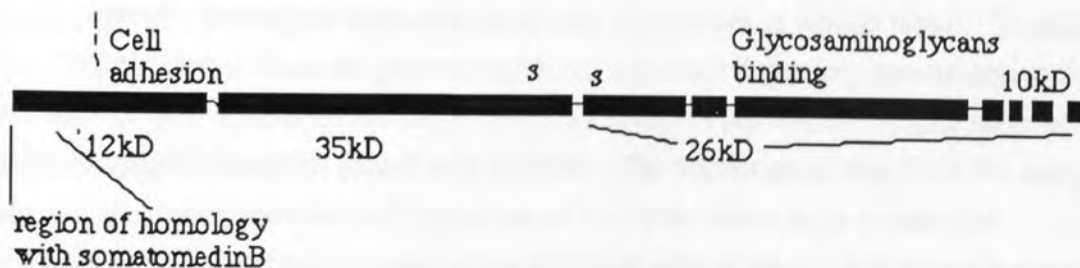


Figure 5. The structure of fibronectin and vitronectin

solution (127). Cellular Fn or plasma Fn adsorbed to surfaces self associates (probably via the 160kD binding region) to form multimers. These fibrillar structures, in association with collagen and proteoglycans can be seen on cell surfaces under EM. Staining of proteins on surfaces also shows the occasional fibrillar nature of this protein. The Homandberg model (128) of plasma Fn tertiary structure was derived from studies of the interactions of the individual domains. It explains the self association of Fn and subsequent formation of fibrils. The model correlates well with later studies by ESR (129) and fluorescence (130) that show the free sulphdryl group near the cell binding region is enclosed in the compact structure of plasma Fn, but becomes exposed upon adhesion.

Fn has binding regions for other proteins and more than one site for cell binding. The LDV sequence is part of the CS1 region of the molecule and provides a binding site for immune cells via the $\alpha 4$ group of integrins (131). There is also new evidence for a cell binding site in the heparin binding region of the molecule (132).

Vitronectin (Vn), (also called S protein and epibolin) is a serum glycoprotein involved in several physiological processes. The function of Vn in these processes is dependent on binding to various matrix components (133), to heparin (134), to C5b7-9 complex of the complement system (135), to cells (136), to thrombin-antithrombin (TAT) complexes (137) and to plasminogen and plasminogen activator inhibitor (PAI) (138, 139). Vn is commonly isolated from humans as two major forms: 75 and 65 kD monomers. Its activity is remarkably resistant to heat or chemical denaturation (140).

Vn has a region of homology with somatomedin B, a hormone of undefined physiological role, close to the RGD cell binding region. PAI binds to Vn via the somatomedin B region, and is stabilised in its active form in the extracellular matrix in this manner. This prevents plasminogen activator from catalysing the production of plasmin from plasminogen, which would result in digestion of extracellular matrix components. Vn also binds directly to plasminogen. Cleavage of Vn by plasmin doubles the binding capacity of Vn for plasminogen but eliminates PAI binding, resulting in upregulation of plasmin formation locally, thereby forming an important regulatory mechanism in wound repair. In addition to this, TAT binding to Vn in the plasma may be an important regulatory mechanism in the clearance of TAT from the circulation. The TAT complex represents the final stage of the clotting cascade, thrombin, bound to its inhibitor. The formation of this TAT-Vn complex exposes the cryptic heparin binding domain of Vn. This seems to be a route for immobilisation of Vn from the plasma into matrix at sites of injury. The plasmin upregulation system described above is then activated in order to clear the clot and remodel the injury site. The immobilised Vn also serves to bind complement and immune cells to the site of injury. In this way Vn plays a pivotal role in wound healing.

Vn is adhesive to cells via an RGD sequence at approximately one tenth of the concentration of Fn (140) and adheres to most materials without displacement from even high levels of competing proteins. It is highly pertinent to ask what effects Vn immobilising materials have on the regulatory systems described above. If Vn adsorbs in association with

other proteins, does this obscure its detection by immunolabelling methods? Does the conformation of material adsorbed Vn alter its susceptibility to plasmin cleavage? This situation is discussed further in Chapter 3.

Variable methods of isolation have led to different Vn isoforms being used in cell adhesion studies. The cryptic heparin binding region towards the COOH terminus can be exposed by urea treatment allowing simple isolation on a heparin column (140). Other isolation methods have produced a non-heparin binding form of Vn that was heat labile with respect to cell binding properties (141). Further research into Vn may yet resolve some of the present anomalies in the literature, and lead to development of Vn resistant materials.

1.3.3 Conformation of adsorbed proteins

It has been emphasised throughout that the amount and conformation of adhesion proteins adsorbed to a surface determines the degree of cell adhesion.

All proteins suffer conformational changes at interfaces. Proteins adsorb and desorb over time, the exact sequence of these events being determined by the type of surface, the concentration and type of proteins present. Very hydrophobic surfaces such as polystyrene tend to be dominated by globular proteins with hydrophobic cores such as albumin. This effect is sometimes referred to as the water exclusion effect, as the protein unfolds its hydrophobic heart against the hydrophobic material surface, water is excluded from the protein structure, and this results in a thermodynamically favourable state. Different serum proteins adsorbing and exchanging on a surface over time, as described by Vroman, is characterised by a peak in fibrinogen concentration at the surface, which is then displaced by activated high molecular weight kininogen. Fibrinogen usually displaces albumins from the surface, but on some very hydrophobic surfaces this effect is missing, as albumin is not easily displaced due to the large conformational changes in its tertiary structure. Where fibrinogen has adsorbed to more hydrophobic surfaces it also undergoes larger conformational changes (89,90).

Adsorbed Fn is commonly displaced by fibrinogen in serum and is rarely significantly adsorbed on surfaces where serum concentrations exceed 0.1% (113,114, 115). Vn, however, adsorbs at high levels relative to its bulk concentration on most surfaces, is not displaced by fibrinogen, and is established as the protein that governs initial adhesion to surfaces in serum containing conditions (114,115). However, it has been demonstrated that on surfaces bearing NH₂ species, rather than OH groups, Fn may be cooperative with Vn in catalysing endothelial cell adhesion (67).

The evidence for differences in conformation of adsorbed adhesion proteins was first observed by Grinnel and Feld (142) investigating the non-cell adhesive polystyrene (PS) and cell adhesive tissue culture plastic (TCP). Monoclonal antibody labelling of Fn adsorbed to PS and TCP showed that although more Fn adsorbed to PS than to TCP at low Fn plasma concentrations, less could be labelled with the antibody. If albumin was included, the

labelling of PS adsorbed Fn was increased, showing that conformation was altered by the presence of albumin. More recent evidence for the role of conformation in adhesive properties of adsorbed adhesion proteins is presented by Underwood et al (143). In these investigations, monoclonal antibodies to Vn showed few differences in conformation of adsorbed Vn, but large differences in adsorbed Fn, on polystyrene and oxidised polystyrene surfaces. It was also found that cation binding to Vn altered its conformation resulting in reduced cell adhesion. The availability of the critical binding sequences in the adhesion protein to the cell surface integrins obviously alters according to adsorbed conformation. This is the way in which surfaces such as polystyrene apparently exert 'nonadhesive' effects even in the presence of adsorbed adhesion proteins. Nonadsorbed adhesion proteins generally have a much lesser binding capacity for integrins (127), but this does not apply to disintegrins. Conformational presentation of integrin binding sequences in snake venom disintegrins results in very high binding affinity between integrin and disintegrin. Snake venoms are thus very effective anticlotting agents, and besides being very effective weapons for irritated snakes, have enormous potential for therapeutic use (144). Disintegrins are further discussed in section 1.34.

Techniques other than immunolabelling for examining protein conformation include Fourier Transform Infrared Spectroscopy (FTIR). Fn secondary structure has been examined in solution (145), and when adsorbed to surfaces (146). It has a beta sheet structure which is diminished on adsorption. Other adsorbed proteins have been observed by FTIR, showing a loss of β sheet structure (147). Vn was also demonstrated to have altered conformation upon surface adsorption (148). However, FTIR on surface adsorbed proteins is subject to many sources of error. The signal to noise ratio is often high, and subtraction of the spectral contributions of water and the base polymer is highly subjective. Tiny amounts of adhesion proteins are adsorbed and signals are very weak. Attempts to identify surface adsorbed Fn to hydrogels in this laboratory were not successful.

1.3.4 Disintegrins; lessons to be learnt about the RGD sequence

The use of RGD related sequences seems to be universally exploited in nature. Disintegrins are a group of short (47-84 amino acids) soluble, snake venom peptides that inhibit platelet aggregation (144). Like the proteolytic fragments of Fn (120), they bind to the adhesive integrins of platelets preventing their adhesion to matrix protein scaffolds. They contain the RGD cell attachment sequence but are incredibly 500 to 1000 times more effective than the simple tripeptide at inhibiting platelet association. The inhibitory properties of integrins have already been explored in a biomaterials context; disintegrins were shown to inhibit platelet adhesion to extracorporeal circuits (149). The great affinity of disintegrins for integrin receptors seems to be due to conformational presentation of the peptide sequence. Cyclisation of RGD peptides is known to enhance affinity (150), and on the side of the integrin, conformation of the binding sequence in the EF hand is known to bestow specificity and regulation of affinity. The disintegrin loop in venoms seems to present RGD to the

integrin in a similar manner to cyclic peptides (151). The design of highly specific integrin antagonists is now being pursued by studying the optimisation of cyclic ring size and adjacent hydrophobic residues with reference to the snake venom barbourin (152,153). Venoms are mostly nonspecific for platelet integrins (except for barbourin (154)) and can block the adhesive functions of all integrin receptors. For example, echistatin and eristocophin (containing the sequences RGDD and RGDW) have been shown to inhibit M21 melanoma cells adhering to Vn surfaces at nanomolar concentrations, but barbourin (KGDW) does not (154). Tergeminin, barbourin and eristocophin all bind to the Fn receptor but echistatin does not. It is interesting to note that such small changes in structure have such effects on function. There is much to be learned from examining the structure and function of these molecules, which are now being considered for their therapeutic potential in the treatment of metastasis.

Finally, a new family of membrane anchored disintegrins have been discovered, which presumably bind to integrins (155). This may begin to explain some previous anomalies in cell-cell adhesion experiments previously attributed to cell surface associated adhesion proteins.

1.3.5 Complement

Complement is an integrated system of about 20 known proteins found in the blood and tears, which together with immunoglobulins, constitutes part of the immune system (79,80). After "recognition" of a foreign body by the complement system (the binding of a specific complement protein), there follows a sequence of protein activating events that leads to a terminal cell lysis complex. This can be reached by two routes known as the classical pathway or the alternative pathway. Neutrophils and monocytes are attracted by products of the activation which can then result in inflammation and tissue damage. Vn (commonly known as S protein in this field of the literature) inhibits complement mediated cytolysis by binding to the C5b7-9 complex and preventing the final polymerisation that would result in a lysis complex. Complement compatibility of a material relies on its resistance to adsorbing or catalysing the production of certain complement proteins such as C3b or C5a. However, very many materials seem to activate the system, for example, polystyrene, cellulose, latex, polypropylene. Even if adsorbed 'Vn - complement' complexes prevent terminal cell lysis, they still provide cell attachment for leukocytes attracted by prior activation of the pathway (135). Recent studies have shown that complement activation occurs on surfaces bearing solely OH or SO₃ groups but is reduced on surfaces carrying equal quantities of these groups (81). There is also evidence to suggest that hydrophobic surfaces bind less complement components than hydrophilic surfaces and binding of C3 to a hydrophobic surface also increases its binding capacity for an inhibitory factor H (88). This implies that hydrophobic surfaces are less likely to activate complement, yet polystyrene is a very potent activator *in vivo*.

1.1 Summary of Introduction

The introduction of the chapter is divided into two parts. The first part discusses the general concept of cell adhesion and its importance in various biological processes. The second part discusses the specific mechanisms of cell adhesion, including the role of cell surface receptors and the influence of various factors such as pH and ionic strength.

The chapter also discusses the role of cell adhesion in various biological processes, such as cell migration, cell division, and cell death. The chapter concludes with a summary of the key points discussed in the introduction.



Key points

- 1) The composition of the immediate environment of the polymer surface governs a process known as "interface conversion" which involves the adsorption of various proteins and other components onto the surface.
- 2) Cells adsorbed from protein free media onto a surface are subject only to physicochemical interactions, not specific adhesive interactions.
- 3) In serum containing media or tissue fluids, proteins adsorb from the medium onto surfaces. Specific adsorption of adhesion proteins in appropriate conformations leads to cell adhesion.
- 4) Adhesion proteins contain specific peptide sequences that interact with cellular integrins.
- 5) Serum proteins such as albumins and macroglobulins compete with integrins during adsorption and hence inhibit cell adhesion.
- 6) Complement activation by materials may be moderated by Vn.

1.4 Summary of Introduction

In this introduction, the complex system in which a cell comes into contact with a polymer surface was explored, possibly in the presence of complex mixtures of proteins, lipids and variations in conditions of temperature, pH and ionic strength. What were the major points?

Firstly, that cells all carry adhesion receptors for specific proteins that may be adsorbed to a surface. The expression of these cellular receptors depends upon the cell type. Intracellular controls regulate the level of expression and subsequent adhesion behaviour.

Secondly, the composition of the medium surrounding the cells whether in culture or *in vivo*, affects adhesion. Specific adhesion proteins such as Fn and Vn must adsorb in sufficient quantities and active conformations for integrin based cell adhesion to occur. Other serum proteins such as albumins compete with adhesion proteins and thereby inhibit cell adhesion. Divalent cations are essential and regulatory.

Thirdly, the physical and chemical properties of a polymer surface affect the amount and type of proteins adsorbed onto it from protein containing solutions. What is the ideal adhesive surface? A surface that is moderately hydrophilic, moderately wettable, with moderately high surface energy and with a moderate concentration of appropriate chemical groups such as amino, hydroxyl or carbonyl groups. Very high or low values for the surface properties discussed usually lead to non cell adhesive surfaces. High water content simple polymers are often non adhesive in the presence of serum proteins. Topology can be manipulated to control cell spreading and adhesion.

This literature review has sought to give a broad overview of the mechanisms involved in cell adhesion to a surface. There are still many gaps in the general understanding of these processes which are being filled in, even as this thesis is written. Some areas of the literature may seem confused or unnecessarily reductionist. The rapid development of understanding in the study of integrin-adhesion protein binding is ready to be exploited for biomaterials applications, which should lead to exciting developments in the next decade.

Much of the understanding of cell adhesion mechanisms is based on cells in culture systems where the substrate is oxidised polystyrene; an abnormal state for the cells, where spreading and phenotype may be altered. It is important to remember that there is a limited ability to mimic the *in vivo* system using classical culture conditions.

The experimental part of this thesis is concerned with the application of cell culture techniques to various materials. Interpretation of the results is based on the model of cell adhesion that was presented in the introduction and literature review. Initially, surfaces are simply assessed for their cell adhesion properties with fibroblasts. Blocking antibodies and competing peptides are introduced into experimental procedures in order to assess the mechanisms of adhesion, and techniques are developed to investigate a variety of surfaces including worn contact lenses.

In the course of this research, in addition to establishing cell adhesion as an assay for vitronectin adsorption, extended drying procedures have been developed to prepare samples of high water content polymers suitable for SEM, and a rapid live staining technique has been developed for investigating cell adhesion to opaque polymers by light microscopy. An established assay for cell quantitation has been modified in order to investigate the effects of divalent cations on cell adhesion to serum coated tissue culture plastic. This assay has further potential for development into a screening procedure for other polymers.

Briefly, in order to encompass the concepts of cell adhesion to surfaces discussed in this introduction, the experimental part of this thesis falls into three major sections.

In chapter 3, cell adhesion to a variety of surfaces is explored, demonstrating the use of cell adhesion in the exploration of a surface. Cell adhesion can visually amplify very small quantities of adhesion proteins adsorbed from serum or tears that would be undetectable by other methods.

Secondly, in chapter 4, the cationic conditions that regulate adhesion are investigated to demonstrate the techniques and logic necessary to examine the adhesion process on a molecular level.

Thirdly, in chapter 5, manipulation of cell response to topology is used as an aid to designing a hydrogel based biomaterial. Porous hydrogel matrices also show interesting possibilities as cell growth substrates for other purposes.

Chapter 2

Materials and Methods

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2.0 Introduction

Experimental techniques to investigate cell surface interactions must be carefully considered and carried out in a highly consistent manner. Cell culture stocks should be passaged regularly and not used at high passage numbers. The fibroblasts and monocyte lines used in this research were not used for assays after confluence. They were used in log phase growth from undepleted medium to ensure maximum cell viability. Cells can be unattached in a number of ways, but in this work all cells were released in a trypsin (0.5g/l)/ EDTA (0.2g/l)/ buffered saline solution, at room temperature for 5 minutes. It was considered important to attempt to standardise the trypsinisation time, as cell surface proteins are degraded during this process which could lead to very different adhesion results. The experiments described in this thesis do not address the strength of adhesion of cells to materials, which constitutes another large field of study.

2.1.0 Culture of cell lines

3T3 swiss mouse fibroblasts were first cultured by Todaro and Green (156). They constitute a well characterised fibroblast line used extensively in toxicological assays and general research. L929 cells are also a well established adhesion dependent mouse fibroblast line.

J744.2 cells are a mouse monocyte line (157). They are marginally adhesion dependent, although a good many cells will dislodge if the flasks are knocked. They produce Interleukin 1 (IL 1), lymphocyte activating factor, and like all immune derived cells, carry Fc receptors, in this case specific for IgG2a and IgG2b.

3T3 swiss mouse fibroblasts and L929 mouse fibroblasts were obtained from ICN Flow (Paisley, Scotland). J744.2 cells were obtained from the ECACC. They were all routinely cultured in 75 cm² flasks, at 37°C in an atmosphere containing 5% CO₂, and passaged at confluence at the recommended split ratios. The nutrient media for 3T3, L929 and J744.2 cells was Minimal Essential Media with Earles salts with L glutamine (ICN flow), 10% foetal calf serum (Advanced Protein Products) and 100 I.U. penicillin and 100µg streptomycin (ICN Flow) per litre.

2.1.1 Isolation and culture of chondrocytes

Articular cartilage was removed aseptically from a recently killed rabbit and cut into small pieces (1mm³) in phosphate buffered saline (PBS). The cartilage was sequentially digested in 1% pronase (Sigma) for 1 hour at 37°C and then in 300 U/ml collagenase (Sigma) for 1-3 hours to release the cells. Both enzymes were diluted in Hams F12 supplemented with 10% foetal calf serum, 1% HEPES and antibiotics as described in section

2.10. The cells were rinsed thoroughly and resuspended in serum containing medium before plating in 75 cm² flasks as above. Nutrient media was Ham's F12 medium supplemented with 10% foetal calf serum, 50µg/ml ascorbic acid, and antibiotics as described in 2.10 (ICN Flow). The viability of the cells was checked every day using aliquots of still suspended cells and trypan blue. The chondrocytes took several days to settle onto the tissue culture plastic and sometimes needed rerinsing to encourage adherence. The cells were cultured at 37°C in an atmosphere containing 5% CO₂ and passaged in the normal way using trypsin/EDTA after the cells had reached confluence. The cells were not used beyond passage 6 to ensure their ability to return to phenotype was not compromised. Cells were frozen down after the first passage and brought up as required.

Pig chondrocytes isolated and cultured as described above, were generously (but not personally) donated by Patrick Lawlor at Department of Anatomy, UWCC in Cardiff.

2.1.2 Preparation of materials for cell adhesion experiments

Polymers were prepared by solution polymerisation. Polymer gels were prepared by polymerising the monomers *in situ* in a mould consisting of two clamped glass plates, covered with Melinex® and separated by 0.25mm polyethylene gaskets. The monomer mixtures were prepared with an appropriate cross linking agent and a free radical initiator (AZBN 0.5wt%) and degassed prior to injection into the mould. Polymerisation was carried out at 60°C for 72 hours followed by 3 hours postcuring at 90°C.

Spincoated polymers were prepared from the polymers described in the text, onto precleaned 13 mm glass coverslips. The coverslips were cleaned by rinsing in ethanol, drying in a dust free environment prior to air plasma treatment for 15 minutes in a Nanotech® plasma unit. All spincoated polyethylene oxide (PEO) containing polymers were prepared from a 38g/100ml solution containing two crosslinking agents; 23% (w/w) glycidyl methacrylate and 2% (w/w) uranyl nitrate. The solvent mixture was 80% methoxyethanol and 20% ethanol. The films were crosslinked by UV irradiation for one hour and baking at 60°C for 6 hours. Spincoated polymers with variable sequence distribution were prepared as above except for the use of 1% N-vinyl carbazole as a film stabilising agent in place of the crosslinkers.

The preparation of porous membrane samples and synthetic cartilage samples is described in section 2.1.3

Substrates from chapter 3 and 5 were all prepared as 13 mm discs and extensively rinsed in buffered saline prior to assay (either cut from a cast gel or spun onto 13 mm coverslips, as described in the text). Synthetic cartilage samples were deemed sterile after autoclaving in dilute HCl and soaked for several days in PBS before use.

Equilibrium water content (EWC) of the hydrogels was calculated by the following equation: $EWC = (\text{wet weight} - \text{dry weight} / \text{wet weight}) \times 100$.

In chapter 3, section 3.5, contact lenses were examined using cell adhesion assays. Contact lenses with two different formulations (G, a polyHEMA lens, and AD, a PEO

substituted lens) were spoiled with 1 ml fibronectin (Gibco) at 20 µg/ml before use in the cell adhesion assay.

Fresh contact lenses worn daily for one week (Acuvue® disposable) were donated by Mr Lyndon Jones from a number of patients in his practice. The lenses were refrigerated and used within one day of removal from the eye in order to avoid bacterial spoilage. Complete lenses were rinsed in HEPES buffered saline before cell adhesion experiments. Preincubation with antibodies to adhesion proteins was carried out for 1 hour at 37°C followed by rinsing for 5 minutes in HBS. Some contact lenses were cut into 4 pieces after preparation as described above.

2.1.3 Preparation of porous membranes

Simple HEMA:EGDM copolymers were polymerised around a crystalline matrix in order to produce porous membranes (the structures of these monomers can be found on pages 59-61). Briefly, the membranes were cast against Melinex® in moulds as described above, from a well dispersed mixture of HEMA : EGDM 90:10 and varying amounts of aqueous phase solvents (water and variable amounts of ethylene glycol), as indicated in the results section. The moulds were either placed on Cardice (slow freeze) or immersed in an acetone Cardice slurry (fast freeze) to form the crystalline matrix in the aqueous phase. The membranes were then UV irradiated for several hours to polymerise the monomers to form the HEMA : EGDM polymer. Membrane samples were hydrated and soaked for several days in repeated changes of water before cell culture experiments. Assay samples were prepared as 13 mm discs.

Studies of cartilage, during the development of suitable materials for synthetic cartilage, indicated that porosity may make materials more compatible with surrounding healthy tissues of the proposed implant. Interpenetrating network polymers (IPNs) were developed with the appropriate mechanical properties, and attempts were made to produce freeze-thaw IPNs in the manner described above. However, these experiments were unsuccessful as the IPN monomer mixture either underwent phase separation and/or precipitated prior to polymerisation around the crystalline aqueous phase. Therefore, dextrin particle pore formers were used to introduce porosity into the IPN polymers. The filler polymer was dissolved in a solution of monomers EGDM 1% w/w and AZBN 0.5% w/w. 20% v/v sized dextrin particles were added and the mixture was poured into a Melinex® mould as described in section 2.12. The gel was cured for 3 days at 60°C and postcured for 2 hours at 90°C. Polymers were swollen in methanol and then water before autoclaving in dilute HCl at pH 1.8 to remove dextrin particles. The samples were then soaked in distilled water and phosphate buffered saline for several days before use. Sodium chloride and sucrose were also used as pore formers in later samples as described in the text.

2.1.4 Cell adhesion assays

Method A) trypsinisation

Marginally subconfluent 3T3 cells (to ensure maximum viability) were trypsinised for 5 minutes and rinsed in PBS. Cells were plated at $2.4 - 5.0 \times 10^5$ cell/ml in 1 ml onto 13mm discs of cast gels in 24 well plate, in Minimal Essential Media (plus antibiotics) with or without 10% FCS. The discs were incubated at 37°C and 5% CO₂ for 6 hours. The discs were rinsed in phosphate buffered saline to remove non adherent cells before trypsinisation for 10 minutes in 1 ml trypsin/EDTA. The adherent cells so released were counted in a conventional blood cell counting chamber and assessed for viability by trypan blue exclusion. In addition to this, tissue culture plates were checked for peripheral cell adhesion on the tissue culture plastic in order to assess any material toxicity.

Method B) adherent cell counts

Cells were plated at $2.4 - 5.0 \times 10^5$ cell/ml in 1 ml onto 13mm discs of gels or spincoated polymers on precleaned coverslips. The polymer discs were preincubated with FCS overnight at 4°C and rinsed thoroughly in PBS prior to use in the cell adhesion assay. After one hours incubation with the cells at 37°C in an atmosphere containing 5% CO₂, samples were rinsed in two changes of 1 ml PBS and fixed in 1% glutaraldehyde. Adherent cells were counted by eye. One sample was assessed for cell viability by trypan blue exclusion prior to fixing.

Method C) for chondrocytes

Rabbit or porcine chondrocytes were seeded onto 13mm discs of porous polymers at 5×10^5 cells per disc, and cultured for a minimum of two days. The primary derived rabbit or porcine chondrocytes were cultured in Hams F12+10% foetal calf serum, HEPES and ascorbate.

Method D) for contact lenses

Contact lenses were worn on a daily basis by patients for one week. The lenses were removed from the eye and used in the assay system after cleaning with conventional contact lens care regimes. The lenses were rinsed in saline before use in the adhesion assay. For the experiment described in figure 14, lenses obtained from 3 patients were cut into four pieces. For the experiment described in figure 15, whole lenses were obtained from 4 patients and randomised prior to the study.

Polyclonal antibodies to vitronectin and fibronectin can successfully block adhesion of 3T3 cells to these adhesion proteins at x10 dilution. This is demonstrated in figures 17A and B. Antifibronectin antibodies did not block cell adhesion to vitronectin, so it is unlikely that nonspecific blocking effects occur with these antibodies on tissue culture plastic surfaces. However, hydrogel surfaces may differ in this respect. In order to assess the possibility of

nonspecific blocking effects due to IgG antibodies, control samples were treated with nonspecific 300µg/ml IgG. This correlated approximately with the blocking antibody titre.

The lenses were preincubated with antiadhesion molecule antibodies at x10 dilution, or control IgG (Cohn fraction V) at 300µg/ml, for 1 hour at 37°C, and rinsed in HEPES buffered saline (HBS) as described in the text. 3T3 cells were trypsinised prior to confluence. 5x10⁴ cells in 1 ml HBS containing 5mM magnesium were seeded onto either whole or quartered contact lenses. The lenses, or lens pieces were incubated with the cells for 1 hour prior to rinsing in two changes of 1 ml HBS. The lenses were fixed in 1% glutaraldehyde. Adherent cells were counted by eye. One sample was assessed for cell viability by trypan blue exclusion prior to fixation.

2.15 Adhesion assay for 96 well plates (for divalent cation investigations)

A) Basic assay system (SRB assay)

This method is a slightly modified version of the National Cancer Institute (NCI) assay developed for toxicological applications (158). Sulphorhodamine β is a highly sensitive protein dye. Use of this dye facilitates quantification of small amounts of cell adhesion. Staining gives a linear response even at supraconfluent levels. As this assay was done manually, plates could not be compared. It was impossible to ensure complete uniformity of washing and staining without automation in this assay system, so the staining intensity varied slightly between experiments performed on different days. Therefore, experiments were planned so that most results were compared only within the plate, and in the case where it was necessary to use more than one plate, cells were plated from exactly the same stock culture. For all experiments, cells were at approximately the same stage of growth (log phase) and plated at approximately the same dilution.

96 well tissue culture treated plates were coated with either whole dialysed serum or fibronectin (Fn) at 1µg or vitronectin (Vn) at 0.1µg per well overnight at 4°C and washed in HEPES buffered saline (HBS). The Fn plates were blocked with heat denatured bovine serum albumin 1mg/ml for one hour at 37°C, and washed in HBS.

3T3 cells and L929 cells were applied to the 96 well plate at 2x10⁴ cells per well in a total volume of 100µl of HBS containing the appropriate concentration of cation or combination of cations. Six replicates of each cation condition were used (J744.2 and rabbit or porcine chondrocytes, being slightly smaller were applied at 6x10⁴ cells per well). The plate was incubated at 37°C for one hour after which the supernatant was removed. The plate was washed with HBS and icecold 10% trichloroacetic acid was applied for 30 minutes. After washing with tap water, the cells were stained with 1% sulphorhodamine β in 1% acetic acid for 20 minutes at room temperature. After extensive washing in 1% acetic acid the dye was eluted from the cells with 100µl 50mM TRIS in phosphate buffered saline. The plates were read at 550 nm.

The figures in chapter 4 describe the results of experiments using this assay system. 'Adhesion' as the y axis of these graphs refers to the optical density of the eluted SRB read at 550nm. Each experiment is self consistent, but quantitative comparisons between experiments should not be made unless stated in the text.

B) Timed competition assay between manganese and zinc.

A competition assay in which Mn mediated adhesion was blocked by Zn was designed. Cells were plated as explained above, in the presence of increasing concentrations of Mn together with 3 different concentrations of Zn. Plates were incubated for either 5,10 or 15 minutes and stained as above. This timed assay yielded a set of curves. The initial slope of the curve where adhesion was not yet affected by occupied sites, corresponded to the initial rate of adhesion. These were plotted as a function of the concentration of Mn as [S] in a classical Lineweaver-Burke plot ($1/\text{rate} \text{ v } 1/[S]$). These plots for different inhibitor (Zn) concentrations give a series of lines fanning out from one point. In the case of direct competition, rather than uncompetitive inhibition or noncompetitive inhibition, the lines meet at or near the same ordinate intercept⁽¹⁵⁹⁾.

2.1.6 Scanning Electron Microscopy

Discs of membranes with or without adherent cells were fixed in 2% glutaraldehyde 0.2M sodium cacodylate for an hour and then dehydrated in graded alcohols before drying from Freon 22 via the critical point of CO₂. Where materials had a high water content the dehydration process was extended to maintain maximum tissue structure. After coating with gold, these samples were mounted and viewed by Cambridge stereoscan SEM. Samples were stored dehydrated.

2.1.7 Live staining of cells with fluorescent labels

Opaque materials with cells could be viewed conveniently and quickly by fluorescence microscopy. The samples were removed from the culture medium and placed in phosphate buffered saline (PBS) containing carboxy fluorescein diacetate (9.6 μ M, Sigma) which stains live cells (esterases release fluorescein within the cell) and ethidium bromide (25 μ M, Sigma) which binds to the DNA of dead cells and is also adsorbed to most hydrogels. When seen under mercury light, the live cells appear green and the structure of the hydrogel a dull red. This live cell stain tends to leach rapidly and results must be recorded quickly. Longer lasting alternatives such as lipid labelling with the cationic lipid probe diI[C18] (1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanin perchlorate) proved prohibitively expensive.

2.1.8 Immunostaining for chondrocyte matrix production

For artificial cartilage experiments, matrix constituents were identified by antibody staining. Thicker, mechanically stable pieces of material proved to be autofluorescent. Very low levels of fluorescence are detected in antibody staining and the background fluorescence overwhelmed any signals (live staining with fluorescein diacetate gives a very strong signal that makes the background fluorescence insignificant). For this reason, thin 30 μ M sections of the materials were cut using a cryostat. The samples were very flexible and cells tended to detach during processing however, cells that remained attached could clearly be seen to produce collagens.

Pig chondrocytes (isolated from the full depth of the cartilage) were plated onto cryostat cut sections of the hydrogel IPNs 'PC97' and 'PC123'. The samples were incubated for one week before fixation in freshly prepared 1% formaldehyde in saline. Samples were then treated in the following manner:

- a) Incubation for 15 minutes in either 5% pig serum (for blocking polyclonal) or rabbit serum (for monoclonals) in TBS (TRIS buffered saline, 0.1% TritonX100).
- b) Rinsed three times in TBS.
- c) Incubation for 45 minutes in undiluted antibody. These included SP1D8, a monoclonal antibody to procollagen I, CICII a monoclonal antibody to collagen type II or P2 a polyclonal antibody to pig type II collagen (1/10 in TBS) (monoclonal antibodies were obtained from the Developmental studies Hybridoma Bank, Dept. Pharmacology and Molecular sciences, John Hopkins University, Baltimore USA MD21205. Polyclonal antibodies were generously donated by Patrick Lawlor, Department of Anatomy, UWCC).
- d) Rinsed in TBS three times for 5 minutes.
- e) Incubation for 45 minutes with secondary antibody, FITC rabbit antimouse IgG.diluted 1/50 in TBS (DAKO).
- f) Rinsed in TBS three times for 5 minutes.
- g) Samples were mounted in glycerol containing antifadent 'DABCO' (SIGMA) before viewing.

Samples were viewed and photographed using a Zeiss photomicroscope III at the Department of Anatomy, UWCC, Wales.

Phalloidin staining of chondrocytes (to label actin cytoskeleton) on these IPN materials was attempted, but, not surprisingly, the phalloidin also bound strongly and nonspecifically to these materials, preventing visualisation of the cells.

2.1.9 Staining for light microscopy

Cells adhered to opaque surfaces were fixed and then stained with toluidine blue (0.5% in 1% sodium tetraborate) and rinsed in water before viewing wet. This technique allows cell counting without trypsinisation (useful for low cell counts) and without the need for live cell staining on marginally opaque hydrogels.

2.2.0 The use of antiadhesion peptides and antibodies

Polyclonal rabbit anti bovine and anti human vitronectin and fibronectin antibodies were obtained from TCS pharmaceuticals via Gibco. When used in assays, the antibodies were diluted in HBS and adsorbed for one hour at 37°C onto the appropriate surfaces. The surfaces were rinsed in HBS before plating cells onto the surface. The antiadhesion peptides GRGDS and GRGES were obtained from Calbiochem. They were used at 1.2 mg/ml in the plating medium for assay as described in the text. Fibronectin and vitronectin were obtained as purified proteins from GIBCO and adsorbed onto surfaces at concentrations stated in the text.

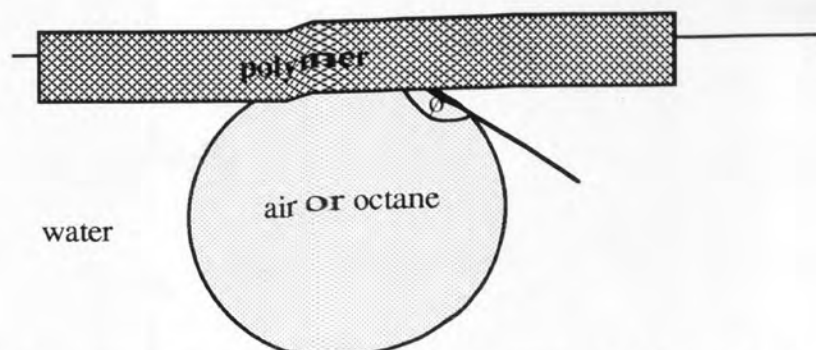
2.2.1 MTT and BCA assays

Cellular growth on porous membranes was assessed by the rate of mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product. 3T3 cells were plated onto 13 mm discs of the porous hydrogels at 2×10^5 cells per disc. 24 hours after plating, the adherent cells were incubated in the presence of MTT for 3 hours in PBS with glucose (phenol red containing medium would affect the final reading). The discs were thoroughly rinsed in PBS before the water insoluble formazan product was solubilised in propanol. The optical density of the formazan product at 550nm was taken as an index of growth rate (160). The results expressed in figure represent actual optical density.

Protein production was assessed by a modified biuret assay based on bicinchoninic acid (161). Two molecules of bicinchoninic acid react with one Cu^{1+} ion to form a water soluble product with maximum adsorption at 562nm. Cu^{1+} is formed by the classical Biuret reaction where protein reacts with Cu^{2+} in alkaline medium to give Cu^{1+} . The results in figure represent optical density of the product rather than mg/ml protein, as it is the relative protein production on the substrates rather than the absolute amounts of protein that are relevant to the study.

2.2.2 Contact angle analysis

This technique serves to evaluate the surface energy and wettability of materials. The underlying principle of this technique is that the extent of spreading of a liquid upon a surface is determined by the balance of forces at the interface between liquid, solid and air. The forces involved in determining the contact angle of a captive bubble are shown below



Owens and Wendt (87) described the balance of forces by the following equation:

$$\cos \theta \cdot \gamma_{lv} = \gamma_{sv} - \gamma_{sl} - \pi_e \quad (1)$$

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. θ is the angle of contact between a bubble and a planar solid surface. π_e is the equilibrium spreading pressure exerted by the surface on the liquid. When $\theta=0$, the liquid is considered to completely wet the solid; and when $\theta \neq 0$, the surface is less wettable.

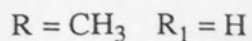
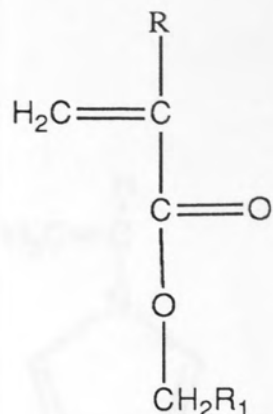
The wettability of a material is favoured by low interfacial free energy which is a result of a combination of high solid-surface free energy, and low liquid-surface free energy. Equation (1) can be further modified to calculate the value of θ as follows:

$$\cos \theta + 1 = 2/\gamma_{lv} \{ (\gamma_{dl} \gamma_{ds})^{1/2} + (\gamma_{pl} \gamma_{ps})^{1/2} \} \quad (2)$$

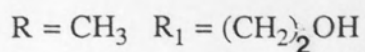
where: γ_{dl} is the dispersive component of the free energy of the liquid
 γ_{ds} is the dispersive component of the free energy of the solid
 γ_{pl} is the polar component of the free energy of the liquid
 γ_{ps} is the polar component of the free energy of the solid

Bubbles of air and octane were introduced onto the underside of hydrated polymers. Contact angles were measured using a calibrated eyepiece, and the polar and dispersive components were calculated from equation (2).

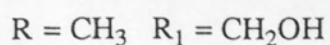
2.23 Structures of monomers



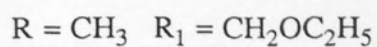
Methyl methacrylate



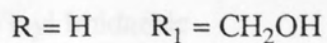
2-Hydroxypropyl methacrylate



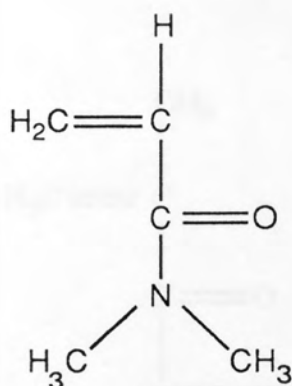
2-Hydroxyethyl methacrylate



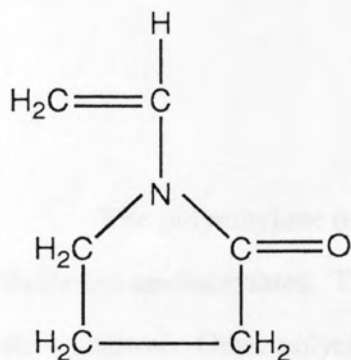
2-Ethoxyethyl methacrylate



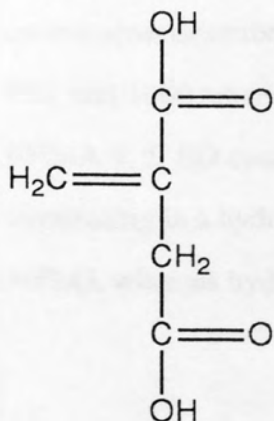
2-Hydroxyethyl acrylate



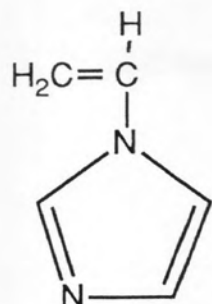
N, N-Dimethyl acrylamide



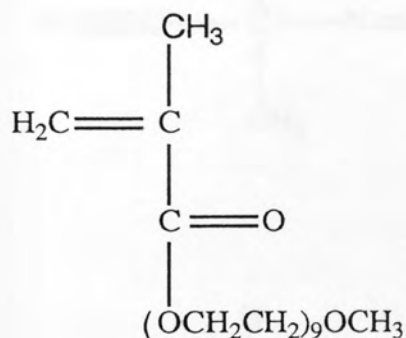
N-Vinyl pyrrolidone



Itaconic acid



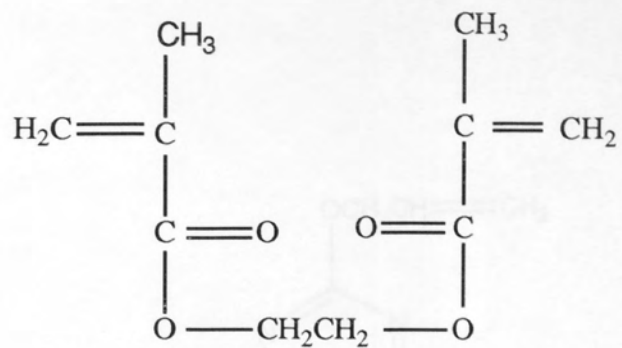
N-Vinyl Imidazole
(NVI)



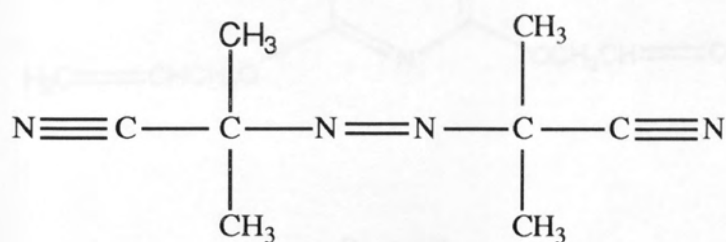
Methoxy Poly(ethylene glycol)₄₀₀
-mono methacrylate

MPEGMA

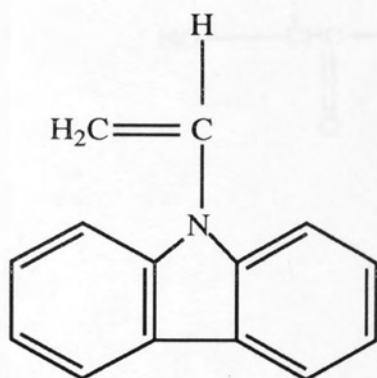
The polyethylene oxide containing monomers used in the experiments described in this thesis are methacrylates. The structure of a methoxy poly (ethylene glycol) methacrylate is shown above. Other polyethylene oxide containing methacrylates (PEGMAs) were used in the experiments described in chapter 3. These included PEGMAs with molecular weights of 200, 550, and 1000 where the PEO chain included respectively, 4, 12, or 22 OCH₂CH₂ units. HEMA 4, 5, EO contains 4 or 5 CH₂CH₂O units but lacks the methoxy endgroup, terminating in a hydroxyl group. Methoxy PEGMAs are referred to as MPEGMA or simply MPEG, whereas hydroxy PEGMAs are referred to as PEGOH throughout the text



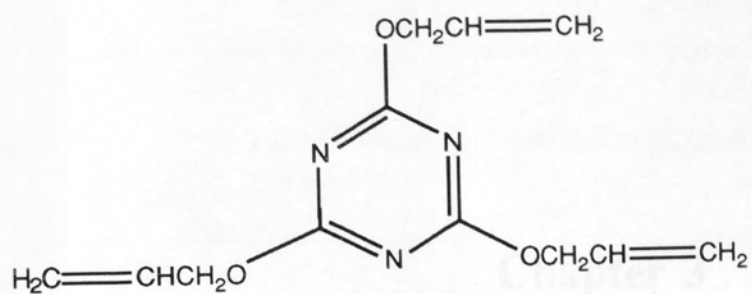
Ethylene glycol-
dimethacrylate
(EGDM)



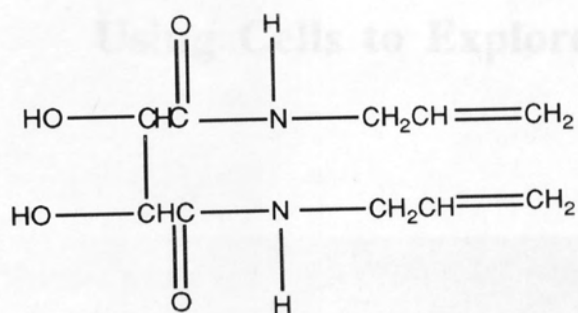
Azo-bis-isobutyronitrile
(AZBN)



N-Vinyl Carbazole
(NVC)



2,4,6-Triallyloxy-s-Triazine



N,N'-Diallyltartardiamide

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3.0 Introduction; cells as a probe

This chapter concerns the use of cell adhesion to 'probe' a surface. In the first part of this chapter, several different groups of materials are examined with reference to their cell adhesion properties, to demonstrate the use of cell adhesion as a means of visually amplifying adsorption of specific adhesion proteins from serum. In the second part of this chapter, cell adhesion to worn contact lenses in the presence of specific antibodies is examined, to determine which adhesion protein deposited on the lens is responsible for cell adhesion.

Protein based spoilation, of contact lenses in the eye for example, is normally considered to be dominated by the nonspecific adsorption of proteins. To what extent does the adsorption of specific adhesion proteins correlate with spoilation *in vivo* or in other biological situations? Firstly, the adsorption of adhesion proteins provides an indicator of the susceptibility of a material to general protein adsorption. Secondly, adhesion proteins have binding regions for other molecules and tend to adsorb strongly to surfaces. Hence irreversible adsorption of even a tiny quantity of an adhesion protein may form the base for further deposition of other serum or tear constituents. In this way, adhesion proteins may contribute to *in vivo* spoilation. Having considered both of these explanations, if adhesion proteins are present on a surface, how can they be identified? It can be very difficult to remove small amounts of proteins from hydrogels, and techniques such as electrophoresis require milligram quantities of proteins. Cell adhesion to materials in the presence of adsorbed proteins is directly related to the amount and biological activity of adsorbed specific adhesion proteins. The use of cell adhesion to visually amplify small amounts of protein which are not removable by other means is a large step in forecasting the likely fouling properties of materials at an early stage in their development. The cell adhesion assays described in this chapter do not relate to cell adhesion to lenses *in vivo*, but to the use of *in vitro* studies as diagnostic aids.

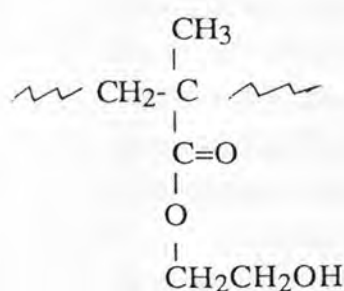
Adsorption of vitronectin (Vn) is the critical factor for the initial adhesion of many connective tissue cells (113,114,115) under serum containing tissue culture conditions. Fibronectin (Fn) from serum containing media does not generally adsorb when serum concentrations exceed 0.1%, although Fn may be cooperative in binding on some modified surfaces (66,67). In the following chapter, the adhesion of the well characterised 3T3 swiss mouse fibroblast is used to characterise a variety of surfaces. 3T3 cells have receptors for Vn, Fn and collagen. The adhesion properties of this cell type with respect to incubation time, the presence of serum and the nature of the substrate will be outlined in order to clarify these points.

The surfaces examined in this chapter are all hydrogel surfaces, where the polymer backbone has been substituted with a variety of pendant side groups. Various chemical functionalities, as outlined in the introduction, influence the water binding properties of the polymer. Furthermore, the expression of these groups at a polymer surface influences the adsorption of adhesion proteins and subsequent cell adhesion from serum containing medium.

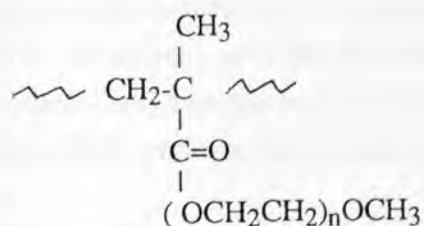
The water binding properties of various functional groups can also be seen in biological situations. For example, the cell glycocalyx, which consists of oligosaccharide chains of membrane glycoproteins and glycolipids functions at two levels. Firstly, the thick glycocalyx, elaborated by cell types such as corneal epithelia, acts as a water structuring umbrella providing a non adhesive coat for the cell. However, the glycocalyx is also a highly sophisticated recognition facility, essential for the functioning of the immune system and recognition of 'self'. The highly specific structures of the oligosaccharides proximal to the cell membrane in most cells gives rise to the ABO blood group typings and the major histocompatibility complex (MHC) typings essential to transplantation procedures. The water structuring ability of the glycocalyx proximal to the cell membrane allows a *selectively* adhesive process on the basis of recognition of specific oligosaccharides to occur; an ability that biomaterial research strives for.

A homogeneous crosslinked polyHEMA gel contains a high proportion of OH groups. This results in a hydrogel with an equilibrium water content of approximately 40%. The surface of a polyHEMA gel such as this is generally nonadhesive to cells in the presence of serum. However, previous work from these laboratories indicated that a slight reduction in the water content, by adding MMA for example, would result in an adhesive surface. The addition of monomers that increased the water content of the gel, but only contained OH pendant groups, resulted in a nonadhesive surface. If monomers with pendant groups containing amino or carboxyl groups were added, in addition to increasing the water content of the gel, the surface became adhesive to cells (64,65)

The hydrogels examined in this chapter include polyethylene oxide (PEO) modified polyHEMA based gels. The structures of HEMA and a PEO methacrylate (PEGMA) are shown below. The HEMA pendant group is OCH₂CH₂OH. The pendant group on a PEGMA is a multiple (OCH₂CH₂)_n ether linked structure that terminates in either OH or OCH₃ (hydroxy or methoxy terminated)



poly HEMA



methoxy PEG methacrylate. eg n=9 is MPEG400

The inclusion of long PEO chains in a HEMA based copolymer has two effects. It increases the water content of a copolymer, and also offers an unsuitable surface for cell adhesion (the long chains act as a 'carpet' of molecular cilia preventing the adsorption of adhesion proteins). The distribution of PEO pendant chains at the surface may be critical, as discontinuities in the 'carpet' of molecular cilia would allow the deposition of adhesion proteins.

In order to present an even and unbroken carpet of 'molecular cilia' the PEO chains must be distributed in an evenly dispersed manner throughout the copolymer. For this reason, PEGMA introduced into the backbone of the polymer during polymerisation seems to endow an adhesion resistant surface at lower PEO chain length than would be achieved by grafting PEO onto a surface after polymerisation. The well dispersed sequence distribution is achieved in the homogeneous HEMA copolymers because reactivity ratios of HEMA and PEGMA are similar and moderate. However, in the case of copolymers including monomers of different reactivity ratios, the sequence distribution may not be regular, and the same proportions of PEGMA in the polymer may not result in a nonadhesive surface.

In the first experiments described in this section, a variety of chain length PEGMA substituted polyHEMA gels were investigated for their cell adhesion properties. Cell adhesion was low on all of these gels, as they have a high water content. Could PEGMA substituted gels with a low water content still resist cell adhesion? In order to answer this question, a set of PEGMA modified HEMA : MMA copolymers were examined for their cell adhesion properties.

Sequence distribution in any copolymer may influence the subsequent adsorption of adhesion proteins and cell adhesion. All of the PEGMA substituted polymers discussed above are composed of methacrylate monomers, which have a similar reactivity ratio. This results in statistically random copolymers, with a well dispersed sequence distribution. However, in copolymers composed of monomers with widely different reactivity ratios, blocks of chemical functionality arise.

The effects of sequence distribution in polymers on biological response have not previously been examined. The prediction and analysis of distribution is now being examined by this research group. Recent investigations in these laboratories have assessed computer modelling predictions of sequence distribution with reference to the actual distribution (assessed by NMR) at various percentage conversions of a number of copolymers. As part of the experimental work described in this chapter, cell adhesion assays were assessed for their usefulness in examining the surfaces of polymers previously prepared for investigation of sequence distribution. Preliminary results presented here indicate that this technique may be a valid method of investigation.

The second part of this chapter relates to the use of cell adhesion to probe a surface that has been adsorbed with proteins (and other components) *in vivo*. Under classical culture conditions in a medium containing 10% serum, vitronectin (Vn) is the adhesion protein adsorbed to the surface that initially leads to cell adhesion (113,114,115). Fibronectin (Fn) is not adsorbed in high quantities to surfaces in the presence of serum concentrations exceeding 0.1% (113,114,115). Fn may be adsorbed in significant quantities where surfaces are modified (66,67). The ocular environment differs in many ways from culture conditions. In serum containing culture conditions, Fn is displaced from surfaces by fibrinogen, yet the healthy ocular environment contains little fibrinogen. Fn is also present in larger quantities in the eye than in serum, so that Fn adsorption on materials is likely to be greater than that seen in

culture conditions. Vn has not previously been studied in the context of the ocular environment. Which adhesion proteins are responsible for the adherence of cells *in vitro* to worn contact lenses? In the final part of this chapter, cell adhesion to worn contact lenses was used to examine their protein deposition properties. The use of blocking antibodies helped to elucidate the proteins responsible for cell adhesion to worn contact lenses.

To summarise, experiments in this chapter show how cell adhesion can be used to examine a variety of polymers bearing functional sidechains. In the first section, cell adhesion to a variety of polymers where long polyethylene oxide chains have been substituted into the backbone of the polymer are examined. Cell adhesion to a variety of polymers originally prepared to examine sequence distribution are also examined.

Contact lens materials have been examined by others for their protein and lipid deposition characteristics in the eye by a variety of methods. In the latter part of this chapter, cell adhesion in the presence of blocking antibodies is described, and used to analyse the possible adsorption of the adhesive proteins Fn and Vn from tears. The implications of the presence of these proteins and their adsorption to contact lens materials is discussed.

In the following work, PEGMA substituted polymers in figures 7-9 were prepared by Dr Helen Oxley and the PEGMA substituted polymers prepared in figure 10 were prepared by Mark Smith. The sequence distributed polymers were prepared by Naweed Ashraf. Worn contact lenses were kindly provided by Lyndon Jones from a number of patients at his practice

3.1 The presence of serum and the effects of time

This short experimental section demonstrates the effect of serum on cell adhesion, as opposed to the physicochemical adsorption of cells to bare polymer surfaces. The incubation period after plating the cells is important in assessing the amount of cell adhesion, as adhesion levels at 5 minutes may vary markedly from those at 1 hour or 6 hours. Many cells produce their own adhesion proteins after periods longer than an hour in culture. As a result of this, six hour incubations can mask the adhesion to a specific adhesion protein. The length of trypsinisation prior to plating the cells is also a major factor in the time taken to adhere. Probably the best way to assess adhesion is to preadsorb the material with serum or preferred protein, to rinse well and then assess cell adhesion after 1 hour in a serum free system. This allows manipulation of ionic conditions for example.

In order to assess the effects of serum on cell adhesion to homogeneous polyHEMA hydrogels, 3T3 cells were plated onto polyHEMA coated coverslips under a variety of conditions for 20 minutes. Figure 6A shows that polyHEMA coated coverslips were *adhesive* to 3T3 cells in the absence of serum and regardless of the presence of divalent cations. These photographs were taken after 20 minutes incubation. (If the cells are metabolically active and producing proteins, they gradually desorb presenting a completely different adhesion profile after 6 hours).

In addition to the experimental results pictured in figure 6A, cell adhesion to polyHEMA in the presence of serum and manganese was assessed. It is known that manganese ions promote adhesion in many situations. Albumin, commonly considered to be a nonadhesive surface can be adhered to by cells in the presence of manganese cations⁽¹⁸⁴⁾. Could manganese promote adhesion to the non adhesive polyHEMA in the presence of serum? Using 3T3 cells, 500 μ M manganese and serum adsorbed polyHEMA coated coverslips, no adhesion was noted even after 6 hours incubation, implying that the nonadhesive qualities of albumin and polyHEMA are exerted by different properties.

The effects of the period of incubation time on cell adhesion was assessed using the SRB assay system described in chapter 2 (page 54, 2.1.5). Briefly, cells were plated onto polystyrene 96 well plates. One plate was pretreated with air plasma for 20 minutes, to mimic commercial tissue culture plastic.

Figure 6B shows the effects of measuring adhesion to the two surfaces under four different conditions after different amounts of time. The treatment groups were:

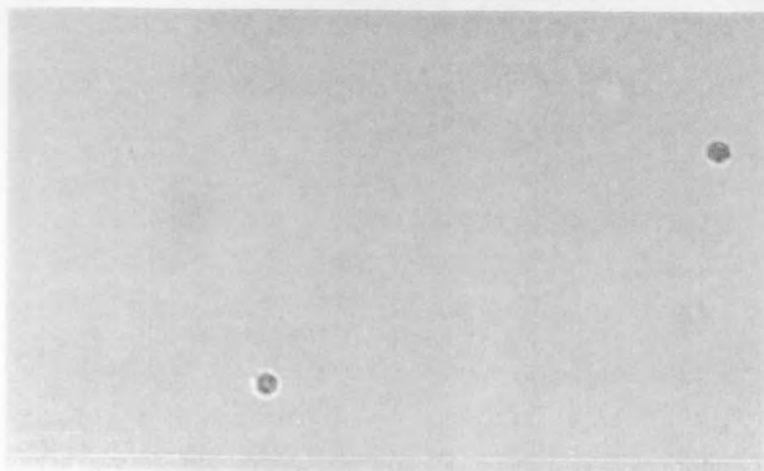
- with serum onto untreated polystyrene (PS),
- with serum onto plasma treated polystyrene (TCP),
- without serum onto PS,
- without serum onto TCP.

After 10 minutes, the greatest amount of cell adhesion is seen in cells without serum to unmodified PS. Over time, several factors are evident. The cells begin to produce protein, making them less adhesive to PS, and the initial low adhesion to TCP becomes greater over

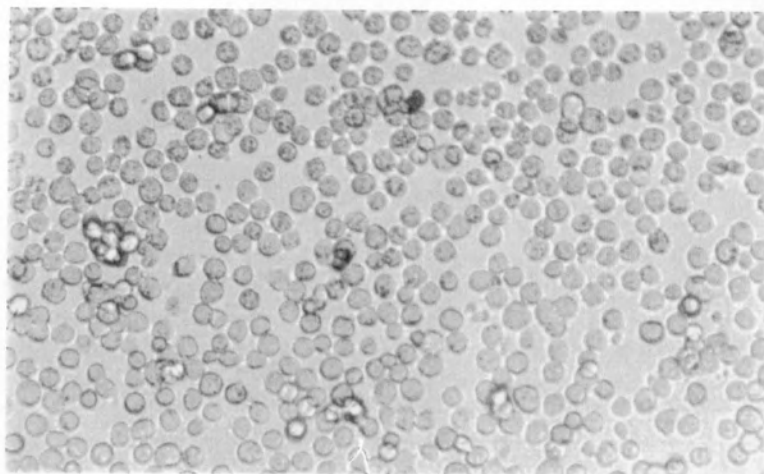
time. This time dependence and serum dependence illustrates the factors involved in physicochemical adsorption as opposed to adhesion protein mediated adsorption. Adhesion proteins adsorbed to PS are not as biologically active as those adsorbed to TCP. In addition to this, less adhesion proteins are adsorbed to PS than to TCP.

The greatest differences in the amounts of cell adhesion are still evident after 6 hours between the TCP + serum group as opposed to all of the other treatment groups. This 6 hour time period is still viable in experiment for serum containing medium, but in order to determine initial adhesion mechanisms, one hour periods are more suitable. Method A, used for the experiments described in figures 7 and 8 assesses cell adhesion after 6 hours. Other experiments described in this chapter were assessed after 1 hour. In chapter 4, where adhesion to various coated surfaces in the presence of divalent cations is investigated, the incubation period is also one hour.

a) 10% serum, 5mM Mg



b) no serum, 5mM Mg



c) no serum, no cations

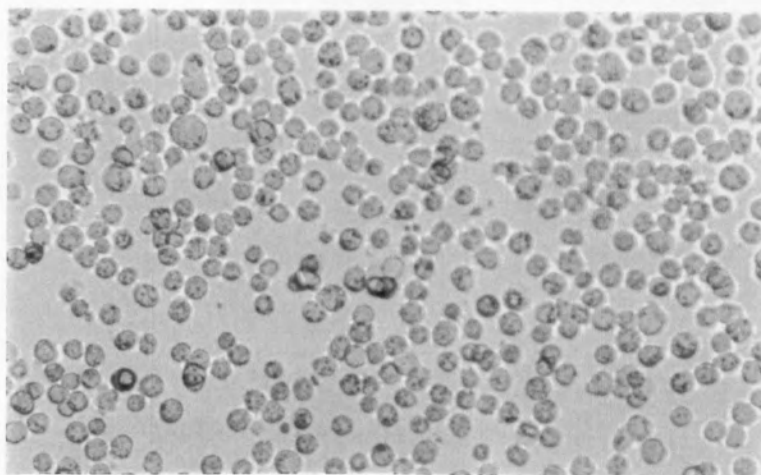


Figure 6A 3T3 Cells adherent to spincoated ppolyHEMA on glass coverslips after 20 minutes incubation in various conditions a) 10% serum, 5mMMg b) no serum, 5mM Mg, c) no serum, no divalent cations

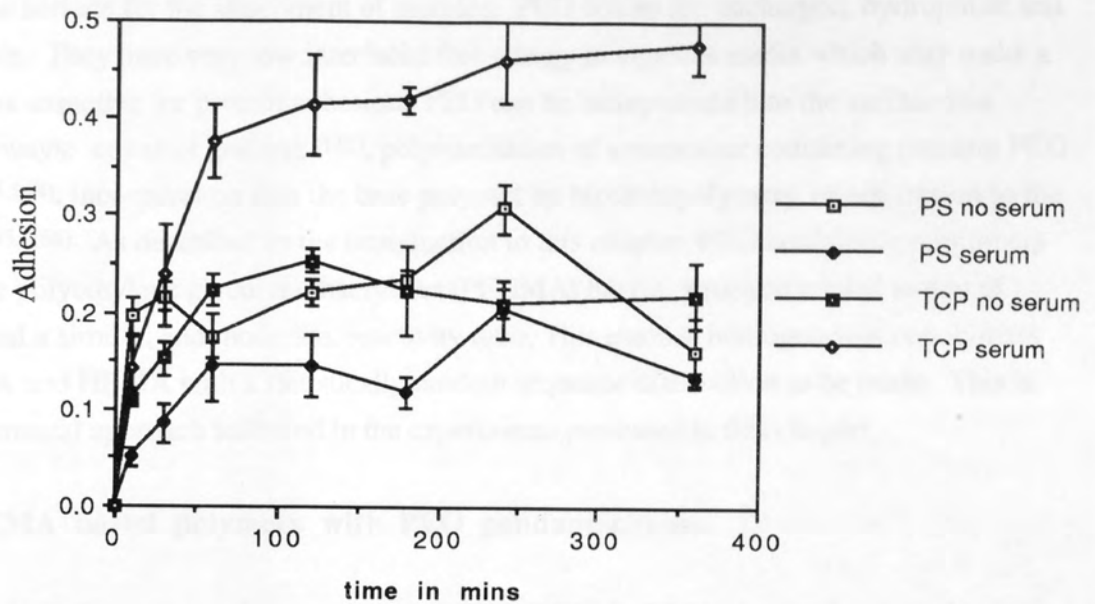


Figure 6B 3T3 cells adherent to polystyrene (PS) or plasma treated polystyrene (TCP) over time, with and without the presence of 10% foetal calf serum in DMEM. Each point represents an average of six measurements with standard deviations represented by the error bars. Cell adhesion is measured by the SRB method.

3.2 Polyethylene oxide modified surfaces

Polyethylene oxide (PEO) modified surfaces have been recognised as protein resistant surfaces in recent years. This is thought to be due to steric occlusion effects. It is, perhaps, easier to visualise the PEO chains forming a carpet of 'molecular cilia' at the surface and thence an unstable surface for the attachment of proteins. PEO chains are uncharged, hydrophilic and very mobile. They have very low interfacial free energy in aqueous media which may make a surface less attractive for protein adhesion. PEO can be incorporated into the surface in a variety of ways: covalent grafting⁽¹⁶²⁾, polymerisation of a monomer containing pendant PEO chains^(163,164), incorporation into the base polymer by block copolymers or adsorption to the surface^(165, 166). As described in the introduction to this chapter, PEO containing monomers such as the polyethylene glycol methacrylates (PEGMA) have a structure related to that of HEMA, and a similar, and moderate, reactivity ratio. This enables homogeneous copolymers of PEGMA and HEMA with a statistically random sequence distribution to be made. This is the experimental approach followed in the experiments presented in this chapter.

3.2.1 HEMA based polymers with PEO pendant chains.

In the following set of experiments HEMA : EGDM : PEGMA copolymers with different polyethylene oxide pendant chains were tested in serum containing conditions for their cell adhesion properties. The first set of experiments were measured after 6 hours and cell adhesion was measured by method A. However, on the spincoated polymer films, overall numbers of cells adhered after the incubation period were always low, so cells were fixed and counted *in situ* at the magnification stated in the figure legends (method B). Spincoated films of linear polymer differ from crosslinked gels in that they are not completely polymerised. Hence water content is often higher in films and this may result in a greater resistance to cell adhesion.

The first set of experiments, referred to in figure 7 and 8, were carried out on cast gels. Figure 7 shows the relative amounts of 3T3 cell adhesion in medium containing 10% (v/v) serum, onto HEMA : EGDM 90:10 containing 20% by weight of polyethylene oxide methacrylates at molecular weights 200, 400 and 1000. (These were all methoxy terminated polyethylene oxide chains (MPEGMAs), except for 4,5, ethylene oxide modified HEMA, which is a hydroxy terminated polyethylene oxide. The structures of these monomers can be found in chapter 2). The trend in cell adhesion was pronounced: as the PEO chain length increases, cell adhesion decreases. Yet, as shown by previous studies from this research group, cell adhesion can be closely correlated with water content. The water content of the 20% PEGMA containing copolymers tested in Figure 7 are respectively, 43, 46, and 49% . Water contents above 40% are known to decrease adhesion where only OH groups are present⁽⁶⁵⁾.

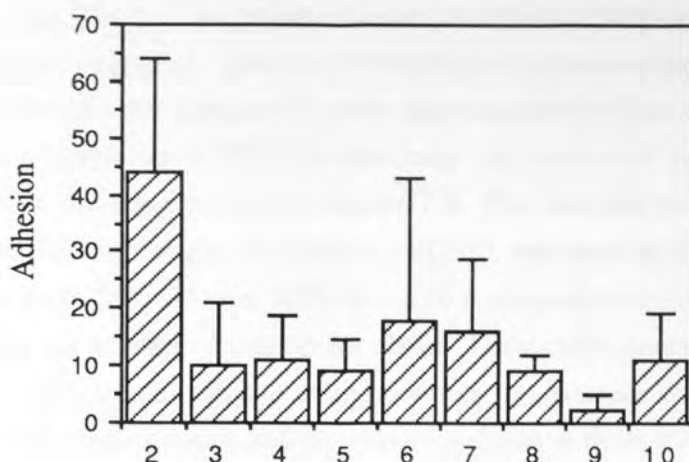
A range of polymers at 40% water content were prepared and assessed in the cell adhesion assay as shown in figure 8. The polymers contained by weight:

- 4, 5, ethylene oxide modified HEMA at 20%,
- 15% 200 MW MPEGMA,
- 10% 400 MW MPEGMA
- 5% 1000 MW MPEGMA.

Figure 8 shows that the effect of increasing chain length is still apparent even with a decreased density of chains at the surface, and a similar water content. The maximum nonadhesion effect was achieved with 400 MW PEO chains. Note that in figures 7 and 8 cell adhesion in the presence of serum was always slightly higher in the serum containing mediums, with the exception of plain HEMA. Whilst many materials are more adhesive in the absence of serum, polymers substituted with polyethylene oxide pendant chains are still non adhesive. This reflects the surface structure of highly mobile PEO chains.

The next set of adhesion experiments was carried out on PEGMA modified polymers spun onto cleaned glass coverslips. Film stability was assessed by soaking coated coverslips in saline for a day prior to experimentation. Unstable films are not suitable for cell adhesion experiments. Maximum film stability was achieved with a combination of optimum film thickness, 2% uranyl nitrate and 23% glycidyl methacrylate as cross linkers. Films were then treated with 20 minutes UV and baked for 2 hours at 60°C. Figure 9 shows the effects of PEO chain lengths on cell adhesion to these spincoated polymers. Cell counts were extremely low on all of these polymers. This is selected by the method for cell counting described in the legend to figure 9. Maximum nonadhesive effects are observed in methoxy terminated PEGMA modified polymers of MW 1000. Hydroxy terminated polymers were less effective. A qualitative assessment revealed that plasma etching of the above polymers for 5 minutes did not increase cell adhesion, suggesting that the pendant chains within the matrix of the polymer can rotate and provide a new surface of mobile PEO chains. Although the chains may be chemically altered at the chain terminus (say, to COOH) it is the chain length that maintains the protein resistant qualities of the surface, so that oxidation by air plasma treatment did not produce a more adhesive surface. It was particularly noteworthy that such low cell counts revealed trends corresponding with the surface chemistry, showing the value of cell adhesion assays in assessing surface properties.

In the next section (3.2.2) hydrogel polymers with a lower water content are investigated, in order to determine whether PEO chains can inhibit adhesion when substituted onto the backbone of a lower water content polymer (HEMA : MMA 50:50) that is very cell adhesive.



Polymer 1 Tissue culture plastic Cells adhered 1000 ± 100 (not shown on graph)
 polymer 2. (HEMA:NVP:MMA 78:10.5:9.5)/MPEG400MA 80:20
 polymer 3. (" ")/PEG10, E,O 80:20
 polymer 4. HEMA:HEMA 4,5,EO 80:20
 polymer 5. HEMA:MPEG200MA 80:20
 polymer 6. HEMA:MPEG400MA 80:20
 polymer 7. HEMA:MPEG 550MA 80:20
 polymer 8. HEMA:MPEG 1000MA 95:5
 polymer 9. HEMA:MPEG 1000MA 90:10
 polymer 10 pHEMA

Figure 9 3T3 cell adhesion to spincoated PEGMA substituted HEMA copolymers. These hydrogels were films crosslinked with glycidyl methacrylate and 2% uranyl nitrate. Spincoated linear polymers often have a higher water content than the some polymer cast as a gel. This is because the gel is 100% cross linked, and the film is only cross linked to achieve stability as a surface coating. The cells counts were very low; very few cells had adhered to these polymers. Cells counts (adhesion on y axis) were therefore given as the sum of cell counts from four fields at x 10 magnification . The trends in cell adhesion with respect to chain length even at these very low levels can be seen.

However, polymer 2 shows relatively high adhesion despite containing 400MA PEG. Unexpectedly, the hydroxy terminated 4,5, EO PEG in polymer 3 exhibits less adhesion. This does not follow the trend observed where hydroxy terminated PEO chains are less effective than methoxy terminated chains at inhibiting cell adhesion. Why should this occur? In this case, the difference in reactivity ratios in the monomers has led to a less well dispersed sequence distribution in the 400MA PEGMA substituted polymer, and inhibition of cell adhesion is less effective.

3.2.2 Lower water content HEMA based polymers with PEO chains

The PEGMA modified polymers described in the preceding section were all very high water content polymers. Does the PEGMA substitution into the polymer backbone still produce nonadhesive surfaces in lower water content polymers? In order to answer this question, a further set of PEGMA substituted polymers were prepared at much lower water content than the sets examined in figures 7-9. The base polymer was HEMA : MMA (50 : 50). 5 to 20% by weight of the stated PEGMA was introduced into the composition. The numbers 2000, 550, 400 and 1000 refer to the molecular weight of the PEGMA monomer rather than the number of units in the chain. These copolymers were cast as gels as described in 2.1.2. 3T3 cell adhesion to these polymers was assessed with respect to the chain length of the PEO, the water content and the surface energies of the materials. The results are presented in table 1 below, and graphically in figure 10.

Composition	Cell adhesion	EWC %	γ_d	γ_p	total surface energy γ mNm ⁻¹
50%Hema50%MMA	160± 30	16.4	36.5	28.1	64.4
5% MPEG550	106± 22	16.9	33.0	32.7	65.7
10% MPEG550	105± 60	17.1	31.0	36.1	67.1
20% MPEG550	64± 7	21.6	27.4	39.3	66.6
5% MPEG2000	110± 15	20.1	24.0	35.0	59.0
10% MPEG 2000	76± 50	23.2	27.4	36.1	63.5
20% MPEG2000	14.5±3.8	33.7	30.0	38.2	68.2
5% PEGOH 1000	268±84	21.4	32.1	33.3	65.4
10% PEGOH 1000	28.3±9.1	24.7	31.2	34.4	65.6
20% PEGOH 1000	152.5±48.3	29.6	28.9	36.6	65.5
5% PEGOH 400	117±39	17.2	31.3	33.9	65.1
10% PEGOH 400	163±62	21.4	36.2	32.7	68.8
20% PEGOH 400	288±61	27.1	31.9	35.5	66.6

Table 1. PEO modified gels.

The results are presented graphically in Figure 10. MPEG refers to methoxy terminated PEGMAs whereas PEGOH refers to hydroxy terminated PEGMAs. Cell adhesion refers to the mean cell counts \pm standard deviations. Cell numbers were assessed by counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 samples (16 fields). Surface energy measurements are in units of mNm⁻¹

Methoxy terminated PEGMA substituted copolymers (figure 10A), are seen to be more effective at inhibiting cell adhesion than the corresponding hydroxy terminated PEGMA substituted polymers of similar chain length (figure 10B). The fall in adhesion with increasing chain length in the methoxy terminated PEGMA copolymers is pronounced. The trend was not observed in the hydroxy terminated PEGMA copolymers. This may be because of impurities in the latter polymers or because of hydrogen bonding interactions of the chain terminating hydroxyl groups.

The water content of the polymer increased with PEGMA of increasing molecular weight. Figure 10C shows that the inhibition of cell adhesion correlates with this increase water content in methoxy terminated gels. However, as illustrated in Figure 10D cell adhesion does not correlate with the water content of the hydroxy terminated PEGMA substituted gels. Surface energy was measured by captive bubble contact angle measurement method. Figure 10E shows that polar surface energy increased with increasing PEO content of the hydrogels in the methoxy terminated PEGMA substituted hydrogels. Total surface energy increased slightly. This also correlated with cell adhesion, although as previously, correlations were only found in the methoxy terminated PEGMA containing hydrogels. Polar surface energy correlated most strongly with cell adhesion as shown in figure 10F.

One of the most striking aspects of the results obtained in the experiments described above was that relatively short PEO chains (molecular weights 400-1000 D) inhibited cell adhesion. Recent work by Desai and Hubbel⁽¹⁶²⁾ found that protein adsorption and cell attachment was significantly reduced on PEO grafted polytetrafluoroethylene surfaces when the grafted PEO was at least 18.5 kD. Due to the different methods of introducing PEO to differing surfaces it is difficult to make comparisons on the molecular weight (and chain length) necessary to decrease protein adsorption or cell adhesion. The base material obviously makes a contribution, as well as density and distribution of chains and the chain mobility. Lee et al^(165, 166) used PEO containing copolymers as surfactants. They reported that although some exchange between water soluble adsorbed PEO copolymers and proteins could be seen, protein resistant behaviour of the adsorbed surfaces could be observed after 100 minutes of exposure to protein. The adsorbent technique used by Lee et al has the advantages of being technically simple, retaining mechanical strength of the base polymer and cheap to do on a large scale, but may be unsuitable for long term applications. For example, long term catheters would also lose coatings by physiological mechanisms, so this technique may be limited to short term applications. Grafted PEO surfaces have already been used with some success by Hubbell et al⁽¹²²⁾ to reduce the nonspecific adherence of cells to a surface containing specific endothelial adhesion peptide moieties. The same graft polymerisation technique has also been successful in reducing tissue response to implanted polyethyleneterephthalate and reducing bacterial interaction^(167, 168). What advantages are offered by the technique used in the experiments described in this chapter? Polymers with a substituted PEGMA as described in the experiments here, offer a system which seems to offer protein resistance at relatively low molecular weights. This may be because an even dispersion of PEO chains

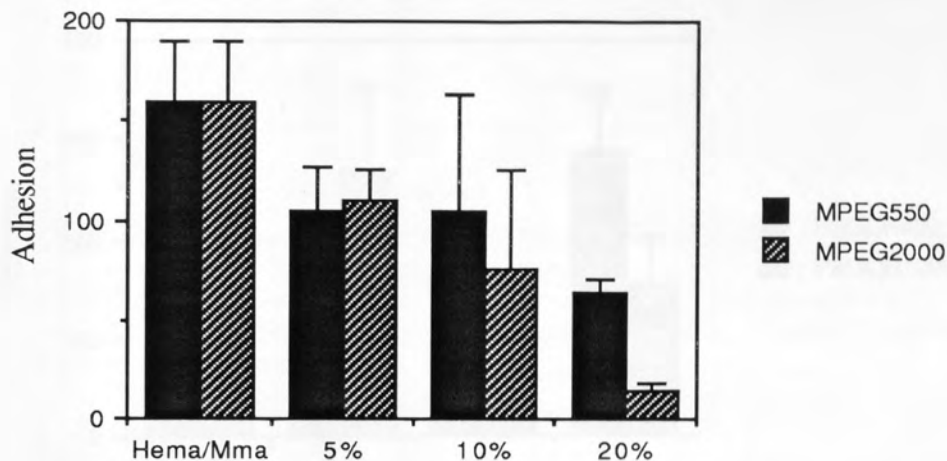


Figure 10A 3T3 cell adhesion to PEO modified polymers with lower water contents. Hema/Mma represents cell adhesion to the unsubstituted polymer. Cell adhesion to methoxy terminated PEGMA substituted polymers falls with an increase in chain length as illustrated above. Cell adhesion is very low on the 20% MPEG2000 substituted polymer, despite a water content that supports adhesion in simple HEMA based gels. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations. Figures 10A and 10B can be directly compared as they were carried out in the same experiment.

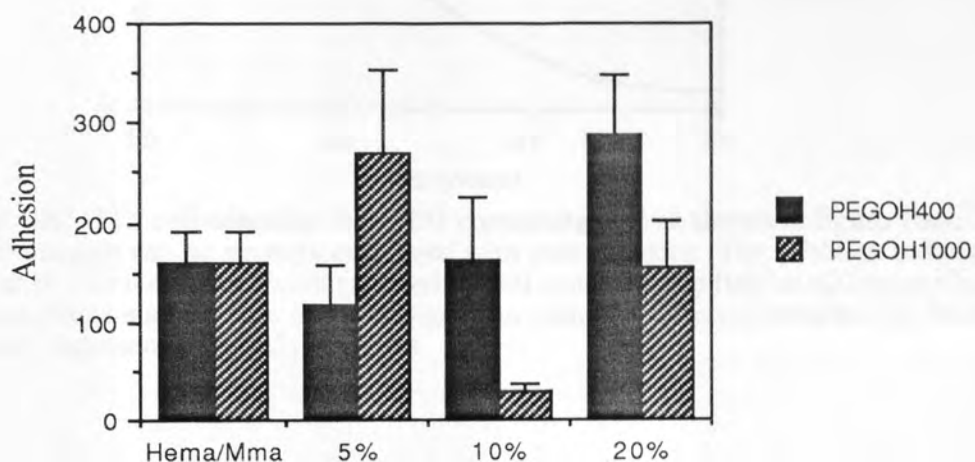


Figure 10B The same correlations between cell adhesion and chain length cannot be observed on polymers with hydroxy terminated PEG, although low cell adhesion is observed on 10% PEGOH 1000. Generally cell adhesion on the hydroxy terminated polymers is higher than on the methoxy terminated polymers with similar chain lengths. This may be because the PEGMA was less pure prior to polymerisation *, or because the OH terminated chains tend to loop back on themselves, hydrogen bonding to groups further down the chain. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations. Figures 10A and 10B can be directly compared as they were carried out in the same experiment.

*These monomers were prepared in house as part of a project on monomer synthesis. Details of the synthesis of these monomers can be found in the thesis of Mark Smith, 1994. Technical factors associated with the preparation and purification of the OH terminated PEGMA monomers may result in slightly higher levels of contamination, which are reflected in any subsequent polymerisations.

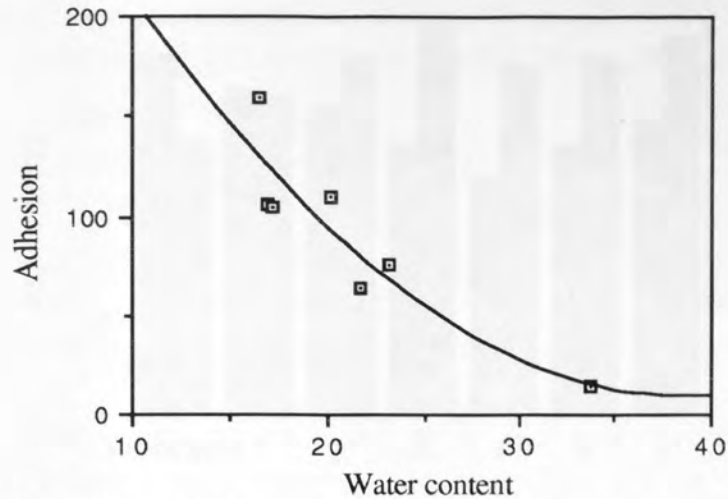


Figure 10C 3T3 cell adhesion to MPEG containing gels as shown in figure 10A. Cell adhesion to gels can be strongly correlated with water content. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

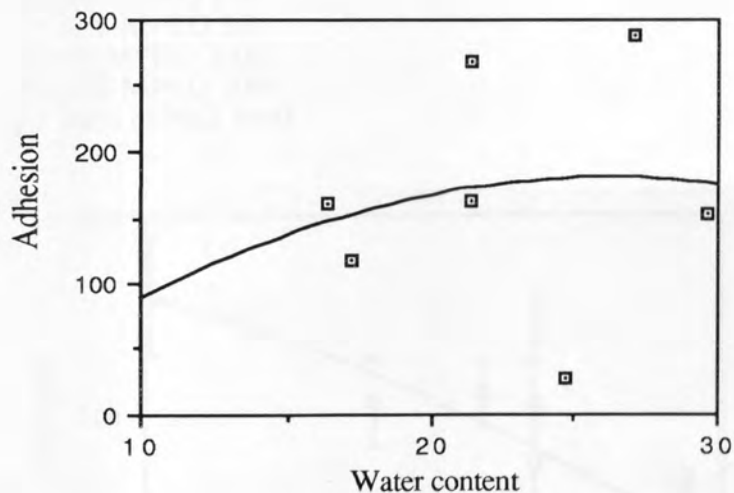


Figure 10D 3T3 cell adhesion to OH PEG gels as shown in figure 10B cannot be well correlated with water content in the same way as for MPEG containing gels. Water content does however correlate with the increase in concentration and chain length of the PEG. Lack of correlation of cell adhesion with the OH PEG is therefore probably due to hydrogen bonding within the chain. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

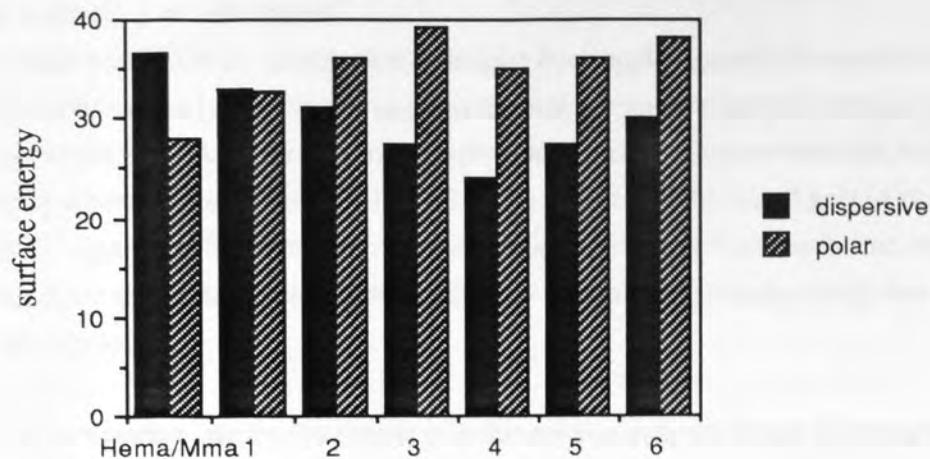


Figure 10E The relationship between increasing PEGMA content of a polymer and surface energy. In this set of methoxy terminated PEGMA containing hydrogels, the dispersive and polar components of surface energy respectively fall and rise in response to increasing amounts of PEO expressed at the surface of the gel. These strong correlations are not exhibited by the hydroxy terminated PEGMA containing hydrogels.

- Key*
- 1) 5% MPEG 550
 - 2) 10% MPEG 550
 - 3) 20% MPEG 550
 - 4) 5% MPEG 2000
 - 5) 10% MPEG 2000
 - 6) 20% MPEG 2000

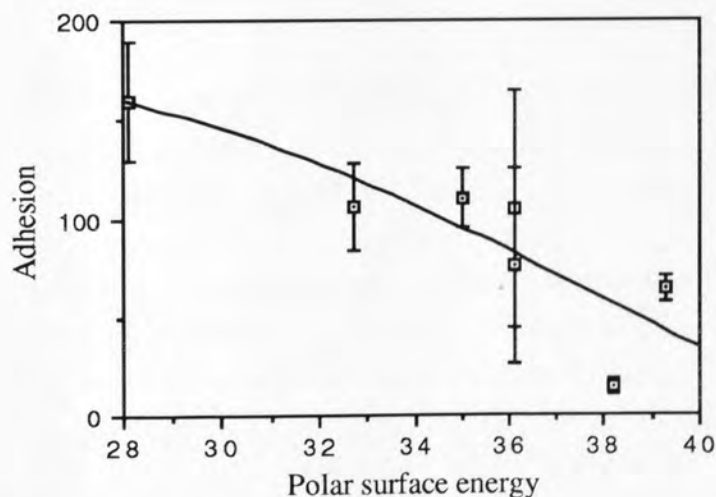


Figure 10F Cell adhesion to methoxy terminated PEGMA containing hydrogels is strongly correlated with polar surface energy. It also correlates with total surface energy, although not as strongly. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

at the surface of the statistically random copolymer results from this method of fabrication as opposed to grafting or adsorption.

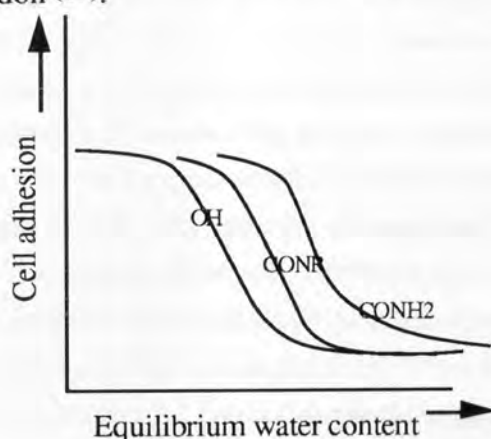
Could this PEGMA substitution technique have applications in biomaterial fabrication? This technique is already used in contact lens technology in an attempt to reduce protein deposition in the eye. Affinity chromatography also requires support materials with a low background adsorption of proteins. PEGMA substituted HEMA based gels in combination with specific ligands offer a route to controlled adsorption, and this may be the most promising route to the design of improved affinity separating techniques with low background protein adsorption.

To summarise, the results obtained in this section indicate trends in cell adhesion that correspond with PEO chain length and terminating group.

- 1) Cell adhesion decreases with increasing chain length and concentration of PEGMA within the copolymer
- 2) Methoxy terminated PEO chains are more effective at inhibiting adhesion than hydroxy terminated chains. This may be because hydroxy terminated chains have a tendency to loop back and interact with the polymer surface.
- 3) Statistically random sequence distribution of the PEO chains along the polymer backbone may be an important feature of the adhesion resistance of these polymers.
- 4) It is largely the mobility of the long chains that inhibits adhesion

3.3 Sequence distribution in polymers; effects on cell adhesion

The pendant functionalities substituted into a polymer backbone can influence the cell adhesive nature of the polymer. These functionalities determine the bulk water content of the gel and also affect the surface properties of the material. For homogeneous polymers substituted with OH pendant functionalities, gels of water content exceeding 40% do not support cell adhesion. Polar pendant groups are very hydrophilic and increase the bulk water content of the gel. However, gels substituted with polar pendant groups will support cell adhesion despite water contents exceeding 40%. The water content 'cutoff point' for cell adhesion depends on the nature of the pendant functionality. The substitution of pendant amide groups results in a cutoff point for cell adhesion at about 60% for example. These experimental results were obtained by Thomas (65) who expanded on the earlier work of Minett and Lydon (69).



The effects of sequence distribution on the cell adhesion to hydrogels of various compositions has not previously been investigated. Polymer surfaces with 'islands' of specific chemical functionality that result from an uneven sequence distribution in the polymer may adsorb more adhesion proteins from a serum solution. Proteins consist of small blocks of charged amino acids that govern their secondary and tertiary structures, and these areas could interact with areas of chemical functionalities on the polymer surface.

How would areas of functionality on a polymer surface arise? Polymerisation of two or more monomers may result in a polymer with long blocky sections, due to the differing reactivity ratios of the monomers and their relative concentration in the reaction mixture during polymerisation. The monomer with the highest reactivity ratio tends to constitute the blocks in the polymer at the start of the polymerisation, whereas the monomer with the smallest reactivity ratio tends to form a large residual block at the end of the polymerisation. Monomers with similar and moderate reactivity ratios form polymers with well dispersed compositions (when concentrations in the reaction mixture are equal).

By understanding the dynamic process of polymerisation and/or stopping reactions before completion, polymers of a controlled dispersed sequence distribution can be achieved even when monomers of dissimilar reactivity ratios are used. This approach has been followed

at Aston. A variety of these polymers were examined in the cell adhesion system in order to assess whether this technique provided a useful assay system for investigating sequence distribution effects.

3.3.1 Results

A variety of polymers were either cast as gels or spincoated onto precleaned glass coverslips as described in the materials and methods section. Samples were preincubated with serum before culturing in serum free medium (MEM) with 3T3 fibroblasts. Adherent fibroblasts were counted at x20 magnification.

The first set of polymers were spincoated copolymers. These copolymers are referred to by their initial concentration in the polymerisation, although this does not reflect their concentration in the final polymer. The polymerisation was stopped at various time intervals which is reflected by the percentage conversion of the initial monomer mixture. This was calculated by a mathematical model and confirmed by NMR analysis. The actual percentage of each monomer in the resulting polymer varies according to percentage conversion. The distribution of the monomers is different to that formed from 100% conversions. The blockiness of either constituent monomer may be reduced.

The second set of polymers were cast as gels. These compositions were 100% polymerised but the introduction of small amounts of different crosslinking agents led to variable final structure in the polymer. These are reflected in both the water content and cell adhesion parameters. Table 2 overleaf shows the results obtained for these polymers. The copolymers are referred to by number convenient for the understanding of the graphs.

Figure 11 illustrates cell adhesion to all of the spincoated linear polymers, whereas Figure 12 illustrates cell adhesion to the 100% polymerised hydrogel membranes. Firstly, Figure 11A demonstrates the effects on cell adhesion of increasing percentage conversion in HEMA : MMA copolymers. Cell adhesion varies very little with the increase in conversion, reflecting the similarity of the reactivity ratios of the two monomers. The composition of the polymer is largely independent of the percentage conversion of the polymer, and cell adhesion shows no trends. Figure 11B illustrates the cell adhesion to MMA : NVP copolymers with increasing percentage conversion. NVP has a lower reactivity ratio than MMA and initially, in polymers 3 and 4, the polymer only contains small amounts of NVP. Cell adhesion was very high on the homogeneous NVP film. This was an anomalous result and can only be explained by the high amounts of cross linker required to stabilise this film. Low adhesion was expected. Polymers 3 and 4 exhibit some cell adhesion where MMA is still a high proportion of the MMA : NVP copolymer. Note that the polymer 5, with the highest sequence conversion and containing most NVP exhibits no cell adhesion at all. This correlates well with the results for 100% conversion of the same copolymer observed by Thomas (65), and also with the hydrogel membrane polymer 11 HEMA : NVP which contains a similar proportion of NVP. (HEMA and MMA have similar reactivity ratios)

Polymer composition (Mole %)	spuncoat (S)	conversion	water content	Adherent
	gel(G)	%		cells \pm SD
pHEMA	S	100	-	3.8 \pm 1.7
Tissue Culture Plastic(TCP)		-	-	300 \pm 30
pNVP	S	100	-	245 \pm 35
1) HEMA/NVP	S	15	-	0.75 \pm 1.3
60:40				
2) HEMA/NVP	S	61	-	0.0 \pm 0.0
60:40				
3) MMA/NVP	S	6	-	70 \pm 17
60:40				
4) MMA/NVP	S	24	-	114 \pm 16
60:40				
5) MMA/NVP	S	50	-	0 \pm 0
60:40				
6) HEMA/MMA	S	38	-	44 \pm 6
50:50				
7) HEMA/MMA	S	72	-	65 \pm 7
50:50				
8) HEMA/MMA	S	76	-	44 \pm 29
50:50				

Table 2. Polymers with variable sequence distribution (continued overleaf)

GROUP AND
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pHEMA	G	-	37	13.5 ± 9.8
9) HEMA/NVP	G	-	54	63 ± 28
60:40				
(1% EGDMA)				
10) HEMA/NVP	G	-	58	3 ± 3.3
60:40				
(1% TAC)				
11) HEMA/NVP	G	-	61	0 ± 0
60:40				
(1% DATDAM)				
12) NVP/NVI	G	-	95	116 ± 23
40:60				
13) NVP/NVI/SPE	G	-	85	68 ± 19
40:40:20				
14) NVP/NVI/SPE	G	-	83	33 ± 20
40:35:25				
15) NVP/ITA/SPE	G	-	62	Toxic
40:35:25				
16) HEA/ITA/PEG	G	-	73	Toxic
25:38:37				
17) HEA/EOEMA/NVP	G	-	67	37 ± 35
50:12:38				

Table 2 Polymers with variable sequence distribution

The results are demonstrated graphically in figures 11 and 12. Cell adhesion was assessed by cell counts of one field at x 20 magnification. Results represent a average of four readings from each of four different samples (16 fields) ± standard deviations.

Figure 12 A demonstrates cell adhesion effects in a set of gels. These gels represent polymers where conversion is 100%. However, small amounts of different cross linkers with decreasing reactivity ratios caused dramatic differences in cell adhesion properties. These could also be correlated with the increasing water content that resulted from the use of these crosslinkers; the equilibrium water content 'cutoff' level for cell adhesion to homogeneous NVP copolymers has previously been shown to be at about 60% (65). Figure 12 B illustrates the correlation between water content of the polymers in 12 A with respect to their cell adhesion.

Figures 12C and D show the cell adhesion to a series of NVP : NVI : SPE copolymers. Although water content actually decreases with the addition of SPE, cell adhesion decreases as well. Cell adhesion usually increases with decreasing water contents so why should SPE cause this effect? NVI introduces a large amount of positive charge into the polymer. The increase in negative charge caused by introducing the zwitterion SPE may have counteracted the effects of this positive charge, which in turn led to a decrease in cell adhesion despite the increase in water content. Other studies have also shown that positively charged substrates are very adhesive to cells (74-78), but the reason for this is not established. Is the increased adhesiveness due to conformation and concentration of adsorbed adhesion proteins or to the direct interactions of the surface with the cell surface? There is potential for further study on this question.

It is relevant at this point to compare the cell adhesion to polymer 17 (HEA : EOEMA : NVP) with the NVP : NVI copolymer. The latter polymer was composed of moderately reactive monomers of similar reactivity ratios that resulted in a fairly statistically random dispersion of the functionalities. HEA : EOEMA : NVP copolymer is composed of monomers with different reactivity ratios that lead to a less even dispersion of functionalities in the polymer. Cell adhesion to the HEA : EOEMA : NVP polymer is only one third of that to NVP : NVI indicating that, in this case, a well dispersed sequence distribution has resulted in increased cell adhesion.

To summarise, cell adhesion has been used to examine a number of copolymer surfaces previously prepared to analyse sequence distribution effects. Results suggested that this technique could be further developed to provide a reliable assay for examining sequence distribution effects in polymers.

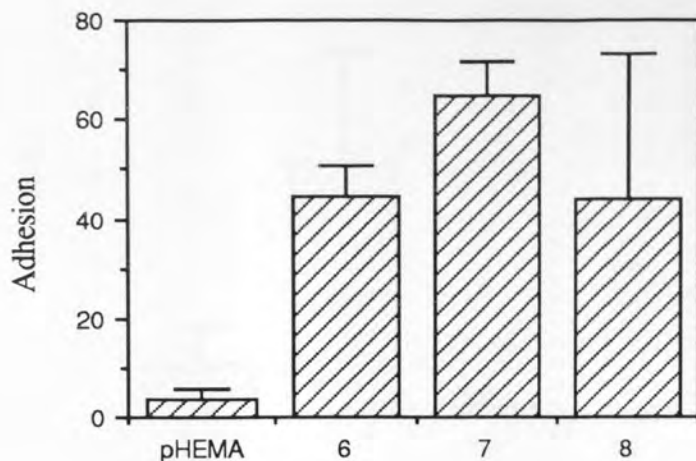


Figure 11A 3T3 cell adhesion to HEMA : MMA copolymers (see table 2 for polymer composition). HEMA and MMA have similar reactivity ratios. There are few differences between the adhesion seen on these copolymers, indicating a similar composition in polymers 6,7,and 8. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

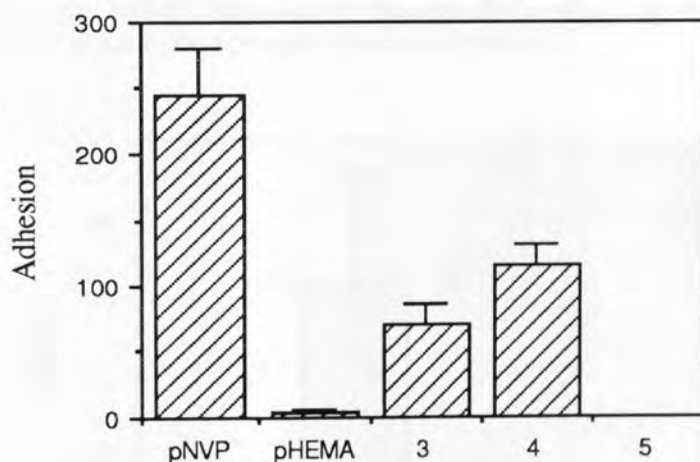


Figure 11B 3T3 cell adhesion to MMA : NVP copolymers. NVP has a lower reactivity ratio than MMA and initially, in polymers 3 and 4, the polymer only contains small amounts of NVP. Cell adhesion is very high on the homogeneous NVP film. This is unexpected and can only be explained by the high amounts of cross linker required to stabilise this film. Polymers 3 and 4 exhibit some cell adhesion where MMA is still a high proportion of the polymer. Note that the polymer 5, with the highest sequence conversion and containing most NVP exhibits no cell adhesion at all. This correlates well with the results for 100% conversion of this copolymer observed by Thomas. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

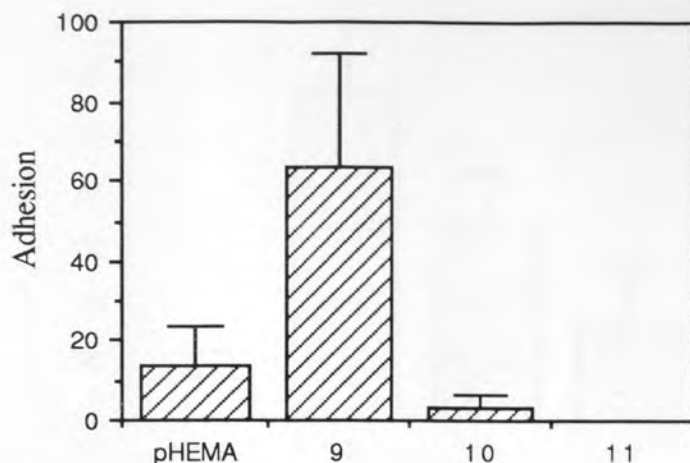


Figure 12A 3T3 cell adhesion to a series of HEMA : NVP copolymers gels with a variety of cross linkers. Notice that adhesion decreases to zero with different cross linkers. Polymers 9, 10 and 11 reflect decreasing reactivity ratios in the cross linker. The cross linker with the lowest reactivity ratio tends to break up the structure of the long block of NVP at the end of the polymer. This results in an increased water content and cell adhesion can actually be related to the water content effect as shown in figure 12B. The water cut off region for a simple copolymer containing NVP is 60% as previously shown by Thomas. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

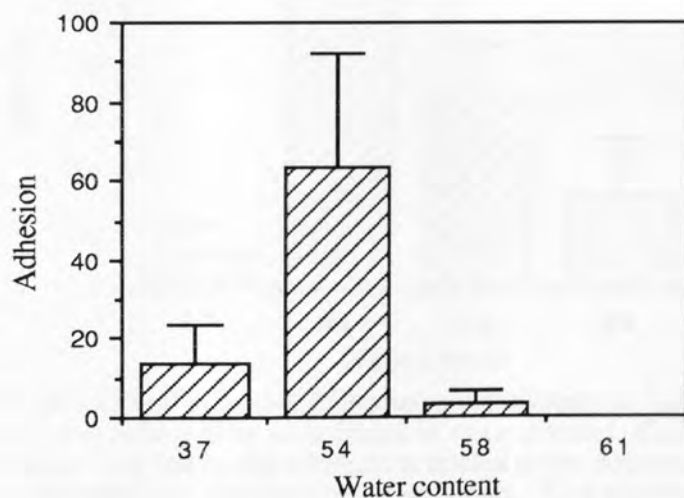


Figure 12B 3T3 cell adhesion to polymers as in 12A: pHEMA, 9, 10, 11. The increasing water content also correlates with the decrease in cell adhesion. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

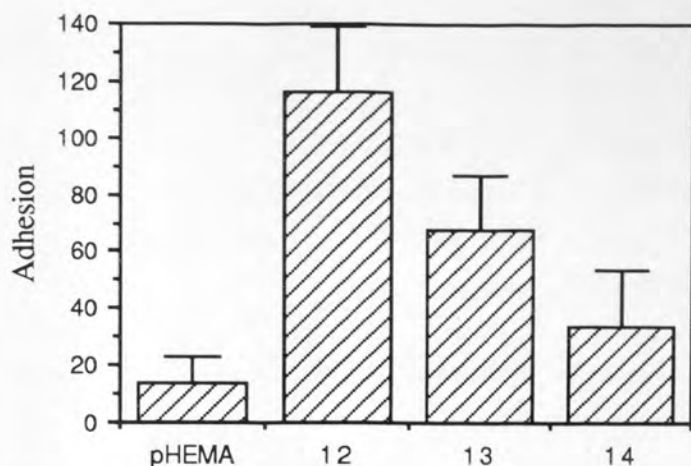


Figure 12C 3T3 cell adhesion to this series of NVP : NVI : SPE copolymers shows that increasing quantities of SPE decrease cell adhesion despite a fall in water content of the polymer as shown in figure 12D. The positive charged NVI results in a high degree of cell adhesion. The introduction of increasing amounts of SPE, counteracts this with negative charge, and cell adhesion decreases. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields).

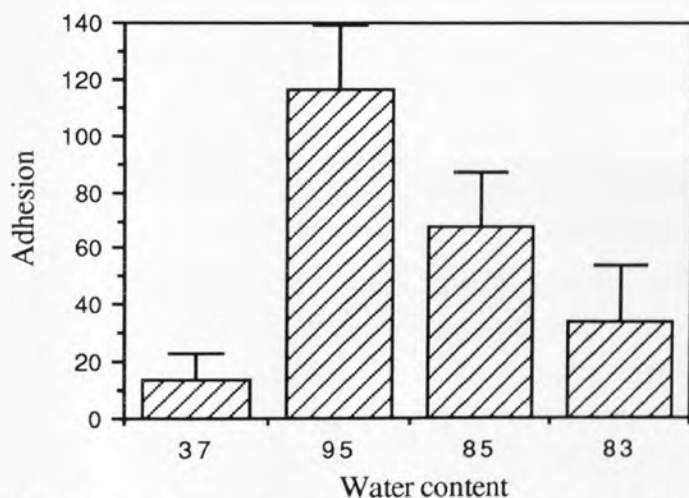


Figure 12 D 3T3 cell adhesion to the same copolymers as 12C. The increase in SPE content from 12 to 14 is reflected by an decrease in water content. Cell adhesion falls with the fall in water content. The fall in cell adhesion is related to the decrease in positive charge at the surface. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

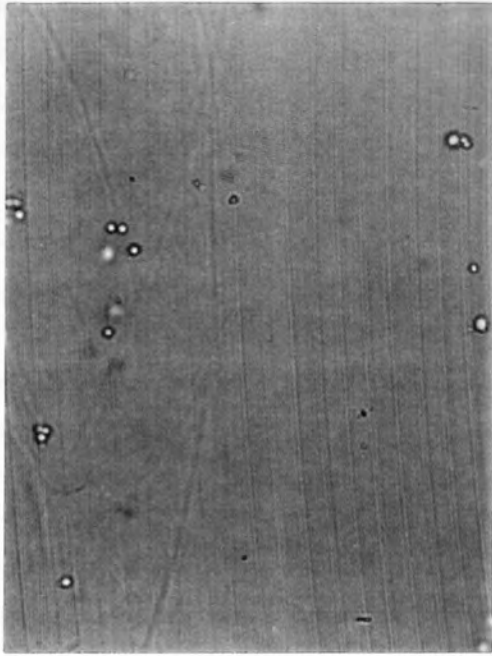
3.4 Contact lens protein deposits; a new approach to analysis

Contact lens wear is associated with changes in the composition of the tear film and build up of proteins and lipids onto the contact lens. Fibronectin (Fn) is a known constituent of the ocular environment (125) that presumably adheres to lens materials. Contact lens spoilage is usually attributed to nonspecific protein adsorption, but adhesion proteins such as Fn have binding sites for other proteins such as heparin, and could initiate protein build up. In addition to this, adsorption of Fn may provide an indicator for general protein adsorption. On this basis the adhesion of fibroblasts to a contact lens surface treated with serum or adhesion proteins *in vitro* gives an indication of lens deposition behaviour *in vivo*. In the first set of experiments described in this section, different lens materials were treated with 1 ml fibronectin (Fn) in phosphate buffered saline at 20µg/ml overnight. Worn lenses of the same composition were also obtained. A cell adhesion assay was carried out on these materials. This experiment generated a set of results that gave an indication of susceptibility to deposition of the lens materials and also gave rise to some questions about the proteins deposited on lenses *in vivo*. Cells adherent to worn lenses were characteristically round rather than spread, whereas cells adherent to Fn usually spread. What other adsorbed tear proteins from the ocular environment could be responsible for cell adhesion?

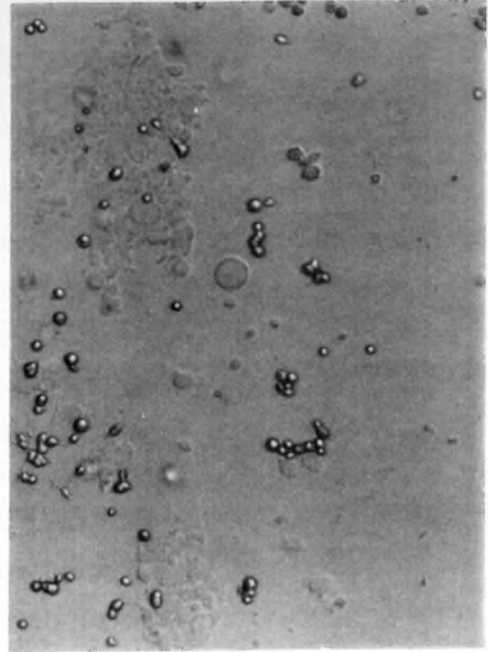
Complement proteins and plasmin are some of the components normally present in tears (Other constituents include lactoferrin, albumins and lysozyme). Levels of the former proteins are known to be raised in contact lens wearers (169). Complement proteins and plasmin are also linked to vitronectin (Vn) in other physiological systems. Whilst there are presently no reports of Vn adsorption to lenses, it is known that Vn levels in tears are raised during sleep (177). As Vn is a 65-75kD protein, it codistributes with albumin on electrophoretic analysis. As levels of Vn would be several orders lower than albumin most systems, Vn would be masked by typical analysis techniques. On the basis of a possible connection between Vn and raised plasmin levels in contact lens wearers, in addition to the appearance of cells adherent to worn contact lenses *in vitro*, the second set of experiments in this section describe cell adhesion assays in the presence of specific antibodies. Freshly obtained lenses worn daily for one week were examined for the presence of Vn and Fn, using a recently available effective polyclonal blocking antibody.

3.4.1 Results

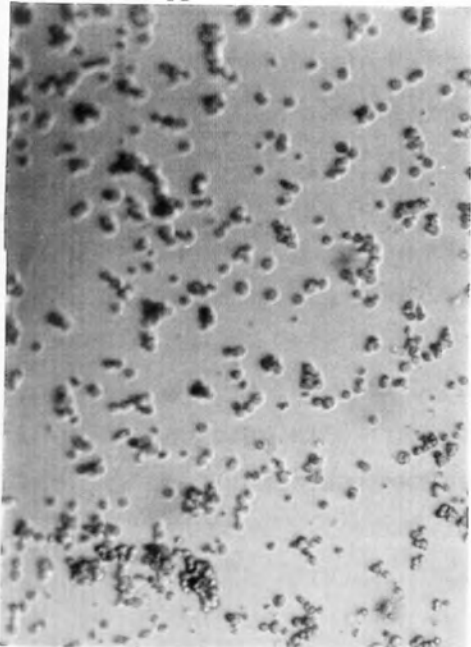
Cell adherence to two types of contact lens was assessed. Figure 13 shows the cell adhesion response to two lens compositions. G is a polyHEMA lens and AD is a PEO copolymer lens. Some lenses were precoated with 20µg/ml Fn as indicated in the figure 13. Variation in the cell adhesion levels to the samples was high. This could have been due to the curvature of the lenses as the lens pieces did not lie flat in the culture wells. In the photographs of figure 13, it can be seen that spreading of cells was greater on the polyHEMA ('G')lens,



A



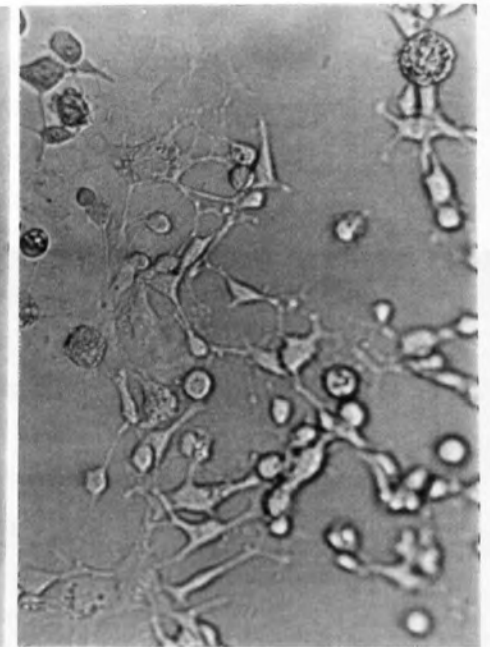
B



C



D



E

Figure 13 3T3 cell adhesion to a set of test lenses in the presence and absence of preadsorbed fibronectin . G is a HEMA based lens, whilst AD is a PEO including lens composition.

A Cells adhered to an unworn AD lens.x40

B Cells adhered to a fibronectin spoiled spoiled AD lens.x40

C Cells adherent to an AD lens that has been worn for one week on a daily basis and rinsed in phosphate buffered saline prior to cell adhesion in serum free medium.x40

D Cells adhered to an unworn G lens. x100

E Cells adhered to a fibronectin spoiled G lens.x100

and lower adhesion and spreading was noted on the PEO copolymer lenses. Preadsorption of material surfaces with Fn increased adhesion in all cases, but the most prominent result was that from a lens worn for one week. Cells were not well spread but had strongly adhered to this worn lens indicating the presence of deposited proteins despite the PEO content of the lens.

It was noted that worn contact lenses exhibited much higher cell adhesion than *unworn* contact lenses treated with serum *in vitro*. Furthermore, the adherent cells were not well spread on the worn contact lenses. This indicated strongly that an adhesion protein other than Fn was involved. Perhaps vitronectin (Vn) was involved? If this theory was correct, adhesion of fibroblasts to the lenses would be blocked in the presence of competing peptides, and a specific blocking antibody to Vn. Attempts to stain sections with monoclonal antibodies to Vn and Fn were unsuccessful due to problems commonly associated with hydrogels (nonspecific absorption of small molecules into the matrix of the gel). Vn is very difficult to remove once adsorbed. Only small quantities of this protein are necessary for cell adhesion so visual amplification by cell adhesion is an ideal method for measurement.

In the following experiments, worn contact lenses were rinsed and used in the 3T3 cell adhesion assay. Initial experiments have shown that adhesion to Fn and Vn can be successfully blocked by polyclonal antibodies (see results in chapter 4, figures 17 and 18). Figure 14A shows the effects of antibovine fibronectin (antiFn) and antibovine vitronectin (antiVn) on cell adhesion to worn Acuvue® lenses. Figure 14 B shows photographic evidence of the results. Cell adhesion is markedly reduced by antiVn and slightly less inhibited by antiFn. Figure 15A shows the effects of antihuman fibronectin and vitronectin on cell adhesion to whole worn Acuvue® lenses. Figure 15B shows photographic evidence of the results. Once again, cell adhesion is strongly inhibited by antiVn and slightly less inhibited by antiFn. These anti human antibodies are marginally more effective at blocking adhesion than the antibovine antibodies.

It is apparent from these blocking experiments that Vn and Fn are important proteins adsorbed to lenses from the ocular environment. There was inhibition by both antivitronection and antifibronectin antibodies. This indicates that either Fn and Vn are cooperative in the adhesion of cells to the polymer or that there is a cross reactivity in the antibodies for Vn and Fn. The control IgG did not inhibit adhesion to the lens, so the inhibitions are specific, and not merely the blocking actions of nonadhesive IgG protein.

The inhibition of adhesion by both antiVn and antiFn may seem anomalous, until the reader considers the explanation of integrin function given in the introduction. Integrins may be 'promiscuous', in that many receptors can interact with several adhesion proteins. In this case, cell receptors responsible for 3T3 adhesion interact with either, or both, Vn and Fn. For this reason, antibodies to either of these proteins have an inhibitory effect on cell adhesion. It is not possible to separate the effects of Vn and Fn mediated adhesion.

As outlined in the introduction to this section, complement proteins and plasmin are tear constituents known to be raised in contact lens wearers. Both of these proteins are known to

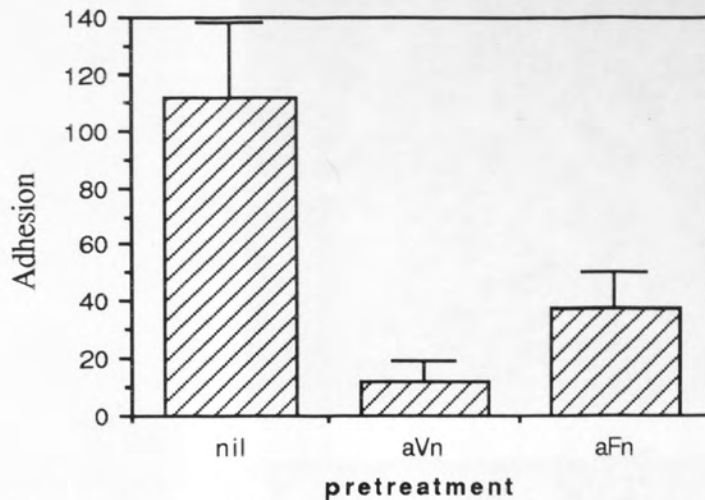
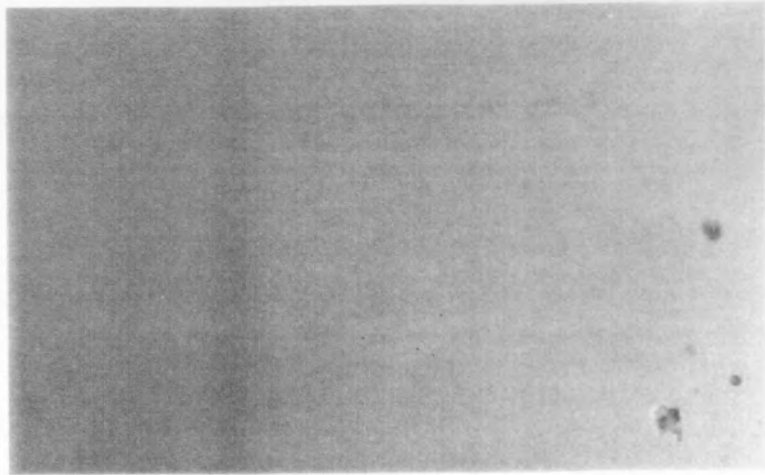


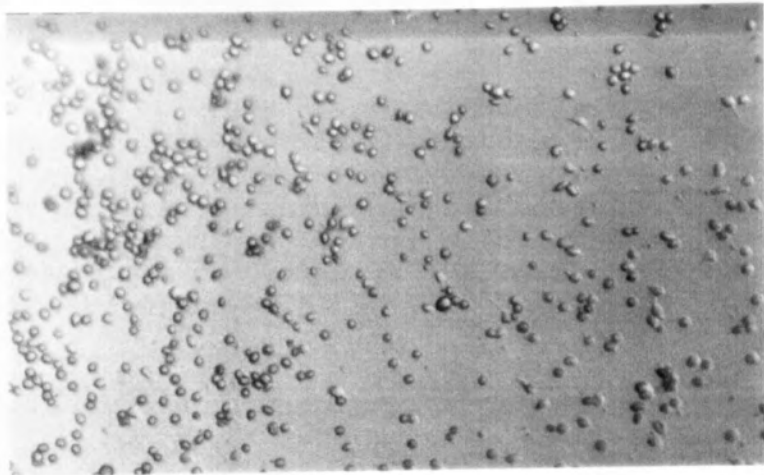
Figure 14A 3T3 cell adhesion to worn Acuvue® lenses in the presence of rabbit-anti bovine vitronectin and fibronectin at x10 dilution. The lenses were cut into four equal sections and incubated with 3T3 cells for one hour. The control sections 'nil' were treated with non immune IgG (Cohn fraction V, Sigma) at 300µg/ml. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different sections (16 fields). The error bars represent standard deviations.

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1) aVn



2) control



3) aFn

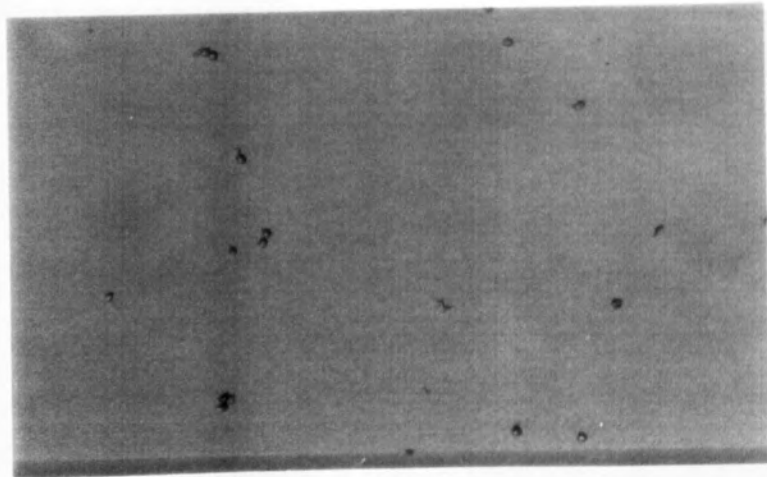


Figure 14B Acuvue® lenses were cut into 4 sections and treated with either control IgG (2) or rabbit anti-bovine vitronectin (1) or rabbit anti-bovine fibronectin (3). 3T3 cell adhesion to the lenses was counted (previous figure) and photographed. x40

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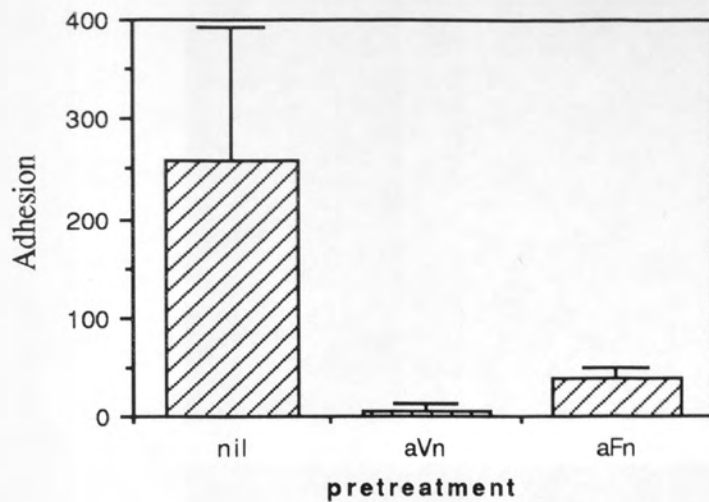
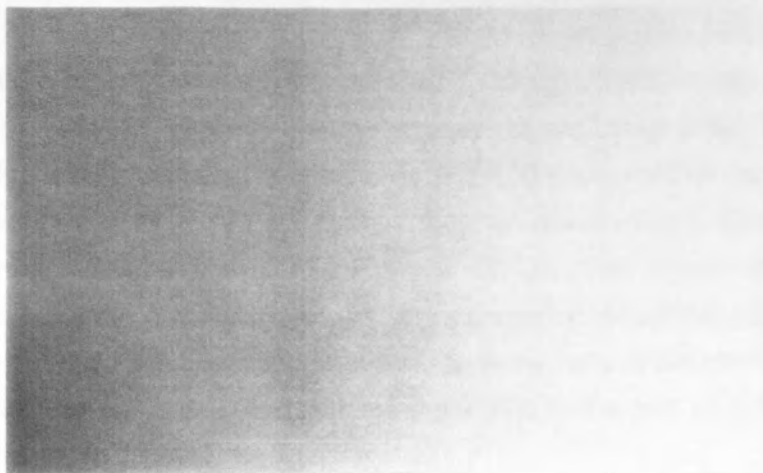
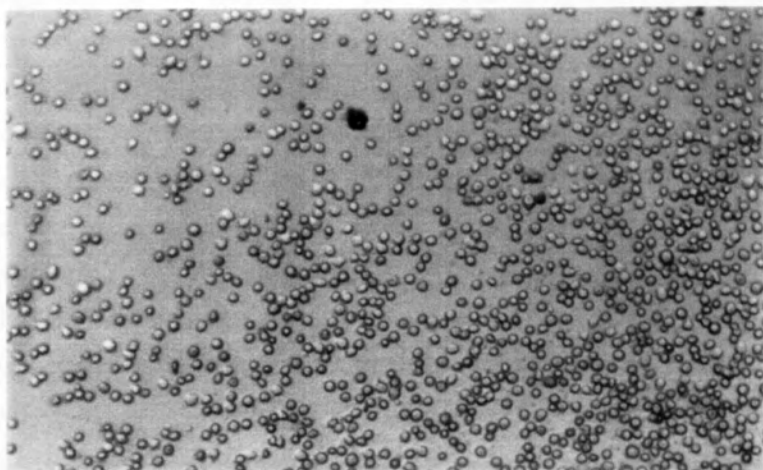


Figure 15A 3T3 cell adhesion to worn Acuvue® lens in the presence of rabbit-antihuman vitronectin and fibronectin. Whole lenses were incubated with 3T3 cells for one hour. The control sections 'nil' were treated with non immune IgG (Cohn fraction V, Sigma) at 300µg/ml. The adhesion index refers to cell counts. Cell numbers were assessed by cell counts of one field at x20 magnification. Results represent an average of 4 readings from each of 4 different samples (16 fields). The error bars represent standard deviations.

1) aVn



2) control



3) aFn

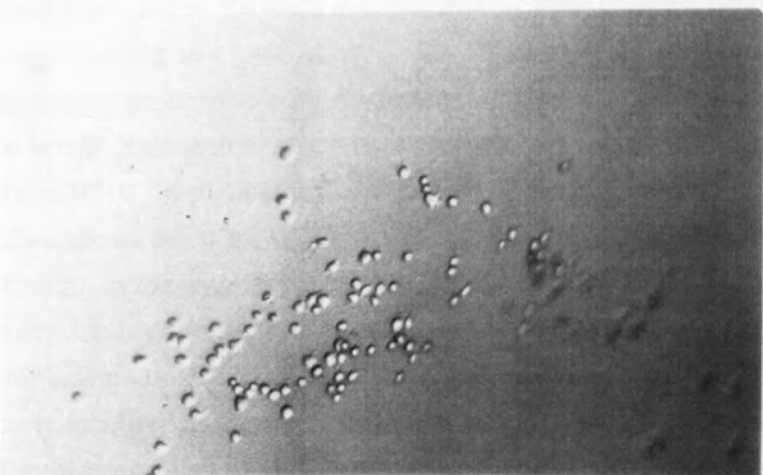


Figure 15B Whole Acuvue lenses were treated with either control IgG (2) or rabbit antihuman vitronectin (1) or rabbit antihuman fibronectin (3). 3T3 cell adhesion to the lenses was counted (previous figure) and photographed.x40

be involved with Vn in other physiological systems. Complement is an essential system of proteins involved in the immune response. Vn binds to complement proteins C5b7-9, preventing the formation of terminal cell lysis complex. This exposes the usually cryptic heparin binding site of Vn which probably leads to its adsorption to injury sites. The immobilised 'Vn C5b complex' is adhesive to cells via the RGD sequence (for example to the polymorphonucleocytes attracted by earlier stages of complement activation). Corneal 'immune rings' sometimes occur in contact lens wearers. Opaque rings form in the cornea as a result of infiltration by polymorphonucleocytes. The presence of these opaque rings in the cornea indirectly result from complement activation (170). In the light of the results obtained from the experiments described in this section, it is suggested here that lens adsorbed Vn may well be an initiating factor in this process.

Plasmin is a serine protease that digests proteins including adhesion proteins like Fn. Possible control mechanisms of plasmin levels were also discussed in the introduction in section 1.32. Fn is an important part of the healing process in the damaged cornea enabling corneal cells at the periphery of an injury to migrate across the defect. Plasmin levels are usually low immediately after injury which presumably prevents digestion of the Fn. Plasmin levels are high in contact lens wearers (169). Plasmin is released by the action of plasminogen activator (PA) on plasminogen which is in turn, inhibited by plasminogen activator inhibitor (PAI). PAI is bound in its active state to the somatomedin B homology region of Vn. (PAI in solution degrades very rapidly). Vn bound PAI prevents digestion of proteins by plasmin type proteases. Thus, it would appear that the presence of lens bound Vn/PAI would prevent *digestion of other lens associated proteins resulting in protein buildup*. However, it is known that preliminary plasmin digestion of Vn leads to a loss of PAI binding and a two fold increase in plasminogen binding, resulting in a local upregulation of plasmin. In other words, proteolysis would be promoted extensively by the presence of lens adsorbed Vn. High plasminogen activator levels peripheral to injury sites are implicated in the pathogenesis of persistent corneal defects (171). To parallel this observation of the eye, chronic wound fluid from venous ulcers often shows complete degradation of Vn and Fn into smaller peptides that prevents local cell adhesion to the wound bed, preventing closure (172). As the normal plasmin upregulation mechanism usually occurs in the absence of foreign materials, plasmin eventually digests proteins into non functional units and the process of upregulation ceases. The process is also fairly localised as there are cellular receptors that bind the various components of the plasmin control system close to injury sites (173, 174, 175). In the presence of surface adsorbed Vn, the process of upregulation may well be extended, perpetuating high levels of plasmin. Furthermore, Fn, also digested by plasmin, yields fragments that are known to induce collagenase production in cartilage (176). It is possible that the same mechanism may occur in the stroma of the cornea.

As a result of the experiments described in this section, it is proposed that Vn is a constituent of the ocular environment that is adsorbed onto contact lenses. This adsorption may be correlated with elevated levels of adsorbed complement proteins and alterations to the

plasmin regulation system found in contact lens wearers. If persistent corneal proteolysis in particular patients is associated with high levels of Vn bound PAI adsorbed to the lens, as suggested here, materials with a reduced affinity for Vn should be developed. Alternatively, supplementary therapeutic treatments could consider blocking PAI binding to Vn. This could be achieved by introducing an excess of somatomedin B into the tears.

To summarise, cell adhesion assays in conjunction with specific antibodies to adhesion proteins have demonstrated the presence of vitronectin and fibronectin on worn Acuvue lenses. These conclusions can be summarised as follows:

- a) Cell adhesion to worn lenses was inhibited by polyclonal antibodies to Vn and Fn.
- b) Vn and Fn are adhesion proteins adsorbed to lenses from the ocular environment.

It is suggested that adsorption of Vn to the lens surface may be correlated with prolonged activation of proteolysis and complement activation at the corneal surface.



Key points

- 1) Fibroblast adhesion to surfaces is influenced by incubation time and the presence of serum.
- 2) Polyethylene oxide pendant chains substituted into the backbone of a polymer increase resistance to cell adhesion and protein deposition. Methoxy terminated polyethylene oxide chains are more effective than hydroxy terminated chains. Resistance to adhesion increases with increases in chain length.
- 3) Cell adhesion assays can provide a useful assessment of polymer composition, demonstrated here with regard to alterations in sequence distribution in polymers
- 4) Cell adherence to worn contact lenses can be blocked by antibodies to adhesion proteins. Vitronectin is an important adhesion protein that adsorbs to contact lens materials. This may be responsible for the perpetuation of high levels of proteolytic enzymes in contact lens wearers.

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Chapter 4

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4.1.1 The role of divalent cations in the cellular adhesion mechanism

In integrin mediated adhesion to surface described adhesion proteins, the presence of divalent cations in the cytosol are essential. Current theory suggests that these cations bind to the cytosolic binding sites on the β tail of the integrin [1]. It is not clear if all of these sites must be occupied for adhesion to occur. The cytosolic binding site is composed of the

4.1 Introduction

In this chapter, the fundamental mechanisms of cell adhesion are examined with reference to the divalent cation requirement (see Figures 1, 2). These investigations constitute explorations of cellular integrin function, an understanding of which is essential for the development of new lines of biomaterials.

The mechanism of adhesion is explored in this chapter in the context of known facts that can be tested in a simple system. Firstly, the adhesion protein governing cell adhesion to serum coated surfaces will be established, and then a sequence of single cations will be introduced to examine their effect on adhesion. Combinations of cations and adhesion proteins will be examined in order to build adhesion profiles for different cell types.

Divalent cations have an essential and regulatory role in the binding of cell surface integrins to adsorbed adhesion proteins^(40,41). The effect of any particular cation depends on the integrin type and the presence of competing cations, as well as on the conformation of the adsorbed adhesion protein. These effects are still being explored, as few cell types have been characterised for integrin type and other regulatory mechanisms are not completely understood. In the course of these investigations, a chance observation during a preliminary experiment led to the discovery that physiological zinc concentrations modulate the adhesion to Vn which is catalysed by other divalent cations. Zinc has not previously been implicated as an important regulatory cation for integrin function and this discovery may partly explain the promotion of wound healing by classical zinc based preparations. It also adds to the body knowledge which may lead to an understanding of the way in which divalent cations are coordinated in the integrin binding sequence.

How can an understanding of divalent cation regulation aid biomaterials design? As available data increases in this field, divalent cation effects could be used to characterise integrin type. The inclusion of leachable divalent cations into polymers could provide a novel basis for cell separation, and aid cell integration into prostheses. For example, it may be possible to encourage preferential epithelial cell growth at a rapid rate onto vascular grafts, and encourage osteoblast adhesion to bone cements. Such techniques would be valuable for their simplicity and relatively low cost. Lastly, an appreciation of the different ionic environments within the body and the implications for cell adhesion processes may aid materials design.

4.11 The role of divalent cations in the cellular adhesion mechanism

In integrin mediated adhesion to surface adsorbed adhesion proteins, the presence of divalent cations in the medium are essential. Current theory strongly suggests that there are three or four cation binding sites on the α unit of the integrin⁽⁴¹⁾. It is not clear if all of these sites must be occupied for adhesion to occur. The cation is only partially coordinated by the

binding cleft, which lacks an aspartate (D) residue in the 12 position. The coordination of the cation is completed by the D residue of the recognition sequence on the appropriate adhesion protein. It is also thought that binding of cations activates the integrin. Perhaps a critical number of binding sites must be occupied for full activation (42,43).

Divalent cations are also regulatory for adsorbed adhesion proteins. Thrombospondin for example is more adhesive in the absence of calcium (118) whereas the J1 neuronal glycoproteins J1-160 and J1-180 are antiadhesive in the absence of cations and incubation with zinc neutralises this effect (117). These effects are related to the structure of the adhesion protein rather than the donation of the cation to the integrin binding site.

Which divalent cations are important? Magnesium (Mg), manganese (Mn) and calcium (Ca) are well recognised for their promotion of receptor-ligand binding in the adhesion of live cells and in cell free systems. The binding of Mg and Mn seems to correlate with the active form of many integrins, whereas Ca, for example, has been shown to regulate Mg catalysed binding at physiological concentrations (178, 179).

Mn seems to universally promote binding regardless of integrin type, and can activate receptor function at very low concentrations (40, 180-184). It is not totally unique in this respect as Ca can activate the Fn receptor in BHK cells at very low levels (183). Mn at high levels has also been shown to promote binding to the "nonadhesive" albumin coated substrata (184). The reasons for the effectiveness of Mn are unclear, as Mn does not have any radically different physical properties to other divalent cations. A recent example of the effects of Mn was presented by Yanai et al, (185) who showed that ligand specificity is overridden by Mn catalysed binding. In the presence of Ca or Mg, Fn placental receptor bound to RGD sequence of Fn but not to the RGD sequence in Vn. The Vn receptor bound to Vn but not Fn. In the presence of Mn however, Vn receptor would bind to Fn RGD region. If Mn was replaced by Ca or Mg, the binding was lost. Mn is found in high levels in tears (186). The presence of Mn in the ocular environment could relate to the very rapid healing activity of the cornea, when epithelia migrate onto damaged areas where a high level of Fn is present.

4.2 Results

A variety of cations were tested in the adhesion of a variety of cell types to serum preadsorbed tissue culture plastic surfaces. A HEPES buffered glucose containing saline was chosen as the base medium to ensure solubility of the various salts. All cations were introduced as the chloride salt. Adhesion experiments were carried out as described in Chapter 2. Briefly, the amount of adhered cells is quantified by staining, elution and reading at 550nm of a highly sensitive protein binding dye. The assay can be carried out in a 96 well plate, which allows statistically robust sample numbers to be assessed.

It has been pointed out that incubation of cultures in a CO₂ atmosphere can lead to precipitation of carbonates of heavy metal salts. The low levels of Zn used in these

experiments did not lead to precipitation during incubation in an atmosphere containing 5% CO₂.

4.2.1 Can adhesion be blocked by adhesion peptides?

Divalent cation catalysed adhesion is thought to be a result of integrin ligand binding. In order to test this assumption, peptides containing the RGD sequence, known to compete for the integrin binding site, were introduced into the culture medium (HBS+5mM MgCl) at 1.2 mg/ml during the adhesion of 3T3 cells to dialysed serum coated surfaces. Figure 16 shows the results of this experiment. GRGDS almost completely abolishes adhesion, whereas GRGES has slightly less effect. This provides good evidence for integrin based adhesion to an RGD containing adhesion protein.

4.2.2 Is vitronectin or fibronectin involved?

Various studies suggest that Vn is the probable adhesion protein involved in initial adhesive interactions onto serum adsorbed polystyrene surfaces. In order to test this assumption, polyclonal rabbit anti-bovine Vn and polyclonal rabbit anti-bovine Fn antibodies were incubated with the serum coated or Vn coated surfaces for 1 hour at 37°C. After rinsing the surfaces, 3T3 adhesion was assessed in the presence of 5mM Mg or Mn. Results are shown in Figure 17 A and B. Adhesion was almost completely abolished by a x10 dilution of the antiVn antibody, and severely inhibited by x50 dilution on both serum adsorbed and Vn adsorbed surfaces. AntiFn antibody had very little effect on adhesion to serum adsorbed surfaces but strongly inhibited adhesion onto Fn adsorbed surfaces at x10 dilution as shown in figure 18.

4.2.3 What effect do cations have on adhesion in a serum free system ?

Adhesion of cells to polystyrene (Ps) and tissue culture plastic (TCP) was carried out in serum free conditions to examine the effect of various cations on physicochemical adhesion between cells and surface. For the cations tested, on polystyrene there were few differences in the amount of adhesion. These results can be seen in figure 19A. This can be directly compared with figure 19B which shows the effects of cations on cell adhesion to dialysed serum adsorbed TCP 96 well plates.

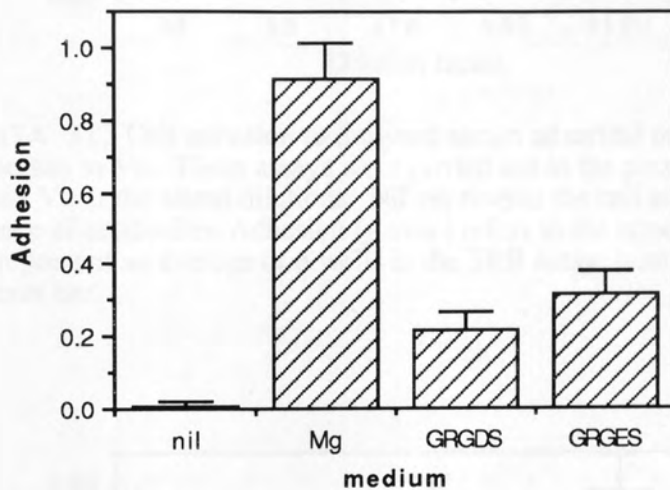


Figure 16 3T3 cell adhesion to dialysed serum coated TCP 96 well plates can be blocked by adhesion peptides. Although 'RGD' is purported to be the active adhesion peptide, 'REG' also had an inhibitory effect. This could be due to a high concentration of the peptide in the medium. Peptides (GRGDS and GRGES) were added at 1.2mg/ml (after Piersbacher et al (120)) in the presence of 5mM Mg. 'Mg' shows cell adhesion to serum in the absence of peptides. 'Nil' shows the adhesion to serum in the absence of Mg and of peptides. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

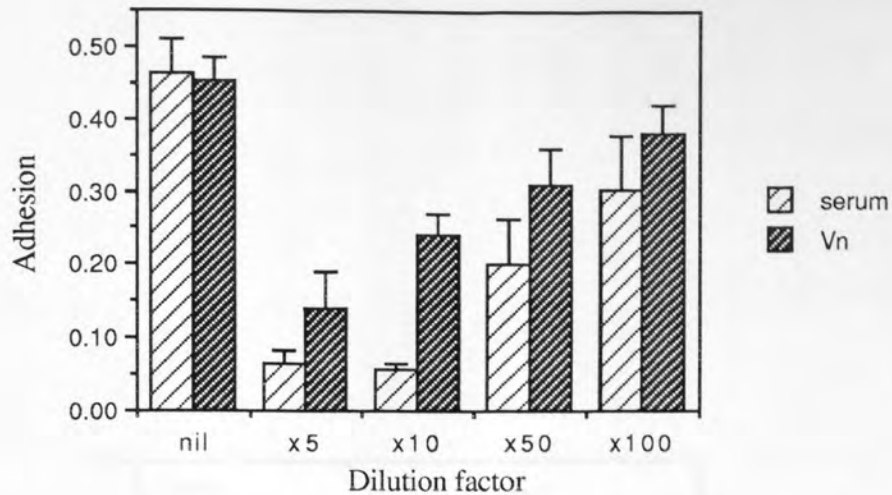


Figure 17A 3T3 Cell adhesion to dialysed serum adsorbed or Vn coated TCP can be blocked by antibodies to Vn. These assays were carried out in the presence of 5mM Mg and rabbit antiovine Vn at the stated dilutions. 'Nil' represents the cell adhesion to the coated surfaces in the absence of antibodies. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

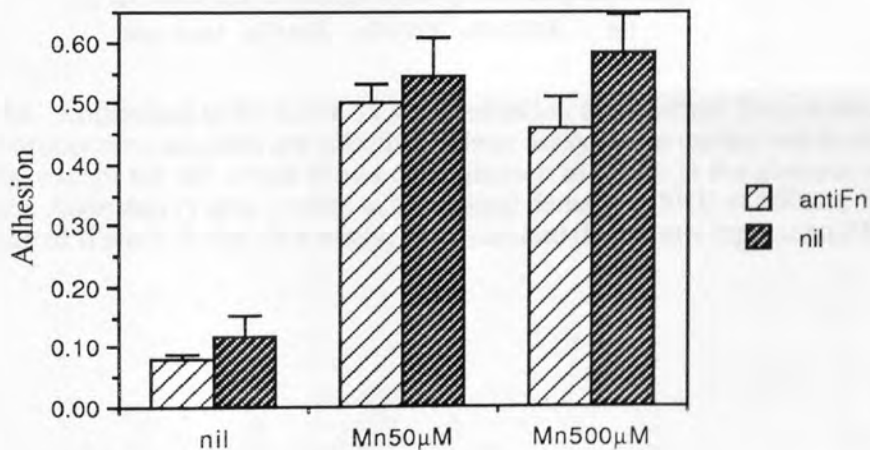


Figure 17B Antifibronectin antibodies did not block adhesion of 3T3 cells to dialysed serum coated surfaces. In fact a small amount of inhibition (significant $P < 0.05$) is seen where Mn is used at 500µM. This assay was carried out in the presence of two concentrations of Mn and rabbit antiovine Fn at x10 dilution. 'Nil' represents cell adhesion to serum in the absence of cations. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

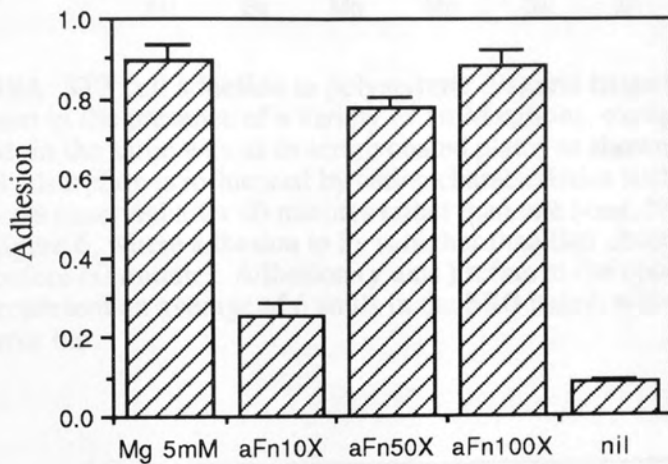


Figure 18 Antibodies to Fn block 3T3 cell adhesion to adsorbed fibronectin, showing that the antifibronectin antibodies are effective. These assays were carried out in the presence of 5mM Mg, except for 'nil' which shows cell adhesion to serum in the absence of Mg and of antibodies. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

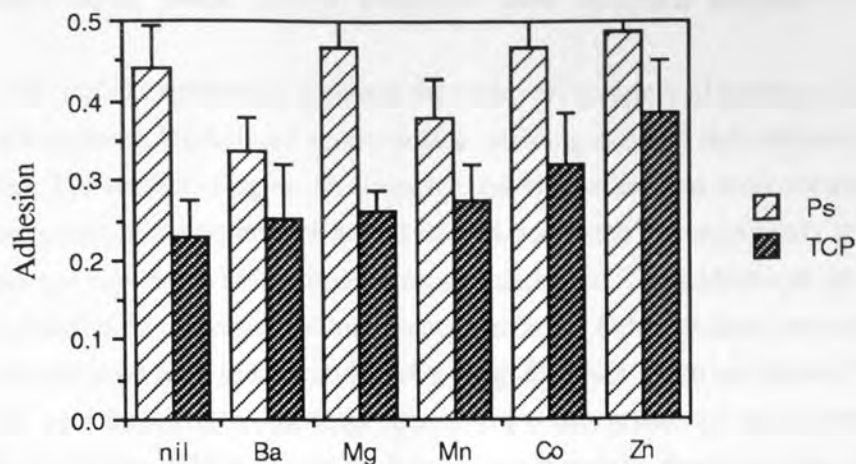


Figure 19A 3T3 cell adhesion to polystyrene (Ps) and tissue culture plastic (TCP) in a serum free system in the presence of a variety of 1mM cations, except for 'nil'. Adhesion was not promoted in the same way as to serum coated plates as shown in figure 19B. This physico chemical adsorption is influenced by cation characteristics such as charge density. These results were observed after 40 minutes rather than one hour. Note that similar results can be seen in figure 6, where adhesion to Ps is higher than that observed on TCP in a serum free system before 60 minutes. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

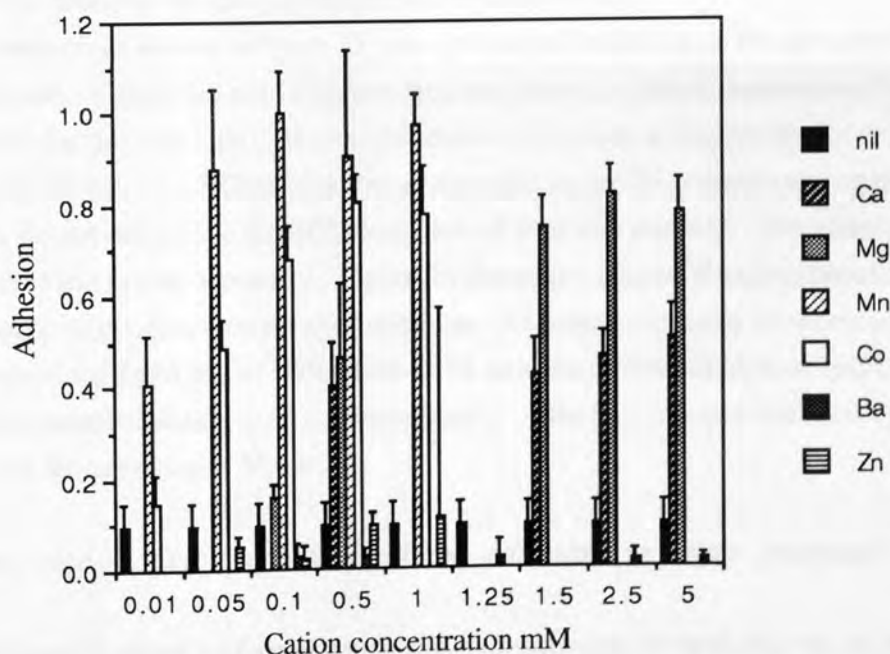


Figure 19B 3T3 cell adhesion to dialysed serum coated TCP plates in the presence of a variety of cations. The adhesion is catalysed most effectively by Mn, followed by Co, Mg, Ca. Ba and Zn have no effect, other than slightly lowering the base level in the case of Ba. Cadmium was proven to be toxic even at very low levels and is not included in these results. All the ions tested above were not toxic after one hours incubation. Viability was >95% in all cases. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

4.2.4 How does whole serum compare with dialysed serum?

The initial experiments assessed the effect of a variety of cations at 500 μ M onto serum preadsorbed plates. Undialysed serum (still containing cations) and dialysed serum were compared. The results in figure 20 show that on the undialysed serum coated plate, cell adhesion occurs in the absence of added cations due to the cations already present in the serum. This does not happen on the dialysed serum coated plate. The addition of Zn causes a dramatic loss of adhesion on undialysed serum coated surfaces. Other cations promote adhesion in the following order on dialysed serum Mn>Co>Mg>Ca>>Zn (also see figure 19B). Barium and cadmium were included in other experiments for which results are not shown. Barium has no effect and cadmium inhibits adhesion, but this could well be due to toxicity of the latter cation.

4.2.5 Do other cell types respond in the same way?

Other cell types, L929 fibroblasts and primary dedifferentiated chondrocytes, showed similar adhesion profiles to 3T3 cells. Zinc did not promote adhesion as a single cation and furthermore, inhibited the adhesion promoted by other divalent ions. Figures 21 and 22 show this effect in porcine chondrocytes and L929 cells (Note that calcium did not promote adhesion in the chondrocytes. Further effects of this cation were not explored, although they are of direct interest to the study of the chondrocyte cell type).

However, as shown in figure 23, zinc *promoted* adhesion of the monocyte line J744.2 whereas calcium did not (Calcium was not shown to inhibit promotion of adhesion by other cations for this cell type). Monocyte adhesion to serum, catalysed by Zn or Mg, was only slightly inhibited by RGDS peptides as shown in figure 24, demonstrating that these monocytes do not adhere via the RGD sequence of adhesion proteins. The adhesion is, however, divalent cation dependent. Figure 25 shows the effects of antivitronectin and antifibronectin antibodies on monocyte adhesion. As monocytes have Fc receptors, the medium contained 5mM Mg or 100 μ M Zn and a saturating concentration of IgG (300 μ g/ml) to block nonspecific binding to Fc on the antibody. Adhesion was not inhibited by the antibodies in the presence of Mg or Zn.

4.2.6 Does zinc inhibit integrin mediated adhesion to other proteins?

Collagen I coated surfaces (where adhesion may also be mediated via an RGD sequence) were used in the adhesion assay with 3T3 cells. In this case Zn (figure 26A) promoted adhesion as a single cation and had an additive affect on adhesion catalysed by other cations. Calcium, however, inhibited adhesion to collagen and has been shown to inhibit adhesion catalysed by Mg and Ca as shown in figure 26B. High cell adhesion in the absence of cations ('nil') may be attributable to residual divalent cations in the collagen I preparation (227).

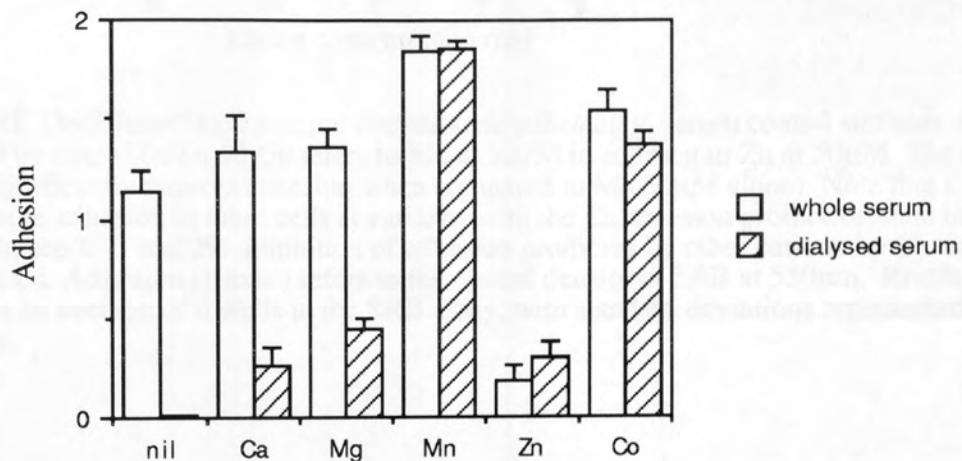


Figure 20. 3T3 Cell adhesion to surfaces adsorbed with dialysed serum or whole serum in the presence of a variety of cations at 500µM except for 'nil'. Note that adhesion occurs in the absence of added cations in the whole serum coated surfaces ('nil') due the presence of cations in the adsorbed serum. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

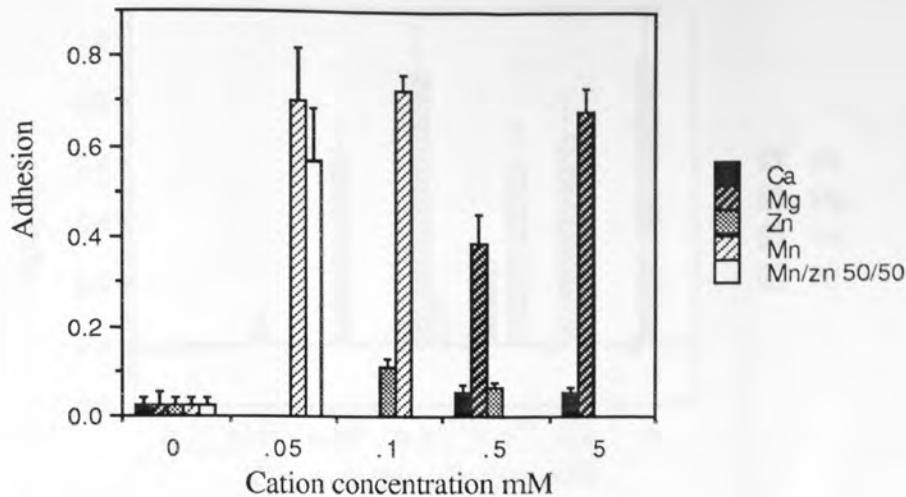


Figure 21 Dedifferentiated porcine chondrocyte adhesion to serum coated surfaces is also inhibited by zinc (Mn/Zn 50/50 refers to Mn at 50 μ M in addition to Zn at 50 μ M. The inclusion of Zn significantly reduces adhesion when compared to Mn 50 μ M alone) Note that Ca does not promote adhesion in these cells at variance with the Ca adhesion promotion seen in 3T3 cells in figure 19B and 29. Inhibition of adhesion promoted by other cations by Ca was not investigated. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

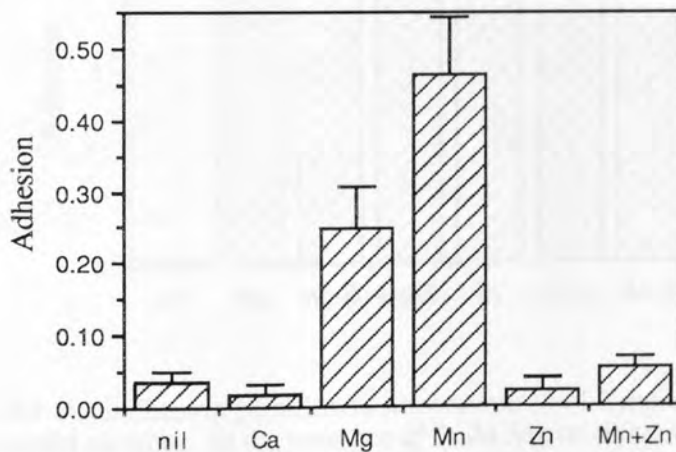


Figure 22 L929 fibroblast adhesion to serum coated surfaces catalysed by Mn is also inhibited by Zn. In this experiment, Ca, Mg are at 5mM, Mn and Zn are used at 100 μ M. Adhesion was very much lower for this cell type than for 3T3 cells generally after 1 hour incubation but the inhibition of Mn catalysed adhesion by Zn is still clear. Note that Ca does not catalyse the adhesion of this cell type, in contrast to the 3T3 cell line seen in figures 19B and 29. Also see graph 21 above, where no Ca promoted adhesion is seen. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

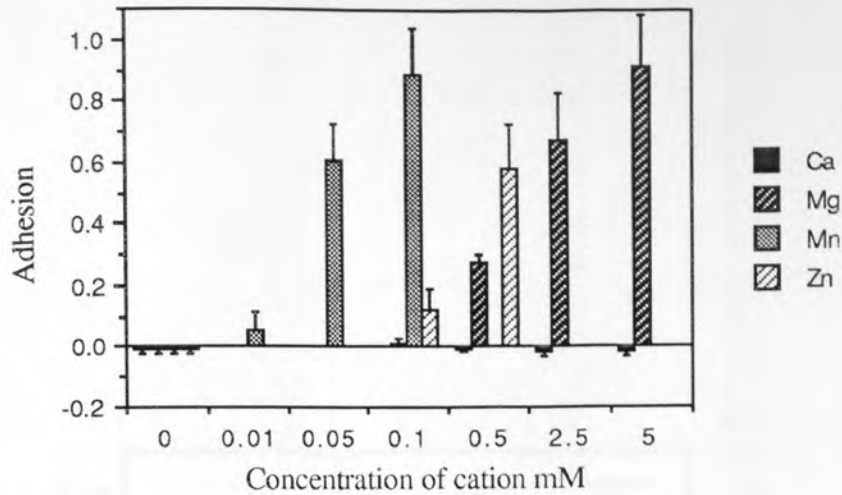


Figure 23 J744.2 monocyte adhesion to serum coated surfaces is promoted by Zn. Ca does not promote adhesion but does not inhibit adhesion promoted by Mn, Mg or Zn (results not shown) Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

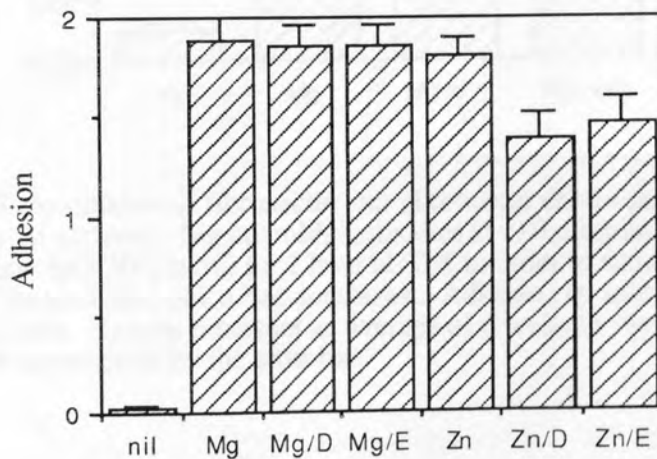


Figure 24 Antiadhesion peptides do not prevent J744.2 monocyte adhesion to dialysed serum coated surfaces. In the presence of 5mM Mg or 100 μ M Zn and either GRGDS (Mg/D and Zn/D) or GRGES (Mg/E and Zn/E). Note that no adhesion occurs without cations present. Very high results for J744.2 here represent an initially high concentration of plated cells. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

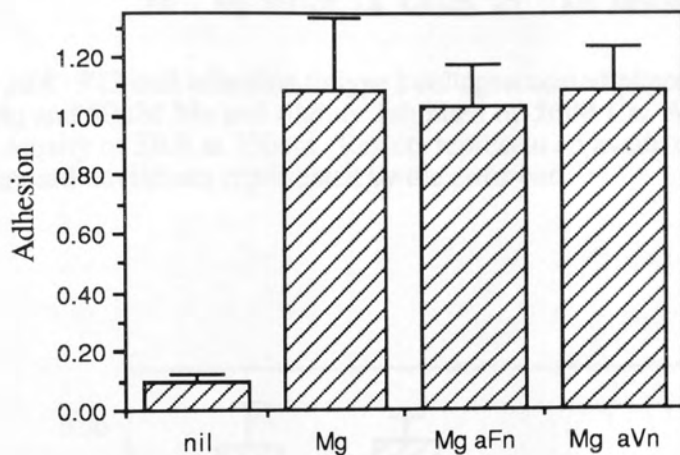


Figure 25 Antibodies to fibronectin and vitronectin do not prevent the J744.2 adhesion to serum coated surfaces. Mg at 5mM, antibodies at x10 dilution. Cells were preincubated with non immune IgG(300µg/ml) for 1 hour at 37°C in order to saturate the Fc receptors. Similar results in the presence of Zn are not shown. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

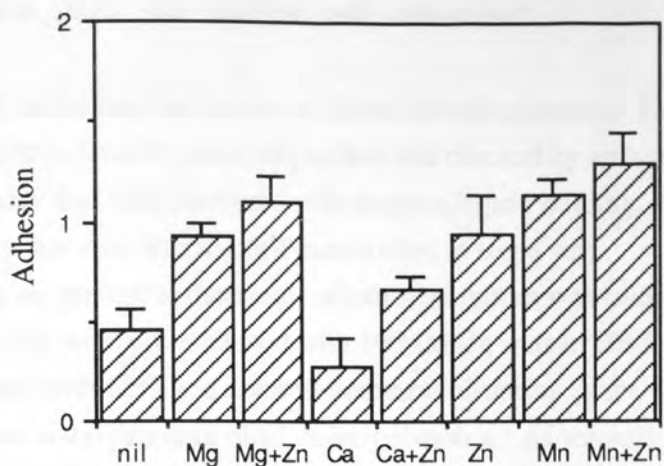


Figure 26A 3T3 cell adhesion to type I collagen coated plates is promoted by 100 μ M Zn, 5mM Mg and 50 μ M Mn and slightly inhibited by 5mM Ca. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

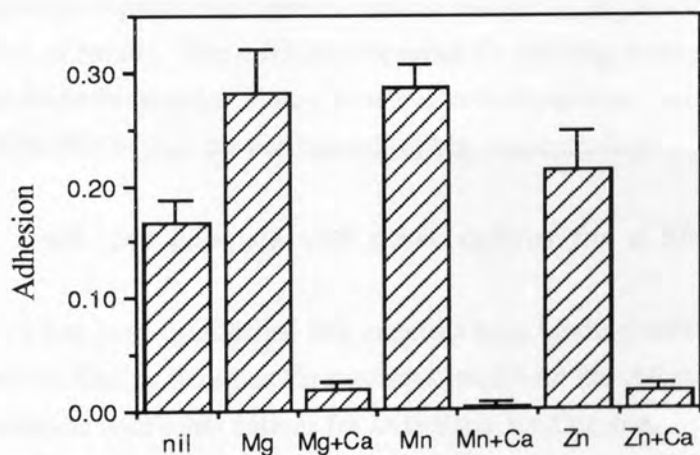


Figure 26B Adhesion of 3T3 cells to type I collagen coated plates. This assay was carried out with a reduced number of cells which accounts for the relatively low results obtained. However, the effects of 5mM Ca in addition to either 5mM Mg or 50 μ M Mn or 100 μ M Zn can be clearly seen. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

4.2.7 How does zinc inhibit cell adhesion?

By what mechanisms does Zn inhibit cell adhesion? Having established that adhesion of 3T3 cells is divalent cation dependent and blocked by antivitronection antibodies, it seemed highly likely that zinc interfered with integrin ligand binding. However it remained a possibility that zinc affected cell metabolism in some way.

In an attempt to determine whether inhibition was caused by Zn associated with the cell surface, cells were preincubated with 100 μ M Zn ions in HBS for 20 minutes at 37°C, and then treated with EDTA, a non cell permeant chelating agent, for 5 minutes at 37°C, before plating out in the presence of adhesion promoting cations such as Ca, Mg and Mn, as outlined above. Figure 27 shows the results of this experiment.

Chelation with EDTA after preincubation in the presence of Zn, reversed the inhibitory effects of Zn when cells were then plated in the presence of other cations. This was an important result, as it showed that Zn was associated with the cell surface; it could be removed by a chelator that could not enter the cell. This experiment also showed that Zn was associated with the cell surface prior to interaction with the surface adsorbed serum proteins. This further implied that Zn may be interacting with binding sites on a cell surface integrin. Competition experiments were carried out using Ca, Mg, and Zn. Figures 28A and B show a selection of results. The difference between Ca and Mg in response to competition with Zn are evident from the graphs. Note that at low concentrations, Ca is more resistant to the Zn inhibition effects than the corresponding Mg concentration.

4.2.8 Does zinc compete with other cations for a binding site ?

It has been established that integrins have binding sites for divalent cations (40,41). In view of the finding here that Zn was associated with the cell surface, it was suggested that Zn may compete with other cations for an integrin binding site.

A timed assay was performed with varying concentrations of Mn and three different concentrations of Zn. These results were plotted by the Lineweaver Burke method as described in chapter 2. This method is commonly used to analyse substrate competition for binding sites of enzymes. Cell adhesion was correlated with 'product' and the cation with 'substrate'. These experiments yielded a set of results that strongly imply direct competition for a binding site, i.e. competition for the same or mutually exclusive sites. If the Zn was associated with the cell surface in another manner, it would be unlikely to produce this set of results. Figure 30 shows the final Lineweaver Burke plot.

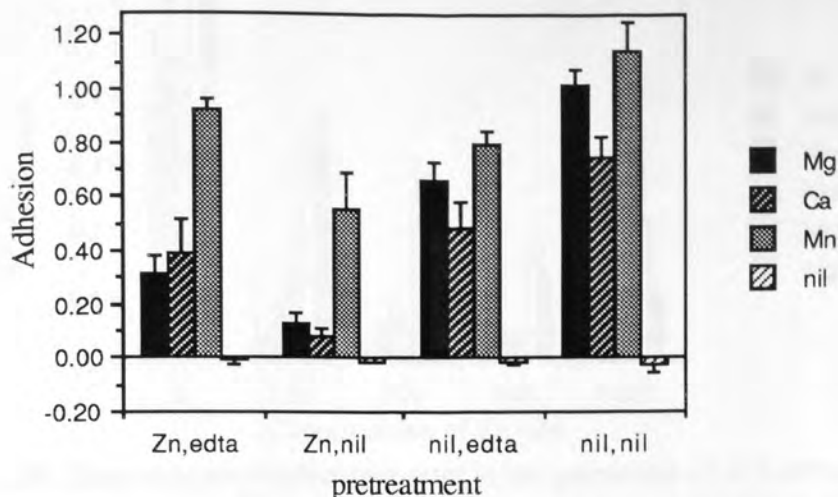


Figure 27 Pretreatment with non cell permeant chelator EDTA shows that zinc is associated with the cell surface.

Cells were trypsinised and separated into four treatment groups. The cells were treated with either 1mM Zn ('Zn') or non cation containing HBS ('nil') for 20 minutes at 37°C. The cells were then either treated for 5 minutes with EDTA ('edta') or HBS ('nil') for 5 minutes at 37°C. The cells were then resuspended in HBS containing 5mM Mg or Ca or 50µM Mn or HBS with no cations and plated onto dialysed serum coated TCP 96 well plates for one hour. Adhesion was assessed by the SRB method

The cells treated with Zn show lower adhesion than the cells not treated with Zn. The cells treated with EDTA after Zn incubation show higher adhesion than the cells treated with Zn alone. This shows that the removal of Zn by the non cell permeant chelator EDTA has returned adhesive properties to the cell. This implies that Zn is associated with the cell surface, and that this surface associated Zn inhibits adhesion in a manner that can be returned by other divalent cations. Note that EDTA has some membrane disrupting effects so that in the group not treated with Zn, EDTA treatment has reduced adhesion. This is because EDTA is a relatively nonspecific chelator and has removed other cations essential for membrane function related to adhesion. Cell viability was >90% at the end of this experiment. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

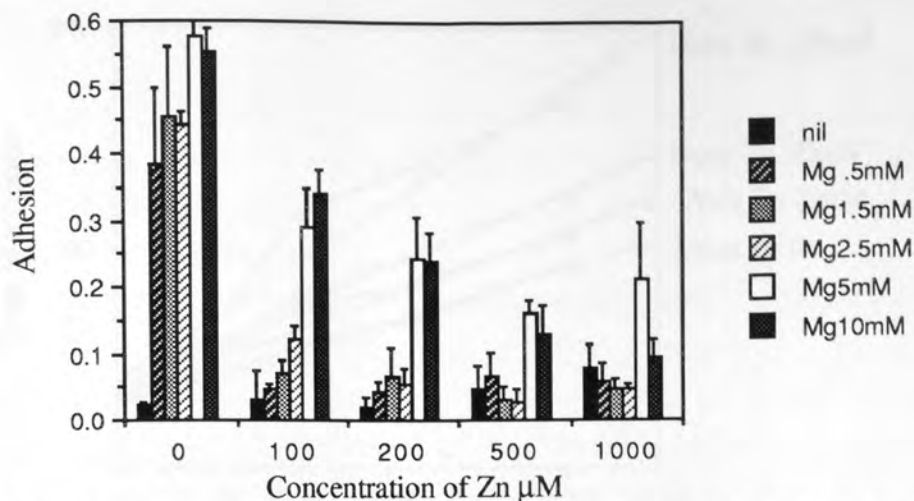


Figure 28* Zinc competes with magnesium in the promotion of 3T3 cell adhesion to dialysed serum coated surfaces. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

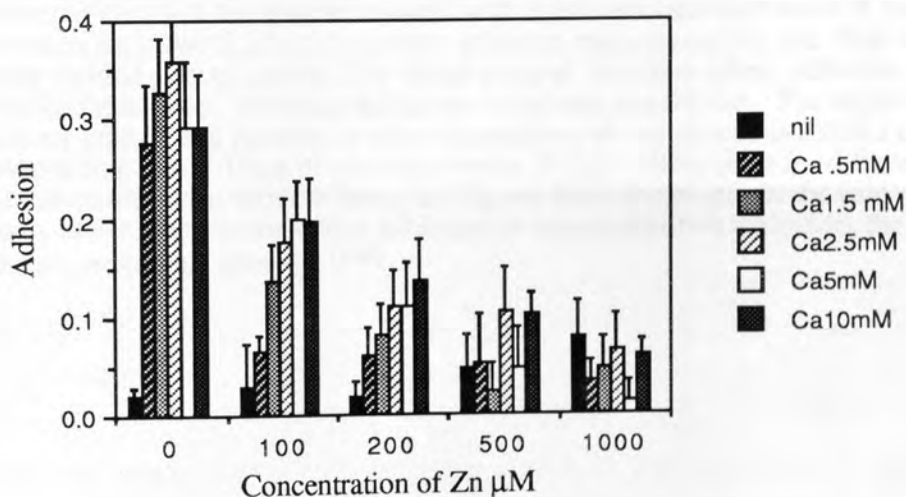


Figure 29* Zinc competes with calcium in the promotion of 3T3 cell adhesion to dialysed serum coated surfaces. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.

* Figures 28 and 29 should be compared with each other. Experiments were carried out with the same lot of cells at the same time. Notice particularly that whilst Ca initially promotes less adhesion than Mg, it is more resistant to the increasing concentrations of Zn. Calcium competes more effectively with zinc for the binding site.

Lineweaver -Burke plot to show competition between manganese and zinc

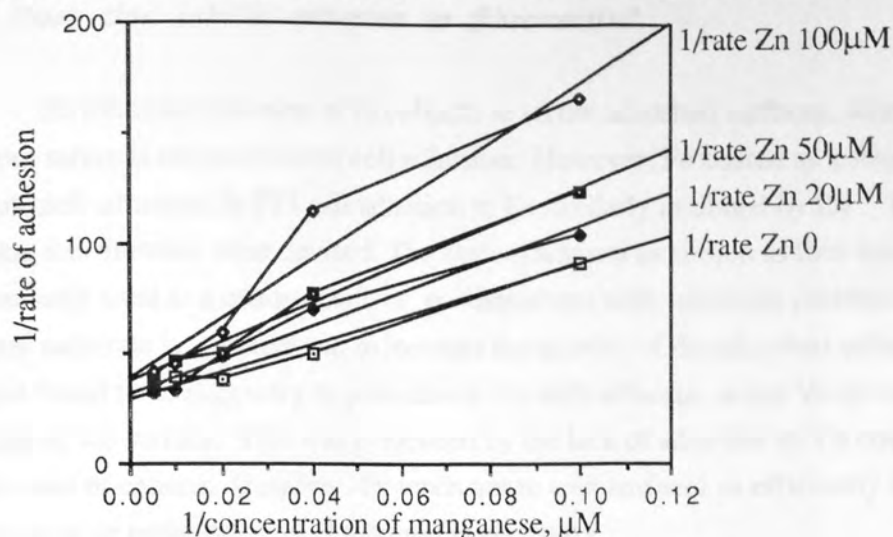


Figure 30 Zinc competes with manganese for a binding site on the cell surface. Direct competition of a binding site is confirmed by a Lineweaver-Burke plot of a timed adhesion experiment.

Briefly, a series of timed adhesion experiments were carried out in the presence of several concentrations of manganese together with 3 different concentrations of zinc. Plates were incubated for either 5, 10 or 15 minutes adhesion was assessed by the SRB method. This timed assay yielded a set of curves. The initial slope of the curve where adhesion was not yet affected by occupied sites, corresponded to the initial rate of adhesion. The initial rates of adhesion were plotted as a function of the concentration of manganese as $[S]$ in a classical Lineweaver-Burke plot. ($1/\text{rate of adhesion}$ versus $1/[S]$) These plots for different inhibitor (Zn) concentrations give a series of lines fanning out from one point. In the case of direct competition, rather than uncompetitive inhibition or noncompetitive inhibition, the lines meet at or near the same ordinate intercept (138).

4.2.9 Does zinc inhibit adhesion to fibronectin?

Zn inhibited adhesion of fibroblasts to serum adsorbed surfaces, where adsorption of Vn from serum is responsible for cell adhesion. However, Fn coated as a single protein can promote cell adhesion. Is 3T3 cell adhesion to Fn similarly inhibited by Zn? Experiments to examine this question were devised. The system was not as simple as first thought. Albumin is commonly used as a coadsorbant or postadsorbent with adhesion proteins to ensure that the base substrate is 'masked' and to increase the activity of the adsorbed adhesion protein. It was not found to be necessary to post adsorb Vn with albumin, as the Vn gives adequate masking of the surface. This was evidenced by the lack of adhesion to Vn coated surfaces in the absence of cations. However, Fn tends not to coat surfaces as efficiently as Vn, so coadsorbing or postadsorbing of albumin is necessary.

Albumin is not thought to have any adhesive activity except in the presence of high levels of Mn. In fact, albumin is known as a nonadhesive protein and is used to passivate surfaces. In the following experiment, it became apparent that Zn promotes cell adhesion to albumin. Whilst Zn was found inhibitory for the adhesion of 3T3 cells to dialysed serum coated plates, Zn promoted adhesion of 3T3 cells to 'Fn + albumin' coated plates. These effects could not be separated from the effects of albumin alone, as Zn promotes fibroblast adhesion to albumin as shown in figure 31.

4.2.10 Does zinc have any other effects on cells?

Morphological differences were noted between the cells adhered in the presence of zinc and other ions. The 3T3 cells adhered to albumin or collagen in the presence of Zn spread without the 'polarity' noted in the presence of other cations. This can be seen in figure 32 as compared to the cells spread in the presence of Mg shown in figure 33. This may be due to other effects on integrin associated components such as phospholipase A2 or protein kinase C. Alternatively it may be due to the nonspecific nature of the adhesion-perhaps there are no integrin stimulated cytoskeletal changes.

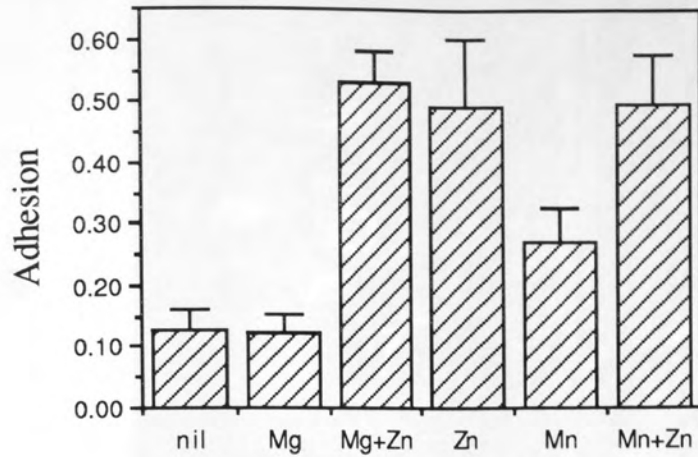
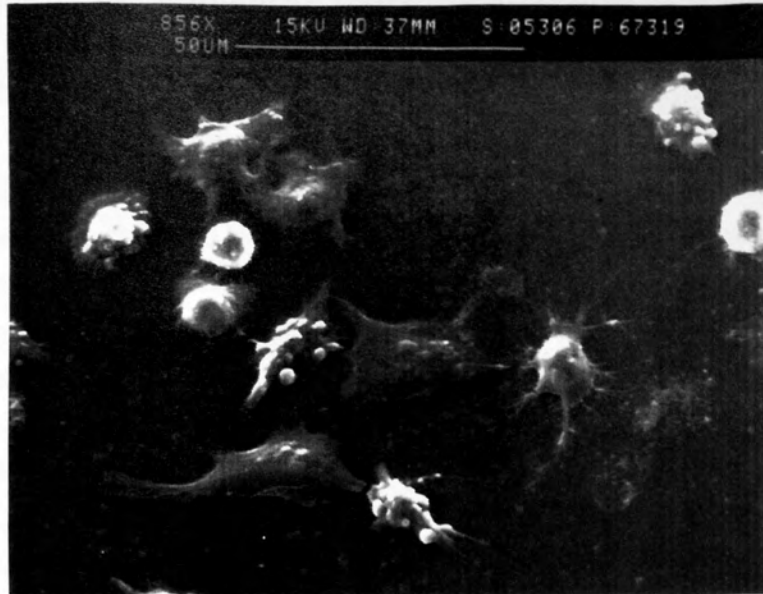
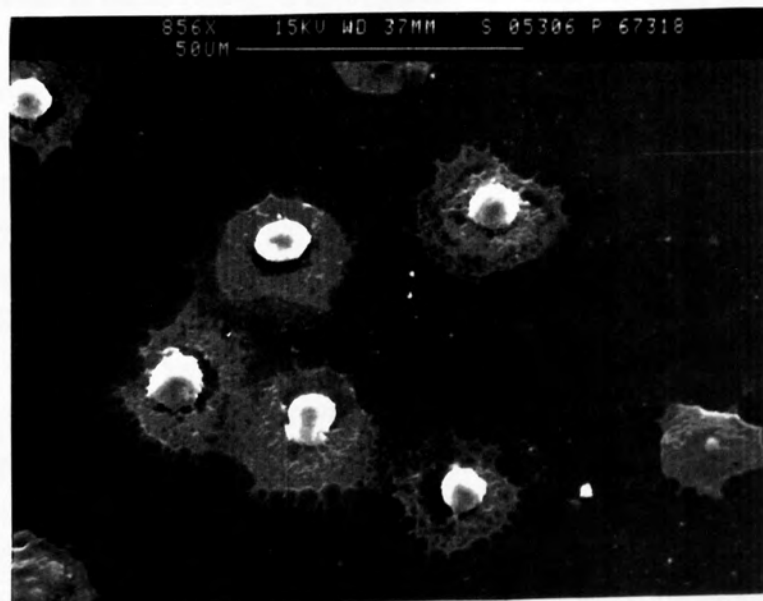


Figure 31 Zinc promotes 3T3 cell adhesion to albumin. Zn 100 μ M, Mg 5mM, Mn 50 μ M in all cases. This albumin was dialysed and purified, but may still have contained residual proteins other than albumin. Note that Mn also promotes a small amount of adhesion. Cells lack receptors for albumin so this effect is likely to be non specific. Zn associates with albumin in serum and also with the cell surface. An alternative explanation could be that the adhesion promoting effects of very small amounts of cell surface associated Fn is potentiated by albumin and the presence of some cations. Adhesion (y axis) refers to the optical density of SRB at 550nm. Results represent an average of 6 wells in the SRB assay, with standard deviations represented by the error bar.



32) Magnesium 5mM



33) Zinc 100µM

Figures 32 and 33. Morphological difference in 3T3 cells plated in the presence of 32) Mg and 33) Zn on adhesive surfaces after 1 hour.

4.3 Discussion and Summary

4.31 Physiological roles of zinc

The results of the experiments in this chapter showed that Zn plays a role in the modulation of adhesion catalysed by other divalent cations. Could this modulation effect be involved in physiological situations? Zn is an essential trace element associated with multiple enzyme functions, notably for alkaline phosphatase and carboxy peptidase A⁽¹⁸⁷⁾. Low levels of Zn intake or absorption are associated with a variety of pathologies, including skeletal retardation in the young, skin abnormalities, depression and impaired wound healing⁽¹⁸⁸⁾. In serum, Zn is mainly associated with albumin and α macroglobulin fractions with a small amount of free Zn, to a combined concentration of about 15 μ M⁽¹⁸⁹⁾. Erythrocytes and white blood cells contain high molar concentrations of membrane bound Zn, which is thought to play a role in the stabilisation of the membranes⁽¹⁹⁰⁾. This stabilisation could be due to the antioxidant properties of Zn which prevent lipid peroxidation⁽¹⁹¹⁾. Zn buffers are conventionally used in the extraction of membranes from other cell components^(192,193).

In vitro studies have shown that Zn supported fibroblast adhesion to 'cCAP'-collagen complex (probably Fn-collagen complex)⁽¹⁹⁴⁾, platelet adhesion to collagen I via the IIbIIIa complex⁽¹⁹⁵⁾ and induced spreading and inhibited motility of macrophages⁽¹⁹⁶⁾. Zn did not support chick embryo fibroblast adhesion to TCP in the presence of serum (ie probably to Vn)⁽¹⁸¹⁾ or the spreading of guinea pig epidermal cells⁽¹⁸²⁾, but has not, to the author's knowledge, been shown to have any other effects with respect to cell adhesion. Zn is required within the cell for DNA synthesis before cells enter S phase of the growth cycle⁽¹⁹⁷⁾ and is mildly toxic to cells at high levels⁽¹⁹⁸⁾. Zn is not, however, toxic with respect to other heavy metals and can be ingested at >2g a day without ill effects as compared to say, copper which is toxic at < 1 mg a day⁽¹⁵⁹⁾.

In the course of the investigations described in this chapter, the effects of Zn on the adhesion of various cell types, in combination with, and in comparison to, other divalent cations were observed. The results obtained indicate a role for Zn as a regulator of cell adhesion to Vn in combination with other cations. In the light of these results it is suggested that cell surface associated Zn may also play a physiological role in the control of Vn mediated adhesion and it is noted that consideration of the ionic environment is vital to the investigation of integrin function.

4.32 Mechanism of Zn mediated inhibition

Evidence outlined in this chapter showed that direct competition for an integrin metal binding site was the likely mechanism for the regulatory effects of Zn. Why should Zn not promote binding in the same manner as Mg? Why does Zn act as an inhibitor? There is

emerging consensus that Ca is also an inhibitor of adhesion for many receptors (demonstrated in this chapter for 3T3 cell adhesion to the collagen I receptor, and also for pig chondrocytes and L929 cells to serum coated surfaces i.e. to vitronectin), and Mn seems to activate all receptors. As discussed in the introduction and in chapter I, subtle differences between integrins, their activation state and ligand offered are all other controlling factors in the adhesion mechanism. The divalent cation is coordinated by the amino acids of the binding pocket and usually (but not necessarily) a D residue from the ligand binding sequence. The size of the hydrated cation may well be a factor in its activity for a given integrin ligand interaction, but the hydrated radius for Zn is similar to that of other metals (199-200), as illustrated in the table 3 below. Mn is a transition metal ion and can exist in several states allowing a variety of chelation conformations to occur. It is thought that Mn can therefore promote adhesion fairly universally in this manner. There is also a compelling amount of evidence to suggest that Mn is an activator of integrins.

Metal ion 2+	crystal ionic radius A	Charge to radius ratio	Ionic radius of aqua ion (six coordination)
Magnesium	0.66	3.0	0.86
Cobalt	0.72	2.7	0.88
Zinc	0.74	2.7	0.89
Manganese	0.80	2.5	0.96
Calcium	0.99	2.0	1.14
Barium	1.34	1.5	NA

Table 3 Ionic radius and charge data for various divalent cations.

Zn may block cell adhesion by being completely coordinated by the binding pocket of the integrin, or by inducing an unfavourable conformational change upon binding to the integrin that prevents interaction with the D residue of the binding sequence in the surface adsorbed Vn. What other possible explanations could be put forward to describe the phenomenon ?

It is known that cellular integrin interactions with adhesion proteins trigger responses in the cytoskeleton via diverse signalling pathways (see figure 3). Current research interest in the signalling pathway and the mechanisms which lead to the formation of focal adhesions centre on a number of regulatory enzymes that localise on the intracellular aspect of the membrane at focal adhesion sites. These include protein kinase C (PKC) (201,202) calmodulin, and GTPases (203). There is substantial evidence that Zn (perhaps membrane bound) increases the activity of PKC at μM concentrations (204, 205) and can even increase its binding to plasma membrane in T lymphocytes (206). Zn is almost certainly involved in the reorganisation of cytoskeleton in cultured cells via its interactions with PKC within the cell (207).

It is proposed that although Zn could be involved in inhibition of cell adhesion in the manner described above, this is unlikely in the face of the evidence presented in this thesis. Firstly, Zn was shown to be inhibitory when associated with the outside of the cell membrane (The non cell permeant chelator EDTA could return adhesion to cells preincubated with Zn). Interaction with PKC requires Zn to be on the intracellular side of the plasma membrane on the evidence to date. Secondly, Zn was shown to compete directly with Mn for a binding site, as illustrated by the Lineweaver-Burke plot. These kinetics would be unlikely if Zn was inhibiting at a site remote from the Mn binding site. Thirdly, Zn was shown to promote adhesion of the same cell type to collagen. This was probably an integrin mediated interaction, and would presumably trigger similar cytoskeletal responses. The appearance of cells incubated in the presence of Zn does however, indicate that some interference with the cytoskeletal system has occurred. This may be a secondary effect, associated with phospholipase A, or PKC, as the Zn may have permeated the membrane after an hour in culture. To complete this study, the radiolabelled receptor must be isolated from the cell membrane and its metal binding characteristics assessed. This requires a large volume of cells to be prepared. Alternatively, further indirect evidence could be obtained by assessing the inhibitory potency of antibodies to various β integrins on cell adhesion to serum coated surfaces.

The mechanisms of adhesion of the monocyte line J744.2 remain a mystery. Whilst adhesion is not blocked by RGD peptides or antibodies to adhesion proteins Vn of Fn, adhesion does require divalent cations and 37°C. It is proposed that adhesion may be reliant on one of the newly discovered nonintegrin receptor classes. Further study requires the assessment of adhesion to various molecular weight fractions from serum to determine the adhesion moiety. It is possible that Fc receptors were insufficiently blocked by nonspecific IgG during the assay, and this avenue of research should also be followed.

To summarise, 3T3 cell adhesion to serum coated tissue culture plastic surfaces is mediated by surface adsorbed Vn. This adhesion is promoted by a number of divalent cations including Mn, Mg and Ca. Zn does not promote adhesion of this cell type to serum, and furthermore, Zn inhibits the adhesion promoted by other cations. This inhibition was caused by the direct competition of Zn with other cations for a binding site on the cell surface. Similar Zn mediated inhibition of adhesion was seen in two other cell types, but Zn promoted the adhesion of monocytes to serum coated surfaces. Monocytes do not adhere to surfaces via the RGD dependent mechanisms seen in fibroblast cells. Zn and Mg and Mn promoted adhesion of 3T3 cells to collagen, but Ca inhibited this adhesion. This mechanism of this Ca mediated inhibition remains to be investigated.

As monocytes and 3T3 cells seemed to exhibit different degrees of adhesion to serum coated plates in the presence of Zn, mixtures of these two cell types should be easily separated on this basis. Experiments were performed where one cell type was labelled with fluorescent dye prior to plating, so that the adherent cells could be distinguished. However, this experiment yielded very variable results, where 3T3 cell adherence seemed to be increasingly

resistant to the inhibitory effects of Zn in proportion to the time incubated with the monocytes. These investigations remain to be followed up, but it is known that IL-1 has adhesion promoting effects on fibroblasts (36) (generally, stimulated monocytes produce cytokines such as IL-1), and this may have been contributory to the variability of the results observed.

Key points

- 1) Fibronectin and vitronectin adsorbed serum coated, TCP surfaces can be blocked by antibodies against their receptors. This adhesion is also inhibited by EDTA provided calcium is the essential integrin receptor based adhesion. Adhesion is distinct calcium dependent.
- 2) Different cell types vary in their ability to promote cell adhesion to serum, collagen or Vitronectin surfaces. The adhesion regulating effect of protein coatings also vary according to cell type.
- 3) Calcium inhibits the adhesion of T_H1 cells to collagen I promoted by Mg, but promotes adhesion to dialysed serum coated surfaces. Zinc inhibits the adhesion of several cell types promoted by Mg, Ca and Na to dialysed serum coated surfaces. However, zinc promotes adhesion of U937-2 monocytes to the same surfaces.
- 4) Zinc inhibition of adhesion to serum coated surfaces can be reversed by treatment with the non cell permeant chelator EDTA which removes calcium receptors a site on the cell surface.
- 5) Competition experiments between integrins on one cell type are possible direct competition for a binding site.



Key points

- 1) Fibroblastic cell adhesion to dialysed serum coated TCP surfaces can be blocked by antiVn but not antiFn antibodies. This adhesion is also inhibited by RGD peptides indicating a vitronectin -integrin receptor based adhesion. Adhesion is divalent cation dependent.
- 2) Divalent cations vary in their ability to promote cell adhesion to serum, collagen or Vn coated surfaces. The adhesion regulating effects of divalent cations also vary according to cell type.
- 3) Calcium inhibits the adhesion of 3T3 cells to collagen I promoted by Mg, but promotes adhesion to dialysed serum coated surfaces. Zinc inhibits the adhesion of several cell types promoted by Mg, Ca and Mn to dialysed serum coated surfaces. However, zinc promotes adhesion of J744.2 monocytes to the same surface.
- 4) Zinc inhibition of adhesion to serum coated surfaces can be reversed by treatment with the non cell permeant chelator EDTA which suggests that zinc occupies a site on the cell surface.
- 5) Competition experiments between manganese and zinc indicate probable direct competition for a binding site.

5.0 Introduction

5.1 Surface structure

5.2 Macroporous hydrogels formed by freeze-thaw technique

5.2.1 Membrane morphology

5.2.2 Cell seeding

5.2.3 Culture

Chapter 5

Macroporous Membranes and the Design of Artificial Cartilage

5.3 Cartilage and replacement

5.3.1 Fabrication of materials

5.3.2 Cellular reactions to polymer

5.3.3 Finding for matrix components

5.3.4 Discussion

5.4 Key points

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5.3 Cartilage and replacement materials

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Key points

5.0 Introduction

This chapter concerns the cell response to physical structure in hydrogels. Cell adhesion, growth and morphology can be manipulated by altering the surface chemistry of a polymer. The surface chemistry is usually governed by the bulk chemistry of the polymer, which in turn, results in particular mechanical properties. Due to constraints on the mechanical performance required of the material, it is not always possible to manipulate the surface chemistry of a material to achieve the desired cell adhesion parameters. However, surface physical structure can also be altered. Cell guidance and behaviour on physically structured surfaces is being researched by others for applications as diverse as nerve guidance and synthetic organ development. Control of cell behaviour on hydrogel based structures is greatly enhanced by the ability to control the surface morphology in addition to surface chemistry.

The experimentation described in this chapter formed part of the programme of work involved in the search for successful strategies in the development of a synthetic cartilage replacement material. Whilst mechanical properties of cartilage had been successfully mimicked in a hydrogel, using interpenetrating network technology, the biological interactions of the resulting polymer had not been addressed. In order to improve the compatibility of this material with surrounding tissue, and increase the permeability of fluids through the material, the introduction of porosity was examined experimentally. In the first instance, freeze-thaw techniques were used to form porous membranes. Although this approach to fabricating porous materials was shown not to be suitable for the fabrication of synthetic cartilage materials, promising results were obtained. The morphology of a variety of freeze-thaw formed membranes was documented. Cell response to these hydrogels was assessed, generating some interesting observations.

The introduction of porosity into the synthetic cartilage polymer was solved by polymerisation in the presence of dextran pore formers, which were then eluted. Cartilage cells (chondrocytes) were isolated and cultured in order to examine the cellular response to materials with a variety of pore sizes. A variety of techniques were developed to observe the behaviour of cells on these materials.

In brief, the experimental work presented in this chapter is divided into two major parts. Firstly, the structure of a variety of simple freeze-thaw membranes is explored. The cell response to these porous gels *in vitro* in a simple non-invasive cell system is described.

Secondly, introduction of porosity into a polymer surface was thought to be important in improving the biocompatibility of a synthetic cartilage material. In the second part of this chapter, the introduction of porosity into this material was examined with respect to the subsequent response of cartilage cells *in vitro*.

5.1 Surface structure

Topology is a fundamental biological guide to cell growth and repair and in embryological development (95). Synthetic planar surfaces exhibit degrees of roughness that influence cell behaviour *in vitro* and *in vivo*. Connective tissue matrices have a complex three-dimensional structure that is harder to quantify. Various cell types grow and migrate within these matrices. Structural and chemical feedback systems arising from the cellular response to these three-dimensional matrices maintain the phenotypic stability of the various tissues of the body. The cellular production and response to various adhesion proteins can be seen not only in terms of the chemical composition of the tissue matrix but also of its structure. This is beautifully illustrated by the elegant descriptions of the structure of the extracellular matrix by Yurchenko et al (95). Biological questions involving cellular response to structured surfaces are presently being examined on simply etched planar substrates (98,100) with a view to determining the abilities of the cell to cross boundaries.

As described in section 1.2.6, the cell response to surface topology is governed by the cell type. Briefly, macrophages and some other large cells tend to adhere to rough hydrophobic surfaces, whereas fibroblasts or other connective tissue cells tend to adhere to smoother more hydrophilic surfaces (99,102). Roughened polymer surfaces and sharp edged implants are associated with an increased inflammatory response *in vivo* (103,104,105). Surface structure of high water content hydrogels is not associated with roughness and sharp edges, as hydrogels tend to be less stiff than other polymers. However, hydrogels do exhibit microporous structure visible at the SEM level. This microporosity (on a scale of about 1 μ m) may have effects on the cell response as it undoubtedly affects the type, conformation and amount of proteins adsorbed at the surface. Larger pores introduced by freeze-thaw techniques or the introduction of pore formers, such as those described in the experimental work that follows, influence the cell adhesion and spreading behaviour because of the limitations of the cell cytoskeleton when focal adhesions are established.

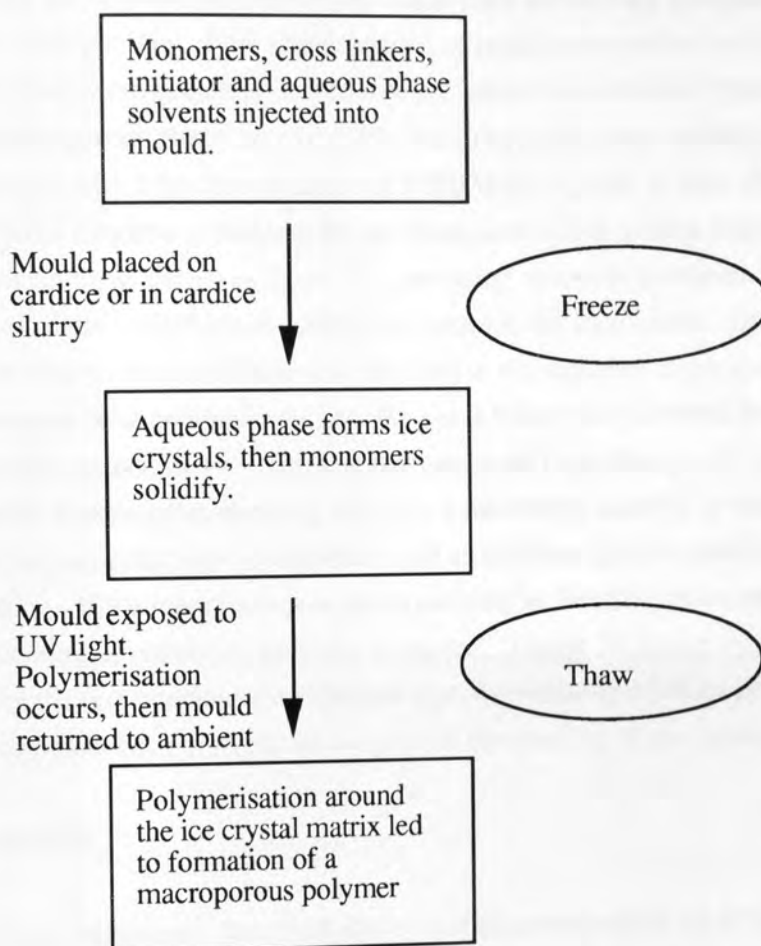
As this field of research progresses, it may be possible to establish a set of ground rules about the likely behaviour of a given cell type.

5.2 Macroporous freeze-thaw formed hydrogels

This section describes experimental work involving macroporous membranes formed by a freeze-thaw method. Membranes were prepared and examined for morphology at the SEM level. Cellular response to these materials was assessed by 3T3 fibroblast growth *in vitro*. Macroporous polymers have been used previously in a variety of biomedical applications; for example in liver support systems (acellular), and in controlled release devices. However, none of the former materials were membranes formed by the freeze-thaw technique. The use of a "freeze-thaw" technologies to synthesise macroporous hydrophilic membranes was first described in 1972 by Haldon and Lee following the initial work describing the

polymerisation of monomers around a crystalline matrix (208,209). Using this technique, the monomers are polymerised around ice crystals to produce a macroporous polymer. Two papers from this research group have recently described the preparation and characterisation of a range of hydrophilic macroporous matrices in some detail concentrating firstly on phase equilibria and permeation studies and secondly on the morphology of the membrane (210, 211).

The flow diagram below illustrates the basic method of producing these membranes.



A number of porous membranes were prepared by this freeze-thaw method, under sterile conditions. The membranes were soaked in phosphate buffered saline for several days before use in the cell culture system. 3T3 cells were plated onto the porous hydrogels. These cells are not 'invasive' and would not be expected to penetrate the pores of the hydrogel.

5.2.1 Membrane morphology

One of the advantages of the freeze-thaw technique is the ability to synthesise membranes with a range of morphologies and pore sizes by varying the ratio of solvent to monomer. There are limitations to this technique however, based on a number of factors; the formation of two monomer phases due to the insolubility of the crosslinker in the the monomer

mixture at different temperatures and concentrations, or the insolubility of the monomers in the aqueous phase at different temperatures and concentrations. Partial phase diagrams can aid the design and prediction of morphology (210, 211)

The clarity of the SEM micrographs of these membranes was achieved by the slow dehydration and drying of the samples. A thin gold coating was evaporated onto the mounted sample. Charge effects were minimised by mounting the samples on very small amounts of graphite putty.

All of the membranes prepared by the freeze-thaw technology described in this chapter exhibited porous morphology. Pore size depended on initial composition of solvents and monomers, but pores were generally interconnected. Membranes formed from compositions including a water aqueous phase or salt (0.1 M NaCl) aqueous phase tended to exhibit a similar morphology with a random structure of 5-20 μ M pores, such as that shown in figure 34. Introduction of ethylene glycol into the aqueous phase led to a more highly organised and smaller pored structure as shown in figure 35. Increasing amounts of ethylene glycol in the freezing aqueous phase caused increasingly small pores in the membrane. Homogeneous morphology throughout the membrane was observed in the majority of compositions. However, in systems with compositions near the phase boundaries at room temperature, small changes in ambient temperature and freezing rate can cause large changes in morphology. Figures 36 and 37 illustrate the 'skinning' effect on a membrane formed by the rapid freezing of a salt containing aqueous phase composition and an ethylene glycol containing aqueous phase composition. If the monomers precipitate out prior to freezing of the aqueous phase, a membrane consisting of connected particles is formed. Figure 38 shows this 'phase inversion effect' caused by the fast freezing of an ethylene glycol containing aqueous phase membrane. In this case, monomers have precipitated out prior to the freezing of the aqueous phase.

5.2.2 Cell studies

The porous membranes described above were all composed of 90:10 HEMA :EGDM, a membrane composition that is adhesive to cells. How would fibroblast adhesion and growth be altered by the porous nature of these membranes in comparison to the nonporous membrane? 3T3 fibroblasts were plated onto the membrane surfaces in medium containing 10% serum. The morphology and growth of the cells was followed by a variety of methods including SEM, fluorescence microscopy and conventional growth assays.

3T3 fibroblasts were able to adhere and grow on all the membranes produced by the freeze-thaw method. This is illustrated in figure 39. Cells grew rapidly on the porous membrane surfaces. At confluence, the cell layer obscured the porosity of the membrane. This effect is shown by SEM in figure 40. The cells tended to be well spread on the macroporous HEMA : EGDM membranes described, and exhibited a less 'even' morphology than cells plated onto non-porous substrates of the same composition. Presumably this

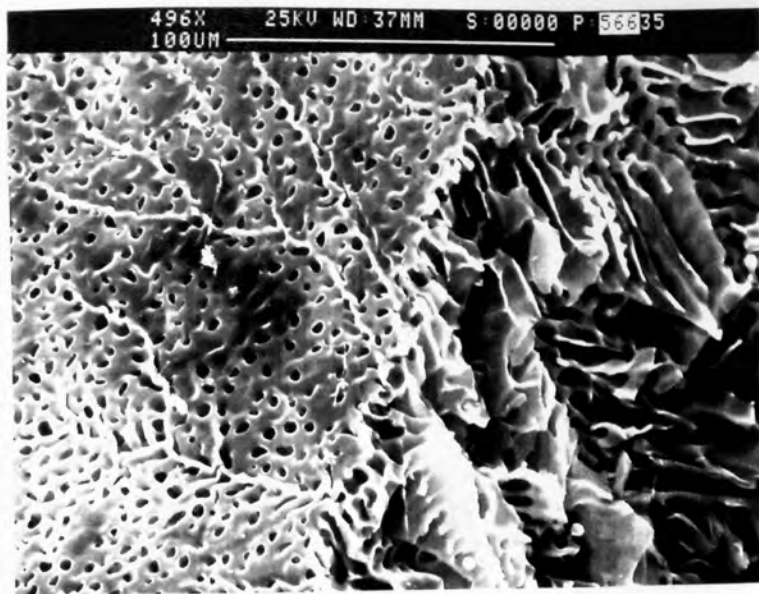


Figure 34 HEMA : EGDM 90 : 10 membrane formed with 0.1 M NaCl as the aqueous phase. The grain of the surface reflects the internal morphology but is subject to a slight skinning effect.

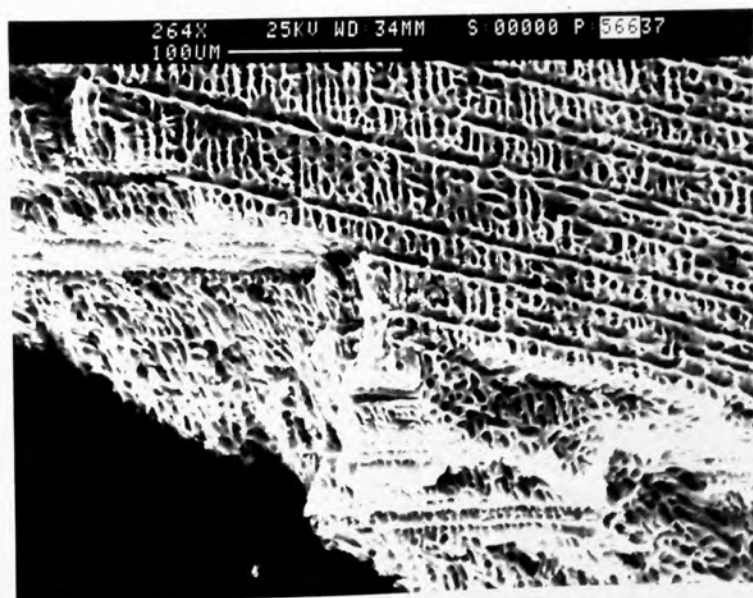


Figure 35 HEMA : EGDM 90 : 10 membrane formed with ethylene glycol:water 40 : 60 as the aqueous phase. Surface and fractured edge view shows a highly organised pore structure with no apparent skinning effect.

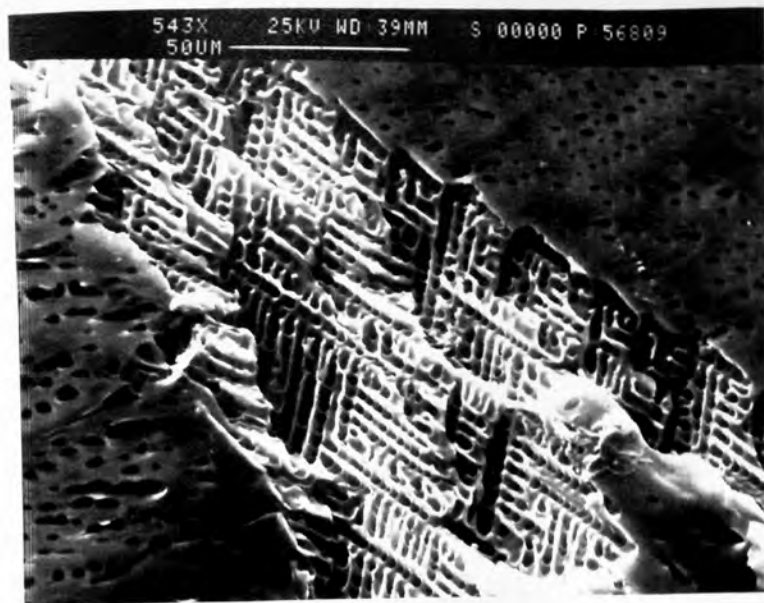


Figure 36 HEMA : EGDM 90 : 10 membrane with ethylene glycol:water 20 : 80 as the aqueous phase. The split surface view shows a denser skinning effect due to localised fast freezing and the highly organised internal pore structure associated with ethylene glycol formed membranes.

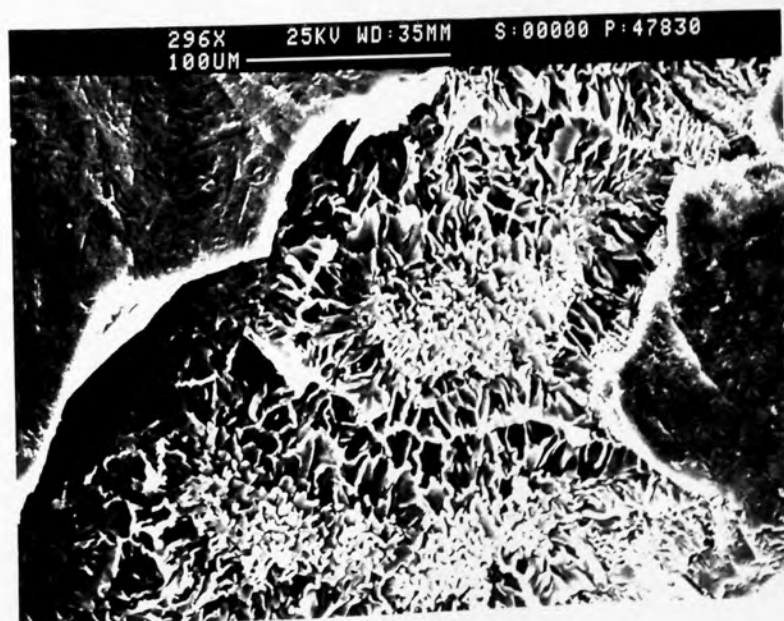


Figure 37 HEMA : EGDM membrane containing 0.1 M NaCl as the aqueous phase. This fast frozen membrane has a cracked surface revealing both a dense surface skin and the underlying porous structure.

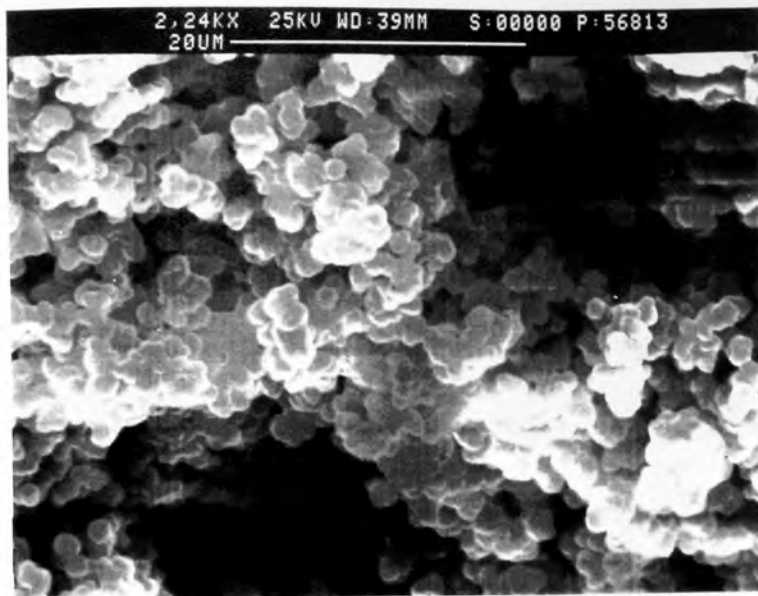


Figure 38 HEMA : EGDM membrane containing ethylene glycol : water 40 : 60 as the aqueous phase. This fast frozen membrane shows the particulate formation that occurs when phase separation occurs before crystal formation in the aqueous phase.

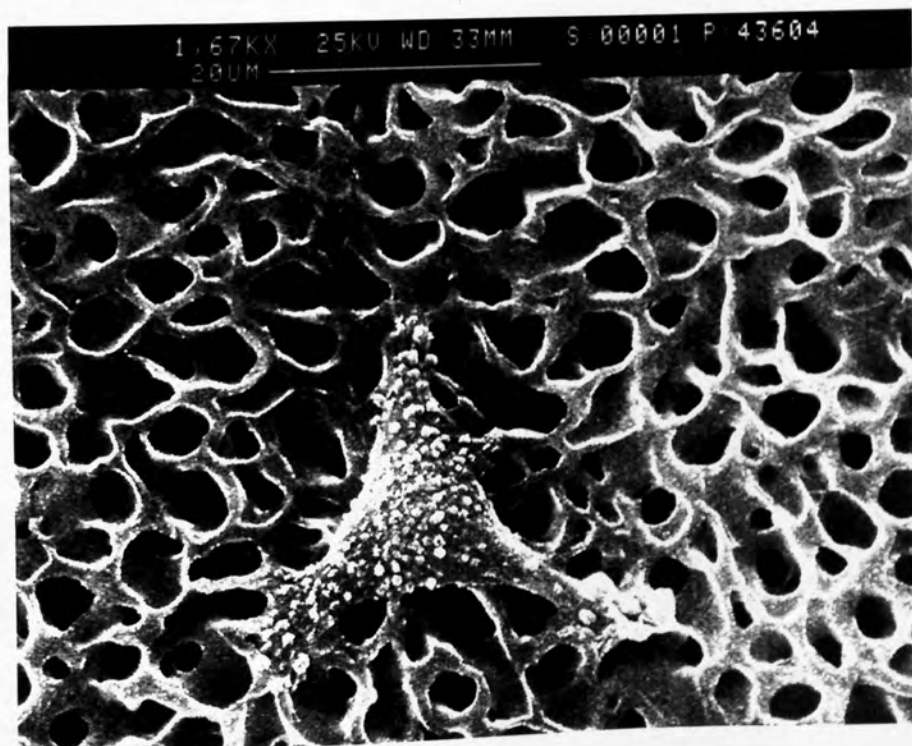


Figure 39 Fibroblast adheres to a HEMA : EGDM 90 : 10 membrane with $5\mu\text{M}$ surface porosity. The fibroblast is spread although focal adhesion is limited by the architecture of the surface

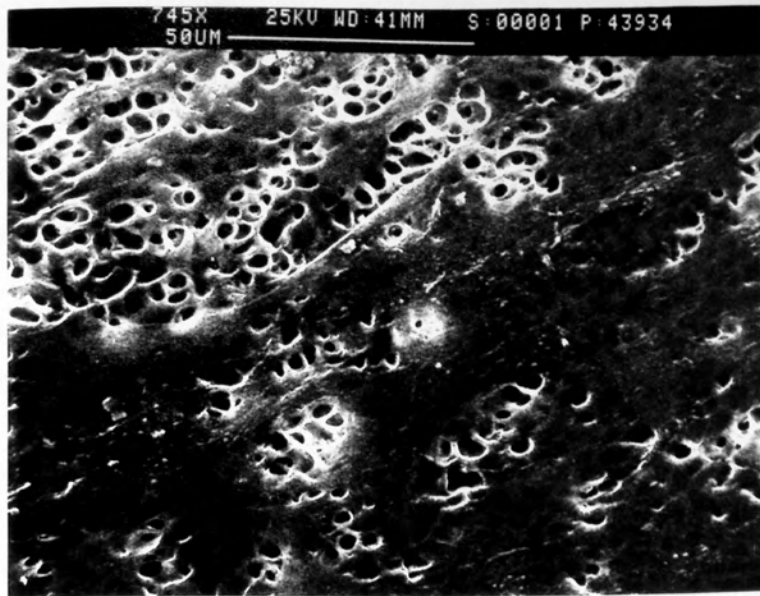


Figure 40 Fibroblast overgrowth after 1 week in culture completely covers porous surface. Cells form their own matrix and spread. Individual cells can no longer be determined by SEM.



Figure 41 Live staining of fibroblasts (green) showing orientation of the cells to the grain of the porous hydrogel (red) X100

difference in cell shape was due to constraints on the cell cytoskeleton, during the process of focal contact formation at the membrane surface. This theory is supported by the observation that cells also tended to orient to the grain of the underlying porous surface. This effect can be seen in figure 41. Cell layers grown on the macroporous membranes were quite stable at confluence. The confluent cell sheets did not detach upon handling the membrane samples. This contrasts with the behaviour of confluent cell sheets on non-porous hydrogels of the same composition. Cell sheets on the latter substrates tended to detach at confluence as shown in figure 42. Membrane areas where pores were eliminated prior to culture did not support cell adhesion or growth in the same manner. This effect can clearly be seen figure 43, where the edges of the membrane were squashed during the cutting of the sample. The cell growth on the membranes was monitored by assessment of the growth rate using the classic MTT method (160) and, secondly, by monitoring total protein production. Figure 44 and 45 show the protein production and growth rates respectively of 3T3 cells grown on porous, skinned, and nonporous membranes based on HEMA:EGDM as described above. Cells grown on porous hydrogels showed the fastest growth rate, which may be attributed to the increased nutrient supplies to the cells. Additionally, the cells can be seen to exhibit a more elongated shape and morphology on the porous membrane. It is proposed that the cell makes fewer focal contacts and is able to replicate more rapidly. This phenomenon has previously been observed in cells adherent to adhesive islands of varying shapes (56)

Discussion

In this section, the morphology and cell growth characteristics of freeze-thaw formed hydrogel membranes have been described. It has been established here that membrane morphology can be varied by the careful control of composition and freezing rate of the membranes. Cell growth characteristics of these membranes show great potential for development. Not only can the direction of cell growth be influenced by the grain of the underlying substrate, but the growth rate of the cells is increased. The increased stability of the confluent cell layer is an additional factor that may make these materials suitable for development as vascular prostheses or artificial organ support materials. These results compare favourably with existing reports in the literature. Previous research reports from other groups have not described morphology or cell growth characteristics of freeze-thaw formed porous membranes. However, the structure and morphology of poly (vinyl alcohol) hydrogels have been described by Japanese workers (212) and this group has also investigated the morphology of macroporous membranes based on 2-hydroxyethyl methacrylate (polyHEMA) formed by other methods (211).

Many studies on cellular response to hydrogel based macroporous implants have been examined *in vivo*, although none of the implants have been formed by the freeze-thaw methodology. Cellular response was found to be determined not only by the nature of the implant but also by the body site location. So far, porous gels have been used *in vivo* for

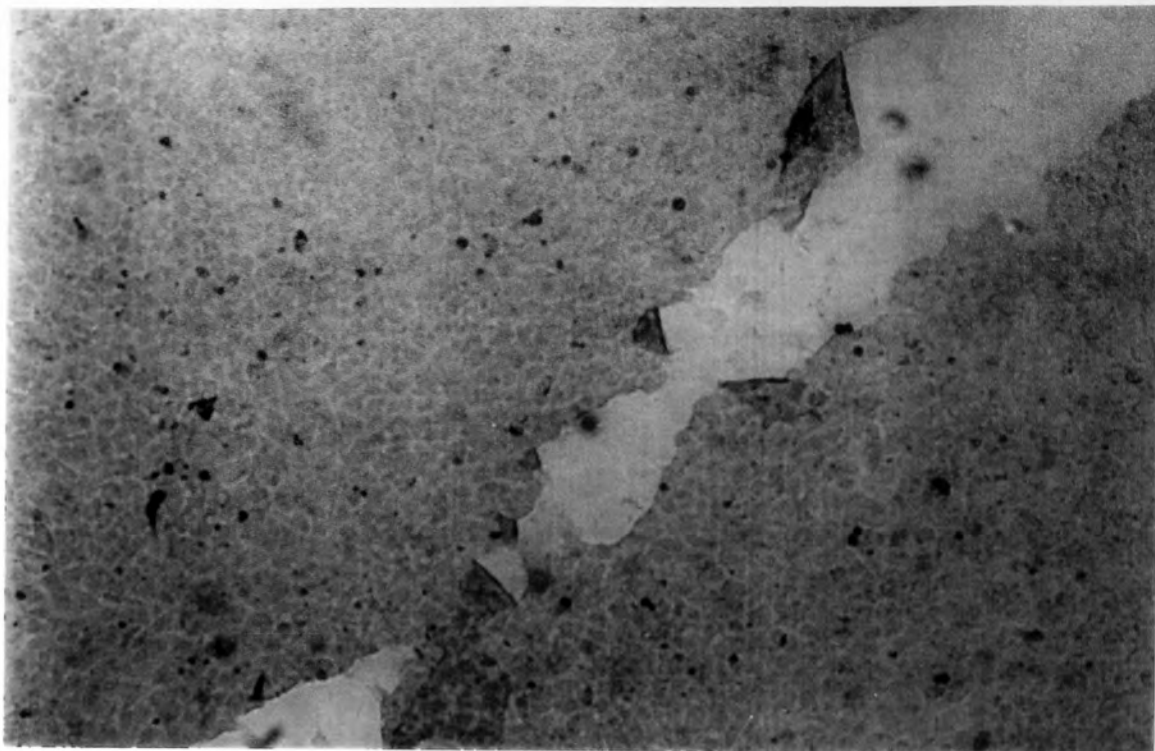


Figure 42 A confluent layer of fibroblasts breaks away easily from a nonporous HEMA : EGDM 90 : 10 membrane in contrast to the strong adhesion seen on macroporous hydrogels. Toluidine blue stain X100

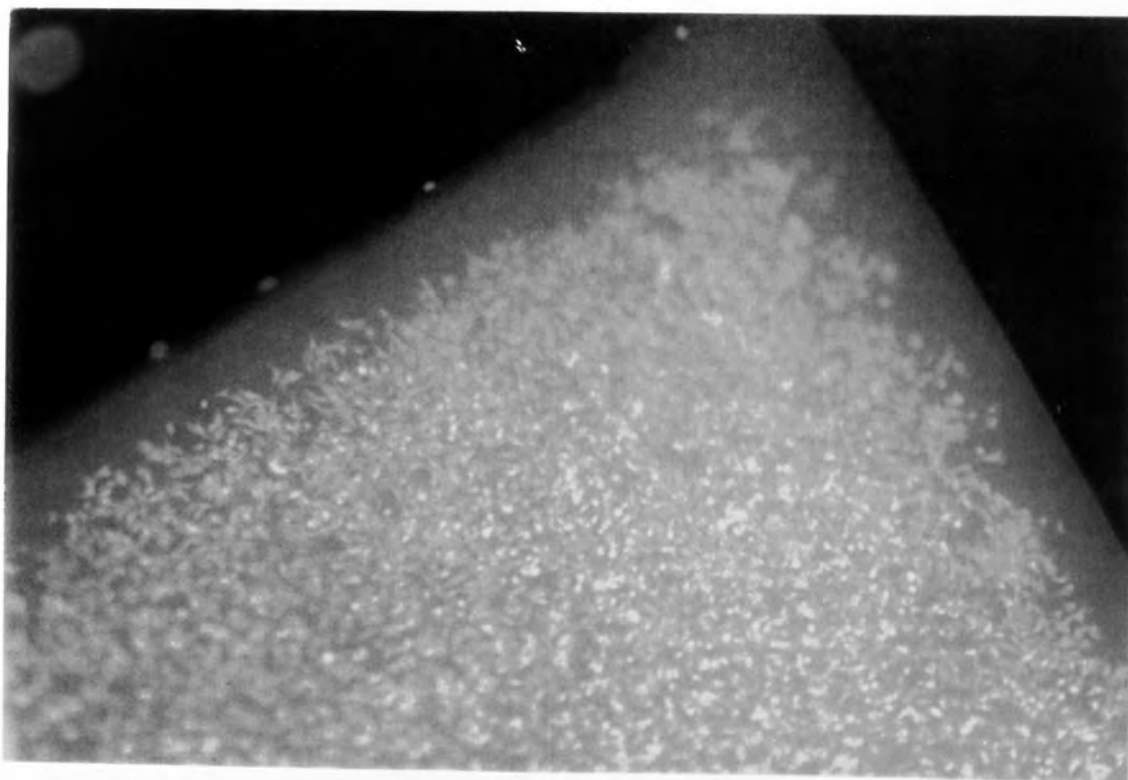


Figure 43 Live staining of a confluent layer of fibroblasts on a porous membrane (HEMA : EGDM 90 : 10, 5 μ M pores) demonstrates the contrasting behaviour of cells on the macroporous centre and the non porous cut edges of the membrane. X40

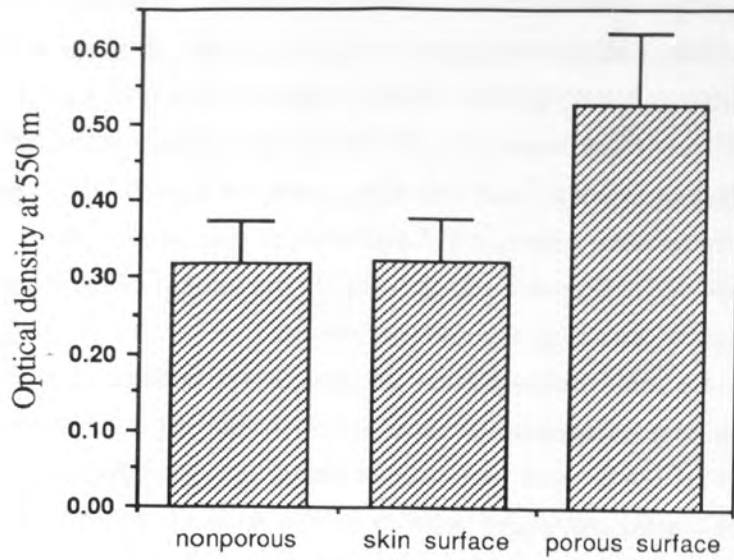


Figure 44 Growth rate of 3T3 fibroblasts grown on porous hydrogels determined by the MTT method. Growth rate is assessed by the mitochondrial reduction of MTT. The optical density of the resulting tetrazolium salt is read at 550 nm

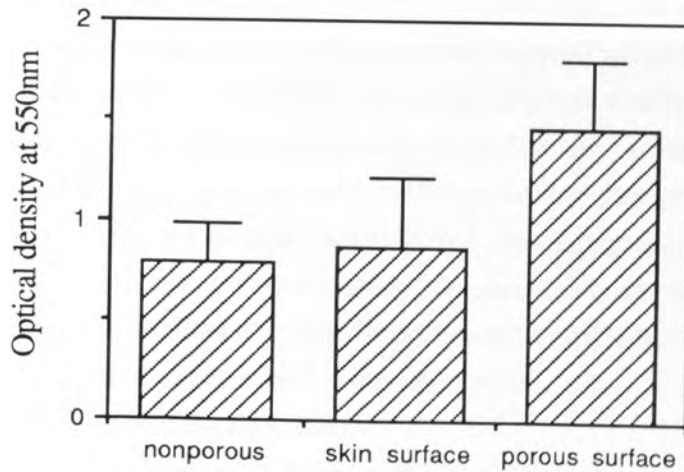


Figure 45 Protein production of 3T3 fibroblasts grown on porous and nonporous hydrogel surfaces assessed by the micro BCA method. Protein production is assessed by the the optical density of a dye product.

breast implants (213), artificial pancreas (214) and brain tissue supports (215). Porous hydrogels make ideal supports for artificial organ culture, but nutritional flow to cells in the centre of the implant is a problem. Cellular necrosis is associated with calcification, which has been shown to occur in implanted sponges and can be directly related to the porosity of the gels (216). However, very highly porous gels permit vascularisation as well as infiltration by tissue cells. Hence, less encapsulation and no calcification occur when very highly porous gels are implanted (217). Would the porous gels described in the experiments presented in this chapter here be suitable for *in vivo* implantation? The porous membranes described here can be cast as thin as 1mm (although the membranes cast for the experiments were 5 mm thick). If membranes such as these were used *in vivo*, tissue growth within, or on, the porous structure could be supplemented by encouraging vascularisation along preformed channels on the membrane surface, perhaps doped with neovascularisation peptides. The gels could be loosely folded into appropriate shapes that would avoid tissue necrosis *in vivo*. Alternatively, cell seeded membranes could be used as external processing plants for say, liver function.

More recent studies of macroporous hydrogels have been directed towards developing an synthetic corneal graft (keratoplasty). Presently, polymers designed for use as synthetic corneal tissue have initiated 'tissue melting' (the gradual but persistent proteolytic degradation of tissue at the implant interface). Chirila et al (218) proposed porosity at the implant periphery as a means of implant stabilisation and the avoidance of tissue melting. It may seem that the freeze thaw technology described in the experiments in this chapter would be useful in this application. However, in chapter 3, Vn function in relation to the proteolytic systems present in the eye was discussed. It was concluded that material adsorbed Vn was probably involved in persistent proteolysis. Therefore, it is suggested here that the continued proteolysis cannot be avoided by simply introducing porosity in the implant periphery. It is rather the surface chemistry and adsorbed proteins on the polymer surface that lead to the extended proteolysis.

The problem of calcification associated with porous implants may be turned to advantage. In the development of bone replacement materials the calcification induced by porosity may be desirable (219). One of the potential problems of developing a cartilage replacement material as described in the next section, is the avoidance of calcification at a body site prone to pathological calcification.

To conclude, porous hydrogels with controllable porosity and structure can be used effectively as substrates for cell growth. The following points summarise the results obtained in this section of chapter 5.

- a) A variety of freeze-thaw HEMA : EGDM membranes with homogeneous pore structures may be formed by altering aqueous phase composition.
- b) Fibroblasts grown on porous membranes have dramatically increased growth rates when compared to fibroblasts grown on nonporous hydrogels of the same composition.
- c) The porous grain of freeze-thaw hydrogels modulates the direction of cellular growth.

d) Confluent cell layers strongly adhere to porous membranes but can detach from nonporous substrates of the same composition.

Freeze-thaw formed macroporous membranes have potential for use in applications that require a fast growing confluent and stable layer of cells. As the membranes are relatively pliable, it is suggested that this technology could be useful in production of vascular prostheses pregrown with endothelia or artificial organ culture. As the underlying structure is porous, a prosthesis based on this technology would still permit extravasion of immune cell types.

5.3 Cartilage and replacement materials

In this section, the fabrication of a synthetic cartilage material is described. Cellular response to this material *in vitro* was utilised to develop a polymer surface likely to exhibit increased compatibility with surrounding healthy tissue *in vivo*.

Normal healthy articular cartilage is a naturally avascular tissue with a slow turnover rate. It consists of a high water content (70-85%) natural hydrogel with components that include collagen II, chondroitin sulphate, hyaluronate, keratan sulphate and other glycosaminoglycans. These components 'knit' together in a complex, natural, interpenetrating network (IPN). The charged proteoglycans serve to bind water. This matrix is produced by chondrocytes which are arranged in small vertical groups surrounded by a capsule of specialised proteoglycans (see figures 45 and 46). These groups are known as chondrons⁽²²⁰⁾. The capsules have a shock absorbing function which prevents damage to the cells during weight-bearing.

Chondrocytes *in vivo* are rounded cells, and produce a specialised adhesion protein similar to Fn called chondronectin. When isolated from cartilage, chondrocytes may be plated over agarose; a nonadhesive naturally occurring hydrogel surface. Plating over agarose ensures the cells remain rounded in suspension culture. Alternatively, chondrocytes may be plated onto tissue culture plastic, where they adhere and spread. The rate of replication of adherent plated chondrocytes is much higher than that of rounded chondrocytes plated over non adhesive agarose⁽²²¹⁾. Matrix production of adherent chondrocytes reverts to a fibroblastic composition. The adherent chondrocytes produce collagen I and exhibit decreased production of collagen II and glycosaminoglycans such as chondroitin sulphate, which are characteristic of chondrocytes *in vivo*⁽²²²⁾. The adherent chondrocytes are said to be dedifferentiated. However, providing they have undergone a limited number of replications, the dedifferentiated chondrocytes can be induced to re-express cartilage specific macromolecules by culturing under conditions which induce a rounded cell shape^(223,224). The production of appropriate matrix can be monitored by monoclonal antibody staining⁽²²¹⁾.

The chondrocyte integrins and other receptors for extracellular matrix are critical parts of the feedback mechanisms that maintain cell shape and phenotype. Chondrocyte receptors are still largely unexplored, partly due to the difficulties in isolating the cell type without inducing dedifferentiation. Chondrocytes respond to the immediate environment i.e. constituents of the pericellular capsule. These include collagen types II, VI, IX and XI and hyaluronan. Type VI collagen is concentrated and anchored to the chondrocyte at the pole of the pericellular capsule. It interacts with the cell either indirectly via interaction with other matrix components such as hyaluronan, or directly via the NG-2 receptor. It is also possible that anchorin receptor and integrins bind to this collagen type⁽²²⁵⁾. Interaction of the chondrocytes with the other capsule components is similarly organised. Interaction of the chondrocyte with collagen type II is via the anchorin receptor (a non-integrin receptor type that shares the EF hand morphology common to integrin)⁽²²⁶⁾. Interactions with Fn and Vn are

possibly critical to the dedifferentiation of chondrocytes. Certainly the chondrocytes express receptors for these adhesion proteins *in vitro* (227), but this area of research is unresolved to date.

Cartilage injury is a common and largely untreated condition. Osteoarthritis, a condition in which the cartilage calcifies in an irregular fashion, damages large areas of the articular surfaces. Due to the slow turnover of the tissue even simple sporting injuries in relatively young people take weeks to heal. Long term, imperfectly healed tissue tends to form fibrocartilage with associated vascularisation and innervation (228). Vascularisation may cause further deterioration of healthy surrounding cartilage. The increased blood supply results in increased oxygenation of the tissue. The change in oxygen tension accompanied by the localised production of wound healing peptides may also cause calcification of the damaged cartilage. Calcified areas at the articular surface may abrade the opposing cartilage, causing further injury. As substance P -the pain peptide! -is abundant in this now innervated area, these injuries are extremely painful and disabling.

Typically, surgical treatment of damaged articular cartilage follows one of three routes: excision and no further treatment, excision followed by replacement with carbon fibre pad, or excision followed by replacement with allograft tissue. The carbon fibre pads induce massive fibrosis to provide supporting tissue. This is far from ideal as the same problems of vascularisation and further deterioration apply. Allograft tissue can be obtained from cadavers (cartilage is relatively non-immunogenic) but may deteriorate quickly *in situ*, and in the present climate of HIV infection, the usual safety problems apply with donor tissue. Experimentally, the most successful allografts have been with growth plate cartilage, as this tends to have higher remodelling capacity (229). Entire allografts of the cartilage and supporting bony plate for severe osteochondral defects have been reported as successful, yet can present immunological rejection problems (230).

The experiments described in this chapter concern the fabrication of a synthetic cartilage replacement material. The cell studies and final preparation of the materials were carried out by the author, whilst the materials were fabricated by Dr Phil Corkhill. Initial work on the development of IPN (interpenetrating network) polymers for use as synthetic articular cartilage was carried out prior to the experiments described in this chapter, and is described by Corkhill et al (231). The research that includes some of the results outlined in this chapter is described by Corkhill et al (232). The synthetic cartilage is based on a semi interpenetrating network hydrogel (sIPN) with a defined macroporous structure. Hydrogels have previously been fabricated in an effort to produce a synthetic cartilage, as described in the discussion to this section, but the mechanical requirements of cartilage were not addressed in the design of these materials. In designing an improved synthetic articular cartilage, several areas were considered. It was thought desirable to provide immediate weight bearing properties, the ability to knit into the surrounding cartilage without fibrosis, and encourage matrix production by chondrocytes peripheral to the implant. In addition to this, the material should have a high

water content and appropriate mechanical properties. The material should also be easy to cut and sterilise in a clinical situation. It should also be possible to take some of the patients own chondrocytes and grow up a layer onto the precut implant before surgery.

The following results outline the direction of the studies leading to a synthetic cartilage material now being assessed in *in vivo* studies.

5.3.1 Fabrication of materials

Natural cartilage was studied as a model for the fabrication of the synthetic cartilage. Various polymers were then produced in order to mimic the mechanical properties of cartilage and provide a biocompatible integrating surface. Armed with the knowledge that chondrocytes dedifferentiate upon spreading, materials were fabricated to reduce the area on which cells could spread.

Figures 46 and 47 show respectively a low (light) and high (SEM) magnification views of rabbit cartilage. Cartilage is an anisotropic material. The phenotype of the chondrocytes and the mechanical properties of the tissue vary progressively throughout the cartilage perpendicular to the articular surface. The bulk of the cartilage in the mid zone contains groups of chondrons perpendicular to the articular surface. Further towards the surface, the chondrons are less defined, and the cells become flatter. Figure 47 shows an SEM view of an acellular piece of midzone cartilage. The shape of the chondrons can clearly be seen. The size of each cell compartment within the chondron is about $30\mu\text{m}$ in diameter. $30\mu\text{m}$ was taken to approximate to a pore size likely to inhibit spreading of chondrocytes proximal to an implant.

On the basis of the former observations, semi interpenetrating networks (sIPNs) were polymerised around dextrin pore formers (sIPN technology had already been established as a suitable method for fabricating a high water content material with the necessary mechanical properties). The dextrin was eluted out of the materials after polymerisation. Several pore sizes were fabricated, in an attempt to produce an environment in which a chondrocyte would be unable to spread and produce fibrous matrix, but instead, would remain rounded and produce cartilage-type matrix. It was found that the optimum dextrin size and incorporation was $38\mu\text{m}$ particles at 20% w/w as this left fewer 'flat' areas on the cut surface for the polymer to permit spreading of chondrocytes. Interconnecting pores were thought to be undesirable; cell growth within a porous matrix has been associated with cell death and calcification. A weight bearing material subject to regular loading would be especially susceptible to this kind of calcification.

The diagram overleaf illustrates the proposed implant material. The cut edges of the implant are porous, whilst the articulating surface is dense and smooth. This partially reflects the anisotropy of natural cartilage, although the progressively variable mechanical properties of natural cartilage may not have not been attained.

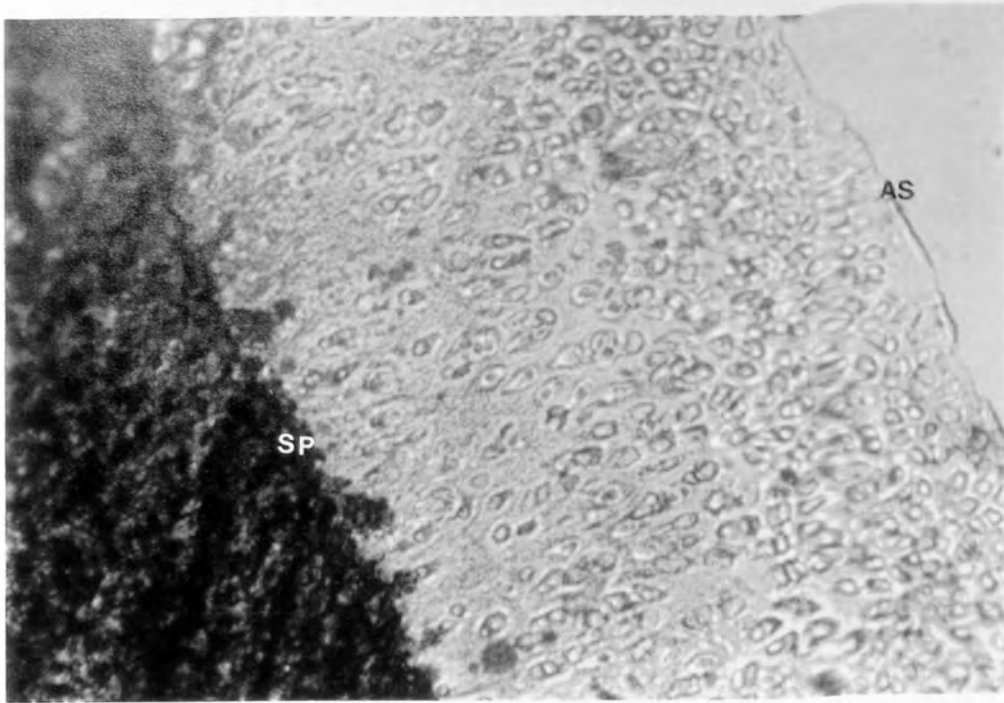


Figure 46 Rabbit cartilage section. (Light micrograph) Notice that the arrangement of chondrons varies throughout the cartilage from the subchondral plate (SP) to the articulating surface (AS).

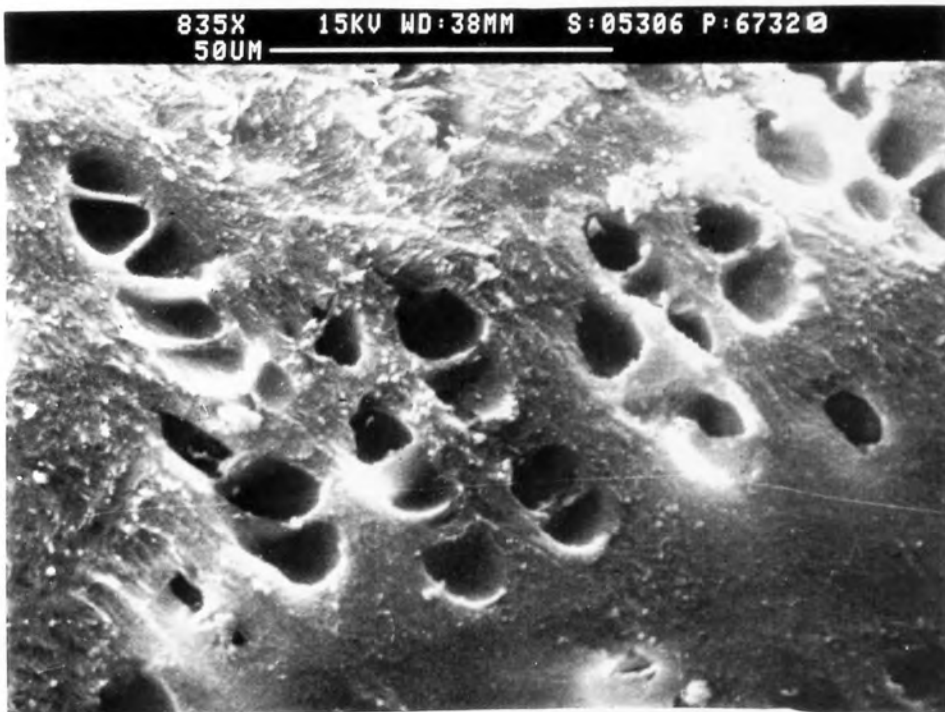
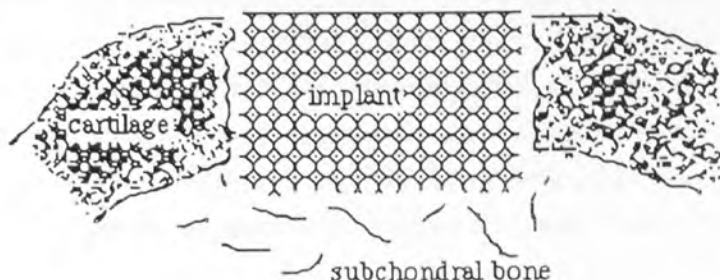


Figure 47 SEM of rabbit cartilage section. Cells were removed from this section prior to sample preparation. 'Empty' chondrons can be seen.



The sIPN macroporous polymer has a smooth articulating surface and rough edges at the implant interfaces with cartilage and bone.



Initial samples were fabricated with a variety of compositions, using unsized dextrin particles. SEM micrographs of some preliminary samples PC86, PC87 and PC90 are shown in Figs 48 A,B and C respectively. The compositions of some of these materials are shown below in table 4. The first three materials were not suitable due to low mechanical performance and interconnecting pore structures, but PC97 was found to have acceptable properties.

PC86	NVP:MMA:Pellathane	54:36:10, with 10 % dextrin (various sizes)
PC87	NVP:MMA:Pellathane	54:36:10, with 20% dextrin (various sizes)
PC90	NVP:MMA:CAB	51:34:15, with 20% dextrin (various sizes)
PC97	NVP:MMA:CAB	56:29:15, with 20 % dextrin sized at 38µm

Table 4 Compositions of some macroporous IPN materials

The mechanical requirements for weight bearing cartilage led to the development of the macroporous sIPN 'PC97' (see tables 4 and 5). At first, the dextrin was removed by several weeks immersion in hydrolysing solutions. This, however, increased time available for infection. Autoclaving in dilute HCl proved to be effective in the removal of the dextrin particles and made no great difference to the mechanical properties of the membrane. Further polymers were developed with the same polymer composition as PC97 using NaCl and sucrose as pore formers instead of dextrin particles.

	Cartilage	HEMA	PC97
tensile strength MPa	10-30	0.5	3.3
initial modulus	10-100	0.5	21
elongation to break	80	180	65
water content	75	40	55

(The variable results for cartilage reflect the progressive anisotropy of the material.)

Table 5 Mechanical properties of natural cartilage, polyHEMA and PC97

The composition of the sIPN 'PC97' resulted in mechanical properties felt to be appropriate for cartilage replacement materials i.e. high initial modulus and a tensile strength approaching that of natural cartilage, whilst maintaining a water content higher than that of a homogeneous polyHEMA gel. The inclusion of sized dextrin particles at 20% (w/w) led to a homogeneous macroporous polymer able to support the adhesion of chondrocytes without inducing cell spreading. Figures 49 A and B show high and low power SEM views of PC97 before removal of dextrin by autoclaving in dilute HCl. Figures 50 A and B show PC97 after elution of the dextrin particles.

5.3.2 Cellular reactions to the polymers

Chondrocytes were isolated as described in chapter 2, section 2.11. Chondrocytes were plated onto the 13 mm² polymer samples at a concentration of 5x10⁵ cells per sample. Samples were incubated at 37°C in an atmosphere containing 5% CO₂ for seven days.

Cells were observed using a variety of methods. Chondrocytes were able to adhere to the surface of the macroporous IPN 'PC97' but did not spread. Live staining showed that chondrocytes tended to form clusters or remained as rounded single cells, in a similar manner to chondrocytes cultured over non adhesive agarose (see figure 51). The technique of live staining proved ideal for studying the cellular response to this opaque material. Whilst problems were later encountered with background fluorescence during immunostaining, the fluorescent levels attained with live staining are high enough for background fluorescence to be unnoticeable.

Spreading occurred on the smooth surfaces of the same composition and on materials with larger pores. SEM micrographs show that whilst chondrocytes spread on a larger pored membrane (figure 52), chondrocytes were able to fall into 38µM pores on the polymer 'PC97', where they remained rounded. Figure 53 also shows chondrocytes nestled in the pores of a 38µM pore formed membrane.

It is notable that fibronectin precoating (1ml 20µg/ml overnight) the sIPN 'PC97' 38µM pores did not induce spreading in the plated chondrocytes. In this case, the effects of the topology of the material were not overridden by the presence of an extra adhesive matrix

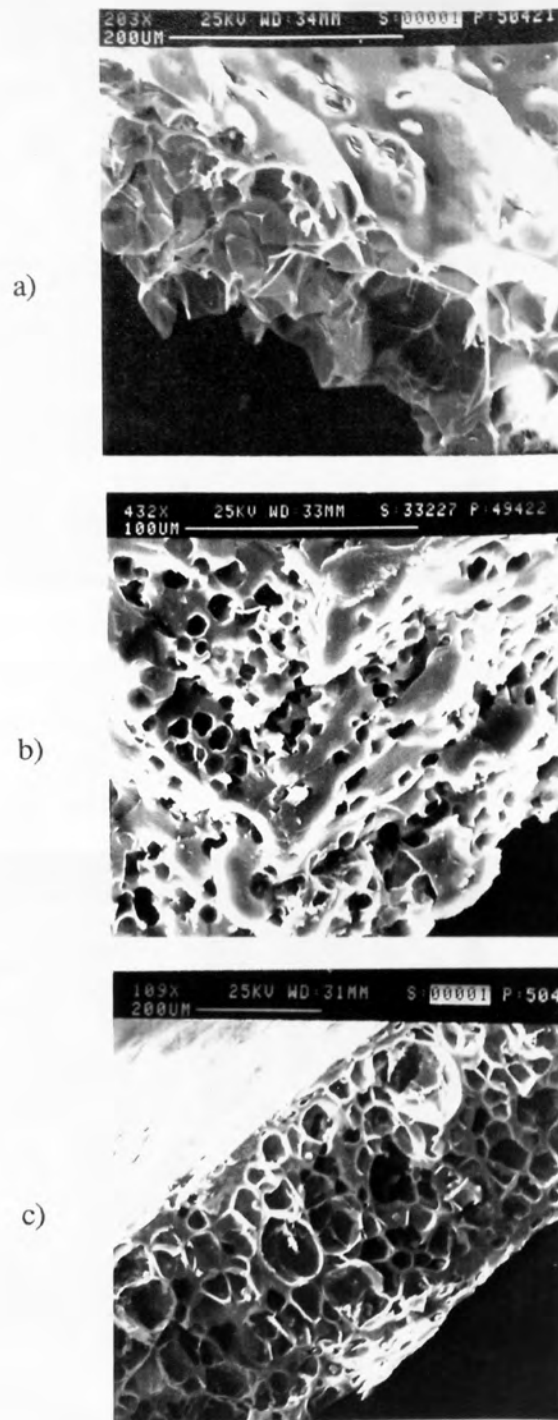


Figure 48 SEM micrographs of the macroporous sIPN materials a) PC86, b) PC87 and c) PC90 are shown here. Compositions of these materials can be found in table 4 (page 148).

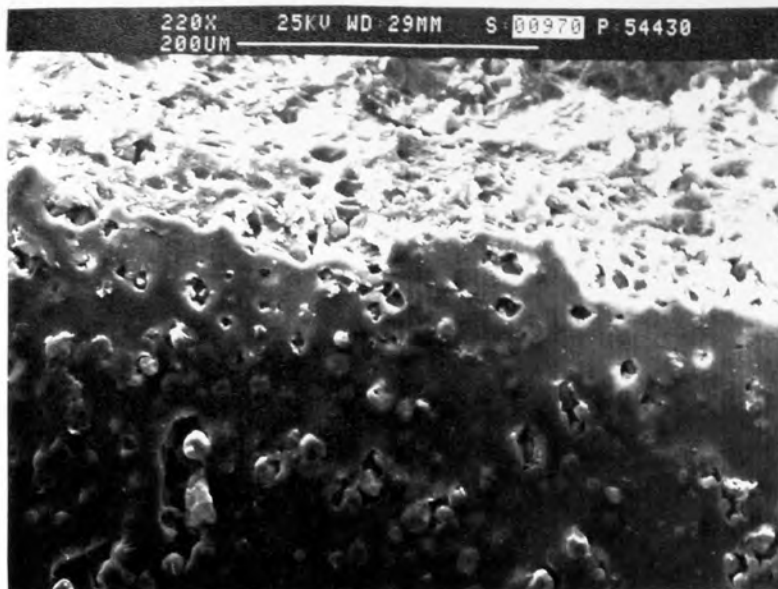


Figure 49A Low power SEM of PC97 in the presence of dextrin particles

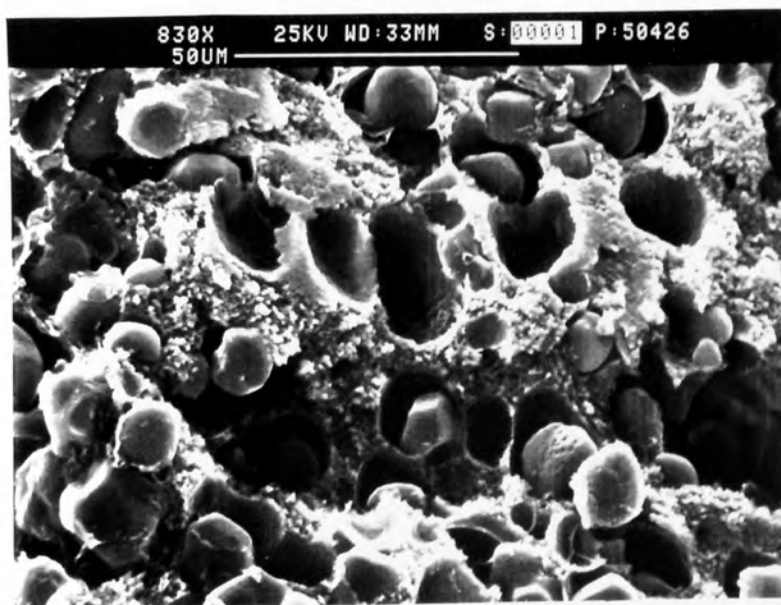


Figure 49B High power SEM of torn edge of the macroporous IPN 'PC97' in the presence of dextrin particles. Lacunae formed by the particles can be seen, and the microporosity of the polymer can also be observed.

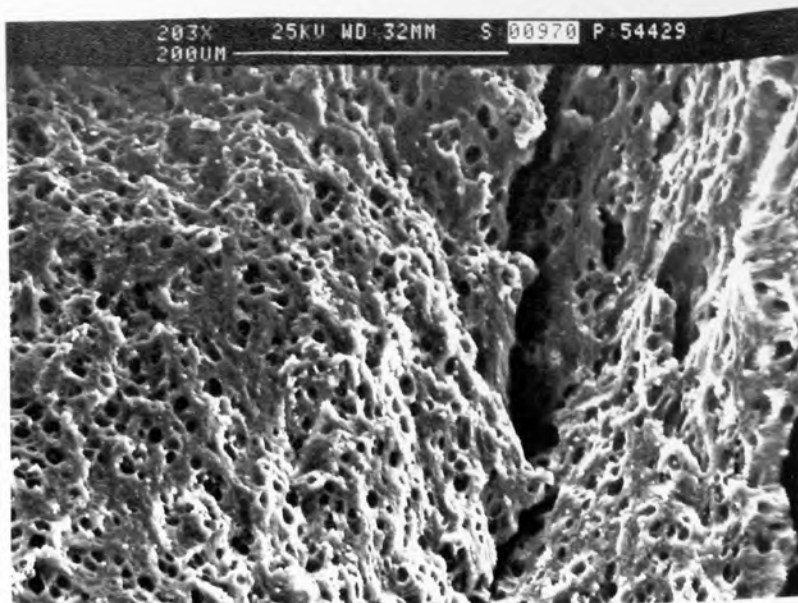


Figure 50 A The macroporous IPN polymer 'PC97' after elution of dextrin particles. Low power SEM

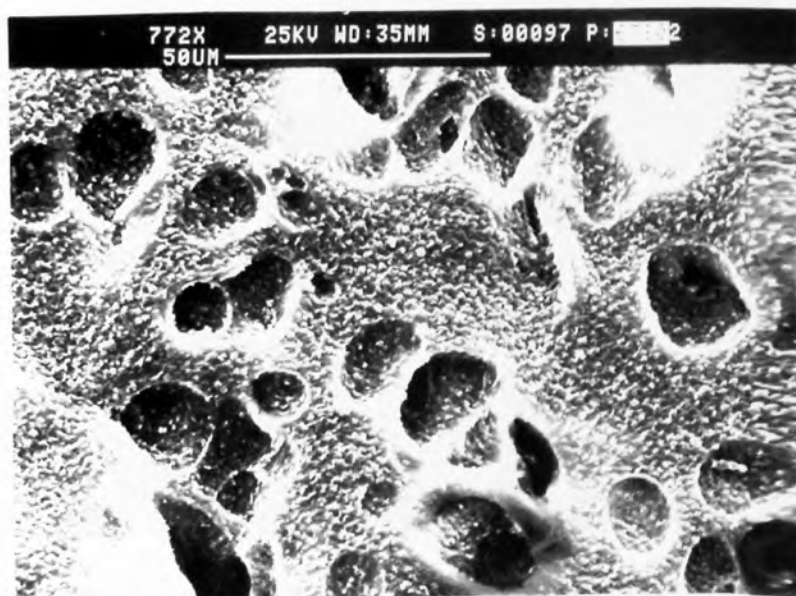


Figure 50B High power SEM of cut surface of the macroporous IPN 'PC97' after elution of dextrin particles.

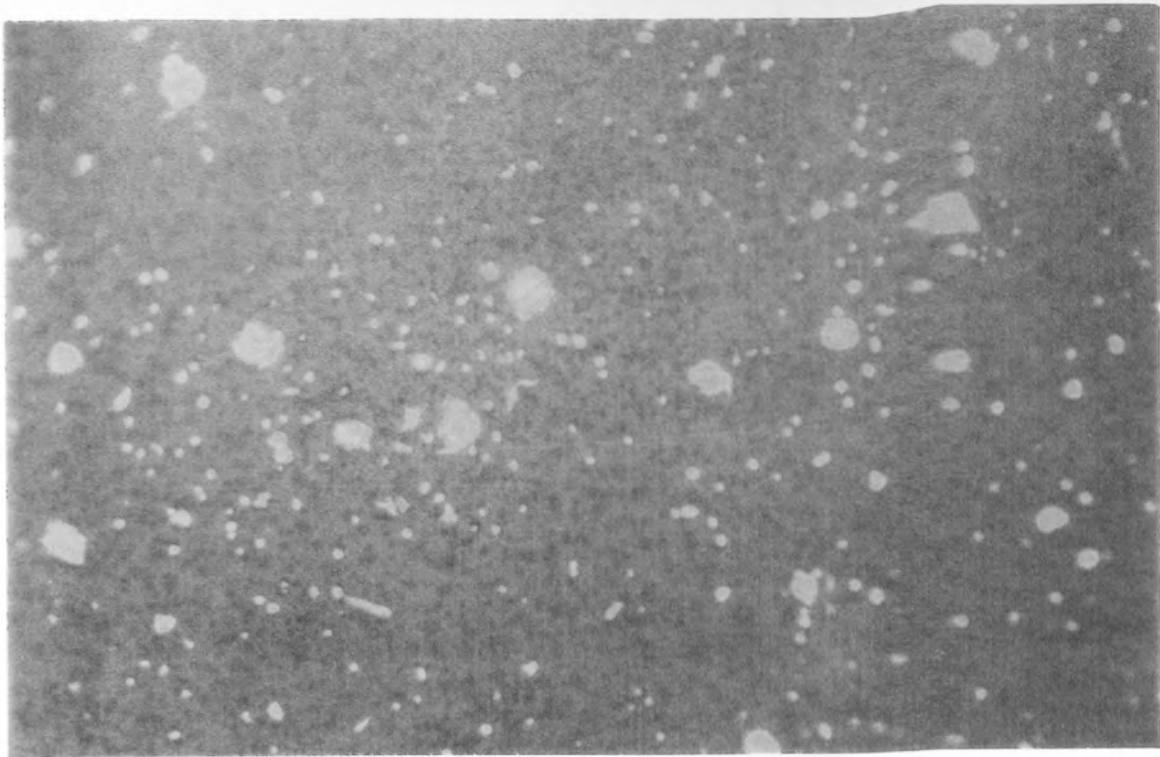


Figure 51A Live staining of rabbit chondrocytes cultured on the macroporous IPN 'PC97'. Note that the cells remain rounded or in rounded clumps. The cells are attached to the polymer as evidenced by the ability to process the specimens for this photograph, **but** the cells do not spread. x40

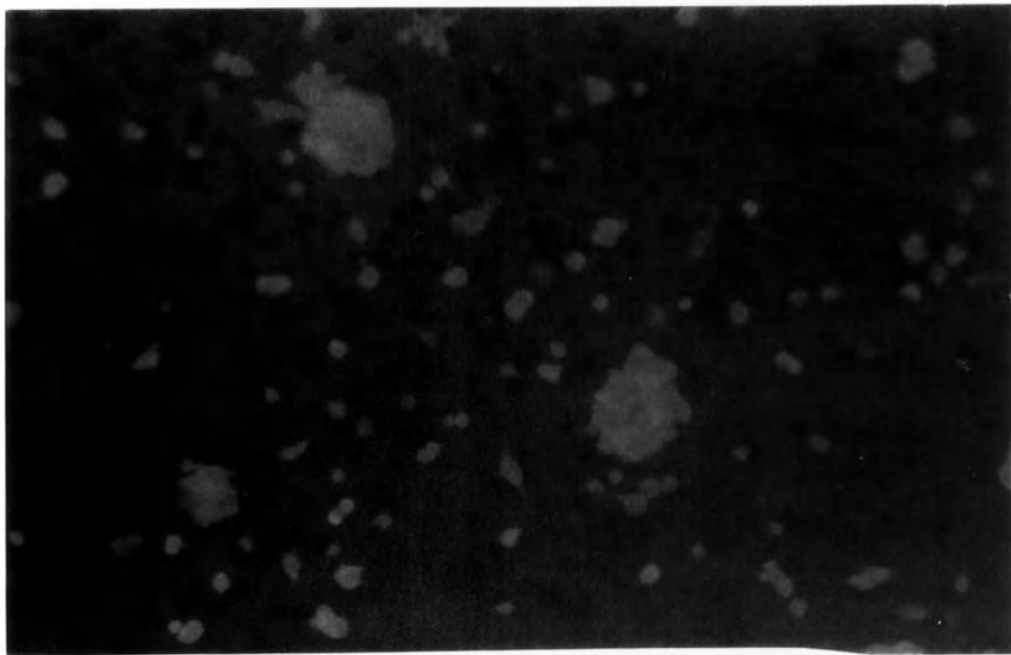


Figure 51B Live staining of rabbit chondrocytes cultured on the macroporous IPN 'PC97'. Note that the cells remain rounded or in rounded clumps. At this higher magnification, the ethidium bromide staining of the background is less pronounced x100

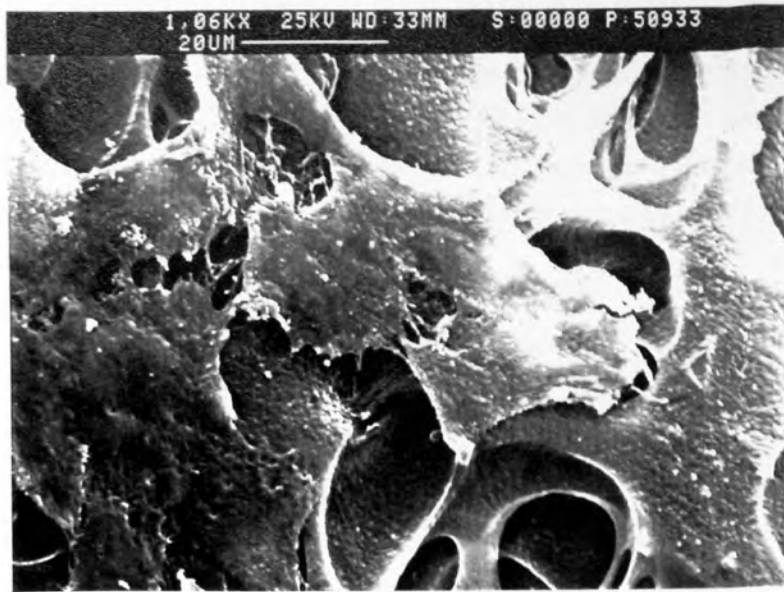


Figure 52 Rabbit chondrocytes cultured on large pore IPN polymer tend to spread.

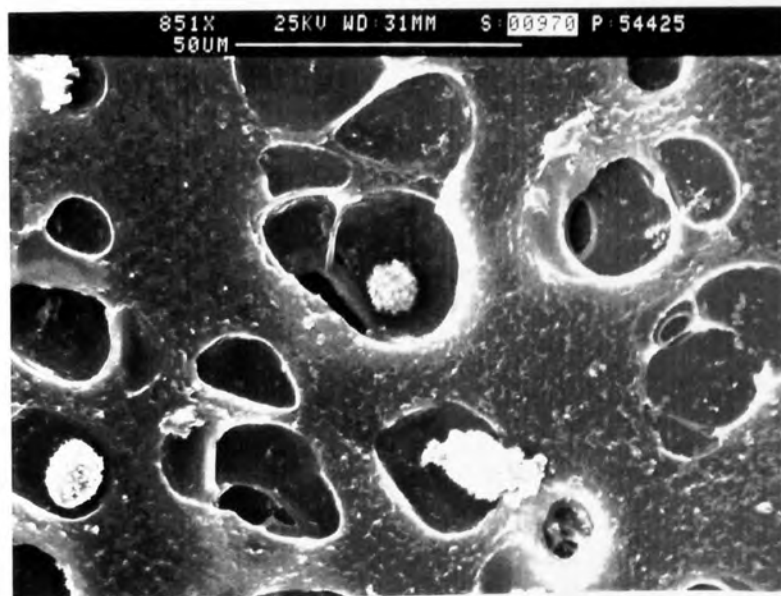


Figure 53 Rabbit chondrocytes cultured on the macroporous IPN 'PC97' tend to remain rounded. Single cells nestle in the surface pores. Clumps of cells similar to those seen when culturing chondrocytes over agarose can also be seen (also see Figure 51).

coating. This implies that the cell response to topological surface features may be able to override the cell response to adhesive proteins.

These cell studies were qualitative rather than quantitative. Cell counts proved impossible as cells tended to form clusters at the material surface. Trypsinisation of the cells was inefficient as released cells tended to remain caught in the porous surface. Further development of chondrocyte studies could include MTT assays⁽¹⁶⁰⁾ to determine growth rates, as described in section 2.2.1, and used earlier in this chapter (figure 44).

5.3.3 Staining for matrix components

In an attempt to elucidate the matrix composition produced by chondrocytes plated onto the sIPN porous material, immunostaining techniques were employed. This proved to be a difficult task, as the material is autofluorescent. The fluorescence of the antibody staining was overwhelmed by the background fluorescence from the material. In an attempt to solve this problem, thin sections (30 μ m) of the materials were cut on a cryostat, and pig chondrocytes plated onto these materials were placed on agarose during incubation (so that the cells would not settle on adhesive tissue culture plastic instead of the material). The cells were cultured for a week before immunostaining and examination by fluorescence microscopy. The major difficulty involved in the experiment was the flexibility of the thin sections of materials.

At this point it may be instructive to reiterate the implications of the presence of type I and II collagen. Generally, type I staining would coincide with dedifferentiating chondrocytes, whereas type II staining would be indicative of maintenance of the chondrocytic phenotype. Successful staining was observed with three antibodies to collagens type I and II. Firstly SP1D8 to procollagen I⁽²³³⁾, secondly, CII to collagen type II⁽²³⁴⁾ and thirdly, a polyclonal antiserum 'P2' to pig collagen type II⁽²³⁵⁾. Since the experiments described here were performed, SP1D8 has been shown to stain type II in addition to type I collagen. Hence, staining can not be reliably attributed to type I collagen expression.

Clusters of chondrocytes on PC97 were both seen to produce collagen I (SP1D8) and collagen II (P2 antibody and CII monoclonal antibody) as shown in figures 54, 55, 56. Single cells also produced type I collagen (SP1D8) and collagen type II (P2) as shown by figures 57 and 58. These figures are all shown with the corresponding bright field photograph which shows the position of the cell or cluster of cells on the polymer.

PC123, a polymer with the same composition as PC97 but with pores formed by 38 μ m NaCl also exhibited chondrocyte matrix production. Figure 59 shows type I collagen production by a single cell. Type II staining was also observed on this material. This is not illustrated due to low levels of fluorescence in the sample.

Type II expression has definitely been observed on chondrocytes plated onto the macroporous sIPN 'PC97', which is an indication that the previously dedifferentiated chondrocytes are phenotypically chondrocytic when grown on this material.

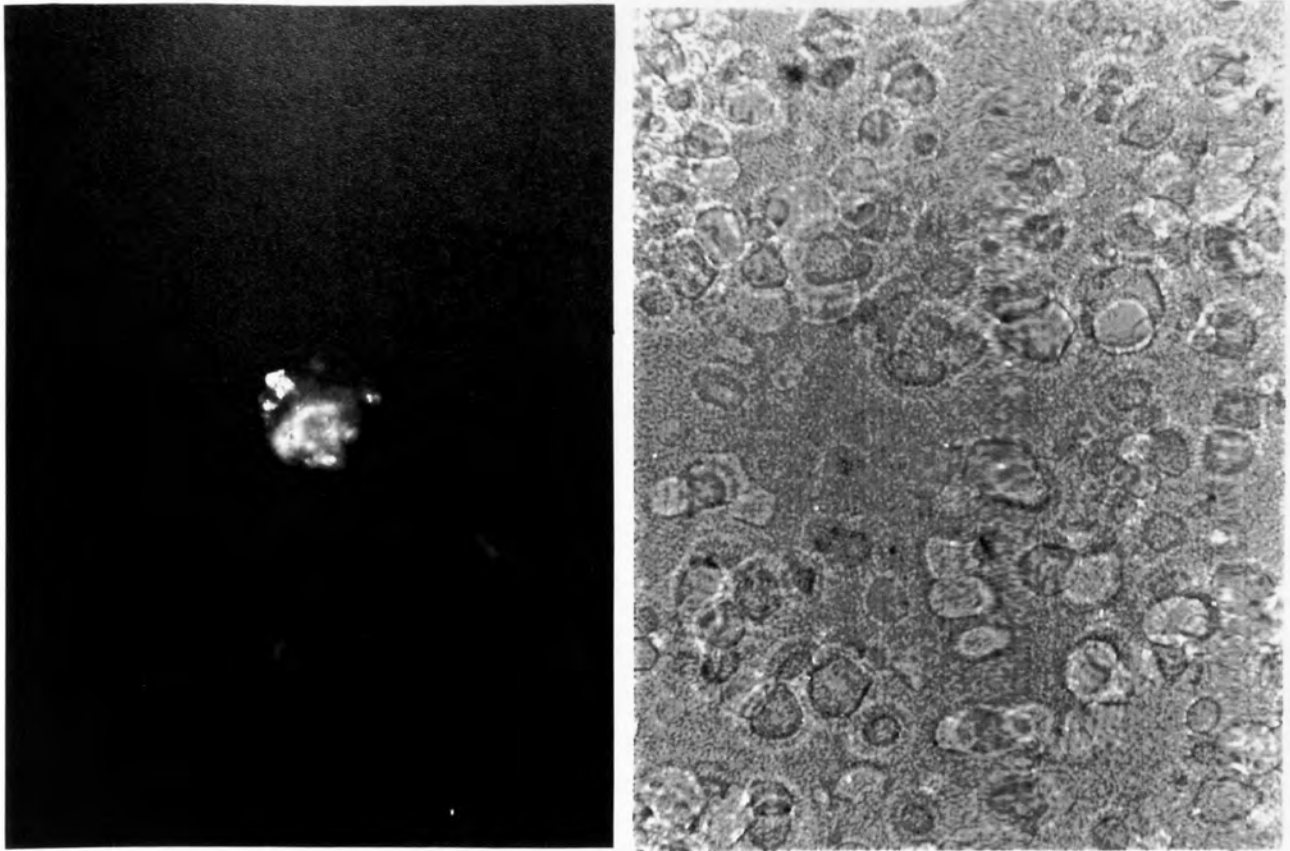


Figure 54 Porcine chondrocytes cultured on the macroporous IPN 'PC97' for one week. This cluster was stained for procollagen type I with MAb SP1D8.

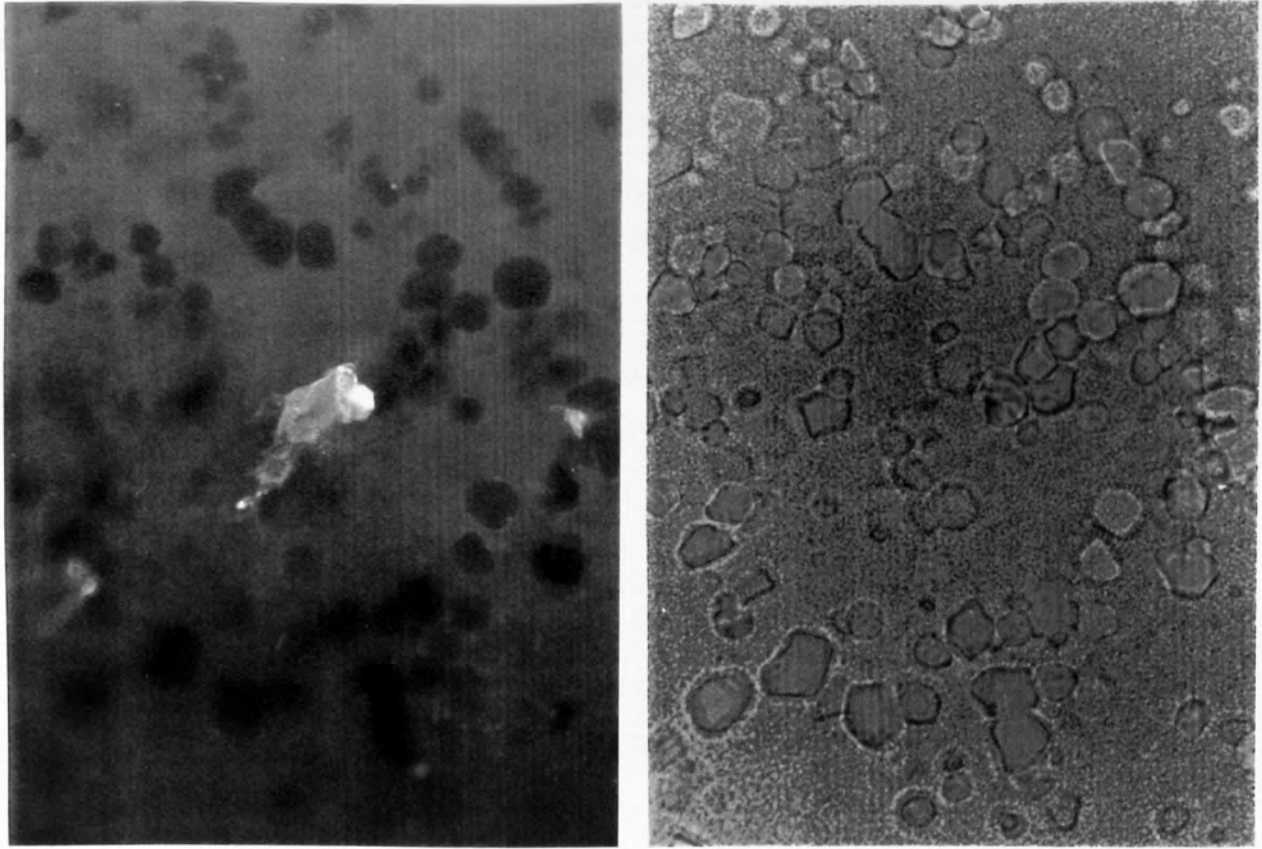


Figure 55 Porcine chondrocytes cultured over the macroporous IPN 'PC97' for one week. This cluster was stained for type II collagen with the polyclonal antibody P2

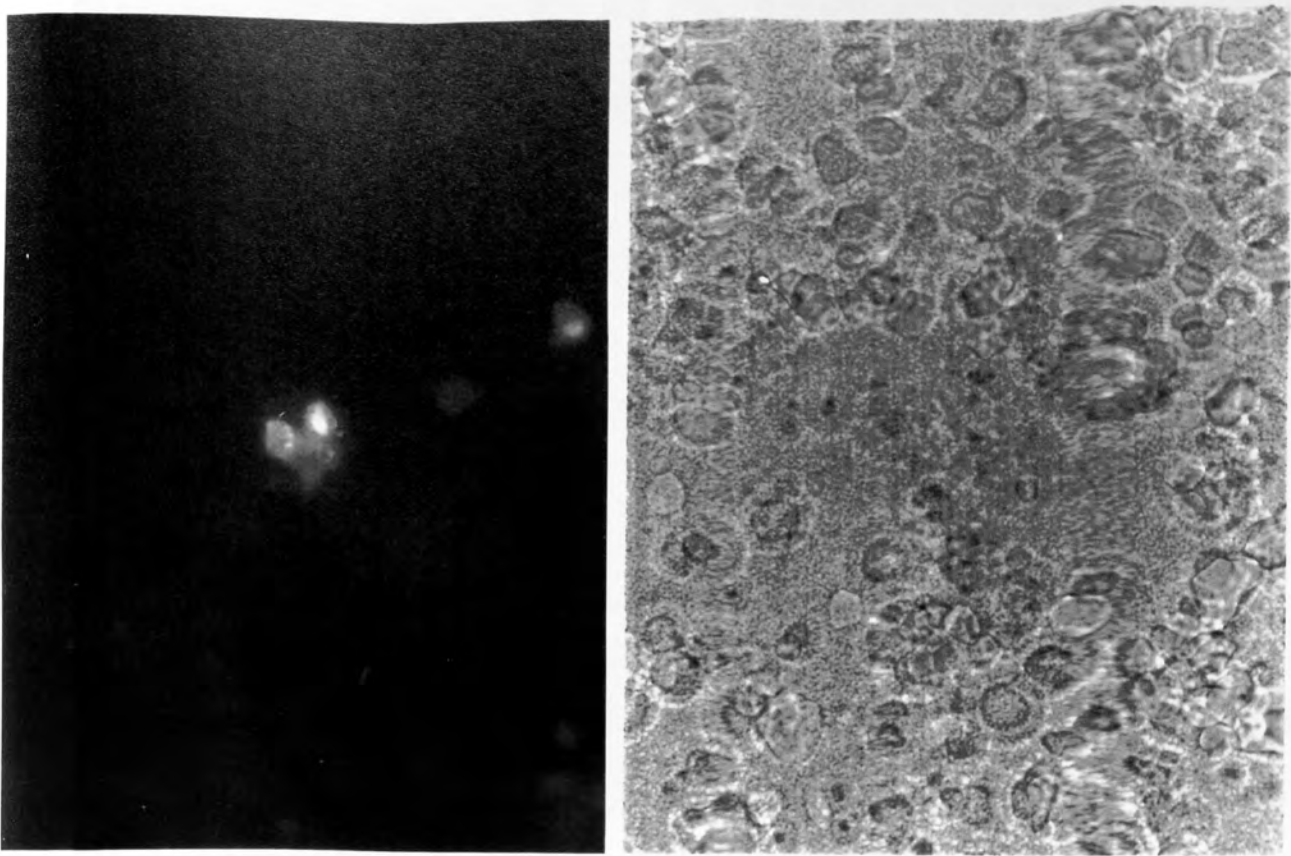


Figure 56 Porcine chondrocytes cultured over the macroporous IPN 'PC97' for one week. This cluster was stained for type II collagen with the MAb CIIC1.

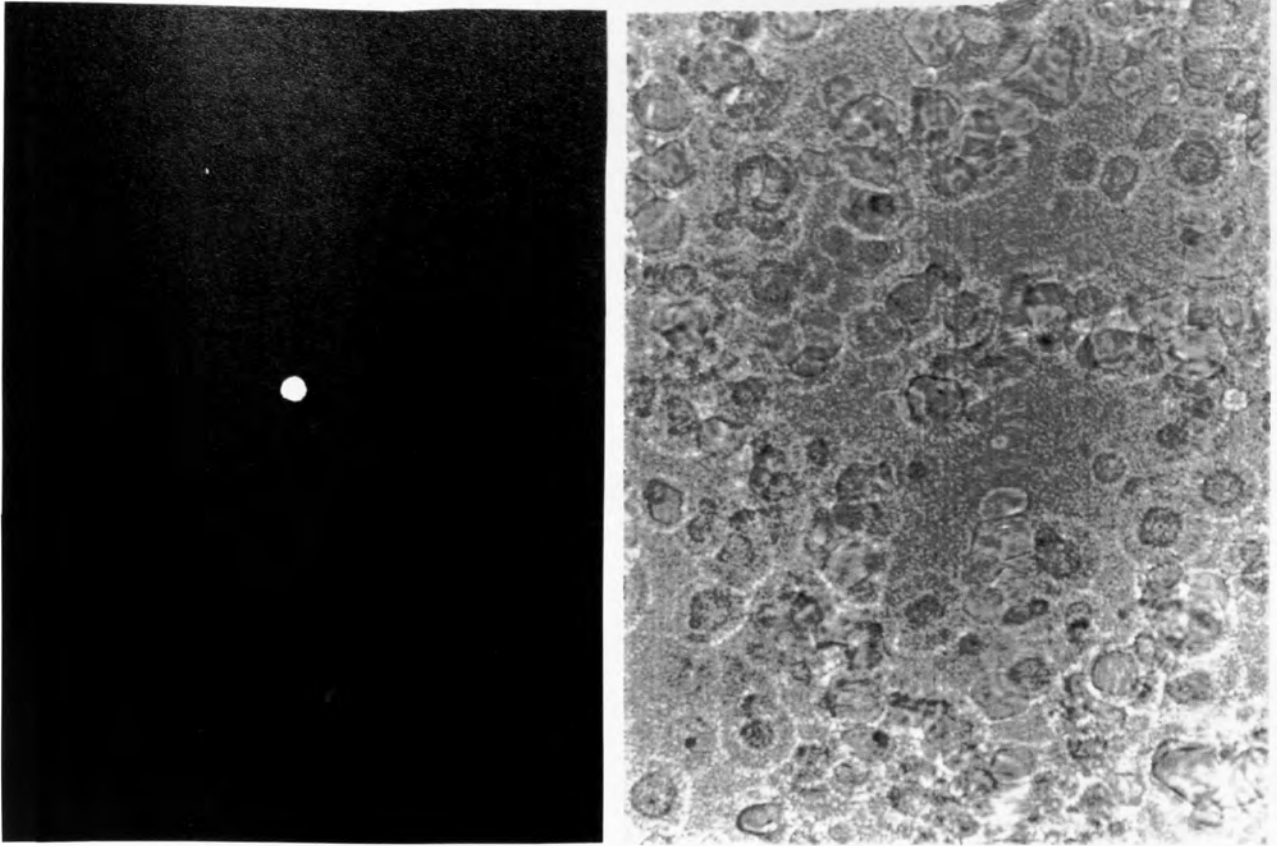


Figure 57 Porcine chondrocytes cultured over the macroporous IPN 'PC97' for one week. This single cell was stained for type I collagen with the MAb SP1D8.

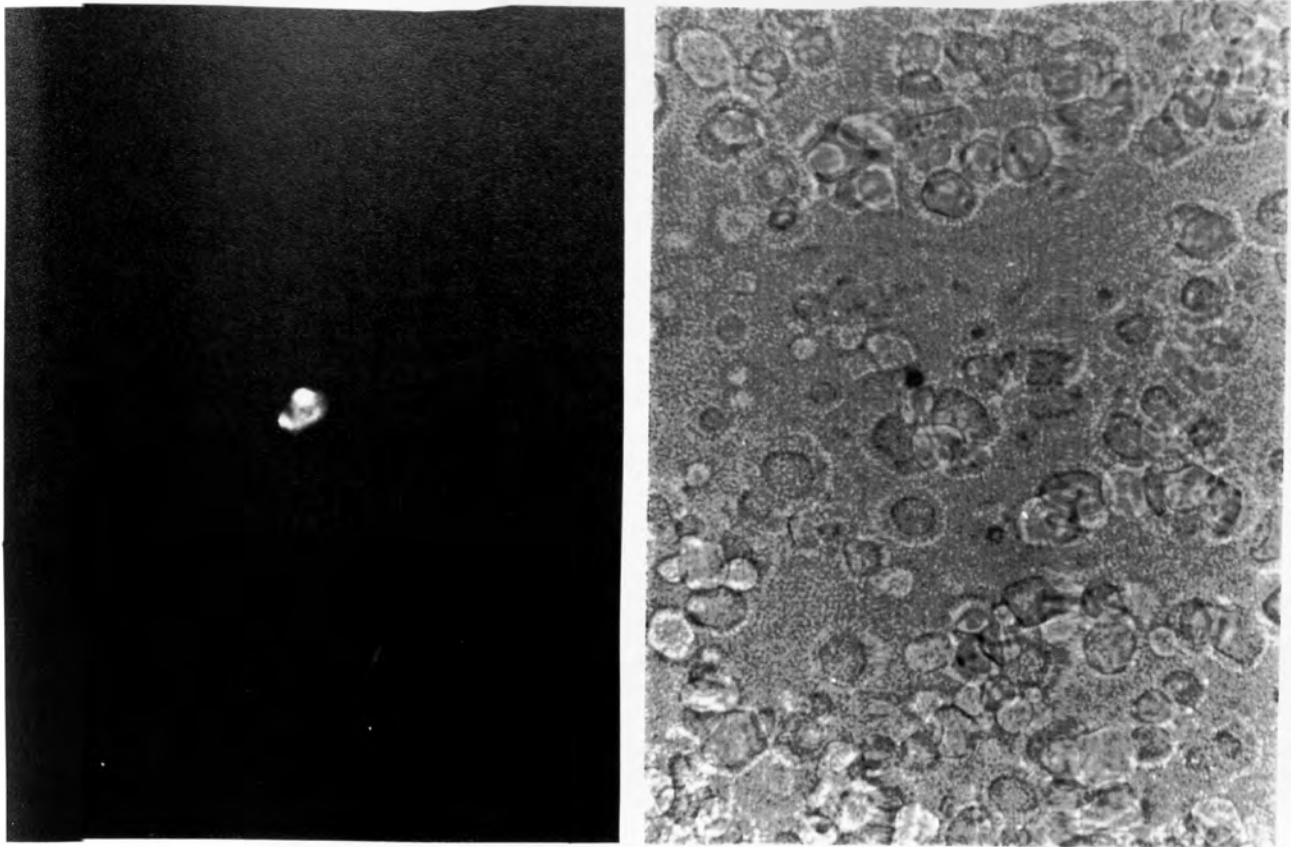


Figure 58 Porcine chondrocytes cultured over the macroporous IPN 'PC97' for one week. This single cell was stained for type II collagen with the polyclonal Ab P2.

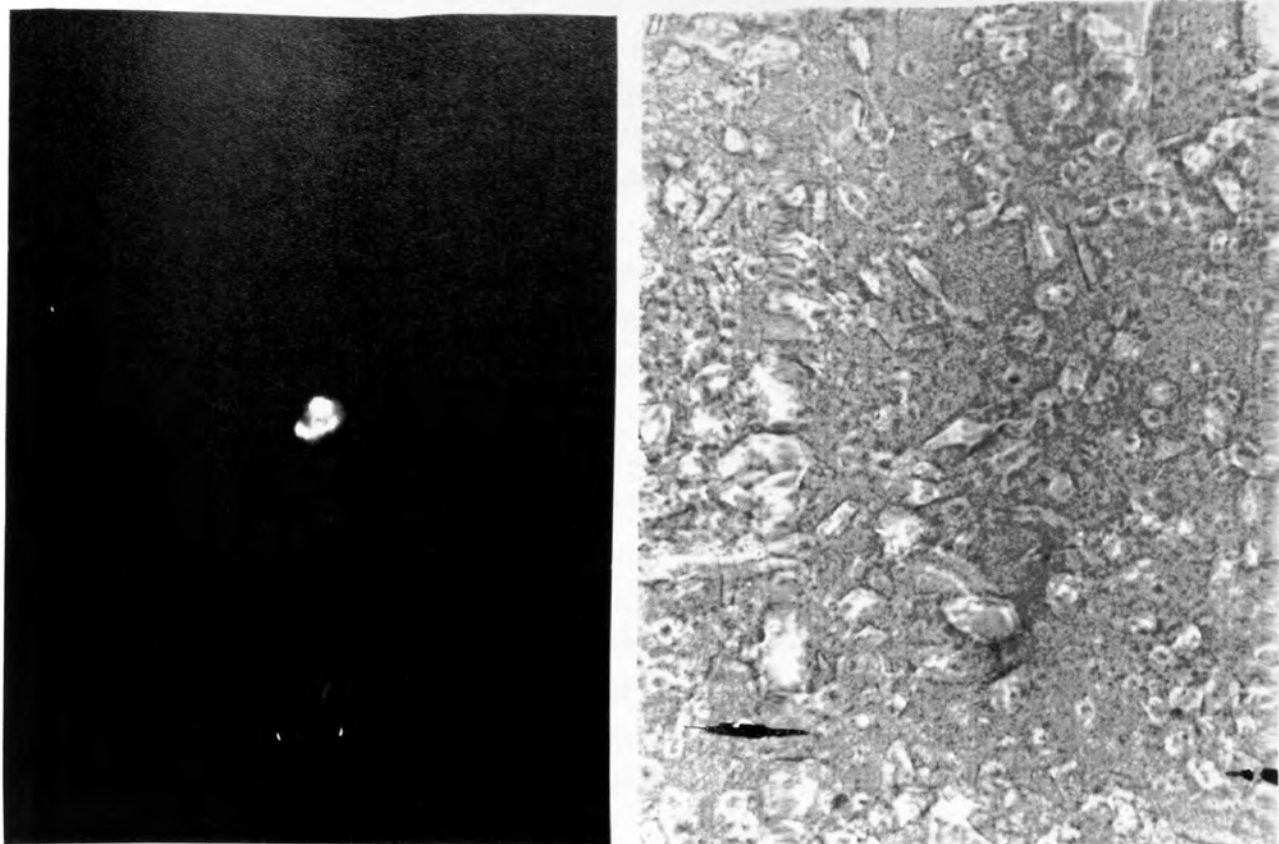


Figure 59 Porcine chondrocytes were cultured over the porous IPN 'PC123' for one week. This small cluster was stained for type I collagen with the MAb SP1D8. Cells also stained for type II collagen but these results were not photographed due to the low levels of fluorescence.

5.3.4 Discussion

The experiments described in this section investigated the chondrocyte response to sIPN hydrogels formed with a variety of pore sizes. It was shown that a macroporous sIPN with 38 μ M diameter pores supported cell adhesion but not spreading *in vitro*. Furthermore, previously dedifferentiated chondrocytes plated onto the porous sIPN'PC97' produced type II collagen indicating that these cells expressed a chondrocytic phenotype when grown on this material.

The introduction of porosity into the material was successful in increasing the *in vitro* compatibility with chondrocytes. In addition to this, the porosity of an implant made from this sIPN increases mechanical stability at the implant interface *in vivo*, and assists lubrication of the smooth articulating implant surface.

Previous approaches to designing a synthetic cartilage have included chondrocytes packed into collagen gels (236) and various polymers (237). The collagen gels packed with living chondrocytes exhibited superior incorporation and remodelling proximal to the implant, but had little weight bearing capacity. Hydrogels have great potential as synthetic cartilage. The high water contents attainable in hydrogels mimics that of cartilage. The first attempts at using hydrogels for synthetic cartilage were in 1973 (238). The use of a polyvinyl alcohol was suggested, grafted with cationic groups at the surface to provide interaction with the hyaluronic acid in the joint capsule. Further improvements in the mechanical properties of this material were made by Peppas et al (239), but there is no biocompatibility information on this material. Murray and Dow (240) found homogeneous polyHEMA gel implants acceptable, as they caused little abrasion in the opposing cartilage. Microporous polyHEMA gels were first suggested by McCutchen (241) and later implants of this type were investigated *in vivo* by Kon and de Visser (242). They used a simple polyHEMA gel and found an optimal pore size of 50 μ m for weight bearing and biocompatibility. There were no further reports on this promising material. More recent developments in polymers for cartilage replacement focus on providing a base for cartilage resurfacing from the bone marrow precursors.

The prospect of implant calcification should always be considered (243). Calcification is actually desirable at the implant interface with the bony supporting area, but calcification elsewhere would be disastrous. The calcification of bone itself is still poorly understood, but it seems that the lack of calcification of healthy cartilage depends partially on the proper lubrication of the joint and hence bathing of the articular cartilage in joint fluids.

This research group has made big improvements on the mechanical properties of suitable high water content polymers using interpenetrating networks (231,232). The potential of these hydrogel composites to support the growth and encourage phenotypic expression of chondrocytes *in vitro* was investigated in the experiments described in this chapter, with a view to developing prostheses capable of biological integration with natural cartilage.

The production of a suitable matrix by chondrocytes is critical to implant integration in cartilage. Dedifferentiation induced by inappropriate materials may lead to fibrosis and implant

loosening, in addition to vascularisation and innervation of natural cartilage peripheral to the implant. For these reasons, chondrocytes in the remaining tissue adjoining an implant should be encouraged to revert to a chondrocytic phenotype as quickly as possible. In this chapter it was shown that topology of the implant interface could be used to manipulate chondrocyte phenotype *in vitro*. A synthetic artificial cartilage material based on a porous sIPN polymer was developed as a result of the *in vitro* experiments. Animal trials were carried out further to the work described in this chapter⁽²⁴⁵⁾. Tibial plateau implants (penetrating subchondral bone) of the sIPN 'PC97' were examined after 3 months. The implants were placed in punctate lesions 3 mm in diameter, and were retained *in situ* for the experimental period. Reaction to the material (giant cell reaction) was mainly caused by an excess of particulate debris introduced into the joint capsule during implantation. Integration with natural cartilage was macroscopically good, but microscopic investigations were marred by inadequate sample preparation. Notably, type II collagen and keratan sulphate antibody staining was positive on some successful sections of the implant, indicating phenotypic stability of the chondrocytes adjacent to the implant. Further development of this synthetic material is now being undertaken.

Whilst punctate defects offer a convenient experimental site, their relevance to clinical situations is questionable. Most clinical conditions requiring cartilage replacement are a result of cartilage abraded over large areas of the surface. In order to create a suitable integrating surface it may be necessary to fabricate total articulating surfaces from synthetic materials and anchor these to the subchondral bone. Alternatively, combined therapies could be developed, coordinating synthetic material incorporation with drug treatments designed to encourage cartilage growth. For example, *in vivo*, it may be possible to further encourage phenotypic expression peripheral to the implant by impregnating the implant transforming growth factor β ⁽²⁴⁴⁾ or a high viscosity collagen II solution.

In conclusion, sIPN materials were successfully developed to form a suitable replacement for articular cartilage. Qualitative assessment methods to monitor chondrocyte behaviour on these materials were developed. Previously dedifferentiated chondrocytes were returned to phenotype after plating onto these materials. The cells remained rounded and demonstrated production of type II collagen, indicating expression of the chondrocyte phenotype.



Key points

- 1) Macroporous hydrogels formed from simple HEMA:EGDM mixtures around an ice crystalline matrix exhibit a number of morphologies depending on the content of the aqueous phase and freezing rate.
- 2) Macroporous hydrogels form suitable substrates for cell growth. Fibroblastic cell growth is accelerated on porous membranes as compared to nonporous membranes of the same composition.
- 3) Cell growth aligns to the grain of the porous surfaces.
- 4) Confluent cell layers adhere strongly to porous membranes, but detach easily from nonporous membranes of the same composition.
- 5) Chondrocytes plated onto SIPN hydrogels suitable for use as synthetic cartilage with pore size $38\mu\text{M}$ retain a rounded phenotype. Larger pore sizes initiate spreading.
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Contents

Introduction

What is a digital business? A business that uses digital technologies to create value for its customers and to improve its internal operations.

Why is digital business important? It is the future of business and it is essential for companies to be successful in the 21st century.

How can companies succeed in a digital business? By embracing digital technologies and creating a digital business strategy.

Chapter 6

Discussion

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6.0 Introduction

This chapter serves to reiterate the results and conclusions of the experimental chapters in this thesis, and discuss them in the context of the model of cell adhesion presented in the introduction.

The introduction brought together recent descriptions of all the factors affecting cellular adhesion to polymer surfaces. It has been emphasised throughout that an understanding of multiple aspects of the adhesion process is important for the proper design of experiments and appreciation of the literature. The largely physicochemical explanations of cell adhesion that characterise the literature of the 1970s have been added to by the great volume of literature in the mid 1980s to date, that explain the nature of cell adhesion receptors and specific adhesion proteins. The fusion of these two major lines of reasoning now begins to build an informed picture of cell adhesion that allows a degree of prediction of the cell response to a specified material. Whilst it is true that many anomalies haunt the model presented here, this thesis seeks to broadly represent the understanding of cell adhesion to surfaces to date, and provide a context for future experimentation. With the increasingly detailed analysis of cell type integrin expression and control available in the literature together with the availability of cell lines of defined integrin expression, antibodies to integrins and to adhesion proteins, and novel peptides, the study of cell response to materials in the 1990s offers some very promising lines of research.

6.1 What were the major experimental conclusions of this thesis? New directions for further studies and some suggestions for specific experiments.

Three main subject areas that comprise this study have been examined experimentally as described in chapters 3,4, and 5.

Chapter 3 considered the use of cell adhesion studies to 'probe' polymer surfaces. Cell adhesion provided an indication of the protein adsorption behaviour of potential biomaterials. Cell adhesion *in vitro* was also used to identify adhesion proteins adsorbed onto lens materials *in vivo*.

The first group of materials examined in chapter 3 were PEGMA substituted polyHEMA gels. Statistically random sequence distribution was achieved in these copolymers as the reactivity ratios of the monomers (HEMA and PEGMA) were moderate and similar. Water content was increased by the presence of PEGMA, but reduced cell adhesion could be attributed to the long PEO chains rather than the increase in water content. Briefly, experimental materials were adsorbed with serum prior to assessing cell adhesion. Cell adhesion was assessed with respect to a number of polymer surfaces exhibiting trends in PEO chain lengths. Decreased amounts of cell adhesion to PEO modified polymers correlated broadly with increases in chain length. Hydroxy terminated PEGMA substituted polymers did

not exhibit the same antiadhesive properties as methoxy terminated PEGMA substituted polymers. This was reflected in the measured physical properties of these polymers, showing that cell adhesion represents a useful method for characterisation of a surface. Decreased cell adhesion was shown at relatively low PEO chain lengths. There are two possible explanations for this. Firstly, the substitution of the PEGMA into the HEMA polymer allowed rotational mobility of PEO chains in the plane of the surface as well as the mobility of the flexible chains perpendicular to the surface. Secondly, the sequence distribution of the PEO chains along the backbone of the polymer is also of paramount importance. An even distribution, achieved by controlling polymerisation with careful choice of monomers and conditions, results in a homogeneous 'carpet' of PEO chains. The effects of surface distribution of PEO in a block copolymer was previously noted by Okano et al⁽⁹³⁾. The increased effectiveness of the PEO chains in the substituted PEGMA polymer in reducing protein adsorption, as compared to, say, adsorbed or grafted PEO chains means that these PEGMA substituted materials are likely to be more robust in functional use. Further experiments could include investigation of optimised non adhesive PEGMA substituted polymers in conjunction with the effects of adhesion proteins or specific chemical groups as ligands. Used as affinity chromatography supports, the relative efficiency of these materials in reducing non specific adsorption could be assessed against the more commonly used polysulphone.

In chapter 3, adhesion assays were then used to assess a number of polymer surfaces originally prepared for analysis of sequence distribution effects. Trends in cell adhesion reflecting the composition of the polymers were observed. In relatively random copolymers such as HEMA : MMA, percentage conversion made little difference to the amount of cell adhesion. However, the increasing amounts of NVP in a HEMA : NVP copolymer were reflected by the reduction in cell adhesion on polymers with a higher percentage conversion. Of particular note, were the effects of introduction of charged monomers, NVI and SPE. The positive charge of the NVI increased cell adhesion but the introduction of SPE, a negative pendant functionality, cell adhesion was decreased. Cell adhesion to most serum coated surfaces is mediated by adsorbed adhesion proteins. However, extremely dense charged surfaces, such as can be achieved by coating with polylysine, can override effects of adhesion protein mediated adhesion. Does this occur in the case of highly charged hydrogels such as these? No cell death was noted in these assays, but cell growth was not assessed after 1 hour. Cell death has previously been noted on highly charged polymer surfaces⁽⁷⁴⁾. The differentiation between direct charge effects and charge effects mediated by adsorbed adhesion proteins remains to be investigated. This line of research could be followed using the techniques described in section 3.4 of chapter 3.

A natural progression from the work described in chapter 3 of this thesis, and the earlier work of Thomas⁽⁶⁵⁾ would be the investigation of the relative contributions of Vn and Fn to cell adhesion. A wide range of polyclonal antibodies suitable for blocking cell adhesion to these adhesion proteins are now available commercially. In conjunction with antibodies directed towards integrin subunits, cell adhesion strategies could be examined. Simple

experiments using polyclonal blocking antibodies were described in chapter 3, section 3.4. Cells were used to probe the surface of worn contact lenses. The aim of this set of experiments was to identify the specific adhesion proteins adsorbed to the surface from the ocular environment. Cell adherence to the lenses was assessed after preadsorbing the lenses with antibodies to either Vn or Fn. This experiment was carefully controlled with nonimmune IgG in order to exclude the measurement of any nonspecific cell adhesion effects. Adhesion was strongly inhibited by antiVn antibodies and less strongly by antiFn antibodies. This indicated the presence of both adhesion proteins on the surface, and showed that the cells were using both adhesion proteins for initial adhesion. This may have reflected the promiscuity of integrin receptors^(15,16) that was discussed in the introduction, section 1.1.3. It was not possible to assign the adhesion to Fn or Vn to any one receptor type. It is most likely that the 3T3 cells bear a number of receptors for Fn and Vn, some of which interact with both proteins. As the adhesion of cells to Acuvue® based contact lenses has been shown to be partly a Vn mediated response, the presence of Vn in tears is confirmed. The adsorption of Vn to lens materials from the ocular environment raises questions about the role of contact lenses in the disruption of the proteolytic regulatory system in the eye. There is great potential for further experimentation in this field. (The role of surface adsorbed Vn is further considered in section 6.5 in this chapter). The following experiments are suggested as an easily achieved limited study:

- a) The correlation of tear Vn levels (assessed via an ELISA assay using the same antibodies as for blocking cell adhesion) with Vn adsorbed to worn lenses assessed by cell adhesion levels. There may be difficulties at adsorbed higher Vn levels in this assay as cell adhesion may reach a maximum prior to maximum adsorption of Vn. This could be checked by a parallel experiment quantifying the amount of adsorbed radiolabelled Vn to lenses *in vitro*.
- b) The assessment of adsorbed Vn onto different lens materials *in vivo*. However, the possible contribution of Fn to adhesion particularly in polymers with an NH₂ pendant functionality cannot be ignored. As antiFn antibodies have also been used effectively here, this presents an interesting line of dual antibody experiments.
- c) Given the role of Vn in the proteolytic system as described in chapter 3, antibodies to a variety of the other components involved, such as plasmin, plasminogen activator and PAI could be used in quantitative assessments of the latter proteins by conventional methods. *In vivo*, contact lenses worn in eyes dosed with amiloride (a PA inhibitor) may yield interesting results in comparison to undosed eyes.

The role of divalent cations in the adhesion process was considered in Chapter 4. The effects of a variety of divalent cations was examined with respect to serum and adhesion protein coated tissue culture plastic. The differential effects of variety of cations were demonstrated. This is physiologically relevant, as cation concentrations *in vivo* are

heterogeneous. High concentrations of manganese are found in tears, and high concentrations of calcium are found in the soft tissues adjacent to calcified tissues.

The major points arising from the results of the experiments in chapter 4 can be summarised as follows. Divalent cations vary in their ability to promote cell adhesion to serum, collagen and vitronectin (Vn) coated surfaces. Cell response to divalent cations also depends on the cell type. Zinc inhibits 3T3 cell adhesion to Vn catalysed by other divalent cations. Strong evidence was presented to show that zinc directly competes with other cations for an integrin binding site. Completion of this study requires the identification of the integrin responsible for adhesion. This may well be a $\beta 3$ integrin. Antibodies to this integrin that blocked cell adhesion to serum coated surfaces would provide further evidence for the role of zinc as an inhibitor of $\beta 3$ integrin function.

It was shown here for the first time that zinc has a regulatory role in cell adhesion. This discovery further emphasised the role of cations *in vivo* and *in vitro* and brought into question the nature of *in vitro* cell culture mediums. Classical cell culture mediums such as Dulbeccos modified Eagles medium or minimal essential medium do not contain zinc salts in their formulations. Zinc salts are present in normal serum and tissue fluids, and this may be one of the many reasons that *in vitro* experiments fail to tally with the *in vivo* response to polymers. The methods of investigation used in chapter 4 to identify the effects of divalent cations on cell adhesion are suitable for further biomaterials studies; further experiments could include an examination of the effects of divalent cations on cell adhesion to adsorbed adhesion proteins on a variety of different polymers. This could be achieved easily if the polymers could be evenly coated onto the bottom of 96 well plates (This could be achieved using a plate spinner attachment on a centrifuge but may prove dangerous due to the use of volatile solvents. Current legislation regarding safety and the use of centrifuges may mean it is necessary to devise a non enclosed plate spinner with safety shields rather than modifying a current centrifuge for the purpose. This coating technique would be well worth establishing as many different experimental regimes could then be achieved using plate reader methods).

In addition to testing the contribution of Vn and Fn to cellular adhesion to groups of hydrogels, it would be valid to examine the divalent cation requirements for cell adhesion to adhesion proteins when adsorbed to various hydrogels. The water structuring effects of surface moieties on hydrogels alters the solubility of cations. Does this affect the integrin-adhesion protein binding? An interesting set of polymers to test might be the HEMA : EMA copolymer and HEMA : NVP copolymer sets with regard to the effects of magnesium, calcium and zinc. The 3T3 cell line could be used in addition to a cell line with a single receptor population.

In chapter 5, cell response to macroporous membranes was examined. Freeze-thaw formed macroporous hydrogel based membranes were assessed with regard to cellular growth and morphology. Aligned cell growth prior to confluence, superior stability of confluent layers of cells and faster growth rates were all features of cells grown on porous

surfaces where pore size was $<10\mu\text{M}$. Perhaps the most interesting feature of the cell growth on these porous materials was the increased growth rate. The cell shapes adopted by spread cells were constrained by the underlying structure of the pores. The cells tended to be elongated. They may have formed fewer focal adhesions, although this was not specifically examined. The increase in growth rate parallels that shown by O'Neil et al.⁽⁵⁶⁾ The latter investigations concerned fibroblasts adherent to various adhesive islands. Maximum incorporation of thymidine (an indicator of growth rate) was found in cell grown on long thin rectangular islands.

Perhaps these porous homogeneous copolymer gels have applications as supports for artificial organ culture? The fabrication of cell seeded vascular prostheses may be a particularly suitable avenue for further research. Endothelial confluent cell layers could be grown on the porous membranes in the same manner as demonstrated in chapter 5 for fibroblasts. The underlying porous structure would allow the extravasion of immune cell types *in vivo*. Porosity has been suggested as a means of stabilising synthetic corneal implants⁽²¹⁸⁾. Synthetic corneal implants are prone to rejection as a result of tissue breakdown at the implant periphery. Whilst the introduction of porosity may initially seem advantageous, a retrospective view of the results of chapter 3 indicates that this approach to implant stabilisation in the eye may not be successful. Briefly, in chapter 3, adsorbed Vn was found on worn contact lens materials. Adsorbed Vn may well be associated with extended proteolysis at the corneal surface of contact lens wearers. This observation may explain the tissue breakdown at the periphery of corneal implant materials. This problem would not be solved by introducing porosity into a material, but only by changing the nature of the polymer.

One of the major disadvantages of using porous gels as support for organs *in vivo* is the increased risk of calcification. Certainly, the main reason for the failure of microporous Hydron® as a breast implant material was calcification⁽²¹³⁾. Calcification is still a poorly understood physiological system, but is associated with necrosed tissue and lack of tissue fluid flow in some pathological states. Continual mechanical loading of tissue can also give rise to calcification, which is a consideration in the design of synthetic cartilage.

In the latter part of chapter 5, the fabrication of semi interpenetrating networks (sIPN) was described. The sIPNs were developed to give the mechanical properties required in synthetic cartilage. Previous attempts to fabricate a hydrogel based synthetic cartilage had not attempted to replicate the mechanical properties of natural cartilage⁽²³³⁻²³⁷⁾. The development of synthetic interpenetrating network hydrogels for biological functions is in its infancy. Further development of such polymers with a view to biomimesis could be aided by viewing the remarkable interpenetrating structures found in basement membranes and other tissues of the body. (Yurchenko et al⁽⁹⁵⁾ have made a comprehensive study of the basement membrane. The chemistry of the assembly of this structure is far more complex than likely to be attempted on a synthetic scale, yet could be instructive).

One of the largest considerations in the development of an implant is compatibility with the natural tissue at the implant interface. In this case, it was felt important that chondrocytes

maintained phenotype at the implant interface. Maintenance of the chondrocytic phenotype is associated with round shape and the production of type II collagen^(221,222,223,224). As the smooth surface of the sIPN materials induced spreading in plated chondrocytes, alteration of surface topology was attempted. *In vitro* experiments were carried out to assess the implant interface most likely to maintain chondrocyte phenotype. Optimum effects using macroporous sIPN polymers were obtained at 38 μ M pore diameter.

It was notable that fibronectin precoating the IPN materials with 38 μ M pores did not induce spreading in the plated chondrocytes. In this case the effects of the topology of the material were not overridden by the presence of an extra adhesive matrix coating. This was an observation on only one material, on one cell type, but implies that topological surface features giving rise to cytoskeletal restrictions that determine cellular morphology may be able to override adhesive nature of materials in other situations. In the introduction, section 1.26, the contribution of surface topology to adhesion protein adsorption was discussed. On many polymer surfaces, cell adhesion in the presence of serum is mediated by adsorbed Vn. Fibronectin (Fn) is only adsorbed in significant amounts from serum concentrations of 0.1%⁽¹²³⁾. However, the usual displacement of Fn during typical Vroman sequence protein adsorption from serum onto a surface does not occur in small spaces⁽¹⁰⁷⁾. This may be because displacing proteins (usually fibrinogen) are too large to enter the spaces. Does this effect occur on material surfaces? Does it occur in hydrogels? If the Fn is not displaceable by fibrinogen, is it available for cell adhesion? This question has potential for further cell assay based investigations.

6.2 The cell surface adhesion process; a complex system with many levels of control.

Adhesion is a complex process in which the cell, the surface and specific adhesion proteins interact. There are many levels of control in this system, which reflects the complex physiological requirements for adhesion *in vivo*.

What broad levels of control can be identified? The reader is referred back to figure 1 where the cell-adhesion protein-surface system is pictured as a whole.

- 1) The first level of control *in vivo* is exerted by systemic and local chemical signalling to the cell.
- 2) The response of the cell represents a second level of control. This varies according to specific receptor expression and prior physiological state. For example, the stage in the cell cycle. Adhesive interactions with adsorbed adhesion proteins then depend on integrin receptor expression and activation of the particular cell type.
- 3) The third level of control is determined by local concentrations of cations and competing proteins that affect the integrin-adhesion protein interaction.
- 4) The fourth level of control arises from the adsorption of specific proteins to the polymer surface. This partly depends on the composition of the aqueous environment.

5) The nature of the polymer surface represents a fifth level of control, as this determines the type, concentration, conformation and activity of any adsorbed proteins.

The recognition of the multiple factors involved in the cell adhesion process leads not only to an understanding of experimental systems but allows consideration of therapeutic intervention in the event of immune response to an implant. Would it be possible to diminish the inflammatory response to a material for example, by introducing local antibodies to adhesion peptides? Could persistent ulceration or chronic wounds be controlled by introduction of material that interferes with the enzyme regulation system that perpetuates these conditions? Positively charged materials seem to induce intimate and enzyme resistant association with cells. Could this be a route to enhancing bone material implants of the future?

6.3 What was *not* considered: emerging views on receptors, adhesion with a little lateral thinking and new thoughts on surfaces.

What was *not* considered in the outline of the cell adhesion process given in the introduction and literature review? Firstly, the cell adhesion receptors were classified as four major sets, of which integrins were of most interest due to their interaction with the classical adhesion proteins. It has recently been shown that some cellular receptors that interact with collagen (76) for example are not integrins, although the anchorins (annexins) do share the EF hand homology that characterises integrins. The discovery of receptors with disintegrin domains is also important (155). These receptors may also have a role in binding to adhesion proteins.

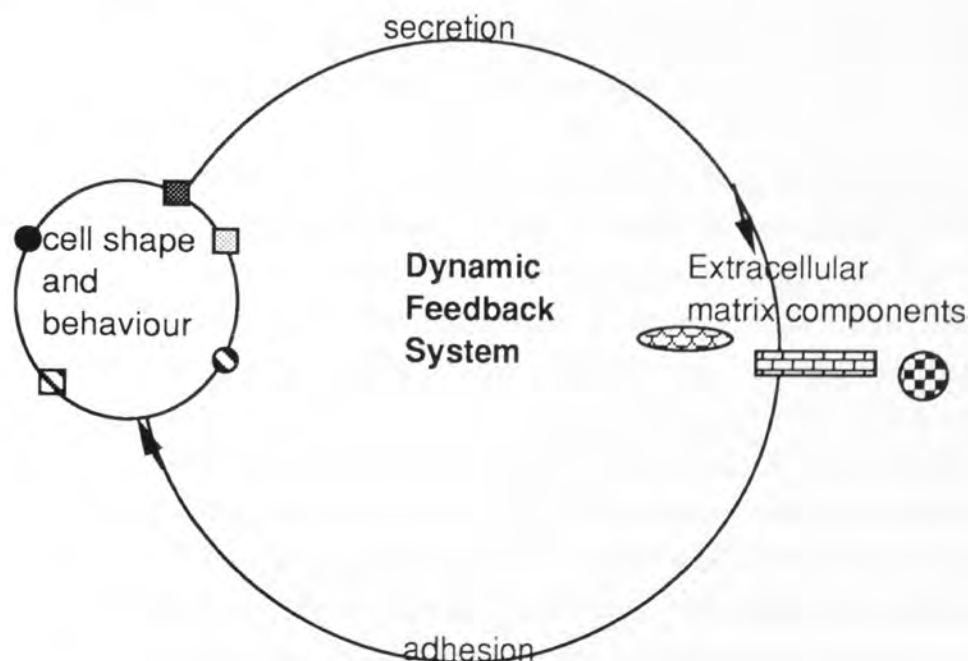
Secondly, during this thesis it has been largely assumed that cell interaction with one surface adsorbed adhesion protein is the only method of adhesion in a serum containing situation (although it was emphasised that charged macromolecules such as polylysine or Cell tak®, or highly charged synthetic surfaces could override the effects of nonadhesive proteins by nonspecific charge interactions with the cell surface). The *in vivo* response is known to differ from the *in vitro* response to materials for many reasons, not least of which is the dynamic physiological environment and complex collection of interacting cell types *in vivo*. It is proposed here that some of the differences are due to the interactions between adhesion proteins. If a cell type lacks receptors for say, Vn, it can still adsorb to a surface that primarily adsorbs Vn because Vn also binds to other adhesion proteins such as collagens for which the cell may have receptors.

Thirdly, surface chemistry and related physical characteristics are often regarded as static features of a system. There is a small amount of evidence to suggest that cell esterases alter surfaces, but this field of study is undersubscribed. Surfaces consisting of polymers with easily hydrolysable backbones, so called 'biodegradable' polymers have been developed specifically for their susceptibility to physiological processes. Given the biological repertoire

of matrix degrading enzymes and free radical production - degrading collagens, carbohydrates, lipids it seems possible that alteration of the 'nondegradable' polymer surface can occur. polyHEMA surfaces examined in this study were susceptible to surface oxidation through age and contamination. The introduction of methacrylic acid groups makes the polyHEMA cell adhesive. This effect is more noticeable on gel cast polyHEMA.

Fourthly, spatial heterogeneity of adsorbed proteins is possible even on homogeneous surfaces (especially hydrophobic surfaces). This phenomenon was demonstrated by several techniques including visual amplification by adherent bacteria (246) and immunolabelling (112,148). This heterogeneity could lead to initial preferential adhesion of cell types or differences in receptor clustering and cell growth. This effect is often ignored and may be relevant particularly where studies reveal differences in cell adhesion or spreading despite similar amounts and conformation of adhesion proteins on two surfaces.

Lastly, this thesis has not concentrated on the longer term response to materials. *In vivo*, regulatory control is based on a feedback system of cellular secretion of matrix which then regulates the phenotype and subsequent matrix turnover by the cell (see diagram below). Systemic or local chemical signalling can alter the state of this system to allow flexibility for say, growth or injury repair. Turnover of the matrix relies on local proteolysis as described in section 6.4. The introduction of a polymer surface into the system may alter several features of this system. Firstly, matrix deposition may be altered at the surface, and hence alter cell phenotype. Secondly, matrix proximal to the surface may be more or less susceptible to proteolysis and turnover. Longterm changes in the surface due to pericellular oxidation may alter the response over a long period of time.



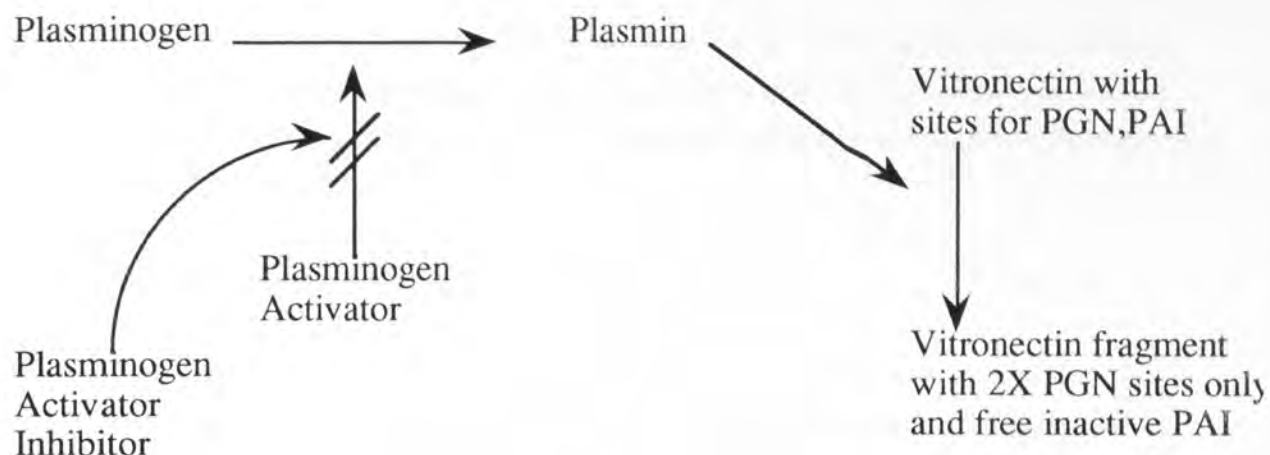
6.4 Biomaterials design from the cellular aspect. New horizons resulting from understanding of cellular processes.

Materials design is rarely a problem of making a material adhesive. As outlined in the introduction, many polymers are adhesive and adhesion proteins such as Vn adhere strongly and irreversibly to surfaces even in the presence of many competing proteins. Rather the problem is making a surface preferentially adhesive to a particular cell type, endothelial cells for example, in preference to fibroblasts or leukocytes, or making a surface totally nonadhesive. High water content hydrogels or PEGMA substituted polymers offer some solutions to the latter case, but high water content should be considered along with other parameters such as sequence distribution in a polymer. High water content gels can be mechanically inappropriate although interpenetrating network (IPN) technology offers mechanical strength combined with a high water content. IPN materials are often cell adhesive which may be due to concentration of particular charged groups at the surface of the polymer. Surface modifications of polymers can also be achieved with plasma modifications, which may increase the adhesive nature of a polymer⁽⁶⁷⁾. Surface modifications can also be achieved with polyethylene oxide chains, either substituted into polymer as described in this thesis, or grafted or adsorbed onto the surface of a polymer. Additionally, selectively adhesive polymers can be produced by exploiting knowledge of the adhesion protein sequences. Whilst RGD type adhesion sequence modifications of material surfaces offer promise *in vitro*, implantation *in vivo* may result in other reactions.

6.5 The role of vitronectin in the response to biomaterials. Maintenance and turnover of cell associated matrix proteins.

This thesis examined the initial cell adhesion to polymer surfaces *in vitro*. As stated in the introduction, initial cell adhesion is only one aspect of the cellular interaction with material surfaces. The strength of cell adhesion and subsequent locomotion and cell replication were not specifically addressed. These factors are also important considerations in the design of a biomaterial for *in vivo* use. The strength of adhesion may reflect the number and distribution of adhesion plaques, and the organisation of the binding complex. The conformation and strength of adhesion of the adhesion protein-surface interaction is obviously another factor in the strength of adhesion between cell and material surface. Cell locomotion and replication both involve the breaking and reformation of adhesive points between the cell and the surface. This may be achieved by localised proteolysis at the cell surface or by specific conformational changes in the integrin via inside out signalling. Extravasation of immune cell types across basement membrane relies on their secretion of matrix degrading enzymes and metastatic cells also cross boundaries using this technique. Matrix degrading enzymes include metalloprotease type collagenases and the serine proteases that digest adhesion proteins like Fn.

Vn is established as the primary adhesion protein for many cells in serum containing media *in vitro*, and probably has the same function *in vivo*. Recent work suggests that Vn also plays an important part in the regulation of localised proteolysis *in vivo*. Localised proteolysis i.e. pericellular proteolysis is a necessary process in the general maintenance and turnover of tissues. Upregulated proteolysis is necessary to dissolve fibrin clots and assist in wound healing.



As described in the introduction and illustrated in the diagram above, Vn binds plasminogen and plasminogen activator inhibitor as well as thrombin, heparin, complement components and cells. PAI is only active when bound to Vn. Vn in the plasma is nearly always bound to thrombin which ensures its localisation in tissues at the site of injury. Cells have high affinity sites for plasminogen activator and plasminogen, which ensure that proteolysis is a localised pericellular event^(173,174,175). Recent work suggests that limited proteolysis of Vn by plasmin results in a loss of binding affinity for PAI but a two fold increase for plasminogen binding⁽¹³⁹⁾. This would result in a localised upregulation of proteolytic activity as Vn is transformed from an antifibrinolytic to a profibrinolytic cofactor. The perpetuation of plasminogen activator at the periphery of corneal epithelial healing wounds leads to further ulceration⁽¹⁷¹⁾, and chronic wound fluid was also found to contain high levels of PA and Vn⁽¹⁷²⁾. Increased collagenase production causing further damage to the stroma was observed in cases of perpetual corneal ulceration. In cartilage disease, upregulation in protease in the synovium digests synovial Fn. The Fn fragments diffuse into the cartilage and initiate the production of collagenase by chondrocytes, leading to cartilage breakdown. Introduction of materials into the synovium can initiate this response which may be perpetuated by material adsorbed Vn⁽¹⁷⁶⁾. It is possible that Fn fragments have a similar activity in the stromal tissue of the cornea, initiating collagenase production. How does this relate to the preferential adsorption of Vn to many biomaterials?

As the conformation of Vn affects its ability to bind to other proteins such as plasminogen, it is possible that binding sites may be obscured or revealed by adsorption to a surface. In the normal situation the upregulation of plasmin activity caused by the increase in

binding of plasminogen and loss of binding sites for inhibitors is burned out when the Vn is finally digested into nonfunctional fragments. If material is present, firstly the total reservoir of Vn (adsorbed and soluble) is likely to be much greater and secondly, the adsorbed Vn may take longer to digest due to conformational changes, drawing out the process. If Vn was still active as a cell adhesion protein, then subsequent localised proteolysis by adherent cells may be affected. This would then affect cell locomotion and replication regardless of the nutritional state of the cells.

It was suggested for the first time in this thesis (section 3.4) that Vn was involved in the plasmin regulation system in the eye, and that the presence of Vn adsorbed onto contact lens materials may be responsible for the raised levels of plasmin found in contact lens wearers and associated pathologies.

6.6 Are adhesion proteins surface active agents ?

The surface adsorption of Vn in high concentrations relative to its concentration in the bulk is puzzling. What aspects of the protein molecule lead to this effect? Surface active protein molecules that lower surface tension, such as lung surfactants, adsorb strongly to surfaces because of a particular tertiary structure. Typically, these structures are α helices where one aspect of the helix contains all the polar or hydrophilic groups and the other contains all of the non polar and hydrophobic residues. These molecules have an amphiphilic nature. It was recently determined that other protein structures have amphiphilic characteristics. These include the π helices of apolipoprotein E⁽²⁴⁷⁾ and an unusual β sheet sequence found in apolipoprotein β ⁽²⁴⁸⁾. Synthetic derivatives of the latter peptide mimic the surface active properties of the whole protein, stabilising lipoprotein particles of 200 A, with a tendency to self associate in aqueous solution.

Vn and Fn are known to be principally β sheet formers and do not contain any regions of classically amphiphilic α helix. The primary structures of Vn and Fn were, however, checked for the characteristic amphiphilic β sheet sequences discussed above. These structures were not found, even in small sections in either protein. Vn does contain a high proportion of hydrophilic residues however, and also normally associates with a large variety of other components of serum. For example, heparin, TAT, PAI, β endorphin and serotonin. It is possible that some of these associated proteins may contain amphiphilic characteristics which explain the adsorption properties of Vn and Fn.

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Appendix

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- I Single letter code for amino acids
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I Single letter code for amino acids

A alanine
C cysteine
D aspartic acid
E glutamic acid
F phenylalanine
G glycine
H histidine
I isoleucine
K lysine
L leucine
M methionine
N asparagine
P proline
Q glutamine
R arginine
S serine
T threonine
V valine
W tryptophan
Y tyrosine

II Equivalent nomenclature in the literature

CD44	hyaluronate receptor, found in many cell types.
C3b, iC3b.....C7-9	refer to various parts of complement sequence. 'i' indicates the activated form of the protein
C5b-9	end of complement activation which is membrane attack complex
CR3=CD11b/CD18	complement receptor 3, a $\beta 2$ integrin, $\alpha M\beta 2$
CR4=CD11c/CD18=p150,95	" 4 " $\alpha x\beta 2$
ELAM-1	endothelial leukocyte adhesion molecule 1, an L selectin
GPIIa IIIb	platelet glycoprotein, $\alpha IIb\beta 3$, a $\beta 3$ integrin
ICAM-1=CD54	intercellular adhesion molecule, an immunoglobulin
J1-160 or 180 or 200	tenascin related neuronal glycoproteins
LAM	leukocyte adhesion molecule, a selectin
LECAM	leukocyte endothelial cell adhesion molecule, a selectin
Leu-CAM	leukocyte cell adhesion molecule, a $\beta 2$ integrin
LFA 1=CD11a/CD18	lymphocyte function related antigen 1, a $\beta 2$ integrin, $\alpha L\beta 2$
LPAM 1=CD49d/CD29	mouse lymphocyte Peyer's patch adhesion molecule, a $\beta 1$ integrin, $\alpha 4\beta 1$
Mac1=Mo1=CD11b/CD18	macrophage, a $\beta 2$ integrin
Osteonectin	also called SPARC protein and BM40, glycoprotein rich in cysteine, can inhibit cell spreading. Bone related adhesive glycoprotein
Tenascin	glycoprotein that can block adhesion to fibronectin, but can promote adhesion under certain conditions
Thrombospondin	adhesion protein with some antiadhesive properties under certain conditions
VCAM=INCAM	vascular cell adhesion molecule, an immunoglobulin
Vitronectin	serum glycoprotein important in cell adhesion also called S protein, epibolin and serum spreading factor
VLA 4=CD49d/CD29	very late activation antigen 4, a $\beta 1$ integrin, as are all VLA group receptors.

III Designing experiments with cells and surfaces; a set of ground rules

The scientific method involves asking a question in the right way so that the answer makes sense. To put it another way, experiments must be designed so that the result has a clear meaning with respect to the present model. Biomaterials science presents a wide array of different viewpoints which can prove confusing. A brief review of the present literature will reveal selections of papers covering fields as diverse as microbiology, haematology and immunology. If one of these subjects is to be researched with respect to a material it is essential to attain a degree of expert knowledge before proceeding with experiments.

General cellular interactions can be investigated *in vitro* quite successfully given an understanding of the present knowledge about cell adhesion receptors and mechanisms. The best results are obtained from experiments where all the variables are known quantities. The following points may provide some clarity useful to persons starting a set of experiments with mammalian cells.

1. Gather as much information as possible on the cell types to be used in experiments. If possible use cells with defined receptor populations. If the research does not require specific cell types use a classic, well used cell line such as the 3T3 swiss mouse fibroblast. Make sure that any blocking or staining antibodies do not arise from the same species as the cells in the experiment. Passage cells routinely with care, as it is easy to select for populations with different adhesion properties using hurried techniques. Cells can easily be synchronised by cold shock at replating.
2. Determine the adhesion protein necessary for initial adhesion in serum coated materials by the use of polyclonal blocking antibodies. When coating surfaces with pure proteins, mask the effects of any surface still exposed by post coating with denatured albumin. This does not apply to whole serum or complex mixtures of proteins, and can be checked against the results for the single proteins. Note the procedures used in chapter 4 of this thesis with particular regard to the divalent cation requirement of the adhesion.
3. Ensure that the material is not toxic to the cells. Establish routine viability tests in every assay. Ensure that materials are not fouled by bacteria, algae or fungi. Whilst these contaminants can be removed by detergents, endotoxins may still be adsorbed to the surface. These living organisms secrete enzymes and may well have made surface modifications that will affect the results of the experiment. Old materials may have oxidised, cell adhesive surfaces. Nonsterile, old polyHEMA based gels are a classic example of this.
4. Cell adhesion in the presence of serum or tissue fluids is nearly always determined by the adhesion proteins adsorbed to the surface. The cellular interactions with adhesion proteins via

the integrins require physiological temperatures, divalent cations and metabolic activity by the cells. A few exceptions to this rule include cell adhesion by charged polylysine coatings and celltak coatings.

In the absence of serum or adhesion proteins cell adhesion is affected purely by the physicochemical interactions between cell surface and material surface. These interactions do not require physiological temperatures or divalent cations. Some research in the current literature still ignores these highly pertinent facts.

Cells secrete adhesion proteins that are loosely associated at their cell surfaces and inadequate trypsinisation may result in cells adhering in the apparent absence of adhesion proteins. Some transformed cells, such as HeLa cells, lack the ability to produce their own matrix and may be useful for investigating adhesion mechanisms in some experiments.

5. Spreading and adhesion are closely connected but divisible events in terms of cell material interactions. It is not clear what can be inferred from the shape of the cells. Spreading usually equates with stronger adhesion and either the presence or production of Fn. Vn is not associated with extensive spreading, but with adhesion. There is an emerging field of literature associated with cell shape and function. Quantify cell numbers by counts, dye binding or radiolabelling, and quantify cell shape by image analysis. Qualitative descriptions of cell shape are valid but pseudoquantifications such as '+++' are largely redundant as forms of useful information, being entirely subjective.

IV Fractal analysis

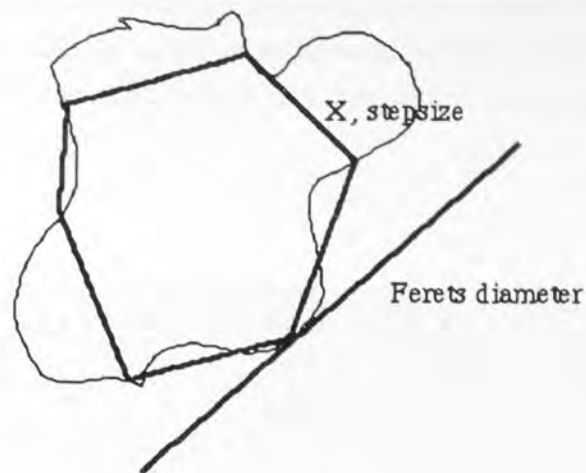
The degree of rugosity of a surface can be described qualitatively or as stepsize estimations. However a large body of work exists describing surfaces quantitatively by means of fractal dimensions. Fractal dimensions provide an ingenious way of describing a texture in a line or a plane. The term was invented by Benoit Mandelbrot, a scientist prominent in the world of chaos and fractals. The Mandelbrot set, a well known image derived from the reiteration Z^2+C on the complex plane, is named after him. In the mathematical world, fractals have the peculiar property of structure on a limitless scale i.e. one can magnify an image infinitely and still find the same degree of structure. This is true for the strange attractors found in many diverse chaotic systems-systems far from equilibrium behaving in a nonlinear fashion- such as heartbeats or weather patterns. However, surfaces and perimeters of bumpy objects are obviously not limitless in structure, but possess a "fractal quality".

Experimental measurements can be taken perhaps by means of light scatter measurements, scanning electron microscopy or even using scanning tunneling microscopy and the various dimensions may then be assessed by calculation. The chemical structure of the surface, which on some smaller scale also contributes to its intrinsic rugosity can also be described in fractal terms. This is especially useful for describing the domain structure on the surface copolymers. Fairly complex programs are normally used to assess the various fractal dimensions of a surface. However, a limited impression can be achieved simply by taking a cross section of the surface, magnifying the image and taking measurements with a ruler! (108)

A point or a line has nominally 1 dimension, whereas a plane has 2, and a cube or globe has 3 dimensions. A line with a few wiggles might have a dimension of say 1.3, and a plane with a rough surface a dimension of 2.45. (In fact there are several fractal dimensions that can be computed for a surface which gave a more complete mathematical description of its nature). In this way one can assign a quantity to the roughness of a surface or the perimeter of a rough particle-perhaps a spreading cell.

Fractal methods are obviously becoming more popular as a way of describing features. Chesters et al assessed the surface roughness of various electropolished and chemically polished surfaces from STM images, quantifying the fractal dimensions by using graph paper overlay techniques on the image profiles (108). Even proteins have fractal qualities. Allen et al (249) found that the roughest part of the molecule responds to its most adhesive part, and Wang et al (250) demonstrated the relationship between fractal dimension and tertiary structure of the molecule. Generally, the more extensible the local structure of a protein was (ie if the beta sheet content was high), the smaller the fractal dimension was.

Assessing the fractal nature of an outline



Perimeter estimates of the object are made using sequential smaller **step** sizes X . The perimeter estimate P is then plotted against the stepsize X logarithmically. **This** is known as a Richardson plot. (Both values are normalised with respect to the maximum **Ferets diameter** of the object and are therefore dimensionless). The slope of the resulting line, **added** to 1, is the fractal dimension of the boundary. Sometimes two or three distinct slopes **can** be found. These delineate 'roughness' at various stepsizes and are known variously as **textural** and **shape** fractal quantities.

Figure 60 shows a light micrograph of a 3T3 fibroblast spread on tissue culture plastic in the presence of serum and divalent cations.

The fractal dimensions of this cell are 1.06 where the stepsize is less **than** 0.08 with respect to the maximum Ferets diameter and 1.28 from stepsize 0.08 to 0.4. **These** figures correspond to the textural and shape fractals respectively. Cells spread or **not** spread under different culture conditions have varying fractal dimensions. Fairly **obviously** rounded cells tend to have shape fractals close to 1.0. The textural fractal then increases **with** the first cell processes on adhesion and the shape fractal increases on flattening of the cell. **Cell** types also vary. For example, in the few photographs examined to date, the regularly **arranged** islands of the SIRC cell line have a dimension closer to 1.0, whereas the 3T3 cell line **tends** to form islands of dimensions approaching 1.2. Obviously the measurements taken **are** dependent on the resolution of the photograph available. SEM can improve resolution but **the** preparation of the specimen tends to increase the roughness of the surface. The fine fibrillar **detail** seen on the average spread fibroblast under SEM would undoubtedly give rise to a much **larger** textural fractal than seen under light microscopy but the shape fractal would remain **essentially** the same. It is easy to make overgenerous assumptions when using any new **technique** and in **this** case there are many pitfalls to be made. For example, when the maximum Ferets diameter

exceeds the minimum Ferets diameter by a factor of 3 or more, the Richardson plot technique is invalid.

In conclusion, there are two major uses for fractal analysis in this field of research. Firstly, it may be worthwhile to investigate the correlation between fractal dimension of the perimeter of a cell when adherent to various substrates. Secondly, correlations of cell behaviour such as cell shape or rates of cell replication with fractal dimensions of a surface could be made.

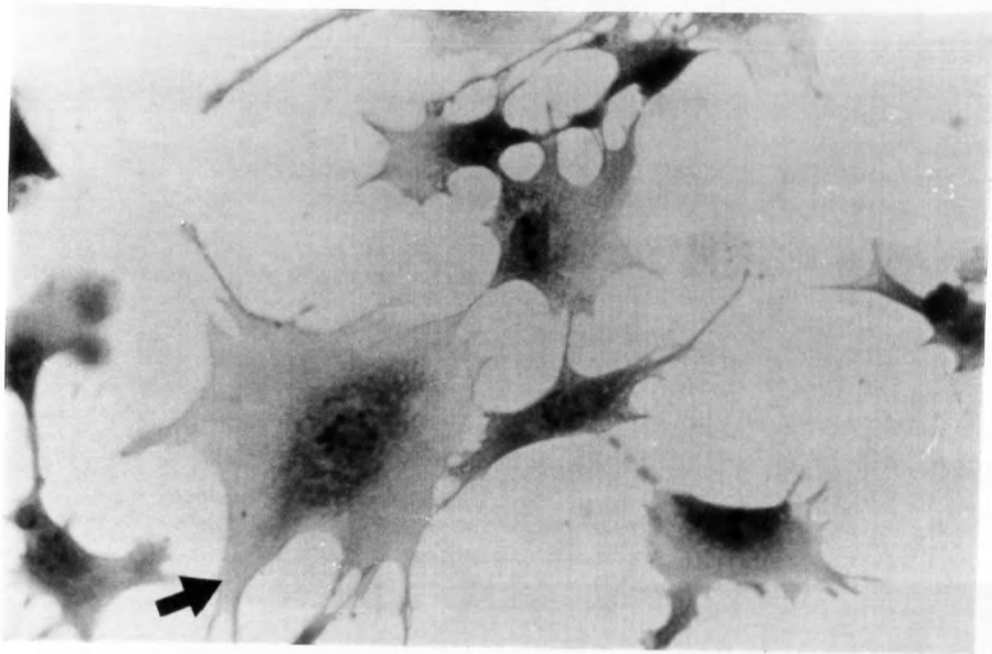


Figure 60 3T3 cells adhered to tissue culture plastic, stained with toluidine blue. x200
Fractal dimensions measured directly from this photograph: textural 1.06
shape 1.28

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