BIOLOGICAL INTERACTIONS WITH SYNTHETIC POLYMERS

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Summary of thesis

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Anchorage dependent cell culture is a useful model for investigating the interface that becomes established when a synthetic polymer is placed in contact with a biological system. The primary aim of this interdisciplinary study was to systematically investigate a number of properties that were already considered to have an influence on cell behaviour and thereby establish the extent of their importance. It is envisaged that investigations such as these will not only further the understanding of the mechanisms that affect cell adhesion but may ultimately lead to the development of improved biomaterials. In this study, surface analysis of materials was carried out in parallel with culture studies using fibroblast cells. Polarity, in it's ability to undergo hydrogen bonding (eg with water and proteins), had an important affect on cell behaviour, although structural arrangement and crystallinity were not found to exert any marked influence. In addition, the extent of oxidation that had occurred during the process of manufacture of substrates was also important. The treatment of polystyrene with a selected series of acids and gas plasmas confirmed the importance of polarity, structural groups and surface charge and it was shown that this polymer was not unique among 'hydrophobic' materials in it's inability to support cell adhesion. The individual water structuring groups within hydrogel polymers were also observed to have controlling effects on cell behaviour. An overall view of the biological response to both hydrogel and non-hydrogel materials highlighted the importance of surface oxidation, polarity, water structuring groups and surface charge.

Initial steps were also taken to analyse foetal calf serum, which is widely used to supplement cell culture media. Using an array of analytical techniques, further experiments were carried out to observe any possible differences in the amounts of lipids and calcium that become deposited to tissue culture and bacteriological grade plastic under cell culture conditions.

Keywords cell adhesion, polymeric substrates, biocompatibility, surface properties, lipids and calcium

To my parents

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Abbreviations

AA	acrylic acid
ACM	acrylamide
BHK	baby hamster kidney
DAACM	diacetone acrylamide
DMEM	Dulbecco's modified Eagle's medium
EDXA	energy dispersive x- ray microanalysis
EWC	equilibrium water content
ESCA	electron spectroscopy for chemical analysis
GLC	gas - liquid chromatography
γ_p	polar component of surface energy
γ_d	dispersive component of surface energy
γ_t	total surface energy
HMACM	hydroxy methacrylamide
HPA	hydroxy propyl acrylate
HPLC	high performance liquid chromatography
$-HSO_3$	sulphonate group
MAA	methacrylic acid
MACM	methacrylamide
NVP	N - vinyl pyrrolidone
N'N'DMA	N'N' dimethyl acrylamide
-OH	hydroxyl group
PBS	phosphate buffered saline
polyEMA	poly(ethyl methacrylate)
polyHEMA	poly(2 - hydroxy ethyl methacrylate)
polyMMA	poly(methyl methacrylate)
PVC	poly(vinyl chloride)
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TLC	thin layer chromatography
VP	vinyl pyrrolidone

CHAPTER 1

Introduction and literature review

1.1. Historical background to cell culture

It has been known for some time that a number of cell types can be kept alive under defined conditions in vitro, that is, in cell culture outside the living organism from which they were derived. The sophisticated cell culture techniques of today have developed greatly from their more primitive origins in the last century. The early pioneers were primarily concerned with the maintenance of embryonic tissue outside the living organism but their experiments were difficult to repeat, not least due to unsatisfactory media, and it was a point of controversy as to whether they were observing genuine survival of tissues or merely the delayed death of cells. However, the observations that were to revolutionise cell and tissue culture came in 1907 when Ross Harrison [1] demonstrated that the normal functional behaviour of nerve fibres could be continued in vitro. His relatively simple experiments highlighted the requirement for asceptic conditions and it was noted, even at this early stage, that some cell types required a solid support for growth in culture [1 - 3]. These observations were soon extended to include other cells [4 - 6] and thus, the foundations were laid for the development of one the most powerful research tools. Originally, workers had maintained cells in warm saline [7] or held the explant in blood or plasma [1 - 4] inverted over a hollow, ground microscope slide sealed with paraffin wax ("hanging drop") [8]. However, gradually the increased interest in this area led to further advances in the techniques. The need for the correct balance of essential nutrients was soon identified [9] which ultimately led to the development of suitable media for use in culture systems [10 - 12]. Interestingly, the importance of adding embryonic extracts to culture systems became recognised [5, 13] and steps were taken to limit the problems of bacterial infection [14]. As these more reliable culture techniques advanced, it was recognised that cell culture systems would have important applications in other many of research. However, routine use of such systems

was not without problems. The vessels used for the maintenance of cell lines were made of glass and unfortunately there were difficulties in continually recleaning and resterilising glassware. The introduction of disposable polystyrene petri dishes in the 1960's partially overcame this drawback, but a further problem lay in that native polystyrene did not allow attachment and spreading of fibroblasts [15 - 17]. However, it was later shown that these dishes could be rendered suitable for use in cell culture following surface treatments to render the polystyrene more hydrophilic [18 - 23]. These early findings eventually led to the development of commercially available tissue-culture plastic and this is the substrate currently used by most laboratories where routine cell-culture is carried out. Whilst manufactures are reluctant to disclose details as to its production, it is considered to be produced by glow discharge treating polystyrene vessels with an oxygen gas plasma [24 - 25].

1.2. Anchorage dependent cells

It is generally accepted that whilst some cell lines (and bacteria) can grow in suspension, others, particularly normal eukaryotic tissue and most primary cells require a suitable substrate to which they can adhere and spread for growth. Stoker et al [26] have introduced the term 'anchorage dependent' to describe those types of cells which need to become attached to and spread on a suitable surface in order to undergo normal cell behaviour in cell culture. Similarly these workers described those cells (transformed cells in culture and those derived from tumours) that have the ability to grow in suspension, unsupported by a surface as 'anchorage independent'.

1.3. The role of cell culture in the field of biomaterials science

Even from the early days the potentiality of tissue and cell culture was recognised in such

such areas as morphogenesis, cancer research and virology. Valuable information was accumulated by embryologists from the beginning, whilst experiments in cancer research almost preceded the development of reliable methods [27]. Thus, the in vitro cell culture system has become recognised as having several advantages over more traditional in vivo techniques. When this study started, most investigations into biocompatibility had involved implanting the biomaterial into a suitable host and examining the prosthetic on removal at a later date. The period of implantation was usually days, but could involve months or years, and was occasionally post mortem. It is now considered that the initial biological responses may govern the ultimate fate of the material and on this criterion alone most in vivo models prove unsuitable for studying biological / synthetic material interactions. Surgeons are reluctant to implant materials only to remove them after a few hours, not least because of the trauma of surgery to the host. In vivo experiments are often difficult to repeat due to the particular characteristics of one subject. However, in vitro experiments can be designed to last a few minutes, hours or days and can be repeated easily under the same conditions. Such models are therefore accessible and can be used to examine the effects of systematically changing one or a number of conditions.

The field of biomaterials science itself is almost unique in the sense that the discovery of successful materials preceded fundamental research as to how and why they were successful. A biomaterial may be described as a material that is used in the treatment of a patient, and which at some stage interfaces with tissue for a 'significant' length of time [28]. This suggests that the interaction between the material and the tissue is important both in the treatment of the patient and in the success of the material. A material can be considered to be biocompatible when it can exist in a physiological environment without it adversely or significantly affecting the biological system, or the biological phase

adversely and significantly affecting the material [28]. However, these aspects are inter-related in that an adverse effect by the material facilitates an adverse response by the tissue. The development of improved biomaterials requires a better understanding of the interactions that occur immediately after contact with the body. However, progress in this area has been limited partly due to the fact that there is no suitable in vitro model available to study this phenomenon. One successful in vitro model that has been used during investigations to observe what happens when a biomaterial interfaces with a biological environment had been that of the contact lens. The lens could be placed into the eye, easily removed after a suitable time period and examined for evidence of spoilation. However, difficulties arise when using this model to observe the effects of altering the surface physico-chemical composition of the lens using human subjects; such investigations could damage the sight of the subject and again responses can depend on the individual.

When a biomaterial, for example contact lens, wound dressing, heart valve and so on, is placed in contact with the living organism the association may, in broad terms, be described as a material of non-physiological origin being placed in contact with a biological environment. Further examples of this association are numerous and include dentures in contact with saliva, ships in the ocean and indeed the situation in cell culture where cells are seeded onto culture substrates. All of these situations where a non-physiological material becomes interfaced with a biological system have one underlying principle in common: there is a rapid interaction between the material and the biological environment which ultimately leads to a layer becoming deposited out of the physiological system onto the material [29] . The composition of the adsorbed layer is influenced directly by the surface properties of the material itself. It is perhaps ironic that

following the adsorption of this layer the material presents a surface with a new set of characteristics, ie those of the adsorbed layer. This is the process that is frequently referred to as interfacial conversion. Thus, whenever a non-physiological surface(eg a contact lens, denture, wound dressing, prosthetic) is placed in contact with a biological system (eg tears, saliva, blood, tissue) interactions between the two take place and there is a rapid deposition of matter from the biological phase [29] . It is via the adsorbed, interfacial layer that the surface properties of the material are 'transmitted' to the biological environment and it is generally believed that subsequent interactions are influenced by this adsorbed 'conditioning' layer [30]. Therefore, contact lens spoilation, marine fouling of ships, dental plaque formation, fibrous encapsulation of implants all result from this interfacial conversion and a similar phenomenon is paralleled during cell culture situations [31]. When cells are seeded onto culture substrates, they are usually suspended in 'complete' culture medium, which is a defined medium (eg. Dulbecco's Modified Eagles Medium) that has been supplemented with animal serum (eg of foetal calf origin). Thus, when the complete medium contacts the culture substrate, there is an active deposition of various biological molecules, eg proteins, onto the substrate and a 'conditioning' layer, approximately 200 Å [29] in depth is set up. In the field of biomaterials research, the ultimate success of the material in a biological environment is considered to depend on the initial biological reactions to it and the nature of the interfacial layer established. It has already been outlined that initial reactions to biomaterials in vivo are difficult to observe. However, the anchorage dependent cell culture system appeared to be a powerful model for studying the types of biological responses that are triggered during interactions with materials. The initial phenomena observed under cell conditions (ie the establishment of an interfacial layer) mimic quite closely that seen in an in vivo environment. Therefore, the study of cell adhesion to

synthetic polymers as a research tool appears potentially exciting in two areas of investigation. First, whilst there have already been several investigations in the field, there is still little known about the mechanisms that govern cell behaviour and, in particular, those physico-chemical properties of the substrate that mediate cell adhesion. Therefore, the study of cellular interactions with substrates where the physico-chemical properties of the material are systematically controlled will improve the current state of awareness about cell behaviour and those factors that trigger cell adhesion. Secondly, a better understanding of the factors that govern the interactions occurring at the interface between materials and biological systems will ultimately prove fundamental in the design of improved biomaterials.

Prior to this study, there had been some investigations of those properties of the substrate that affected cell behaviour and indeed, some properties of the substrate had been suggested as being more important than others. However, from the literature, the general, overall picture was one of confusion and there were a number of conflicting reports among the findings of several workers. The important properties of the substrate and the contradictory reports over cell adhesion will be highlighted later in this chapter. The reason for this state of affairs was partly due to the fact that the greatest proportion of research in this area had been pieced together from studies in a number of disciplines, for example, from materials and polymer science, cell biology and physical chemistry. Because of this multi-disciplinary approach, the situation was that cell biologists were often restricted to studying cellular reactions to a fairly limited, arbitrary array of polymer substrates, about which they knew very little in terms of history and physico-chemical characteristics. Similarly, whilst polymer, materials and physical scientists were more able at substrate characterisation, they had little perception of the

techniques available to investigate the effects on a biological environment. Therefore, from an examination of the literature a sound conclusion could be drawn. Fundamental research into interfacial conversion and it's effect on cell behaviour requires an interdisciplinary approach whereby some aspects of polymer chemistry, physical and materials science are combined with a knowledge of cell biology and biochemistry. Whilst this type of approach is seldom found in research organisations, work of this nature has been carried out both in our own laboratories and those at Unilever and ConvaTec throughout the last decade. In order for the reader to understand the logic behind carrying out certain experiments, there are various important aspects that need to be introduced.

1.4. Fibroblast cells

Fibroblast cells are a family of collagen secreting cells that are present in virtually all tissues. These cells are important during wound repair processes where their functions include the deposition, maintenance, degradation and rearrangement of the extracellular matrix. This type of cell is, therefore, specialised for the establishment and maintenance of the matrix structure [32] . Fibroblasts are anchorage dependent cells and their shape in normal cell culture is well characterised. Prior to cell attachment they are spherical (Figure 1.1) but following attachment to suitable substrates they undergo spread morphology which has often been described as 'kite'-shaped (Figure 1.2). The types of fibroblasts used during studies here are of baby hamster kidney (BHK) origin which are useful in those studies that require a biological probe. Whilst all cells are complex organisms in their own right, BHK fibroblasts are non-differentiated and are hardy in terms of maintenance. The in vitro investigations of this cell type may also provide an indication of the success of materials on contact with the body since they play an

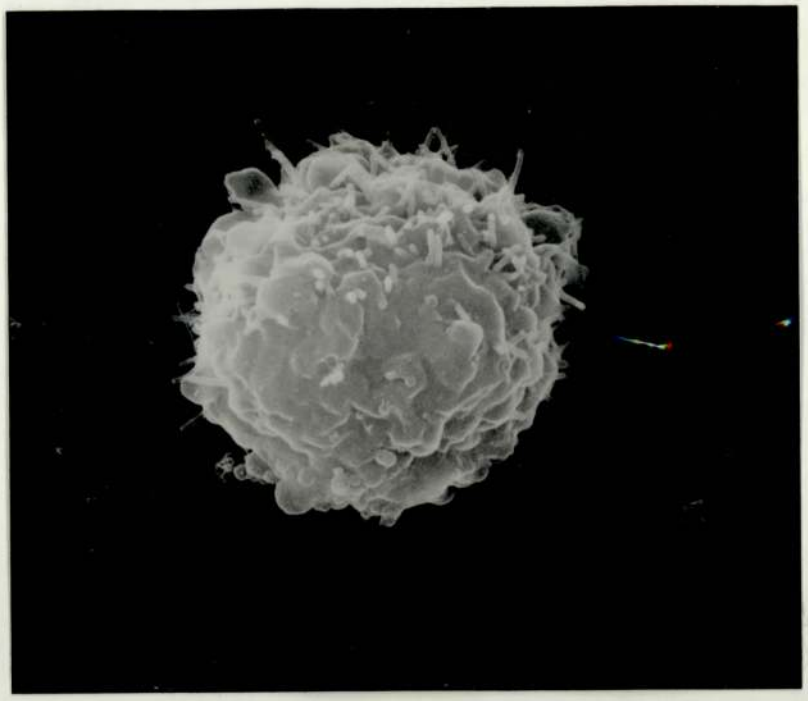


Figure 1.1 Rounded BHK cell in culture (x 6,000)

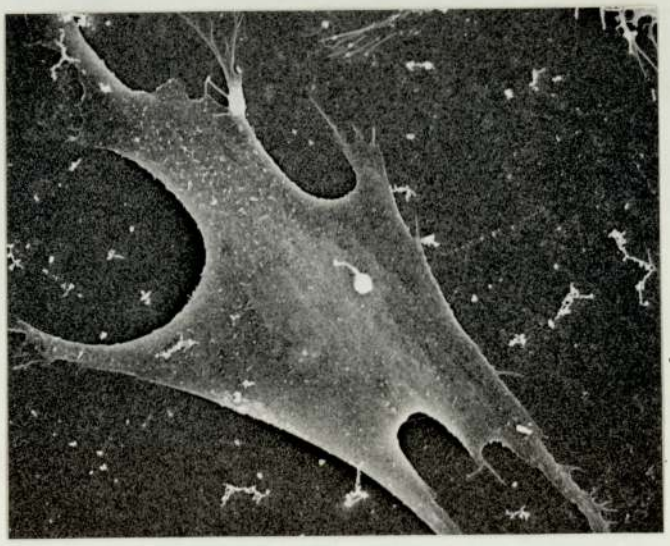


Figure 1.2. Spread BHK cell in culture (x 750)

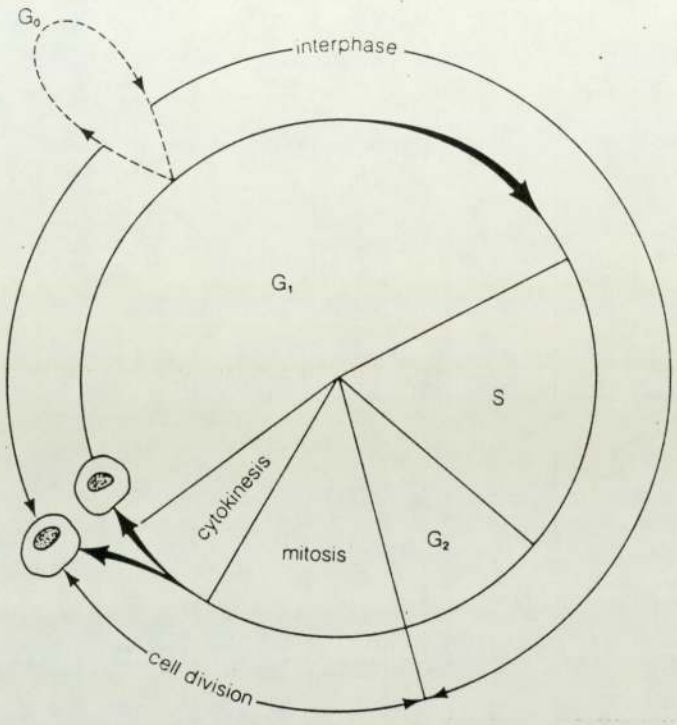
important role in the biological response to biomaterials in vivo. They have been shown to be important in the formation of dense tissue capsule which frequently forms around implanted prostheses [33] . Indeed, it has been reported that the widespread use of polymers necessitates the need for knowledge of the biomaterial interaction with fibroblasts [32].

1.5. Cell adhesion

Cell adhesion is a dynamic process, the rate of which depends on how quickly the cells will interact with the substrate and the subsequent formation of attachment bonds via the interactions of serum fractions, divalent ions, surface charges, peptides, electric double layers, van der Waals forces and alterations in the physical structure of the surface of contact [34 - 37]. Ultimately, an equilibrium state is reached and the cells attain a certain morphology and strength of attachment depending on the number and organisation of attachment bond and cytoskeletal structures [38] . These will be discussed later in this chapter.

When cell adhesion is observed using light or scanning electron microscopies, there is a dramatic change in the overall cell shape [39, 40] . As mentioned above, cells in suspension and on early contact with the substrate are spherical, but during cell attachment to favourable substrates they become flattened with 'concave' borders [39 - 43] . Cells remain in this fully spread state until the mitotic phase of the cell division cycle (Figure 1.3). Cells round up prior to division and the two daughter cells commence the spreading process again. Unless cells undergo this change in shape to fully spread then they do not pass through the G_1 phase in their cell cycle [44] and consequently do not divide; it is widely accepted that rounded anchorage dependent cells do not undergo

Figure 1.3. Brief description of the cell cycle



The above diagram shows a typical cell cycle with it's four successive stages. After the M phase consisting of nuclear division (mitosis) and cytoplasmic division (cytokinesis), the daughter cells begin a new cycle. The period between M and the start of DNA synthesis is called the G_1 phase (G = gap) and the period between the completion of DNA synthesis and the next M phase is called the G_2 phase. Interphase is thus composed of successive G_1, S and G_2 phases and it normally comprises 90% or more of the cell cycle.During contact inhibition, cells appear to be arrested in G_1 where cells maintain themselves in a non-dividing state. However, prolongation of G_1 may result in the cell entering one or more hypothetical G_0 shunts.

division. Ben-ze'ev et al [45] have since shown that anchorage dependent cells not only need to attach for protein synthesis, but that DNA, RNA and mRNA synthesis was closely linked to cell shape. Prolonging the G_1 stage may result in a cell entering G_0 (Figure 1.3), a type of hypothetical shunt and this may happen when a cell is presented with an unsuitable substrate [46]. Cell spreading has been shown to be maximal during mid-G_1 and S periods [47, 48], and during mitosis cells round up [49]. Another significant cause of G_1 arrest in cultured animal cells is confluence, that is, the condition achieved when cultured cells come into close contact. This 'contact inhibition' of division is believed to be a normal cell response that maintains fully developed animal tissues in a non-dividing state [46].

Cell adhesion is a fundamental cell property playing an important part in a number of cellular functions. These include cell migration during embryogenesis; homeostatic processes, including tissue and organ stability, thrombosis and wound healing. It also has a role to play in the invasive and metastatic behaviour of some cell types and in disorders of leucocyte and platelet function [15].

There have been two approaches to studying cell adhesion, namely the adhesion of cells to each other or the adhesion of cells to an extracellular substrate. This thesis will be concerned with the latter situation.

1.5.1. Theories of the cell adhesion process

The DLVO theory [50] of colloidal stability postulated a balance between long range electrostatic repulsion arising from negative charges on the cell surface and electrostatic

attraction due to fluctuations in dipole moments, both on the cell surface and in the interfacial medium. This first theory was concerned with long range continuum forces, and did not consider any direct interparticulate contact. The resultant picture is that of a cell suspended above the substrate at 10 -100 Å. An alternative theory, the 'contact hypothesis', proposed that close-range intermolecular forces, including hydrogen and 'hydrophilic' bonds in addition to dipole and electrostatic bonds [51, 52] are essential for cell adhesion. This second hypothesis acknowledges the impracticality of a completely theoretical theory [53] and offers a semi-empirical approach where it is assumed that the forces involved in close interfacial contact also contribute to surface tension. Consequently, surfaces could be graded according to surface energy parameters measured by the wettability of various liquids. This latter theory suggested therefore, that cells will adhere preferably to hydrophilic than to hydrophobic surfaces. Indeed, this theory held true when initial experiments showed that platelets [54] and fibroblasts, in both serum free [55] and full culture conditions [20 - 22, 36, 56 - 64] adhered to hydrophilic surfaces. This hypothesis is further supported by the fact that native, hydrophobic polystyrene does not support cell adhesion, but that after treatment with H_2SO_4 [21, 55, 61], the resultant more hydrophilic surface is cell adhesive. It was shown using dye binding assays that there was an increase in the substrate's surface charge after the sulphuric acid treatment, and this was assumed to be due to the introduction of sulphonate groups at the polystyrene surface [21]. These groups have a negative charge and so the cell adhesion response was seen to correlate with an increase in surface negativity. This observation could not be explained by the DLVO theory and because of this and other phenomena such as the resistance of cells to shear force [62] and further evidence from both electron microscopy [65] <u>in vitro</u> and immunofluorescence studies [66], led to the 'contact hypothesis' becoming the more widely accepted theory.

1.5.2. Cell contacts with the substrate during the process of adhesion

The contacts the cells make with the substrate is of fundamental importance to their behavioral response [67] . The contacts made are affected by a number of factors namely the type of cell , [68, 69] , type of substrate [15, 21, 53, 60, 70] , the properties of the surrounding medium, the time for which contact has been in existence [71] , and the cell's motile state [72] . With the identification that some cells were anchorage dependent, attention was focussed on the events occurring within the cell and at the cell/substrate interface after initial seeding in culture. The phenomenon of cell spreading would be of direct relevance to cell growth in culture and so cell spreading represents an ideal system for the study of cell adhesion and growth requirements. Once the cells have become fully attached and spreading is completed the cells move about prior to division. The direction in and speed with which they move depends on the lifespan of the cell-substrate and cell-cell contacts; the latter may result in the phenomenon referred to as contact inhibition.

Research using interference reflection microscopy (IRM) [73] on fibroblast spreading in vitro has revealed specialised cell-substrate associations. These adhesions are similar in shape to junctions between cells in that microfilament bundles terminate at the cytoplasmic face in an electron dense plaque. It has been possible to distinguish between the adhesion plaques in terms of their size, composition, morphology, their distance from the substrate and the time for which they exist [72 - 75] . There are four main types of contact thus far identified:

focal adhesions or **focal plaques** are characterised by the close contact between the cell membrane and the adsorbed layer. When observed using transmission electron

microscopy they are seen to be 10-20nm from the substrate. Such adhesions also possess a bundle of actin filaments which terminate at an electron dense plaque at the cytoplasmic face of the membrane and have also been seen to associate with actinin, vinculin and talin [77] . These focal adhesions can exist for as long as 72 hours in culture and they are typically found at the margins of stationary cells [72] . The development of focal adhesions have been observed by some workers to be accompanied by a change in the cells from a migratory phase to a stationary phase of proliferation [72] ;

focal contacts are similar to focal adhesions but , having dimensions of 0.25 - 0.5 μm **wide and 2 -10 μm long, they are smaller in area. These associations are characteristic of** mobile cells and have a lifetime of only minutes [72, 74] ;

close contacts are the third kind of contacts observed between cell and substrate. These cover a larger area of substrate and have an estimated distance of 30nm from the substrate. The cytoplasmic organisation is less prominent [72] and if they are not stabilised by an adjacent adhesion they may have a lifespan of only a few seconds. It has been suggested [79] that cell spreading is associated with close contacts, whilst focal contacts have a role in the contractility of cells.

Lastly, **non-contacting plaque** structures found in membranes are similar to focal contacts but are found only in cell - cell contacts [80] .

There have been some investigations into the biochemistry of cell adhesion sites formed during cell adhesion. Treating cells with EGTA causes them to round up and leave the adhesion sites with some substrate attachment material (SAM) being left behind [81] .

Marchant used labelling techniques, including immunofluorescence, to analyse these substrate attachment sites in situ; alternative techniques involve their removal by eg SDS followed by analysis by a number of biochemical techniques, including electrophoresis. However, whilst techniques such as these have been used extensively to investigate the components and biochemical structure of adhesion sites [82 - 84], their drawbacks include the facts that there are difficulties in ensuring all cells are removed and preventing further cell debris from depositing on the exposed sites.

1.6. The effect of the substrate on cell behaviour

Research of the nature just described ultimately led to investigations into the role played by the substrate in governing cell behaviour. As mentioned earlier, such fundamental research requires a knowledge of not only cell biology and biochemistry, but also an understanding of some aspects of synthetic polymer science, including structure-function relationships and physico - chemical properties. Such an interdisciplinary approach is seldom found in research establishments and this has been suggested as one reason why progress in this area has been limited [85]. Also, work in this area has often been restricted to a small and arbitrary range of substrate materials representing only a fraction of the potential array of properties provided by modern synthetic polymer chemistry. Thus, there is no consensus of opinion as to which physico-chemical properties of a surface are necessary for cell adhesion, or as to the biophysical mechanisms which may have an overiding influence on cell adhesion in culture.

As mentioned above, cells seeded onto polystyrene substrates will only attach and spread provided there has been pre-treatment of the surface. Workers have also investigated the attachment of cells on modified polystyrene surfaces [18 - 25]. Klemperer and Knox [22]

treated polystyrene not only with sulphuric acid, but also with chromic acid (10M CrO_2 in water) and a mixture of the two (0.01M CrO_2 in H_2SO_4). The resulting surfaces were analysed using crystal violet dye binding and revealed that sulphuric acid treatment produced strongly acidic groups (-HSO_3) at the surface and chromate treatment introduced weak acid groups (-OH). Mixtures of the two resulted in a surface expressing both moieties. The cells used for culture assays were BHK fibroblasts and adult rat liver cells. BHK cells grew in monolayers on all treated surfaces, whilst the liver cells were successfully cultured only on those surfaces that had been pretreated with chromate or the mixed acid reagent. Thus, it was suggested that weak acid groups (assumed to be hydroxyl) may be important in addition to or independent of strong acid groups.

Maroudas suggested that a plateau is established on those surfaces expressing between 1.8 and 17.2 charges per 100sq.Å [62] , considered to be due to the introduction of sulphonate groups. This work concluded that a 'contact hypothesis' could predict this plateau of cell adhesion and it was postulated that the mechanism responsible for cell adhesion involved the structuring of surface water by charged groups. This would ultimately lead to protein deposition onto the substrate thus providing the necessary attachment forces and plannar rigidity. The same group of workers later concluded that a cell adhesion substrate must be charged to the desired extent, be reasonably inflexible and dense.

Further experiments by Curtis et al [20] have also explored the effects of treated polystyrene on cell behaviour. Some of the results published by this group confirm the results of other workers whilst others contradict. Curtis et al treated polystyrene surfaces with H_2SO_4, CrO_2, ozone and other chemical reagents and assessed the ability of these

surfaces to support the adhesion of BHK fibroblasts and leucocyte cell-cultures. These were compared with the cell response to both tissue culture- and bacteriological- grade plastic dishes and the surface chemistry was assessed using X-Ray Photoelectron Spectroscopy (XPS) and chemical blocking techniques. They hypothesised that the acid treatments introduced hydroxyl and carboxyl groups onto the polystyrene surface which they chemically blocked before examining the effects on cell behaviour. When the -OH groups were chemically blocked using trimethylsilylimidazole there was inhibition of cell adhesion, but blocking the carboxyl groups by esterification with alcohols had no effect on the biological response. Whilst these workers did not comment on the role played by sulphonate groups, their findings are in accordance with Klemperer and Knox [22]. It is important to comment on the fact that the agent used by Curtis [20] et al to block the surface moieties introduced onto the polystyrene would have further modified the surface prior to cell adhesion studies. For example, chemically blocking the carboxyl group with a compound with similar structure and other properties would not have successfully blocked the effects of the -COOH groups. These workers were merely substituting one type of polarity for another and so Curtis was not unambiguously observing the effects of the blocked carboxyl groups. Similarly, trimethylsilylimidazole is itself a very hydrophobic substance and the use of this to block the -OH groups would create a surface with different surface properties to that produced by the acid treatments which would have markedly affected the cell response. This group, therefore, concluded that hydroxylation was the overiding factor that will lead to cell adhesion.

1.6.1. Surface properties of synthetic substrates that influence cell behaviour.

As previously mentioned most work in this area had been as a result of a

multidisciplinary approach. Scientists from a variety of disciplines had contributed to this field of research, which may be one reason why the overall view of the interactions of cells with synthetic polymer substrates has been confusing and conflicting. For example, cell biologists had endeavoured to understand cell adhesion mechanisms [86, 87], control cell growth [16, 18, 19, 88 - 90], or produce surfaces suitable for use in cell culture [20 - 22]. Chemists have employed the cell- culture system to study biological interfaces; biomaterials scientists have observed cell culture models along with in vivo systems in order to draw a parallel between the two [81, 91]. Complete surface characterisation is a complex process requiring an integration of a number of techniques and whilst a number of different physico- chemical properties had been put forward as having an effect on cell behaviour, there had been few systematic investigations of these factors. Because of this division of the sciences, workers did not realise that observations in the field of cell biology had applications in the field of biomaterials science, or that exploring the interactions between material and body may have the same underlying principle as found in cell-culture systems. Consequently, there was no overall consensus of opinion as to the requirement for a cell adhesive surface, or as to the biophysical mechanisms which may have an overiding influence upon cell adhesion in culture. Once the mechanisms governing cell adhesion are fully understood, the ability to then control subsequent cell behaviour is advantageous in a number of areas. For example, the advantages in the field of biomaterials science have already been discussed but in addition the prevention of cell adhesion to surfaces under controlled conditions has long been a goal in the development of surfaces that are non-adhesive to blood components, marine micro-organisms and bacteria.

Several surface physicochemical properties have been proposed as having an effect on

cell behaviour and these include the chemical composition of the substrate [92 -95], the chemical group expression at it's surface [92 - 96], surface energy (including fractional polarity) [94, 97 - 99], hydrophobicity / hydrophilicity [36, 61, 97, 100 - 104], equilibrium water content [85, 98], rugosity (ie. the degree of roughness of the surface) [93, 95, 105], surface charge [18, 21, 62, 106, 107], and substrate rigidity [61, 108]. However, there is no overall agreement of their relative importance, how they interact and no generally accepted hypothesis as to which of these properties (if any) has a more dominant part to play in governing cell behaviour. Previously the 'individual science' approach meant that cell biologists were exploring a small range of materials for evidence of how these properties were affecting cell behaviour. However, an understanding of some aspects of polymer science enables the cell biologist to explore a wider spectrum of substrates with controlled differences in surface, physical or chemical properties. In the field of polymer chemistry their potential as biomaterials has already been recognised partly due to ease of manufacture, variation in mechanical properties and, when selected properly, their chemical inertness. They therefore prove valuable in investigations of those properties affecting interfacial conversion and ultimately cell adhesion.

1.6.1.1. Surface wettability and polarity.

Weiss was the first to report the importance of substrate wettability on cell adhesion [36], although this was later supported by other workers [52, 57, 61, 102 - 104], However, some workers believe that wettability exerts it's effect via the nature of the adsorbed protein layer [109 - 110]. Indeed, it has been shown that protein adsorption is greater on hydrophobic surfaces [111], but it is the binding of specific proteins with the correct configuration that ultimately leads to cell adhesion. This can be illustrated by the work of Bentley et al [112] who showed that there was a greater extent of fibronectin binding to

bacteriological grade plastic, which is widely considered unable to support cell adhesion, than to tissue culture plastic. However, the number of active sites exposed on the former surface was markedly less. This suggests that there was a difference in the conformation of the fibronectin that had adsorbed to the two surfaces.

Klebe and co-workers [101] have proposed that a surface needs to be hydrophobic in order to support adhesion of anchorage dependent cell lines, as portrayed by their studies using poly(ethyl methacrylate) (polyEMA) and poly(methyl methacrylate) (polyMMA). This hypothesis explains the 'inhibition' of cell attachment to poly(2- hydroxyethyl methacrylate) (polyHEMA) unless it is blended with the non-hydrophilic polymers poly(vinyl acetate) [92], polystyrene, or polyEMA [93]. However, this theory is in direct conflict with the findings that non-polar, native polystyrene does not support cell adhesion, yet it is rendered more cell adhesive after treatments to render it more wettable.

The terms 'hydrophobic' and 'hydrophilic' have been used extensively in the field of biomaterials science to describe the wettability of a number of materials. However, these terms are fairly arbitrary and do not go beyond the macroscopic wetting behaviour of these materials in air. Thus, tissue-culture plastic is often classified as 'hydrophilic' because a droplet of water at it's surface will spread. In contrast, bacteriological grade polystyrene is described as 'hydrophobic' because water droplets show little spreading at the surface of this material. However, it should be noted that non-wettable surfaces are not always 100% 'hydrophobic' and may contain polar groups which are capable of attracting water molecules. Therefore, the terms 'polar' or 'non-polar' may more fundamentally describe the wetting nature of the surface of a material [113].

In a liquid or solid the atoms at the surface undergo interactions with each other which in turn gives the surface energy composed of polar and dispersive forces. The polar forces may become ionised, interact by ionic forces and / or form permanent dipoles. These dipole moments are capable of undergoing hydrogen bonding, orientation and induction reactions. Consequently, it is via similar interactions with water that the polar forces present at the surface of a polymer are capable of affecting the wettability of a surface. In contrast, the surfaces of non-polar materials, such as polystyrene, possess few permanent dipoles and these types of materials generally interact with water or other polar compounds mainly via dispersive or hydrophobic interactions with only a small orientation and a negligible induction interaction. A biomaterial / water interface with ionic groups or strong dipoles on the solid surface will tend to bind water molecules strongly and to orientate or polarise them around the ionic or polar groups. In contrast, surfaces which are predominantly hydrophobic in character will cause water molecules to structure in an ice-like conformation on or near the surface [113].

One group of workers have proposed the 'Minimum Interfacial Energy' hypothesis [113] which suggests that materials exhibit a low interfacial tension (≤5 dynes / cm) with the biological system so that maximum compatibility is achieved. It is of interest that hydrogel -type materials can minimise the interfacial free energy in contact with water [115, 116] and these materials have a good compatibility with blood and tissues. There will be a fuller discussion of this unique group of polymers in Chapter 5.

An alternative approach to the 'Minimum Interfacial Energy ' hypothesis is the 'moderate surface energy' theory [114] . Workers found that prostheses coated with a thin film of

glycoprotein exhibited thromboresistance after implantation in regions of high thrombogenicity. These implants had a critical surface tension of between 20 and 30 dynes / cm, and this team also derived the same value for the inner surfaces of blood vessels which were dominated by end methyl groupings. They therefore suggested that for a material to be thromboresistant (ie biocompatible when in contact with blood) it must have a surface tension of 20 to 30 dynes / cm. It must be stressed, however, that this theory is based on in vitro measurements of a material that would be in a partially dehydrated state and therefore the in vivo surface would be more wettable than the above measurements predict. It should also be highlighted that this theory was applied only to materials for applications in contact with blood, not for all biomaterials per se. From the literature, it is often very easy to confuse the terms of 'thromboresistant' and 'biocompatible' with the cell adhesive properties of materials.

1.7. Proteins and cell adhesion

As previously mentioned when a non-physiological (foreign) surface is placed in contact with a biological environment there is an active deposition from the biological system which occurs within seconds [29] . This event will, therefore, alter the physicochemical properties and texture originally observed at the contacting surface. A great deal of work in the literature has identified that proteins become adsorbed during this interfacial conversion almost excluding the identification of any other species that may be involved. Consequently, there will now be an overview of protein deposition at the polymer / biological border and those proteins that are considered to influence the biological response.

Once the adsorbed layer at the contacting surface is approximately 20nm in thickness, it

does not remain static, but is constantly being altered [117, 118] by, for example, other plasma proteins [119]. The composition and configuration of this layer depends upon the material, and it is via this layer that the surface characteristics are transmitted to the biological system. Thus interfacial conversion means that the material will take on the 'new' surface properties of the adsorbed layer. Indeed, it has been shown that after adsorption, hydrophobic surfaces can become more wettable, whereas hydrophilic surfaces become less so [120, 121]. It is generally believed therefore, not least in the field of biomaterials research, that subsequent responses are influenced by this adsorbed 'conditioning layer' [30].

A variety of different proteins of different size, configuration, and molecular weight have been shown to have a profound effect on cell behaviour. Protein adsorption has been hypothesised as being either hydrophilic, exothermic and reversible or hydrophobic, endothermic and strongly bound (ie. irreversible). The conformational states and anchorage sites of adsorbed proteins have been shown to differ according to temperature, pH and concentration of the surrounding medium.

Whilst the actual components of the interfacial layer have been incompletely characterised, those proteins thus far identified as having an important effect on cell behaviour will be reviewed here.

1.7.1. Fibronectin

The most extensively studied glycoprotein has been fibronectin [122] which represents a group of high molecular weight glycoproteins present in extracellular matrices, basement membranes and plasma [123]. This protein consists of two similar subunits, each of

approximately 250 kD. Each subunit is folded into an elongated and flexible arm approximately 60 nm long, and the 2 subunits are joined by a di-sulphide bond very near their C-terminus. Within each subunit is a series of tightly folded globular domains, each specialised for it's binding to other molecules or to cells [124]. Fibronectin has been shown to exist in 2 forms; one type circulates in the plasma, whilst the other is bound to cell surfaces [125]. However, the primary structure of interstitial and plasma fibronectins vary due to differential splicing of fibronectin pre-mRNA [126]. Workers have shown that fibronectin is important during cell adhesion to surfaces of different cell adhesiveness [127] and spreading on untreated polystyrene does not occur unless very high levels of fibronectin have previously been bound [124]. Researchers have also shown that whilst more fibronectin adhered to hydrophobic, bacteriological grade plastic than to hydrophilic tissue-culture ware, the activity on the former surface was lower [112]. This suggests that the conformation of fibronectin on these surfaces was different and this work highlights the fact that surface properties like wettability can influence the adsorbed layer, which in turn affects the cell response. Grinnel and Feld [124] implied that events on tissue culture plastic stimulated cultured cells to produce still more fibronectin, which was not the case on bacteriological plastic. However, one drawback of these findings is that testing surfaces by precoating them with specific proteins prior to testing the biological response does not closely mimic the in vivo situation.

Fibronectin has proven to be of great interest in blood studies, where it has been shown to bind covalently to fibrin through the action of factor XIII [128, 129] and is required for fibroblast adhesion to fibrin in vitro [130]. Fibronectin has been found in platelets [131] and is released following stimulation [132] at which time saturable receptors for fibronectin appear at the platelet surface [133]. While these receptors may be important in

the ability of fibroblasts to promote platelet spreading [134] and clot retraction [135], fibronectin has little effect on platelet attachment per se [136]. In dermal wounds, fibronectin-coated fibrin acts as the substrate for the ingrowth of fibroblasts and endothelial cells during the formation of granulation tissue [137]. Fibronectin also binds to a number of other materials, including denatured collagen [128, 138] and may act as a general opsonin in the phagocytosis of debris by the reticuloendothelial system [123]. Two domains with high affinity and additional domains with lower affinity for heparin have been characterised [139 - 143]. The high affinity domains are located at the NH_2 terminal and between the cell binding and the COOH- terminal fibrin binding domains. It is currently controversial as to whether or not heparin and heparan sulphate are bound at physiological pH and at physiological ionic strength [143 - 145]. It has also been shown that isolated heparin-binding domains may interact with dermatan sulphate, chondroitin sulphate and hyaluronate at low ionic strength [143]. More recently, the cell attachment site within the fibronectin molecule has been identified, sequenced and reproduced by peptide synthesis [145]. Analysis of progressively smaller synthetic peptides enabled the active portion to be narrowed down to a tetrapeptide composed of serine, arginine, glycine and aspartic acid. The arginine, glycine and aspartic acid residues were found to be essential for activity, but it was considered possible that the serine residue could be replaced by other amino acids [145].

1.7.2. Vitronectin

It has been reported that fibronectin is not significantly important during early adhesion processes [124, 147]. An alternative candidate is the 70kD spreading factor vitronectin, [148, 149] also referred to as alpha one protein [150], serum spreading factor (SSF) [151] or epibolin [152]. This protein was initially identified in human serum and has been

confirmed as a component of foetal calf serum [149] . Vitronectin is a glycoprotein, or family of glycoproteins, of molecular weight 65-80 kD. It is found in mammalian sera and is also distributed throughout a variety of tissues eg muscle, lung, kidney, skin [147]. The major source of circulating vitronectin is probably the liver [151] although it may also be synthesised by some human fibroblasts in culture [149]. Vitronectin is a cell spreading factor in culture and contains the arg-gly-asp sequence [153] which is considered to be the cell attachment tripeptide of fibronectin [145] . Another common feature of both these proteins is their ability to bind the polysaccharide heparin . However, this is where their similarity ends as there is no other homologous sequence with fibronectin other than the arg-gly-asp portion. Furthermore, the distribution of these proteins in tissue is not identical and there is some evidence to demonstrate the exclusivity of their actions as spreading factors in culture [151, 154] and other in vitro systems [155] . The complete amino acid sequence of vitronectin has been derived from cDNA clones made from human liver mRNA [153] . An interesting feature of this molecule is that it contains the entire 44-amino acid sequence of somatomedin B peptide at it's NH_2- terminus. The biological significance of this is still unclear. Somatomedin B is a growth hormone-responsive peptide while vitronectin is not, and vitronectin preparations do not contain the biological activity associated with somatomedin B (stimulation of DNA synthesis [151, 156]). The spreading factor properties of human vitronectin have been described by a number of workers [148 - 152] .

1.7.3. Laminin

Laminin is a glycoprotein of high molecular weight found specifically in the lamina lucida of the basement membrane [158] . In vivo, this protein is found as an extracellular matrix that enfolds epithelial, endothelial, muscle, nerve and fat cells [159] . Laminin with

an approximate molecular weight of ~900 kD is significantly larger than fibronectin and is a cross-shaped multifunctional molecule. It consists of two non - identical B chains of molecular weight 220 kD and a heavier A chain of molecular weight ~440 kD [160]. Whilst the exact role and location of the various domains is still in dispute it is known to possess a domain structure within which the functions are dispersed.The molecular mechanisms by which cells interact with laminin has been of great interest and the protein is known to alter the cell shape and to promote matrix deposition [161, 162]. It has been shown [163] that laminin, binding only type IV collagen, can mediate the attachment of fibroblastic cells to plastic and glass although it's action was seen to be less effective than fibronectin. In this context, laminin may promote fibroblast attachment and spreading via it's binding to heparan sulphate proteoglycan at the fibroblast surface. In another situation, namely ganglion neurite outgrowth laminin has been shown to be a more effective substrate than fibronectin [164, 165]. In contrast, fibronectin and laminin may act competitively for binding type IV collagen in preventing fibroblasts from invading the epithelial side of basement membranes and in the loss of fibronectin from basement membranes as the tissue matures [166]. However, laminin is not a good substrate for epidermal cell migration [167].

1.7.4 Heparin

A glycosaminoglycan which has recently been shown to bind several cell adhesion proteins, including fibronectin, vitronectin and laminin is heparin [168]. Although the functional significance of heparin interaction with cell adhesion proteins and growth factors is not clear, this polysaccharide has been shown to bind numerous growth factors, including fibroblast growth factor [169]. While heparin is not a cell surface component per se, it is a degradation product of the cell surface macromolecular heparin

sulphate [170, 171] . It also shares the disaccharide repeat unit of heparan sulphate, the major cell surface proteoglycan of fibroblastic cells. Thus, it might be expected that both cell adhesion proteins and growth factors would associate with heparin-related molecules on the surface. It has also been shown that pharmacological doses of heparin can alter cell growth rate, cellular morphology and cell migration. [172] . These same workers postulated that the effect of heparin on cell morphology and migration is probably mediated by one or more cell adhesion proteins and they established that the effect on cell growth is mediated by growth factors.

1.7.5. Vinculin

Vinculin is a cytoskeletal protein of relative molecular mass 130 kD [173] and has been localised in focal contacts [174] and intercellular adherens junctions [175] (ie. areas where cells make contact with the substrate or other cells). The assembly of vinculin into a membrane bound junctional plaque seems to be one of the earliest cellular responses to contact with exogenous substrates ultimately leading to the local assembly of actin rich microfilament bundles [173] .

It was mentioned earlier that anchorage -dependent cells need to attach and undergo full spread morphology on a suitable surface in order to pass through the G_1 phase of their life cycle. Research involved with the synthesis of vinculin by rounded, non-proliferating and fully spread cells led Unger et al [173] to suggest that the production of vinculin may be involved in the transmission of contact-dependent growth-related signals from the exterior. Indeed, the same workers observed that transformed cells (non-anchorage dependent) show marked alterations in both vinculin and actin organisation, reflected in changes in their cell shape and adhesiveness; these observations were

paralleled when tumour promoters were added to an anchorage dependent cell culture system.

1.7.6 Actin

Actin is a protein that is attached to the plasma membrane where the cells attach to the substrate and each other [174 - 178] . At these regions cells establish a transmembrane linkage between components of the extracellular matrix and the actin rich cytoskeleton again referred to as the adherens junction.

Whilst this list is by no means complete, it can be seen that there has been considerable work carried out to identify some of the proteins that have a role in the interfacial conversion process and this work is still ongoing. However, the specific, overall importance of each of these proteins in mediating cell behaviour remains generally unclear. In addition to these proteins, the complete culture medium may contain others along with a whole array of other biological components, although the composition of foetal calf serum has been poorly characterised. Human serum, however, has been more clearly defined and has been shown to contain a variety of ions, nutrients, waste products, gases, in addition to other biological species such as proteins and lipids. Serum certainly has some effect in cell culture as there are differences observed for those cells cultured in serum free medium. For example, cells that adhere to the substrate when serum is absent from the culture medium are not disrupted by proteases or chaelating agents to the same extent as when serum is present [15] . Therefore, it is important that at least some of the components of foetal calf serum are defined in order to give those researching into cell adhesion mechanisms an idea of the types of biological species that are available to the cell during culture. It may be possible that in addition to proteins,

some other biological component, eg. of lipid origin may be active during interfacial conversion. Certainly, work in the field of contact lens research has suggested that fatty acids become adsorbed to the lens during spoilation processes in vivo although there has been little work undertaken to observe whether a similar phenomenon occurs during cell-substrate interactions.

This chapter has suggested that anchorage dependent cell culture is a powerful research technique having applications in a variety of areas. An investigation of certain aspects of this type of culture system is important in order to further the understanding of those factors that mediate cell adhesion in vitro. Whilst there has already been some research in this area, there is no overall consensus of opinion as to what governs the attachment, and ultimately spreading, of anchorage dependent cells. In addition, the development of improved biomaterials requires a better understanding of the initial biological responses to materials. The ultimate success of materials in a physiological, environment is dependent on these initial reactions but at present, there is no suitable in vivo model available to study these interactions and the processes involved during interfacial conversion. In this respect, the study of the anchorage dependent cell culture model as a biological probe seems to be of significant value, since the initial events in culture seem to mimic quite closely the events often observed in vivo after implantation. An improved understanding of those properties that govern the biological response, eg. adhesion of fibroblast cells to substrates, will no doubt aid in the development of further biomaterials. Investigations such as these require an interdisciplinary approach where the barriers between cell biology, polymer, physical and materials science are broken down, and it is along these lines that work in our laboratories has progressed for some time. This approach was originally adopted by Minett [85] who, as part of his work, catalogued

the cell responses to a well defined series of carefully selected polymers. For example, it has previously been mentioned that polystyrene is a highly hydrophobic polymer that is widely recognised for being unable to support cell adhesion in culture. Minett carried out cell culture investigations on other hydrophobic polymers which included polypropylene, poly (4-methyl pent-1-ene) (TPX), in addition to polystyrene. Klebe [101] had shown that the 'hydrophobic' polymers poly (methyl methacrylate) (PMMA) and poly(ethyl methacrylate) (polyEMA) were both cell adhesive and Minett found that polystyrene was the only non-hydrophilic polymer that did not support high levels of cell attachment and spreading. This situation was strange because whilst PMMA and polyEMA are more polar than polystyrene, both polypropylene and TPX are less polar. Thus, as is often the case, experimentation in one area often leads to more questions being raised and in this case it was left unanswered the reason why polystyrene was unique. However, of the polymers that Minett had investigated, polystyrene was the only one that had been in the amorphous form, all the others had been studied in the isotactic, more crystalline forms. Thus, the expression of a regular crystalline array at the surface may have promoted areas of surface polarization, a phenomenon which would not occur in an amorphous polymer, although it was not possible for Minett to test this hypothesis within the time constraints of his study.

Prior to Minett's work, it had been shown that whilst polystyrene in its native, untreated form supported little cell attachment and was widely considered as non-adhesive, there were quite simple techniques available to render it a more suitable substrate for use in cell culture. Among these techniques was the treatment of polystyrene by sulphuric acid [20 - 22] used by some cell biologists to economise on tissue culture ware [22]. Other workers had shown quite a dramatic effect following this type of treatment [95]. The treatment of

half a bacteriological grade petri dish with sulphuric acid prior to cell culture studies showed that the cell response could be quite striking. There were few cells on the untreated half, but the section that had been treated with sulphuric acid was extensively covered with a high number of cells with a large proportion having undergone some spreading and there was a clearly defined boundary between the two sections. In addition, on closer examination by SEM, it was seen that not only were cells reluctant to cross the demarkation to the untreated portion, but some cells had aligned themselves along the boundary almost as though they were turning away from the untreated half. It was clear that some property (or properties) of the untreated polystyrene were grossly unfavourable for cell attachment but that in comparison the treated portion was was highly suitable. What properties of the polystyrene had been modified following the sulphuric acid treatment to produce such a dramatic effect? Some work had been carried out to answer this question [20, 21] and whilst it was noted that the polystyrene was rendered more wettable, it was also hypothesised that the introduction of different surface groups was governing the cell response. However, it became clear from the literature that there were conflicting views as to which surface groups had been introduced into the polystyrene to render it cell adhesive. Two groups of workers assumed that the effect was due to the introduction of sulphonate groups ($-HSO_3$), [21] or weak acid groups [22] whilst others suggested that sulphuric acid treatment of polystyrene lead to an hydroxylation (ie the addition of -OH groups) [20] or weak acid groups [22] Clearly, the effects of H_2SO_4 treatment on polystyrene had not been identified.

A further technique to render polystyrene cell adhesive is that of glow discharge treatment, often termed plasma etching. Minett had shown [85] that the treatment of this polymer with either oxygen (reactive), nitrogen, or argon (inert) gas plasmas lead to the

production of a substrate capable of supporting confluent cell attachment and spreading. Whilst Minett had observed the phenomena of producing a cell adhesive surface, there had been little characterisation of the total surface properties following gas plasma etching. The increased rugosity of the surface may also have increased it's wettability [179] and the exposure to different types of gas plasma will affect the introduction of groups at the material's surface. Thus, the route by which polystyrene was made cell adhesive by gas plasma treatment is unclear.

The work of Minett [85] was extended to include a unique family of polymers termed hydrogels which are essentially water swollen polymer networks. Hydrogel polymers can be made from one type of monomer (for example, poly[2-hydroxy ethyl methacrylate], [polyHEMA]) or monomers can be co-polymerised (eg poly (HEMA / styrene) and they may be characterised in terms of their ability to absorb water up to a point of equilibrium (ie. equilibrium water content, EWC). In addition to a range of hydroxyl-containing hydrogels, Minett also studied a range of both purpose synthesised and commercially available polymeric materials and despite their differences in chemical structure, morphology and crystallinity between all of the materials, they were taken together and compared in terms of their effect on the cell response. A broad attempt to arrange the polymers in order of decreasing EWC (or decreasing polarity of the non-hydrogels) showed an interesting transition in cell adhesive behaviour. Of the materials he had studied, no polymer with an EWC of between 35 - 60% supported cell attachment. Indeed, all the polymers in the range 2 - 35% did allow high levels of cell attachment and spreading to take place. It was implied that cell adhesion to those polymers between 60 and 90% EWC was not straight forward because whilst there was some cell attachment to the hydrogels in this region, it was not always accompanied by

cell spreading. The effects of these high EWC hydrogels may have reflected a more complex set of possibilities for the structure of the hydrogel itself and the effect on the subsequent structure of water held at the surface. The relationship between cell adhesion and EWC in these high water hydrogels represents a potentially exciting area of research. At a high water content a more complex array of interactions may be coming into force than merely those due to EWC alone although there have been few investigations of this possibility since the work by Minett. Work in this area may prove of great value because of the great potentiality for the use of hydrogels as such biomaterials as contact lenses and wound dressings.

In an attempt to improve the understanding of those mechanisms effecting anchorage dependent cell adhesion, it was decided to adopt the inter-disciplinary approach that had been initiated by Minett. Therefore, it seemed reasonable to design a research program for the systematical investigation of those physico-chemical properties that had previously been arbitrarily proposed as having an effect on cell behaviour and to undertake an exploration of the adsorbed interfacial layer.

1.8. Scope of this study

i) To investigate the possibility that polymer structure, morphology and crystallinity may have an affect on cell behaviour. This attempt may also ascertain whether polystyrene is unique among hydrophobic polymers in it's inability to support cell adhesion.

ii) To identify whether the treatment of polystyrene by sulphuric acid at ambient temperatures introduces hydroxyl or sulphonate groups at the polymer surface and to attempt to ascertain the importance of other physico-chemical properties on the cell response.

iii) To observe the effects of high equilibrium water content hydrogels on the biological response.

iv) To identify some of the biological components present in foetal calf serum. Broad attempts will also be made to investigate whether lipids and calcium have a role to play in the interfacial conversion of cell culture substrates and ultimately the cell adhesion response.

CHAPTER 2

Materials and experimental techniques.

2.1. Polymers

The polymers used in this study were purchased as follows:

poly (p-tert butyl styrene), poly(α-methyl styrene), poly(4-isporopyl styrene), poly(4-methyl pent -1 -ene), isotactic polypropylene and poly(vinyl chloride) from Aldrich, Dorset, England; atactic polystyrene from I.C.I., Runcorn; chlorinated rubber from Polymer Consultants Ltd., London; amorphous polypropylene from Hercules Powder Company Ltd, Wilmington, Delaware.

The calendered polypropylene and the calendered poly (4-methyl pent -1 -ene) were kindly donated by I.C.I. plastics, Welwyn Garden City whilst both the calendered poly(vinyl chloride) and polystyrene were available in house.

The hydrogel polymers used in Chapter 5 were kindly donated by Mr. P. Corkhill [180].

2.2. Materials for cell culture

Tissue-culture and bacteriological grade ware were obtained either from those stocked at Unilever, Colworth or were purchased from Sterilin. The baby hamster kidney (BHK) fibroblasts were also a donation from Unilever but the original cell line had been purchased from Flow Laboratories, Irvine, Scotland. The following culture media was also purchased from Flow:

Dulbecco's Modified Eagles Medium (DMEM, composition shown in Appendix 1); phosphate buffered saline (PBS, both with and without calcium and magnesium ions, recipe given in Appendix 2); trypsin-EDTA; penicillin / streptomycin mixture.

The foetal calf serum was purchased from Gibco Laboratories, Paisley, Scotland.

2.3. Solvents

Unless otherwise stated, all solvents were obtained from Fisons and were of HPLC standard.

2.4. Sample preparation

2.4.1. Preparation of pressed films

Discs of films were prepared by compression moulding using an electrically heated, self-contained upstroking hydraulic press (N° 205/SC, Bradley and Turton Ltd., Kidderminster, U.K.). Pressed discs of polymer were prepared using a mould (2cm. thick steel plate with 1cm. diameter holes evenly spaced) placed between two steel plates. The mould was filled with polymer 'grains', and the backing plates lined with cellophane (used as a mould release). The 'sandwich' was then preheated to the appropriate temperature (Table 2.1) for 2.5 minutes (at zero pressure) to allow the material to flow. The pressure was increased to 25 tons in^{-2} and maintained for 2 minutes. After this time the platens were cooled to room temperature whilst maintaining full pressure. The pressed film discs were removed from the cellophane sheets.

Samples were then washed in 1% poly(oxy- ethylene sorbitan monolaurate) (ie. 1 % Tween 20) for 24 hrs after which time they were rinsed thoroughly in distilled water and air dried prior to cell culture studies.

Table 2.1. Temperature of platens during compression moulding

Polymer	Temperature of moulding (°C)
Amorphous polystyrene	194
Isotactic polystyrene	193
Amorphous polypropylene	191
Isotactic polypropylene	174
Poly(vinyl chloride)	250
Poly(4-isopropylstyrene)	190
Poly (α -methyl styrene)	190
Poly (p-tert butyl styrene)	200
Poly (but -1- ene)	145
Poly(4 - methyl pent -1 - ene)	200

2.4.2. Preparation of polystyrene samples

Using a heated cork borer of diameter 1.5 cm samples were cut out of bacteriological grade plastic and were either used untreated or treated in different ways prior to cell culture studies. In a similar way, samples were also cut out of tissue culture plastic.

2.4.3. Preparation of hydrogels prior to cell culture studies

All hydrogels were washed in 1% Tween 20 and rinsed thoroughly in distilled water. Prior to their use as cell culture substrate they were equilibrated overnight in phosphate buffered saline with added $Ca^{2+}$and Mg^{2+} (PBS +).

2.5. Surface treatment

2.5.1. Acid treatment

Samples of bacteriological grade plastic were immersed in one of the following acids.

i) 98% sulphuric acid (BDH) at 20°C,

ii) 98% sulphuric acid at 60°C,

iii) chlorosulphonic acid vapour (BDH),

iv) oleum (fuming sulphuric acid) (BDH)

for the times shown in Chapter 4. After treatment the surfaces were washed thoroughly in distilled water and air dried prior to use in cell culture studies.

2.5.2. Plasma (glow discharge) Treatment

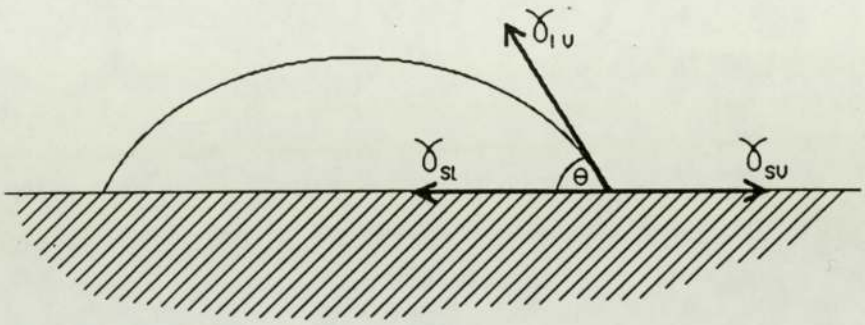
As above, samples were cut out of bacteriological grade plastic and exposed to glow discharge treatment in barrel or parallel plate type plasma reactors. Discharge conditions were standardised at 150 kV under a vacuum of 0.05 Torr. Samples were exposed to reactive (oxygen) or unreactive (argon , nitrogen) gases at a flow rate of 15 - 20 cc/min for the times shown.

2.6. Surface analysis

2.6.1. Contact angle measurements

Contact angle measurements were considered of value in this study because they have been shown to be a useful technique to evaluate the surface energy and wettability of materials [181] . Furthermore, contact angles can be easily measured and the principle behind this technique is based on the fact that most liquids on contact with a solid will spread. The extent of this spreading is determined by the balance of forces at the boundary line between liquid, solid and air, as shown in Figure 2.1:

Figure 2.1. The forces involved in determining the contact angle, θ, of a liquid drop on a surface.



The balance of forces outlined above may be further described by the following equation[182]:

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

where, γ_{lv}, γ_{sv} and γ_{sl} are the free energies of the liquid and solid against their saturated vapour and of the interface between liquid and solid respectively and π_e is the equilibrium pressure on the solid. θ is the angle of contact between a liquid droplet and a plane solid surface. 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, high solid surface free energy and low liquid - surface free energy [182]. Further modification of equation (1) allowed Owens and Wendt to calculate the

value of θ as follows

$$\text{Cos } \theta + 1 = 2/\gamma_{lv} \left((\gamma^{d}_{l} \gamma^{d}_{s})^{1/2} + (\gamma^{p}_{l} \gamma^{p}_{s})^{1/2} \right) \qquad (2)$$

where, γ^{d}_{l} is the dispersive component of the liquid,

γ^{d}_{s} is the dispersive component of the solid,

γ^{p}_{l} is the polar component of the liquid

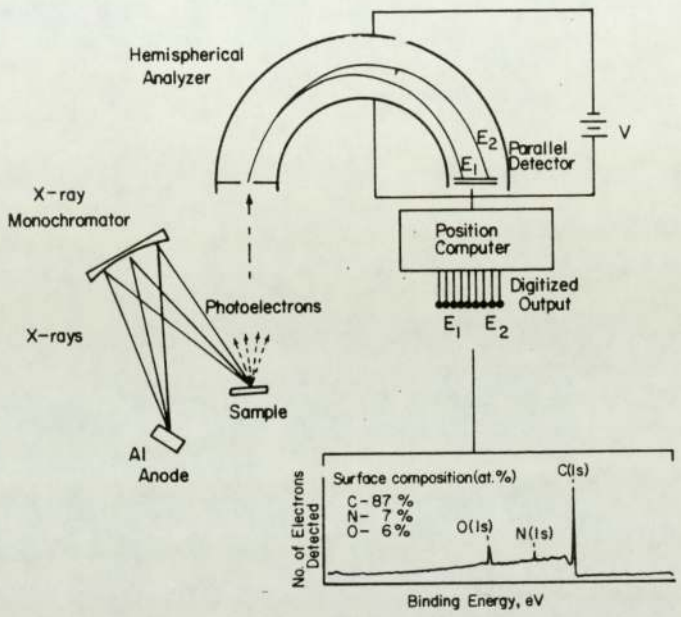
and γ^{p}_{s} is the polar component of the solid.

The value for the contact angle is affected by the atoms that are exposed in the upper 10 Å of the material surface. Thus, in this study, contact angles were measured by the sessile drop method in air, as described by Owens and Wendt [182]. However, prior to analysis by this technique, all samples were washed in 1% Tween 20, rinsed thoroughly in distilled water and air dried. Drops of the wetting liquids (water and methylene iodide) were then in turn placed onto separate areas of the substrate. Each drop was then viewed through a rotating eyepiece calibrated in degrees, and the contact angle, θ, at each side of the drop was measured. From the mean values of θ, measured using both water and methylene iodide, the polar and dispersive components were calculated using equation (2)

2.6.2. Electron spectroscopy for chemical analysis (ESCA)

This analytical technique may also be referred to as X-ray photoelectron spectroscopy (XPS) and was used in the surface elemental analysis of the samples as shown in the individual chapters. A diagram to represent the apparatus used is as shown in Figure 2.2

Figure 2.2. Schematic diagram of an ESCA spectrometer.



Samples were irradiated with monoenergetic MgK_{α} X-rays (1253.6 eV) under a vacuum of 10^{-9} Torr using a Kratos XSAM 400 spectroscope. The X-rays penetrated 10 Å into the polymer surface, interacted with the atoms in this region which lead to the photoelectric effect and the emission of electrons. The kinetic energies (KE) of these electrons is given by [183] :-

$$\mathbf{K.E. = hV - B.E. - s} \qquad \mathbf{(3)}$$

where **hV** is the energy of the photon,

B.E. is the binding energy of the atomic orbital from which the electron originated (Appendix 3)

and **s** is the spectrometer work function.

In addition to the photoelectrons emitted during the photoelectron process, Auger electrons were also emitted due to the relaxation of the energetic ions after photoemission, and this would have occurred approximately 10^{-14} seconds after the photoelectric event.

The electrons leaving the sample are detected by the electron spectrometer according to their kinetic energies. The analyser normally operates as an energy 'window', accepting only those electrons having an energy within the range of this fixed boundary. Scanning for different energies is accomplished by applying a variable electrostatic field before the analyser is reached. This retardation voltage may vary from zero, up to the photon energy. Electrons are detected as discrete events and the number of electrons for a given detection time and the values for their energy is stored digitally or recorded using analogue circuitry. Elements can be identified according to their binding energies.

Using the following equation the quantity of each element present can be calculated as a percentage:

$$C_X = I_X / S_X \; / \sum I_i / S_i \; . \; 100\% \qquad (4)$$

where, **C** is the atomic fraction of any constituent in a sample,

I gives the number of photoelectrons emitted per second in a specific spectrum peak, denoted by the peak height,

S is the atomic sensitivity factor (Appendix 4)

x refers to a particular element

and, **i** refers to all the peaks in one spectrum.

Whilst this technique is valuable in the identification of the elements present at the surface of a material, it was difficult in this study to comment on the reproducibility of the results. The quantification method required a measurement of the peak heights obtained in a spectrum which may be subject to error, particularly during the measurement of the smaller peaks.

2.7. Cell culture techniques

Baby hamster kidney (BHK) fibroblast cell lines were routinely maintained at 37°C in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1 % L-glutamine (Gibco) and 1% 0.02M penicillin/ streptomycin (Gibco). Following establishment of confluent monolayers the culture medium was aspirated the cells washed with phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS-), and 15-20ml trypsin-EDTA added to detach cells. The cells were then resuspended in complete DMEM and subcultured into tissue-culture flasks. Prior to and following cell culture studies, a viability test was carried out on the cells whereby 0.1ml of trypan blue dye (Sigma) was added per 1ml of cell suspension and using a haemocytometer the ratio of viable cells to total cells was calculated.

During observations of the cell response to specific materials, cells were seeded at a concentration of 1×10^6 onto test substrates. All tests on these substrate were carried out in duplicate and throughout these experiments samples of both tissue culture and

bacteriological grade plastic were used as the controls, since the cell response to each of these substrates is well characterised. After 6 hours in culture cells were either observed using light microscopy prior to trypsinisation and counted using a haemocytometer or fixed and prepared for observation by scanning electron microscopy.

2.7.1. Trypsinisation and cell counts.

Following cell culture, each substrate was washed in phosphate buffered saline (without calcium or magnesium ions) and placed into one well of a 24 - well multiplate. 1ml of trypsin-EDTA was added to each substrate and after approximately 10 minutes the surface was examined under the microscope to ensure that all the cells were removed.

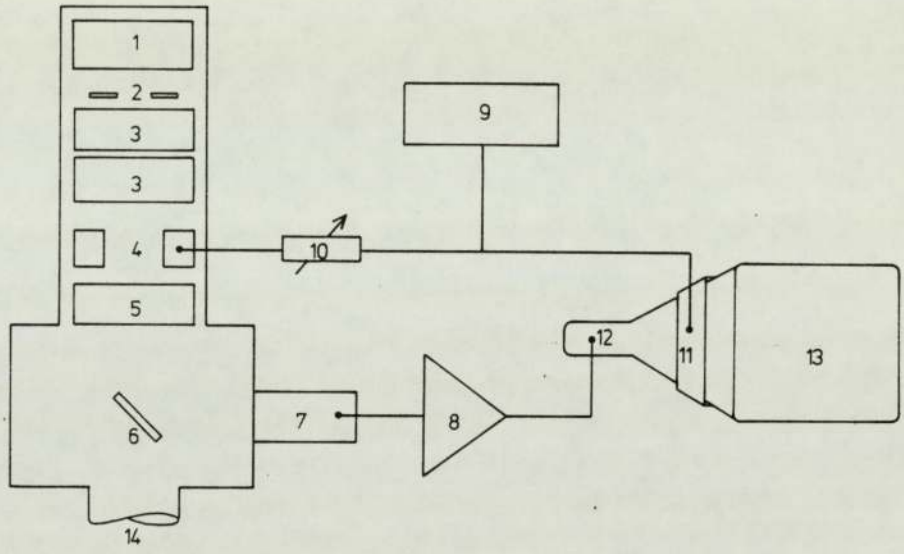
2.7.2. Preparation for scanning electron microscopy (SEM)

The following fixative was used prior to observations using scanning electron microscopy:

25ml 0.2% sodium cacodylate was added to 6ml 25%$_{(aq)}$ glutaraldehyde; the mixture was made up to 50ml with distilled water and the pH adjusted to 7.2. The cells were first washed with PBS+ and the fixative applied for at least 30 minutes. Samples were post fixed in 1% osmium tetroxide in 0.1M cacodylate buffer. To reduce the amount of shrinkage which can result from critical point drying, samples were further treated with 1% tannic acid in 0.1M sodium cacodylate [184] . After fixation the samples were dehydrated through a series of ethanol-water mixtures (50%, 70%, 100%, x 3) for 10 minutes each. Samples were transferred to a Polaron E3100 II critical point drying apparatus and dried under liquid CO_2. The samples were then mounted onto aluminium

stubs using Durafix (Rawlplug) and coated with 15nm of gold/palladium in a Polaron E5100 'Cool' Sputter Coating Unit at 1kV and 20mA. Samples were examined in a JEOL JSM-35 scanning electron microscope (Unilever Laboratories) at a tilt angle of 45 degrees which is represented in Figure 2.3:

Figure 2.3. Layout of a scanning electron microscope



Electron optical column

1. Electron gun
2. Anode disc
3. Condenser lens
4. Scan coils
5. Objective lens
6. Specimen
7. Detector

Display/electronics

8. Signal amplifier
9. Waveform generator
10. Magnification control
11. Scan coils
12. CRT brightness control
13. CRT display screen
14. Vacuum Connection

2.8. Preparation of glassware for use in lipid analysis

All glassware was soaked for up to 24 hours in a phosphate free detergent (Decon Labs. Ltd.) after which time it was rinsed thoroughly with distilled water and heat dried. Prior to use glassware was rinsed with a chloroform / methanol mixture (2:1) and to limit contamination gloves were worn at all stages of analysis.

2.9. Analysis of lipids

2.9.1. Whole foetal calf serum

2.9.1.1. Cholesterol and glyceride content

The total glyceride component of foetal calf serum was determined using a Boehringer Mannheim Diagnostic kit. Similarly, the values for the total and free cholesterol content were also derived and from these results the quantity of esterified cholesterol was calculated. Using the same techniques, comparisons were made with human serum.

2.9.1.2. Lipoprotein analysis

Lipoprotein analysis of both FCS and human serum was carried out using a 'Paragon' lipoprotein electrophoresis kit (Beckman). This kit composed an agarose gel system and lipids were visualised using an Oil Red 'O' based stain.

2.9.1.3. Analysis of other serum components

Using an ASTRA analytical instrument (Good Hope Hospital, Sutton Coldfield) an array of other foetal calf serum components were also identified.

2.9.2. Lipid extracts

2.9.2.1. Foetal calf serum (FCS)

To 25 ml of FCS 100ml of chloroform / methanol mixture (2:1) were added. The two layers were separated using a separating funnel, and the upper, non-lipid containing layer discarded.

The lipid containing fraction was filtered (Whatman filter paper 541) and the filtrant washed through with chloroform / methanol (2:1). The filtrate was washed with 100ml 0.88M KCl [185] (Folch wash) x 3, the upper layer was discarded after each application. The remaining lipid fraction was then dried down under nitrogen and frozen prior to use. Controls were also carried out where the procedure was undertaken in the absence of serum.

2.9.2.2. Adsorbed layers to tissue culture and bacteriological grade plastic

30 ml of complete DMEM were added to both bacteriological and tissue culture grade Petri dishes. Again controls were set up where the same procedures were carried out but in the absence of serum in the culture medium. All plates were incubated at 37°C for 6 hours after which time they were washed with PBS; care was taken to remove all the washing buffer. 1 ml of methanol was added to the dishes for 0.5 hours (x3); after each time the solvent was removed and dried down under nitrogen. Samples were frozen prior to use. In order to achieve an overall outline of the lipid content of the extracts, a number of different techniques were combined.

2.9.2.3. Thin Layer Chromatography (TLC)

Analysis of both FCS and adsorbed layer extracts was carried out in duplicate. Using this

technique, the adsorbent was held on a glass plate as a thin layer. Silica gel plates (Anachem Merc) measuring 20cm x 5cm were heat activated at 80° C for 1 hour after which time a 2mm margin was scraped off either side of the plate. Standards and samples, dissolved in chloroform, were applied as discrete spots 1.5 -2 cm above the bottom of the plate. The following plates were prepared:

2.9.2.3.1. Plate 1: Neutral lipids

Solvent:	diethyl ether	petroleum	formic acid
	20	80	1

Standards:		
	monoglyceride	cholesterol
	diglyceride	sterol esters
	triglyceride	phospholipid
	free fatty acid	

2.9.2.3.2. Plate 2: Polar lipids

Solvent	chloroform	methanol	acetic acid	water
	85	15	10	3.5

Standards		
	cholesterol	phosphatidyl ethanolamine
	cholesterol esters	" inositol
	monoglyceride	" choline
	triglyceride	" serine

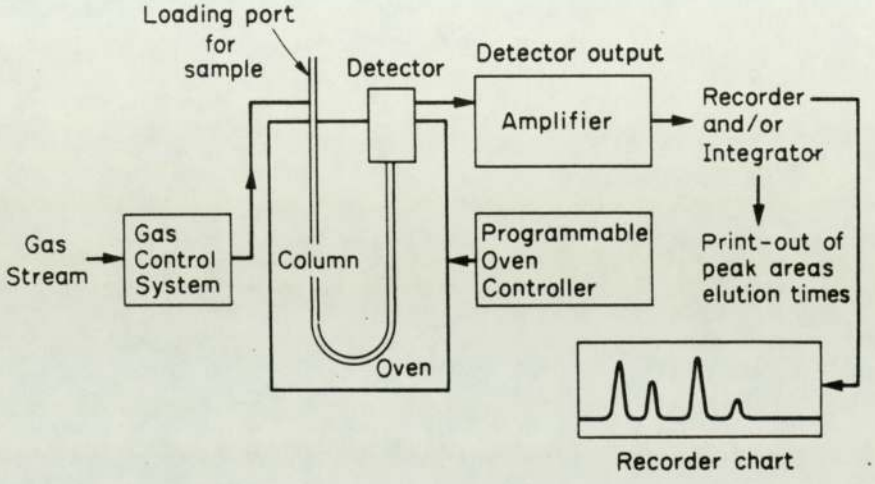
2.9.2.3.3. Procedure for TLC

Plates were placed into the appropriate solvent mixtures and allowed to run until the front was 5cm from the top of the plate (approximately 0.5 hour). Both plates were then air dried, sprayed with 50% sulphuric acid until slightly damp and placed in an oven for approximately 2 minutes. After this time a colorimetric reaction was observed where cholesterol showed red and cholesterol esters were pink. The circumference of each spot were outlined by indentions into the silica gel. The plate was returned to the oven for a further 10 minutes to char all lipids.

2.9.2.4. Gas-liquid chromatography (GLC) of fatty acid methyl esters

Following the extraction of lipids from foetal calf serum or those layers adsorbed to tissue culture and bacteriological plastic, the free fatty acids were methylated using a sulphuric acid : toluene : methanol (1 : 10 : 20 v/v) mixture. The reaction was carried out by refluxing the extract in the reagent for 1 hour. After this time, water (5 ml) and hexane (5ml) were added and the resultant lower aqueous phase was aspirated to waste. The upper layer was then dried under nitrogen and the samples were redissolved in 200µl of pentane before injecting onto the GLC column. Fatty acid methyl esters were separated on 6 ft (1.8m) columns of 10% diethyleneglycol succinate) on Chromabsorb 101 using a Pye series 304 Gas Chromatograph equipped with a dual-flame ionisation detector and computing integrator. A diagram representing this apparatus is shown in Figure 2.4. Nitrogen (40ml per minute) was used as the carrier gas at a column temperature of 190°C. Samples taken up in pentane were identified with those standards routinely used at the collaborating body (Unilever, Colworth).

Figure 2.4. Schematic diagram of a gas chromatograph.



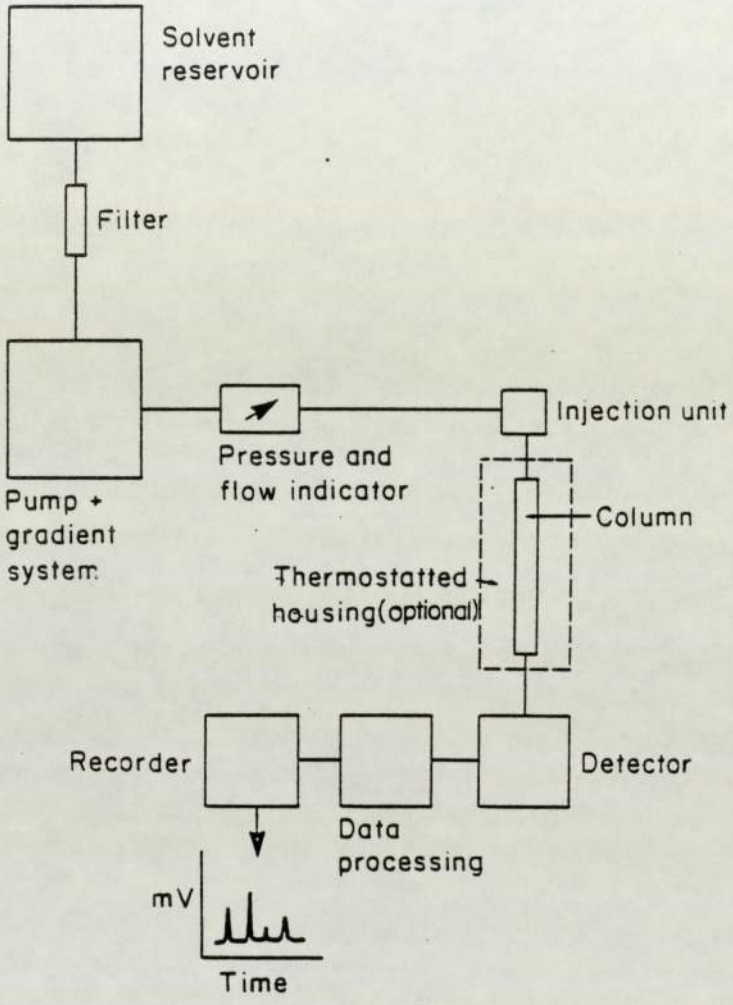
2.9.2.5. High performance liquid chromatography (HPLC)

Separation of materials by high performance liquid chromatography depends on the relative affinities of the solute molecules for the stationary and mobile phases. Samples injected onto the column head (stationary phase) are flushed along by the mobile phase and identified according to their retention times compared with known standards.

In this study lipids were identified using a Perkin-Elmer HPLC system coupled to an LKB UV detector (at 210nm), with Knauer pumps and chart recorder and this apparatus

is represented by Figure 2.5. All solvents were degassed using a Millipore - Waters system.

Figure 2.5. Block diagram of a high performance liquid chromatograph



The following mobile phase flowing at 40 ml per minute was chosen to maximise the separation of fatty acids and cholesterol, two groups of lipids normally exhibiting similar retention times. Known standards, dissolved in mobile phase were also applied to the columns.

Mobile Phase 1000ml hexane

5ml propan -2-ol

1ml glacial acetic acid

2.9.2.6. Calcium analysis

Samples measuring 0.5 cm in diameter were cut from both tissue-culture and bacteriological grade dishes. The appropriate surfaces were coated with complete DMEM and incubated for 6 hours at 37°C. After this time they were prepared for energy dispersive X-ray microanalysis (EDXA) as follows:-

2.9.2.6.1. Method 1 for preparation of samples for EDXA analysis

The samples were prepared following the protocol previously outlined for SEM preparation.

2.9.2.6.2. Method 2 for preparation of samples for EDXA analysis

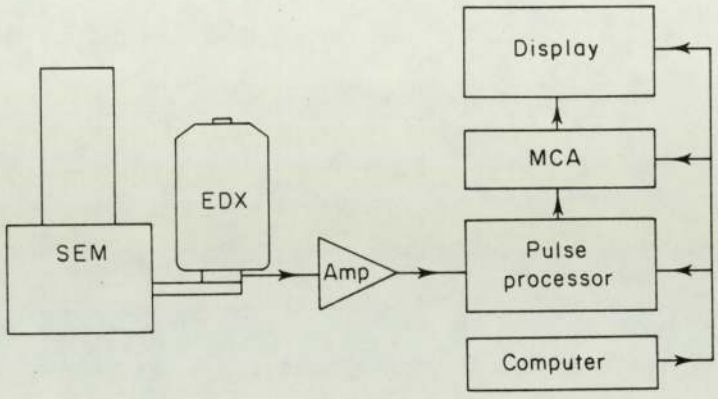
Samples were washed with PBS- and dried overnight in an oven.

Samples prepared by methods 1 and 2 were mounted onto aluminium stubs, sprayed with conducting aerosol paint prior to analysis by EDXA.

2.9.2.6.3. Energy dispersive X-ray microanalysis (EDXA)

This surface technique was used to analyse any calcium deposited onto the plastic surfaces. These investigations were carried out using a Link System Autoanalyser, mounted on a Cambridge Stereoscan electron microscope. A diagrammatical representation of the apparatus used is shown in Figure 2.6. Optimum results were achieved using an electron beam voltage of 20keV and a sample collection time of 100 seconds and the critical excitation potentials for the elements identified during this analysis are shown in Appendix 6.

Figure 2.6. Layout of an overall EDXA system



CHAPTER 3

The effects of polymer structure, crystallinity, polarity and methods of manufacture on cell adhesion.

3.1. Introduction

The interaction of eukaryotic 'anchorage dependent' cells is of considerable importance both in it's own right and in the field of biomaterials science. Progress within the biomaterials area relies on a knowledge of those properties of the material that influence the biological response, together with an improved understanding of the events that occur at the interface between the material and the biological environment.

There is a great deal of debate about the structural factors and surface properties associated with the ability of polymers to support adhesion and spreading of BHK fibroblast cells. Most of this attention has been centred on the role of functional groups (such as hydroxyl groups [20 - 22, 85,]) and the contribution of such properties as surface energy and wettability. By far the greatest proportion of this work is concerned with polymers that do show an ability to support cell adhesion.

The position of unmodified polystyrene in all this debate appears to be unique in that it is the only polymer amongst the exceptionally wide range of thermoplastic and thermosetting materials which have been studied that is unambiguously non-cell adhesive. The position of hydrogel polymers, a unique group of water swollen hydrophilic polymer networks, is unusual. Amongst this family of materials, which are quite different in their surface characteristics from conventional polymers, poly (-2-hydroxyethyl methacrylate) (polyHEMA) has been identified as non cell adhesive [85, 93, 98, 186, 211] . This is a somewhat contentious point and the peculiarities of polyHEMA and other hydrogel polymers have been previously discussed [85, 93, 98, 186] . Previous interest in polystyrene has centred upon the ease with which surface modification of the polymer can render it cell adhesive. Thus, bacteriological grade plastic, which is essentially unmodified polystyrene is converted by the introduction of

polar oxygen-containing groups into tissue culture plastic, which is the standard polymeric substrate used for the routine maintenance of anchorage dependent cells. Many workers have paid attention to this conversion and to the nature of those polar groups that are most efficient in promoting cell adhesion. The points that have not been previously addressed are:

- why is polystyrene non-adhesive to cells?
- is its position unique in this respect?
- what is the smallest change in surface structure that will induce polystyrene to become cell adhesive?

In order to investigate these points a series of polymers having similar surface energies to that of polystyrene were investigated together with a group of modified polystyrenes containing structural features known to be present in other polymers that do support cell adhesion. In particular the role of the effectiveness of polar atoms other than oxygen was felt to be important, together with morphological changes that result from changes in stereoregularity and therefore crystallinity of the polymers.

3.2. Discussion of results

3.2.1. Dependence of cell adhesion on substrate structure, crystallinity and manufacture.

Although the position of polystyrene appeared to be unique from an appraisal of results presented in the literature, this uniqueness in part reflects the fact that very few polymers that would be broadly regarded as 'hydrophobic' have been examined in terms of the cell response that they induce. Whilst polymers such as polypropylene for example have been characterised in terms of their cytotoxic effects, the standard tests used to determine cytotoxicity are not carried out in the same way as those for anchorage dependency. An initial experiment was therefore carried out in which four polymers commonly regarded as hydrophobic were examined in commercially available forms. These were polystyrene (bacteriological plastic) and poly(vinyl chloride) (PVC) in the form of moulded specimens together with polypropylene and poly(4 - methyl pent - 1 - ene) in film form.

The structures of these four polymers are shown in Structures 3.1 - 3.4. Samples of the polymers were washed in a surfactant (1% Tween 20), thoroughly rinsed in distilled water and maintained in sterile phosphate buffered saline prior to cell adhesion studies. All the samples were subsequently handled with sterile forceps in a laminar flow cell culture hood and then used in cell culture studies of BHK fibroblast cells. The results after 6 hours in culture are shown in Table 3.1 and these results raise several questions. Although these polymers are all broadly hydrophobic, differences exist between them:

The two cell adhesive polymers are stereoregular whereas the other two not;

The two cell adhesive polymers are crystalline whereas the other two are not;

Table 3.1. The relative ability of various synthetic polymer substrates to support BHK fibroblast cell adhesion after 6 hours in culture; confluent cell adhesion (+++); no cell adhesion (--)

Polymer (form)	Cell adhesion
Polystyrene (moulded specimen)#	--
Poly (vinyl chloride) (moulded specimen)	--
Poly (4 methyl pent - 1 - ene) (commercial film)	+++
Polypropylene (commercial film)	+++
Tissue culture plastic	+++

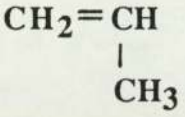
Bacteriological grade plastic

The two cell adhesive polymers were in film form whereas the the two non adhesive polymers were examined as melt or mould forms.

In order to examine the effect of stereoregularity a comparison was carried out between two polymers, one which was cell adhesive (polypropylene, Structure 3.1) and the other non cell adhesive (polystyrene, Structure 3.3) according to the results shown in Table 3.1. Since polystyrene is normally available as the atactic, amorphous polymer, the crystalline, isotactic form was obtained and examined. Similarly, since polypropylene is predominantly used as a stereoregular, isotactic and in consequence highly crystalline polymer, it was appropriate to examine the effects of the atactic polymer on cell behaviour in culture studies. In order to eliminate possible effects arising from differences in melt processing techniques, the four samples were prepared for cell studies in the form of solvent cast films. Of the techniques available to prepare samples of this type we have found that spin coating offers the greatest control of film thickness, uniformity and surface profile (ie. smoothness) [85, 93] . None of the four polymers examined in this way (atactic and isotactic polypropylene, atactic and isotactic polystyrene) showed any evidence of being cell adhesive under the conditions previously described. In a control experiment, poly (methyl methacrylate) (Structure 3.5) which is well known to be moderately cell adhesive [85, 101] was prepared for cell culture studies in the form of a spun film. The resultant surface was found to reflect the degree of cell adhesiveness normally associated with this polymer.

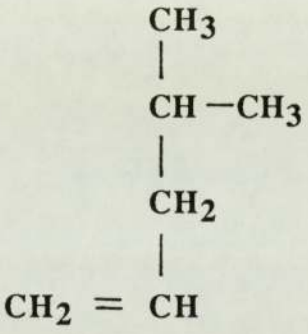
Although the atactic and isotactic forms of polystyrene and polypropylene are not equally susceptible to melt processing techniques it is possible with some care to produce melt pressed samples of each polymer. These were examined by the cell culture techniques previously described and again were found to be unable to support cell attachment and

Propylene



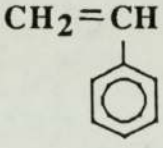
Structure 3.1

4 - methyl pent - 1 - ene



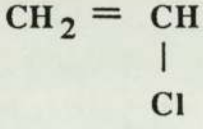
Structure 3.2

Styrene



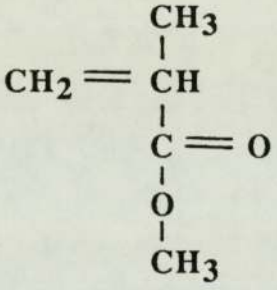
Structure 3.3

Vinyl chloride



Structure 3.4

Methyl methacrylate



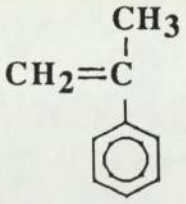
Structure 3.5

spreading. The evidence of the experiments described above enables structural comparisons to be made between polymers prepared under two sets of identical conditions. This evidence strongly indicates that neither the specific presence or absence of pendant aliphatic hydrocarbon groups nor the tacticity of the polymer chain or indeed the consequent crystallinity thereby induced influences the ability of a polymer substrate to be rendered cell adhesive.

Further groups of experiments were therefore necessary to support these contentions and in particular to examine the effects of inducing small structural changes by the introduction of both branched and unbranched aliphatic hydrocarbon groups and of polar atoms other than oxygen, into the styrene repeat unit. A sequence of structural changes designed to reflect these requirements and to provide further structural information relating to the apparent uniqueness of polystyrene in the cell biology literature is shown below (Structures 3.6 - 3.9).

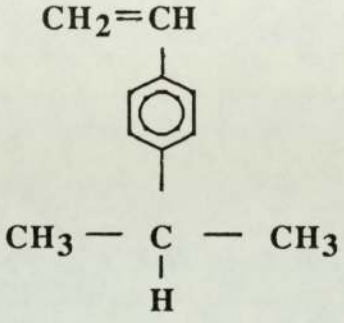
In poly(α - methyl styrene) (Structure 3.6) a pendant methyl group reflecting that found in polypropylene has been introduced directly onto the polymer backbone, whereas in both poly (p - isopropyl styrene) (3.7) and poly (p - tert butyl styrene) (3.8) branched aliphatic hydrocarbon groups reflecting the structure of poly (4 - methyl pent - 1 - ene) have been introduced directly into the aromatic ring of polystyrene. Finally, in poly (p bromo styrene) (Structure 3.9) a halogen atom with a larger dipolar effect than that of chlorine in poly (vinyl chloride) (PVC, Structure 3.4) has been introduced as a substituent. Spun films and melt pressed discs of these polymers were prepared although it was not possible to present each polymer in both forms. All of the four polymers (Structures 3.6 to 3.9) were used in the atactic form and as a result the samples prepared were amorphous with no detectable crystallinity. The samples were prepared for cell

α – methyl styrene



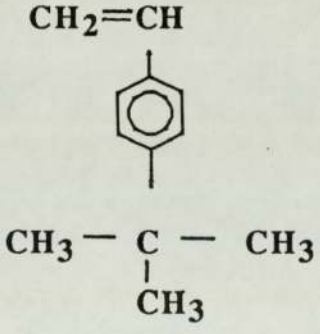
Structure 3.6

4 - isopropyl styrene



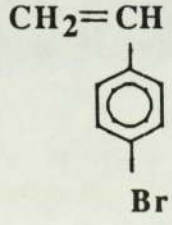
Structure 3.7

Para - tert butyl styrene



Structure 3.8

para bromo styrene



Structure 3.9

adhesion studies as described above and the cell interactions monitored for a period of six hours in culture. None of the substrates showed any tendency to support cell adhesion after this time. The overwhelming evidence from the previous results taken together is that no specific structural features associated with polypropylene and poly (4 -methyl pent - 1- ene) are responsible for the cell adhesiveness reflected in the results of Table 3.1. Similarly the inability of both poly (p - bromo styrene) (3.9) and poly (vinyl chloride) (3.4) to support cell adhesion indicates that the confluent cell adhesion supported by tissue culture polystyrene is not simply a consequence of the dipole moment induced by the presence of polar atoms.

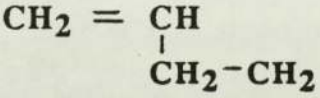
The question of processing techniques is obviously important and is related to the stereoregularity of the polymer together with the ultimate crystallinity and morphology of the surface as prepared. The fact that the only cell adhesive samples in any of the previous experiments had been prepared commercially in the form of films is significant. The technique that had been used to produce these films was calendering, which involves drawing the polymer through heated rollers at a temperature in the region of it's melting point. The significant differences between this technique and melt pressing, injection moulding or film spinning are that it offers greater potential for surface oxidation of the polymers and at the same time is capable of inducing higher degrees of crystallinity by a concurrent axial orientation process [187] . It is possible, therefore, that the cell adhesiveness of isotactic polypropylene and isotactic poly(4 - methyl pent - 1 - ene) reflected in Table 3.1 is due to enhanced surface oxidation or to the enhanced morphological effects induced during calendering (or both). In a group of experiments designed to investigate these possibilities, methods of sample preparation of five polymers were examined more closely. These were isotactic polypropylene (Structure 3.1), isotactic poly (4 - methyl pent - 1 -ene) (Structure 3.2), isotactic poly(but - 1 - ene)

(Structure 3.10) together with both isotactic and atactic polystyrene (Structure 3.3). All five samples were melt pressed under conditions that allowed optimum crystallinity to develop (ie. slow rates of mould cooling). In addition, calendered films of two of the polymers (atactic polystyrene and isotactic polypropylene) were prepared in the laboratory. These were compared with isotactic polypropylene and poly (4 - methyl pent - 1 - ene) previously studied and referred to in Table 3.1. Again samples were cleaned in 1% Tween 20, rinsed in distilled water and left to soak in sterile phosphate buffered saline prior to their use as culture substrates of BHK fibroblast cells. Of this group of samples, none of the melt pressed films showed any tendency to support cell adhesion whereas all four calendered films supported cell adhesion.

The clear implication is that the morphological aspects of polymer production played no discernable part in inducing cell adhesion. This conclusion is supported by two observations. Firstly the sample of atactic polystyrene became cell adhesive when the processing technique was changed from melt pressing to calendering although the polymer was amorphous. Secondly the different isotactic polymers showed no tendency to become progressively more cell adhesive as the crystallinity of the processed samples was increased. There was rather a step change in behaviour in passing from melt pressing to calendering, presumably associated with the greatly enhanced oxidation associated with the calendering process.

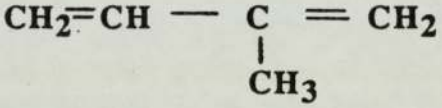
One final material which reflects some of the structural effects discussed in relation to polymer repeat unit structure was examined. This is chlorinated rubber which is produced by passing chlorine through a solution of cis - poly - isoprene (Structure 3.11) thereby inducing extensive chlorination with some oxidation [188] . The fact that a dipolar atom such as chlorine or bromine was unable to induce cell adhesion whereas dipolar oxygen

But - 1 ene



Structure 3.10

Isoprene



Structure 3.11

obviously can might perhaps be due to some inhibitory effect of the halogen atom. Studies of the cell adhesiveness of chlorinated rubber demonstrated however that this polymer was extensively cell adhesive. Similarly although solvent cast and melt pressed discs of poly(vinyl chloride) are non cell adhesive, production of PVC film by calendering in the laboratory yielded a material that was cell adhesive. It is clear, therefore, that the introduction of the polar halogen atoms, chlorine and bromine, is of itself unable to induce cell adhesion. On the other hand, the presence of the polar oxygen atom in the form of, for example, ester, hydroxyl and carboxyl groups is known to produce adhesive substrates [20 - 22,101] [poly(methyl methacrylate), polystyrene]. The presence of chlorine and oxygen together induced cell adhesion (chlorinated rubber and calendered poly(vinyl chloride). It is clear, therefore, that polarity alone is unable to induce cell adhesion and equally clear that polar halogen atoms are unable to totally inhibit it. A summary of the response of cells to the various substrates examined in this study is contained in Table 3.2.

3.2.2. Surface characterisation

In parallel to the cell adhesion studies, analytical techniques were also employed to characterise those physico-chemical properties of the surface, such as polarity and surface energy, that had previously been shown to affect cell behaviour. The technique used to determine the polar and dispersive components of the surface energy was the sessile drop contact angle technique of Owens and Wendt [182] which has been described in the literature as a valuable technique for measuring surface energy and wetting [181]. This method involved placing droplets of two wetting liquids, namely water (polar) and methylene iodide (non-polar), onto separate areas of the substrate. The value of the contact angle θ is averaged from measurements at both sides of several droplets and the polar and dispersive components are determined using the equation shown in Chapter 2. The mean values of θ for both water and methylene iodide are given in Table 3.3 (n=4)

Table 3.2. Effects of polymer structure, tacticity, polarity and methods of manufacture on BHK fibroblast cell adhesion; confluent cell attachment and spreading(+++); cell attachment with some spreading (++); no cell attachment (--)

		Cell adhesion		
Polymer	**Tacticity***	**Spun**	**Calendered**	**Pressed**
Polystyrene	Atactic	--	+++	--
	Isotactic			--
Polypropylene	Atactic	--		--
	Isotactic	--	+++	--
Poly (vinyl chloride)	Isotactic	--	++	--
Chlorinated rubber	Atactic	++		
Poly (p bromo styrene)	Atactic	--		
Poly (4-methyl pent-1ene)	Isotactic		+++	--

Table 3.2. (continued) Effects of polymer structure, tacticity, polarity and methods of manufacture on BHK fibroblast cell adhesion.

		Cell adhesion		
Polymer	**Tacticity***	**Spun**	**Calendered**	**Pressed**
Poly (p tert butyl styrene)	Atactic	--		--
Poly(4 isopropyl styrene)	Atactic			--
Poly (α - methyl styrene)	Atactic			--
Poly (but-1-ene)	Isotactic			--
Controls				
Atactic poly(methyl methacrylate)		++		++
Tissue culture plastic (moulded) +++				
Bacteriological grade plastic (moulded) --				

Table 3.2. (continued) Effects of polymer structure, tacticity, polarity and methods of manufacture on BHK fibroblast cell adhesion.

*** Tacticity**

Isotactic - pendant groups of each polymer repeat unit regularly present themselves on the same side of the polymer backbone. The molecules in this type of polymer are packed more closely and in a more ordered fashion. These polymers are therefore more crystalline.

Syndiotactic - the pendant groups of each polymer repeat unit present themselves on alternate sides of the backbone.

Atactic - the pendant groups do not present themselves with any regularity. Consequently the molecules are poorly packed together and the polymer is amorphous.

and the polar, dispersive and total components of the surface energy are given in Table 3.4. Whilst comparisons of the values for water contact angles can be made as a useful indication of polarity/hydrophilicity, a more accurate interpretation can be made from a comparison of the polar surface energy component.

In general, the smaller the contact angle with water, the higher the polar contribution at the surface. This is reflected in the results for the calendered films of atactic polystyrene, poly (4 - methyl pent - 1 - ene), poly(vinyl chloride) and isotactic polypropylene, which all have water contact angles between between 41° and 69° and polar components between 10.1 mN m^{-1} and 24.2 mN m^{-1}. Tissue culture plastic with a water contact angle of 50° and a polar contribution of 17.9 mN m^{-1} lies within these ranges. In contrast, spun films of atactic polystyrene and isotactic polypropylene show a marked increase in their water contact angles (both 82°) which is reflected in lower values for their polar components of 2.5 mN m^{-1} and 4.0 mN m^{-1} respectively. These values confirm that there is greatest oxidation at those polymer surfaces that were produced by the calendering process. Those surfaces that were markedly less polar were unable to support normal anchorage dependent cell behaviour.

A further technique which may be used to analyse a surface is electron spectroscopy for chemical analysis, ESCA. This technique involves the irradiation of the surface with monoenergetic MgK_{α} x-rays (1258.6 eV) which results in the emission of an electron from the upper 10 Å of the sample. These electrons are detected according to their kinetic energies; the analyser normally operates as an energy 'window' accepting only those electrons having an energy within the range of this fixed boundary. Prior to analysis the samples were handled with forceps and stored in airtight polythene bags.

Table 3.3. Results of contact angle measurements calculated using water and methylene iodide as wetting agents. (n=4)

Polymer	Form	Water (°)	Methylene Iodide (°)
Tissue culture plastic	Moulded	50	19
Atactic polystyrene	Moulded#	91	22
	Spun	82	30
	Calendered	41	22
Isotactic polystyrene	Pressed	95	25
Atactic polypropylene	Pressed	87	42
Isotactic polypropylene	Spun	82	45
	Calendered	60	40
	Pressed	93	46
Poly(vinyl chloride)	Calendered	63	25
Chlorinated rubber	Spun	71	43
Poly (4-methyl pent 1 ene)	Calendered	69	30
Poly (p-tert butyl styrene)	Spun	71	35
Poly (4-isopropyl styrene)	Pressed	86	30
Poly (but - 1 - ene)	Pressed	91	40
Poly (methyl methacrylate)	Pressed	66	24

Bacteriological grade plastic

Table 3.4. To show the effect of method of manufacture on fractional polarity as measured by sessile drop contact angle techniques.

		mN m^{-1}		
Polymer	**Form**	**Polar(γ_p)**	**Dispersive(γ_d)**	**Total(γ_t)**
Tissue culture plastic	Moulded	17.9	40.2	58.1
Atactic polystyrene	Moulded#	0.3	47.3	47.6
	Spun	2.5	42.0	44.5
	Calendered	24.2	38.0	62.2
Isotactic polystyrene	Pressed	0.02	47.0	47.0

Bacteriological grade plastic

Table 3.4. (continued) To show the effect of method of manufacture on fractional polarity as measured by sessile drop contact angle techniques.

		mN m^{-1}		
Polymer	**Form**	**Polar(γ_p)**	**Dispersive(γ_d)**	**Total(γ_t)**
Isotactic polypropylene	Spun	4.0	34.1	38.1
	Calendered	15.1	33.0	48.1
	Pressed	0.9	34.1	35.0
Atactic polypropylene	Pressed	2.0	36.8	38.8
Poly (vinyl chloride)	Calendered	10.6	40.4	51.0
Chlorinated rubber	Spun	8.9	33.2	42.2
Poly (4 -methyl pent -1 - ene)	Calendered	10.1	38.7	48.8
Poly (4- isopropyl styrene)	Pressed	1.4	42.8	44.2

Table 3.4. (continued) To show the effect of method of manufacture on fractional polarity as measured by sessile drop contact angle techniques.

		mN m^{-1}		
Polymer	**Form**	**Polar(γ_p)**	**Dispersive(γ_d)**	**Total(γ_t)**
Poly (but-1-ene)	Pressed	0.8	38.7	39.6
Poly (methyl methacrylate)		8.8	41.4	50.2

Using the equation in Chapter 2 the relative percentages of those elements present were determined and ultimately the carbon : oxygen (C : O) ratios shown in Table 3.5 were calculated. The higher the C : O ratio, the lesser the extent of oxidation of the sample surface and similarly the lower values reflect a greater extent of oxidation. It must be pointed out that there was little known about the % error that may have had an affect on the quantification of ESCA results. However, the outstanding feature seen in Table 3.5 is that those surfaces that showed the greatest oxidation were those surfaces that had been manufactured by the calendering technique. Polymers that had previously been thought of as being hydrophobic became more hydrophilic after the calendering process. Therefore, similar conclusions to those derived from analysis of surface energy measurements are paralleled in the results for C:O ratios. Lower C : O ratios of 7.6, 9.3, 12.3 were obtained for calendered polystyrene, poly (4 - methyl pent - 1 - ene) and polypropylene respectively, as compared to the values of 46.6, 49.0 and 48.0 for the non cell adhesive surfaces of moulded and spun atactic polystyrene. The results show that tissue-culture plastic had similar values to that of calendered polystyrene in terms of surface energy and in contrast, tissue culture plastic had quite different values to either spun or moulded forms of atactic polystyrene.

Figure 3.1. ESCA analysis of tissue culture plastic

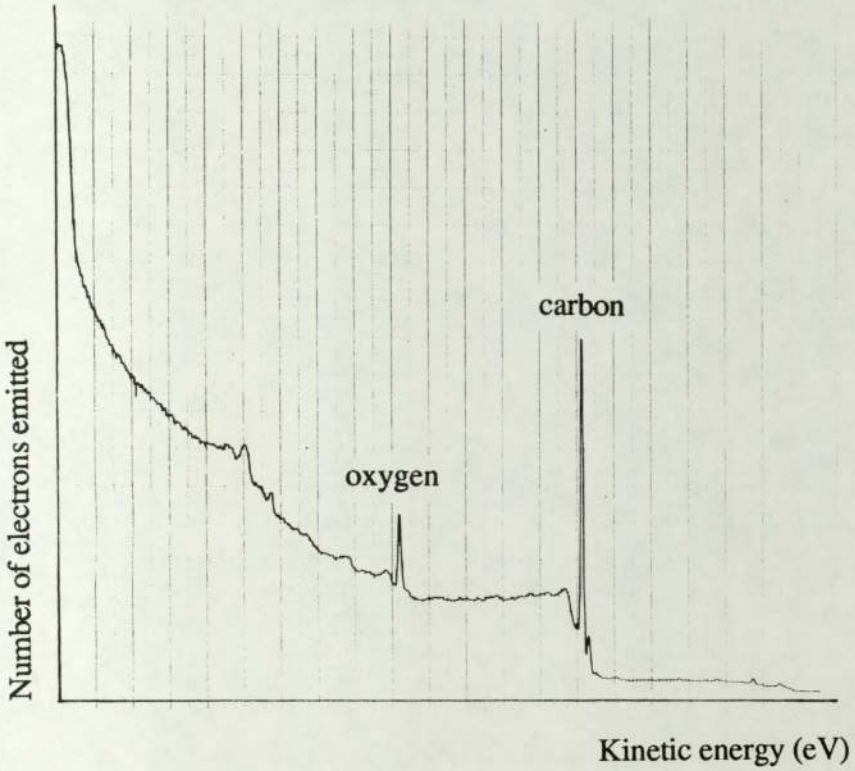


Figure 3.2. ESCA analysis of bacteriological grade plastic

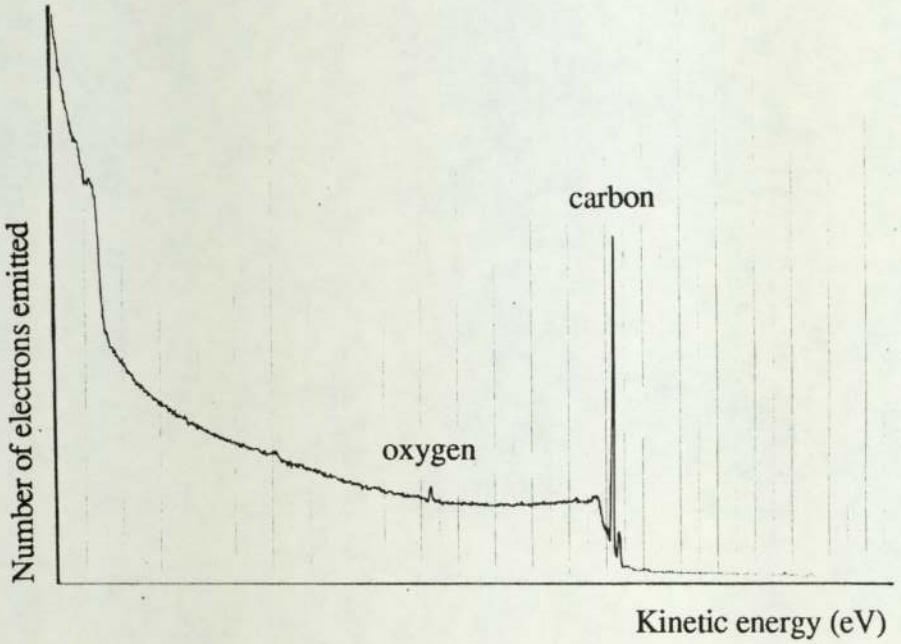


Figure 3.3. ESCA analysis of calendered polystyrene

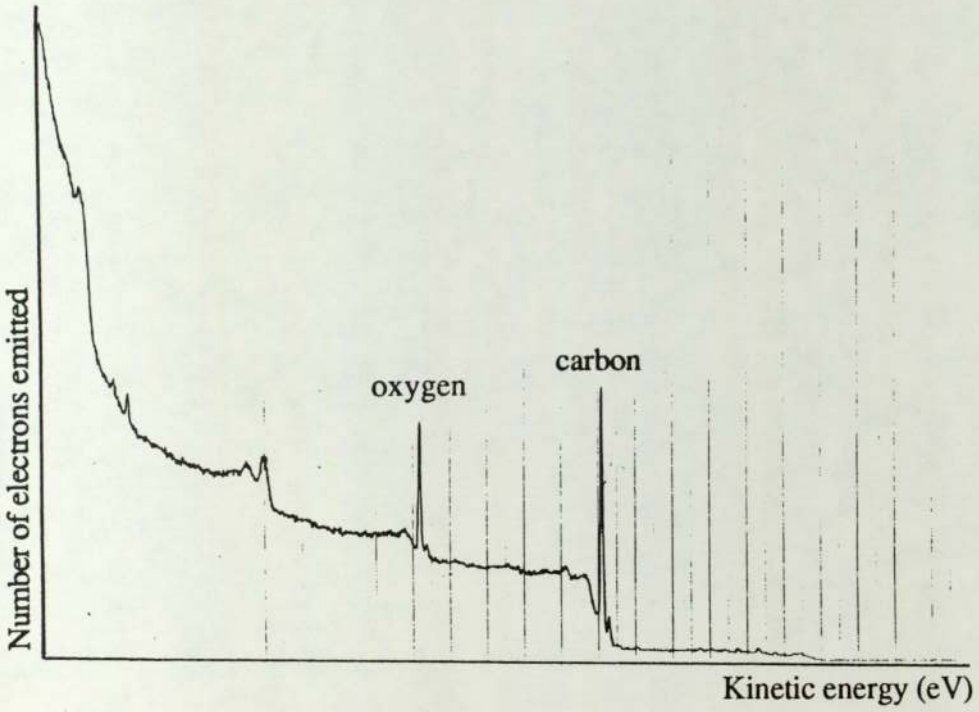


Figure 3.4. ESCA analysis of calendered poly (vinyl chloride)

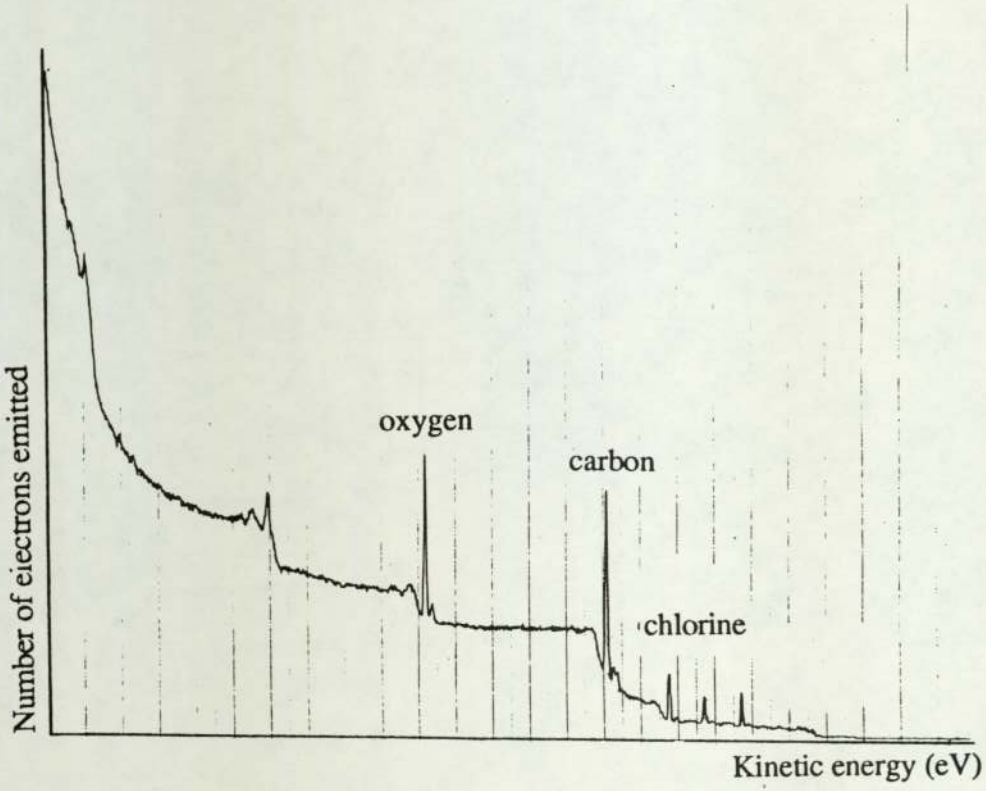


Table 3.5. Carbon:oxygen ratios, determined using ESCA techniques, for a selection of polymers

Polymer	Form	C:O
Tissue culture plastic	Moulded	11.5
Atactic polystyrene	Moulded[#]	46.6
	Spun	49.0
	Calendered	7.6
	Pressed	22.8
Isotactic polystyrene	Pressed	44.5
Isotactic polypropylene	Pressed	48.0
	Calendered	12.3
Atactic polypropylene	Pressed	18.6
Poly(vinyl chloride)	Calendered	3.5
Chlorinated rubber	Spun	6.6
Poly (4 methyl pent-1-ene)	Calendered	9.3
Poly(4 - isopropyl styrene)	Pressed	14.9
Poly (α-methyl styrene)	Pressed	27.6

[#] Bacteriological grade plastic

3.3. Concluding discussion

Prior to this study, polystyrene occupied a unique position among non-hydrogel materials in that it was the only 'hydrophobic' material that was recognised as being non-adhesive for BHK fibroblast cells in culture, a position which appeared to represent something of an anomaly. Substrates more hydrophilic than polystyrene, eg poly(ethyl methacrylate) and tissue culture plastic are recognised as being cell adhesive materials. However, it was also known [85] that materials generally regarded as being similar in polarity to polystyrene, eg. poly(4 - methyl pent - 1 - ene) (TPX) and polypropylene supported high levels of cell adhesion. It had been thought that since these latter two polymers had previously been studied in isotactic forms then tacticity, and ultimately crystallinity and morphology, may be responsible for cell adhesion. It had been suggested [85] that the expression of a regular array at a surface may promote cell adhesion through microscopic areas of surface regularity and such interactions may not have been possible with an atactic, less polar polymer. However, an inspection of the results presented in Table 3.2 shows that pressed films of polypropylene and polystyrene in both atactic and isotactic forms did not support cell attachment. These results indicate that crystallinity alone was not the sole criterion for the cell adhesive response. It can be seen from structures 3.1 and 3.2 that the predominant structural feature of the cell adhesive polypropylene and TPX was the presence of the unbranched and branched alkyl group. However, this present study has revealed that the substitution of such alkyl groups onto the aromatic ring of polystyrene did not lead to a substrate that was capable of supporting cell attachment. Thus, such structural features are not in themselves important in mediating cell adhesion.

This study has also shown that TPX and polypropylene are cell adhesive only in the form of calendered films whereas spun or pressed films of these two polymers are non-cell adhesive. An investigation of the surface energy components of the cell adhesive

polymers (Table 3.4) showed that they had a significant polar contribution and studies of their carbon : oxygen ratios revealed that there was more oxygen at the surfaces of the calendered films than those manufactured using other techniques. Surfaces with halogens present show similar dipolar moments as those with oxygen. The importance of oxygen at a surface had been highlighted by Curtis et al [20], Maroudas [21] and Klemperer et al [22]. The selection of halogenated polymers chosen for this study included poly(vinyl chloride), poly (p-bromostyrene), and chlorinated rubber. Interestingly, spun films of poly (p-bromostyrene) and both spun and pressed films of poly(vinyl chloride) did not support cell attachment. However, the presence of the halogen did not inhibit cell adhesion since both poly(vinyl chloride) (calendered film) and chlorinated rubber (spun) exhibited extensive cell attachment and spreading. It is clear, therefore, that the presence of a dipolar moment produced by the presence of a polar atom substitutent does not in itself produce cell adhesion.

Further inspection of Table 3.2 shows that the only polymers capable of supporting good cell attachment and spreading are polypropylene, polystyrene, poly(vinyl chloride) and poly(4 - methyl pent - 1 - ene) (TPX) in the form of calendered films, and spun films of chlorinated rubber. The method of fabrication or manufacture of polymer samples is one variable rarely discussed during investigations into the effects of substrates on cell behaviour. The production of commercially available calendered films, such as polypropylene and TPX exposes the molten polymer to atmospheric oxygen which would ultimately lead to oxidation of the polymer. The values of γ_p for both calendered polypropylene and TPX were relatively higher than those for other forms. This higher polar component was coupled with a lower C:O ratio, indicating there had been surface oxidation which could have arisen as a result of manufacturing techniques. There is, therefore, substantial supporting evidence for the proposition that oxidation is responsible

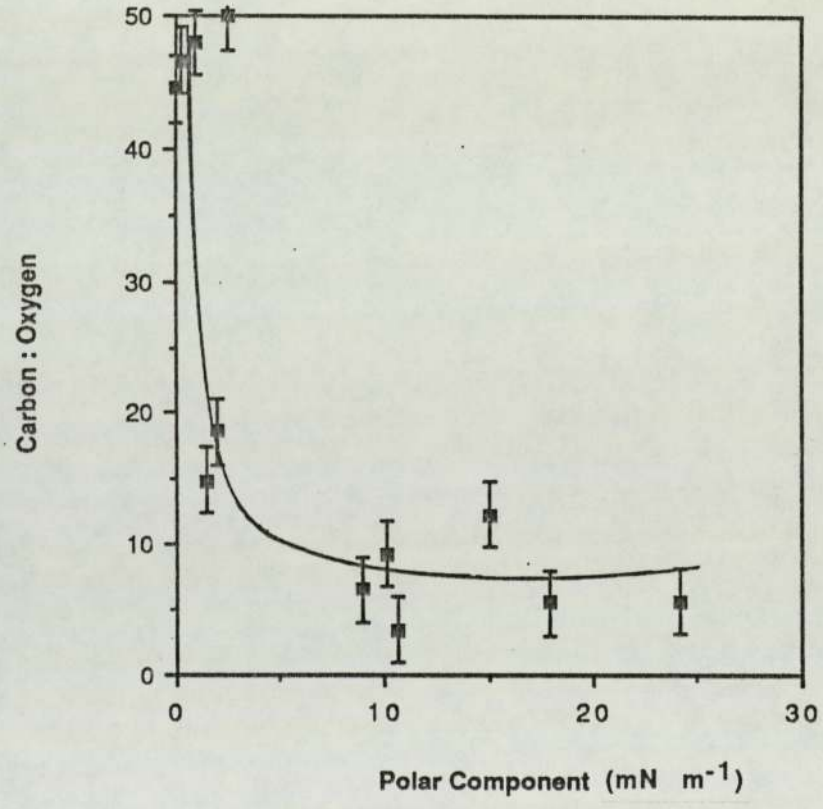
for the cell adhesive response seen on the calendered films.

In contrast, little oxidation had been produced on those polymer films produced by melt pressing and these surfaces did not support attachment of BHK fibroblast cells. Values of γ_p were markedly lower for pressed and, with the exception of chlorinated rubber, spun films. This suggests that these polymers are less polar and this was coupled with higher values for carbon : oxygen ratios (less oxidation) for these surfaces, and Figure 3.5 shows that there did appear to be some correlation between these two parameters. One further interesting feature to note is that whilst chlorinated rubber contains over 66% chlorine it is partially oxidised during it's manufacture [188] and this was reflected in a significant polar contribution to the surface energy and an oxidised surface (Table 3.5). Again, this oxidation could have affected the cellular response.

Oxygen at the surface may be important because of it's ability to mediate hydrogen bonding. This becomes important when considering the possible adsorption of proteins (via their amide groups) or fatty acids (via carboxyl groups) during the process of interfacial conversion and the role of water in these processes. It is possible that a certain critical oxygen concentration is needed to induce cell adhesion. This threshold level may be important spatially in that the distances between oxidised domains may be important or the size of these 'patches' of oxygen may be the deciding factor. However, the aims of this chapter were to identify some of the underlying physico-chemical factors of materials that may be important in triggering the biological response and investigations at the molecular level are outside it's scope.

This study also highlights the fact that cell attachment and spreading is not an 'all or nothing' phenomenon but that there may be an array of responses produced, as

Figure 3.5. Plot of carbon : oxygen ratios against the polar surface energy component for the selected series of polymers



and spreading usually associated with tissue-culture plastic after 6 hours under culture conditions. It can be seen that after this time a confluent monolayer of spread cells was set up with few rounded cells present. However, it can be seen in Figure 3.7 that after the same length of time on bacteriological grade plastic there are few cells attached. A similar response was observed on a pressed film of atactic polystyrene (Figures 3.8 and 3.9) but a confluent monolayer of well spread cells was observed on the calendered films of atactic polystyrene (Figure 3.10). Some polymers are able to trigger responses between these extremes as in the case of spun films of chlorinated rubber (Figures 3.12 and 3.13) and calendered poly(vinyl chloride) (Figure 3.14). On the former polymer, it can be seen that a number of cells have attached and the majority of these have spread. A similar number have attached to calendered poly(vinyl chloride) but approximately half of these have spread.

In addition, this study confirms that polymers such as poly(methyl methacrylate) have a considerable polar contribution to the surface energy whereas some workers had previously considered them to be 'hydrophobic' [101]. This illustrates that the terms 'hydrophilic' and 'hydrophobic' are commonly used in a fairly descriptive and arbitrary manner. This had not been taken into account by Klebe et al [101] when after they found that poly(ethyl methacrylate) and poly(methyl methacrylate) were cell adhesive, they hypothesised that polymers needed to be hydrophobic in order to support cell adhesion. Whilst polyEMA and polyMMA are less wettable than tissue-culture plastic, they do express a polar surface energy component and it is this that influences specific protein attachment and ultimately cell adhesion. Polystyrene is an extremely non-wettable material in which any polar contribution can only be derived from asymmetry of the aromatic ring about the polymer backbone. By comparison, a polymer such as polyEMA (Structure 3.12) is only moderately hydrophobic due to significant polar contributions

Table 3.6. Summary of the cellular response to a selection of polymers after 6 hours in culture.

Polymer	Form	Cell behaviour
Tissue culture plastic	Moulded	Confluent monolayer of well spread cells.
Atactic polystyrene	Moulded[#]	Very few cells attached and incomplete spreading. This surface is unable to support normal spread cell morphology
	Spun	A few cells attached but little spreading.
	Pressed	Very few cells attached; some attempt at spreading.
	Calendered	Confluent monolayer of well spread cells.
Isotactic polystyrene	Pressed	Little attachment, no spreading.
Atactic polypropylene	Pressed	Some cell attachment (number less than bacteriological plastic) little spreading.

[#] Bacteriological grade plastic

Table 3.6. (continued) Summary of the cellular response to a selection of polymers after 6 hours under culture conditions.

Polymer	Form	Cell behaviour
Atactic polypropylene	Spun	Some cell attachment (less than on bacteriological grade plastic), little spreading
Isotactic polypropylene	Pressed	Very few cells attached, no spreading.
	Calendered	Confluent monolayer of well spread cells. Comparable to tissue culture plastic
Poly (vinyl chloride)	Spun	Very few cells attached, little spreading
	Calendered	The number of cells attached was comparable to tissue culture plastic but not all cells have spread
Chlorinated rubber	Spun	Although not totally confluent there was extensive cell attachment and spreading

Table 3.6. (continued) Summary of the cellular response to a selection of polymers after 6 hours in culture.(continued)

Polymer	Form	Cell behaviour
Poly (p bromo styrene)	Spun	Few cells attached, little spreading
Poly (p tert butyl styrene)	Pressed	Very few cells attached, little spreading
Poly (α- methyl styrene)	Pressed	No cell attachment
Poly (4 - isopropyl styrene)	Pressed	No cell attachment.
Poly (methyl methacrylate)	Spun	The number of cells attached was comparable to tissue culture plastic, but not all the cells have spread.

from the ester linkage.

If a polymer surface is more polar then the interfacial tension with aqueous solutions is lower and this is reflected in the nature of the protein adsorption that occurs during contact with biological environments. Whilst less wettable surfaces can produce a marked response in adsorbing proteins, the deposition resulting from hydrophobic bonding is relatively poorly organised and not favourable for cell adhesion. In contrast, more polar surfaces tend to adsorb more specific proteins with suitable configurations which favour cell attachment and spreading.

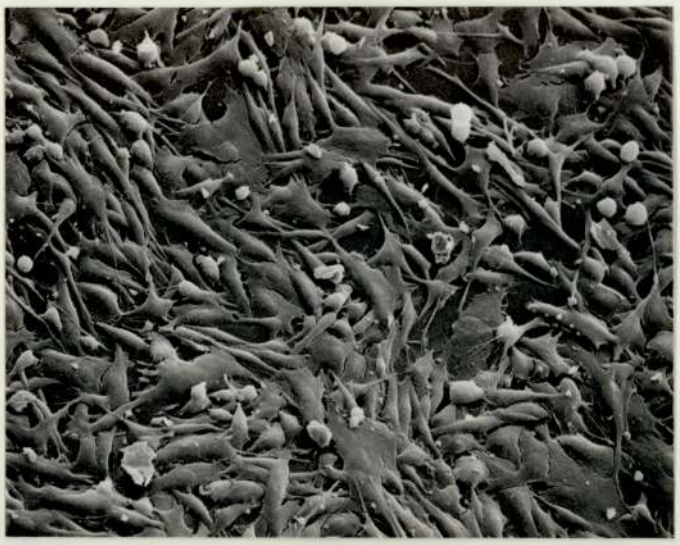


Figure 3.6. Cell response to tissue culture plastic after 6 hours in culture (x 200)

Figure 3.7. Cell response to bacteriological grade plastic after 6 hours in culture (x 200)

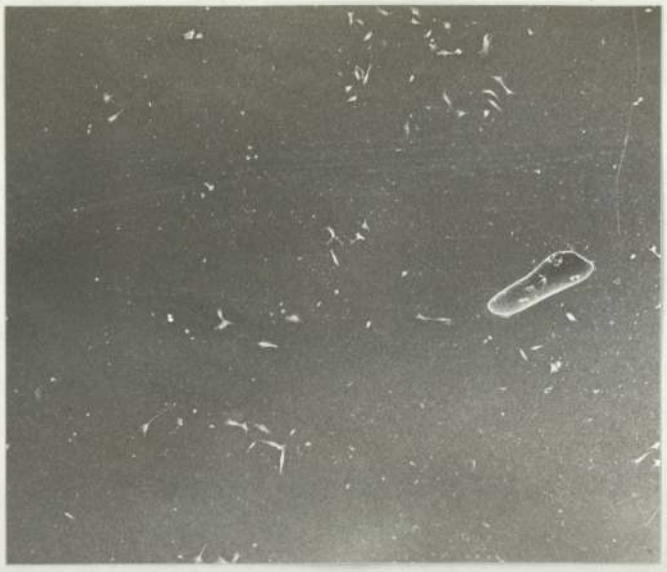


Figure 3.8. Cell response to pressed film of atactic polystyrene after 6 hours in culture (x 20)

Figure 3.9. Cell response to pressed film of atactic polystyrene after 6 hours in culture (x 200)

Figure 3.10. Cell response to calendered film of atactic polystyrene after 6 hours in culture (x 100)

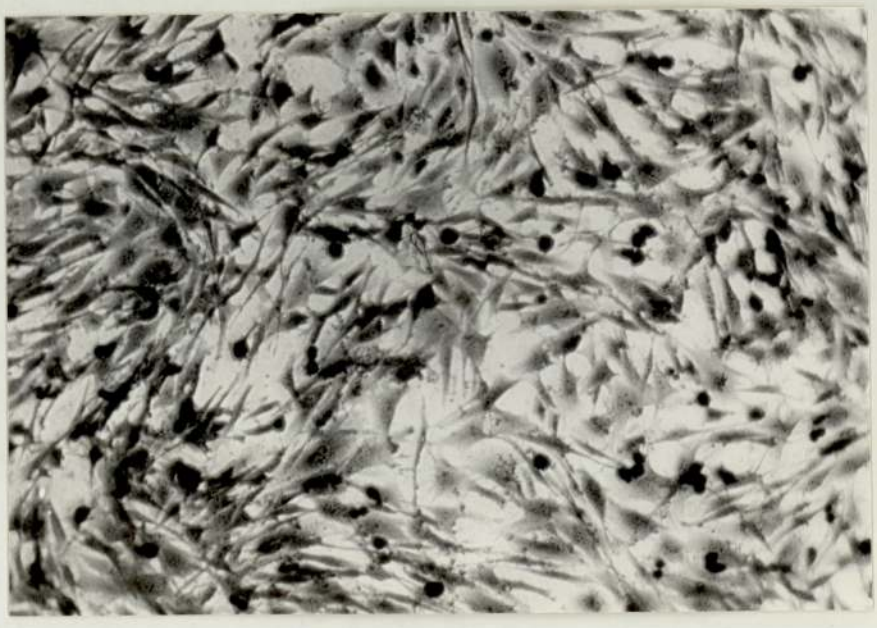


Figure 3.11. Cell response to calendered film of isotactic polypropylene after 6 hours in culture (x 100)

Figure 3.12. Cell response to spun chlorinated rubber after 6 hours in culture (x 200)

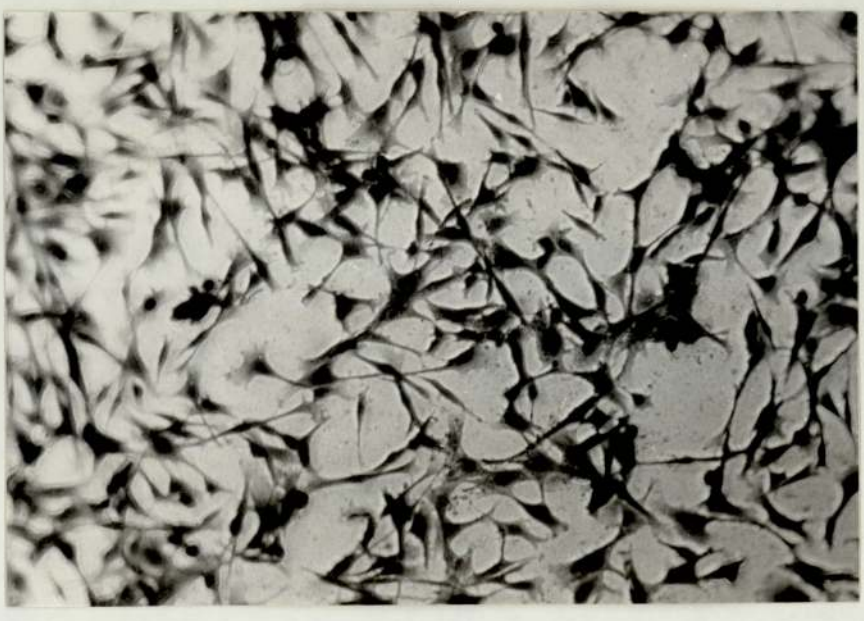


Figure 3.13. Cell response to spun chlorinated rubber after 6 hours in culture (x 100)

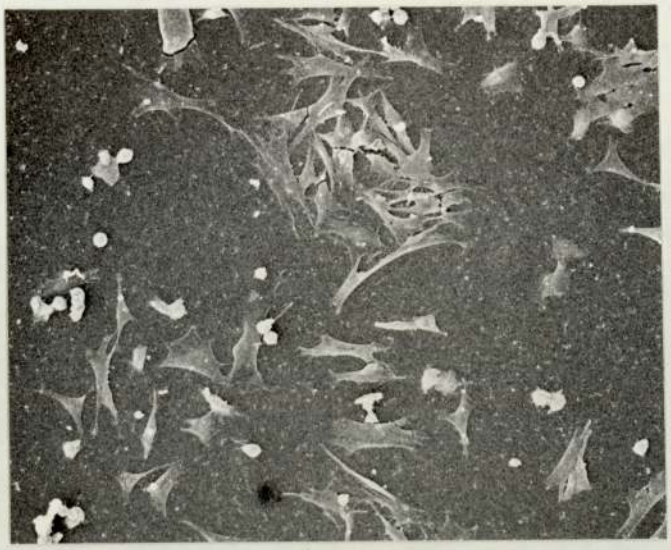
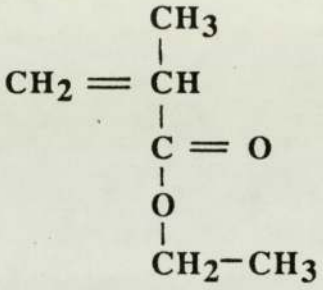


Figure 3.14 Cell response to calendered poly (vinyl chloride) after 6 hours in culture (x 100)

Ethyl methacrylate



Structure 3.12

CHAPTER 4

The surface modification of polystyrene for use in cell culture.

4.1 Introduction

The continuing development of biomaterials depends on an improved understanding of interactions at the interface. A useful fundamental model system should mimic, as closely as possible, the in vivo responses to synthetic materials and in this respect, anchorage dependent cell culture would seem to be a useful approach [92, 189].

Anchorage dependent cells require a suitable substratum for adhesion, spreading and growth [26] and several important biomaterials have already proven valuable in studies probing those properties of the substratum that influence cell behaviour [93, 98, 186]. Model systems in which the physico-chemical properties can be controlled are of potential value in the development of improved biomaterials [92]. Whilst there have been some investigations carried out on some of the physico-chemical properties considered to affect cell behaviour, the reports in the literature have often been confusing. For example, at the time of this study, untreated polystyrene in the form of a moulded article (for example bacteriological grade plastic) was widely recognised in its inability to support cell attachment and spreading of BHK fibroblast cells in culture. On the other hand it had been identified by Maroudas, Klemperer et al and Curtis et al [20 - 22] that the relatively simple treatment of the surface with sulphuric acid under ambient conditions resulted in the polystyrene being rendered suitable for use in cell culture. However, some controversy reigned amongst these groups of workers in that it was unclear as to which surface groupings were allowing the cell adhesion response to be mediated. For example, Maroudas [21] hypothesised that cell adhesion was being effected by sulphonate groups having been inserted into the surface, whilst Curtis et al [20] proposed that it had been via the introduction of hydroxyl groups. Klemperer at al [22] hypothesised that it was the

introduction of 'weak acid' groups (assumed to be hydroxyl) at the surface that were responsible for the observed cell behaviour and work by Gibson et al [190] had suggested that sulphonation of the aromatic ring of polystyrene following sulphuric acid treatment occurred only after long periods of time and under certain conditions.

In an attempt to explain this controversy it was decided to design a series of experiments whereby, in the first instance, the surface of polystyrene following mild treatment with sulphuric acid would be analysed using ESCA techniques. It was hoped that this analytical technique would ascertain whether treatment in this way facilitated the introduction of hydroxyl or sulphonate groups at the surface. In addition, it was also considered of interest to observe whether a different type of oxygen containing group, the sulphonate group, would produce a similar response on cell behaviour. Therefore, an array of acid treatments, described later in this chapter, were selected whereby sulphonate groups would be introduced at the surface via more rigorous treatment of the polystyrene and their effects on cell behaviour observed.

4.2.Discussion of results

4.2.1. Mild treatment of polystyrene by sulphuric acid at 20°C

It was decided to carry out a series of investigations in an attempt to ascertain which surface groupings were responsible for the effects observed on cell behaviour following treatment of polystyrene with sulphuric acid. Polystyrene is a valuable polymer to use for investigations of those properties of the substrate that affect cell behaviour. It is the fundamental material of both bacteriological and tissue-culture grade ware yet these materials exhibit quite different cell responses. Thus, using a heated cork borer, samples of polystyrene (diameter approximately. 1.5 cm) were removed from bacteriological grade petri dishes. These samples were then treated with sulphuric acid at room temperature for the times shown in Table 4.1 and then washed thoroughly in distilled water prior to their use in cell culture studies of BHK fibroblast cells. Control surfaces of untreated tissue culture plastic were also set up and some polystyrene samples were left untreated. After 6 hours in culture, all samples were washed in phosphate buffered saline and observed under the light microscope. The cells were then removed from the samples using trypsin -EDTA and counted using a haemocytometer following an examination of the surfaces to ensure that all the cells had been removed. The results of the cellular response to the treated polystyrene is shown in Figure 4.1 and the cell counts are shown in Table 4.1.

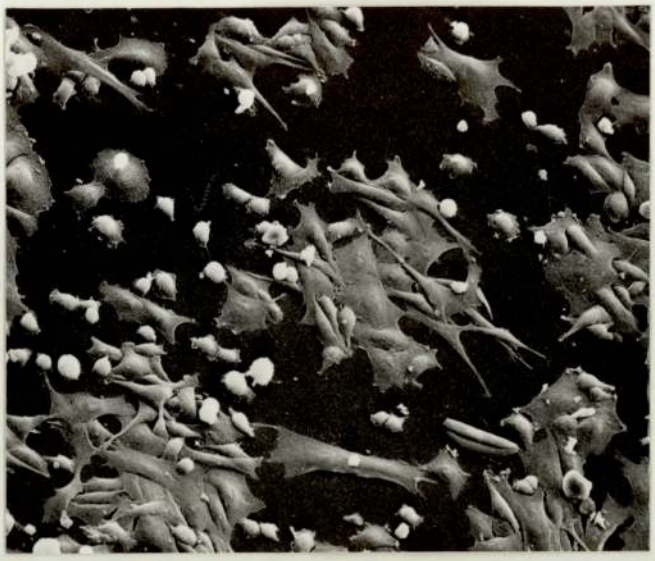


Figure 4.1. Six hour cell response to polystyrene following mild treatment with H_2SO_4 (20°C, 36 hours) (x 200)

Table 4.1. Cell counts on polystyrene following treatment by sulphuric acid at 20°C. (n=4 for each treatment)

Substrate	Time (minutes)	Cell count	% cell count comparedto tissue culture plastic.
Treated polystyrene			
H_2SO_4 (20°C)	10	2.3×10^4	38
Controls			
Tissue culture plastic	-	6.0×10^4	100
Bacteriological grade plastic	-	5.0×10^2	0.8

In addition to observations on the cell response, treated and untreated samples were investigated using ESCA techniques and from these results (Figures 4.2 -4.3), the carbon to oxygen, oxygen to sulphur and carbon to sulphur ratios were calculated (Table 4.2). The lower the C:O ratio, the greater the extent of oxidation that has occurred and similarly, the lower the C:S or O:S ratios, the greater the sulphonation. Unfortunately, it was not possible to detect -OH specifically since these groups become incorporated into the peak observed for oxygen in the ESCA spectrum. However, any evidence of sulphonation will be shown by the presence of a sulphur peak (Figure 4.3) and an increase in the amount of oxygen. It is important to note, however, that the percentage error that may have occurred during the quantitation of the results obtained using this technique, was unknown. Despite this, ESCA is a useful technique for estimating the presence of certain elements at the surface of a material. Thus, following ESCA analysis it was observed that the C:O for the untreated polystyrene was 46.6 but that following treatment with sulphuric acid for 36 hours this value decreased to 5.0. The value for the C:O for tissue culture plastic was 3.6.

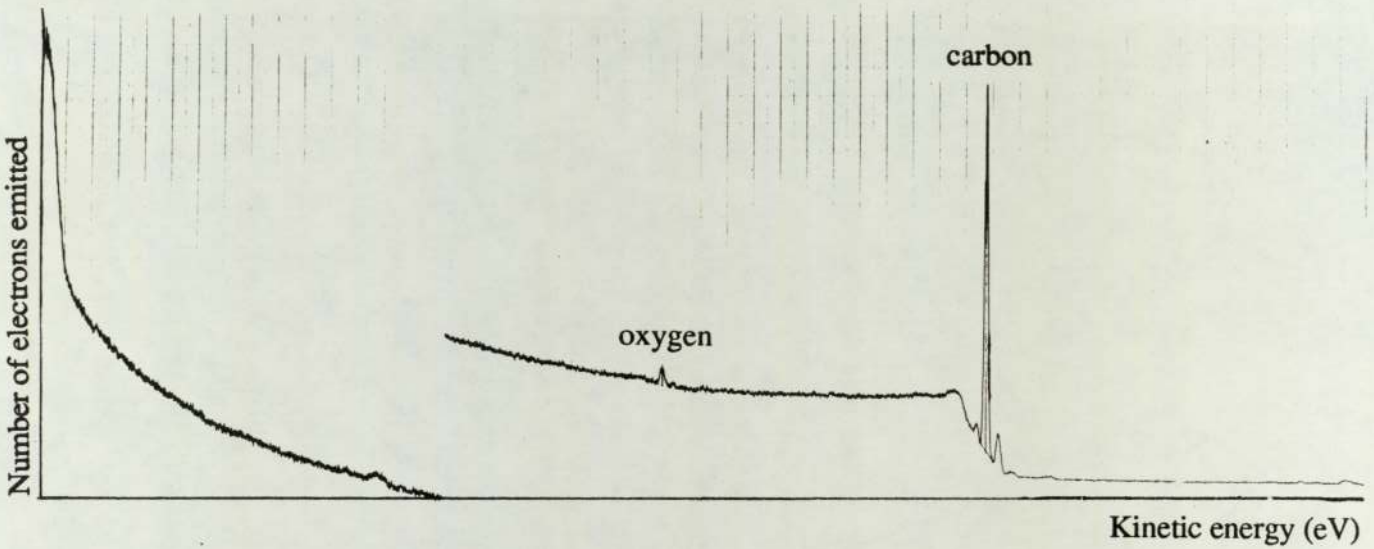


Figure 4.2.
ESCA analysis of bacteriological grade plastic

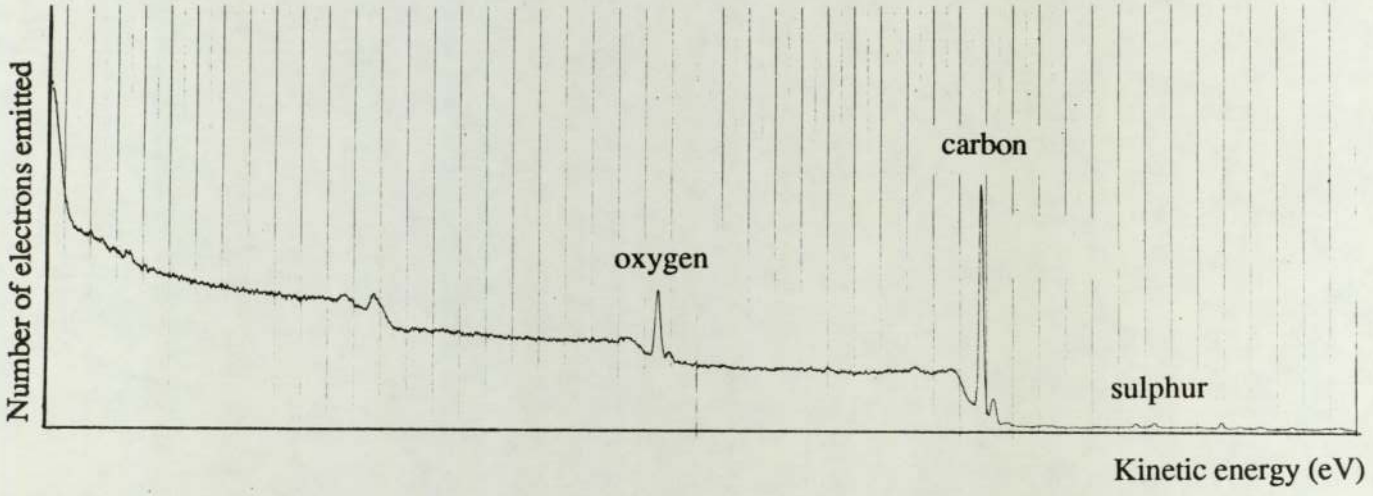


Figure 4.3.
ESCA analysis of polystyrene following mild treatment with H_2SO_4 (20°C, 10 minutes)

It is perhaps of interest to note that there had been little sulphonation of the surface, since the values for C:S are relatively high.

Table 4.2. Results for C:O, O:S, C:S ratios for polystyrene following mild treatment with sulphuric acid at 20° C.

Substrate	Treatment	C:O	O:S	C:S
Bacteriological grade plastic	H_2SO_4 (20°C, 36hours)	5.0	3.6	18.0
Controls				
Bacteriological grade plastic	Untreated	46.6	--	--
Tissue culture plastic	Untreated	3.6	--	--

The untreated and treated polystyrene samples and tissue culture plastic were also characterised by the contact angle techniques of Owens and Wendt [182] . This involved placing droplets of the wetting agents (water and methylene iodide) onto the surface of the samples and measuring the angle of contact θ as outlined in Chapter 2 and the results for these values are shown in Table 4.3.

Table 4.3. Results of contact angle measurements following mild treatment of polystyrene by H_2SO_4 at 20°C (n=4)

Substrate	Treatment	Water(°)	Methylene iodide(°)
Bacteriological grade plastic	H_2SO_4 (20°C, 36 hours)	57	15
Controls			
Bacteriological grade plastic	Untreated	75	18
Tissue culture plastic	Untreated	45	14

These results enabled the polar, dispersive and total components of the surface energy to be calculated as shown in Table 4.4.

Table 4.4. Results for total, dispersive and total surface energy components following mild treatment of polystyrene by H_2SO_4 at 20°C

		Surface energy (mN m^{-1})		
Substrate	Treatment	(γ_p)	(γ_d)	(γ_t)
Bacteriological grade plastic	H_2SO_4 (20°C, 36 hours)	13.4	42.3	55.6
Controls				
Bacteriological grade plastic	Untreated	1.3	51.5	52.8
Tissue culture plastic	Untreated	21.9	41.6	63.5

where γ_p, γ_d and γ_t are the values for polar, dispersive and total surface energy components respectively.

The least polar surface was the untreated polystyrene and as seen with the results for oxidation, following sulphuric acid treatment the surface became markedly more polar, although not as greatly as tissue culture plastic. Interestingly, these results were reflected in a range of cell responses after 6 hours in cell culture. For example, on the least oxidised and least polar surface, the untreated polystyrene, there was little cell attachment and very little spreading. As the surface of this polymer became more polar and there was an increase in the extent of surface oxidation (ie a decrease in C:O ratio) following sulphuric acid treatment, so the number of cells that became attached to the surface markedly increased which was accompanied by a high degree of cell spreading . This was not to the same extent as that seen on the tissue culture plastic, the most polar and most oxidised surface, where after 6 hours in culture there was a confluent monolayer of well spread cells established (see Figure 3.6 in Chapter 3).

Thus, it can be seen that treatment of polystyrene with sulphuric acid resulted in the production of a more oxidised surface that was able to support high levels of cell attachment and spreading. Due to the little evidence of sulphonation, it appears that this treatment resulted predominantly in the introduction of hydroxyl groups at the polystyrene surface. However, it must also be noted that the degree of cell spreading was not to the same extent as that observed on tissue-culture plastic. Therefore, it was considered of interest to observe the effect of increasing the amount of sulphonate groups at the surface.

4.2.2 Further attempts to sulphonate polystyrene

The introduction of sulphonate groups at a polymer surface is another method of oxidising polystyrene surfaces. Investigations into the effect of these groups on cell behaviour would help cell biologists understand whether any oxidation can produce the cell adhesion response or whether the cells have a preference for certain groups. Indeed, the importance of oxidation has already been highlighted in Chapter 3 where it may be recalled that those polymer materials that had become oxidised during manufacture ie by calendered processes, supported far greater extents of cell attachment and spreading than those articles that had been melt pressed or spun coated onto glass coverslips. However, no attempts had been made to identify the types of groups that had been introduced as a result of the oxidation process. Therefore, a further selection of acid treatments were chosen which all had the ability to introduce sulphonate groups at the surface of polystyrene. It was known that heating sulphuric acid to approximately 60°C would increase it's sulphonating ability. In addition, both chlorosulphonic acid and oleum (fuming sulphuric acid) were chosen since they are both known sulphonating reagents. Therefore, the treatment of polystyrene with 'hot' sulphuric acid (60°C), oleum and chlorosulphonic acid would allow the effects of surface sulphonate groupings on cell behaviour to be observed.

As before, samples were cut out of bacteriological grade polystyrene petri dishes which were then treated for 10 minutes with either 'hot' (60°C) sulphuric acid or chlorosulphonic acid or for 5 minutes with oleum and washed thoroughly in distilled water before their use in cell culture studies. Unfortunately, the chlorosulphonic acid treatment proved to be too harsh and resulted in a 'buckling' of the whole sample (Figure 4.4). Therefore, as a compromise, samples were exposed to chlorosulphonic acid vapour, which did not distort

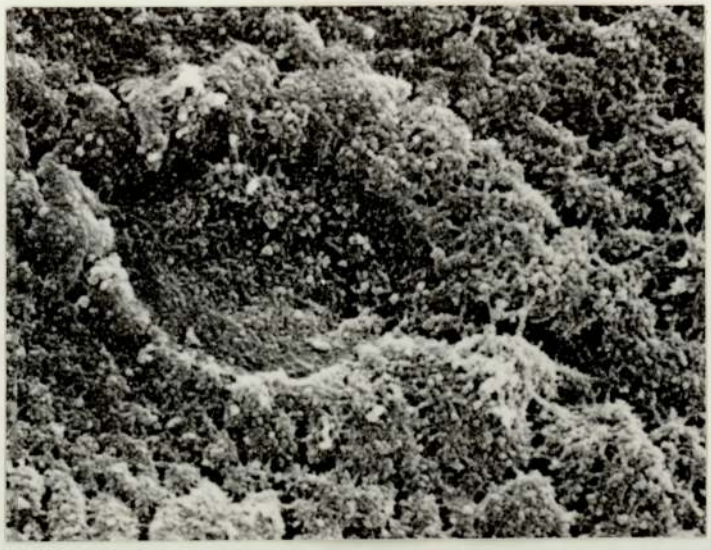


Figure 4.4. To show the polystyrene surface following treatment with undiluted chlorosulphonic acid for 10 minutes (x 10,000)

the sample, for the time shown. Exposure of the polystyrene samples to oleum for lengths of time exceeding 5 minutes also appeared to be too rigorous. As in the previous experiment, untreated samples of bacteriological and tissue-culture grade polystyrene were used as control substrates since the cell response to each of these is well characterised. After 6 hours in cell culture the surfaces were rinsed in phosphate buffered saline and following examination by light microscopy, the cells removed by trypsin-EDTA and counted using a haemocytometer. The surfaces were again checked to ensure that all the cells had been removed and the results of the cell counts are shown in the following table:

Table 4.5. Cell counts on polystyrene following attempts to sulphonate the surface by acid treatment. (n=4 for each treatment)

Substrate	Time (minutes)	Cell count	% cell count compared to tissue culture plastic.
Treated bacteriological grade plastic.			
H_2SO_4 (60°C)	10	3.9×10^4	65
Chlorosulphonic acid vapour	10	4.3×10^4	72
Oleum	5	3.6×10^3	6
Controls			
Bacteriological grade plastic		5.0×10^2	0.8
Tissue culture plastic		6.0×10^4	100

The cellular response is also illustrated in figures 4.5 to 4.9.

Figure 4.5. Six hour cell response to polystyrene following harsher treatment with H_2SO_4 (60^oC, 10 minutes) (x 200)

Figure 4.6. Six hour cell response to polystyrene following harsher treatment with H_2SO_4 (60^oC, 10 minutes) (x 330)

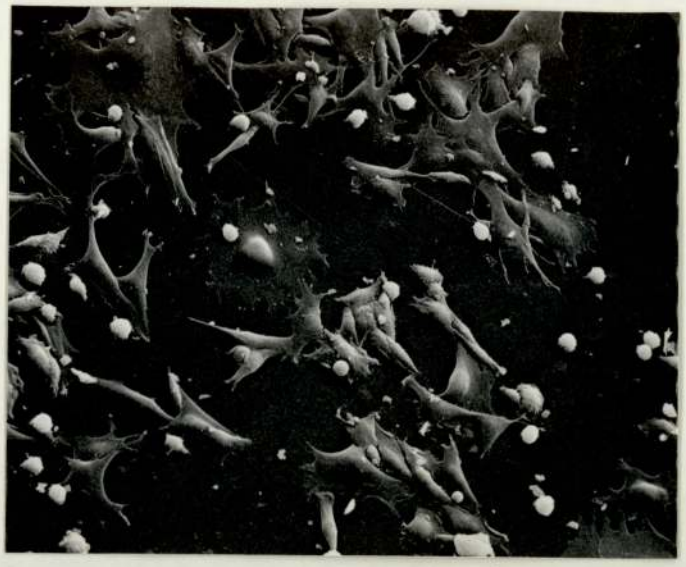


Figure 4.7. Six hour cell response to polystyrene following treatment with chlorosulphonic acid vapour for 10 minutes (x 200)

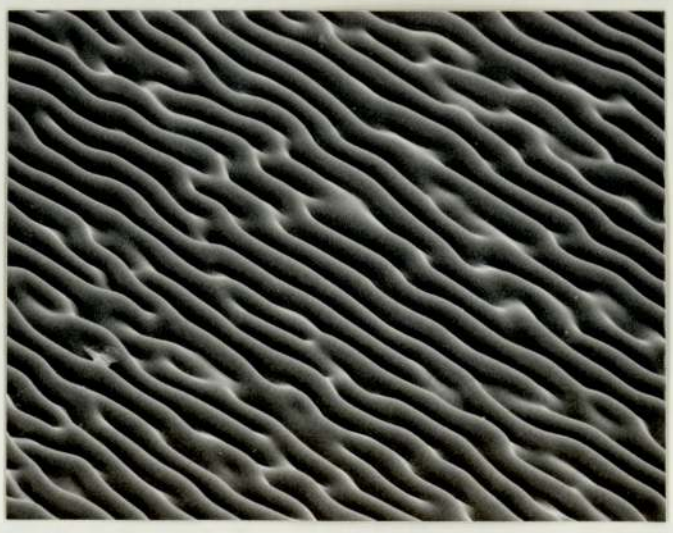


Figure 4.8. To show the surface of polystyrene following treatment with oleum (x 200)

Figure 4.9. Six hour cell response to polystyrene following treatment with oleum for 5 minutes (x 200)

In addition to the cell culture studies a variety of analytical techniques were carried out and taken together in order to characterise a number of surface physico-chemical properties of the treated and untreated samples. Thus, as before, surface elemental analysis was carried out using ESCA techniques. The sensitivity of the spectrometer to each element (Appendix 4) was taken into account and this allowed the percentages for carbon, oxygen and sulphur to be calculated from the spectra obtained (Figures 4.10 - 4.12). The values for the carbon:oxygen were determined as before, but also this technique had the added advantage of detecting whether there had been any sulphonation of the surface, expressed by the presence of a sulphur peak. Thus, in a similar way as the C:O ratios were calculated, so values for the carbon:sulphur and the oxygen:sulphur ratios were determined. These values are expressed in Table 4.6. Again it was not possible to analyse specific groupings such as hydroxyl groups from the ESCA traces since -OH groups become incorporated into the oxygen peak. The smaller the value for the carbon : oxygen ratio the greater the extent of oxidation of the surface. Similarly the smaller the O:S or C:S ratios, the greater the degree of sulphonation that has taken place.

Table 4.6. Results for C:O, O:S, C:S ratios for polystyrene following attempts to sulphonate the surface.

Substrate	Treatment	C:O	O:S	C:S
Bacteriological grade plastic	H_2SO_4 (60°C, 10minutes)	2.8	2.1	5.9
	Chlorosulphonic acid vapour (10 minutes)	6.0	2.6	15.6
	Oleum (5minutes)	3.3	1.9	6.3
Controls				
Bacteriological grade plastic	Untreated	46.6	--	--
Tissue culture	Untreated	3.6	--	--

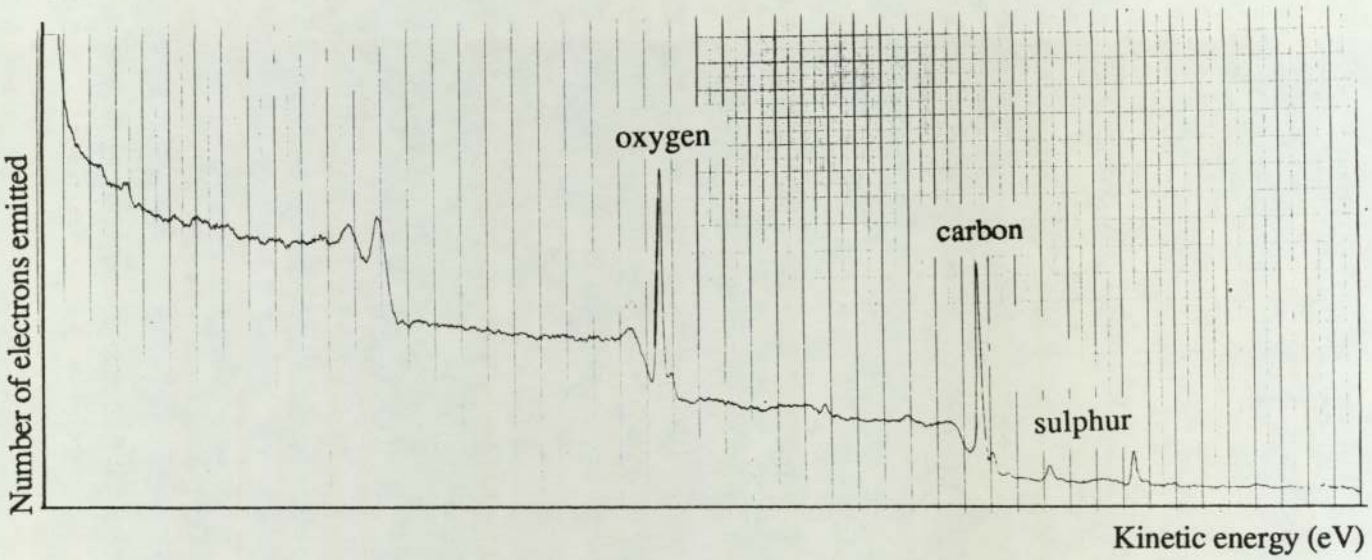


Figure 4.10.
ESCA analysis of polystyrene following harsher treatment with H_2SO_4 (60°C, 10 minutes)

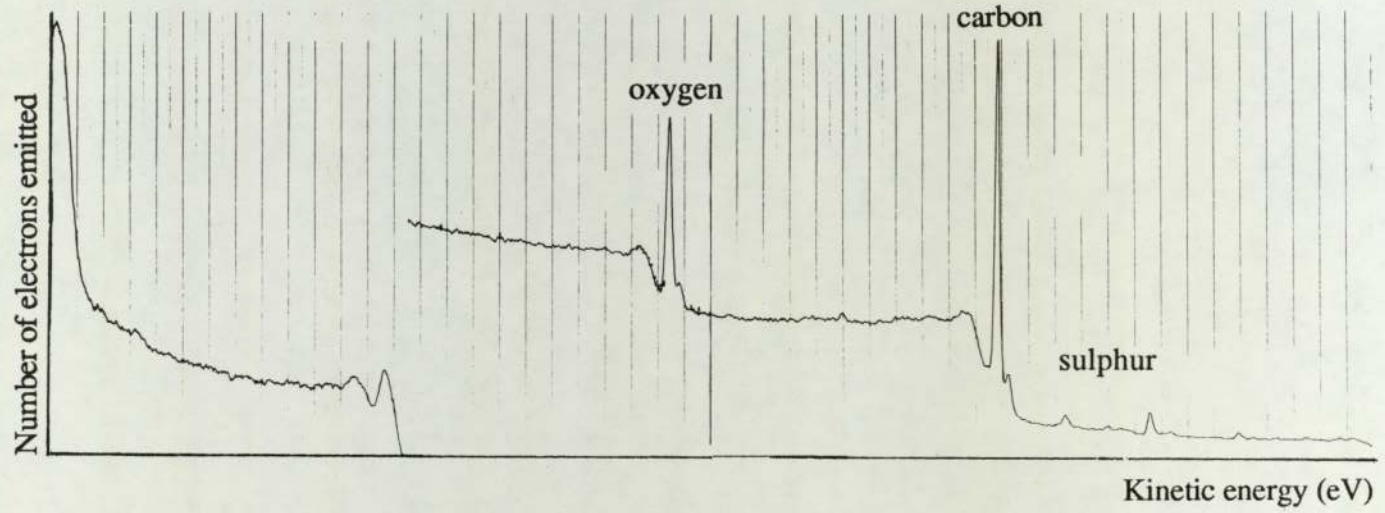


Figure 4.11.
ESCA analysis of polystyrene following treatment with chlorosulphonic acid vapour for 10 minutes

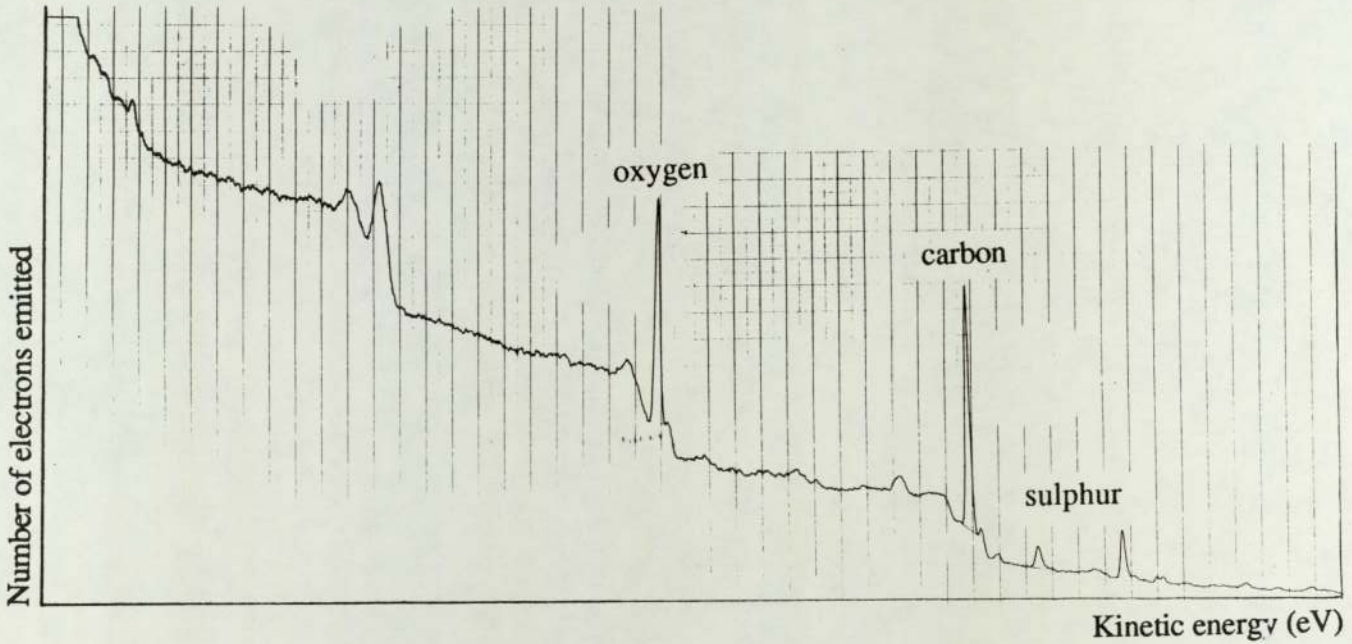


Figure 4.12.
ESCA analysis of polystyrene following treatment with oleum for 5 minutes

Using the sessile drop contact angle technique of Owens and Wendt as previously described enabled the polar and dispersive components of the surface energy to be determined. The mean values for both water and methylene iodide contact angles are given in Table 4.7 (n=4) and the polar, dispersive and total components of the surface energy are given in Table 4.8.

Table 4.7 Results of contact angle measurements for polystyrene following attempts to sulphonate the surface (n=4)

Substrate	**Treatment**	**Water(°)**	**Methylene iodide(°)**
Bacteriological grade plastic	H_2SO_4 (60°C, 10 minutes)	44	36
	Chlorosulphonic acid vapour (10 minutes)	27	46
	Oleum	Readings not possible	
Controls			
Bacteriological grade plastic	Untreated	75	182
Tissue culture plastic	Untreated	45	14

Table 4.8. Results for polar, total and total energy components of polystyrene following attempts to sulphonate the surface

Substrate	Treatment	Surface energy (mNm^{-1}) (γ_p)	(γ_d)	(γ_t)
Bacteriological grade plastic	H_2SO_4 (60°C, 10 minutes)	24.7	33.0	57.7
	Chlorosulphonic acid vapour (10 minutes)	39.6	26.2	65.8
Controls				
Bacteriological grade plastic	Untreated	1.3	51.5	52.8
Tissue culture plastic	Untreated	21.9	41.6	63.5

where, γ_p, γ_d and γ_t are the values for the polar, dispersive and total components of the surface energy.

Thus these values give an indication of the polarity of the surfaces and increasing values of γ_p signify an increase in surface wettability.

Heating sulphuric acid to 60°C prior to treatment rendered it a more powerful sulphonating and oxidising reagent, as shown by the lower values for C:O, O:S and C:S ratios. From these results it appeared that the treatment by hot sulphuric acid produced the greatest surface oxidation of the polystyrene. In addition this surface had undergone markedly more

sulphonation than had occurred following treatment with H_2SO_4 at room temperature. The polarity of this treated surface was also increased, although not as extensively as that which followed treatment with sulphuric acid at 20°C. Whilst the number of cells adhering to this surface (Table 4.5.) was greater than to the polystyrene that had been treated with sulphuric acid under mild conditions, the extent of spreading was, in general, markedly reduced to approximately 5% of that seen on tissue culture plastic.

Chlorosulphonic acid vapour did not appear to be as strong a sulphonating reagent as would have been expected for the acid itself, although there was the presence of some sulphur but not to the same extent as after treatment with hot H_2SO_4. There had been an oxidation of the surface and this treatment resulted in a surface with high polar component being produced. Whilst there were a high number of cells attached spreading was markedly less than that observed on tissue culture plastic.

The treatment of bacteriological polystyrene by oleum resulted in an increase in rugosity, even at low magnifications (Figure 4.8). From Table 4.6 it can be seen that oleum treatment resulted in the greatest extent of sulphonation and this surface was also markedly oxidised However, whilst there was some cell attachment to this treated surface, cells were reluctant to spread, as shown in Figure 4.9. The macroscopic roughness introduced into this surface prevented contact angle measurements from being successfully carried out since both the wetting liquids became 'absorbed' into the sample, possibly by capillary action.

The conclusion that can be drawn from the series of experiments presented so far is that those surfaces that had become more oxidised by the introduction of predominantly

sulphonate groups (eg. following oleum treatment) supported cell attachment, but the cells were unable to spread. In contrast, the surface that had been oxidised via the introduction of mainly hydroxyl groups (ie. following sulphuric acid treatments) supported high levels of cell attachment and high numbers of well spread cells. In between these two extremes lay those surfaces that had been treated by 'hot' sulphuric acid or chlorosulphonic acid vapour. Following treatment by oleum, H_2SO_4 at 60°C or chlorosulphonic acid vapour, it remained unclear as to the extent of concurrent hydroxylation that may have occurred in addition to the sulphonation which may have effected the cell response.

4.2.3. Glow discharge treatment of polystyrene

A further technique that renders polystyrene suitable for use in anchorage dependent cell culture studies is glow discharge treatment. In general terms this technique involves the exposure of polystyrene samples to a gas plasma. A gas plasma may be simply described as a low temperature (ie. room temperature) ionised gas and it's ability to render polystyrene cell adhesive is often attributed to the fact that it increases the substrate wettability [18, 19, 24, 25].

The use of glow discharge treatment was considered particularly useful in this study because the treatment of polystyrene by an oxygen plasma would facilitate the introduction of hydroxyl groups into the surface. Thus, this treatment would allow the effects of a purely hydroxylised surface on cell behaviour to be examined. However, Minett had reported [85] that this type of treatment also roughens the surface which in itself may affect cell behaviour. It was decided that in order to investigate the effects of increased rugosity on cell behaviour, polystyrene would be exposed to a nitrogen or argon plasmas which would be expected to roughen the surface but not oxidise it.

Therefore, again samples were cut out of bacteriological grade plastic and exposed to either oxygen, nitrogen or argon gas plasmas for the times shown in Table 4.9. These surfaces were then used in 6 hour cell studies using BHK fibroblast cells after which time the cell response was examined by light microscopy. The cells were removed from the samples and counted using the haemocytometer (Table 4.9) and the precaution of checking the surfaces was carried out as before. It was observed that all of these surfaces had been rendered as adhesive as tissue culture plastic in that they all supported a confluent monolayer of well spread cells.

Table 4.9. Cell counts on polystyrene following glow discharge treatment (n=4 for each treatment)

Substrate	Time (minutes)	Cell count	% cell count compared to tissue culture plastic.
Treated polystyrene			
Oxygen plasma	5	7.1×10^4	117
	60	8.0×10^4	133
Nitrogen plasma	5	3.1×10^4	52
	60	4.8×10^4	80
Argon plasma	5	3.1×10^4	52
	60	6.1×10^4	100
Controls			
Bacteriological grade plastic	-	5.0×10^2	0.8
Tissue culture plastic	-	6.0×10^4	100

As before, the surfaces were analysed using ESCA and contact angle techniques, from which the carbon : oxygen ratios and surface energy respectively were calculated (Tables 4.10 - 4.12 and Figure 4.13).

Table 4.10. Results of carbon : oxygen ratios for polystyrene following treatment with oxygen, nitrogen and argon gas plasma.

Substrate	**Treatment**	**C:O**
Bacteriological grade plastic	Oxygen plasma (5 minutes)	2.0
	Nitrogen plasma (5 minutes)	3.7
	Argon plasma (5 minutes)	4.9
Controls		
Bacteriological grade plastic	Untreated	46.6
Tissue culture plastic	Untreated	3.6

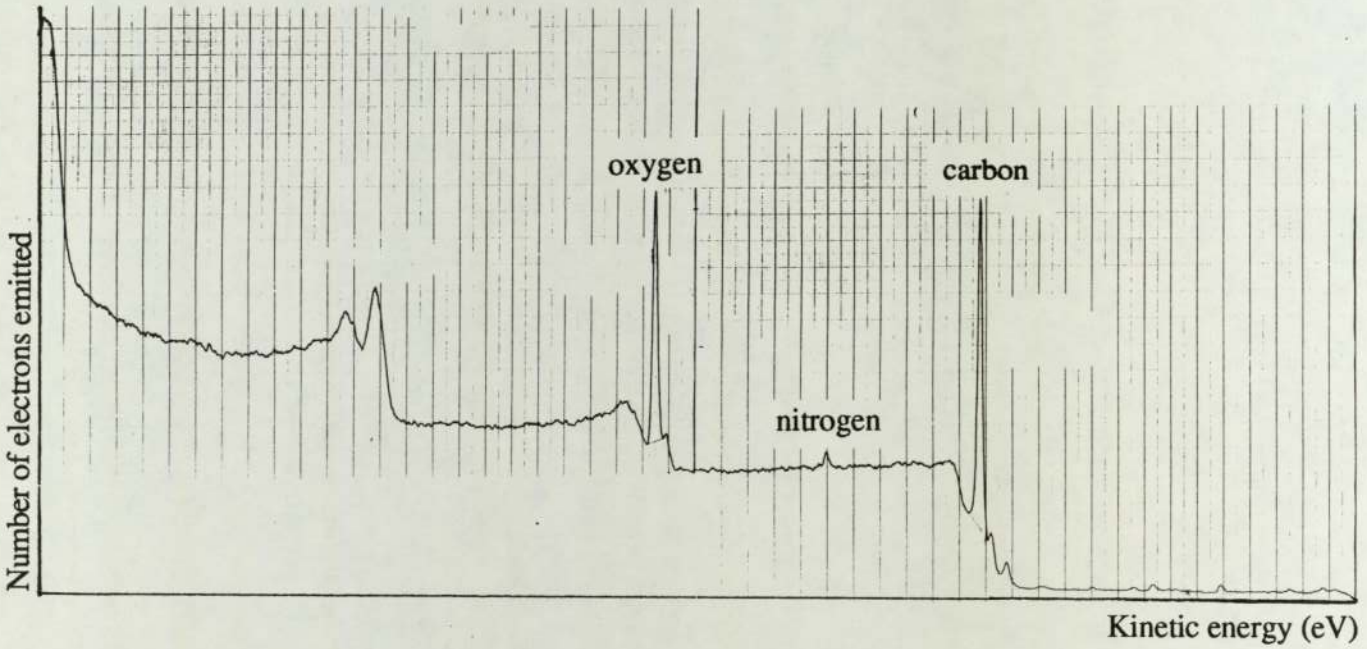


Figure 4.13.
ESCA analysis of polystyrene following exposure to a nitrogen gas plasma for 5 minutes

Table 4.11 Results of contact angle measurements for polystyrene following treatment with oxygen and nitrogen gas plasmas. (n=4)

Substrate	Treatment	Water(°)	Methylene iodide(°)
Bacteriological grade plastic	Oxygen plasma	46	29
	Nitrogen plasma	49	40
Controls			
Bacteriological grade plastic	Untreated	75	18
Tissue culture plastic	Untreated	45	14

Table 4.12.Results for polar, total and total energy components of polystyrene following treatment with oxygen and nitrogen gas plasmas

		Surface energy ($mN\ m^{-1}$)		
Substrate	**Treatment**	(γ_p)	(γ_d)	(γ_t)
Bacteriological grade plastic	Oxygen plasma (5 minutes)	22.2	36.0	58.2
	Nitrogen plasma (5 minutes)	23.0	31.5	54.4
Controls				
Bacteriological grade plastic	Untreated	1.3	51.5	52.8
Tissue culture plastic	Untreated	21.9	41.6	63.5

where γ_p, γ_d and γ_t are the values for the polar, dispersive and total surface energy components respectively.

Table 4.10 shows that the greatest extent of oxidation had occurred after glow discharge treatment with an oxygen (reactive) plasma for 5 minutes. However, those samples that had been treated with nitrogen or argon gases were also oxidised (Table 4.10). It had been hoped to investigate separately the effects of rugosity and oxidation using the inert gases since they would not normally be expected to oxidise the surface. It is not known exactly why this occurred, there are several possibilities that may have led to this phenomenon. First, even though samples were maintained in an inert gas atmosphere for some time (up to 36 hours) after treatment, some reactive species may have remained at the surface which may then have reacted with environmental oxygen. Another alternative may be that the levels of oxygen present in the nitrogen and argon used might have been higher than that stated by the manufacturers. A further possibility is that whilst the surface of bacteriological grade plastic is little oxidised, there may be some dissolved oxygen in the polymer as a result of manufacturing processes. In addition, it is possible that there may have been a leak of environmental oxygen into the reactor. Thus, after etching with argon or nitrogen gas plasmas, this oxygen may become exposed at the surface which may have facilitated the cell adhesive response. Despite this, the greatest extent of oxidation occurred after treatment with an oxygen plasma and this was reflected in the highest degree of cell attachment and spreading. This is an indication that there was the specific introduction of oxygen into the structure of polystyrene following treatment with this reactive gas plasma. Figures 4.14 and 4.15 show that prior to any treatment the surfaces of both tissue culture and bacteriological grades plastic were smooth.

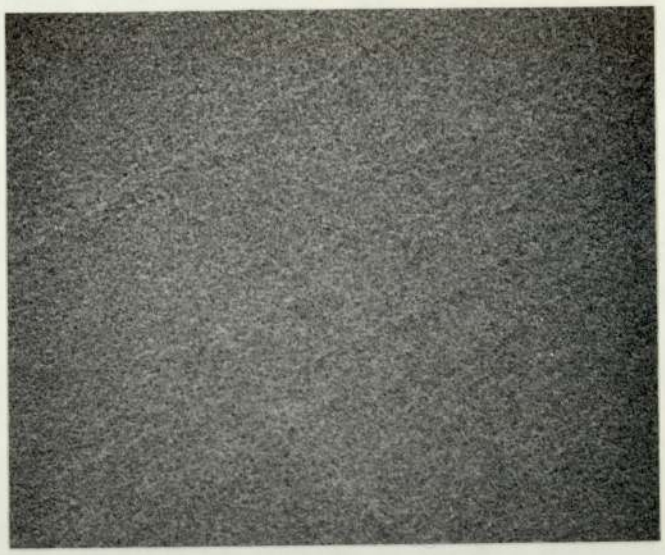


Figure 4.14. To show the surface of untreated tissue culture plastic (x 20,000)

Figure 4.15. To show the surface of untreated bacteriological grade plastic (x 20,000)

Figure 4.16. To show the surface of bacteriological grade plastic following exposure to an oxygen plasma for 5 minutes (x 20,000)

Figure 4.16 shows that whilst the surface of bacteriological polystyrene was slightly 'bumpier' following the oxygen plasma treatment than the untreated surface this may have represented the type of etching produced by this treatment. However, 'rugosity' is a fairly arbitrary term and it is a property that is difficult to quantify. Consequently, there is little known about the degree of roughness which must exist before the cell adhesion response is triggered.

It therefore appeared that it was not the oxidation per se that mediated cell adhesion and spreading, but more specifically this response was being effected by the introduction of an hydroxyl groups, as in the case of the oxygen plasma treated surfaces. However, whilst the oxidation of the polystyrene surface by the introduction of sulphonate groups allowed cell attachment to take place, these groups appeared to be less favourable in facilitating the spreading response. The greater the extent of sulphonation, the lesser the degree of cell spreading. In a further attempt to investigate the effects of sulphonate groups on cell behaviour, tissue culture plastic which is normally associated with being able to support confluent cell attachment and spreading was subjected to acid treatments with 'hot' and 'cold' sulphuric acids and oleum.

4.2.4. Acid treatment of tissue culture plastic

In order to further investigate the effects of hydroxyl and sulphonate groups on cell behaviour, it was decided to expose tissue culture plastic to both mild and acid conditions. Thus, using a cork borer, samples of tissue culture plastic were cut out and were treated treated with sulphuric acid (at both 20°C, 36 hours and 60°C, 10 minutes) and oleum . As before, the samples were also surface analysed by ESCA and contact angle techniques. The results for carbon : oxygen ratios, contact angle readings and surface energy are shown in

Tables 4.13 - 4.15 respectively.

Table 4.13. Results of C:O, C:S, O:S ratios for tissue culture plastic following a selected range of acid treatments.

Substrate	Treatment	C:O	C:S	O:S
Tissue culture plastic	H_2SO_4 (20°C, 36hrs.)	3.9	13.3	3.4
	H_2SO_4 (60°C, 10mins)	3.3	7.6	2.3
	Oleum (5 minutes)	2.9	5.2	1.8
Controls				
Bacteriological grade plastic	Untreated	46.6	--	--
Tissue culture plastic	Untreated	3.6	--	--

Table 4.14. Results of contact angle measurements for tissue-culture plastic following a selected range of acid treatments (n=4)

Substrate	Treatment	Water(°)	Methylene iodide(°)
Tissue culture plastic	H_2SO_4(20°C, 36 hrs)	45	18
	H_2SO_4 (60°C, 10 mins)	50	28
Controls			
Bacteriological grade plastic	Untreated	75	18
Tissue culture plastic	Untreated	45	14

Table 4.15. Results for polar, dispersive and total surface energy for tissue culture plastic following a selected range of acid treatments

		Surface energy (mNm^{-1})		
Substrate	**Treatment of**	(γ_p)	(γ_d)	(γ_t)
Tissue culture plastic	H_2SO_4 (20°C, 36hrs)	20.6	39.9	60.6
	H_2SO_4 (60°C, 10mins)	19.2	36.9	56.9
	Oleum (5 minutes)		Readings not possible	
Controls				
Bacteriological grade plastic	Untreated	1.3	51.5	52.8
Tissue culture plastic	Untreated	21.9	41.6	63.5

Figure 4.17 shows that while the cell layer was less confluent than that usually associated with untreated tissue culture plastic, most of the attached cells have spread. Thus treating tissue culture plastic with cold H_2SO_4 for 36 hours does decrease the number of cells attaching, but does not inhibit cell spreading. Table 4.4 shows that this surface was slightly oxidised after this time and there was some sulphur introduced at the surface. This suggests that there was some sulphonation, which may explain the decrease in cell numbers adhering to the surface.

Increasing the degree of sulphonation by heating the sulphuric acid to 60°C decreased both the number of cells attaching and their ability to undergo full spread morphology (Figure 4.18). This correlated with similar results for sulphonated polystyrene, as previously discussed. Harsher treatment to sulphonate the surface of tissue culture plastic by oleum

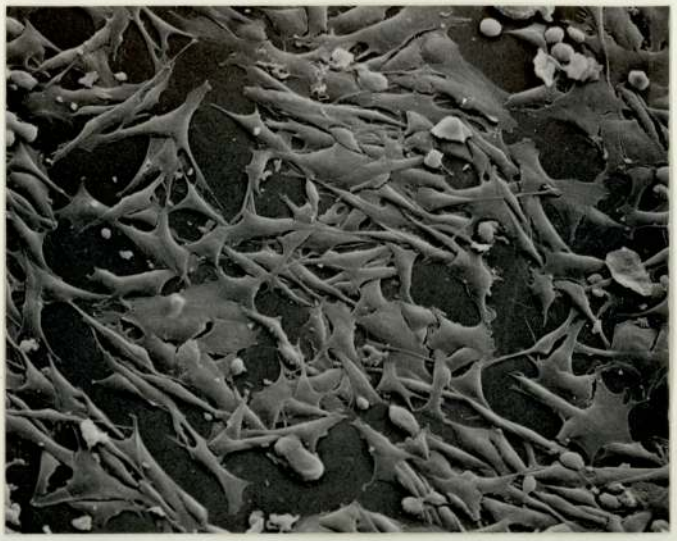


Figure 4.17. Six hour cell response to tissue culture plastic following mild treatment with H_2SO_4 (20°C, 36 hrs) (x 200)

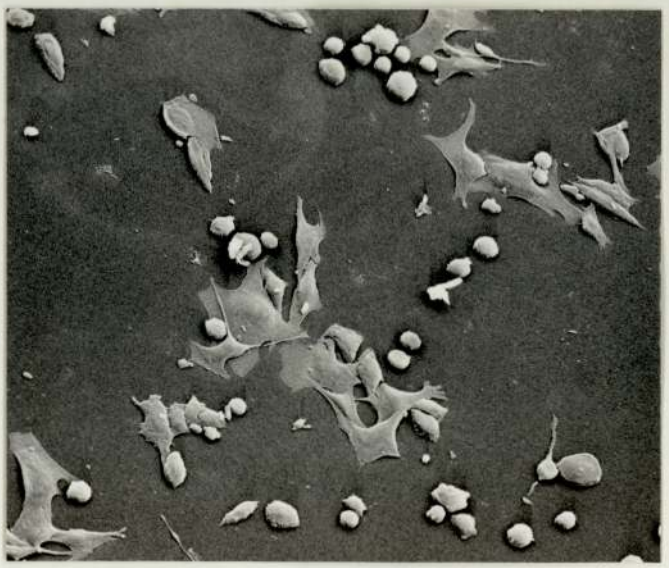


Figure 4.18. Six hour cell response to tissue culture plastic following harsher treatment with H_2SO_4 (60°C, 10 minutes) (x 200)

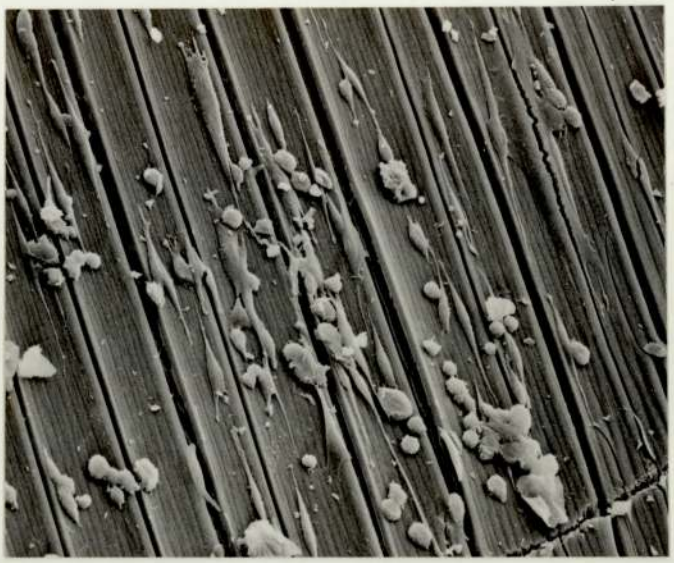


Figure 4.19. Six hour cell response to tissue culture plastic following treatment for 5 minutes with oleum (x 200)

(again reflected in the O:S and C:S ratios in Table 4.13) markedly inhibited the ability of the cells to undergo spreading (Figure 4.18).

4.3 Concluding discussion

One of the essential aims of this study was to improve the understanding as to which surface chemical groupings might be important in mediating not only cell attachment but also spreading. It may be recalled from Chapter 1 that cells only undergo full spread morphology on suitable substrates which is a useful indicator of normal cell behaviour. Cells will only spread on those surfaces that allow the adsorption of specific proteins in appropiate configurations. The cell response following each of the treatments is summarised in Table 4.16.

This study has shown that the exposure of polystyrene to 'cold' sulphuric acid, even for periods of up to 36 hours, produced minimal sulphonation. This was shown by the higher O:S and C:S values, although the C:O ratio suggests that there was oxidation of the surface. Thus, it appears that the increase in cell adhesion and spreading seen on this surface was due predominently to the introduction of hydroxyl groups at the surface which resolved the earlier mentioned controversy. It had also been suggested [85] that hydroxyl groups were introduced into the aromatic ring of polystyrene, although it seems more likely that polystyrene that has been treated with 'cold' sulphuric acid would experience hydroxylation of the backbone. It was observed that while cells attached and spread fully within six hours on this surface, cells cultured on other substrates displayed a range of responses. As shown in Figures 4.1, treatment of polystyrene with sulphuric acid at ambient temperatures rendered the surface cell adhesive and while quite a few cells had attached, approximately 75% of these had spread. This is a useful point because this is the treatment sometimes employed by cell biologists to economise on tissue-culture vessels [22]. However, rarely does this treatment render polystyrene as cell adhesive as tissue-culture plastic since there are differences in the abilities of these substrates to support cell spreading

Table 4.16 Summary of the cell response to treated and untreated bacteriological and tissue culture plastic after 6 hours in culture.

Polymer	Treatment	Cell behaviour
Bacteriological grade plastic	Untreated (control)	Very few cells attached and incomplete spreading. This surface is unable to support normal spread cell morphology
	H_2SO_4 (20^{O}C, 10 minutes)	Fewer cells attached than to the untreated tissue culture plastic and of these approximately 3/4 of them have undergone full spread morphology
	H_2SO_4 (60^{o}C, 10 minutes)	More cells attached than to the surface treated with cold sulphuric acid, but the extent of cell spreading was low.
	Chlorosulphonic acid vapour (10 minutes)	High cell attachment but extent of cell spreading was low.
	Oleum (5 minutes)	Few cells attached; no spreading

Table 4.16 (continued) Summary of the cell response to treated and untreated bacteriological and tissue culture plastic after 6 hours in culture.

Polymer	Treatment	Cell behaviour
Bacteriological grade plastic	Oxygen plasma (5 minutes)	Confluent monolayer of fully spread cells
	" (1 hour)	Confluent monolayer of fully spread cells
	Nitrogen plasma (5 minutes)	A number of cells attached all with full spread morphology
	" (1 hour)	Confluent monolayer of fully spread cells
	Argon plasma (5 minutes)	A number of cells attached all with full spread morphology
	" (1 hour)	Confluent monolayer of fully spread cells

Table 4.16 (continued) Summary of the cell response to treated and untreated bacteriological and tissue culture plastic following 6 hours in culture

Polymer	Treatment	Cell behaviour
Tissue culture plastic	Untreated (control)	Confluent monolayer of full spread cells
	H_2SO_4 (20°C, 10 minutes)	Less cells attached, but those that have adhered are all well spread
	H_2SO_4 (60°, 10 minutes)	Less cells attached than on control surface and fewer have number have undergone spreading
	Chlorosulphonic acid vapour (10 mins.)	Again, fewer cells attached and spreading markedly reduced
	Oleum (5 mins)	The number of cells attached is markedly reduced and of these, few have attempted spreading

Heating H_2SO_4 renders it a more powerful sulphonating reagent, as shown in Tables 4.6 and 4.13. The greater the extent of sulphonation the lower the O:S ratio; similarly, the lower the C:O value, the greater the extent of oxidation.Treating polystyrene with hot sulphuric acid caused a greater extent of sulphonation probably due to the introduction of $-HSO_3$ groups into the aromatic ring although it is possible that there was some hydroxylation of the polystyrene backbone. This was reflected in a surface that had the ability to support cell attachment, but not spreading.

After treating both tissue culture and bacteriological grades plastic with chlorosulphonic acid vapour there was cell attachment but again cell spreading was reduced. Chlorosulphonic acid would have predominantly sulphonated the aromatic ring at the expense of backbone hydroxylation via a process known as electrophilic substitution .

Treatment with oleum produced maximal sulphonation of the aromatic ring of polystyrene and whilst there were some cells adhered to this surface they were reluctant to spread.

There was an increase in the cell adhesiveness of the polystyrene after treatment with each of the gas plasmas (Table 4.9) and this was coupled with a decrease in both C:O ratio (an indication that oxidation had occurred) and an increase in rugosity (the degree of roughness). Exposure of polystyrene to an oxygen plasma for 5 minutes produced a surface that was comparable to tissue culture plastic. Whilst manufacturers are reluctant to disclose their techniques it is widely assumed that this is the method used during the commercial production of tissue-culture ware.

It is believed that treatment of polystyrene by an oxygen plasma hydroxylates not only the

backbone, as in the case of sulphuric acid treatment, but it has the added unique chemical feature of hydroxylating the aromatic ring [192]. The fact that treatments such as these lead to a roughened surface had led to the suggestion that cell adhesion may be due to a 'Wenzel effect' [179]. Wenzel hypothesised that roughened surfaces are more wettable due to a resultant 'capillary' -like action from the etched surface, rather than an effect of surface chemical structure. However, as shown in Table 4.3 and Figure 4.15, polystyrene that has been treated by H_2SO_4 shows an increase in the wettability of the substrate but not in the rugosity; after this treatment the surface remains smooth, as supported by Lydon et al [95]. Also treating the surfaces with oleum produced a surface with macroscopic 'grooves' and whilst it allowed some cell attachment, cells did not spread on this surface.

It can be seen from the summary in Table 4.17 that all the treatments rendered polystyrene cell adhesive but to varying amounts. All the treatments increased the wettability, oxidation and fractional polarity of the polystyrene. Glow discharge and oleum treatments increased the rugosity of the surface but it appears that both cell adhesion and spreading is maximised after the addition of hydroxyl groups.

This study highlights the fact that the introduction of -OH groups renders polystyrene surfaces suitable for cell adhesion. However, this research has also suggested that that the surface polarity is also an important factor in governing the adhesion response. Following all the treatments polystyrene was rendered more polar due to the introduction of oxygen containing groups. This gave the surface the ability to structure water and have an effect on protein binding. However, the introduction of a negatively charged group, the sulphonate group, decreased the ability of the cell to undergo normal 'anchorage dependent' cell behaviour. Thus, as the extent of sulphonation was increased, the ability of

Table 4.17. To show the effects of chemical treatments on oxidation, polarity, chemical group expression, charge and cell adhesion (confluent cell adhesion and spreading +++; cell attachment, some cell spreading ++; cell attachment, no spreading +; no cell attachment --).

Material	Treatment	C:O	Polarity	Chemical group expression	Charge	Cell adhesion
Tissue culture plastic	Untreated	4.8	↑		?	+++
Bacteriological plastic	Untreated	46.6	-		?	--
	H_2SO_4 (20°C, 36 hours)	5.0	↑	↑ -OH	↑ -ve	++
	H_2SO_4 (60°C, 10 minutes)	2.8	↑	↑ -HSO_3/ ↑ OH	↑↑ -ve	+
	Chlorosulphonic acid vapour(10mins)	6.0	↑	↑ -HSO_3/ ↑ OH	↑↑ -ve	+
	Oleum (5 minutes)	3.3	↑	↑ -HSO_3/ ↑ OH	↑ -ve	+
	Oxygen plasma (5 minutes)	2.0	↑	↑ -OH	↑ -ve	+++
	Nitrogen plasma (5minutes)	3.7	↑	↑ -OH	↑ -ve	+++
	Argon plasma (5 minutes)	4.9	↑	↑ -OH	↑ -ve	+++

the cells to undergo spreading was markedly reduced (eg. following oleum treatment), although small 'islands' or domains of the substrate were able to support some cell attachment. Sulphonate groups themselves are able to interact with and bind water strongly, but their high, negative charge may interfere with any affect this may have on a cell culture system. Hydroxyl groups are also able to structure water, but to a lesser extent. However, these groups, as introduced during this study, are possibly neutral. The cell surface possesses an overall negative charge due to eg carbohydrate moieties, possessing hydroxyl groups, extending into the extracellular matrix. The strong sulphonate groups possessing a high amount of negative charge may exert a strong force of repulsion. Such a phenomenon would not occur following the introduction of neutral, hydroxyl groups. Cells would, therefore, find it easier to become attached to hydroxylated surfaces than sulphonated substrates; more cells would attach to surfaces expressing -OH groups and therefore be available to undergo spreading. This theory would confirm the hypothesis put forward by Grinnell [15] that since cells carry a net negative charge at physiological pH (as in cell culture) there will be an electrostatic barrier set up between them and a negatively charged substrate. The cells appear to overcome the force from hydroxyl groups but not from the more powerful sulphonate groups.

It also appears from the literature that sulphonated surfaces do not allow the correct adsorption of the appropiate proteins that facilitate spreading. This is illustrated by the fact that whilst a few cells have attached to the substrate that has been sulphonated by oleum, no cell spreading has occurred. This capacity to adsorb only certain proteins may be responsible for the anticoagulant properties of sulphonated polystyrene, first shown by Lovelock et al in 1951 [193], although these workers did not comment as to how these properties manifested these effects.

It may be possible that the introduction of -OH groups led to small areas becoming oxidised at the surface and it may be the size of these domains that ultimately effect protein adsorption and ultimately cell adhesion and spreading.

CHAPTER 5

Cellular interactions with synthetic hydrogel polymers.

5.1. Introduction

In the preceding chapter it was shown that polystyrene, a non-wettable polymer, was unable to support cell attachment and spreading of BHK fibroblast cells. However, it was also demonstrated that after treatment by chemical and glow discharge techniques, both the the polarity and wettability of polystyrene surfaces were increased and these phenomena were reflected in an increase in cell attachment and spreading. Therefore in this chapter, the behaviour of cells on hydrogel surfaces is investigated since this group of materials are both by nature wettable and capable of inducing different responses of BHK fibroblast cells. Hydrogels are a family of hydrophilic polymers that are swollen by, but do not dissolve, in water. This phenomenon is usually achieved by a low degree of cross-linking which means that hydrogels are effectively water swollen polymer networks [170]. These types of materials can occur naturally, as in the case of cartilage or extracellular matrices [194] but polymer technology has allowed synthetic hydrogels with distinctive properties to be developed which has enhanced their practical utility. Synthetic hydrogels have found a wide range of biomedical applications [195] including contact lenses [117], liver support systems [196], controlled drug delivery systems [197] replacement blood vessels [192], wound dressings [198 - 202] among a variety of other related and potential uses.

The water adsorbed by a hydrogel network is quantitatively represented by the EWC which is the ratio of the weight of water in the hydrogel to the weight of the hydrogel at equilibrium hydration [180] expressed as a percentage:

$$E.W.C = \frac{\text{weight of water in the gel}}{\text{total weight of hydrated gel}} \times 100\%$$

Equilibrium water content (EWC) is a useful indication of the hydrophilicity and polarity

of hydrogel polymers.Hydrogels as a group of polymers are generally well tolerated after in vivo implantation. This success lies partially in their superficial resemblance to tissue, a property attributable to their relatively high water content (~ 20 - 99 % depending on the degree of cross linking). The water acts as a plasticiser, a transport medium for dissolved species (eg. oxygen) and a 'bridge' across the difference in surface energies between the polymer matrix and biological system. The fact that they often exhibit low interfacial tensions with aqueous environments [203] becomes important in considering their compatibility as blood contacting devices where minimal interfacial tension has been related to thromboresistance (ie. 'blood compatibility') [99, 144] . This phenomenon also results in minimal frictional irritation of surrounding tissues [92] . Thus, in a general sense, the most important aspect of synthetic hydrogels as biomaterials is that they show favourable interfacial properties.

Considering their suitability in vivo as biomaterials, it is logical that there is an interest in their use for investigations of cell-substrate interactions. There have been some reports of their successful applications to the construction of model substrates to mimic the extracellular matrix [204 - 209] . The work by Folkman and Moscona [186] and later Benze'ev [45] and Belmont [210] reported that continuous modulation of cell spreading and subsequent cellular metabolism and growth could be effected by coating commercial tissue culture surfaces with increasing thicknesses of the hydrogel poly (hydroxy - 2-ethyl methacrylate) (polyHEMA). However, it has since been reported that spun polyHEMA presented a surface with identical physico - chemical properties independent of polymer thickness in the range attainable by conventional spin film forming techniques [92] . It was further shown that the thinner coatings that Folkman and Moscona [186] had produced by

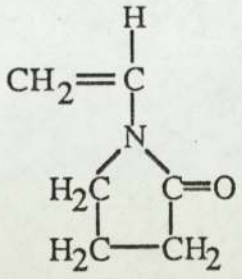
evaporation techniques were discontinuous [85, 93] and that the exposed underlying substrate was effecting cell adhesion. Further work with polyHEMA has shown that cells can remain in a fully rounded form without exhibiting spreading [211] . The majority of papers reviewed here are examples of the use of hydrogels in cell culture studies. Most of these workers took advantage of the fact that the hydrogels can be exploited to produce certain phenomena, eg the fact that polyHEMA supports little cell adhesion. However, these researchers had carried out little research to investigate what properties of the hydrogels were responsible for these phenomena and how the effects were brought about. This point has been common to the field of biomaterials research for some time and as mentioned in Chapter 1 the advent of materials for use in contact with biological systems almost preceded the fundamental research as to how and why some materials were more successful than others.

At the outset of the work described in this thesis an interesting overview of the ability of hydrogels to support cell adhesion had been produced [85] . This work, based largely on the studies of Minett whose experimental observations were confirmed in laboratories at Aston, Unilever and Mill Hill, indicated that polyHEMA was not unique among hydrogels in it's inability to support cell adhesion and spreading. Very detailed observations were carried out using several cell lines on a range of hydrogel co-polymers in which the EWC of poly HEMA was progressively decreased by the introduction of non-hydrophilic monomers such as styrene, MMA and EMA. These studies clearly demonstrated that hydrogels containing between 5 and approximately 35 % water were uniformly cell adhesive. At higher (>35%) water contents, the cell response decreased suddenly and dramatically to produce a non-adhesive zone for hydrogels with an EWC of between 40 and 60%. A limited number of observations were made on higher EWC hydrogels and

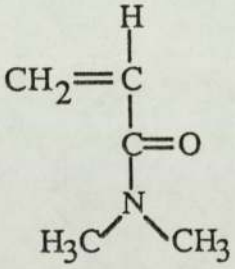
these indicated that cell attachment was restored to some extent at an elevated water content. Although the 'switch-off' point (at around 35% EWC) for cell adhesion was clearly demarked, no corresponding return to the full cell morphology following attachment was observed at higher water contents. In contrast, the limited number of experiments performed on higher EWC hydrogels did not provide convincing evidence of a return to normal 'anchorage dependent' cell behaviour. This work by Minett was not extended for evidence that a non- adhesive band between 40 and 60 % EWC could be duplicated in terms of cell response with other types of structuring group other than those found in polyHEMA (ie hydroxyl). The essential aim of this chapter, therefore, is to investigate a wider range of higher EWC hydrogels which contain a different dominant functional group and to 'probe' whether these neutral water structuring groups have an effect on cell behaviour.

5.2. Discussion of results

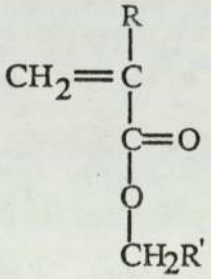
In this study a broad range of synthetic hydrogels was used in cell adhesion studies in an attempt to further the understanding of those substrate characteristics that effect cell behaviour. As previously mentioned, hydrogels are a unique family of polymers that are swollen by, but do not dissolve in water. Consequently, they serve as particularly useful substrates to study the effects of polymer wettability on cell adhesion [36, 61, 85, 97, 98, 100 - 104], just one physico-chemical property considered to have an effect on cell behaviour. Due to the inter-disciplinary nature of this study, it was possible to obtain a variety of hydrogels that had been synthesised in the laboratory by the co-polymerisation of well characterised monomers, namely N-vinyl pyrrolidone (NVP, Structure 5.1) N'N' dimethyl acrylamide (NNDMA, Structure 5.2), methyl methacrylate (MMA) and lauryl methacrylate (LMA, Structure 5.3). In addition a further series of hydrogels were also made obtainable, namely those that had been synthesised as interpenetrating polymer networks . A selection of hydrogels from this array enabled the effects of a broad range of % water contents on cell behaviour to be investigated. In addition, it was possible to obtain further information about these polymers in terms of their strength, the relative proportions of 'free' and 'bound' water and their surface energies [212]. Using a cork borer, samples (diameter 1.5 cm) of each of the hydrogels listed in Table 5.1 were cut and washed in 1% Tween 20, rinsed in distilled water and equilibrated overnight in phosphate buffered saline (PBS). In addition, samples were cut out of tissue culture and bacteriological grade plastic dishes for use as control substrates, since the cell behaviour on each of these surfaces is well characterised. Both hydrogel and control substrates were then used in cell adhesion studies. Following 6 hours in culture, the substrates were rinsed in PBS (made up without calcium or magnesium ions) and placed into a 24-well plate. 1 ml of trypsin EDTA was added to each substrate and the number of cells that had attached



Structure 5.1 N - vinyl pyrrolidone



Structure 5.2 N'N' dimethyl acrylamide



Structure 5.3

$R = CH_3$ $R' = (CH_2)_{10}CH_3$ Lauryl methacrylate

Structure 5.4

$R = CH_3$ $R' = CH_2OH$ 2 - Hydroxyethyl methacrylate

Table 5.1. Cell behaviour on a selected group of hydrogel polymers presented in order of EWC (+++ full spread morphology; ++ less well spread; + some spreading, mainly rounded cells; -- no spreading, classified as non-adhesive)

Hydrogel	Ratio of co-monomers	EWC (%)	No cells attached	No cells expressed as % of those on TC	Cell spreading
Poly HEMA	--	37.6	5.1×10^3	3.9	--
NVP :MMA	50:50	38.6	3.8×10^4	24.6	+
NVP : LMA	60:40	51.2	3.1×10^4	24.0	+
NNDMA : LMA	60:40	53.6	1.2×10^4	9.2	--
NVP : MMA	60:40	55.6	1.1×10^4	8.5	--
NNDMA : LMA	70:30	63.7	4.3×10^4	3.0	--
NNDMA : PMMA	80:20	66.5	1.3×10^4	10.0	+
NNDMA : Pellethane	90:10	76.2	1.3×10^4	10.0	+
NNDMA : LMA	90:10	78.0	1.1×10^3	0.8	--

Table 5.1 (continued) Cell behaviour on a selected group of hydrogel polymers (+++ full spread morphology; ++ less well spread; + some spreading, mainly rounded cells; -- no spreading, classified as non-adhesive)

Hydrogel	Ratio of co-monomers	EWC (%)	No cells attached	No cells expressed as % of those on TC	Cell spreading
NVP : LMA	90:10	80.0	3.9×10^3	3.0	--
NNDMA : HPU25	95:5	81.7	1.7×10^3	1.3	--
NNDMA : Pellethane	95:5	82.4	1.4×10^4	10.8	+
NNDMA : HPU25	90:10	86.6	2.8×10^3	2.2	--
NNDMA : MMA	80:20	83.1	7.8×10^3	6.0	--
NNDMA : Biomer	95:5	83.7	7.1×10^3	5.5	--
NNDMA : PVAc	80:20	84.6	4.4×10^4	33.9	+
NNDMA : MMA	90:10	86.2	1.7×10^3	1.3	--

Table 5.1(continued) Cell behaviour on a selected group of hydrogel polymers (+++ full spread morphology; ++ less well spread; + some spreading, mainly rounded cells; -- no spreading, classified as non-adhesive)

Polymer			No cells attached	No cells expressed as % of those on TC	Cell spreading
Poly NNDMA	--	88.4	4.4×10^3	3.4	--
Controls					
Tissue culture plastic			1.3×10^5	100	+++
Bacteriological grade plastic			4.9×10^3	3.8	--

Abbreviations

PolyHEMA	poly (2 - hydroxy ethyl methacrylate)
NVP	N - vinyl pyrrolidone
MMA	methyl methacrylate
LMA	lauryl methacrylate
NNDMA	N'N' dimethyl acrylamide
PVAc	poly(vinyl acetate)

were counted using a haemocytometer. Prior to counting, each surface was examined by optical microscopy to ensure that all the cells had been removed. The cell count observed is shown in Table 5.1. Whilst there was a confluent monolayer of cells observed on tissue culture plastic, in general the number of cells attached to each hydrogel surface was low and few had more cells attached than bacteriological grade plastic. In addition, since few of the surfaces allowed any cell spreading to take place viability tests were carried out before and after cell culture to investigate whether the hydrogels had proven toxic to the cells. The test used in the viability study works on the principle that dead, or non-viable cells take up a dye (trypan blue) whereas the viable cells do not. Therefore, 0.1 ml of the dye was added to 1ml of the cell suspension prior to seeding or following cell studies to the supernatant from each petri dish that had contained the hydrogels. The number of non-viable and total cells were counted using a haemocytometer and the percentage of viable cells were calculated. The viability before and after culture was approximately 98% which shows that the hydrogels themselves were non-toxic. This implied that some property of the hydrogels had been responsible for the cell behaviour observed. The relative percentages of the cell number compared to that for tissue-culture plastic (taken as 100%) were calculated. A 5 % error band was assumed for these counting techniques.

5.2.1. Preliminary attempts to correlate cell adhesion and hydrogel properties.

Minett's work had implied that there was a relationship between cell adhesion and EWC. In the light of this, the values for EWC against % cell number obtained during this study were plotted (Figure 5.1), but there was little correlation between these two parameters. As previously mentioned, the hydrogels studied here were also well characterised in terms

Figure 5.1.Percentage cell count against EWC

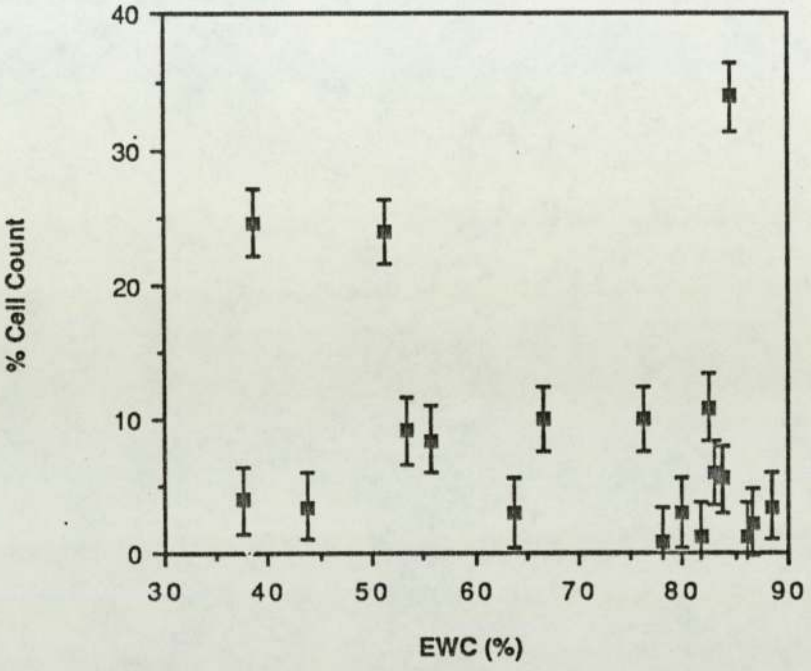
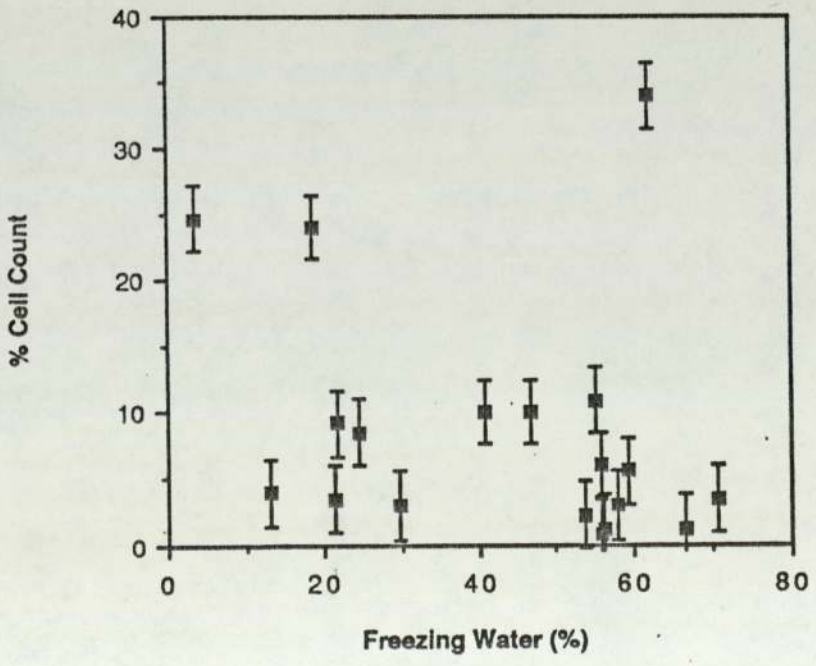


Figure 5.2. Percentage cell count against freezing water



of their surface energies for both hydrated and dehydrated form, for Young's modulus, tensile strength, freezing and non-freezing water contents (Appendix 5). Therefore the % cell count was plotted against each of these physico-chemical and mechanical properties (Figures 5.2 -5.7) and whilst there was a slight increase in the number of cells attached to those surfaces with higher values for Young's modulus and tensile strengths, there was no single over-riding factor that governed cell adhesion. In addition to the above properties, investigations using ESCA techniques of the C:O ratios for each hydrogel were also carried out. Results in the previous chapters had suggested that surfaces with lower values for carbon : oxygen ratios, reflecting that were more oxidised, supported increased cell adhesion. Because surface analysis by ESCA is carried out under vacuum it was not possible to use this technique on hydrated hydrogels. Thus, hydrogel and control (tissue culture and bacteriological grade plastic) samples were washed as before in 1% Tween 20, rinsed in distilled water, equilibrated in phosphate buffered saline and dried in a vacuum oven prior to ESCA analysis. From the spectra produced, it was possible to calculate the % concentrations of carbon, nitrogen and oxygen and from these values the C:O, C:N and O:N ratios also shown in Appendix 5 were derived. The values for these ratios were plotted against cell count as shown in Figure 5.8 and again there was little correlation between these components. Indeed, it can be seen that the C:O values lie within a tight 'band' and it is perhaps surprising that the value for tissue culture plastic, which supported confluent cell attachment and spreading, lay within this region. Therefore there was no simple correlation between cell adhesion and any of the hydrogel properties.

Figure 5.3. Percentage cell count against grams water / grams polymer

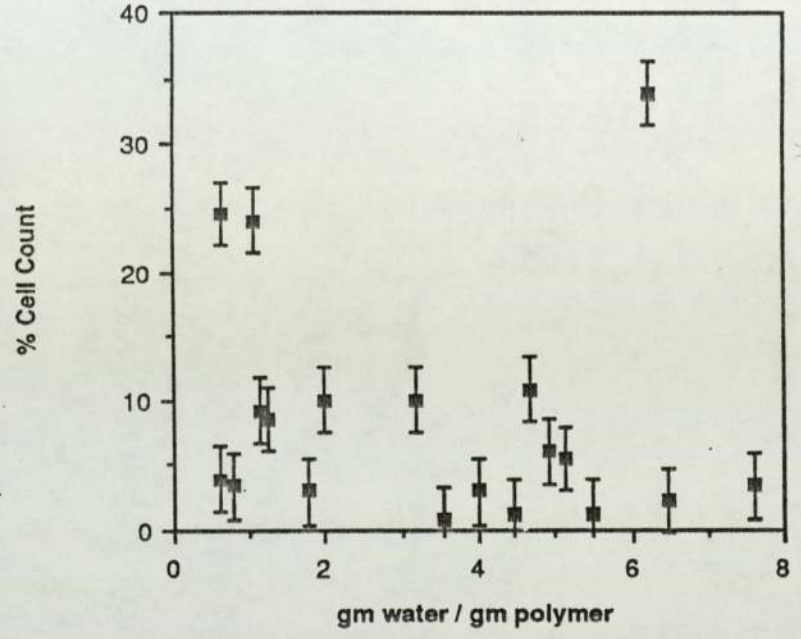


Figure 5.4. Percentage cell count against hydrated surface energy

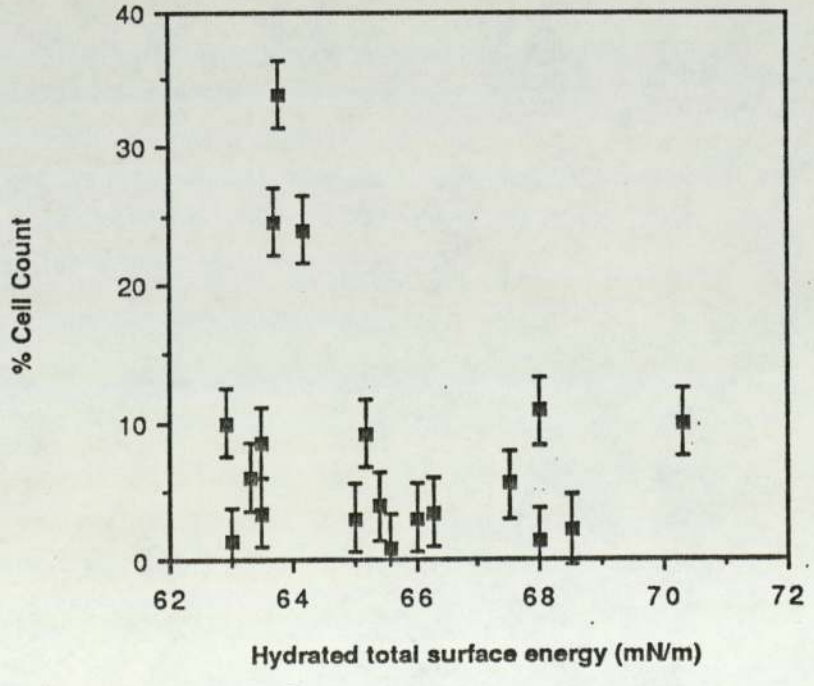


Figure 5.5. % cell count against hydrated polar component

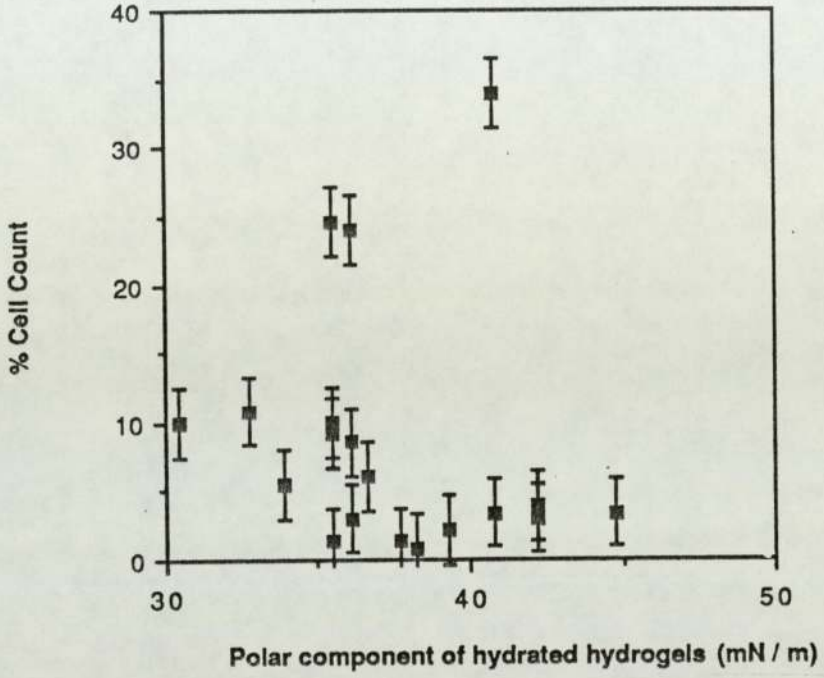


Figure 5.6. Percentage cell count against Young's modulus

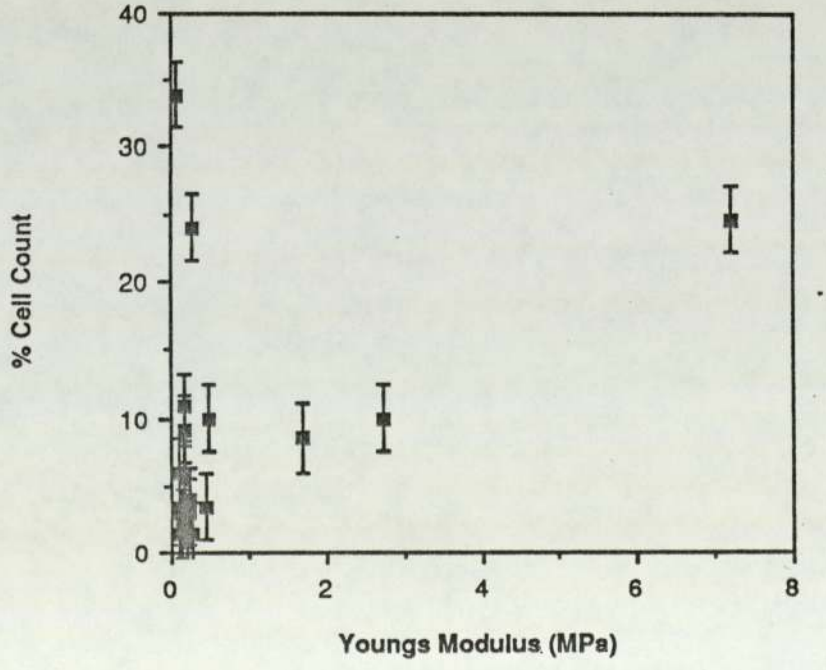


Figure 5.7. Percentage cell count against tensile strength

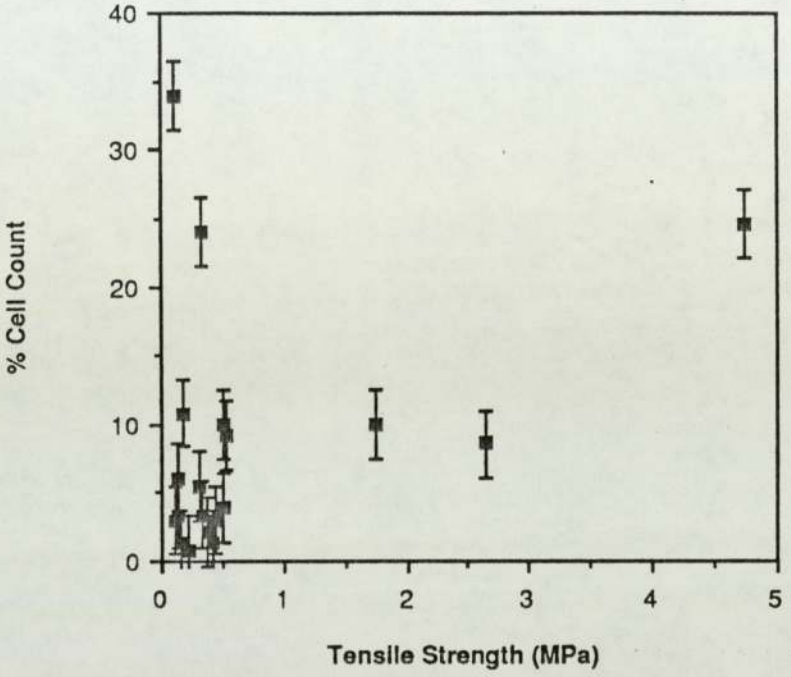
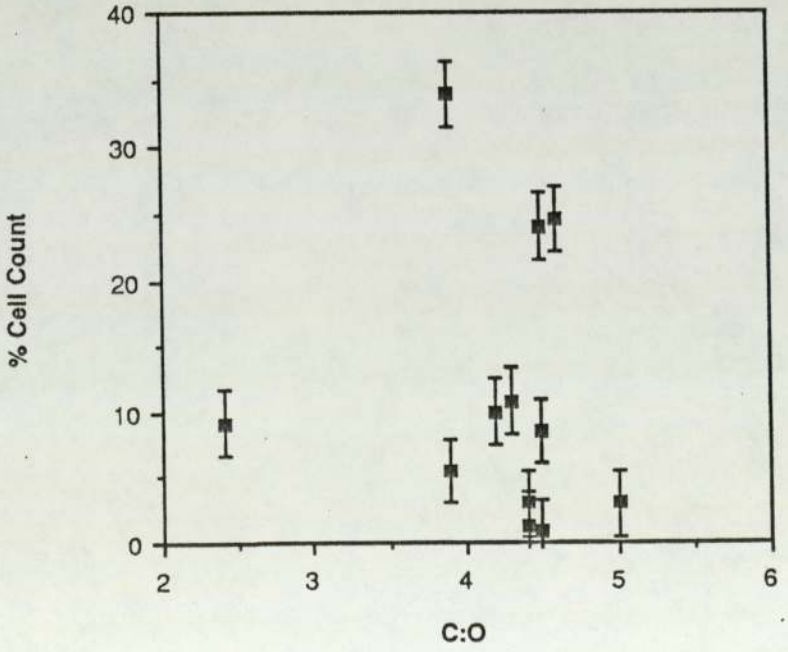


Figure 5.8. Percentage cell count against carbon : oxygen ratio



5.2.2. Correlation of cell adhesion with hydrogel structure

Examination of the structural components for each of the hydrogels made it possible to separate out certain families and these are listed in Table 5.2. 'Family' 1 contains those hydrogels that had been produced following the co-polymerisation of NVP with varying amounts of MMA or LMA. Similarly, 'family' 2 groups together those hydrogels containing co-polymers of NNDMA with MMA or LMA. Co-polymerisation of either NVP or NNDMA with the more hydrophobic monomers of MMA or LMA results in a decrease in EWC as shown in Table 5.1. Both NVP and NNDMA are monomers that tend to be basic, rather than acidic, in nature and the fact that they contain nitrogen at their surface (Appendix 5) gives them the ability to structure water via hydrogen bonding. These hydrogels are homogeneous and clear. 'Family' 3 are those hydrogels composed of interpenetrating networks. These were produced by dissolving poly (vinyl acetate), poly MMA or polyurethanes in N'N'DMA and then polymerising the NNDMA [212]. Hydrogels such as these are heterogeneous and they contain microdomains, or 'islands' at their surface which effect their surface characteristics. These latter hydrogels are generally translucent because these regions tend to be larger than the wavelength of light.

Thus, the % cell number against EWC was plotted out again, but separately for each individual family. Figure 5.9 is the graph produced for EWC versus % cell number for 'family' 1 and it was of interest that the overall shape of the curve was similar for that observed at the onset of Minett's non-adhesive trough. However, whereas Minett found that the non-adhesive zone started at 40%, the curve in Figure 5.9 reaches it's lowest point at approximately 60%. Thus, the results for this group of hydrogels suggest that Minett's curve has shifted slightly to the right in terms of EWC.

Table 5.2 The categorisation of 'families' 1 - 3.

	Co-polymers	Ratio of monomers
Family 1	NVP : MMA	50:50
	NVP : MMA	60:40
	NVP : LMA	60:40
	NVP : LMA	90:10
Family 2	NNDMA : MMA	80:20
	NNDMA : MMA	90:10
	NNDMA : LMA	60:40
	NNDMA : LMA	70:30
	NNDMA : LMA	90:10
	Poly NNDMA only	--
Family 3	NNDMA : HPU25	95:5
	NNDMA : HPU25	90:10
	NNDMA : Pellathane	90:10
	NNDMA : Pellathane	95 : 5
	NNDMA : Biomer	95:5
	NNDNMA :PMMA	80:20
	NNDMA : PVAc	80 : 20

Figure 5.9. Percentage cell count against EWC for 'family' 1

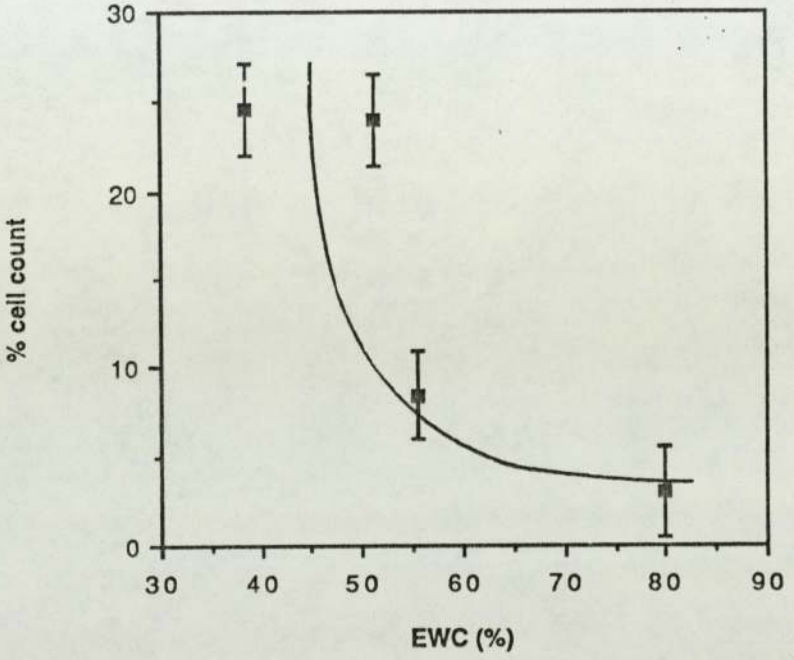
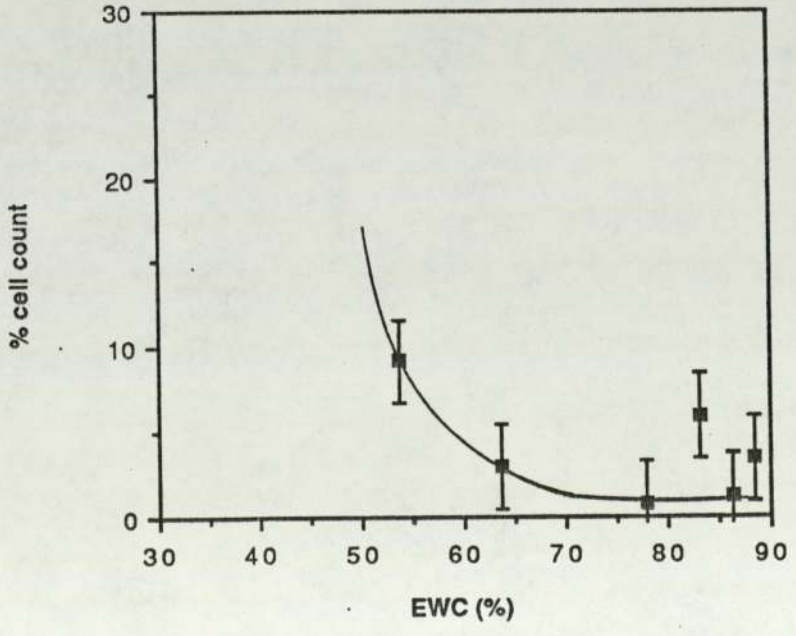


Figure 5.10. Percentage cell count against EWC for 'family' 2



In a similar fashion, the results of % cell count were plotted for 'family' 2 (Figure 5.10) and the curve was very similar to that for 'family' 1 and this was overlaid onto Figure 5.9. It can be seen, therefore, in Figure 5.11 that a trough was reached at 60% which did not appear to rise up at a later stage and it appeared that the Minett curve had again shifted to the right.

There appear to exist therefore 3 curves of similar shape, each one characteristic of an individual water structuring monomer. The 3 water structuring monomers were an hydroxyl (as in polyHEMA, structure 5.4) and and two nitrogen containing monomers (NVP, structure 5.1 and N'N'DMA, structure 5.2). These monomers had then been co-polymerised with varying amounts of non- hydrophilic monomers and the position of the non-adhesive trough for 'families' 1 and 2, ie. those containing monomers 5.1 and 5.2 are similar to each other but quite different from Minett's (those hydrogels that contained structure 5.4). A visual comparison of the nature of the water structuring group confirms the similarity of the hydrophilic centres in 5.1 and 5.2 and their joint distinctness from 5.4. A very important principle hitherto undiscovered appears to emerge. Investigations of the cellular response to the water swollen hydrogels is not dependent on the EWC itself. The effects of this property appear to be over-ridden by the presence of a water structuring chemical group, for example an -OH or nitrogenous group.

When a curve for EWC against % cell count was plotted for 'family' 3 (figure 5.12) there was no simple correlation between these two parameters. However, these polymers are more complex because the fact that they are interpenetrating networks (mostly translucent / opaque) and water at the surface is structured in small groups, or microdomains.

Figure 5.11. Overlay plot of the EWC for families 1 and 2

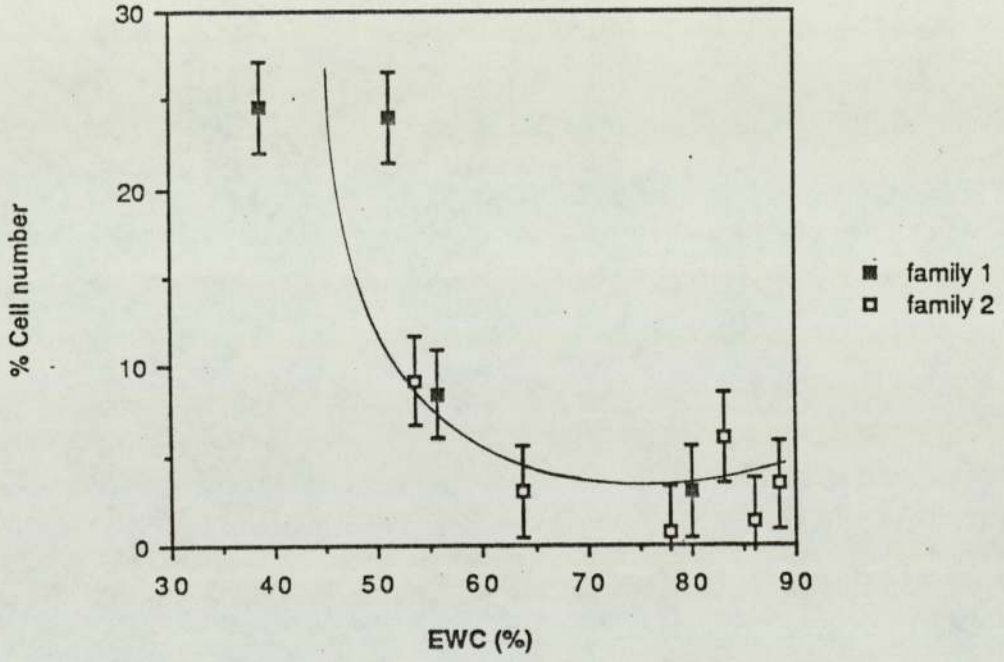


Figure 5.12. % cell count against EWC for the hydrogels in 'family' 3

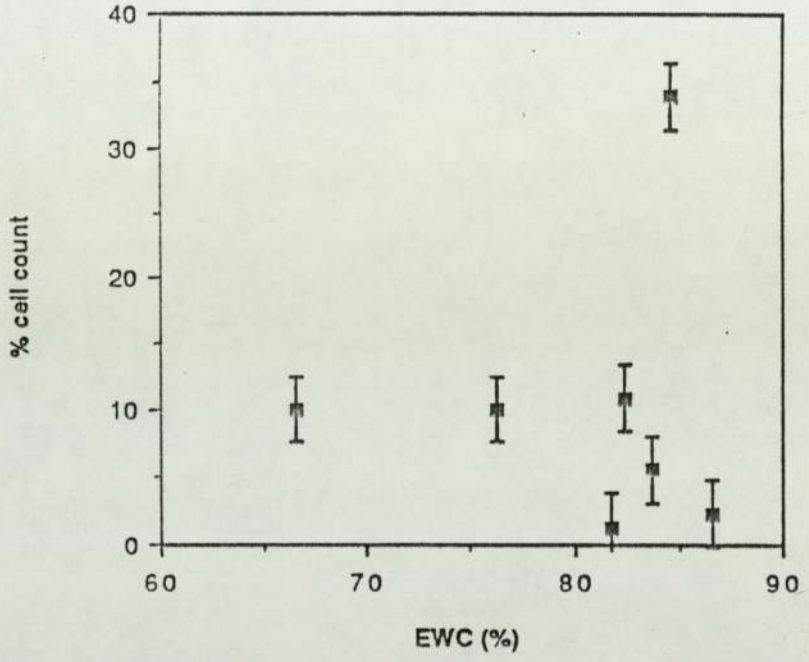
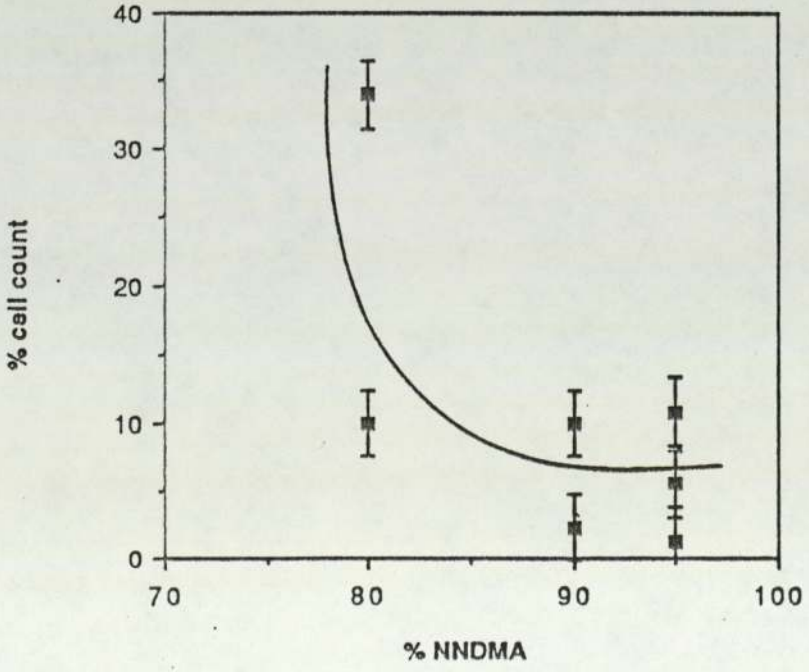


Figure 5.13. Percentage cell count against % NNDMA for 'family' 3



Biological events occur at a molecular level and the intermediary processes that occur in the serum layer involve biologically active molecules, eg proteins such as fibronectin and vitronectin. The nature of the interpenetrated polymer or dispersed phase is most important in relation to this biological process. By analogy with the previous discussion, it is reasonable to imagine these systems as dispersions of polymer segments in an otherwise non-adhesive hydrogel. (From Figure 5.13 it is apparent that polyNNDMA is non-adhesive, a point confirmed in Table 5.1.). The results for 'family' 3 can, therefore, be presented as a % of the dispersed phase in modifying surface energy (Figure 5.14). The information on these materials is very limited because compatibility problems (ie the solubility of the dispersed polymer in the liquid NNDMA monomer before polymerisation) reduce the range of materials that can be prepared. Certainly, more experimental information is required before the interpenetration offered here can be confirmed.

A new interpretation of Minett's data now appears. After his non-adhesive zone he tentatively indicated that cell adhesion began to increase again at an EWC of approximately 70%. It is important to note that in the light of the experiment presented here that these moderately adhesive materials were not based on the -OH structural group which is so clearly delineated at the beginning of the non-adhesive zone but on a range of randomly chosen co-polymers that were available to him at that time. Figure 5.15 sketches the form of Minett's interpretation of his investigations whereas Figure 5.16 sketches an alternative view, consistent with the results presented here. The essential differences between the two interpretations is that Minett saw his curve rising again through modestly adhesive hydrogels, whereas the results presented here suggest that those materials were demarking the edge of a non-adhesive zone for functional monomers of the type that they contain.

Figure 5.14. Percentage cell count against polarity for 'family' 3

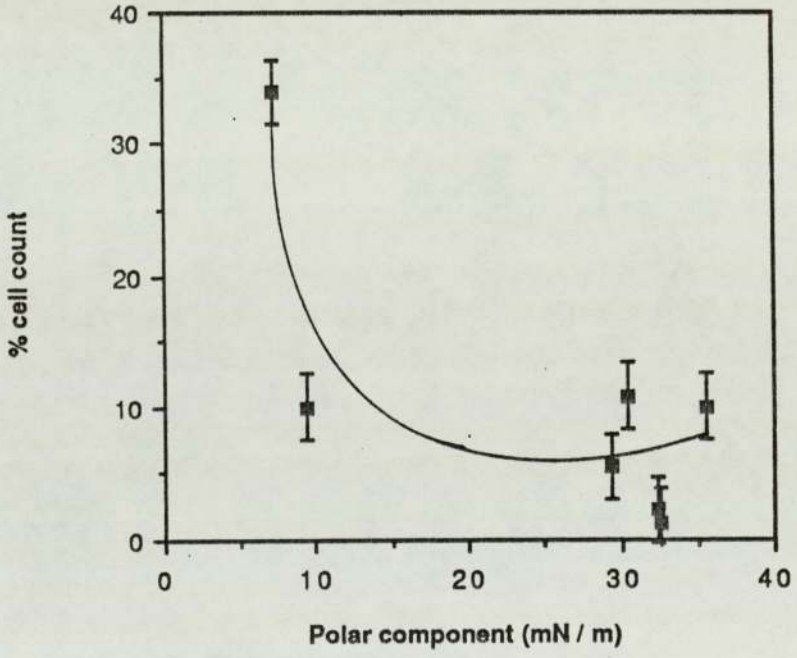


Figure 5.15. Sketch of the 'Minett' curve

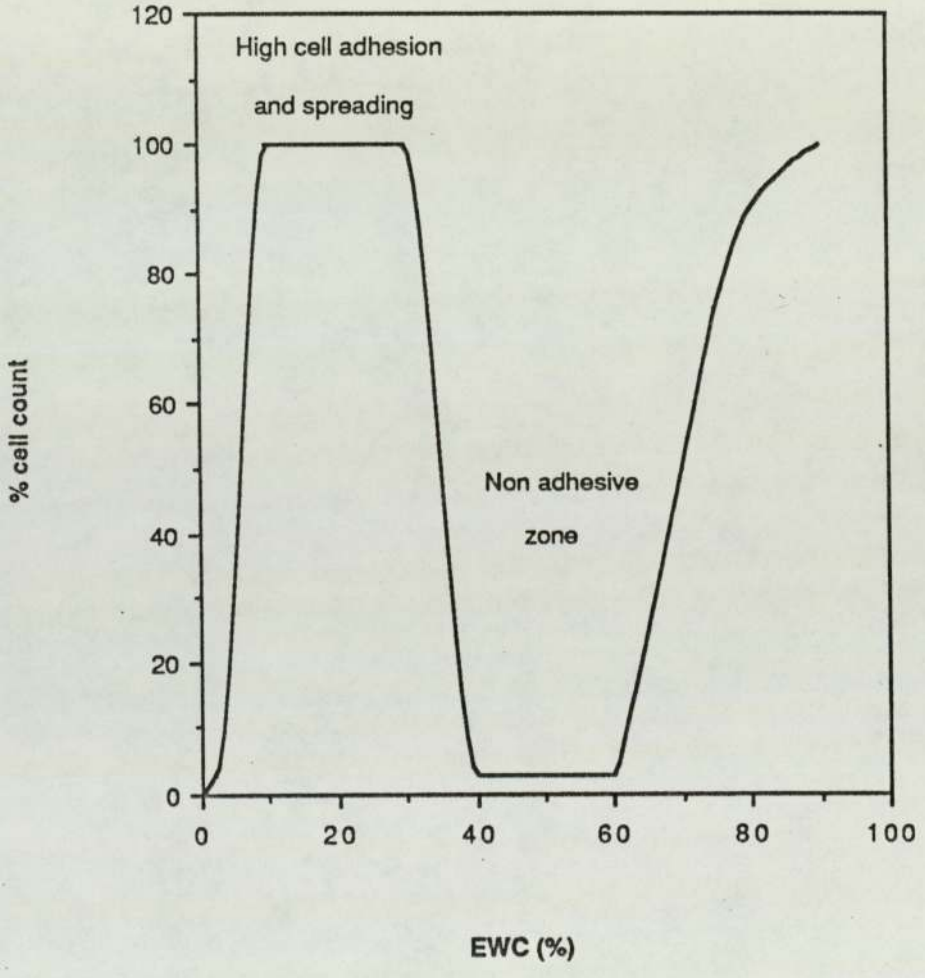
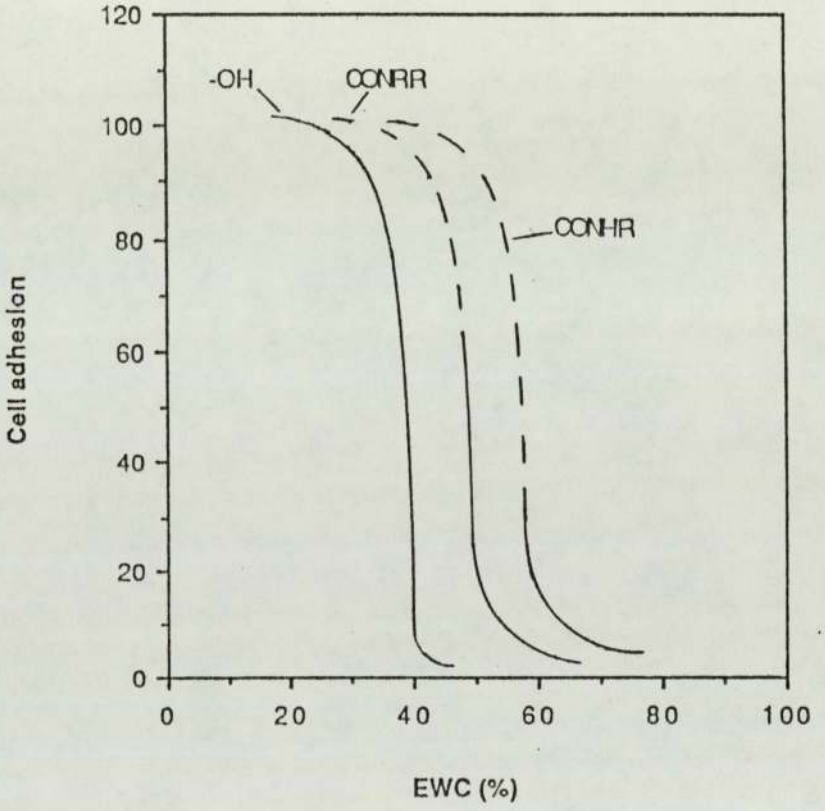


Figure 5.16. Sketch of the three water structuring groups



Since they were for the most part complex co-polymers another important issue is raised. This point is the extent to which individual monomers contribute to the cell adhesive function of a hydrogel and the extent to which the presence of different functional co-monomers modify their ability to undergo hydrogen bonding complexation between the individual functional groups. Whilst no definitive analysis of this complex question was attempted within the time constraints of this project some interesting observations were made on a group of acrylamide and substituted co-polymers. These were again selected from available materials within the laboratory in order to provide a broad overview of substituted acrylamide co-polymers. In retrospect, it is obvious that more detailed and systematic variations in composition would be necessary in order to unravel the complexities of cell behaviour.

In a similar study, the cellular response to a further series of hydrogels was observed using light microscopic techniques (Table 5.3). This series was based on acrylamide co-polymers which could again be separated into groups. 'Family' 4 contained a series of hydrogels where the hydrophilic monomer acrylamide (ACM) was co-polymerised with HEMA and / or the negatively charged methacrylic acid (MAA). The EWC of each of these hydrogels was high (88.9 - 95.9 %) and whilst there was little spreading there were quite high numbers of cells attached. 'Family' 5 contained copolymers of the moderately hydrophilic monomer diacetone acrylamide (DAACM) and HEMA, acrylic acid (AA), NVP, or hydroxy propyl acrylate. This family contained some hydrogels with low EWC (24.4 - 30.8) whilst a mixture of DAACM and NVP had an EWC of 78.5. All these surfaces supported reasonable amounts of cell adhesion and on the lower EWC gels this was accompanied by some spreading. The situation within 'family' 6 was interesting

Table 5.3. Cell behaviour on a selected group of hydrogel polymers (+++ high number of cells attached / spread; ++ less cells attached / spread; + few cells attached / spread; -- no cells attached / spread)

	Polymer	Ratio of co-monomers	EWC (%)	Cell attachment	Cell spreading
Family 4	ACM :MAA	90:10	92.3	++	--
	ACM : HEMA	75:25	95.9	++	--
	ACM : HEMA : MAA	90:5:5	88.9	+++	--
	ACM : HEMA	90:10	89.8	+++	--
Family 5	DAACM : HEMA	33:67	26.4	++	+
	DAACM : AA	50:50	24.4	++	+
	DAACM : NVP	40:60	78.5	+	-
	DAACM : HPA	25:75	30.8	++	--

Table 5.3 (continued) Cell behaviour on a selected group of hydrogel polymers (+++ high number of cells attached / spread; ++ less cells attached / spread; + few cells attached / spread; -- no cells attached / spread)

	Polymer	Ratio of co-monomers	EWC (%)	Cell attachment	Cell Spreading
Family 6	MAA : DAACM	75 : 25	30.0	--	--
	MAA : DAACM	67 : 33	36.5	--	--
	MAA : MACM	67 : 33	36.2	--	--
	HMACM : AA	70 : 30	65.0	+++	--
Family 7	HMACM:MAA:VP:HPA	60:10:20:10	64.0	+	--
	HMACM:MAA:VP:HPA	33:15:42:10	68.9	+	--
	HMACM:VP:MAA:HPA	55:20:15:10	63.2	+	--

Table 5.3 (continued) Cell behaviour on a selected group of hydrogel polymers (+++ high number of cells attached / spread; ++ less cells attached / spread; + few cells attached / spread; -- no cells attached / spread)

	Polymer	Ratio of co-monomers	EWC (%)	Cell attachment	Cell spreading
Family 8	HEMA : MACM	75:25	42.1	--	--
	HEMA : NVP	80:20	43.8	--	--
	HEMA : HMACM	50:50	45.6	++	+
	HEMA : DAACM	50:50	27.5	++	+
	Controls				
	Tissue culture plastic			+++	+++
	Bacteriological grade plastic			--	--

Abbreviations

ACM	acrylamide
MAA	methacrylic acid
HEMA	hydroxy ethyl methacrylate
DAACM	diacetone acrylamide
AA	acrylic acid
NVP	N - vinyl pyrrolidone
HPA	hydroxy propyl acrylate
DAACM	diacetone acrylamide
MACM	methacrylamide
HMACM	hydroxy methacrylamide
VP	vinyl pyrrolidone

because there was no cell attachment whatsoever on these MAA copolymers. MAA is a strongly negatively charged monomer. It has been mentioned in both Chapters 1 and 4 that the cell surface has an overall net negative charge. Therefore, when a negatively charged surface is encountered there may be some force of repulsion which would decrease the possibility of cell attachment. There was some attachment to the hydrogels in 'family' 7 a family containing HMACM as the principle monomer but where a small amount of negative charge in the form of MAA had been introduced (ie <10%) into the hydrogel but increasing the amounts of MAA >60% prevents cell attachment from taking place. It appears, then, that there is a point of equilibrium as far as the effect of negatively charged surfaces on cell behaviour. Below this point, the cells can overcome the 'force of repulsion' or in some cases the negative charge can promote adhesion (eg small amounts of MAA, or following the introduction of -OH groups following acid treatment of polystyrene [chapter 4] of polyHEMA. However, if the substrate becomes too highly negatively charged, (as in increasing the proportion of MAA or sulphonated surfaces) then there is a decrease in the number of cells that are able to attach themselves to the substrate.

An investigation of this second group of hydrogels shows that when the dominant structural group is changed from an -OH or a nitrogen to an acrylamide, there is a further shift in the Minett curve.Therefore the prominent message that comes out of this chapter is that it is not the EWC or the other physico-chemical characteristics of the hydrogels themselves that govern the cell response, but the dominant functional. The evidence is that acrylamide and substituted acrylamide monomers, for example, are capable of producing cell adhesiveness at a water content appreciably above that at which a similar level of adhesion is observed by NVP co-polymers and is certainly vastly in excess of the HEMA

based co-polymers. In conclusion, it appears that the ability to support cell adhesion follows the following trend:

hydroxy alkyl methacrylates < NVP ~ NNDMA < N methylol acrylamide

~ diacetone acrylamide < acrylamide

ie. $-OH < \sim CON(R) < \sim CONHR < \sim CONH_2$

Both acrylamide compounds and hydroxyl groups contain oxygen. Therefore, since oxygen is able to mediate protein adsorption via hydrogen bonding this may a way in which these groups had an effect on cell behaviour in addition to those via water structuring.

Minett had showed that the copolymerisation of polyHEMA with less hydrophilic monomers, such as styrene or ethyl methacrylate, decreased the EWC to between 8 and 33 % which produced a cell adhesive surface [85]. Similarly, in this study when NVP or NNDMA were co-polymerised with the less hydrophilic MMA or LMA, then that too resulted in a lower EWC and a surface with more cells attached (although not spread). Minett had used poly (hydroxyethyl acrylate) and poly (hydroxypropyl acrylate) to ascertain the non-adhesive zone whose functional group, like that of polyHEMA was an -OH group. The hydrogels in the region of >60 % water content did not support the same levels of cell attachment in terms of number and extent of cell spreading as in the case of the <35% EWC hydrogels. There is no conclusive evidence presented in this study to suggest that a reduction of the EWC of NNDMA / NVP hydrogels to around 10 to 40 %

by the introduction of increasing amounts of the alkyl methacrylates will produce a cell adhesive surface similar to those observed for HEMA based copolymers. Similarly, there is no conclusive evidence to suggest that they will not.

Minett had suggested that the inability of the higher water content hydrogels to support cell adhesion to the same capacity as those with lower EWC was due to the mechanical instability of those with a EWC greater 30 %. These latter hydrogels may have lead to an inhibition to spreading of those cells that exert higher tensile stress [213] . Indeed, as suggested earlier, there did appear to be a trend for slightly higher cell attachment on the stiffer hydrogels. (Figures 5.6 and 5.7). Despite this, the hydrogels contained in 'family' 4 all contain a high EWC and all supported extensive cell attachment, although there was little spreading.

As a group of polymers, hydrogels are more complex than other polymers in that interactions can occur within the hydrogel structure itself which ultimately affect the surface properties. Their surfaces represent a unique environment characterised by high polymer chain mobility within the gel [214 - 217], heterogeneity (as in the case of 'family' 3 which were dominated by the presence of interpenetrating networks) and unique water structuring [218 - 220] . These phenomena contribute to the difficulties in precisely analysing and characterising hydrogel surfaces and ultimately further difficulties arise during interpretations of cell responses.

As mentioned in Chapter 1 surface charge has been sited as having an effect on cell adhesion [18, 21, 62, 106, 107]. However, the effects of this property on cell behaviour are difficult to investigate mainly due to the fact that changes in surface charge, eg by acid

treatment, ultimately effect some other property, eg. wettability, surface group expression. It has been shown during this current study that it is possible to produce hydrogels with a charge expressed at the surface. It may be possible to produce a range of charged hydrogels that possess a functional group, but which lay within the non- adhesive zone in terms of EWC for that group (eg. between 40 and 60 for the -OH hydrogel series). It would then be of interest to observe the effects on cell adhesion. It is interesting and possibly significant that the introduction of MAA into the hydrogel progressively decreases the extent of cell attachment that occurs. This is illustrated by the fact that MAA present at a concentration of 15% had little affect on the extent of cell adhesion, yet at a level >65%, the cell attachment response was completely inhibited.

This study has shown that cell attachment is not directly dependent on EWC as was implied by Minett's work. However, the presence of a dominating structural group, as found in N'N'DMA , NVP (nitrogen), a di-, mono- or un-substituted amide or indeed an hydroxyl group, which has the ability to structure water held at the surface, can over-ride any of the effects due to water content. In addition, the introduction of charged groups, as shown by the introduction of MAA, can in turn over-ride the effects of the water structuring complex. However, it is probable that there is a threshold value or balance point for this surface charge, which below a certain concentration has a limited affect on the cell behaviour but above this value can profoundly determine the overall cell response.

CHAPTER 6

An investigation of the adsorbed interfacial layer;

lipids and calcium

6.1. Introduction

It has been previously outlined in this thesis that whenever a material contacts a biological environment there is an interaction between the two systems resulting in the establishment of an interfacial layer. In the cell culture situation, this layer results from the adsorption of components from the culture fluid, which is essentially physiological medium supplemented with foetal calf serum. Serum is important in cell culture for a number of reasons. It is the source for growth factors (inhibiting and promoting), polypeptides, proteins, hormones, minerals, metabolites and lipids which are all essential for normal cell behaviour.

A great deal of literature exists concerning the adsorption of protein to materials during interfacial conversion. The greatest attention has concentrated on protein adsorption and it's role in governing the thrombogenicity of blood - contact devices. In this capacity, most research has involved precoating substrates or supplementing culture medium with particular proteins or examining the effects of deproteinised culture medium. However, there have been few recorded investigations to 'unravel' the conversion layer by extraction techniques and the subsequent identification of species in this layer. The little work that has been carried out in this way has primarily been concerned with the identification of proteins.

There is now some evidence to suggest that in addition to proteins, lipids may also play a role in the interfacial conversion process [221] . However, as with many other aspects of material - biological interactions, there have been few systematic investigations to discover how lipids may be involved in this capacity and consequently there is no overall consensus as to their importance. There are a number of different lipid classes

and a brief overview of these will be given here.

The term 'lipid' has traditionally been used to describe a wide variety of natural products, including fatty acids and their derivatives, steroids, terpenes, carotenoids and bile acids, all of which have one thing in common: they are all soluble in organic solvents such as chloroform, hexane, diethyl ether, methanol and benzene, to name but a few [222]. Elsewhere [223, 224] lipids have also been defined as water insoluble organic molecules. There are a variety of different lipid families and they all have several important biological functions including structural components of membranes [225, 226], storage and transport forms of metabolic fuel, as protective coatings of many organisms and as cell and surface components in cell recognition [227], tissue immunity and species specificity.

The principal lipid classes found in animal fluids and tissues may be listed as follows:

fatty acids, in both saturated (eg. palmitic acid) and unsaturated (eg. oleic acid) forms;

triglycerides, eg. tripalmitin, which are composed of fatty acid esters of glycerol. Mono- (eg. monopalmitin) and di- (eg. dipalmitin) glycerides also exist, but in lesser quantities;

cholesterol, the commonest member of a group of steroids with a tetracyclic ring system;

cholesterol esters, where fatty acids are esterified to the cholesterol ring,

eg. cholesterol palmitate;

phospholipids, which include any lipid that contains phosphoric acid as a mono- or di- ester eg. phosphatidyl inositol;

lipoproteins, which contain both lipids and protein, eg. chylomicrons;

glycolipids, which contain both carbohydrate and lipid moieties, eg. galactosyldiacylglycerol.

It is possible that lipids are important during interfacial conversion since they are able to modify both hydrophilic and hydrophobic surfaces. For example, adsorption of fatty acids to hydrophilic surfaces may occur via attachment of polar head groups, leaving the 'hydrophobic' tails uppermost. On the other hand, attachment to marked less polar materials may occur via the tail region to produce a more hydrophilic surface. The adsorbed lipids may then be stabilised by complexing with calcium ions.

The following overview has been collected via reports in a number of different areas of research but the three relevant categories include the interactions of lipids with polymers (eg. in the petrochemical and prosthetic industries), the affects of lipids on cell biochemistry (eg. platelet function but includes some work of the affects on adhesion) and the interactions of lipids with calcium.

6.1.1. Interactions of lipids with polymers

During contact lens wear there is a deposition of a number of species from tear fluid (biological phase) onto the lens (non-biological phase). The build up of this layer ultimately determines the success of the lens in terms of the biological response. In some patients discrete, elevated deposits build up over a period of time (days, months etc). Previously, these 'white spots' were widely considered to be composed of calcium [228, 229] but histological analysis of these deposits has recently shown that they are principally composed of several lipid species [221]. The density of unsaturated

lipids was maximal at the deposit - hydrogel matrix interface, suggesting that these species are the first to become adsorbed onto the hydrogel lens surface.

Apart from their binding to contact lens materials during spoilation processes, lipids have also been demonstrated to adsorb to other polymers. Lipids have proven to be active in polymer environments [230] and investigations performed in the 1960's on silicone heart valve poppets [231] demonstrated that poly(dimethyl ethyl siloxanes) with low cross-link densities and low silica filler underwent significant lipid uptake in vivo. Other workers [232] have shown that swelling and obstruction of silastic heart valve poppets was principally due to the infiltration of complex lipids.

One research group attempted to characterise those polymers that adsorbed cholesterol and other lipids from micellar solutions [233] . They concluded that amorphous, cross-linked block co-polymers having network chains with an affinity for both oils and water adsorbed bile lipids from micellar solutions. This adsorption was hypothesised as occurring due to the organisation of water, lipid and polymer segments.

6.1.2. The effects of lipids on cell behaviour

Ivanova and Margolis [43] showed that precoating the surface with brain phospholipids produced a surface incapable of supporting cell adhesion. Whilst there was no further characterisation of the phospholipids they used they did highlight the importance of the electronegativity of the phospholipid film produced. They suggested that the interaction of the film with fibroblast cell membranes was a model of cell to cell membrane interactions. This is illustrated by the fact that fibroblast - substrate adhesion is greater than fibroblast cell - cell bonding. Thus, if this hypothesis held true, then a fibroblast

cell in culture would view an adsorbed phospholipid layer as another cell and not adsorb with as great an efficiency as when this lipid is absent. The same workers showed that surfaces coated with paraffin, fatty acids and cholesterol had a capacity similar to each other in their ability to support cell adhesion of mouse fibroblasts.

There have been other studies implicating phospholipids as important in mediating cell attachment. Curtis et al [234] deduced that the state of cell membrane lipids affected cell adhesion. All biological membranes have a common overall structure in that they are essentially assemblies of lipid and protein molecules held together by non-covalent interactions [235] . The lipid molecules are arranged as a continuous double layer 4 - 5 nm which serves as a relatively impermeable barrier to the flow of most water soluble molecules. The protein molecules are 'dissolved' in the lipid bi-layer and mediate the various functions of the membrane. Some serve to transport specific molecules into and out of the cell, others are enzymes that catalyse membrane-associated reactions. Still others serve as structural links between the cell's cytoskeleton and the extracellular matrix, or as receptors for receiving or transducing chemical signals from the cell's environment. Cell membranes are not static but are dynamic, fluid structures [225] and most of their lipid and protein compositions are able to move rapidly in the plane of the membrane. The presence of short or unsaturated double bonds are considered to lower the phase transition point, below which the membrane becomes more fluid [235] . It is with the cell membrane, in particular the 'liquid - mosaic' model that the work of Curtis et al [234] was concerned. Conditions where reacylation was stimulated increased cell adhesion; similarly, conditions leading to the accumulation of lysolipids in the cell surface diminished cell adhesion (a lysolipid is produced when a phosphatidyl compound in the plasmalemma is lysed). The same workers postulated that rapid cell

membrane lipid turnover is required to maintain an adhesive state, where the majority of the plasmalemmal lipids were present in the di-acyl form and not in the lyso state.

Further work by this group led to the incubation of cell cultures with fatty acids added to the culture medium. They assumed that the lipids would become incorporated into the cell membrane, affect the fluidity of the plasmalemma and ultimately cell adhesion. Their findings were confirmed by Hoover and co-workers [236] who in summary showed that incubating cells with fatty acids (linoleic, linolenic and arachidonic acids) decreased cell adhesion of BHK and chinese hamster ovary cells to the substrate. Similarly, incubation with oleic, palmitic or stearic acids had little effect. It is important to remember that these workers assumed that these lipids were inserted into the membrane, and they explained their effects as follows. When unsaturated fatty acids are substituted into the membrane they may lower the phase transition temperature thus leading to a more fluid membrane. Considering the fluid mosaic model of Singer and Nicholson [225] this would enhance lateral diffusion of the cell membrane. Shields et al [237] hypothesised that cell adhesion binding sites must first cluster together in a membrane to form a strong 'adhesive patch ' to exert an adhesive bond. In a more fluid membrane it would, therefore, be difficult to maintain patches large enough to sustain an adhesive bond, whilst in a more viscous membrane the clusters may remain and enhance adhesion. These latter researchers assumed that the adhesive 'patch' had already formed prior to the insertion of the fatty acids. However, if these more 'rigid' fatty acids were inserted before this patch was formed, then again adhesion may not occur due to the membrane being too stiff for an adhesive cap to be formed by lateral movement.

Similarly, other workers [238] showed that the adhesion of anchorage dependent cells decreased following exposure to membrane fluidising reagents (local anaesthetics, non-ionic detergents, aliphatic alcohols). However, these workers did not comment on any toxic effects these treatments had on the cells themselves.

This theory is interesting in that there is some information to suggest that transformed and malignant cells have more fluid membranes than normal cells [239, 240] . This is supported by the hypothesis mentioned in Chapter 1 that transformed and malignant cells are both non-anchorage dependent.

Whilst all of these workers investigated the effects of adding lipids to cells, they did not comment on the phenomena occurring during the physiological culture situation where lipids are present in foetal calf serum.

Cavenee et al [241] showed that incubation of both single cells and cultured monolayers with cholesterol decreased cell-substrate adhesion. Again it was considered to be via an affect on membrane fluidity and it had been demonstrated that cholesterol renders the plasma membrane more viscous [242] . However, this is in direct confliction with the previously mentioned work by Ivanova and Margolis who showed that surfaces precoated with cholesterol did not affect cell adhesion.

It had been shown [243] that cells adhered directly to glycosphingolipids that had been separated on TLC plates. The cells adhered only to areas where there were appropriate glycosphingolipids.

Huang et al [244] reported that lipids could mediate adhesion of animal cells. The lipids most able to do this were those containing galactose, or four sugar molecules at their terminals [245].

Whilst lipoproteins have been shown to influence a number of cellular processes, for example cholesterol biosynthesis [246], receptor biosynthesis [246, 247], membrane signalling events (Ca^{2+} fluxes) [248, 249] and DNA synthesis [246], their effects on cell adhesion has not been documented.

The relationship of polyunsaturated fatty acids to the role of platelets in atherosclerosis and thrombosis has been of great interest for some time and the metabolism of fatty acids to thrombosis and prostoglandins in platelets and vascular tissue has been uncovered [250, 251]. Further research in the field of atherosclerosis has suggested that certain polyunsaturated fatty acids of nutritional significance may interfere with platelet functions; some long chain unsaturated fatty acids (eg. linolate or linolenate) at low concentrations may inhibit platelet aggregation [252 - 254]. Atherosclerosis is a disease that leads to 'fatty' lesions called atheromatous plaques on the inner surfaces of arteries. The early stage in the development of this disease is believed to be in the damage of endothelial cells and underlying intima. Once this damage has occurred, smooth muscle cells proliferate and migrate from the media of the arteries into the lesions. Soon thereafter lipids, especially cholesterol, begin to deposit from the blood onto these proliferating cells, forming the atheromatous plaques, simply often called cholesterol deposits. In the later stages of the lesions, fibroblasts infiltrate the degenerative areas and cause progressive sclerosis (fibrosis) of the arteries. However, it is not clear whether this migration of fibroblasts occurs as a result of the lipid deposition or

because of the muscle damage. In addition to these processes, calcium often precipitates with the lipids to develop calcified plaques, leading to atherosclerosis, the disease often referred to as 'the hardening of the arteries'. Despite the extreme prevalence of atherosclerosis, little is known about it's cause. In basic terms, the disease involves the deposition of a lipid layer on to a modified surface (the intima of the arteries following muscle damage) followed by an infiltration of fibroblast cells. Throughout this thesis, the behaviour of BHK fibroblasts has been well characterised on tissue culture and bacteriological grade plastic; after 6 hours in culture a confluent monolayer of cells is established on the former surface, whilst bacteriological grade ware is widely recognised for it's inability to support cell adhesion. Also in this work, some physico-chemical surface properties of both these forms of polystyrene have been established. Therefore, the anchorage dependent cell culture model may have a role to play in the research field of atherosclerosis. Any differences in the types or amount of lipids, or calcium, adsorbed to tissue culture or bacteriological plastic surfaces may aid the understanding of those properties that may be governing the establishment of atherosclerosis.

6.1.3. The effect of calcium in biological environments

The patchwork of ideas reviewed thus far implies that lipids may have a role in cell adhesion in cell culture, although there have been few investigations of physiological situations. Often in the literature the effects of lipid deposition are linked with the adsorption of calcium at the interface. This may be due to the action that this cation may possess as a stabiliser for adsorbed lipids. However, calcium itself is known to mediate it's own effects on cellular processes. Indeed it has been shown that calcium is important in maintaining normal intercellular contacts in some cells [255] and depletion of

calcium from physiological medium is a means of tissue disaggregation. Thus at the time of this study, calcium was known to affect a number of cellular mechanisms including secretary processes [256, 257], contraction of muscle cells [258 - 259] and membrane permeability [261 - 262]. Today it is commonly accepted today that under physiological conditions, cells require Ca^{2+} for spreading [263]. It is therefore possible that adsorbed lipids may provide anchorage sites for calcium which in turn may provide anchorage sites for cell attachment. Certainly work in the field of contact lens spoilation has shown that deposited lipids complex with calcium from tear fluid to stabilise 'white spots'.

Most cells in vivo are surrounded by biological fluid whose composition is kept constant by various neural and endocrinal mechanisms. Calcium also carries charge across biological membranes of excitable cells and influences excitability by affecting the kinetics of sodium and potassium permeabilities [264]. Calcium is essential for the functioning of ionic channels by binding to fixed negative charges on the cell surface [265] and has also been implicated in the optimisation of cell surface receptor responses to membrane active agents. In addition it has also been shown to be important in cellular motility and contractility [266 - 268]. Interestingly as far as cell - cell interactions are concerned, the presence of calcium has been shown to be crucial for the adherence of cells to one another; calcium deprivation causes cells to uncouple, compromising their ability to communicate with one another [269]. Such a phenomenon occurs when trypsin-EDTA is added to cells to remove them from surfaces. Calcium appears to have a relatively high affinity for negatively charged sites on the cell surface [265] and this has been considered to help explain the unique role that this cation plays in influencing excitable membranes, cell permeability and cell communication [270].

Calcium is known to deposit on a number of materials under physiological conditions but the greatest information in the literature has been concerned with it's deposition to cardiovascular devices [271, 272]. It appears form these reports, however that this calcification occurred after an interfacial layer had previously been established [272, 273].

This chapter will involve an investigation of the lipid classes that are present in foetal calf serum, the serum that optimises the conditions for BHK fibroblast growth. Attempts will be made to identify the lipids and calcium that may become adsorbed to bacteriological and tissue-culture plastic under physiological conditions.

6.2. Discussion of results

It had been known for some time that when polymeric materials contact a biological environment there is an active adsorption of protein from the biological system onto the material. This phenomenon was briefly outlined in Chapter 1. There are many systems where this situation is illustrated and examples include the contact lens in the eye, dentures in the mouth, prostheses inside the body and cells in culture. However, more recent work in the field of contact lens research has shown that lipids also become deposited out of the biological fluid (tears) onto the lens material during spoilation processes [221]. A number of analogies have already been observed between the synthetic/biological models mentioned above in terms of their physiological responses eg. protein deposition and micro-organism attachment. It was, therefore, considered important to carry out investigations to observe whether lipids became adsorbed onto polymeric materials during cell culture. If so, was it possible that they may mediate an effect on cell behaviour? As reviewed in the introduction to this section, lipids had been shown able to exert some affect on cell attachment when they had been precoated onto surfaces [43, 241, 243] or when cells were incubated with, for example, fatty acids [235 - 237]. However, experiments such as these are not a true representative of the physiological situation. It was, therefore, considered important to undertake initial experiments in the identification of those lipid components that become adsorbed to tissue culture and bacteriological grade polystyrene. These two polymer surfaces were chosen because throughout this thesis it has been well established that whilst tissue culture plastic supports confluent cell attachment and spreading, bacteriological grade ware does not.

Under cell culture conditions, culture medium is supplemented with serum. Whilst

there are a number of sera available for cell culture, the addition of foetal calf serum to the culture medium optimises the conditions for BHK fibroblast cells. This type of serum is prepared commercially following the collection of whole blood by cardiac puncture from a number of bovine foetuses. The blood is then pooled, chilled and allowed to clot. After centrifugation the serum is aspirated and frozen prior to use in cell culture [275].

6.2.1. Analysis of whole foetal calf serum

At the time of this study, there was little information available from either the literature or the manufacturers as to the relative concentrations of a number of components in foetal calf serum (FCS), particularly the lipid fraction. Therefore, it was considered necessary to carry out a series of investigations on FCS itself before any attempts could be made to investigate those species that may become adsorbed to the surface of the substrate during cell adhesion studies. FCS was subjected to a variety of analytical techniques that are carried out routinely by hospital biochemistry laboratories to clinically assist in the diagnosis of several disorders. The results of these tests are shown in Table 6.1 and represented in Figures 6.1 and 6.2. In this study, human serum was used as a control since values for the 'normal' or 'expected' range of results are easily available as shown in parentheses.

6.2.1.1. Cholesterol

Table 6.1 shows that the value for total cholesterol content in foetal calf serum was one eighth that for human serum. The most abundant use for cholesterol in the body is to form cholic acid and ultimately bile salts in the liver, although cholesterol is also a precursor for many steroids an animal tissues (eg. androgens, oestrogens,

Table 6.1. An analysis of a number of components present in foetal calf and adult human sera (values in parentheses are the normal or expected values)

Component	FCS	Human serum[e]
Sodium	136 (130-148)[a] mmol/l	141 (134 - 144) mmol/l
Potassium	> 5.2 (5.0-7.7)[a] mmol/l	5.1 (3.5 - 5.2) mmol/l
Creatinine	275 (42-71)[b] µmol/l	88 (50-125) µmol/l
Urea	5.7 (1.4-6.4)[a] mmol/l	3.8 (2.5 - 7.5) mmol/l
Total protein	39 (45-70)[a] g/l	75 (57 - 82) g/l
Albumin	22 (<28)[a] g/l	45 (28-46) g/l
Bilirubin	11 (<34)[a] µmol/l	10 (<17) µmol/l
Calcium	3.41 (1.5-2.5)[c] mmol/l	2.5 (2.2-2.6) mmol/l
Total lipid	1.6 g/l	N.D.
Total Cholesterol	0.75 (1.3-3.2)[a] mmol/l	5.7 (3.1-6.2) mmol/l
Free cholesterol	0.20 (0.4-1.1)[a] mmol/l	1.5 (1.1-2.1) mmol/l
Esterified cholesterol	0.55 (0.8-2.2)[a] mmol/l	4.2 (2.2-4.2) mmol/l
Total glyceride	0.55 (1.1)[d] mmol/l	0.5 (1.8) mmol/l

[a] values for neonates

[b] value for young at 2 - 3 weeks

[c] value for young at 0 - 7 days

[d] value at birth

[e] values for normal adults

Figure 6.1.Comparison of the lipid components of the two sera

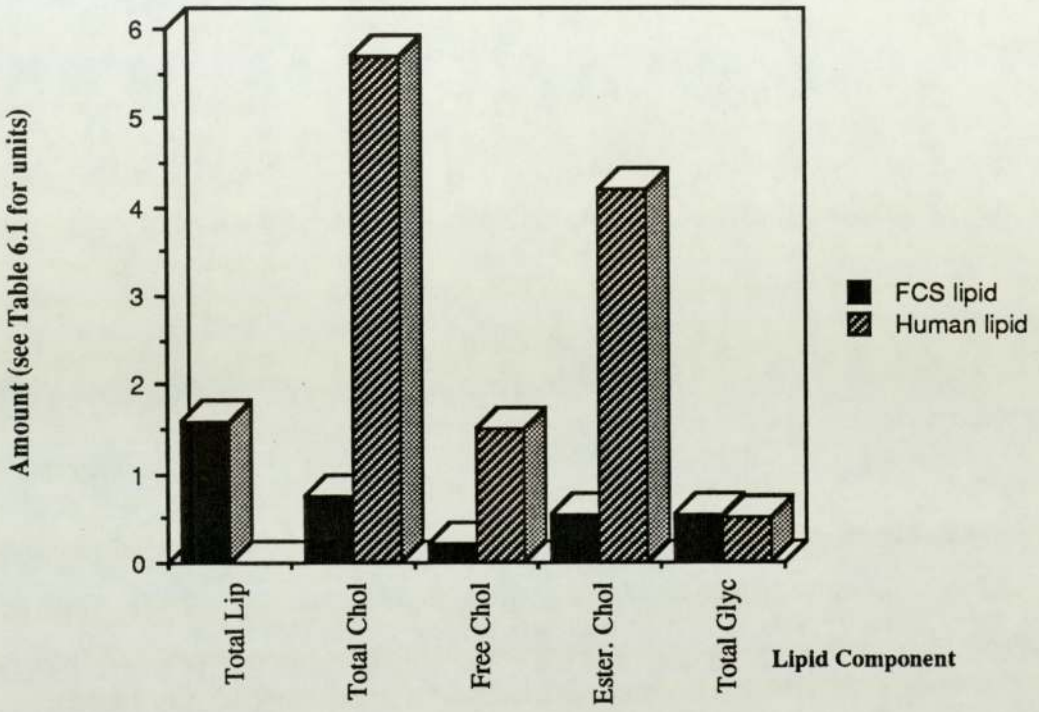
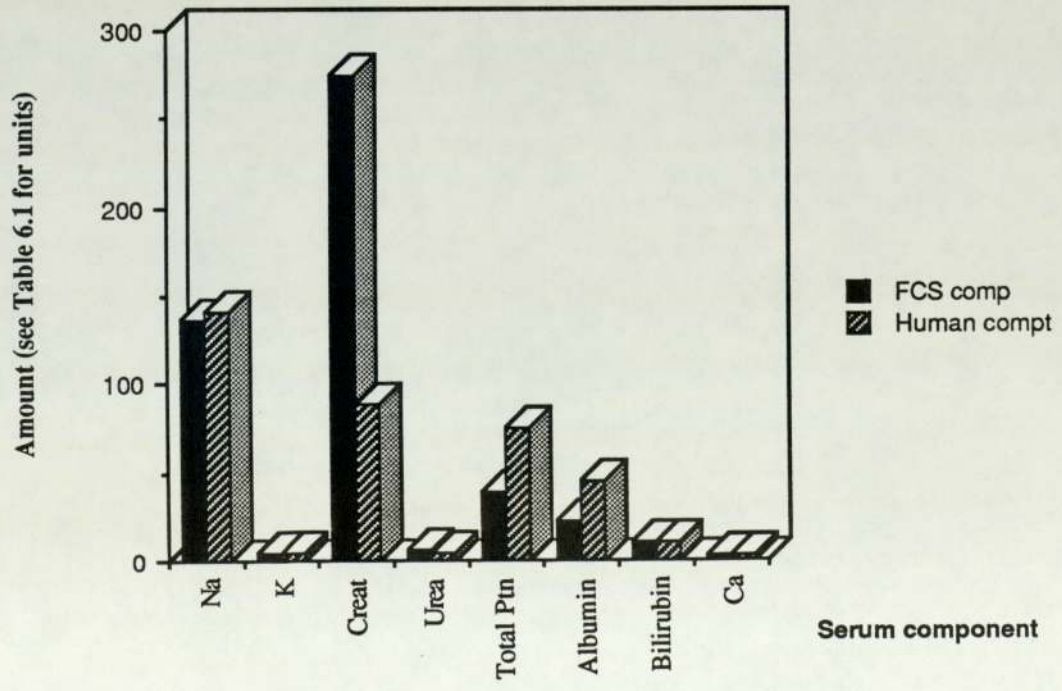


Figure 6.2.Comparison of the non-lipid components of the two sera



progesterones and adrenocortical hormones, although these would be of lesser importance in the foetus). However, as much as 80% of the cholesterol is converted into cholic acid, which is then conjugated with other substances in the hepatic cells to form bile salts to promote digestion and absorption of fats. Whilst the foetus utilises mainly glucose for energy, it has a high rate of storage of fat and protein, but most of the fat is synthesised from glucose rather than being absorbed from the mothers blood. This may explain why the amount of cholesterol circulating in the foetal serum is markedly less than that of the adult. In addition, there may be variations in the cholesterol content due to differences in species. The value for the free cholesterol (0.20 mmol/l FCS; 1.50 mmol/l human) was determined using an enzymatic colorimetric test. In vitro, the proportion of cholesterol that circulates in esterified form has been reported to be approximately 75% [276] of the total concentration, and the results in this study (0.55 mmol/l FCS; 4.20 mmol/l human) correlate with this value.

6.2.1.2. Total glyceride content

The total glyceride content (ie. the value for mono-, di- and tri- glycerides) was also measured using a high performance enzymatic colorimetric test and a higher value was obtained for FCS (0.55 mmol/l) than human serum (0.50 mmol/l). Glycerides, in particular triglycerides, are a ready energy source for the various metabolic processes that occur in vivo, a function which triglycerides share almost equally with carbohydrates. However, as mentioned above, it is glucose that normally acts as an energy source to the foetus.

6.2.1.3. Lipoproteins

Over 95% of all lipids that circulate in the blood of all animals are in the form of

lipoproteins. The protein in the mixture averages approximately a quarter to a third of the total constituents (the remaining fraction is lipid) for humans. However, there are no such values available for the situation in the foetus. The lipoproteins present in both foetal calf and human sera were analysed using an agarose gel electrophoresis system (zone electrophoresis) and the gels were stained with an Oil Red 'O' based dye Electrophoresis involves the motion of dissolved or suspended material under the influence of an applied electric field. Zone electrophoresis refers to the fact that the migration of the charged particles is supported by a negatively inert and homogeneous solid or gel framework in order to minimise convectional disturbances. The four lipoprotein classes are as follows:

chylomicrons (approximately 2000 Å), composed chiefly of triglycerides (90%) but also contain approximately 4% phospholipid, 6% cholesterol and 1% apoprotein B. These remained at the origin on the agarose gel;

β (low density lipoproteins, LDL;) with an approximate molecular weight of 1,300,000 and 100 - 300 Å in size. They contain relatively little triglyceride (7%) but a high percentage of cholesterol (35%). Following electrophoresis this group were found just anodic to the chylomicrons;

pre-β (very low density lipoproteins, VLDL) approximately 2000 Å in size which would contain high concentrations of both phospholipids (25%) and cholesterol (35%). Following electrophoresis this group were anodal to the β - lipoproteins.

α (high density lipoproteins, HDL) with an approximate molecular weight of 200,000 and a size of between 70 and 100 Å. They contain mostly protein (49%) with smaller concentrations of lipids. This group travelled the greatest distance.

All four of these classes were identified in human serum. There were traces (ie. << than human serum) of the α, β and pre- β lipoproteins in the foetal calf serum but no chylomicrons. Chylomicrons are important in the transfer of triglycerides from the intestine during digestion processes. However, the foetus derives it's nutrition directly across the placenta from the mother's blood as opposed to via an alimentary canal. Therefore chylomicrons are of little importance to the foetus and so, if they are present, may exist in small, non-detectable quantities.

6.2.1.4. Albumin and creatinine

It is shown in Table 6.1 that albumin composed approximately 56% of the total protein in FCS which is of interest because this protein is also a carrier for lipids. When fatty acids are bound they are referred to as non-esterified or 'free' fatty acids to distinguish them from those esterified with glycerol or cholesterol. As a rule, 3 fatty acid molecules combine with 1 molecule of albumin, although up to 30 fatty acid molecules can be carried if needed. It can also be seen in Table 6.1 that the concentration of creatinine, produced as a result of creatine (an important energy store) degradation [223] in the muscle, was high. Such high values are often attributed to high concentrations of vitamin C [277] although the value for this was not determined.

The above, relatively simple analytical tests were carried out with little preparative processes being carried out. However, techniques such as thin layer- (TLC), gas- (GLC) and high performance (HPLC) chromatographies require the lipid components to be extracted prior to investigation.

6.2.2. Analysis of the lipids extracted from foetal calf serum

6.2.2.1. Extraction techniques

Lipids have been previously defined as species that are soluble in organic solvents. The two main features of lipids which affect their solubility in organic solvents are the non-polar hydrocarbon chains of the fatty acids or other aliphatic moieties and any other functional groups, such as phosphate or sugar residues [278] . Lipids which contain no markedly polar groups, for example triglycerides or cholesterol esters are highly soluble in hydrocarbon solvents such as benzene or cyclohexane and slightly more polar solvents such as chloroform or diethyl ether. They are, however, less soluble in more polar solvents, for example methanol, although polar lipids dissolve readily in this solvent. Thus, the ideal solvent, or solvent mixture, used to extract animal lipids should be sufficiently polar to remove all lipids from any associations they may have. Because of this, it is generally believed that no single solvent is suitable as a general purpose lipid extraction. Therefore, mixtures of chloroform : methanol (2:1 v/v) and chloroform : isopropanol [279] (1:1 v/v) were added to foetal calf serum since these had previously been reported as being successful. After adding the solvents,a cloudy layer, which contained the remaining serum components floated above a clear lipid containing layer. However, the demarkation between these layers was more clearly defined after separation by the chloroform:methanol solvent system thus making it easier to remove the lipid containing fraction. It has been reported elsewhere [222] that this solvent system is most widely accepted in the field of lipid analysis and this mixture extracts lipids more exhaustively from all animal, plant and bacterial material than other solvent systems. To ensure maximum lipid extraction had occurred, fresh solvent mixture was added to the FCS three times, after which the extract was given a Folch wash [185] (x3) and was then dried under nitrogen. Following the extraction

Folch wash [185] (x3) and was then dried under nitrogen. Following the extraction process, the smallest glass containers as possible were used at all subsequent stages to prevent the lipids from drying as a thin film over a large area of the vessel. After drying down, care was also taken to minimise the length of time that the lipids were left in air and so samples were frozen under nitrogen. This latter precaution was taken to prevent the autooxidation of any polyunsaturated fatty acids which may occur rapidly if they are left unprotected in air. As mentioned in Chapter 2, care was taken to minimise any contamination as a result of handling of glassware. All glass vessels had previously been washed in a phosphate free detergent, rinsed thoroughly in distilled water before heat drying. Gloves were worn throughout lipid investigations, and solvents of research grade were stocked specifically for lipid analysis.

The differences in weight of the FCS before and after the extraction procedure allowed the concentration (by weight) of the total lipid fraction to be determined. The value of 3g/l for this is shown in Table 6.1.

The samples were then further analysed investigated using chromatographic techniques.

6.2.2.2. Thin layer chromatography (TLC)

Following the extraction of lipids from foetal calf serum using a chloroform:methanol (2:1 v/v) mixture, the lipid containing fraction was placed in a small glass vial, dried under nitrogen and frozen. Prior to analysis by TLC 200µl of chloroform were added to each vial and 50µl of this were applied to heat activated TLC plates. Neutral lipids as discrete spots and polar lipids as small bands. Two different solvent systems were used in the analysis of polar (diethyl ether : petroleum ether : formic acid 20:80:1) and neutral

lipids (chloroform : methanol : acetic acid : water 85:15:10:3.5). In addition to the samples of foetal calf serum, known standards were also applied to the plates. Each plate was placed into the appropriate solvent mixture for a period of 20 - 30 minutes after which time they were removed, air dried, sprayed with sulphuric acid and placed into a hot oven. After 3 - 5 minutes the plates were removed and several pink/red spots were observed. These corresponded to cholesterol and cholesterol esters [280] and their position was outlined by small indentations being made into the silica gel on the plate. The plates were then replaced in the oven and allowed to char (approximately 20 minutes) after which the R_f values for each of the sample spots and the standards were determined (Tables 6.2 and 6.3).

Table 6.2. R_f values for the neutral lipid standards used in the TLC analysis of foetal calf serum. (Distance moved by solvent = 15.3 cm)

Standard	Distance Moved (cm)	R_f
Sterol esters	11.5	0.8
Triglyceride	9.5	0.6
Free fatty acid	6.7	0.4
Cholesterol	2.8	0.2
1,3 Diglyceride	2.4	0.2
1,2 Diglyceride	1.9	0.1
Monoglyceride	0.7	0.05
Phospholipid	Remains at origin	

Table 6.3. R_f values for neutral lipid spots of foetal calf serum after TLC analysis (Distance moved by solvent = 15.3 cm)

Distance moved by spot(cm)	R_f	Lipid
11.7	0.8	Sterol esters
9.4	0.6	Triglyceride
6.7	0.4	Free fatty acid
2.7	0.2	Cholesterol
1.9	0.1	1,2 Diglyceride
Origin		Phospholipid

Table 6.4. R_f values of polar lipid standards used in the TLC analysis of foetal calf serum. (Distance moved by solvent = 13.5 cm)

Standard	Distance moved (cm)	R_f
Cholesterol, triglyceride cholesterol esters	11.6	0.9
Monoglyceride	10.1	0.8
Phosphatidyl ethanolamine	5.4	0.4
Phosphatidyl choline	2.7	0.2
Phosphatidyl inositol	1.7	0.1
Phosphatidyl serine	1.4	0.1

Table.6.5. R_f values of polar lipid spots of foetal calf serum after TLC analysis (Distance moved by solvent = 13.5 cm)

Distance moved by spot (cm)	R_f	Lipid
11.7	0.9	Cholesterol, triglyceride cholesterol esters
10.2	0.8	Monoglyceride
2.9	0.2	Phosphatidyl choline
1.7	0.1	Phosphatidyl inositol
1.4	0.1	Phosphatidyl serine

It can be seen from Tables 6.2 - 6.5, therefore, that foetal calf serum is chiefly composed of the following lipid classes:

Cholesterol, cholesterol esters, monoglyceride, 1,2 diglyceride and triglyceride, phosphatidyl -choline, -inositol and -serine, free fatty acids and phospholipids.

6.2.2.3. Gas-liquid Chromatography (GLC) of free fatty acids.

Following the extraction of total lipids from foetal calf serum by chloroform:methanol (2:1) the free fatty acids were converted to their methyl esters and identified using gas-liquid chromatography. Each peak in the spectrum (Figure 6.3), that corresponded to a free fatty acid was identified according to known standards available at the collaborating sponsors of this research (Unilever, Colworth). They may be listed as follows:

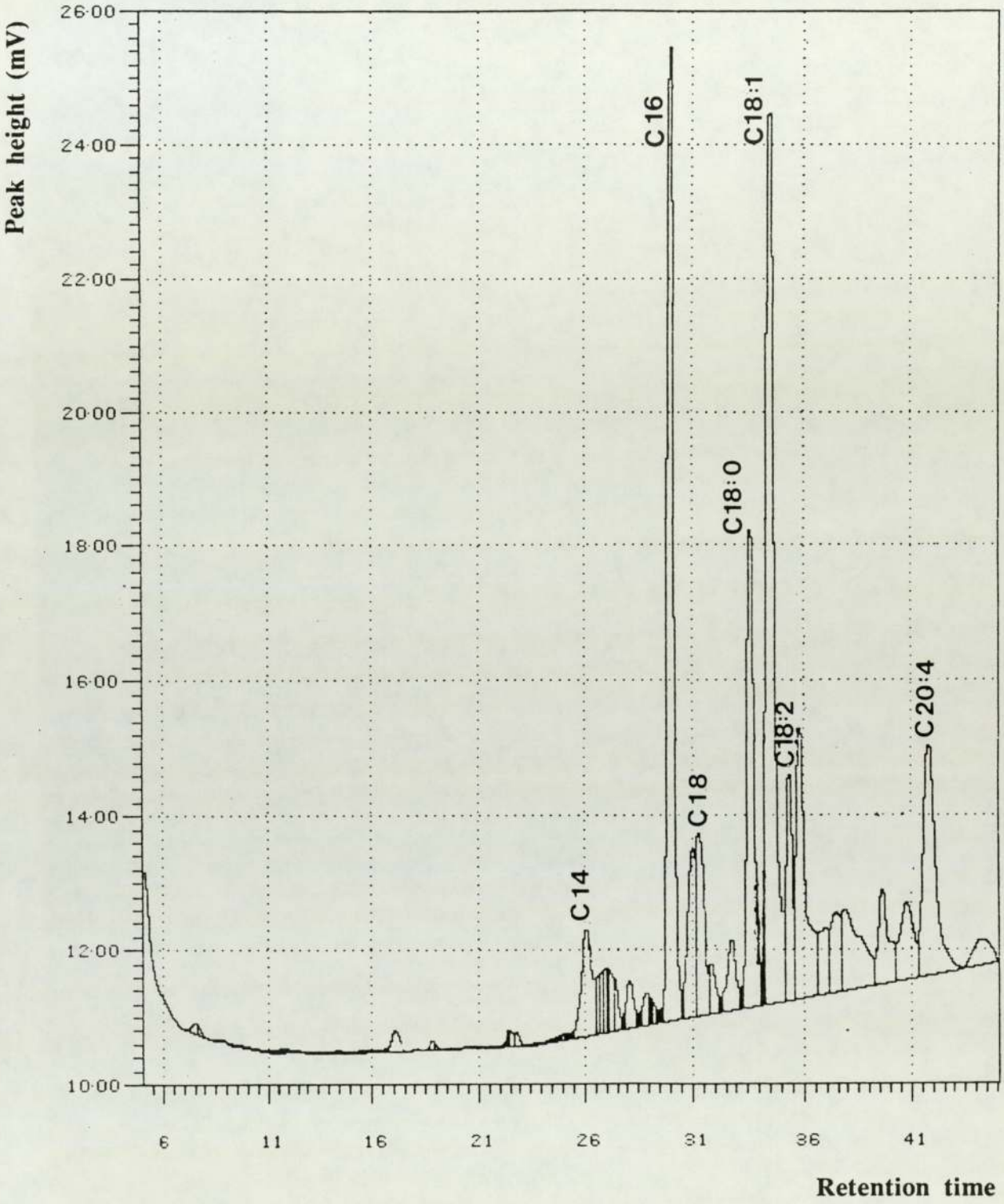
C14	(myristic)	C16	(palmitic)	C18:0	(stearic)
C18:1	(oleic)	C18:2	(linoleic)	C20:4	(aracidonic)

6.2.2.4. High performance liquid chromatography (HPLC)

The normal phase system used for HPLC analysis was that originally pioneered by Hamilton and Comai [281] . Thus the mobile phase used was hexane : propanol : acetic acid (1000:5:1).

Following the extraction of lipids from foetal calf serum (FCS) using a chloroform:methanol (2:1) mixture, the lipid containing fraction was placed in a small glass vial, dried under nitrogen and frozen. In addition, several controls were studied, where each procedure was carried out in a similar fashion but without the presence of

Figure 6.3. Chromatograph of GLC analysis of the methylated free fatty acids in foetal calf serum



foetal calf serum. Prior to HPLC analysis, 200µl of mobile phase were added to all the glass vials containing the frozen lipid and control extracts and 50µl were injected onto the column and the values of their retention times are shown in Table 6.6. In addition to these samples, 50µl standards of known concentration were also applied to the column. Both ultra violet (λ = 210nm) and fluorescence detectors were used in the analysis of the lipids. Lipids are detected by UV due to the presence of double bonds in the fatty acid or other functional groups eg. carbonyl, carboxyl, phosphate, amino and quaternary ammonium groups [224] . Fluoresence detectors identify those lipids with ester linkages, eg cholesterol esters, but not fatty acids or triglycerides. There were no peaks observed for the control runs (ie. those samples where all steps etc had been carried out in the absence of serum) but a typical HPLC trace obtained for FCS is shown in Figure 6.4 and a list of the retention times and area for each peak is given in Table 6.7. There was some difficulty experienced in comparing the retention times for these peaks with those for the known standards. There are two possibilities for this. First, the range of standards shown were selected as representatives of the lipid classes and are by no means a complete, comprehensive catalogue. Secondly, since the lipid extracts had been in contact with more solvents than the standards, this may have had a bearing on their retention times. No peaks correlated exactly, but some quantitation was carried out for a peak that matched most closely with the standards. By comparing the area of the peak for a known volume of sample, the amount of lipid for each of the classes found in the FCS sample was determined and results for this are shown in Table 6.7.The principal components of the serum were cholesterol esters, fatty acids and triglyceride. Cholesterol was not shown to be present by the HPLC technique, but from thin layer chromatography and the other analytical tests already carried out in this study this class is known to be a component in FCS. There are a number of

Figure 6.4. HPLC chromatograph of the lipids extracted from foetal calf serum

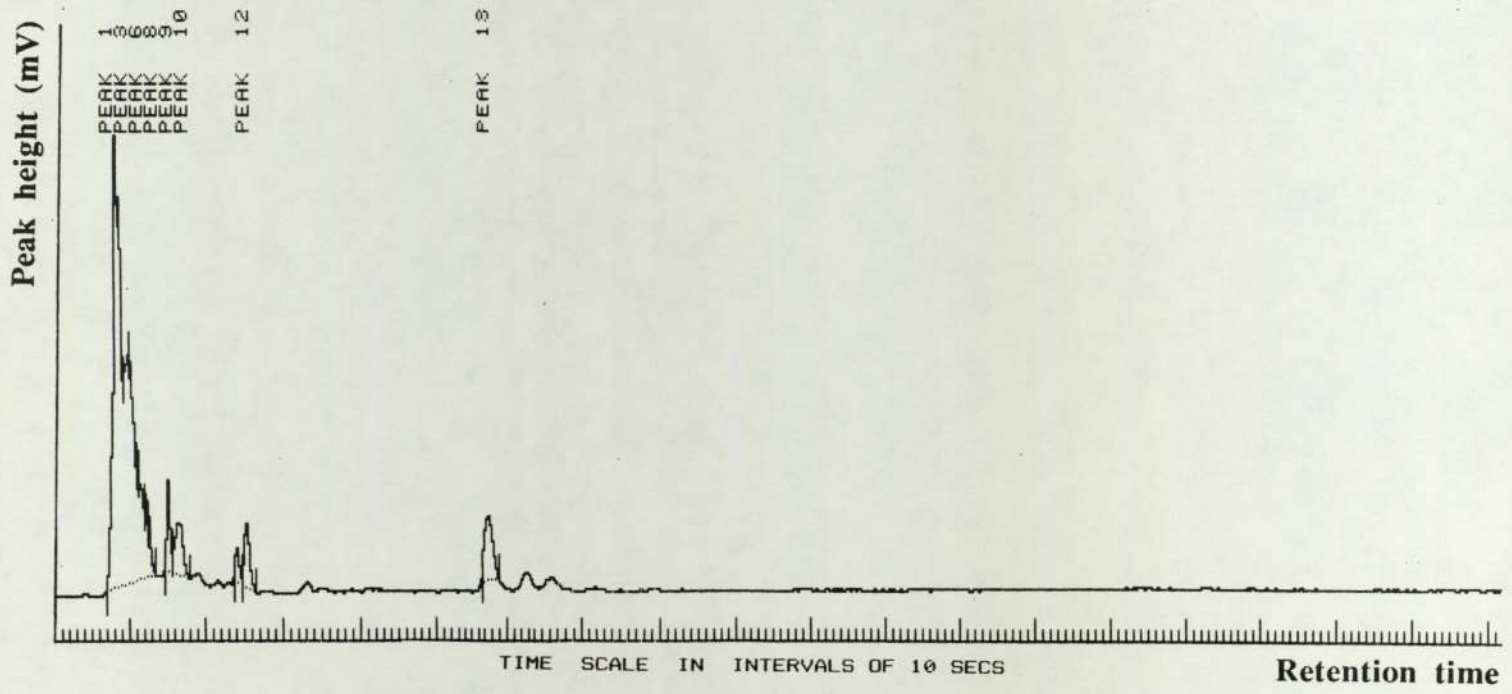


Table 6.6. Comparison of the retention times and areas determined by HPLC for a selection of lipids standards

Standard	Amount injected onto column (mg)	Retention time (secs)	Area
Cholesterol palmitate	0.067	76.61 - 100.10	11556.29
Trilaurin	2.5	121.26 - 169.96	102857.91
Triolein	0.042	121.91 - 143.85	10798.74
Arachidic acid	0.625	108.93 - 135.67	49545.21
Linolenic acid	2.5	114.07 - 129.53 270.03 - 290.65	922.00 3208.60
Oleic acid	2.5	158.08 - 189.76 263.69 - 329.04	71959.85 142733.62
Palmitic acid	1.25	170.63 - 205.15	13604.98
Stearic acid	2.0	225.45 - 274.08	
Linoleic	2.5	253.81 - 288.64	68879.02

Table 6.6. (continued) Comparison of the retention times and areas determined by HPLC for a selection of standards

Standard	Amount injected onto column (mg)	Retention time (secs)	Area
Phosphatidyl inositol	2.5	151.26 - 175.45	2563.77
Cholesterol	0.5	933.00 - 1039.08	214973.87

Table 6.7. HPLC analysis of those lipids extracted from foetal calf serum

Retention time (secs)	Lipid	Area	Amount per 50µl	Amount per 1200µl	Amount per litre
66.66 - 80.52	Cholesterol esters	16488.32			
80.52 - 87.78	"	15655.79			
87.78 - 93.06	"	7508.58			
93.06 - 104.62	"	13632.15			
104.62 - 105.94	"	1086.81			
105,94 - 112.54	Fatty acid	4051.38			
112.54 - 117.49	Arachidic acid ?	2458.08	0.05 mg	0.2 mg	1 g
117.49 - 128.05	Triglycerides	2032.54			
138.94 - 148.84	Triolein ?	2834.13	0.01 mg	0.04 mg	80 mg
148.84 - 169.63	Fatty acid	4010.27			
225.43 - 233.33	"	1346.72			
233.33 - 250.49	"	3221.43			
529.70 - 554.45	?	5056.45			

possibilities why this phenomenon occurred. First, the level present may be too low to be detected by HPLC. It was shown in Table 6.1 that the amount present in the free form was approximately one third that of the total cholesterol. In comparison, 75% was present in the esterified form. Secondly, since in vivo, cholesterol esters are an abundant source of cholesterol [246] it may be that this is the form in which this class of lipid was present in the FCS sample. Cholesterol esters come off the column quite rapidly using this solvent system [280] and the HPLC trace for FCS (Figure 6.4) shows a number of peaks at the beginning. In the whole animal there is an equilibrium set up between the ratio of cholesterol to cholesterol esters. It may, then, be possible that under the pressure from the HPLC system the equilibrium was pushed in the direction of the cholesterol esters. Also, cholesterol may not have been so readily soluble in the mobile phase used for HPLC and so adsorbed onto the walls of the glass vial. On the other hand it is readily soluble in chloroform [222] and so was extracted more easily off the glassware used prior to TLC analysis.

From the data presented so far it has been shown that there are a number of lipid classes present in foetal calf serum and it was possible that in culture some of these may modify the surface and/or have an effect on cell behaviour. For example it had already been stated in the literature that cholesterol esters were an important source of cholesterol and possibly fatty acids for fibroblast cells [246] . Therefore, since foetal calf serum was a rich source of cholesterol esters then this may have been just one way in which FCS exerts an effect on BHK fibroblast cells in culture. Also investigations into the fatty acid content by their conversion to methylated esters showed that there was an array of both saturated and unsaturated free fatty acids present in the culture serum. It

had been postulated by some workers that unsaturated fatty acids decreased cell adhesion whilst saturated fatty acids increased both cell attachment and spreading [235 - 237] . Thus it seemed worthwhile to investigate whether there were any variations in the adsorption of lipids to surfaces with different capacities of supporting cell adhesion. As previously mentioned, the two surfaces most widely characterised for their effects on cells under culture conditions are tissue culture and bacteriological dishes. It has been highlighted throughout this research that the former surface supports confluent cell attachment and spreading whilst bacteriological grade plastic does not. Therefore, steps were taken to prepare an adsorbed layer onto petri dishes made from tissue culture and bacteriological polystyrene, the lipids were extracted and analysed by TLC and HPLC.

6.2.3. Analysis of those lipids adsorbed to tissue culture and bacteriological grades plastic under cell culture conditions

6.2.3.1. Preparation of adsorbed layers

20 ml of Dulbecco's Modified Eagles Medium that had been supplemented with 10% foetal calf serum (FCS) were placed into tissue culture and bacteriological grade petri dishes. Control dishes were also set up where serum was absent but all the following steps were still carried out. All dishes were incubated at 37°C for 6 hours after which time they were rinsed with phosphate buffered saline (PBS). Care was taken to remove all the PBS since any residual water can markedly affect the properties of any organic solvents used in the extraction procedure.

6.2.3.2. Extraction of lipids from the layers adsorbed to tissue culture and bacteriological grade plastic under culture conditions

The properties of lipids that affect their extraction have been outlined above and it was stated that use of one solvent only is not usually the most effective in removing all lipids from their associations. Solvents really need to be able to extract both polar and neutral lipids. Therefore, in an attempt to extract any lipoidal material that may have become adsorbed to the two types of dishes under culture conditions, the following solvent systems were investigated:

carbon tetrachloride	hexane	methanol
diethyl ether	chloroform:methanol (2:1)	

However it was methanol alone that proved to be the most successful solvent. All the others tended to interact with the substrate itself and resulted in the dissolving of the polystyrene. Other workers have also experienced this problem when using 'plastic' ware during extraction processes [222]. The interactions between the material and solvent result in the leaching out of plasticisers from the polymer. However one of the problems that was anticipated to occur was that methanol might not be successful for the extraction of less polar lipids, eg cholesterol. Despite this, 3 x 10ml of methanol was added to the sample and control dishes followed by a Folch wash. The extracts fom 5 tissue culture, bacteriological or control dishes were pooled and the samples were dried under nitrogen. The samples frozen for minimal time prior to analysis.

6.2.3.3. TLC analysis of extracted lipids.

200 µl of chloroform was added to each of the dried samples and 50µl of each along

with known standards were applied to activated TLC plates in the same way as for foetal calf serum. Again known standards were applied and the same solvent systems for the polar and neutral were used as before and the plates were run for 20-30 mins. After this time they were removed from the solvents, air dried, sprayed with sulphuric acid and then placed in an oven for 2-3 mins. The pink/red spots were marked as before and both plates were then replaced in the oven to completely char.

A comparison of the R_f values for the standards (Table 6.8) against those for the spots obtained for the extracted lipids off both tissue culture and bacteriological plastic (Tables 6.9 and 6.10) revealed that there was no real difference in the classes of lipids adsorbed to the surfaces and the chief classes adsorbing were shown to be cholesterol, cholesterol esters, triglyceride and free fatty acids. There was no polar lipid extracted off either tissue culture or bacteriological grade plastic which was interesting because these type of lipids would be more readily soluble in methanol than the less polar lipids.

Table 6.8. R_f values of the polar and neutral lipid standards used in the analysis of lipids extracted from adsorbed layers to tissue culture and bacteriological grades polystyrene.

Neutral lipids (Distance moved by solvent = 17.0 cm)

Standard	Distance moved (cm)	R_f
Cholesterol esters	14.5	0.9
Triglyceride	12.1	0.7
Fatty acid	8.1	0.5
Cholesterol	3.3	0.2
Phospholipid	remains at origin	

Polar lipids (distance moved by solvent = 13.3 cm)

Standard	Distance moved (cm)	R_f
Cholesterol,triglyceride cholesterol esters	10.5	0.8
Phosphatidyl ethanolamine	8.2	0.6
Lecithin	5.5	0.4
Phosphatidyl choline	1.8	0.1

Table 6.9. TLC analysis of the neutral lipids extracted from the layers adsorbed to tissue culture and bacteriological grade plastic under cell culture conditions (Distance moved by solvent front = 17.0 cm)

Tissue culture plastic

Distance moved by spots (cm)		Mean distance (cm)	R_f	Lipid
14.5	14.5	14.5	0.9	Cholesterol esters
11.1	11.1	11.1	0.7	Triglyceride
7.6	7.7	7.8	0.5	Fatty acid
3.0	3.0	3.0	0.2	Cholesterol
Origin	Origin	Origin		Phospholipid

Bacteriological grade plastic

Distance moved by spots (cm)		Mean distance(cm)	R_f	Lipid
14.5	14.5	14.5	0.9	Cholesterol esters
11.1	11.1	11.1	0.7	Triglyceride
7.7	7.8	7.8	0.5	Fatty acid
3.0	3.1	3.1	0.2	Cholesterol
Origin	Origin	Origin		Phospholipid

Table 6.10. TLC analysis of the polar lipids extracted from the adsorbed layers to tissue culture and bacteriological grade plastic under cell culture conditions.(Distance moved by solvent = 13.3 cm)

Tissue culture plastic

Distance moved by spot (cm)		Mean	R_f	Lipid
10.5	10.5	10.5	0.8	Cholesterol, triglyceride and cholesterol esters

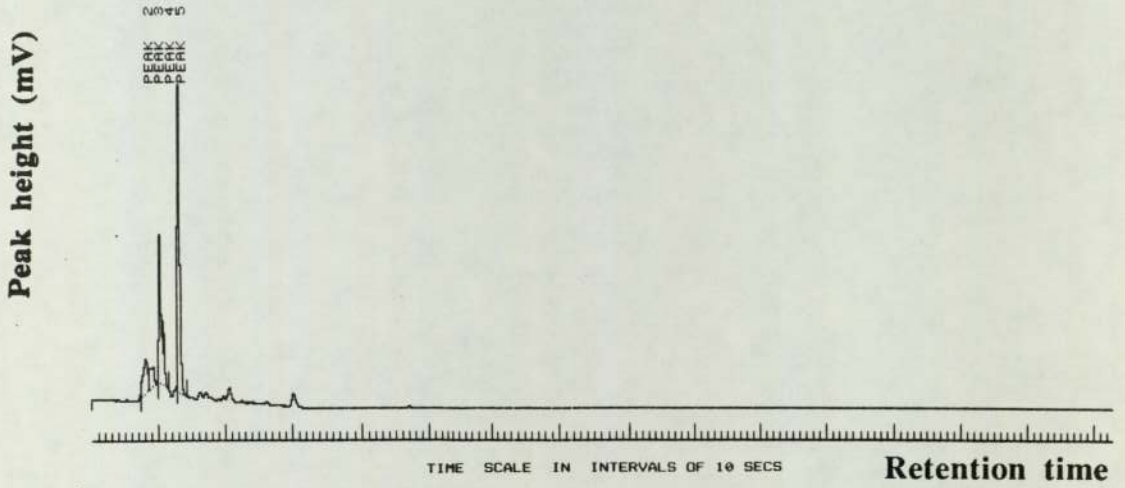
Bacteriological grade plastic

Distance moved by spot (cm)		Mean	R_f	Lipid
10.5	10.5	10.5	0.8	Cholesterol, triglyceride and cholesterol esters

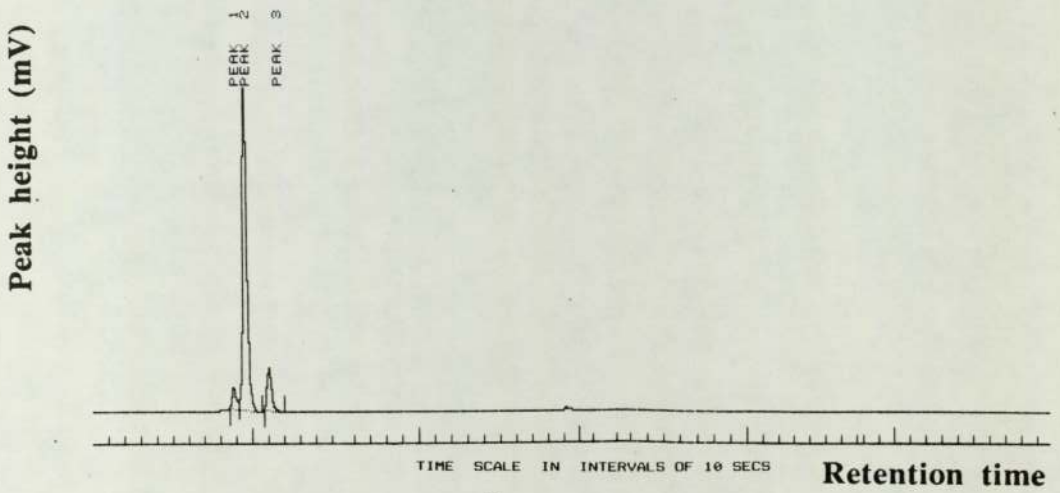
6.2.3.4. HPLC analysis of extracted lipids

As for those from foetal calf serum, the 200µl of mobile phase was added to the dried lipid extracts from the adsorbed layers on tissue culture and bacteriological grades plastic. 50µl was then applied to an HPLC system and the results of the retention times is shown in Tables 6.11 and 6.12. The chromatographs of lipids extracted from the two experimental substrates are shown in Figure 6.5. A variety of standards representing a

Figure 6.5. HPLC chromatographs of the lipids extracted from bacteriological and tissue culture grade plastic following cell culture conditions



bacteriological grade plastic



tissue culture plastic

Table 6.11. HPLC analysis following the extraction of those lipids that adsorbed onto tissue culture plastic during cell culture conditions

Retention time (secs)	Lipid	Area	Amount per 50µl	Amount per 200µl (=5 dishes)	Amount per dish (mg) (~ 55cm^2)
83.79 - 89.29	Cholesterol palmitate ?	651.16	0.07 mg	0.28 mg	56µg
89.27 - 103.12	Cholesterol ester	10352.17			
105.06 - 117.63	Arachidic acid ?	1551.89	0.02 mg	0.08 mg	16µg
123.22 - 137.66	Triolein ?	12482.47	0.05 mg	0.2 mg	40µg

Table 6.12. HPLC analysis following extraction of those lipids that adsorbed onto bacteriological grade plastic during cell culture conditions

-

Retention time (secs)	Lipid	Area	Amount per 50µl (mg)	Amount per 200µl (mg) (=5 dishes)	Amount per dish (mg) (~ 55cm^2)
71.26 - 83.52	Cholesterol ester	2850.00			
83.52 - 96.42	Cholesterol palmitate ?	1980.18	0.3 mg	1.2 mg	240 µg
98.03 - 104.48	Cholesterol ester	5654.71			
104.48 - 113.51	Arachidic acid ?	2408.88	30.4 mg	0.52mg	3µg
123.83 - 138.34	Triolein ?	13661.47	0.05 mg	0.2 mg	40 µg

number of lipid classes were selected and dissolved in mobile phase to give the concentrations shown in Table 6.6. 50µl of each standard were also applied to the HPLC column and the results for their retention times and areas are also recorded in Table 6.6.

6.2.4. Investigation of calcium deposits onto bacteriological and tissue culture plastic

The two polymeric materials chosen for this study were tissue culture and bacteriological grade polystyrene since the cell response to each of these has been well characterised throughout this study. Tissue culture plastic is associated with being able to support confluent cell adhesion and spreading after 6 hours in culture, whilst bacteriological grade plastic, essentially untreated polystyrene is non-cell adhesive after this time. There had been suggestions in the literature that calcium becomes adsorbed to biomaterials during mineralisation processes and calcium is a well known mediator in a number of cellular functions. It has also been shown in this study that foetal calf serum is a rich source of calcium; the value of 3.41 mmol/l is above the expected range for both adult and infant human serum. It was, therefore, considered reasonable to investigate whether calcium played a part in the adhesion of BHK fibroblast cells to substrates via a role in interfacial conversion processes. To investigate this possibility, samples of both types of polymeric material were cut out of petri dishes using a heated cork borer. The samples were then incubated at 37°C for 6 hours, after which time they were rinsed with phosphate buffered saline (made up without calcium ions). It had been shown [221] that one technique, namely X-ray micro analysis (EDXA), was very successful in the examination of calcium deposition onto contact lenses and optimum

results had been produced when the samples had first been prepared using the same techniques as those for scanning electron microscopy. Thus, after washing the samples in phosphate buffered saline they were fixed in a sodium cacodylate/glutaraldehyde buffer and the procedure described in Chapter 2 was followed. Prior to examination by EDXA the samples were mounted onto aluminium stubs by means of double sided sticky tape and examined for 100 seconds. Control specimens were also studied, whereby samples of the two types of material were cut out but were incubated without the presence of serum. All the other steps were carried out as for the experimental samples.

A comparison of the results of the controls with the experimental surfaces showed that there was little detected in terms of adsorbed elements which suggested that any deposited species had been removed during the preparatory stages. Thus a second technique was derived whereby after incubation the samples were rinsed in phosphate buffered saline (without calcium ions), dried in an oven, mounted onto stubs and viewed as before. Following this technique, it can be seen (Tables 6.13 and 6.14, Figures 6.6 and 6.7) that there was more calcium deposited onto the tissue culture surface than onto the bacteriological grade surface. In addition, as represented in Figure 6.8, there were also differences in the quantities of sodium, chlorine and potassium that became adsorbed all of which are important in cell function. For example, in all cells the sodium - potassium pump allows sodium to pass out of the cell and potassium in so that the correct osmotic balance can be maintained across the cell. Calcium and magnesium may be transported in a similar way, although there was no magnesium detected on the samples. As a comparison, it can be seen that apart from silica (possible remains of mould release) there was little else detected on the control surfaces.

Table 6.13. The quantities of elements detected by EDXA (expressed as counts per 100 secs) deposited onto tissue culture plastic under cell culture conditions.

Surface	Element	Counts per 100 secs		Mean
Control	Sodium	0	0	0
	Silica	30	48	39
	Phosphorus	0	0	0
	Chlorine	0	0	0
	Potassium	0	0	0
	Calcium	0	0	0
+ Culture medium	Sodium	16	6	11
	Silica	66	14	40
	Phosphorus	72	32	52
	Chlorine	342	106	224
	Potassium	13	8	11
	Calcium	186	62	124

Table 6.14. The quantities of elements detected by EDXA (expressed as counts per 100 secs) deposited onto bacteriological grade plastic under cell culture conditions

Surface	Element	Counts per 100 secs		Mean
Control	Sodium	0	0	0
	Silica	10	14	12
	Phosphorus	0	0	0
	Chlorine	0	0	0
	Potassium	0	0	0
	Calcium	0	0	0
+ Culture medium	Sodium	11	31	21
	Silica	9	61	35
	Phosphorus	11	85	96
	Chlorine	134	458	296
	Potassium	0	0	0
	Calcium	22	126	74

Figure 6.6. EDXA analysis of the surface of bacteriological grade plastic following cell culture conditions

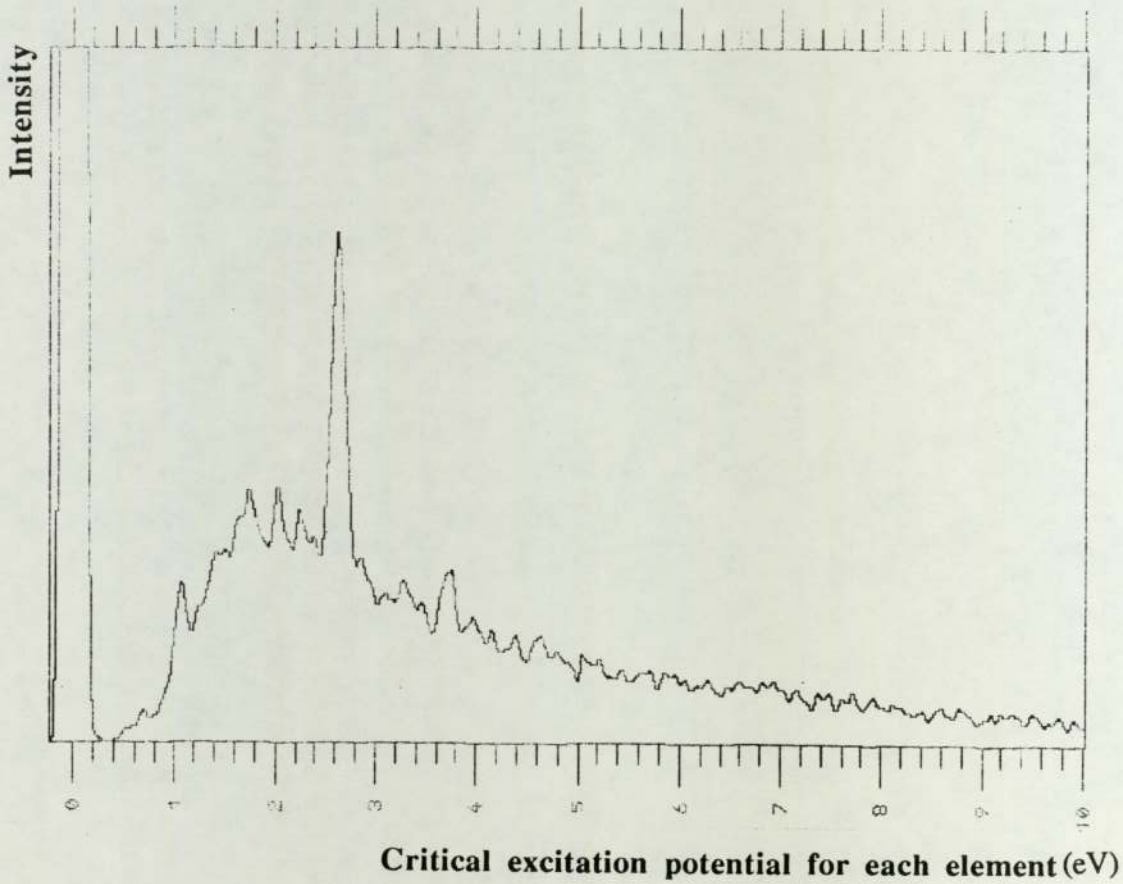


Figure 6.7. EDXA analysis of the surface of tissue culture plastic following cell culture conditions

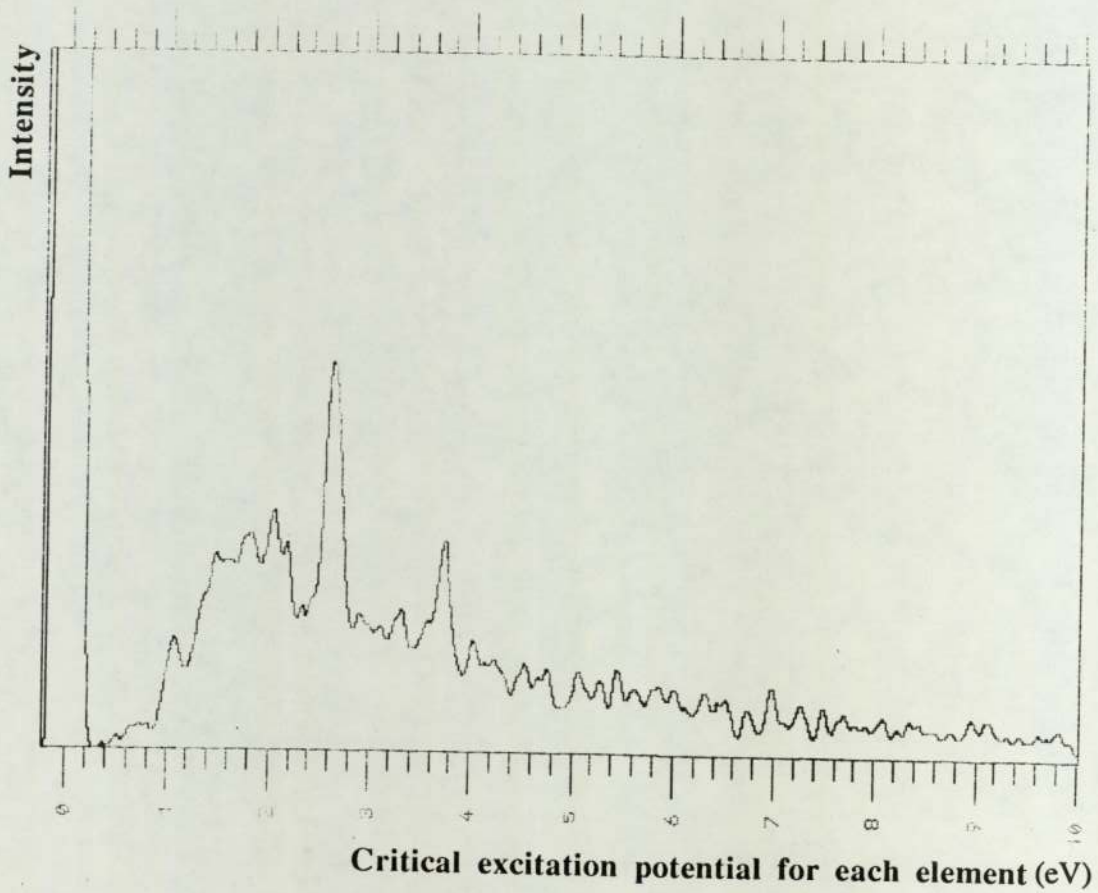
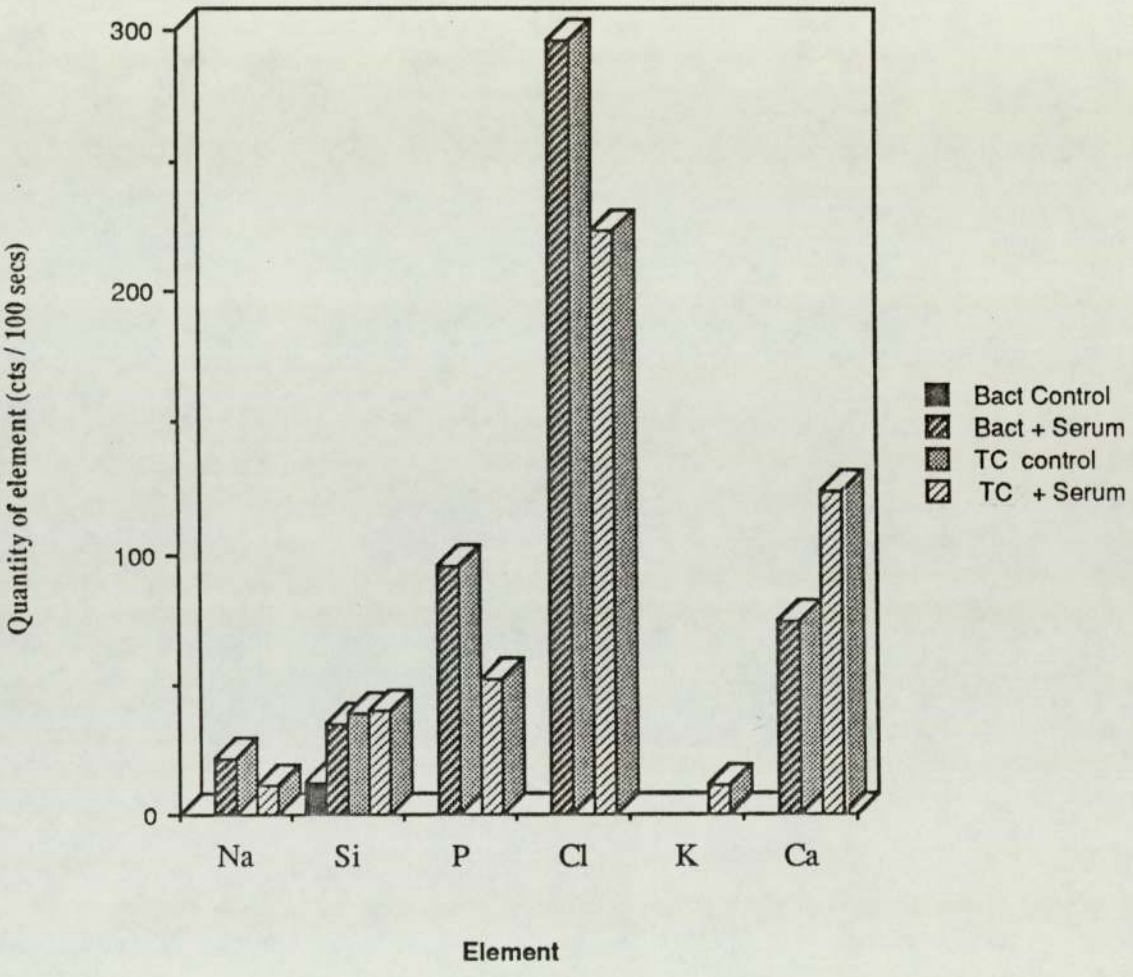


Figure 6.8. To show the deposition of elements onto tissue culture and bacteriological grade plastic following cell culture conditions, and for control samples



6.3. Concluding discussion

One important step forward made as a result of this study is that a profile of some of the components of foetal calf serum has been determined. At the time of this research, information of this nature was not available to the cell biologist from either the literature or the manufacturer. However, since these results have been achieved, a few details on some foetal calf serum components have become available on request from the manufacturer, but only for limited serum batches. The profile produced during this current research is by no means complete as there are a number of other factors which are present in other sera, eg magnesium, phosphate, etc that have not been measured here, some, or all, of which may also effect cell behaviour. However, those components that have been identified and quantitated are of fundamental importance to all researchers investigating those properties of the foetal calf serum that may mediate an effect on cells in culture. It had been suggested in the literature that both lipids and calcium may be important in effecting the cellular response [43, 234, 236 - 253]. These components may have mediated their effects prior or during cell adhesion either directly on the cells themselves, or may have acted via their adherence to the culture substrate. This study has shown that foetal calf serum, that type which promotes maximum attachment and spreading of BHK fibroblast cells under ambient conditions, is a rich source of lipids (1.6 g/l) and calcium (3.41 mmol/l). During the analysis of the lipid components, it was shown that there was no single analytical technique available that would successfully identify and quantify all the lipid components. However, it was shown that the incorporation of a variety of methods allowed a general overview to be gradually built up. The most successful solvent system for the extraction of lipids from the foetal calf serum was shown to be a chloroform : methanol (2:1 v/v) mixture. The use of a range of chromatographic and other analytical techniques showed that for foetal

calf serum itself the main lipid classes present may be listed as follows:

fatty acids, both saturated and unsaturated;

glycerides, mono-, di- and tri- glycerides;

cholesterol, predominantly in the form of cholesterol esters (approximately 75%) and

phospholipids, predominantly phosphatidyl choline, but also traces of phosphatidyl--serine and -inositol.

It was also shown that there were differences in the values of some of these components for adult human serum. This was interesting because human serum itself has previously used to supplement cell culture medium of human cell lines, although nowadays other sera are routinely used. The differences between the foetal calf and human sera may have arisen not only to differences in age and species but also to the duration time between which the sample was taken and it's analysis. The manufacturers have little idea of the length of time it takes before serum reaches the customer from the source or at what stage of gestation the serum is withdrawn [282].

This study shows that lipids are extractable from the interfacial layer following treatment with methanol. A number of solvents were also tested for their extraction capabilities and these included chloroform : methanol (2:1 v/v), carbon tetrachloride, hexane, diethyl ether and methanol but the main drawback was that these solvents interacted with the polystyrene itself. Thus, methanol proved to be the most successful although it is possible that this solvent alone was not powerful enough to remove all adsorbed lipid species. Further difficulties arose in that because the quantities of lipids

extracted were present in such low quantities the analytical techniques were at their limit of sensitivity. However, this problem was partly overcome by combining extracts from five dishes (either tissue-culture or bacteriological plastic or controls) prior to drying under nitrogen. Of those lipids that were extracted from tissue culture and bacteriological grades plastic, there were no gross differences in the lipid classes between the two substrates. This may have been due to the fact that the techniques used were not sensitive enough to identify any small differences. There were difficulties in reproducing spectra for those lipids eg. cholesterol that were known to be present and any subtle variations between the two surfaces may have been masked. However, this study has enabled an overall idea of the types of lipids that become adsorbed to be highlighted and the results show that under cell culture conditions, some lipids are adsorbed to both tissue culture and bacteriological grades plastic. However, there is inconclusive evidence to allow a definite decision to be made about the influence that lipids have in mediating either cell attachment or spreading.

One feature common to all the chromatographic techniques employed in this study is that they all involved the transfer of samples between a number of different glass vessels. It is possible that some lipids had a greater affinity for the glassware than they may have had for the solvents which may have resulted in some loss during this transfer.

One of the obstacles encountered during this study was that it remained unclear as to whether methanol was a strong enough solvent to remove all the lipoidal material out from the adsorbed interfacial layer. In further experiments where the extraction of lipids from culture substrates is required it may be more advantageous to replace polystyrene

ware with hydrogel polymers. The work of Minett [85], as earlier mentioned in Chapter 5, characterised that whilst poly(2-hydroxy ethyl methacrylate) (polyHEMA) itself showed similar cell adhesion responses to bacteriological grade plastic, it's co-polymerisation with, eg. styrene, methyl methacrylate (MMA) or ethyl methacrylate (EMA) rendered this hydrogel markedly more cell adhesive. Thus, substrates made of polyHEMA with poly (HEMA / styrene), poly(HEMA / MMA) or poly (HEMA / EMA) substrates instead of bacteriological and tissue culture plastic in studies may be able to withstand more 'harsh' solvent extraction mixtures because of their cross linked structure. In addition, these hydrogel substrates have the added advantage that following the extraction procedures, they can be examined for complete lipid removal by spectrofluorometric techniques.

It is shown in Table 6.1 that FCS is rich in calcium, as are a number of other biological fluids [283]. These include sea water, saliva and tears which have all mentioned in Chapter 1 as having the capability to interact with non-biological materials and ultimately lead to the adsorption of an interfacial layer. Calcium was reviewed in section 6.1.3 of this study as being an important mediator in cellular processes and it is possible that the presence of this cation in foetal calf serum may allow the maintenance of cellular processes after adhesion has occurred.

It had previously been shown that the preparatory techniques for SEM studies provided optimum results for the investigation by EDXA of calcium deposition onto contact lenses [221]. However, this method did not prove to be the most successful in this study since the preparation of tissue culture and bacteriological polystyrene samples in this way appeared to remove any adsorbed layer. However, following their incubation

at ambient temperatures, samples were rinsed in phosphate buffered saline (without Ca^{2+} or Mg^{2+}) and dried prior to analysis. Tables 6.13 and 6.14 and Figure 6.8 show that following this preparation of surfaces differences were observed in the amounts of calcium, and other elements, that became adsorbed to both tissue culture and bacteriological grades plastic. Thus, it is possible that in addition to the above suggestion that the presence of this cation may maintain cellular processes after attachment has occurred, it may play an earlier role in the adhesion process. In addition, it can also be seen (Tables 6.13 and 6.14; Figure 6.7) that since other elements became adsorbed to the substrates and it may be worthwhile conducting further research as to whether these may affect cell behaviour under similar culture conditions to those investigated in this study.

CHAPTER 7

Conclusions and suggestions for further work.

One of the most important features that has been highlighted throughout the last three years of research is that an interdisciplinary programme, incorporating some areas of both cell biology and polymer science, is one of the most powerful and fruitful approaches for the systematic investigation of the effects of synthetic polymers on biological systems. Prior to this study the majority of investigations had involved cell biologists investigating the cellular reactions often to a limited range of polymers about which they knew very little. In addition, materials and polymer scientists often had little understanding of the biological interactions with the materials they had produced. Whilst a number of physico-chemical properties had been been put forward as having a role in mediating cell behaviour (for example wettability [36, 61, 97, 100 -104], rugosity [93, 95, 105], polarity [94, 97 - 99], surface charge [18, 21, 62, 106, 107], crystallinity [85] and chemical group expression [92 - 96]) there was no overall consensus as to the relative importance of each of these factors in triggering the cell adhesion response of anchorage dependent cells. Indeed, the overall state of opinion about those mechanisms that governed cell adhesion behaviour was one of confusion and there were conflicting reports in the literature. Certainly part of this confusion was due to the multi- rather than inter- disciplinary nature of the studies that had previously been carried out. Therefore, the combination of certain aspects of polymer and physical science together with selected areas of cell biology in order to systematically investigate some of those properties that affect the biological response, as in this study, appears to be a successful approach.

Whilst the results of this investigation do not highlight one sole physico-chemical property as being important in governing cell behaviour, it was shown that some factors are more important than others. For example, this study showed that it was not the morphology or the crystallinity of certain cell adhesive polymers that were triggering this biological behaviour. Indeed, it was also shown that for a carefully selected range of

polymers, cell adhesion did not depend on the structure of these polymers or the presence of dipolar moments per se. During this series of investigations it was revealed that interactions that had occurred between the molten polymer and environment oxygen during manufacturing processes had a strong influence on cell adhesion. It was discovered that when polymer substrates had been produced by the calendering process they were able to support high levels of cell adhesion and spreading. In contrast, when the same polymers were manufactured into articles using such techniques as spin-coating or melt pressing, there was little cell adhesion observed. Interestingly, further investigations by ESCA and contact angle techniques revealed that those surfaces that had been produced by calendering processes had more oxygen at their surfaces and were more polar than those substrates that had been manufactured by the other methods. During the calendering processes the molten polymer is subjected to high, shear forces and is exposed to environmental oxygen which would ultimately lead to a more oxidised surface, phenomena which would not occur using the spin coating or melt pressing techniques. Thus, the presence of an oxidised surface is an important property for allowing cell adhesion to take place.

It was shown during this research programme that the treatment of polystyrene by sulphuric acid under quite mild conditions predominantly led to a hydroxyl groups (-OH groups) being introduced at the surface. This was an important finding because there had previously been some controversy as to which surface groupings had been produced following this treatment [20 - 21] . It was also of interest that when the polystyrene was treated more harshly to facilitate the introduction of negatively charged sulphonate groups ($-HSO_3$), cell spreading was markedly inhibited. However, the acid treatments that had been used allow this sulphonation to take place may also have led to a concurrent hydroxylation. Thus, these latter surfaces would have contained domains of

both hydroxylated and sulphonated groups and it may have the relative proportion and positions of these 'islands' that facilitated the cell response. This phenomenon may be explained by the following hypothesis:

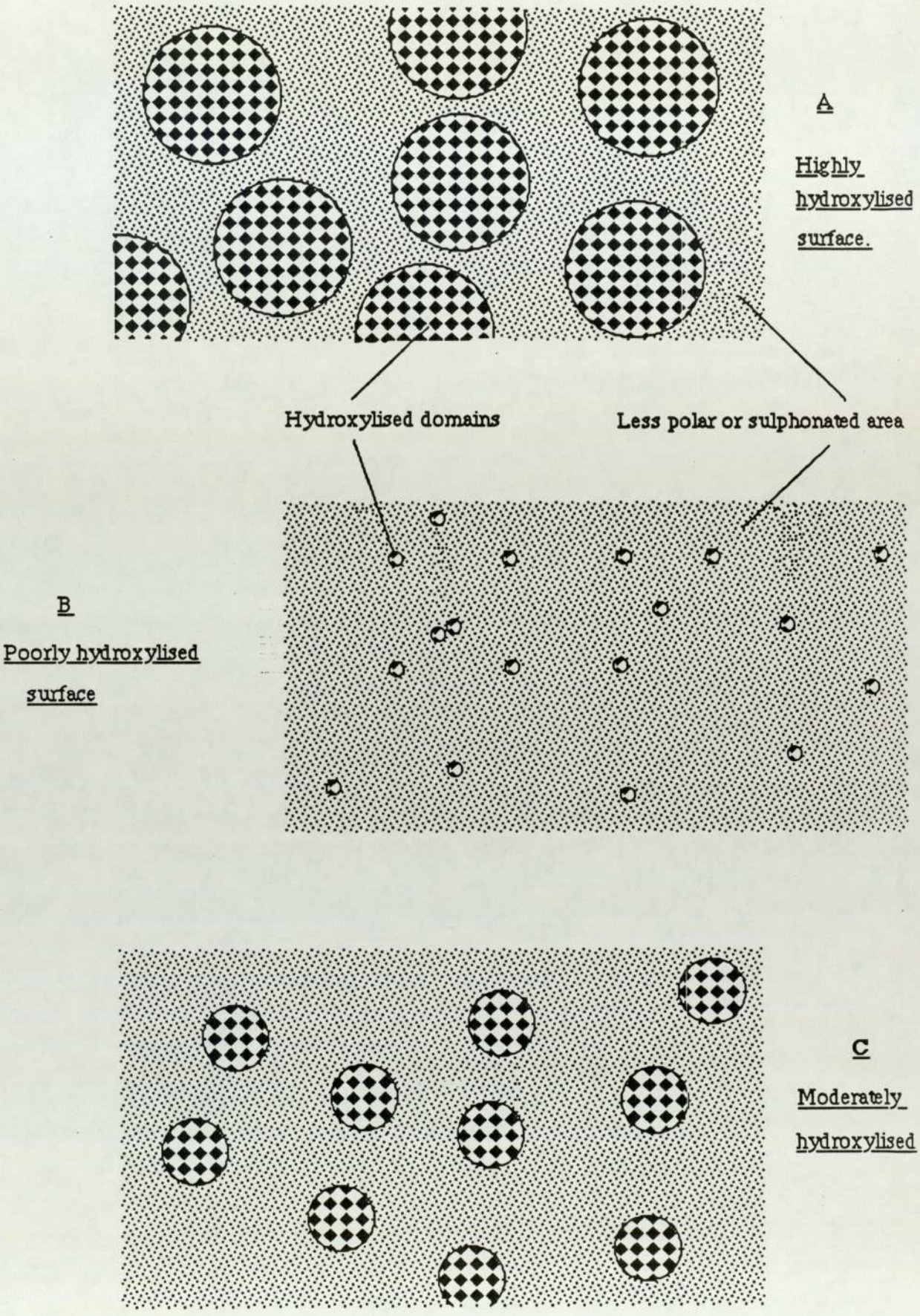
7.1. A molecular domain hypothesis

Surface A in Figure 7.1 represents a surface that has been treated by an oxygen gas plasma and a cell cultured onto this surface has a greater chance of encountering a polar, hydroxylised domain than a non-polar area of substrate. In order to undergo spreading, the cellular extensions will not have to extend very far before experiencing another suitable domain; some domains may be large enough to contain a fully spread cell (~ 60μm^2). Thus, as a surface becomes more highly hydroxylated, the greater the extent of cell attachment and spreading that would be seen on this type of surface. Treatment with sulphuric acid may have also led to a surface similar to A, but it is probable that the hydroxylised domains would be smaller and / or further apart.

However, cells encountering surface B in Figure 7.1 (possibly the type produced after oleum treatment) have more chance of 'landing' on a strongly negatively charged portion which would prove less suitable for cell attachment and spreading. However, should a cell encounter a more suitable, hydroxylised domain, then any pseudopodial extensions in the quest to undergo spreading would have to cross a vast, less favourable, boundary and so the spreading process may be less marked.

Cells cultured onto surface C, as in the case of polystyrene that has been treated with 'hot' sulphuric acid, or chlorosulphonic acid vapour, possibly have equal chances of landing on a hydroxylated as a sulphonated domain. Therefore, cell attachment is less than in case A

Figure 7.1. Diagrammatical representation of the molecular domain hypothesis



and greater than in B and depending on the distances between each domain will affect the cell spreading, as highlighted above.

From work carried out prior to this study there appeared to be a correlation between cell attachment and equilibrium water content (EWC) for a selection of hydrogel polymers. Cell adhesion in the region EWC 2 - 35% was high, but fell sharply to a non- adhesive 'window' between 40 and 60 % EWC whereby there was little or no cell adhesion. The range of hydrogels studied to produce this behaviour all contained hydroxyl groups as their dominant water structuring group. However, the current research programme had examined a further series of hydrogels where the water structuring group was in the form of a nitrogen or acrylamide compound and it was found that there was a shift in the position of the non-adhesive 'window'. Small amounts of negative charge (in the form of MAA) introduced at the surface of the hydrogel had little effect on cell behaviour. However, as the concentration was increased, the extent of cell attachment was markedly decreased.

This study has highlighted a number of properties that are important in cell-substrate interactions. Non-polar ('hydrophobic') materials such as untreated polystyrene are unable to trigger the cell adhesion response of BHK fibroblast cells in culture. On the other hand, surfaces that are highly polar ('hydrophilic') and capable of structuring vast amounts of water at their surface also prove unsuitable for supporting anchorage-dependent cell adhesion. However, surfaces that produce a balance between these two extremes of surface polarity, for example tissue-culture plastic, are able to support confluent monolayers of fibroblast cells. Surface polarity is an important parameter because of it's ability to attract water molecules which will tend to bind strongly, orientated around the polar groups. Less polar surfaces structure water in an ice-like fashion on or near the surface. The binding of these water molecules in both these types of surfaces imparts a

significant thermodynamic driving force for interfacial adsorption of surface active molecules, eg proteins with a simultaneous release of water. Therefore, it appears that the quantity of water that is held at a surface and the way in which it is structured can have an vast affects on cell behaviour. The presence of polar, water structuring groups such as hydroxyl (as produced at polystyrene surfaces following mild sulphuric acid treatment), nitrogen or acrylamide compounds can have quite profound affects on cell behaviour and are capable of overriding the responses due to EWC alone. However, the presence of charged groups (for example MAA or sulphonate groups, both of which are negatively charged) appears to have an even stronger influence on cell adhesion. In low quantities it's effects can go un-noticed, but once a certain critical threshold has been reached, surface charge can become the dominant, deciding factor as to whether cell attachment and spreading can be carried out.

By careful selection of monomers, it is possible to manufacture series of hydrogel polymers possessing different surface properties, for example in terms of their chemical group expression, water structuring ability and surface charge. Further investigations need to be carried out whereby the cell response to a series of nitrogen containing hydrogels of EWC less than 35% is investigated in order to observe whether these hydrogels allow the same extent of cell attachment and spreading as those observed by Minett in the region 2 - 35% EWC. This would ascertain at which point the non-adhesive window begins for the those hydrogels containing nitrogen as the dominating water structuring group. The effect of surface charge on cell behaviour is often difficult to investigate because it's introduction at the surface often leads to effects being manifested on some other surface property, for example, wettability, polarity or chemical group expression. However, it would be of interest to investigate the possibility of producing an array of hydrogels with different amounts of negative and indeed positive surface charge and to observe their affect on cell

behaviour.

This study has also laid some important foundations for the analysis of foetal calf serum (FCS) and in the identification of the lipid species that become adsorbed onto culture substrates during cell culture. It was shown that FCS was composed of a number of biological components including proteins, lipids, waste products, calcium and other ions. Using thin-layer, gas-liquid and high performance liquid chromatographic techniques, it was shown that the lipid profile of foetal calf serum was composed of mono-, di- and tri-glyceride, cholesterol, cholesterol esters, fatty acid (both saturated and unsaturated) and phospholipid. Information of this nature had not been available to the cell biologist prior to this study but it would be of importance in most investigations where FCS is used in cell culture studies. During this research some basic techniques were described for the analysis of some species that become adsorbed to surfaces with different abilities to support cell attachment under cell culture conditions. In particular, the difficulties associated with such techniques were underlined and attempts were made to overcome them. For example it was shown that methanol was the most successful solvent that could be used in contact with polystyrene for the extraction of the lipids. Similarly, it was also shown that extracts from a number of dishes had to be pooled in order to partly overcome problems of sensitivity. Thus, it was shown that some of the lipid species found in foetal calf serum, namely cholesterol esters, fatty acid and triglyceride become adsorbed onto both tissue culture and bacteriological grade plastic under cell culture conditions. However, there were no vast differences observed between these surfaces in terms of the quantity or classes of lipids that they adsorbed. Therefore, any differences in the adsorption of these biological species may be more subtle than the techniques used here were able to identify. It may, therefore, be more advantageous if, in further experiments, substrates with different cell adhesion capacities were selected but which did not adsorb as much, say, cholesterol esters as those

surfaces analysed here. This would help identify the small differences that may be having an affect on cell behaviour that may have been masked by the high amounts of some lipids extracted off the surfaces in this study. There are also other ways in which the techniques used here may be improved upon. For example, it may be of interest to use columns of untreated or oxygen plasma etched polystyrene beads to represent bacteriological and tissue-culture grades plastic respectively. This would markedly increase the surface area from which lipid species, or other species may be extracted. In addition, the lipid content of the culture medium before and after contact with the beads could be determined which may be one further method of analysing the classes adsorbed. Also, it may be possible to aspirate the excess culture medium after contact with the plasma treated polystyrene has occurred to investigate whether the components necessary for cell culture have been removed via their adsorption to the beads. This could be detected via the ability of this excess medium to support adhesion and spreading of anchorage dependent cells in culture.

It may also prove fruitful to use other polymers instead of those based on moulded polystyrene in order to limit the problems experienced when using solvents for extraction purposes. To this end, any cross linked polymer may prove more suitable but it is possible that this may be another situation where hydrogels may prove of value. It has been shown during this and other research that there are differences within these polymers in their ability to support cell adhesion. These polymers also have the added advantage that because their refractive indices are close to that of water, following solvent extraction they can be examined using fluorescence techniques to ensure that removal of biological species has occurred.

This study also established that using relatively simple techniques there was slightly more calcium adsorbed onto the tissue culture surface than the bacteriological grade plastic,

which may aid the initial cell adhesion to this surface. However, in order to ascertain whether this surface actively facilitated the adsorption of this cation, further experimentation is needed, possibly using radiolabelling techniques. It may also be worthwhile further investigating whether the differences observed for the other ionic species are important in cell behaviour.

Whilst no single physico-chemical property has been isolated as the single most important factor for mediating cell attachment of anchorage dependent cells, this study has shown that some features of the substrate are more important than others. Indeed, although not yet complete, a kind of hierarchy has initially been established whereby the importance of polarity, water structuring and surface charge have been highlighted. Similar inter-disciplinary investigations may gradually lead to a better understanding of the 'ranking order' of other physico-chemical properties in governing the cell response and ultimately to the design of improved biomaterials.

APPENDICES

APPENDIX 1

Formulation of Dulbecco's Modification of Eagles Medium

Formulation of Dulbecco's Modified Eagles Medium (DMEM)

(made up in balanced salt solution containing glucose and phenol red)

Amino Acids	**mg/l**
L- arginine	84.0
L- glutamine	580.0
L- histidine HCl. H_2O	42.0
L- isoleucine	105.0
L- leucine	105.0
L- lysine HCl	146.0
L-.methionine	30.0
L- phenylalanine	66.0
L- threonine	95.0
L- tryptophan	16.0
L- valine	94.0
Glycine	30.0
L- serine	42.0
L- cystine	48.0
L- tyrosine	72.0

Vitamins	
Choline	4.0
Nicotinamide	4.0
D- Ca-pantothenate	4.0
Pyridoxal HCl	4.0
Thiamine HCl	4.0
Riboflavin	0.40
Folic acid	4.0
i-inositol	7.20
Fe (NO_2) 9 H_2O	0.1

APPENDIX 2

Formulation of phosphate buffered saline.

Formulation of phosphate buffered saline

Substance	g/l
NaCl	8.00
KCl	0.2
$CaCl_2$	0.1
$MgCl_2\ 6H_2O$	0.1
$Na_2HPO_4\ 2H_2O$	1.15
KH_2PO_4	0.2
Gas phase	air

APPENDIX 3

Binding energies of the electrons emitted during ESCA analysis

Element	Atomic No.	Range (eV)	Photoelectron Lines[b] 1s	2s	2p₁	2p₃	3s	3p₁	3p₃	3d₃	3d₅	4s	4p₁	4p₃	Range (eV)	Auger Lines KL₁L₁	KL₁L₂₃	KL₂₃L₂₃[c]
Li	3		56															
Be	4	4	113															1082
B	5	8	191															993
C	6	12	287															875
N	7	9	402												12			
O	8	4	531	23											10	779	764	743
F	9	6	686	30											8	645	626	599
Ne	10	0	863	41	14										0	491	468	435
Na	11	2	1072	64	31										4	332	303	264
Mg	12	2		90	51													
Al	13	4		119	74													
Si	14	6		153	103	102												
P	15	8		191	134	133	14											

Element	Range (eV)	$L_1M_{23}M_{23}$[d]	$L_2M_{23}M_{23}$[d]	$L_3M_{23}M_{45}$ ^1P	$L_3M_{23}M_{45}$ ^3P	$L_2M_{23}M_{45}$ ^1P	$L_1M_{45}M_{45}$[e]	$L_2M_{45}M_{45}$
S	6		1103					
Cl	4		1071					
Ar	0	1037		1035				
K		1005		1003				
Ca		964		961				
Sc	8		920	892				
Ti	10		873	839				
V	6		822	784				
Cr			767	729				
Mn	6		715	670			620	
Fe	6		659	608			553	
Co	7	604	597	546	541		483	468
Ni	7	548	542	482	476		410	393
Cu	5	486	479	416	408	396	337	317
Zn	7	429	422	352	343	329	265	242
Ga	7	368	361	284	275	257	189	162
Ge	10	305	297	215	205	184	113	82
As								
Se								
Br								
Kr								
Rb								
Sr								
Y								
Zr								

Element	Atomic No.	Range (eV)	Photoelectron Lines[b] 1s	2s	2p₁	2p₃	3s	3p₁	3p₃	3d₃	3d₅	4s	4p₁	4p₃
As	33	7					207	148	143	45	44			
Se	34	8					232	169	163	58	57			
Br	35	7					256	189	182	70	69			
Kr	36	0					287	216	208	89	88	22		
Rb	37	1					322	247	238	111	110	29		14
Sr	38						358	280	269	135	133	37		20
Y	39						395	313	301	160	158	45		25
Zr	40	6					431	345	331	183	181	51		29

Line positions of binding energies.

Kinetic energies may be calculated using the equation:

Kinetic energy = hν - binding energy - spectrometer work function (neg)

a) Lines enclosed in boxes are the most intense and are the most suitable for use of line energies in identifying chemical states

b) For brevity $2p_3$ equals $2p_{3/2}$, $3d_5$ equals $3d_{5/2}$, etc

c) Includes KVV designation when L_{23} is not a core level

d) Designation is oversimplified

e) Includes LVV when M levels are not in core, and MVV when N levels are not in core

f) No simple $4p_{1/2}$ line exists for this group of elements

g) The 4d doublet for these elements is complex and is variable with chemical state because of multiplet splitting and multi electron processes

APPENDIX 4

Atomic sensitivity factors of the XSAM spectrometer during ESCA analysis.

$S_{F1s} = 1.00$. This table is based upon calculated cross-sections corrected for the kinetic energy dependence of the spectrometer detection efficiency and an average value for the dependence of λ on kinetic energy The values are only valid for, and should only be applied, when the electron energy analyzer used has the transmission characteristics of the double-pass cylindrical-mirror type analyzer supplied by Physical Electronics. Data are for Mg x-rays except for those in parentheses that are calculated for Al x-rays. Otherwise, the atomic sensitivity factors for Mg and Al agree within ten percent.

Z	Element	Line	ASF (Area)
3	Li	1s	.012
4	Be	1s	.039
5	B	1s	.088
6	C	1s	.205
7	N	1s	.38
8	O	1s	.63
9	F	1s	1.00
10	Ne	1s	1.54
11	Na	1s	2.51
	Na	1s	(2.27)
12	Mg	1s	(3.65)
	Mg	2p	.07
13	Al	2p	.11
14	Si	2p	.17
15	P	2p	.25
16	S	2p	.35
17	Cl	2p	.48
18	Ar	$2p_{3/2}$	.42
19	K	$2p_{3/2}$	.55
20	Ca	$2p_{3/2}$	.71
21	Sc	$2p_{3/2}$	.90
22	Ti	$2p_{3/2}$	1.1
23	V	$2p_{3/2}$	1.4
24	Cr	$2p_{3/2}$	1.7
25	Mn	$2p_{3/2}$	2.1
26	Fe	2p[a]	3.8
27	Co	2p[a]	4.5
28	Ni	2p[a]	5.4
29	Cu	$2p_{3/2}$	4.3
30	Zn	$2p_{3/2}$	5.3
31	Ga	$2p_{3/2}$	6.9
		$2p_{3/2}$	(5.8)
32	Ge	$2p_{3/2}$	9.2
		$2p_{3/2}$	(7.2)
		3d	.30
33	As	$2p_{3/2}$	(9.1)
		3d	.38
34	Se	3d	.48
35	Br	3d	.59
36	Kr	3d	.72
37	Rb	3d	.88
38	Sr	3d	1.05
39	Y	3d	1.25
40	Zr	$3d_{5/2}$	.87
41	Nb	$3d_{5/2}$	1.00
42	Mo	$3d_{5/2}$	1.2
43	Tc	$3d_{5/2}$	1.35
44	Ru	$3d_{5/2}$	1.55
45	Rh	$3d_{5/2}$	1.75
46	Pd	$3d_{5/2}$	2.0
47	Ag	$3d_{5/2}$	2.25
48	Cd	$3d_{5/2}$	2.55
49	In	$3d_{5/2}$	2.85
50	Sn	$3d_{5/2}$	3.2
51	Sb	$3d_{5/2}$	3.55
52	Te	$3d_{5/2}$	4.0
53	I	$3d_{5/2}$	4.4
54	Xe	$3d_{5/2}$	4.9
55	Cs	$3d_{5/2}$	5.5
56	Ba	$3d_{5/2}$	6.1
57	La	$3d_{5/2}$	6.7
		4d[a]	1.22
58	Ce[a]	3d	12.5
		4d	1.29
59	Pr[a]	3d	14.0
		4d	1.38
60	Nd[a]	3d	15.7
		4d	1.48
61	Pm[a]	3d	17.6
		4d	1.57
62	Sm[a]	3d	20.3
		4d	1.66
63	Eu	3d	23.8
		3d	(20.2)
		4d	1.76
64	Gd[a]	3d	29.4
		3d	(22.6)
		4d	1.84
65	Tb[a]	3d	(26.7)
		4d	1.93
66	Dy[a]	3d	(30.0)
		4d	2.03
67	Ho[a]	4d	2.12
68	Er[a]	4d	2.19
69	Tm[a]	4d	2.28
70	Yb[a]	4d	2.36
71	Lu[a]	4d	2.45
72	Hf	4f	1.55
73	Ta	4f	1.75
74	W	4f	2.0
75	Re	$4f_{7/2}$	1.25
76	Os	$4f_{7/2}$	1.4
77	Ir	$4f_{7/2}$	1.55
78	Pt	$4f_{7/2}$	1.75
79	Au	$4f_{7/2}$	1.9
80	Hg	$4f_{7/2}$	2.1
81	Tl	$4f_{7/2}$	2.3
82	Pb	$4f_{7/2}$	2.55
83	Bi	$4f_{7/2}$	2.8
90	Th	$4f_{7/2}$	4.8
92	U	$4f_{7/2}$	5.6

a) Variable and complex pattern makes it usually desirable to measure areas of entire doublet region.

APPENDIX 5

Tables of results of the characterisation of a series of hydrogels

To show the values for the polar, dispersive and total components of the hydrated surfaces energy of a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	γ_p (mNm^{-1})	γ_d (mNm^{-1})	γ_t (mNm^{-1})
Poly HEMA	--	37.6	42.2	23.2	65.4
NVP :MMA	50:50	38.6	35.5	28.2	63.7
NVP : LMA	60:40	51.2	36.1	28.1	64.2
NNDMA : LMA	60:40	53.6	35.5	29.7	65.2
NVP : MMA	60:40	55.6	36.1	27.4	63.5
NNDMA : LMA	70:30	63.7	36.1	28.9	64.9
NNDMA : PMMA	80:20	66.5	35.5	27.4	62.9
NNDMA : Pellathane	90:10	76.2	30.4	39.9	70.3
NNDMA : LMA	90:10	78.0	38.2	27.4	65.6
NVP : LMA	90:10	80.0	42.2	23.8	66.0
NNDMA : HPU25	95:5	81.7	35.5	32.5	68.0

To show the values for the polar, dispersive and total components of the hydrated surfaces energy (continued) of a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	γ_p (mNm^{-1})	γ_d (mNm^{-1})	γ_t (mNm^{-1})
NNDMA : Pellathane	95:5	82.4	32.7	35.3	68.0
NNDMA : HPU25	90:10	86.6	39.3	29.2	68.4
NNDMA : MMA	80:20	83.1	36.6	26.7	63.3
NNDMA : Biomer	95:5	83.7	33.9	33.6	67.4
NNDMA : PVAc	80:20	84.6	40.8	23.0	63.7
NNDMA : MMA	90:10	86.2	37.7	25.3	63.0
Poly NNDMA	--	88.4	40.8	25.5	66.3
Tissue culture plastic			17.9	40.2	58.1
Bacteriological plastic			0.3	47.3	47.6

To show the values for the polar, dispersive and total surface energy components of the dehydrated surfaces energy of a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	γ_p (mNm^{-1})	γ_d (mNm^{-1})	γ_t (mNm^{-1})
Poly HEMA	--	37.6	21.5	29.0	50.5
NVP :MMA	50:50	38.6	6.7	39.0	45.7
NVP : LMA	60:40	51.2	7.3	37.0	44.3
NNDMA : LMA	60:40	53.6	10.6	36.0	46.6
NVP : MMA	60:40	55.6	7.4	38.2	45.6
NNDMA : LMA	70:30	63.7	11.4	35.3	46.7
NNDMA : PMMA	80:20	66.5	9.3	38.1	47.4
NNDMA : Pellathane	90:10	76.2	35.5	30.9	65.4
NNDMA : LMA	90:10	78.0	15.6	33.4	49.0
NVP : LMA	90:10	80.0	9.3	35.2	44.5

To show the values for the polar, dispersive and total surface energy components (continued) of the dehydrated surfaces energy of a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	γ_p (mNm^{-1})	γ_d (mNm^{-1})	γ_t (mNm^{-1})
NNDMA : HPU25	95:5	81.7	32.5	33.5	65.8
NNDMA : Pellathane	95:5	82.4	30.4	32.1	62.4
NNDMA : HPU25	90:10	86.6	32.4	31.7	64.1
NNDMA : MMA	80:20	83.1	9.8	36.7	46.5
NNDMA : Biomer	95:5	83.7	29.4	34.7	64.1
NNDMA : PVAc	80:20	84.6	7.3	40.2	47.5
NNDMA : MMA	90:10	86.2	12.0	35.0	47.0
Poly NNDMA	--	88.4	32.0	18.7	50.7
Tissue culture plastic			17.9	40.2	58.1
Bacteriological grade plastic			0.3	47.3	47.6

To show the values for tensile strength, Youngs modulus and appearance of a selected group of hydrogel polymers.

Hydrogel	Ratio of co-monomers	EWC (%)	Youngs modulus (MPa)	Tensile strength (MPa)	Appearance
Poly HEMA	--	37.6	0.250	0.495	Clear
NVP :MMA	50:50	38.6	7.210	4.746	Clear
NVP : LMA	60:40	51.2	0.277	0.324	Clear
NNDMA : LMA	60:40	53.6	0.168	0.533	Clear
NVP : MMA	60:40	55.6	1.689	2.651	Clear
NNDMA : LMA	70:30	63.7	0.249	0.433	Clear
NNDMA : MMA	80:20	66.5	2.719	1.740	Opaque
NNDMA : Pellathane	90:10	76.2	0.469	0.506	Clear
NNDMA : LMA	90:10	78.0	0.201	0.211	Clear
NVP : LMA	90:10	80.0	0.088	0.103	Clear

To show the values for tensile strength, Youngs modulus and appearance (continued) of a selected group of hydrogel polymers.

Hydrogel	Ratio of co-monomers	EWC (%)	Youngs modulus (MPa)	Tensile strength (MPa)	Appearance
NNDMA : HPU25	95:5	81.7	0.281	0.410	Translucent
NNDMA : Pellathane	95:5	82.4	0.172	0.170	Clear
NNDMA : HPU25	90:10	86.6	0.149	0.322	Translucent
NNDMA : MMA	80:20	83.1	0.103	0.127	Clear
NNDMA : Biomer	95:5	83.7	0.169	0.308	Translucent
NNDMA : PVAc	80:20	84.6	0.083	0.111	Opaque
NNDMA : MMA	90:10	86.2	0.095	0.161	Clear
Poly NNDMA	--	88.4	0.097	0.136	Clear

To show the values for carbon:oxygen, carbon:nitrogen and oxygen to nitrogen for a selected group of hydrogel polymers.

Hydrogel	Ratio of co-monomers	EWC (%)	C:O	C:N	O:N
Poly HEMA	--	37.6			
NVP :MMA	50:50	38.6	4.6	--	--
NVP : LMA	60:40	51.2	4.5	42.0	9.3
NNDMA : LMA	60:40	53.6	2.4	77.3	31.9
NVP : MMA	60:40	55.6	4.5	34.3	7.6
NNDMA : LMA	70:30	63.7	4.4	--	--
NNDMA : PMMA	80:20	66.5	5.5	16.6	3.0
NNDMA : Pellathane	90:10	76.2	4.2	49.6	11.8
NNDMA : LMA	90:10	78.0	4.5	8.1	1.8
NVP : LMA	90:10	80.0	5.0	6.9	1.4

To show the values for carbon:oxygen, carbon:nitrogen and oxygen to nitrogen (continued) for a selected group of hydrogel polymers.

Hydrogel	Ratio of co-monomers	EWC (%)	C:O	C:N	O:N
NNDMA : HPU25	95:5	81.7	5.9	36.3	6.1
NNDMA : Pellathane	95:5	82.4	4.3	9.5	2.2
NNDMA : HPU25	90:10	86.6	4.5	25.6	5.7
NNDMA : MMA	80:20	83.1	5.3	25.4	4.8
NNDMA : Biomer	95:5	83.7	3.9	28.1	7.3
NNDMA : PVA	80:20	84.6	4.4	33.3	7.5
NNDMA : MMA	90:10	86.2	3.8	15.6	4.2
Poly NNDMA	--	88.4	6.7	32.6	4.9

To show the values for freezing, non- freezing and grams water / gram of polymer for a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	Freezing water (%)	Non-freezing water (%)	g water / g polymer
Poly HEMA	--	37.6	13.2	24.4	0.603
NVP :MMA	50:50	38.6	3.5	35.1	0.629
NVP : LMA	60:40	51.2	18.8	32.4	1.049
NNDMA : LMA	60:40	53.6	21.7	31.8	1.150
NVP : MMA	60:40	55.6	24.6	31.0	1.252
NNDMA : LMA	70:30	63.7	ND	ND	ND
NNDMA : PMMA	80:20	66.5	40.8	25.7	1.985
NNDMA : Pellathane	90:10	76.2	46.8	29.4	3.202
NNDMA : LMA	90:10	78.0	55.6	22.4	3.545
NVP : LMA	90:10	80.0	57.8	22.2	4.000
NNDMA : HPU25	95:5	81.7	56.0	25.7	4.464

To show the values for the freezing, non- freezing and grams water / gram of polymer (continued) of a selected group of hydrogel polymers as determined by contact angle techniques

Hydrogel	Ratio of co-monomers	EWC (%)	Freezing water (%)	Non-freezing water (%)	g water g polymer
NNDMA : Pellathane	95:5	82.4	54.9	27.5	4.682
NNDMA : HPU25	90:10	86.6	53.7	32.9	6.463
NNDMA : MMA	80:20	83.1	55.7	27.4	4.917
NNDMA : Biomer	95:5	83.7	59.2	24.5	5.135
NNDMA : PVAc	80:20	84.6	N.D	N.D	N.D
NNDMA : MMA	90:10	86.2	62.0	24.2	6.246
Poly NNDMA	--	88.4	70.7	17.7	7.621

Appendix 6

Critical excitation potentials for those elements detected during EDXA analysis.

Critical excitation potentials of those elements detected using EDXA

Element	Critical excitation potential (eV)
Sodium	1.041
Silicon	1.739
Phosphorous	2.013
Chlorine	2.621
Potassium	3.312
Calcium	3.690

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