Cell adhesion on synthetic polymer substrates.

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Doctor of Philosophy

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Summary of Thesis

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This thesis investigates the role of synthetic plastic substrates in determining the adhesion and subsequent growth of anchorage dependent cells in vitro. This involved cell culture on specific substrate materials and the use of quantimet analysis, both light and scanning electron microscopy, X ray, photoelectron, spectrographic, surface chemical analysis and surface energetic measurment, in order to monitor and accuratly characterise substrate properties

Attention has been paid to the relative properties of those materials which are claimed to be cell adhesive, and nonadhesive respectively, and in addition to the claims that coatings of poly2hydroxyethyl methacrylate are capable of modulating the extent of cell adhesion and growth. A systematic series of commercial and purpose synthesised copolymer substrates are utilized to determine the relative role of specific chemical groups and physicochemical parameters in cell adhesion. This work coupled with the effect of gas plasma treatment on polystyrene substrates led to the proposal of a model system in which the cell adhesion characteristics of substrate materials can be predicted on the basis of fractional polarity measurements. This represents the first attempt to unify the factors affecting cell adhesion.

The applications of this model system and the relevence of cell culture to the assesment of biomedical implant materials is discussed.

Keywords:- Cell adhesion, polymer, substrate, hydrophilic, hydrophobic, hydroxyl groups, Equilibrium water content, fractional polarity, biocompatibility.

This thesis is dedicated to

My Father.

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Chapter One

Introduction and Literature review

Introduction and Literature Review

Over fifty years of research were required to develop the techniques and systems necessary for routine cell maintenance in vitro. Since that time (approximately 1965) many cell biologists and biochemists alike have been dedicated to determining cell growth requirements and mechanisms. The development of in vitro cell culture was an important step as this technique offers several fundamental advantages over traditional in vivo methods.

Experiments performed in vitro can be precisely monitored controlled and repeated. In vivo results are more difficult to obtain and are often unrepeatable due to the particular characteristics of one subject. Furthermore in vitro experimentation allows simple monitoring of control values under precisely the same conditions or experimental variables such circumstances are rare with in vivo techniques. In vitro experimentation also removes many of the practical problems involved with performing a large number of determinations thus dramatically increasing the speed of research.

Maintaining cells, tissues or organs outside the body was not easy to achieve. Early studies (ref 1) found problems in maintaining sterility and providing the correct balance of essential nutrients in an isotonic environment. These first experiments used droplets of blood, blood plasma or relatively simple isotonic salt solutions in which cells were suspended. Even at this early stage the preference of some cell types for solid supports were noted (ref 2-4). With the further development of tissue culture the mobility and growth in culture

of individual fibroblast cell types was first observed. These cells were shown to move out of tissues in culture and form cell monolayers (ref 5). Subsequently the requirement for a solid substrate to allow fibroblast anchorage and growth was determined (ref 6) and hence the concept of anchorage dependent cell growth in culture established.

The establishment of anchorage dependence focussed attention on to the events occuring within the cell and at the cell/substratum interface after initial cell seeding in culture. During cell attachment to a favourable substrate the shape of the attaching cell which is spherical when in suspension, changes to a flattened morphology with concave cell borders (ref 7-12)(see fig 1a & b). Cells remain in the "fully spread" state until the mitotic phase of the cell division cycle is reached and then round up prior to cell division after which the two daughter cells commence the spreading process again. The significance of these observations is that cells become committed to the cell division cycle during the spreading process (cells prevented from spreading, enter G_o- see fig 1.2).

In view of this, the mechanism and biochemistry of cell spreading would seem to be of direct relevance to cell growth control in culture. Hence cell spreading represents an ideal system for the study of cell growth requirements.

At the same time as the potential of cell culture was being realized, culture practice and materials were being advanced and developed. Commercial cell culture fluids were developed from Dulbecos minimum essential media (ref 13).

Figure 1.1a

Typical rounded cell morphology

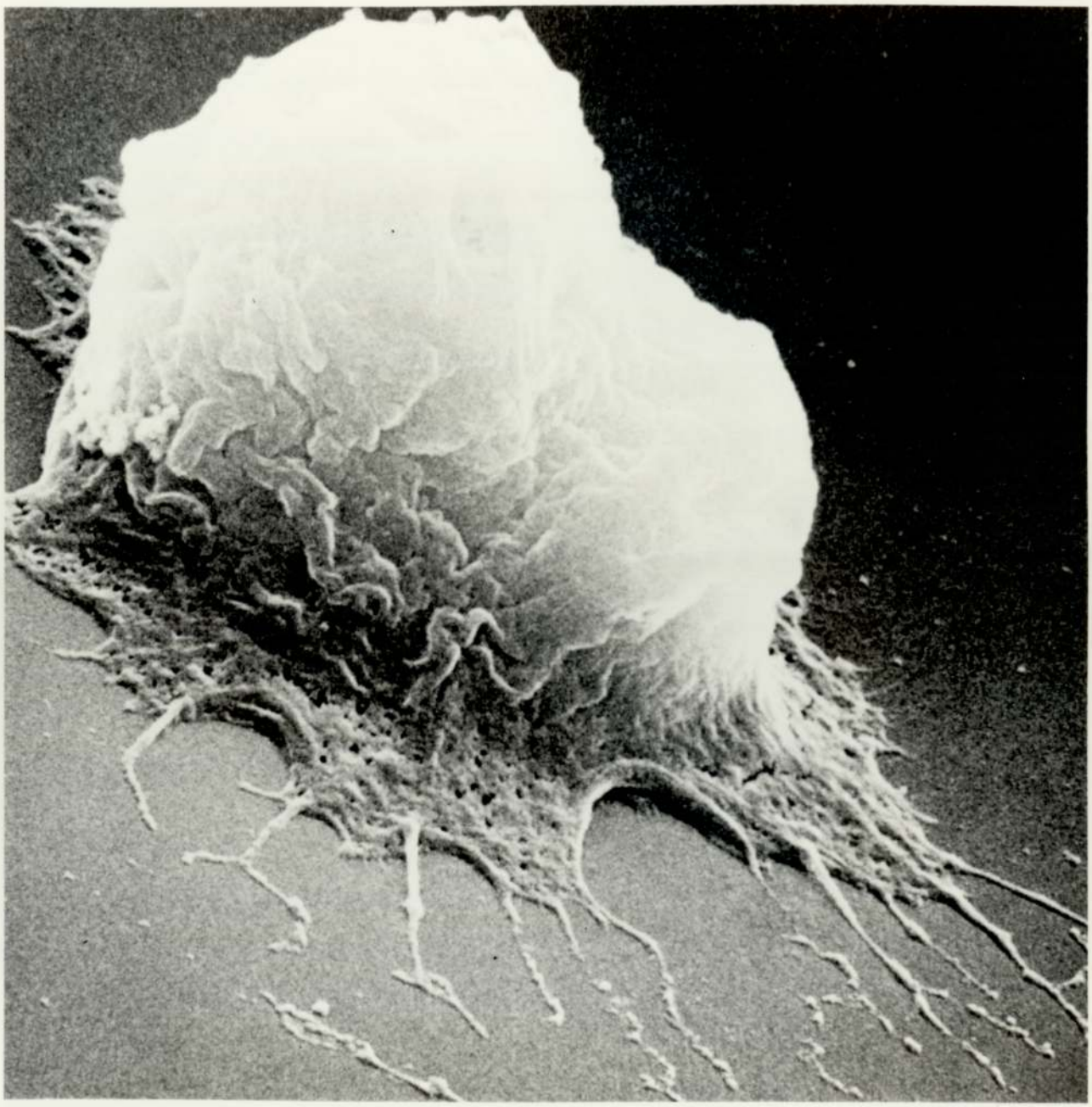


Figure 1.1b

Typical spread cell morphology

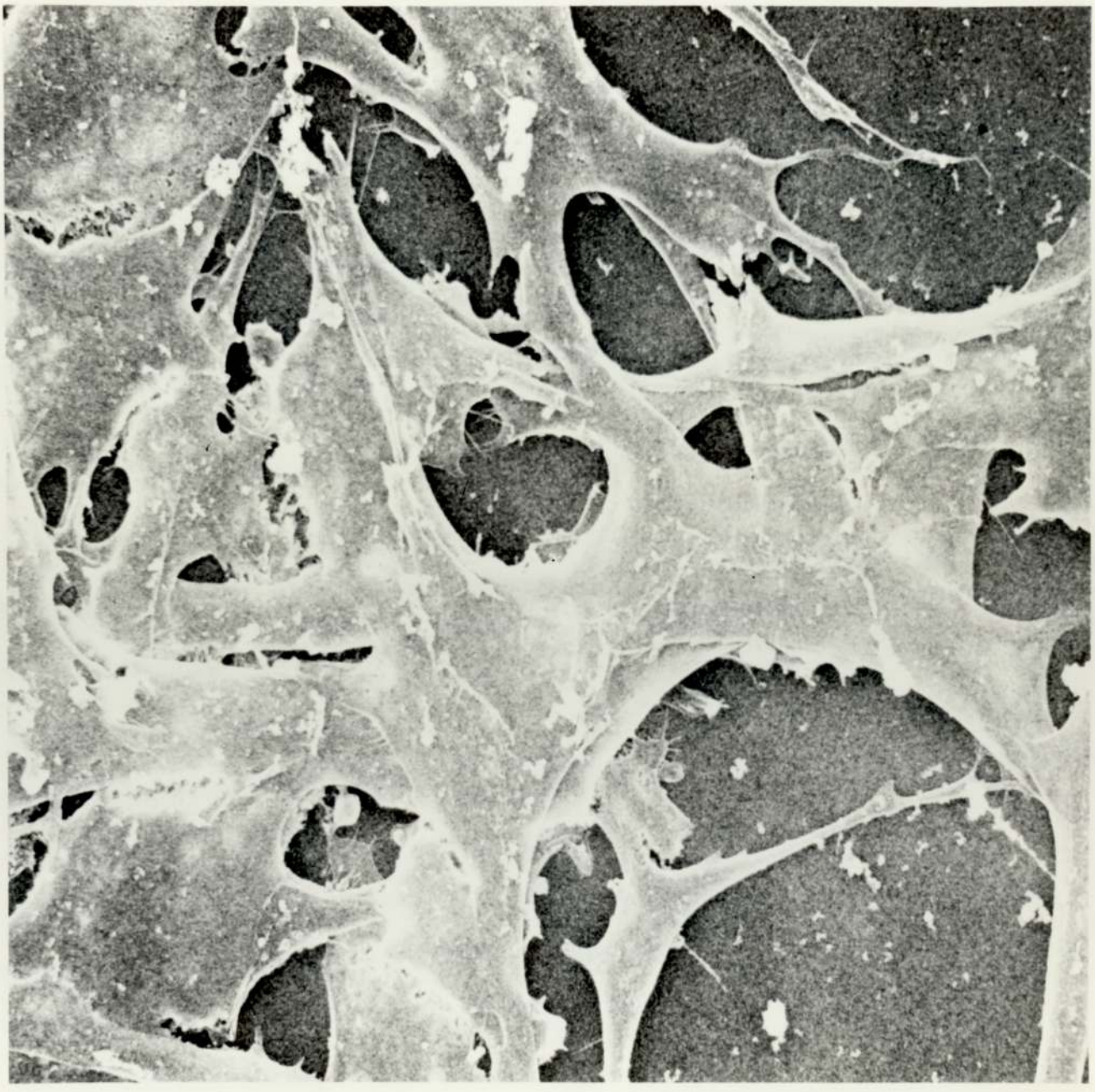
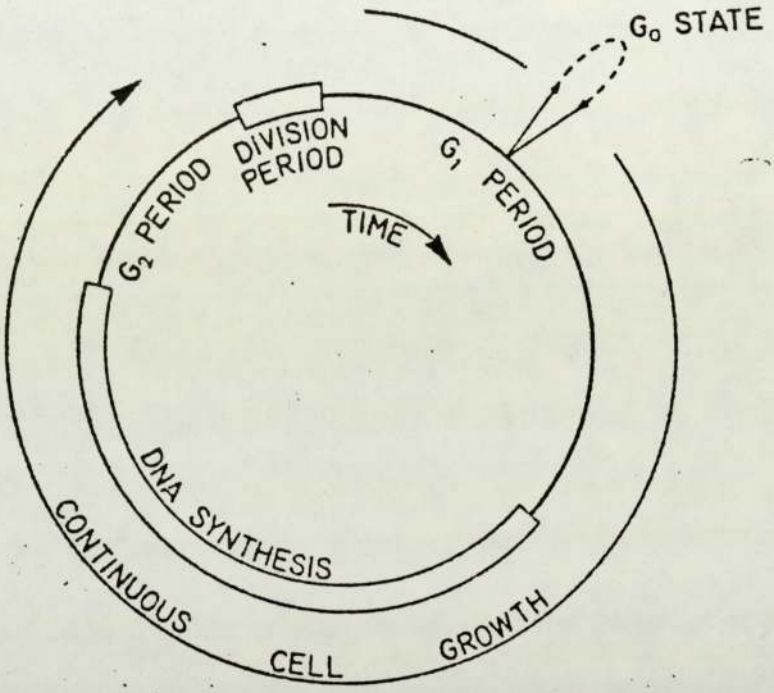


Figure 1.2

Major features of cell life cycle.



The major features of the cell life cycle. The relative proportions of the cycle may vary considerably from one type of cell to another, but the reproduction of every cell consists of growth coupled with DNA replication followed by cell division. A mammalian cell growing in culture with a generation time of 16 hours, for example, will have a G_1 = 5 hours, S = 7 hours, G_2 = 3 hours, and D = 1 hour. G_o is the state into which cells are postulated to move when the cell cycle is arrested in G_1 by various kinds of environmental conditions. (ie unsuitable cell culture substrate.)

which could be supplemented with foetal calf serum in order to sustain cell growth. Under these culture conditions fibroblasts could be grown reliably on glass substrata.

With the development of reliable and practical cell culture methods, the popularity of cell culture as a technique for biological and biochemical research increased rapidly. The routine use of cell culture raised new problems associated with the continual recleaning and resterilization of glassware. Around 1965, disposable polystyrene dishes were introduced. Although these vessels solved any sterilization problems, in its native form polystyrene did not allow attachment or spreading of fibroblasts in the same way as glass and did not support cell growth (ref 14-16). Early workers (ref 17-19) showed that surface treatment of polystyrene with sulphuric acid gave rise to surfaces suitable for cell culture. These findings probably lead to the development of commercial tissue culture plastic, produced by the surface treatment of polystyrene vessels by a corona discharge (ref 20-22). This material represents the cell culture substrate used routinely in thousands of laboratories at the present time.

The actual mechanisms of cell adhesion to glass or surface treated polystyrene had been widely discussed in the literature (ref 23-81). This discussion centered on two rival theories of cell adhesion mechanism; firstly the D.L.V.O. model of colloidal stability (ref 82-83) which postulates a balance between long range electrostatic repulsion arising from negative charges on the cell surface and electromagnetic attraction due to fluctuations in dipole moments both on the cell surface and

in the intervening medium (ref 23-24). These are all long range continuum forces which involve no direct interparticle contact. The resultant picture is that of a cell suspended above the substrate at 10 - 100 A°.

The alternative less rigorous theory, the "contact hypothesis" proposed that close-range intermolecular forces including not only diopole and electrostatic forces but also hydrogen bonds and "hydrophilic" bonds (ref 78) are essential for cell adhesion. It acknowledges the impracticability of a completely theoretical treatment (ref 71). Instead a semi empirical approach can be made in which it is assumed that the forces involved in close interfacial contact also contribute to surface tension, consequently surfaces could be graded according to a surface energy parameters measured by wettability by various liquids. This approach therefore suggests that hydrophilic surfaces will be preferred to hydrophobic surfaces (ref 51-61, 64). Initial experiments using platlets (ref 84) and fibroblasts in both serum free (ref 70) and full culture conditions (ref 44,53,54,57-64) supported the "contact hypothysis" by demonstrating that more cells adhere to hydrophilic surfaces. In fact both Harris (ref 70) and Maroudas (ref 60-63) showed that increasing treatment of polystyrene with sulphuric acid led to increased cell adhesion.

The increase in surface charge over this range of substrate was shown by dye binding assays and was assumed to represent sulphonate group presence at the polystyrene surface. Increased cell adhesion on substrata of increasing negative charge could not be explained by the D.V.L.O approach. This and other

factors, such as the resistance of cells to shear forces (ref 70) and later evidence from electron microscopy (EM),(ref 85,86) as well as immunofluorescence studies lead to the acceptance of a contact model for cell adhesion. This effectively focussed cell adhesion research into three broad areas. The investigation by biochemists of the extracellular matrix (glycocalix) and its role in cell adhesion processes. Associated with this first area of study was the investigation of intracellular and cell membrane organization associated with cell adhesion. Both these approaches represent a further application for techniques already routinely practiced in most establishments routinely maintaining cells in culture. This includes a wide range of biochemical separation and purification techniques such as gel chromatography, immuno blotting, affinity chromatography and cell biology techniques such as immunofluorescence and other biochemical labelling techniques. These two areas have therefore received a high level of attention.

The third avenue for research is that of the role of the culture substrate in cell adhesion behaviour. Purposeful research in this area requires a knowledge of not only cell biology and biochemistry but also synthetic polymer chemistry, including structure function relationships and physiochemical properties. This represents a combination rarely found in biological research establishments. It is the authors view that it is mainly due to these multidisciplinary requirements that progress in this field has been slower than other areas.

Quite obviously it is this third area which forms the main subject matter of this thesis and therefore the subject of an

extensive literature review. The other areas will however, be briefly reviewed to provide a more complete introduction to this area of study. In particular a realization that certain biochemical species are known to be involved in cell adhesion mechanisms, form a useful basis for the subsequent consideration of the implications of the physicochemical aspects of these substrata. It is inevitable that future studies (beyond the scope of this thesis and indeed any current understanding) will address questions of specificity and conformational aspects of the interaction of these molecules with synthetic substrates in the presents of cells and culture media.

1.1 The role of extracellular materials in cell adhesion; a brief literature review.

Interactions of cells with extracellular materials are critically important events during embryonic development, growth regulation and maintenance of normal tissue function. Considering the biological importance of these interactions surprisingly little was known about the mechanisms by which these extracellular molecules interact with cells and other extracellular molecules. Recent progress has resulted from the purification of specific proteins involved in such interactions, and the use of model systems in order to explain complex biological events in terms of the combined action of specific structural and functional domains of these molecules.

In general terms cell adhesion to substrates of such adhesion active molecules has proved one of the more fruitful of

several approaches used to determine the interactions of cells with such molecules. Findings from these studies therefore are not only relevant to cell adhesion and growth control but also useful for studying more general cell surface interaction phenomena.

Proteins bind tightly and non specifically in low amounts to either native polystyrene or tissue, culture plastic, the adsorption can be confirmed by isotopic labelling and immunological methods. Cells are seeded onto such surfaces and are examined for attachment and the morphological effects of the absorbed molecules. The specific activity and cell type specificity of adhesion molecules and fragments of adhesion molecules can be determined rapidly in these systems (ref 87). There are however some difficulties in obtaining reliable results from this system. Firstly the necessary use of serum free medium in order to ascertain the effect of the adsorbed protein, limits cell viability to a few hours. These considerations clearly limit the relevance that any findings may have to cell growth phenomena. Furthermore the choice of substrata (modified or unmodified polystyrene) has been shown to alter the activity of absorbed proteins (ref 88) and protein fragments (ref 89). For this reason it is preferable to use tissue culture plastic and hence retain continuity with normal cell culture. Cells can attach directly to tissue culture surfaces in serum free medium and although such binding is considered non-physiological (ref 90)(eg interactions are not disrupted by proteases or chelating agents as in normal cell culture) such adhesion can still significantly raise negative

control values and hence mask the activity of some moderately active proteins and fragments.

Early investigations in this area identified glycoprotein of molecular weight 440,000 daltons as a cell adhesion factor (ref 91-102). Under serum free culture conditions cells would attach to substrates coated with the glycoprotein or attach to plastic substrata if the glycoprotein was present in the culture fluid (ref 103). This glycoprotein is now known as fibronectin. The addition of excess levels of fibronectin to culture fluid can change cell morphology and restore anchorage dependence in transformed cell lines (ref 104) or induce adhesion in growth cycle defective mutants (ref 105-107).

Fibronectin is present in the cytoplasm and on the plasma membrane surface of fibroblasts (ref 93-102), it is also present in plasma (ref 91,93-102) and the extracellular matrix (ref 108-113). Fibronectin is synthesized by a variety of cell types including fibroblasts (ref 93-102) certain epitheial cells (ref 93-102) and hepatocytes (ref 111-118).

The glycoprotein is composed of two 220,000 dalton subunits linked by two disulphide bridges (ref 95-102). The two subunits are essentially identical and have relatively little alpha helix or beta sheet when examined by circular dicroism (ref 119-121), although infrared spectroscopy suggests the presence of 35% beta sheet structure (ref 122). Within these subunits are functional and structural domains which showed tertiary structuring when circular dicroism studied were performed on proteolytic fragments (ref 119,121,123).

The sedimentation constant for fibronectin decreases

initially with increasing ph suggesting that structured areas (probably forming functional domains) are separated by flexible regions which permit the molecule to unfold under certain electrostatic conditions (ref 123-125). The domain structure of fibronectin allows the molecule to bind to the cell surface and a range of other extracellular molecules many of which are known to be involved in cell adhesion.

The cell binding domain can be separated by proteoyltic enzyme digestion of fibronectin. Initial studies showed a 33k region which would allow cell attachment to plastic surfaces (ref 126). Subsequent investigations identified smaller fragments with cell attachment activity. Recently however subsequent studies of this region have shown that a polypeptide chain containing only three subunits (argenine, glycine and aspartate acid) will support cell attachment (ref 127). The nature of the interaction between the cell binding domain and the cell surface remain unclear. To date no single receptor molecule within the cell membrane has been identified and it seems likely that this binding is of a more complex nature.

Fibronectin can bind to all types of natural (ref 128-134) and denatured (ref 130-136)collagen, surprisingly the later type being more strongly bound. Fibronectin can therefore mediate the attachment of cells to collagen in the presence of divalent cations (ref 136). It is believed that cells bind only to fibronectin in fibronectin/collagen complexes (ref 137,138). Fibronectin has four heparin/heparan sulphate binding sites (ref 139-151) two of which are of significantly lower activity. In cell adhesion it seems that the binding of fibronectin to

heparan sulphate proteoglycan could provide structural organization (ref 151). Fibronectin can also find other proteoglycans hyaluronic acid (ref 151-161) and chondrotin sulphate (ref 156). In general it seems that proteoglycans may mediate in fibronectin to cell and fibronectin to extracellular matrix interactions.

Fibronectin can also bind fibrin fibrinogen (ref 160-166) and the cytoskeletal proteins, actin, (ref 167-169) myosin and actinin (ref 170). Finally fibronectin can also associate with itself in order to form polymers (ref 101).

The existence of specific functional domains arrayed in a modular fashion along the polypeptide subunits of fibronectin can begin to explain it's many ligand binding and biological activities. To date, the known interactions of fibronectin with the cell surface can be attributed to binding at the cell binding domain. Proteolytic fragments (or synthetic polypeptides) containing only this region are sufficient to mediate cell adhesion onto polystyrene surfaces, probably through non specific binding of the fragment to the plastic surface (ref 126). Antibodies to this region block cell adhesion and cell migration (ref 126,171-175). To obtain cell attachment to other ligands bound by fibronectin requires the cell binding and ligand binding domain to be linked together in one intact polypeptide (ref 176,177). Generally ligard binding domain antibodies do not block cell attachment to plastic culture dishes (ref 178). It therefore seems likely that fibronectin acts as a bridging molecule which is instrumental in cell adhesion. In culture however it is likely that cell

adhesion is usually facilitated by complexes containing fibronectin and other cell adhesion molecules (This is discussed later in this chapter.)

Other spreading factors aside from fibronectin have been identified, some from serum, including chondronectin (ref 179-182) and other as yet unpurified serum spreading factors (ref 180-184). Further spreading factors have been obtained from other sources, eg laminin (ref 185), collagen (ref 186) plus the proteoglycans and glycosamino glycans of heparan sulphate (ref 151) chondrotin sulphate (ref 156-9) and hyluronic acid (ref 151-156).

Laminin is a glycoprotein known to mediate the attachment of epitheial cells to basement membrane in vivo (ref 87,185-190). Laminin is found only in basement membrane (ref 191-195) and is significantly larger than fibronectin, being composed of three alpha chains of 200,000 daltons each and one beta chain of 400,000 daltons (ref 191-199). The molecule forms a cross when visualized by rotary shadowing (ref 197-199). At the present time knowledge of the domain structure of laminin is far from complete. It has been demonstrated however, that heparin and cell binding domains exist within the molecule (ref 197-199).

In culture laminin can mediate the attachment of not only epitheial, but also fibroblastic cell to plastic or glass (ref 185), although it may be substantially less effective than fibronectin for fibroblasts. Equally certain epithelial cells can utilize fibronectin for adhesion (ref 189,200). Laminin also a more effective substrate for ganglion neurite out-growth than fibronectin (ref 201-202). The binding of heparan sulphate

proteoglycan by laminin is undoubtedly important in vivo as both are present in substantially quantities in basement membrane (ref 195). This could also be an important factor in cell attachment to laminin in culture.

Chondronectin is a glycoprotein isolated from chick serum, which specifically mediates the attachment of chondrocytes to collagen type II in vitro (ref 179-181). It exists as a disulphide linked multimer of 180,000 daltons (ref 180) and has a binding capacity for most glycosamino glycans (ref 179). Chondronectin is specific for chondrocytes and shows minimal attachment activity for fibroblastic or epithelial cells (ref 180).

Besides fibronectin and condronectin other spreading factors have been identified within serum (ref 180-184). A spreading factor for both fibronectin and epithelial cells of 62-70,000 daltons has been characterized recently (ref 183). This promotes attachment several fold slower than fibronectin or laminin and requires protein synthesis by attaching cells (ref 183).

Another 70,000 dalton spreading factor promotes the spreading of cells in serum free medium (ref 203-206). Epibolin is a 65,000 dalton glycoprotein which promotes the attachment of epithelial cells. Finally a fibroblast spreading factor form chick serum of 140,000 daltons remains to be characterized (ref 181).

The proteoglycans heparan sulphate, chondrotin sulphate and hyluronic acid are abundant in the extracellular matrix (ref 151), although the role of these species in cell adhesion is as

yet uncertain. It is well established however, that they are present at adhesion sites and bind to other extracellular materials not least fibronectin . The role of proteoglycans at adhesive sites is reviewed later in this chapter.

1.2 The structure and biochemistry of cell substrata adhesion sites; a brief literature review.

When spread in culture fibroblasts form specialized adhesions of different types. These adhesions show clear morphological similarities to cell-cell intermediate junctions in that microfilament bundles terminate at the cytoplasmic face in an electron dense plaque. These typically contain muscle accessory proteins vinculin and actinin (ref 207-212). These adhesions can be identified in terms of separation distance between the plasma membrane and the substratum and in the time for which they persist, in apparent composition and in some details of morphology (ref 213-217). There is however, reasonable agreement with the following classification of adhesion structures.

Focal Adhesions are characterized by close apposition of the plasma membrane to the protein layer absorbed to the substratum (10-20nm) and also by a bundle of actin filaments terminating at an electron dense plaque at the cytoplasmic face of the membrane (ref 211). The associations of these structures with actinin vinculin and other muscle proteins are well documented (ref 207,209,211,212). These adhesions are also characterized by a long lifetime since they can persist for many hours in culture (ref 214). There also seems to be an

association with areas of clathrin coated membrane close to these adhesions (ref 218).

Focal Contacts are similar to focal adhesions but are smaller in area; they are characteristic of mobile cells and have a life time of only minutes (213,214).

Close Contact Areas were first defined in terms of a type of image observed using interference reflection microscopy (ref 216). The separation distance is 10nm or over and often varies rapidly. The area of these adhesions is larger than focal adhesions (ref 214) but cytoplastic organization is less prominent. The life time of these adhesions can be as short as several seconds if they are not stabilized by an adjacent focal adhesion (ref 214)

Non contacting plaque structures are a membrane feature very similar to focal contacts but in an area of no cell substratum (or cell-cell) contact (ref 219).

The relationship between adhesion structure formation and cell growth is not clear as yet. Attempts to use other spreading factors to promote cell spreading without focal adhesions (ref 220) need further verification.

In order to investigate the biochemistry of cell adhesion sites techniques were developed to separate these structures from the cell. The treatment of spread cells with the chelator EGTA causes spread cells to round up and pull away from the adhesion sites which attached it to the substrata (ref 151). The cell adhesion sites are left on the substrate and are termed substrate attached material (SAM). SAM can be analysised in situ with immunofluorescence and other labeling techniques (ref 151)

or can be removed by physical means and analysised using almost any biochemical technique. Critics of these SAM preparations point out that it is difficult to ensure that all cells are removed and to prevent cell debris from adhering to the exposed adhesion sites. Use of the technique has nevertheless been quite extensive (ref 221-232) and has given an insight into not only the components (ref 231-232) but more recently the biochemical structure of adhesion structures.

The composition of SAM in terms of proteins (ref 223-233) phospholipids (ref 226) and polysaccharides (ref 224) is significantly different to that of complete plasma membrane preparations. SAM contains 1-2% of the total cell protein which is mainly composed of fibronectin , myosin, actinin and vinculin (ref 234-237). An uncharacterized nonglycosylated protein of 52-55,000 daltons has also been identified this protein is present under a wide range of conditions in both transformed and normal cell lines, it is usually found in a constant ratio to actin (ref 223,231-2,238) except in new adhesions where it is more plentiful.

The most striking feature of SAM composition is the enrichment of glycosamino glycans (GAGS) both as monomers and proteoglycans (ref 223,228,239). In fact SAM contains between 5 and 10% of total cell polysacaride over half in the form of G.A.G.S. In general there is a high concentration of chondrotin-4-sulphate (ref 240), hyluronic acid monomers (ref 241), heparan sulphate proteoglycan and monomer (ref 242), but a low concentration of dermatin sulphate (ref 234). Furthermore heparan sulphate proteglycan is three times enriched in early

cultures (1 hour) before morphologically district adhesion sites develop. After which time chondroitin sulphate and hyluronic acid levels increase in parallel (ref 151).(suggesting a similar relationship to that found in cartilage.

A model for cell-substrate adhesion has been proposed based on these findings (ref 151) in which heparan sulphate proteoglycan mediates between fibronectin and substrate bound serum (ref 223,243,244). The fibronectin then binds to the cell surface to form the adhesion site. Such an adhesion complex is then modified by heparan sulphate monomer, hyaluronic acid and chondroitin sulphate which compete with heparan sulphate proteoglycan for fibronectin binding (ref 238,245). This may cause maturation of the adhesion site and hence allow cell motility or growth. Increasing hyluromic acid levels have already been demonstrated to be important in this context (ref 246-247).

1.3 Cellular Interactions with synthetic polymer surfaces in culture; A literature survey.

This area of research is unusual in that scientists from widely differing backgrounds, with various research objectives have made significant contributions. Cell biologists have endeavoured to understand cell adhesion mechanisms (ref 23-30), or produce cell culture surfaces suitable for differing cell types (ref eg 79) and ultimately control cell growth (ref 13-17,19). Some chemists have used cell culture as a convenient biological interface (ref 248-250) and researchers in biomaterials have

looked at cell culture in parallel with in vivo studies (ref 251,252). It is perhaps due to the dichotomy of these approaches that the findings and conclusions expressed are not only wide ranging but somewhat conflicting and confusing. It is therefore not surprising that there is no overall consensus of opinion as to the requirements for a cell adhesive surface or as to the biophysical mechanisms which may have an over riding influence upon cell adhesion in culture. This survey will review these differing areas of research and will finally present an appraisal of the overall situation. Earlier in this chapter the development of D.L.V.O. (ref 30-35) and contact hypotheses (ref 57-65) for cell attachment; and the use of modified polystyrene substrates to study the relative merits of the two theories were discussed (ref 60). At that time the contact hypothesis was accepted due to the wide ranging evidence (ref 57-70) in favour of such an approach and forms the basis of most present research. It had been demonstrated that when seeded onto polystyrene substrates cells only attach and spread on surfaces which had been treated with concentrated sulphuric acid. The negative surface charge generated by this treatment was monitored with die binding assays. A cell adhesion plateau was observed on surfaces expressing (the term "expressed" is used in cell biology to indicate or describe a group or function that is present or available at a surface)between 1.8 and 17.2 charges per 100 sq $\mathring{A}$. This surface charge was assumed to be due to the presence of sulphonate groups (ref 63). The conclusion of this work was that a contact hypothesis could predict this plateau of cell adhesion. The mechanism for this adhesion was postulated to

involve the structuring of surface water by charged groups resulting in the relative immobility of protein molecules at the surface thus giving rise to the necessary attachment forces and plannar rigidity. The same group later showed that for mesenchymal cells cultured in serum free medium, sulphuric acid treated (assumed sulphonated) polystyrene gave superior cell adhesion to commercial tissue culture plastic. The results again showed a plateau for cell adhesion within the range 1 to 102 charges per nm^2. The decline in cell adhesion beyond this point was thought to be due to the formation of a hydrogel structure at the surface of the polystyrene and the resultant polymer exclusion phenomena. It was concluded therefore that a cell adhesion substrate must be charged to the desired extent, be reasonably inflexible and dense.

Other workers were also investigating the attachment of cells on modified polystyrene surfaces (ref 79,253). Klemper and Knox (ref 79) treated the surface of polystyrene not only with sulphuric acid but also chromic acid (10m CrO_2 in water) and with a mixture of two acids (0.01 in CrO_2 in H_2SO_4). The resulting surfaces were analysised by crystal violet dye binding. This revealed that while H_2SO_4 treatment produced only strongly acidic groups at the substrate surface and chromate treatment produced only weak acid groups, a mixture of the two reagents resulted in a surface expressing both moeties.

BHK (Baby Hamster Kidney) and adult rat liver cell cultures were used for adhesion assays. BHK cells formed growing monolayer cultures on all surfaces. The liver cell cultures however, were only successful on surfaces pretreated with

chromate or the mixed acid reagent. A role was therefore proposed for weak acid groups (presumed to be hydroxyl moeties) in addition to or independent of strong acid groups (assumed to be sulphonate).

The preceding description demonstrates how two similar studies can lead to differing conclusions. Both of these studies represent a departure from the earlier conclusions of Maroudas (ref 63)(see initial introduction) that surface wettability was the overriding criterion in the adhesiveness of a surface for cells.

Cell adhesion on modified polystyrene surfaces was the subject of a recent (1983) study by Curtis et al.(ref 253). Although this work clarifies some points it also produces further anomalies. Polystyrene surfaces were treated with sulphuric and chloric acids, ozone and other chemical agents. The adhesiveness of the resulting surfaces was assayed using BHK fibroblast and leucocyte cultures and compared with T.C.P. and bacteriological grade culture surfaces. Surface chemistry was accessed using X.P.S. (X ray photo electron spectroscopy) and chemical blocking techniques.

It was demonstrated that all acid treatments produced only hydroxyl and carboxyl moeties on the surface of polystyrene . Chemical blocking of hydroxyl groups was shown to inhibit cell adhesion whereas carboxyl group availability had little effect on adhesiveness. These results obviously undermine the previous assumptions regarding the importance of sulphonate groups in cell adhesion. The experimental results of Klemper and Knox are quite clearly in agreement with these findings. Although the

earlier study (ref 79) showed some cell line specificity (ref 79) which is not observed by Curtis et al (ref 253).

A further point made by Curtis et al is that bacteriological plastic dishes do express hydroxyl groups (probably produced during the moulding process) and will support low levels of cell attachment after the removal of silicone mould release agent which is usual left on the surface to inhibit cell adhesion.

In conclusion Curtis et al proposes a fundamental, specific and overriding role for hydroxyl groups in cell adhesion to both synthetic and biological surfaces. Detailed attention is paid to these proposals in chapters four and five of this thesis.

As well as modified polystyrene other synthetic materials have been tested as culture substrates in an attempt to establish properties compatible with cell adhesion (ref 254-272). In order for such an approach to be successful it was necessary for substrates to be found which both allow and inhibit cell adhesion. This proved to be a considerable problem, because under full serum culture conditions cells adhere to a wide range of substrates including Teflon (ref 53,254-256), metals (ref 66,257-8), Cellophane (ref 258), glass , polysaccarides (ref 44), proteins (ref eg 87), polycarbonate (ref 258), perspex (ref 53,258-260), melinex (ref 26), even phopholipid interfaces (ref 262-3). Substrates such as parafin wax, siliconized glass and polystyrene were shown to be non adhesive but only the latter is easily usable in culture.

In a broad study of fibronectin absorption and consequent cell spreading on synthetic and biological substrata Klebe (ref 258) confirmed the adhesiveness of cells for a wide range of surfaces. A notable exception was the hydrophilic hydrogel poly(2 hydroxyethyl methacrylate), commonly known as poly (HEMA), which allowed no fibronectin adsorption or cell adhesion. Furthermore blending of poly(HEMA) with PVA (poly vinyl acetate) produced cell adhesive substrates. This lead Klebe to the conclusion that hydrophobic materials are most suitable for cell culture. This view is not only in conflict with the well established non adhesive behaviour of polystyrene , but is also in conflict with several other reports. These studies (ref 44,57,63,) had shown a general increasing cell adhesion with increasing surface energy between P.T.F.E. and T.C.P. Similarly a comprehensive study of fibronectin absorption on polystyrene and T.C.P. surfaces (ref 273) shows that only the hydrophilic substrate allows the glycoprotein to absorb in a cell adhesive conformation.

The non adhesive nature of poly(HEMA) has been confirmed by several other studies (ref 274-279). This recent work together with the recent claim that modulation of cell spreading has been achieved by the use of varying concentrations of poly(HEMA) (ref 280-282), this is reviewed in detail in chapter three.

More diverse research into cell adhesion has revealed some interesting phenomena. Cells seeded onto linear and plannar anchorages of limiting dimensions show cell adhesion only on particles of over 40um and conventional phagocytosis of all

particles of less than 20um (ref 283-285). These findings are further evidence of a link between cell adhesion and phagocytosis. Adhering fibroblasts have been shown to align with the axis of least stress when attached to elastic substrata (ref 267). The effects upon cell adhesion of grossly grooved surfaces (ref 286), surfaces of differential adhesiveness (ref 287) and hydrogen bonding (ref 252) have all been the subject of some research.

The effect of charge on eurcariotic cell adhesion has continued to be of interest to some researchers (ref 288-289). It seems likely that positively charged surfaces are able to promote cell adhesion (ref 288), although the physiological relevance of the kind of adhesion is as yet unclear. It seems that the long range forces produced by cell adhesion to negatively charged surfaces may play a role in the formation of close contact areas but have no effect on focal adhesions (ref 289).

A further area in which cell substrate relationships may be important is that of Biocompatibility of synthetic materials and the potential applications of these materials for implant and prosthesis manufacture. In vivo studies of these materials have been extensive (reviewed in ref 290), a comprehensive review of this area is therefore beyond the scope of this thesis. Comparison of in vivo results and cell culture studies of potential implant materials have to date been relatively inconclusive (ref 249-250). Due in part to the difficulties involved in comparing short term cell studies to longer term implant research; and also the complexity of some implant

materials makes Comparison of cell adhesion results with known cell culture parameters difficult (ref 290). Therefore although parameters governing the adhesion of platlets (ref 291) and other non eucaryotic cell types (ref 248) seem to be to some extent established, the factors involved in eurcorotic cell adhesion are as yet unclear. The relationship between in vitro and in vivo results can not therefore be established and represents a potentially exciting research area.

It seemed reasonable therefore to undertake a research program which could elucidate the requirements for cell adhesion which would allow the better application of this potentially powerful research technique to the areas such as biocompatibility. Such an approach could lead to the development of substrata systems capable of controlling cell growth in culture and thus allowing the rapid advance of research concerning the biochemical initiation of cell growth. There can however be no fundamental advance in the establishment of any commonality between in vivo performance of biomaterials and in vitro cell culture studies, until there is a clear understanding of the relationship between the in vitro adhesion of anchorage dependent cells and the properties of the substrate.

1.4 Objects and scope of the present work

1. To investigate the claims that poly(HEMA) films of differing thicknesses could modulate the cell spreading process.
2. Attempt to investigate the relative roles of specific chemical groups thought to be important in cell adhesion, specifically hydroxyl and sulphonate moeties.
3. To investigate the physicochemical forces relevant to cell adhesion, by investigating a wide range of commercially available materials, custom synthesized copolymers and glow discharge treated polystyrene substrata.
4. To utilize the above investigations in order to produce a model system which would allow the identification of adhesive and nonadhesive substrates in terms of some physicochemical parameter.

Chapter Two

Materials and methods

Materials and Methods

Materials

Most polymers used in this study were custom synthesized materials, produced precisely for that purpose. Some polymers were however obtained from other sources:-

1.) Polypropylene, Polyethylene, Poly tetra fluoroethylene and polyglycerol methacrylate were kindly donated by Dr P. Skelly.

2.) Polybutylene terephthalate films were kindly donated by Mr Yasin.

3.) Polystyrene (research grade) was obtained from polysciences and was used as supplied.

4.) Poly ethylene terephthalate was kindly donated by I.C.I. Ltd.

5.) Tissue culture plastic and Bacteriological plastic were used from stocks of dishes maintained routinely at Unilever Colworth and M.R.C. Research.

2.1 Monomers

Supply and Purification:

Monomers were obtained from several suppliers and were purified as described previously (ref 292). Exceptions to this are as follows:-

1.) 3 Sulphopropyl methacrylate and 3 sulphopropylacrylate were donated by D Bennett Chemicals acting on behalf of Rashig Ltd and were used as supplied.

2.) 2 Hydroxylethyl methacrylate was supplied as purified optical grade material by Victor-Woolf of Manchester and was used as supplied.

METHODS

2.2 Solution polymerization

Poly(2-hydroxyethyl methacrylate) was produced in a non-cross-linked form by solution polymerization of redistilled HEMA (2-hydroxyethyl methacrylate) in ethanol. The polymerization was preformed at 40°C under a nitrogen atmosphere for four days during which time the mixture was continuously stirred.

Initiation was by t-butyl cyclohexyl per dicarbonate. The polymer was precipitated into di-ethyl ether and dried for four days in a vacuum oven at 400C.

2.3 Bulk Polymerization

(a) Prepolymerization

Monomer mixtures were initiated at the 0.1% level with cyclohexylpercarbonate. These mixtures were then prepolymerized at 40°C until suitable viscosity was attained. This perpolymerization ensures that the loss of volatile monomers during polymerization is reduced to insignificant levels.

(b) Polymerization

The prepolymerized mixtures were transfered to polymerization cells as previously described (ref 293). Polymerization was then continued over four days at 50°C.

(c) Post Cure Treatment

Where suitable all polymers were post cured for 3 hours at 90°C.

2.4 Spun coatings

Spun coatings were produced using a Dage Precima International photoresist coater model P.R.S.14.E. Cover slips (either 22mm diameter or 22mm x 22mm square) were centrifugally coated with poly(HEMA) solutions of between 2 and 40% (wt/vol) dissolved in 2-ethoxyethanol. This medium was chosen because initial experiments had shown a requirement for the use of a slow evaporating solvent.

Spin speeds were varied between 900 and 3500 rpm to produce a range of coating thickness for a given solution concentration (Appendix 1). Coating produced were assayed for coating thickness using the intrephaco method of measurement on a Zeis interference microscope. This involves scratching the polymer film, (being careful not to scratch the underlying mirrored surface (silicon wafer)) and measuring the number of wavelengths by which the image of the scratch is displaced using interference fringes and the micrometer on the microscope. This method of measurement is accurate to 0.15um dependent on the quality of the scratch in the polymer film. Poly(HEMA) Spun coatings were produced over a range of thicknesses between 0.025um and 5.0um. Polystyrene Spun coatings were produced from a 15% solution in toluene at a spin speed of 2000 rpm.

The desired spin speed was maintained for one minute in the case of all polymer coatings in order to allow complete evaporation of solvent from the polymer film.

2.5 Evaporation Coatings

These coatings were preformed as previously described by Folkman and Moscona. Poly(HEMA) solutions were made up in ethanol which was allowed to evaporate at 37°C overnight. Using their terminology (ref 280) the range og poly(HEMA) concentrations employed were

1, 1 x 10-1, 2 x 10-2, 1 x 10-2, 8 x 10-3, 6.6 x 10-3, 6 x 10-3, 5 x 10-3, 4 x 20-3, 3.5 x 10-3, 2.5 x 10-3, 2 x 10-3, 1.75 x 10-3, 1.45 x 10-3, 1.125 x 10-3, 1.11 x 10-3, 1 x 10-3, 8 x 10-4, 6.6 x 10-4, 6 x 10-4

This terminology refers to the number of dilutions of a 12% (wt/vol) poly(HEMA) solution, ie 8 x 10-2 = 125 x dilution of 12% (wt/vol) solution. It is the convention in this field to use the units of the original workers (ref 280) and these will be termed "F and M units" for the purpose of this thesis.

2.6 Plasma treatment of polystyrene surfaces.

Polystyrene Spun coatings were exposed to oxygen, nitrogen or argon plasmas in a Nanotech plasetch 250. Discharge conditions were standardized at 150 KV under a vacuum of 10^{-2} torr. Samples were treated for either 20, 30, 60, 120 or 600 seconds.

Analysis of gases (as produced by BOC) used for plasma treatment is shown in table 2.1.

Table 2.1

(a) Oxygen

B.O.C. Research grade

Specification		Typical analysis	
Oxygen	99.97% (3.7)	Oxygen	99.98%
Nitrogen	200vpm	Nitrogen	100vpm
Hydrogen	2vpm	Hydrogen	1vpm
Argon	75vpm	Argon	50vpm
Carbon Dioxide	1vpm	Carbon Dioxide	1vpm
Carbon Monoxide	1vpm	Carbon Monoxide	not detected
Hydrocarbons	2vpm	Hydrocarbons	1vpm
Moisture	5vpm	Moisture	1vpm
		Physical Form	gas
		Specific Volume	0.755m3/kg at 20°C 1 bar

Table 2.1 continued

(b) Nitrogen

B.O.C. Research grade

Specification		Typical analysis	
Nitrogen	99.994% (4.4)	Nitrogen	99.997%
Oxygen	2vpm	Oxygen	1vpm
Argon	50vpm	Argon	25vpm
Carbon Dioxide	1vpm	Carbon Dioxide	1vpm
Hydrocarbons	1vpm	Hydrocarbons	1vpm
Neon	2vpm	Neno	1vpm
Moisture	2vpm	Moisture	1vpm
		Physical Form	gas
		Specific Volume	0.861m3/kg at 20°C 1 bar

(c) Argon

B.O.C. Research grade

Specification		Typical analysis	
Argon	99.9995% (5.5)	Argon	99.9997%
Oxygen	2vpm	Oxygen	1vpm
Nitrogen	5vpm	Nitrogen	2vpm
Hydrogen	1vpm	Hydrogen	1vpm
Carbon Dioxide	1vpm	Carbon Dioxide	1vpm
Hydrocarbons	1vpm	Hydrocarbons	1vpm
Moisture	2vpm	Moisture	1vpm
		Physical Form	gas
		Specific Volume	0.604m3/kg at 20°C 1 bar

2.7 Surface Energy measurements

(a) On Dehydrated Polymer Surfaces

The sessile drop technique (ref 293,294) was utilized with water and Diodomethane as test liquids. From these measurements surface energies were calculated as previously described (ref 294). All polymers were measured in a sterile condition ready for cell culture.

(b) Fractional Polarity

Primary experimental results were used to calculate fractional polarities by a modification of the methods first proposed by Owen and Wendt (ref 295) and Hamilton (ref 296).Using the following formula:-

$$\text{fractional polarity} = \left(\frac{\gamma_p}{\gamma_p + \gamma_d} \right)$$

2.8 X-ray Photoelectron Spectroscopy (X.P.S.)

An Xsam 800 (Creatos) was used to examine plasma treated and native polystyrene surfaces. Utilizing magnesium Kα radiation under a vacuum of 10^{-9} Torr.

2.9 Equilibrium Water Content (E.W.C.)

Equilibrium water contents were estimated from the results of Jolly (ref 297) using methods described by Pedley and Tighe (ref 293).

2.10 Transmission Electron Microscopy (T.E.M.)

Sample Preparation

Cell cultures were fixed with 3% gluturaldehyde in 0.1m sodium cacodylate buffer, pH 7.2, for 15 minutes. They were then subjected to the following steps:

(1) Washed in 0.1m sodium ceroid buffer.

(2) Dehydrated in a graded series of alcohols (ethanol): 50%, 70%, 90% for 5 minutes each. Followed by 3 x 100% (absolute ethanol) for 15 minutes each.

(3) Air dried from amyl acetate.

(4) Coated with platinum at 30°C.

(5) Carbon coating at 90°C.

(6) Cover slips floated off in HF.

(7) Washed in distilled water.

2.11 Scanning Electron Microscopy (S.E.M.)

Preparation of samples

The cover slips were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 30 minutes, followed by two washes in buffer. The cover slips were then dehydrated through a graded series of ethanol water solutions (50%, 70%, 90% and 100% x 3) for 15 minutes each. The samples were then critical point dried via liquid CO_2 in a Poloron E3100 II critical point bomb.

The cover slips were mounted on brass specimen stubs using colloidal silver and coated with 100 A^{o} of gold/palladium in a Polaron E5100 'Cool' Splatter Coating Unit at 800v and 20mA. The samples were examined in a JEOL-35X at 25kV accelerating voltage.

2.12 Cell Culture

All cell lines were routinely maintained at 37°C in Dulbecco's MEM (Gibco Europe) supplemented with L-glutamine, pen strep (Gibco Europe) and 10% foetal bovine serum (Flow Labs). For the attachment and spreading assays in the presence of serum, cells were detached by trypsinization, resuspended to 1 x 10^{6} cells/ml in complete medium and 2 ml of cell suspension seeded onto dishes or coverslips. Cultures were maintained for up to 6 hours at 37°C and examined by light microscopy (x400 magnification).

In the case of cell spreading assays performed in serum free medium. pbs(-), or pbs(+) the action of the trypsin was

stopped by trysin inhibitor. The cells were washed repeatedly in the medium in which they were to be cultured. Cells were then suspended at 1 x 10^5 cell/ml in the relevant medium and 2 ml of cell suspension seeded onto the test substrata.

2.13 Growth Studies

Baby Hamster Kidney (BHK) were harvested as above and seeded at 5.5 x 10^3 cells/cm^2 of culture substrate in 2ml of complete medium. Cells were harvested at 24 hour intervals using trypsinization. Vigorous agitation was used to reduce cell clumping and substates were checked using light microscopy for remaining adherent cells. The harvested cells were counted in a Coulter counter.

2.14 Cell Lines

The cell lines used in this study are as follows:-

BHK	=	Flow Baby Hamster Kidney 21/C13
16C	=	Spontaneously transformed rat dermal fibroblast obtained from Unilever Research Colworth Labs. Bedfordshire.
CEF1^o	=	Primary chicken embryo fibroblast obtained from chick embryo homogenate.

MRC5 = Normal dipoid human embryonic lung fibroblast.

REF = Primary rat embryo fibroblast (from 12 day embryos). Obtained from Unilever Research Colworth Labs. Bedfordshire.

Hep2 = Epithelial cell line derived from human carcinoma of the larynx

This choice of cell lines enables differences in adhesion characteristics arising from cell phenotype or transformation states to be ascertained

2.15 Cell Fixation

Subsequent to cell spreading assays cells were fixed using 3% Gluteraldelyde.

measurement of cell spreading

2.16 Quantiment Analysis

Wherever possible measurement of cell spreading was performed on a Cambridge Instruments quantiment at Unilever Labs, Colworth.

Rawdat software was utilized to yield the maximum information. Derivation of the largest ferets diameter and a typical print out are shown in Appendix 2.

Preliminary experiments were performed to establish

optimum cell staining conditions as a 2% solution of amido black in a solution of water, acetic acid and ethanol in the proportions of 5:1:5.

Cell parameters were determined from at least 6 randomly chosen fields from any one cell population.

2.17 Cell Counting

For each cell line fully spread cell morphology was determined by comparison with stock cultures maintained on tissue culture grade surfaces (corning, flow or nunc). The percentage of fully spread cells in a population was established by manually counting several hundred cells in at least 6 fields selected "blind" by the microscope operator.

Chapter Three

Characterization of cell spreading responses on poly(HEMA) substrate

3.1 Introduction

Intensive research into anchorage dependent cell growth over several years, has lead to only a partial understanding of the processes involved. Specific roles have been attributed to a variety of macromolecules (fibronectin laminin, heparan sulphate), and the apparent differentiation of the cell membrane to form focal adhesions are thought to be important. But the nature of interactions both at the cell/substratum interface and between the plasma membrane and structures formed within the cytoplasm (eg actin bundles) are still relatively unclear. In many respects research in this area has been hampered by the dynamic nature of the cell spreading process.

The findings of Folkman and Moscona therefore were of paramount significance to any researchers in the field of cell adhesion. The paper, (published in Nature in 1978) (ref 280) showed that the spreading process of anchorage dependent fibroblasts and epithelial cells could be continually modulated by an uncomplicated system of substrates. These were produced by coating tissue culture plastic microwells with the biocompatible hydrogel poly(HEMA). The paper described how by coating tissue culture plastic substrata with firstly a 12% poly(HEMA) solution and then taking dilutions of this solution up to 10,000 fold, a series of substrata of differential cell adhesiveness could be produced.

Furthermore, cells were not held on these surfaces in simply increasing numbers but the mean cell diameter of the cell populations were shown to increase with decreasing poly(HEMA) film thickness (increasing dilution of original poly(HEMA) solution). Cell shape was thus proposed as a powerful regulator of cell growth. This technique appeared to be a fundamental step forward as it allowed cells to be held at any point in the cell spreading process, and provided the ideal method by which to study biochemical changes occuring during cell spreading. These biochemical reactions are of particular interest due to the pertinance of cell spreading to the cell division process.

Surprisingly in the subsequent three years no other work was produced using this system. In 1981 however, a publication by Ben-Ze ev, Penman and Farmer further utilized the poly(HEMA) system. In their experiments it was shown that only cell attachment was required for protein synthesis but that DNA, RNA and rRNA synthesis was closely linked to cell shape. Some of these findings were of particular interest to researchers at Unilever Research (Colworth), who had invested considerable research effort into defining the structure and role of cell surface attachment sites, in particular focal adhesions("feet"). Focal adhesions are areas of cell membrane which come within 15nm of the substratum, and appear to be stabilized by interactions with actin bundles within the cytoplasm and to be associated with specialization within the extra cellular matrix if not the cell membrane (ref 207-220). The exact significance of focal adhesions to the cell spreading or growth process however was still unclear.

Ben-Ze ev at al showed that very low poly(HEMA) concentrations, (greater than 1000 fold dilution of the 12% poly(HEMA) solution) which did not affect mean cell diameter (ie cells remain fully spread) significantly reduced DNA synthesis. It seemed feasible therefore, that the onset of DNA synthesis within fully spread cell populations, could identify a role for specific cell substrate interactions, (ie focal adhesions). It was therefore decided to utilize the Folkman and Moscona system of substrates to investigate the role of focal adhesions in cell spreading and division processes. There were difficulties however, in order to observe the cell-substanum interface, it is necessary to use interference reflection microscopy. This technique requires that the substrate be relatively thin, with a smooth, flat surface. As this microscopy is sensitive to changes in cell to substratum distances of the order of 10nm. This precluded a simple evaporation coating technique onto tissue culture plastic as used by the original workers.(ref 280).
It became increasingly obvious that expertise in the area of hydrogel polymers (indeed synthetic polymer chemistry generally) was required. Workers at the University of Aston in Birmingham had been investigating the behaviour of a wide range of synthetic polymer under varying conditions, and particularly the behaviour of polymers in biological enviroments. Initial discussions lead to the proposal to utilize a spin coating technique already in use at Aston for the production of photoresist coatings.

Initial experimentation showed that a suitable combination of spin speed, polymer concentration and solvent regime, smooth consistent poly(HEMA) coatings could be produced on glass coverslips. At this stage it seemed entirely feasible to use this range of poly(HEMA) coatings to ascertain the relevance of focal adhesions and other cell substratum interactions to cell spreading and subsequent growth processes.

3.2 Results

Polymer coatings; Techniques and terminology

3.3 Evaporation coatings

Polymer films refered to as "evaporation" coatings were prepared as described in the original research (ref 280). That is the production of a 12% (w/v) poly(HEMA) solution in ethanol which is then diluted to a variable degree up to 10,000 fold. These solutions are then added to microwells or T.C.P. dishes and the ethanol is allowed to evaporate overnight at 37°c. This process is claimed to produce coatings of between 35 and 0.0035 um, such figures are based on a theoretical calculation assuming even polymer distribution across the coated surface. The terminology for the thickness of these polymer coatings was used by the original workers and is based upon the degree of dilution of the stock solution, which is required to produce certain coatings, ie. A 1 in 50 dilution of the stock solution is termed a $5x10^{-1}$ poly(HEMA) coating and has a theoretical coating thickness of 0.7um. The correlation between dilution and nominal coating thickness is shown in table 3.1

3.4 SPUN COATINGS

This refers to films produced by the centrifugal spreading of a polymer solution over the surface to be coated.This method of coating is standard practice for the production of photoresist

films. The thickness of polymer films produced by this method is influenced by not only the polymer solution concentration but also by spread of rotation and accleration to that spin speed (see Chapter 2 and Appendix 1.).

3.5 Cell spreading on Poly(HEMA) coatings produced by the spinning technique.

Initial cell spreading assays were performed using poly(HEMA) coatings produced by the spinning technique detailed in the previous section. These studies used BHK fibroblasts cultured in medium containing 10% foetal calf serum. It came as a considerable surprise to find that no cell attachment or spreading was observed regardless of polymer film thickness (table 3.2)

3.6 Cell spreading assays on poly(HEMA) coatings produced by the evaporation technique.

In view of the previous findings it seemed prudent to perform cell spreading assays upon poly(HEMA) coatings produced as described in the Folkman and Moscoana paper. These cell spreading assays highlighted some cell spreading at lower poly(HEMA) concentrations (eg thinner coatings) (table 1). Initial observations also suggested the existence of intermediate cell spreading responses on poly(HEMA) films of $5x10^2$ and $8x10^2$ dilutions.

Table 3.1

comparison of actual poly(HEMA) dilution, "F and M unit" and theoretical estimation of polymer film thickness.

Dilution Ratio	Terminology "F and M units"	Theoretical estimation of polymer film thickness (um)
1	1	35
1:50	$5x10^{-1}$	0.7
1:100	$1x10^{-2}$	0.35
1.200	$2x10^{-2}$	0.18
1:500	$5x10^{-2}$	0.07
1:1000	$1x10^{-3}$	0.035

It became obvious that differences existed between poly(HEMA) coatings produced by spinning and evaporation techniques, even if such coatings were nominally of the same thickness. As a first step we choose to investigate the surface topography of these surfaces using scanning electron microscopy(SEM).

3.7 S.E.M. topology of poly(HEMA) films.

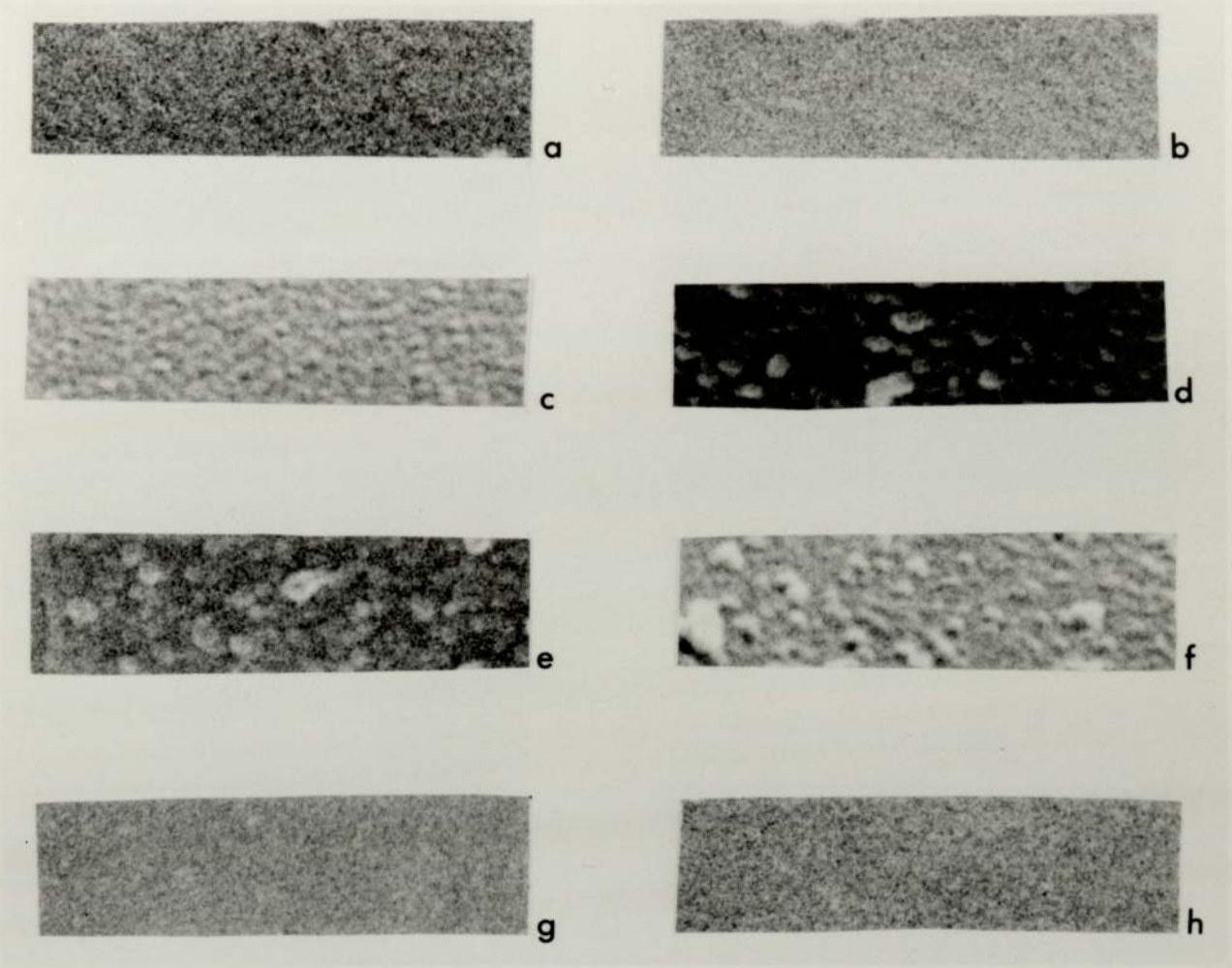
S.E.M. investigations of poly(HEMA) coatings produced by both "evaporation" and "spinning" techniques reveal distinct differences in surface topography. In the case of evaporation coatings there is clear evidence of polymer film discontinuity at poly(HEMA) concentrations of less than $5x10^{2}$. This corresponds to a polymer film thickness of 0.7um (approximately).(figure 3.1 and table 3.2). In contrast poly(HEMA) films prepared by the spinning technique were smooth and complete at all thickness(see figure 3.1 and table 3.2). Transmission electron microscopy investigations of polymer films which have not been subjected to cell culture conditions confirm these findings (Appendix 3).

3.8 Analysis of cell spreading responses on poly(HEMA) surfaces

Interestingly, cell spreading activity on evaporation poly(HEMA) films seemed to be associated with polymer film discontinuity. In that cell attachment (but not spreading) occurs at moderate (eg $5x10^{-1}$ and $1x10^{-2}$) polymer dilutions and cell spreading occurs at greater dilutions (eg $5x10^{-2}$ and $1x10^{-3}$).

Figure 3.1

S.E.M. Micrographs of evaporation and spun coatings (x20,000)



a) Evaporation coating. Dilution 1 estimated thickness 35um
b) Evaporation coating. Dilution $5x10^{-1}$ estimated thickness 0.7um
c) Evaporation coating. Dilution $1x10^{-2}$ estimated thickness 0.35um
d) Evaporation coating. Dilution $2x10^{-1}$ estimated thickness 0.18um
e) Evaporation coating. Dilution $5x10^{-2}$ estimated thickness 0.07um
f) Evaporation coating. Dilution $1x10^{-3}$ estimated thickness 0.035um
g) Spun coating measured thickness 0.2um
h) Spun coating measured thickness 1.2um

It was important to quantify this spreading response in order to ascertain if this system of poly(HEMA) evaporation coatings could fulfill the original expectations. That is to provide a system of substrates which could modulate cell spreading. Such quantification was achieved by the use of a quantimet 720 image analyser.(Appendix 2). Cell populations used for size analysis were prepared by staining with 2% amido black. Approximately 150 cells were measured per field of view (ie all cells within a particular field). Several fields were measured for each cell population. The results were analysed using the Rawdat computer program, which produces fundamental size and shape distribution information (Appendix 2). Several size distribution parameters are provided by this program ie mean and model cell diameters, largest ferets diameter, standard deviation etc.

Consideration of only mean cell diameter gives results similar to those reported previously (ref 280-1) whereby values for mean cell diameter increase gradually with increasing poly(HEMA) dilution over the range $5x10^{-1}$ to $8x10^{-2}$. There is however, a drastic increase in mean cell diameter values for cell populations cultured on $1x10^{-3}$ poly(HEMA) films. Model cell diameter values on the other hand, show remarkable consistency over a range of poly(HEMA) substrates of between $5x10^{-1}$ and $8x10^{-2}$ increasing noticeably for cells cultured on $1x10^{-3}$ poly(HEMA) coatings (figures 3.2, 3.3).

Skew analysis (table 3.3) of cell size distribution measurements obtained from the quantimet showed very significant positive skew for all cell size distributions except those grown on glass control substrata.

Table 3.2
Cell spreading responses on poly(HEMA) substrata

Culture Substrate	Polymer Concentration /film thickness	Culture Conditions	Cell Morphology	Polymer Surface Topography
Spun coatings	0.01um	Complete Medium	No Adherence	Smooth, Complete
Evaporation coatings on tissue culture plastic. Dilutions of 12% stock solution.	1 to 1:10	Complete Medium	No Adherence	Smooth, Complete
	1:50 to 1:200	Complete Medium	Rounded	Rugose
	1:500 to 1:889	Complete Medium	Partially	Rugose
	1:1000 to 1:1667	Complete Medium	Fully Spread	Clearly Broken
Evaporation coatings on siliconized glass	All dilutions	Complete Medium	No Adherence	Discontinuous at dilutions 1:50
Evaporation coatings on bacteriological grade plastic. Dilutions of 12% stock solution.	1 to 1:10	Complete Medium	No Adherence	
	1:50 to 1:200	Complete Medium	Rounded	
	1:500 to 1:889	Complete Medium	Aderrant spreading	N.D.
	1:1000 to 1:1667	Complete Medium	Aderrant spreading	
Evaporation coatings on fibronectin coated substrates. Dilutions of 12% stock solution.	1 to 1:10	Serum Free Medium	No Adherence	
	1:50 to 1:200	Serum Free Medium	Rounded	
	1:500 to 1:889	Serum Free Medium	Partially Spread	N.D.
	1:1000 to 1:1667	Serum Free Medium	Fully Spread	
	Ethanol-treated fibronectin	Serum Free Medium	Fully Spread	

This would seem to show that the increasing mean cell diameter measurements seen with decreasing poly(HEMA) concentration between $5x10^{-1}$ and $8x10^{-3}$ can be explained in terms of the influence of relatively few fully spread cells.
The relatively constant model cell size values in this range shows that the majority of cells are not changing shape but that the mean diameter figure is influenced by a small but increasing number of fully spread cells.

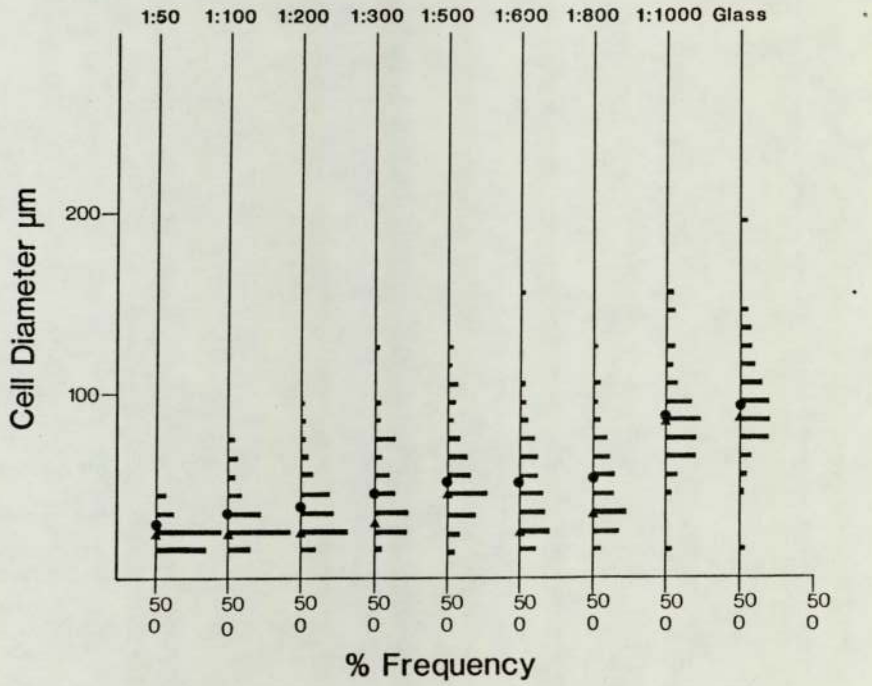
Why do these fully spread cells exist? In view of the S.E.M. evidence presented in this chapter it would seem reasonable to propose that the fully spread cells exist in areas of particularly high poly(HEMA) film discontinuity. The S.E.M. Evidence shows that we can expect the number of such sites to increase with decreasing poly(HEMA) concentrations. These findings clearly present a very different situation to that proposed previously (ref 280-1) and also proposes a role for the surface underlying the poly(HEMA) in determining cell spreading responses on this substrate system.

3.9 The role of the underlying substrate.

To ascertain the effect of differing underlying substrata, on cell populations cultured on poly(HEMA) evaporation coatings. It was necessary to select a series of substrata known to express easily recognized cell spreading characteristics.

Three substrates known to exhibit characteristic cell spreading responses were chosen, siliconized glass, bacteriologyical plastic and glass substrata coated with the

Figure 3.2
Quantification of cell spreading responses on poly(HEMA) evaporation coatings

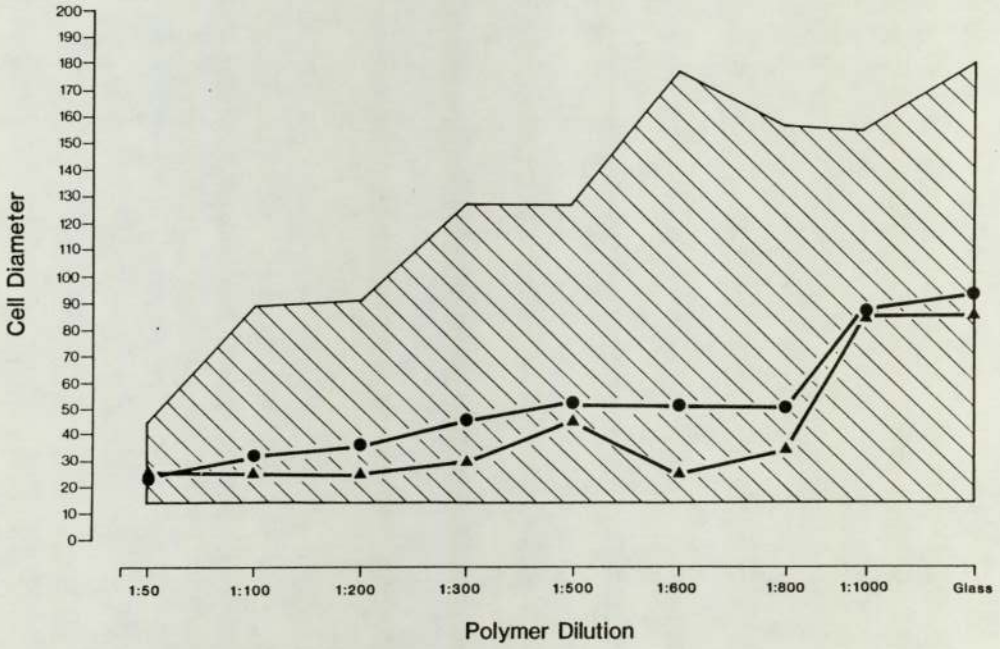


Mean Cell Diameter

Modle cell Diameter

Figure 3.3

Quantification of cell spreading responses on poly(HEMA) evaporation coatings



Total cell population

Table 3.3

Distributions of largest Feret's diameter analysis of skewness.

Poly(HEMA) Dilution	b_1	Significance
Glass	0.0374	Not significant
1:1000	0.5577	P 0.05
1:800	1.2625	P 0.01
1:600	1.4049	P 0.01
1:500	0.8610	P 0.01
1:300	0.8902	P 0.01
1:200	1.0675	P 0.01
1:100	1.6541	P 0.01
1:50	0.8612	P 0.01

$$b_1 = m_3/m_2^{3/2}$$

$$m_3 = (x-x)^3$$

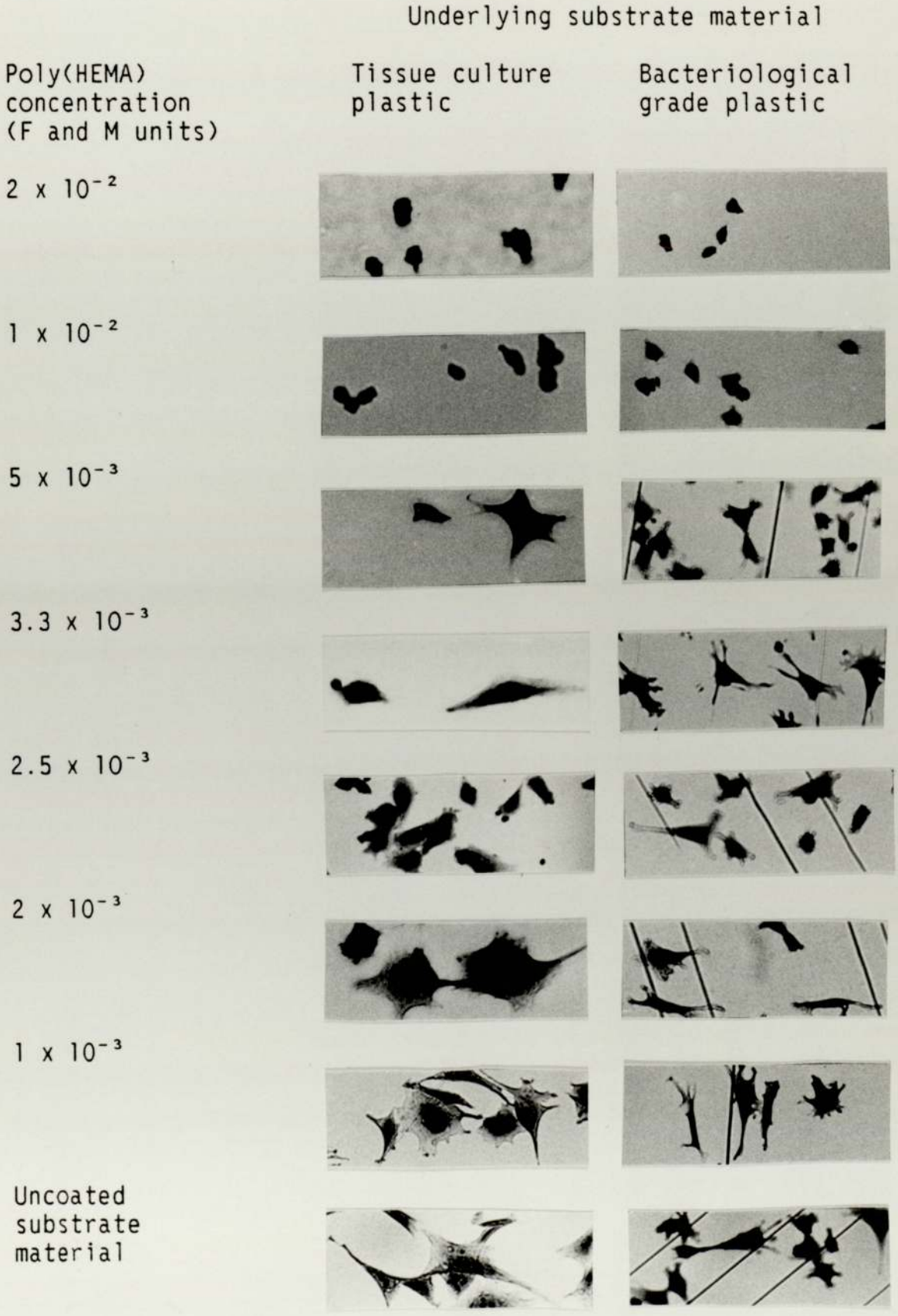
m_2 = variance

Departure of b_1 from zero is an indication of skewness in the frequency function of the sample population.

Figure 3.4

The effect of the underlying substrate.

Comparision of cell spreading on poly(HEMA) evaporation coatings with underlying substrata of tissue culture and bacteriological grade materials.



cell adhesive glycoprotein fibronectin. All these substrata have characteristic cell spreading responses.

Siliconized glass allows no cell attachment under any culture conditions. Poly)HEMA) "evaporation " coating of all concentrations were prepared on siliconized glass cover slips. In subsequent cell attachment assays performed under full serum conditions, no cell attachment was observed at any polymer concentration (table 3.2).

Bacteriological plastic substrates can allow reasonable levels of cell attachment and often cell spreading but the morphology of cells attached to such surfaces is characteristically aberrent. The complete range of poly(HEMA) evaporation coating ($1x10^{-1}$ to $1x10^{-3}$) were prepared on bacteriological plastic substrates. When cells were cultured on these surfaces under full serum conditions cell adhesion was observed on surfaces coated with $5x10^{-1}$ and $1x10^{-2}$ poly(HEMA). Further cell spreading was observed on surfaces coated with $5x10^{-2}$ and $8x10^{-2}$ poly(HEMA). However, in all cases the morphology of the cells was characteristically aberrent (table 3.2 and figure 3.4).

Fibronectin is a glycoprotein which is well established to allow rapid and complete cell spreading in the absence of serum (and therefore serum spreading factors). The third approach therefore was to coat glass coverslips with fibronectin and subsequently coat the glycoprotein with poly(HEMA) evaporation coatings. B.H.K. fibroblasts were cultured on these surfaces in serum free medium. The cell response was exactly the the same as control experiments run concurrently on the normal "evaporation"

coating substrate system under full serum conditions (table 3.2). There seems therefore to be substantial evidence for the role of the underlying substrate in determining cell spreading responses on this substrate system.

3.10 Discussion

Substantial evidence has been presented in this chapter to support the following statements. Firstly on a system of poly(HEMA) "evaporation" coatings the hydrogel affects cell adhesion and spreading by simply masking the properties of the adhesive underlying substrate. Indeed, cells can only attach to poly(HEMA) coated surfaces if the hydrogel film is discontinuous. Fibroblasts do not attach to poly(HEMA) coatings produced by the spinning method, and these films are shown to be smooth and complete when examined by S.E.M. Cell only attach or spread on evaporation coatings which appear to be rugous or discontinuous by S.E.M. Cell populations cultured on poly(HEMA) evaporation coatings accurately reflect the characteristics of a variety of underlying substrata. A system of poly(HEMA) evaporation coating does not represent a useful system for the modulation of the cell spreading process. Detailed statistical analysis of cell populations cultured on poly(HEMA) evaporation coatings show that gradually increasing values for mean cell diameter reflect only an increasing number of fully spread cells in areas of high poly(HEMA) discontinuity.

It is entirely feasible to explain the results of both the original workers (ref 280) and Ben-Ze ev et al (ref 281) in terms of these findings. These two previous publications show a some what different approach. In the case of Folkman and Moscona (ref 280) cell populations were harvested with EDTA-Tripsin in the normal manner and subsequently cultured on poly(HEMA) substrata.

Whereas Ben-Ze ev et al suspended the cell populations for long periods prior to culturing in order to observe the recovery of normal cell metabolism. Poly(HEMA) substrates were used in an attempt to hold cells at various stages of the attachment and spreading process. Both publications report that DNA synthesis is inhibited over the range of poly(HEMA) concentrations which also affect mean diameter. It is of course well established that fully rounded (non-transformed anchorage dependent) cells do not synthesize DNA whereas fully spread cells would be required to synthesize DNA during S phase to allow mitosis to occur. Therefore the results of both groups are completely commensurate with a population of mainly rounded cells, but with a steadily increasing number of cells in a fully spread morphology. Further more Ben-Ze ev et al shows the kinetics of DNA synthesis to be unaffected by concentrations of poly(HEMA) which reduce mean cell diameter. Surely this is good evidence for simply the presence of fewer spread cells? The original workers also show that DNA synthesis is equivalent in cell populations held at the same mean height (equivalent to mean cell diameter) either by poly(HEMA) or by contact inhibition in Cultures allowed to grow to confluence. Both these phenomena can be explained by the influence of fully spread cells within the population. We now know that these exist on poly(HEMA) surfaces and surely fully spread cells must exist in a population of cells grown on tissue culture plastic which has been allowed to grow to (or close to) confluence. Benzere et al also proposes cell shape control for the synthesis of ribosomal RNA synthesis, this is also quite simply explained by increasing numbers of spread cells, when

cultured on decreasing concentrations of poly(HEMA).

Other interesting points emerge upon close examination of the two papers. Folkman and Moscona (ref 280) themselves state that, " as cells of various lines are brought from the existing flat shape toward a spheroidal conformation fewer cells incorporate H^3 thymidire ". Surely evidence for a lower number of fully spread cells. The paper also states that, factors other than cell shape may in fact control both cell proliferation and cell shape and hence relationship between the two factors may not be of a cause and effect nature. This seems to be a most logical argument given the known importance of cell growth factors and other influences upon the cell adhesion and growth process. This is certainly the viewpoint which this author favours.

Interestingly the original author also utilized SEM to examine the surface of poly(HEMA) evaporation coatings although no magnification details are given. It is briefly postulated however, that "tiny spicules of plastic protude as multiple contact points to which cells stick." Ben-Ze ev (ref 281) makes no attempt to explain how poly(HEMA) may control cell shape.

Finally a point of further interest is the proposal that cell shape may regulate an increase in the rate of cell division at the edge of wound sites. This phenomena has previously been shown by Dulbeco, by monitoring the increased labelling index of such cells. The use of labelling index in order to ascertain the level of DNA synthesis within individual cells in populations cultured on poly(HEMA) would have provided very strong evidence for universal cell shape modulation, but this technique was

employed by neither by Folkman nor Ben-Ze ev.

The findings within this chapter indicate that discussion of results, obtained from cell populations cultured on this poly(HEMA) substrate system, in terms of cell shape control and the significance of cell shape to metabolic processes is unfounded. However, it is now established that poly(HEMA) represents a completely non adhesive surface for fibroblasts under full serum culture conditions. This is an important finding in view of the difficulties associated with finding practicable materials to which fibroblasts would not adhere in the presence of feotal calf serum. There are exceptions to this of course, ie siliconized glass and polystyrene. Poly(HEMA) however,not only offers potential as a biocompatible non-cell adhesive biomaterial. It also offers (possibly through copolymerization) the opportunity for subtle chemical modification of both surface and bulk properties and hence represents an excellent starting point from which to investigate the chemical requirements for cell adhesion, or indeed, to produce a reliable series of substrates which could modulate the cell spreading process, making possible the control of cell growth in culture. It was therefore decided to pursue this avenue of research.

Chapter Four

Cell spreading on poly(HEMA/EMA) and poly(HEMA/STY) copolymers

4.1 Introduction

Having established poly(HEMA) to be non-adhesive for fibroblasts in culture, it was decided to consider a wider range of synthetic polymers and the interactions of these materials with cells in vitro. This study was intended to determine properties of relevance to the adhesiveness of synthetic polymer substrates for cells. In the past several substrate properties have been proposed to influence cell behaviour in culture. These include chemical group expression (ref 63,79), physical and chemical anisotropy (ref 298) and substratum contractility (ref 267). Some attempts have also been made to relate cell attachment to substratum wettability or hydrophilicity.

Unfortunately, work in this area has been limited to a small and often arbitrary, range of substrate materials representing only a fraction of the potential array of properties provided by modern synthetic polymer chemistry. A further problem lies in the use of the terms hydrophilicity and hydrophobicity, which are broad relative terms and insufficiently definitive to reflect the range of polarities encompassed by the array of polymers referred to in the published work. Consequently considerable fundamental conflict exists within the literature, such that there is no consensus of opinion as to the relative importance of these physical and chemical properties to cell adhesion and spreading.

Consideration of some work in this field illustrates the confusion. For example Klebe et al (ref 258) have proposed that

'hydrophobic' surfaces are required for cell adhesion and growth. These conclusions were based on a series of observations involving a wide range of polymers. Thus hydrophilic poly-2-hydroxyethyl methacrylate (polyHEMA) was non-adhesive for CHO cells whereas introduction of non-hydrophilic poly(vinyl acetate) into blends with polyHEMA resulted in increased cell attachment. In addition, the methacrylates poly(methyl methacrylate) (polyMMA) and poly(ethylmethacrylate)(polyEMA) were also shown to support cell growth. The work of at least two other groups seems to substantiate this proposal. (ref 260,270). However, if hydrophobicity represents the sole criterion for cell adhesion, one might expect simple polystyrene (or commercial bacteriological-grade plastic) to support cell growth. This is not the case. In a recent series of studies, Grinnell and co-workers (ref 273) have emphasized the relative hydrophilicity of tissue culture grade plastic (surface-modified polystyrene) compared to polystyrene dishes. Nevertheless hydrophilicity per se is neither necessary (ref 258,260,270) nor sufficient for cell adhesion; several studies have shown that modification of poly(HEMA) either by blending with more hydrophobic species as discussed (ref 258,260,270) or by entrapment of biological molocules with known adhesion and growth promoting activities is required for cell attachment to this hydrophilic polymer .

The search for an overall understanding of the substratum requirements for cell adhesion has been further complicated by the work of Curtis et al (ref 253) demonstrating that sulphuric acid treatment of polystyrene (known to render such substrates

suitable for cell culture (ref 57-63,70,79,80) results predominately in hydroxylation of aromatic rings at the substratum surface. The relevance of hydroxyl group expression in this case was demonstrated by the inhibition of cell adhesion when these groups were blocked. A fundamental role for hydroxyl groups in promoting cell adhesion was proposed. In view of the cell adhesion characteristics of such materials as poly(HEMA),poly(EMA), and poly(MMA) it is difficult to regard such a criterion as a complete explanation of the requirements for cell adhesion. Poly(EMA) and poly(MMA) have no hydroxyl groups yet apparently allow cell attachment (ref 258,260,270) while adhesion is inhibited on hydroxyl rich poly(HEMA)

We undertook to investigate cell behaviour on a wide range of synthetic polymers of well defined structure, chemistry and physicochemical properties. This chapter reports cell spreading behaviour on polyHEMA/EMA and polyHEMA/styrene copolymers. These substrates allowed the study of several factors which may influence cell adhesion and spreading. A particular aim was the elucidation of the relative importance of hyroxylation levels and the hydrophobic/hydrophilic balance within the culture substrate.

4.2 Results

4.3 Poly(HEMA/EMA) copolymer series

These results relate to the investigation of cell adhesion behaviour on two copolymer series in which hydroxyl group expression was systematically controlled, the work involved the choice of three well-characterized homopolymers(figure 1)of known cell adhesiveness: poly(EMA), poly(HEMA) and polystyrene. Comparison of the respective monomer subunit structures of EMA and HEMA reveals a simple substitution of a hydroxyl group in place of hydrogen on the pendant ethyl chain representing a straight forward structural translation from a substantially hydrophobic to a moderately hydrophilic polymer. A series of copolymers of EMA and HEMA therefore provides an ideal system for a study of the relative contributions to cell spreading activity deriving from the hydroxyl content and the physicochemical balance of the substrate. Cell spreading responses on this copolymer series confirmed our earlier observations that poly(HEMA) is nonadhesive for mammalian cell lines (figures 4.1,4.2). Inclusion of 10% EMA in such polyHEMA/EMA copolymers, however, was found to promote full cell spreading in a high percentage of cell after six hours in culture. (table 4.1, fig. 4.1)

Perhaps surprisingly, there was no continuous modulation of cell response to changing hydroxylation levels. A more detailed study of BHK cell spreading on copolymers containing from 1 to 9 % EMA showed complete inhibition of cell attachment on

copolymers containing less than 8% EMA, but full cell spreading on copolymers expressing between 8 and 100 % EMA (table 4.1 & 4.3, figure 4.1). No intermediate spreading responses were observed

4.4 Poly(HEMA/STY) Copolymer series

As a comparison with poly(EMA), which is known to support cell adhesion as a homopolymer (ref 270), cell spreading on copolymers of polyHEMA and polystyrene(figure 4.2, table 4.2 & 4.3) both of which have been shown to be nonadhesive for cultured cells was investigated. Polystyrene like poly(EMA), is a hydrophobic material, in contrast to the hydrogel poly(HEMA). As expected, results (figure 4.2, table 4.2) showed no cell adhesion to poly(HEMA) or polystyrene. However full cell spreading is seen on all copolymers in the range 10% HEMA / 90% STY to 90% HEMA / 10% STY (figure 4.2). A more detailed investigation of BHK cell responses on copolymers in the range of 1 to 9% STY was carried out (table 4.3). In this case it was found that only 4% STY in the copolymers was required for cell spreading. Again no modulation of cell spreading was observed; most attached cells adopt a full-spread morphology.

4.5 Polymer characterization: Equilibrium water content (EWC)

The polymers prepared for this study range from hydrophobic poly(EMA) and polystyrene to hydrophilic poly(HEMA). The hydrophilicity of homopolymers or copolymers may be assessed by measurement of the Equilibrium Water Content (E.W.C.). Which may

Table 4.1

Cell spreading on poly(HEMA/EMA) copolymers

(various cell lines)

% EMA in copolymer	% of cell population showing fully spread morphology					
	CEF1°	16C	BHK	REF	MRC5	HEP2
0	No Cell Adhesion					
10	95	95	91	85	90	68
20	97	78	89	82	86	-
30	96	75	84	79	85	-
40	90	76	92	68	89	63
50	81	71	92	77	78	79
60	87	74	74	82	77	85
70	93	86	59	83	78	97
80	91	78	54	88	74	90
90	81	78	72	76	82	70
100	77	75	70	72	78	70

Table 4.2

Cell spreading on poly(HEMA/STY) copolymers

(various cell lines)

% STY in copolymer	% of cell population showing fully spread morphology					
	CEF1°	16C	BHK	REF	MRC5	HEP2
0	No Cell Adhesion					
10	86	95	73	95	93	68
20	92	-	82	79	92	69
30	78	81	84	74	91	91
40	-	80	91	69	96	83
50	69	71	74	83	90	63
60	86	83	79	86	92	82
70	92	86	82	91	89	79
80	-	92	86	74	82	71
90	71	82	74	84	92	69
100	14	9	12	7	17	4

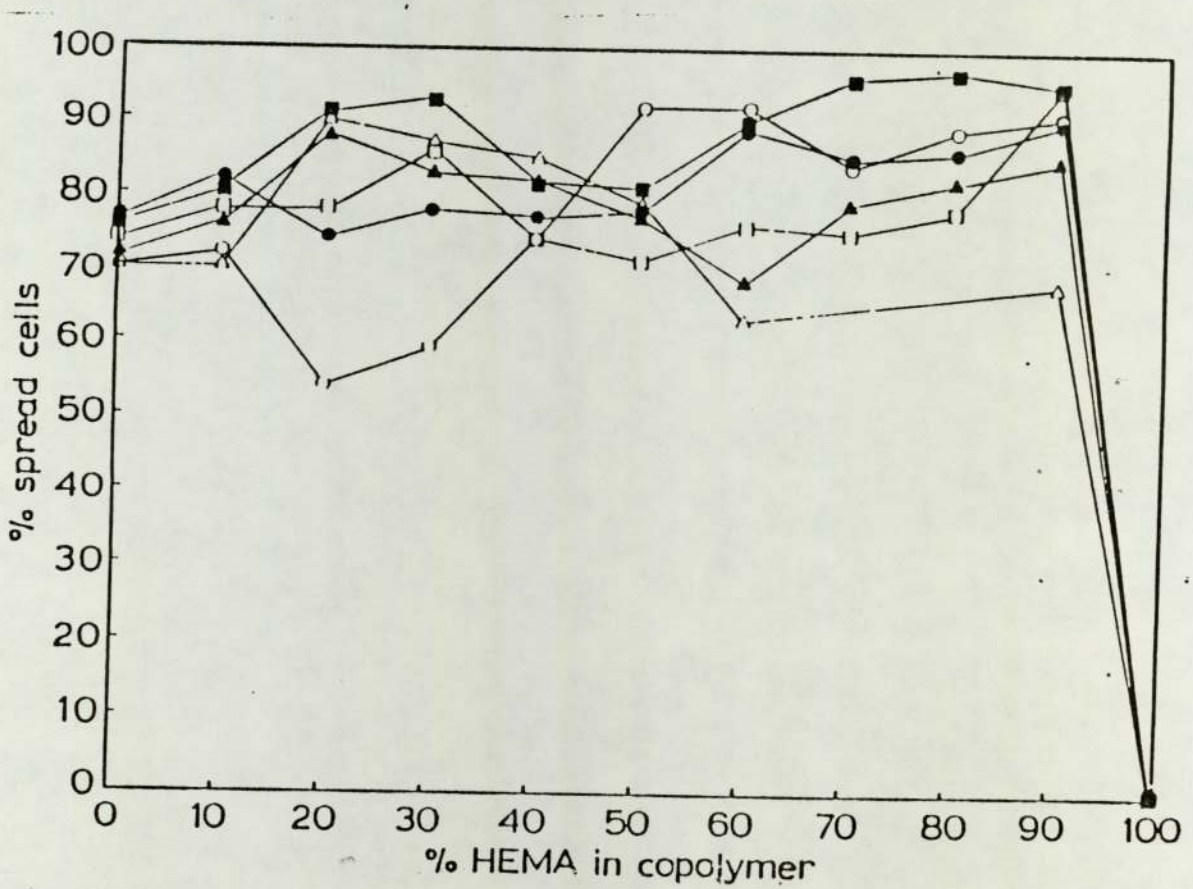
Table 4.3

BHK Cell spreading on poly(HEMA/STY) copolymers

% HEMA in copolymer	% of cell population showing fully spread morphology	
	Poly(HEMA/STY)	Poly(HEMA/EMA)
99	*	*
98	*	*
97	*	*
96	72	*
95	69	*
94	71	*
93	76	*
92	77	90
91	70	93

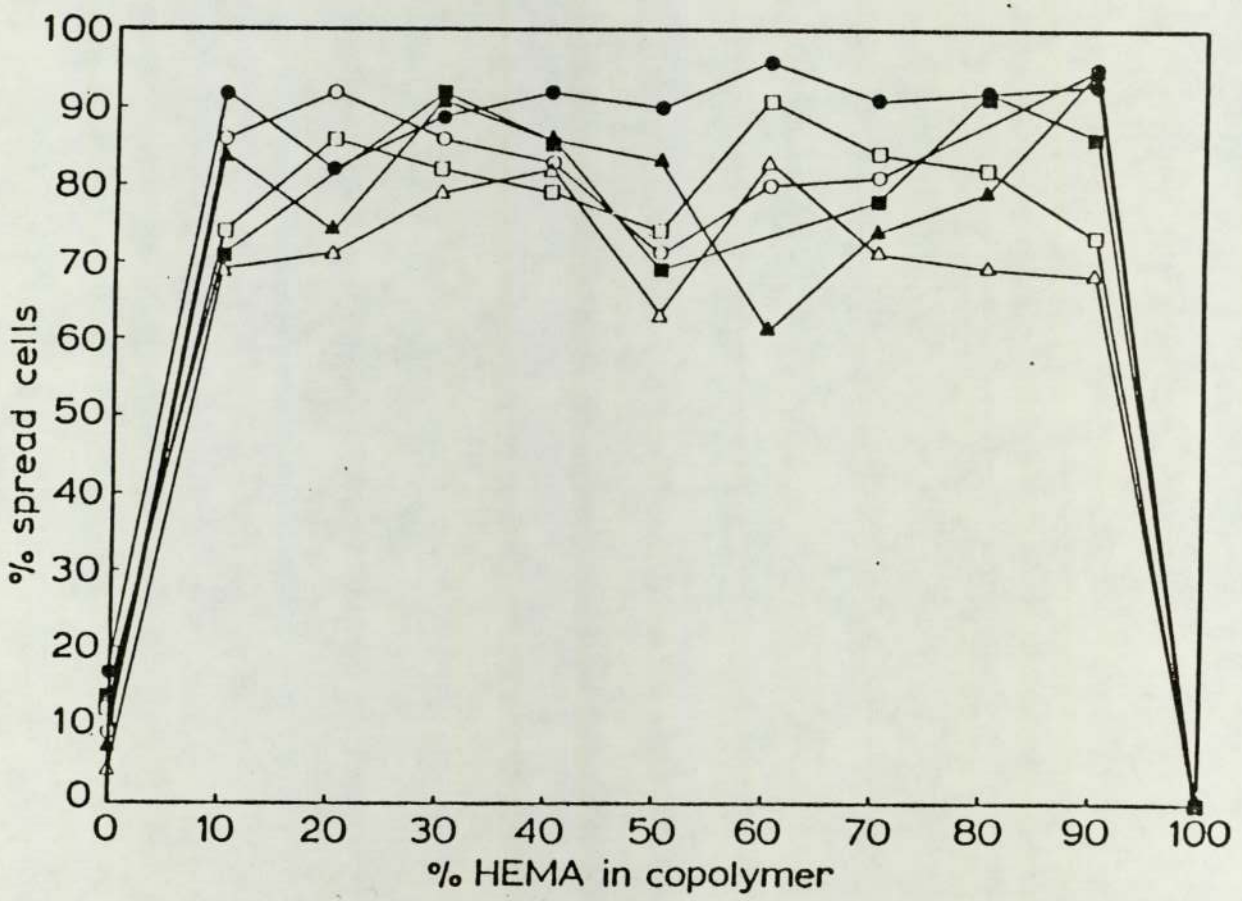
* = No cell attachment

Figure 4.1
Cell spreading behaviour on poly(HEMA/EMA) copolymers



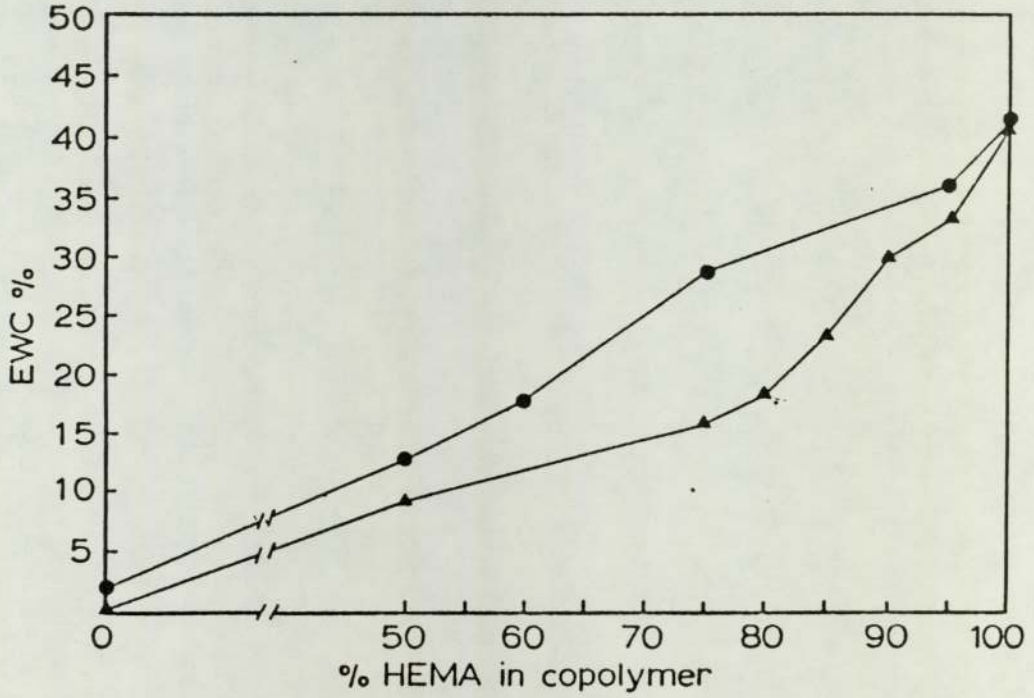
(○) *BHK*, (□) *16C*, (△) *H.Ep-2*, (●) *MRC5*, (■) *CEF*, (▲) *REF*.

Figure 4.2
Cell behaviour on poly(HEMA/STY) copolymers



(○) *BHK*, (□) *16C*, (△) *H.Ep-2*, (●) *MRC5*, (■) *CEF*, (▲) *REF*.

Figure 4.3
Equilibrium Water content of poly(HEMA/EMA) and poly(HEMA/STY) coploymers.



EWC of polyHEMA/EMA (●) and polyHEMA/STY (▲) copolymers

be determined as described in chapter two. The E.W.C. of the polymers used in this particular study are shown in figure 4.3.

It is interesting to note here that while the relative proportions of styrene/HEMA and EMA/HEMA critical for cell adhesion are different they each represent similar E.W.C.s in the range approx. 34.5% to 35.5%, suggesting that the water absorption properties of these particular substrates may be related in some way to cell adhesion.

4.6 Discussion

The results at this stage were at least in part able to explain the confusion arising in the literature regarding chemical and physicochemical properties necessary for cell adhesion to synthetic substrates. This was largely due to the use of polymers which encompass changes in such a wide range of properties that interpretation becomes difficult, or conversely an unrepresentatively narrow range of substrates.

As a first step, two properties were considered which may contribute to the adhesiveness of polymers for cells in culture: the notional role of hydroxyl groups at the polymer surface (ref 253) and the hydrophobic/hydrophilic balance of the substrate (ref 63,79,258,273). We chose three homopolymers, of known structure and physicochemical nature, which have already been widely used in studies of cell adhesion: poly(HEMA) (ref 79,258,274-281), poly(EMA)(ref 258,270) and polystyrene(ref 57,63,79).

Poly(HEMA) is a moderately hydrophilic hydrogel which contains one hydroxyl group per monomer unit. Poly(EMA) and polystyrene are both hydrophobic polymers containing no hydroxyl groups. There are important differences, however between the two hydrophobic homopolymers. Polystyrene is an extremely hydrophobic material in which any polar contribution can only be derived from asymmetry of the aromatic ring about the polymer backbone. Poly(EMA) by comparison is only moderately hydrophobic, due to significant polar contributions from the ester linkage.

While poly(HEMA) and polystyrene are now known to be non-adhesive for cells in their pure forms (ref 57-63,79,274-281), there is good evidence, both in the literature (ref 258,270) and in the data presented here, that poly(EMA) presents a much more adhesive substrate.

In addition to the three homopolymers, we have synthesized copolymer series of poly(HEMA)/EMA and poly(HEMA)/STY. These copolymer series express a range of hydroxyl concentrations as well as a transition from hydrophobic to moderately hydrophilic substrates. Further to this, the respective monomer subunit structures of polystyrene and poly(EMA), and the consequent different positions occupied by these polymers on a "hydrophobicity" scale, makes comparison of cell spreading behaviour on the two copolymer series very informative as to the relevance of such properties.

In this study, we have found, for all cell lines tested, that both poly(HEMA)/EMA) and poly(HEMA/STY) containing 10% to 90% HEMA are capable of supporting a large fraction of the cell population in a fully spread morphology. In view of the very different cell adhesion properties of polystyrene and poly(EMA) homopolymers, it would seem that their role in promoting cell adhesion when copolymerised with HEMA is not a function of their intrinsic cell adhesion characteristics. Rather, it is clear that some modulation of the physical and/or chemical properties of poly(HEMA) by copolymerization with hydrophobic polymers brings that substrate into a "window" within which conditions are favourable for anchorage dependent cell attachment. The results presented in this report allow consideration of some of these possibilities.

4.7 Hydroxyl group expression

Analysis of poly(HEMA/STY) copolymers showed a critical level of 4% styrene for cell adhesion compared to 8% EMA in the poly(HEMA/EMA) series. These figures corresponded to mole fractions of 0.05 for polystyrene and 0.09 in the case of EMA. There is no single upper level of hydroxylation. therefore, which is inhibitory for cell adhesion; if hydroxyl concentration at the polymer surface alone determines cell-substrate adhesiveness the two copolymer series would be expected to behave identically.

Moreover, changes in hydroxyl content between 90% and 10% have no effect on cell behaviour in either copolymer series. Indeed, the fact that poly(EMA) (these results and ref 258,270) and poly(HEMA) (these results and ref 274-281) are adhesive and non-adhesive for cells respectively would preclude any hypothesis that the simple presence of hydroxyl groups is necessary or sufficient for cell adhesion. In view of these considerations it is evident that chemical specificity of the type proposed by Curtis (ref 253) may not provide a comprehensive basis for the interpretation of cell-substrate adhesiveness, and that some secondary effect dependent upon consequences of monomer structure is decisive.

4.8 The hydrophobic/hydrophilic balance.

Steric exclusion of water from within hydrogel networks by the pendant aromatic ring of styrene (figure 1) has the effect of

reducing the E.W.C. by a greater margin than a molar equivalent amount of EMA. With this in mind, and given that a smaller ratio of styrene:HEMA than EMA:HEMA is required for cell adhesion, it is appropriate compare E.W.C. values for the two copolymers series. It is interesting to note that, in each case, cell spreading is initiated at an E.W.C. of approximately 34% (corresponding to 8% EMA or 4% styrene) conversely it can be supported by substrates with an E.W.C. of as little as 2% (poly(EMA)). It is apparent, then, that a wide range of materials possessing low to moderate hydrophilic character are adhesive for cells, although the physicochemical requirements for adhesion on polymers outside this "window" cannot be explained so simplistically.

It would be naive to suggest that E.W.C. alone determines cell-substrate interactions. For example, steric exclusion of water from the polymer bulk by the styrene monomer brings about changes in the organization states of water within the hydrogel (ref 294,297) and may affect polymer chain orientation and hence chemical group expression at the polymer/culture interface. Also polymers having an E.W.C. less than 20% cannot be considered as true hydrogels (i.e water swollen networks) such materials do however contain water near the polymer surface which thus contributes to the interfacial behaviour. These polymers must therefore be realistically considered as extending the observed continuum of behaviour. The differing relative proportions of HEMA:STY and HEMA:EMA critical for cell adhesion may suggest that water absorption and possibly water binding properties may be related in some way to cellular interactions.

In summary it seems reasonable therefore, to propose that a direct relationship between cell-substrate interactions and hydroxyl group expression does not exist. Polymers of varying hydroxyl content (or indeed no hydroxyl content) with low or moderate hydrophilic character seem to be adhesive for cells. Whereas materials more hydrophobic than poly(EMA) or more hydrophilic than poly(92 HEMA/8 EMA)(or poly(96 HEMA/4 STY)) are non adhesive. E.W.C. provides a useful means by which to describe this cell adhesion window. setting lower and upper limits of 2% and 34% E.W.C. respectively. It is clear however that E.W.C. does not provide a reasonable description of the properties of more hydrophobic polymers and hence is not suitable for use as a universal measure of cell adhesion in vitro. It seems likely that some aspect of surface energetics could be utilized to adequately describe the adhesiveness of substrates for cells.

It was clear therefore that it could be fruitful to investigate the role of physiochemical forces in cell adhesion; and to subsequently further extend the range of substrate materials studied.

Chapter Five

Cell spreading on glow discharge treated polystyrene surfaces

5.1 Introduction

The importance of wettability in allowing the correct functioning of biological surfaces has long been appreciated (ref 25-26). In the previous chapter the possibility of an important role was discussed for the surface energetics of the substrate in determining cell spreading behaviour on a range of hydrogel copolymers.

There also seems to be some evidence for the importance of such phenomena when considering mammalian cell culture on rigid polystyrene or glass based substrata (ref 53-63)

Native polystyrene for instance is non cell adhesive whereas polystyrene exposed to various surface treatment processes allows full cell spreading and growth. These findings are of more significance when it is considered that native polystyrene can be assumed to represent bacteriological grade (hydrophobic non cell adhesive) plastic, and treated polystyrene represents tissue culture grade (hydrophilic and cell adhesive) commercial substrata. Many treatments have been shown to render polystyrene suitable for cell culture, primarily the action of strong acids (ref 14-19) and glow discharge treatments (ref 20-22).the later method being used on a commercial scale and involves the use of a gas plasma (usually air) to produce a surface modification of the polystyrene, (ie oxidation). While there is no doubt that these processes render polystyrene wettable, the precise mechanisms of this phenomenon are as yet undefined.

Maroudas for example (ref 57,63) assumed that the treatment of polystyrene substrate with concentrated sulphuric acid

resulted in the formation of sulphonate groups at the polymer surface. Recent work however (ref 253) has shown that such a process leads to a predominance of hydroxyl groups, as indeed do treatments with chloric acid or ozone. In contrast it seems that treatment with chromic acid produces a high degree of carboxylation at the polymer surface. Andrade et al (ref 298) utilized X-ray photo electron spectroscopy to investigate the surface chemistry of tissue culture grade plastic. This investigation revealed not only significant oxidation on tissue culture grade materials but also on bacteriological grade materials after the removal of the siloxane based mould release agent.

Interestingly a new type of tissue culture plastic now available (Primaria) claims to inhibit fibroblast growth and encourage primary cell division by the incorporation of amine (primary, secondary, or tertiary) moeties at the culture surface. This follows research work showing that type 1 collagen, a favourite substrate for primary cell culture strongly expresses amine groups.

In view of the variety of processes proven to make polystyrene cell adhesive, it would seem reasonable to attribute such phenomenon to some fundamental change in the physicochemical properties of the polymer, ie increased wettability (ref 44,57). However many workers outline a role for specific chemical groups (ref 53,63,79). Furthering this line of investigation Curtis et al (ref 253) has proposed a specific and fundamental role for hydroxyl groups in cell adhesion to synthetic substrata. The situation is further complicated by the

view of Gringel and Vince (ref 81) who suggested surface roughness as an alternative explanation of some of the conflicting literature (ref 81). Some workers have also suggested a role for interfacial energetics (ref 25,248,265). Problems arise however from the use of chemically and structurally different polymers (ref 248-272) or alterations in the properties of the medium (ref 248). Although these variations in substrata and medium allow the manipulation of surface and interfacial energetics conclusive interpretation is made difficult. It has therefore been impossible to positively define a role for surface energetic considerations in determining cell spreading behaviour.

A fact that seems to be appreciated by only a few workers (ref 81,300), is the effect of surface rugosity upon the wettability of a polymer surface as described by Wenzel (ref 300). In general terms surface rugosity formed from features larger than 500 A°(ref 300) will increase the observed wettability of a polymer surface. Topography of this type can be produced at a polystyrene surface by plasma treatment mainly due to the etching of the polymer which occurs under these conditions. In the presence of an inert gas plasma only etching of the polymer occurs, but in the presence of an oxygen plasma surface oxidation would also take place. It was decided therefore, to undertake the separate treatment of polystyrene substrata with oxygen, nitrogen and argon plasmas to attempt to induce changes in surface energetics by means of surface oxidation and by surface rugosity, through the Wenzel Effect (ref 300). Hence attain some further information as to the

relative roles of surface chemistry, surface energetics and surface rugostity.

5.2 Results

5.3 Analysis of surface chemistry by X-ray photoelectron spectroscopy

Surface chemical analysis of native polystyrene (unmodified polystyrene spun coatings) shows the presence of a large carbon (C-1s) peak and a small oxygen (O-1s) peak (figure 5.1a). Analysis of the same surfaces which have been treated with nitrogen and argon plasmas show very similar chemistry to that of the untreated surface (figure 5.1 a,c,d). The small O-1s peak in all these spectra (figures 1a,c,d)is due to the slight oxidation that occurs during the commercial manufacture of polystyrene. In the sample of argon treated polystyrene (figure 5.1d), the oxygen peak is slightly enlarged due to the detection of the siliconized glass substrata as evidenced by the silicon 2s and 2p peaks (figure 5.1d)

Treatment of polystyrene surfaces with an oxygen plasma (figure 5.1b) results in extensive oxidation of the polymer surface, this is evidenced by the enhanced O-1s peak (figure 5.1b).

Figure 5.1a
X.P.S. Surface chemical analysis of native polystyrene

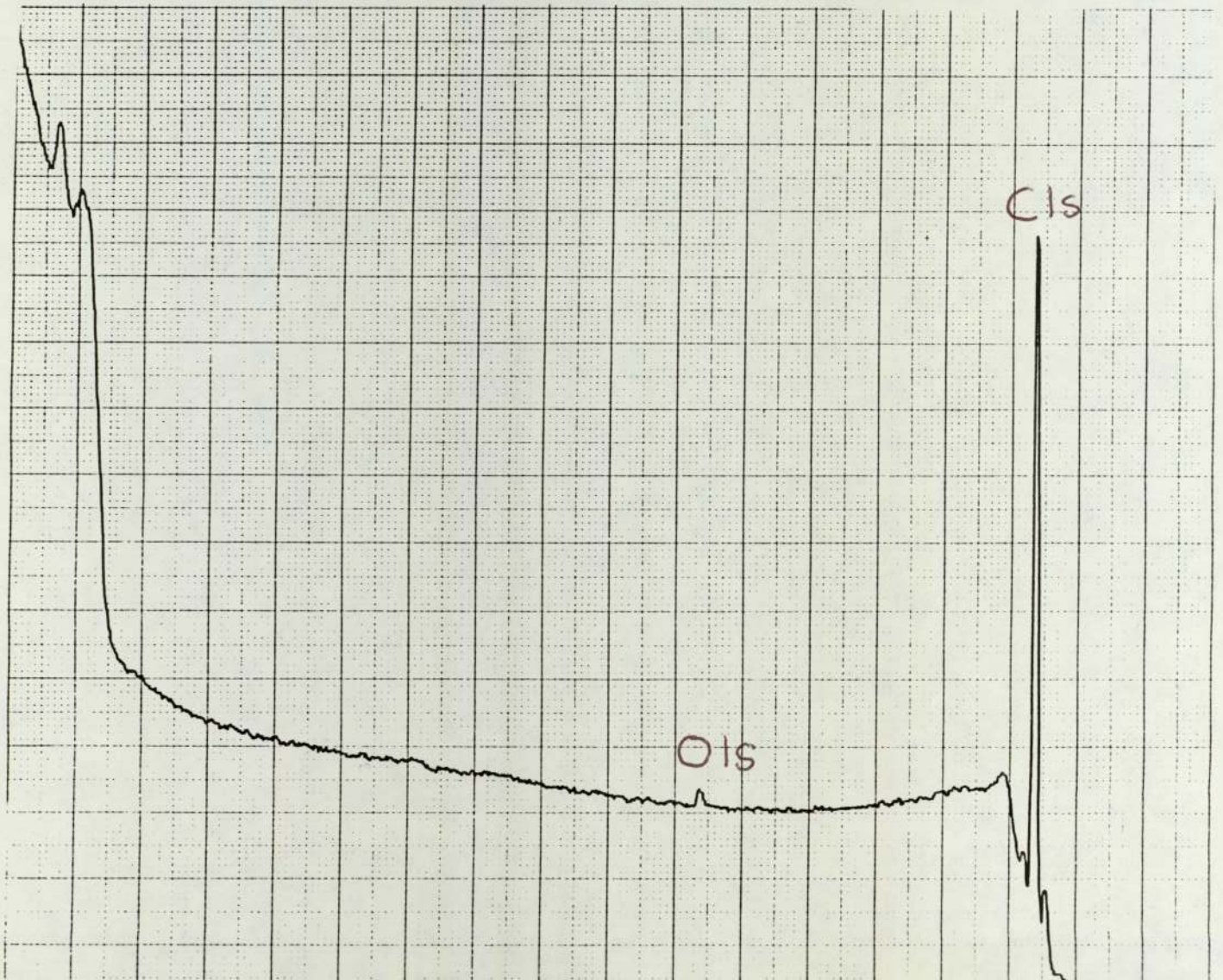


Figure 5.1b
X.P.S. Surface chemical analysis of Oxygen plasma treated polystyrene.

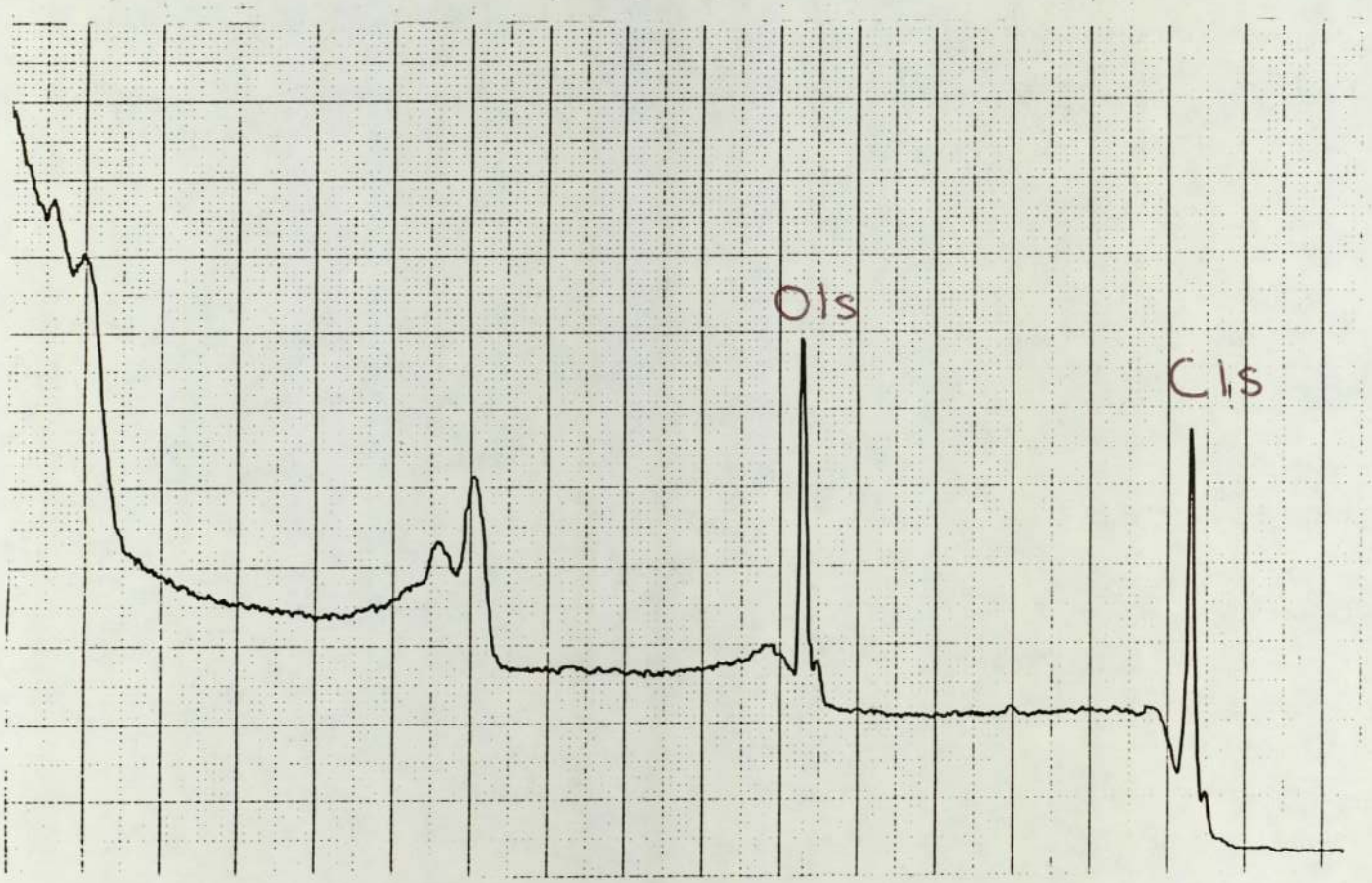


Figure 5.1c
X.P.S Surface chemical analysis of Nitrogen plasma treated polystyrene.

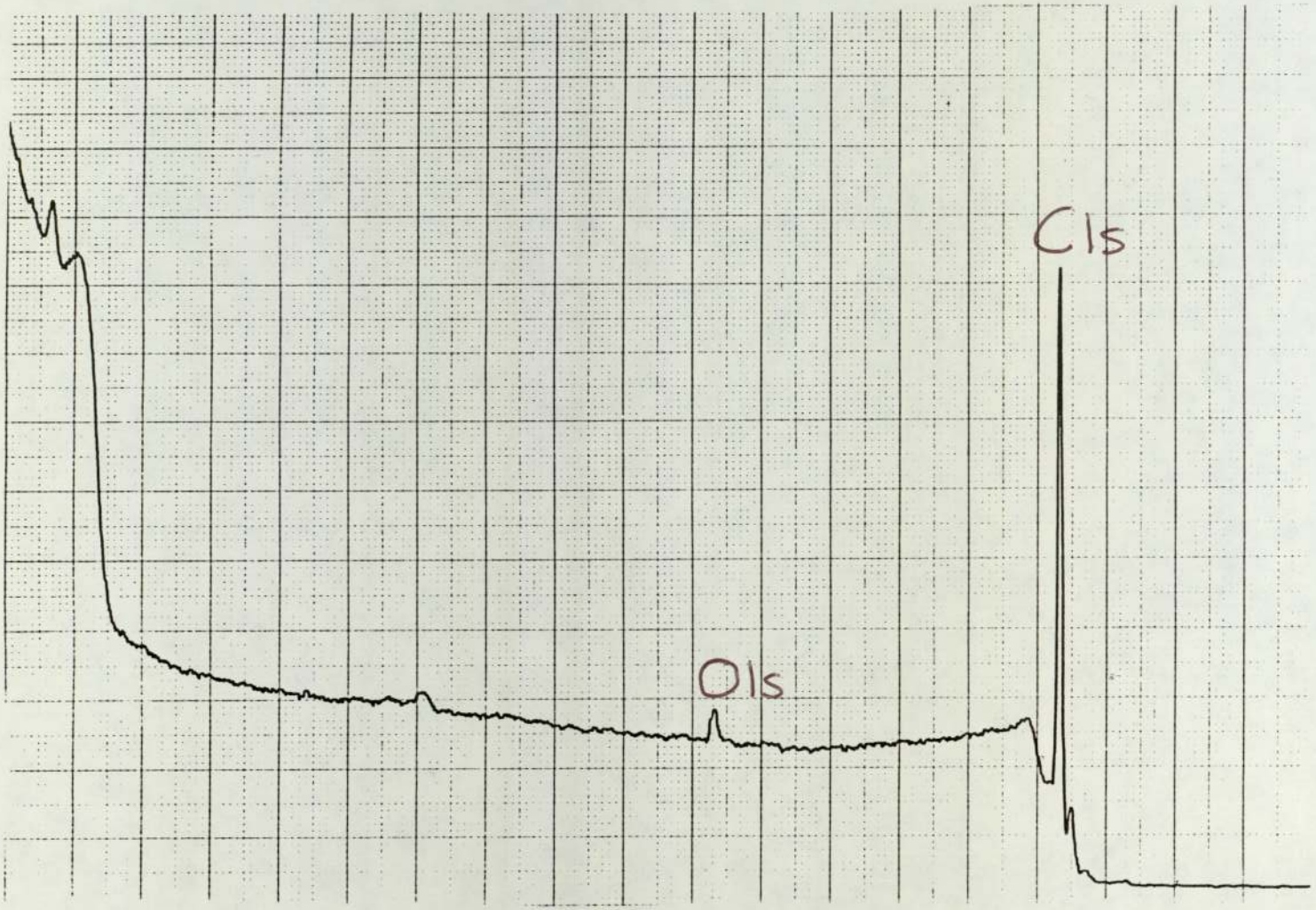
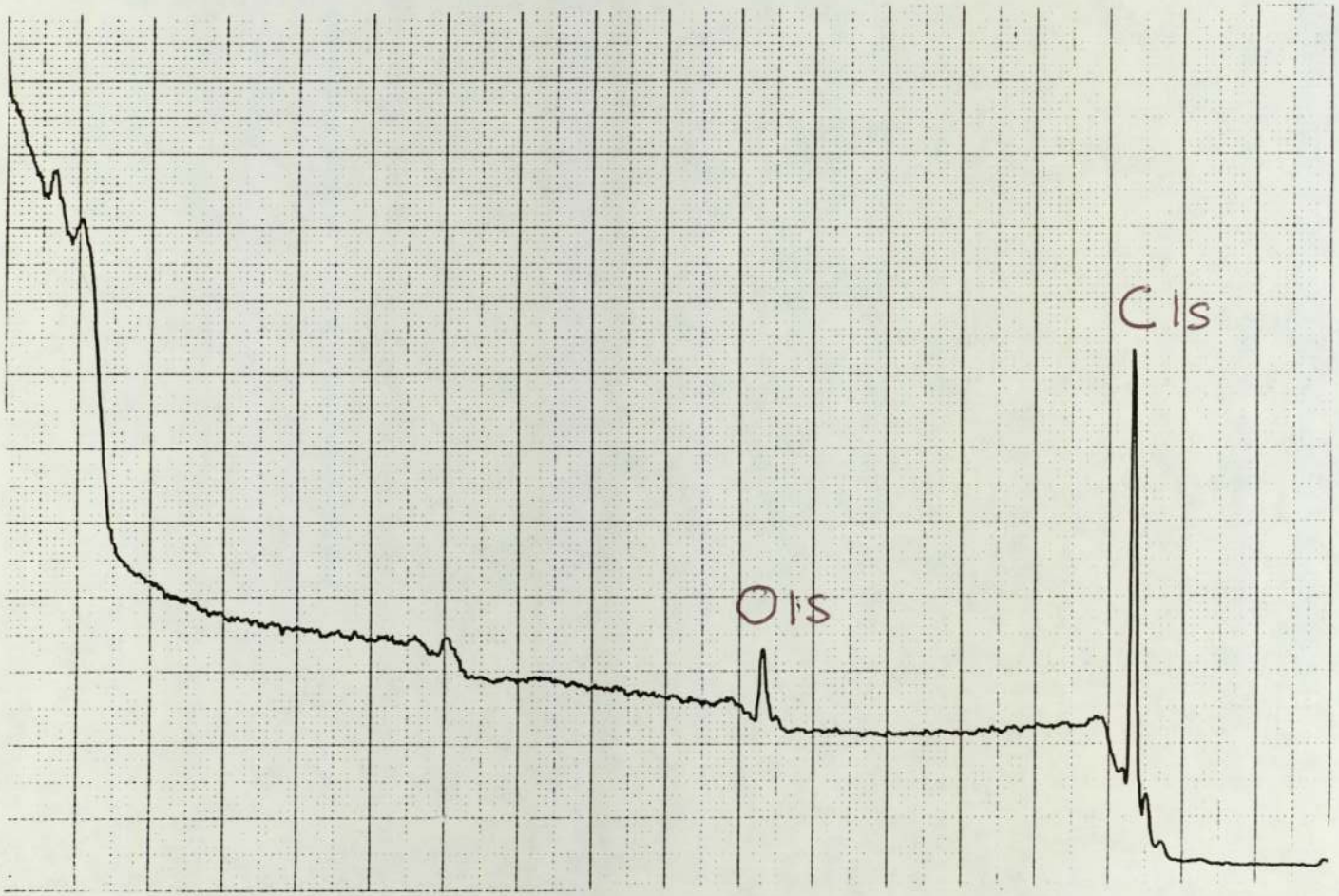


Figure 5.1d
X.P.S. Surface chemical analysis of Argon plasma treated polystyrene.



5.4 S.E.M. Study of surface topography

Both native and plasma treated polystyrene surfaces were examined using S.E.M. after exposure to one of two different environments:-

a) Full serum cell culture for 6 hours

b) 4 hours at 37°C in phosphate buffered saline (P.B.S.)

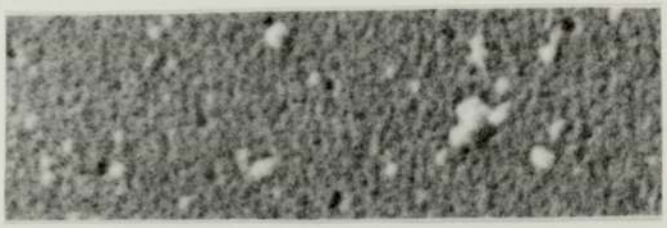
In all cases untreated polystyrene spun coatings are smooth and complete (figure 5.2a,3a,4a). After exposure to cell culture or P.B.S. all plasma treated surfaces show surface rugosity (figure 5.2b,c,d and figure 3b,c,d). All S.E.M. micrographs are shown at 20,000x magnification.

Figure 5.2:

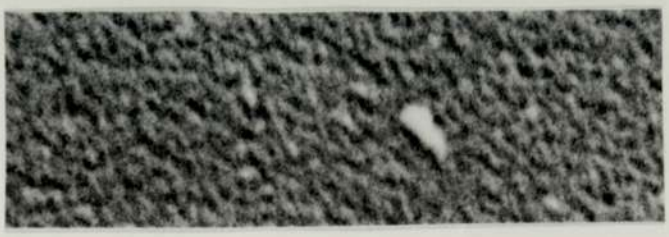
SEM Studies of polystyrene spun coatings after cell culture.

a) Poly(styrene) spun coating.

b) Oxygen plasma treated (10 minutes) polystyrene spun coating.



c) Nitrogen plasma treated (10 minutes) polystyrene spun coatings.



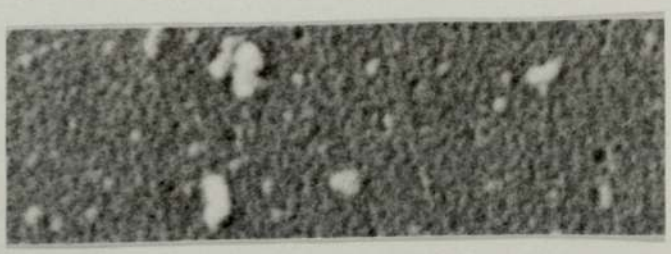
d) Argon plasma treated (10 minutes) polystyrene spun coatings.

Figure 5.3:

S.E.M. Studies of polystyrene spun coatings after exposure to PBS(-) for 4 hours.

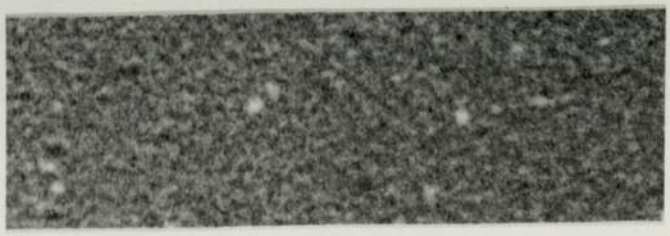
a) Poly(styrene) spun coating

b) Oxygen plasma treated (10 minutes) polystyrene spun coating.



c) Mitrogen plasma treated (10 minutes) polystyrene spun coatings

d) Argon plasma treated (10 minutes) polystyrene spun coatings



5.5 Surface Energy analysis:

Contact angle measurements reveal that the treatment of polystyrene with either oxygen, nitrogen or argon plasmas renders the polymer surface considerably more hydrophilic (table 5.1 a,f,g,h,). The dispersive component (γ_d) of polystyrene is marginally reduced by plasma treatment (table 5.1 a,f,g,h,) whereas the polar component (γ_p) is significantly increased (table 5.1 a,f,g,h).

If these results are compared with those for commercial cell culture materials it is clear that the γ_p value for polystyrene is intermediate to the values for falcon and sterilin bacteriological plastics(table 5.1 a,b,c,) the γ_d values for these substrates are also very similar (table 5.1 a,b,c,). The γ_p of tissue culture grade materials is increased significantly compared to those of bacteriological grade materials (table 5.1 b,c,d,e,). The γ_p values of all plasma treated surfaces are intermediate to those of Nunclion tissue culture grade plastic and Primaria, a new material designed for use with primary cell lines (table 5.1 d,e,f,g,h,).

Table 5.1

Contact angle measurements and surface energetic analysis of commercial tissue culture surfaces and plasma treated polystyrene.

	Contact angles(°) H_2O	CI_2H_2	Surface Energy(dynes/cm²) γ_p	γ_d	
Polystyrene	85°	35°	1.9	40.2	42.2
Sterilin bacteriological grade plastic	90°	15°	0.25	49.2	49.5
Falcon 1004 "untreated" petri discharge.	74°	22°	4.5	43.8	48.3
Nunclion Tissue culture grade	54°	32°	17.0	36.2	53.3
Primuria	41°	20°	38.6	23.7	62.3
Argon plasma treated polystyrene	29°	30°	32.9	33.7	66.6
Nitrogen plasma treated polystyrene	33°	34°	32.0	32.3	64.3
Oxygen plasma treated polystyrene	20°	30°	37.6	33.0	70.6

5.6 Cell spreading assays

(i) In complete medium

As expected untreated polystyrene spun coatings support only very limited cell attachment and no cell spreading (table 5.2,a).

All plasma treated surfaces support a level of cell attachment and spreading comparable of that of tissue culture plastic; regardless of type of plasma or duration of treatment (table 5.2 b,c,d)

(ii) In the absence of serum

It was decided to test cell adhesion to argon plasma treated polystyrene in the absence of serum. This step was taken because under these protein free conditions the adhesiveness of tissue culture plastic relative to other substrate materials is increased and hence a comparison with argon treated polystyrene is of interest. In addition to serum free medium cell adhesion was also assayed in both positively and negatively charged phosphate buffered saline (ie P.B.S. (+) and P.B.S. (-) respectively). These experiments would provide some information regarding the effect of charged ions upon cell - polystyrene interactions.

(a) Serum free medium

Both argon plasma treated polystyrene and tissue culture plastic support the attachment of approximately seven times the number of cells found on bacteriological grade plastic. In terms of

cell spreading tissue culture plastic and argon treated polystyrene support a few spread cells, which are not present on bacteriological plastic and this is reflected in mean cell diameter values (table 5.3a).

The three surfaces however, support predominantly rounded cells, hence the modal cell diameters recorded for the cell populations cultured on these substrata are identical.

(b) In P.D.S.(+)

In this medium tissue culture plastic and argon plasma treated polystyrene support the adhesion of an identical number of cells, whereas bacteriological grade plastic allows the attachment of only one third of this number (table 5.3b). No cell spreading is observed on any of the substrates tested under these conditions. Hence, cell populations cultured on these surfaces show very similar cell size distributions in terms of both mean and modal cell diameters (table 5.3b).

(c) In P.B.S.(-)

Under these conditions tissue culture plastic supports the attachment of twice as many cells as argon plasma treated polystyrene, the number of cells attached to bacteriological plastic being in between these values (table 5.3c). No cell spreading is observed on any substrata in this medium and therefore mean and modal cell diameters are similar to those recorded in P.B.S(+) (table 5.3 b,c)

Table 5.2

Cell spreading on native and plasma treated polystyrene

(a) Controls

Material	No. of cell adhered as % of control	% of cell population spread	Morphology
Tissue Culture Plastic	100	91	full spread
Poly styrene (untreated)	14	0	rounded

(b) Oxygen plasma treated polystyrene

Treatment Time	No. of cells adhered as % of control	% of cell population spread	Morphology of cells
20 Secs.	96	90	fully spread
30 Secs.	102	96	fully spread
1 min	104	87	fully spread
2 min.	95	92	fully spread
10 min.	100	93	fully spread

Table 5.2 continued

(c) Nitrogen treated polystyrene

Treatment Time	No. of cells adhered as % of control	% of cell population spread	Morphology of cells
20 Secs.	87	94	fully spread
30 Secs.	103	84	fully spread
1 min	89	91	fully spread
2 min.	90	93	fully spread
10 min.	104	90	fully spread

(d) Argon treated polystyrene surfaces

Treatment Time	No. of cells adhered as % of control	% of cell population spread	Morphology of cells
20 Secs.	90	91	fully spread
30 Secs.	92	90	fully spread
1 min	101	82	fully spread
2 min.	103	93	fully spread
10 min.	102	91	fully spread

Table 5.3

Cell spreading assays in the absence of serum

(a) Cell spreading assays in serum free medium

Substrate Material	No. of cells adhered as % of control	Arithmetic mean cell diameter (um)	Model cell diameter (um)
Tissue Culture plastic	100	29.9	15
Argon plasma treated (10 mins) poly(styrene)	129.6	27.9	15
Sterilin bacteriological grade plastic	19.9	16.3	15

(b) Cell spreading assays in P.B.S (+)

Substrate Material	No. of cells adhered as % of control	Arithmetic mean cell diameter (um)	Model cell diameter (um)
Tissue Culture plastic	100	21.0	15
Argon plasma treated (10 mins) poly(styrene)	99.2	18.7	15
Sterilin bacterilogical grade plastic	34.7	16.0	15

Table 5.3 continued.

(c) Cell spreading assays in P.B.S (-)

Substrate Material	No. of cells adhered as % of control	Arithmetic mean cell diameter (um)	Model cell diameter (um)
Tissue Culture plastic	100	15.7	15
Argon plasma treated (10 mins) poly(styrene)	45.8	18.6	15
Sterilin bacterilogical grade plastic	61.4	16.3	15

5.7 Cell Growth Assays

Growth rates were recorded for B.H.K. fibroblasts over an eight day period. Cells were cultured on three commercial tissue culture grade surfaces (Nunclion, Corning, Primaria) and argon treated polystyrene. The growth rate was recorded per unit area of the substrata. Very similar growth rates were recorded for the four substrata tested. (table 5.4 & figure 5.5).

Table 5.4

Cell growth on commercial T.C.P. and Argon plasma treated polystyrene

	Nunc*	Bact*	Corning	Argon*	Primaria
Cells/cm2	0.55x104	0.55x104	.055x104	0.55x104	0.55x104
Day 1					
Coulter	10943	5929	11744	8084	14416
Readings	10831	5776	11715	8091	14022
Cell No/cm2	2.42x105	1.3x105	2.6x105	3.6x105	3.16x105
Day 2					
Coulter	32127	6619	25218	27005	40329
Readings	32106	6665	25655	27035	40515
Cell No/cm2	7.14x105	1.48x105	5.6x105	1.2x105	8.98x105
Day 3					
Coulter	56980	41900	51747	53897	34012
Readings	56905	42329	51303	54836	54910
Cell No/cm2	1.26x106		1.15x106	1.6x106	1.12x106
Day 4					
Coulter	55166		51571	53643	53597
Readings	55452		51808	53940	53603
Cell No/cm2	1.15x106		1.06x106	1.71x106	1.11x106
Day 8					
Coulter	41277		44311	41792	42384
Readings	41807		44373	42355	42727
Cell No/cm2	8.63x105		9.2x105	1.2x106	8.85x105

Footnote:- Two coulter counter readings per sample per day are shown plus the resultant average cell density figure.

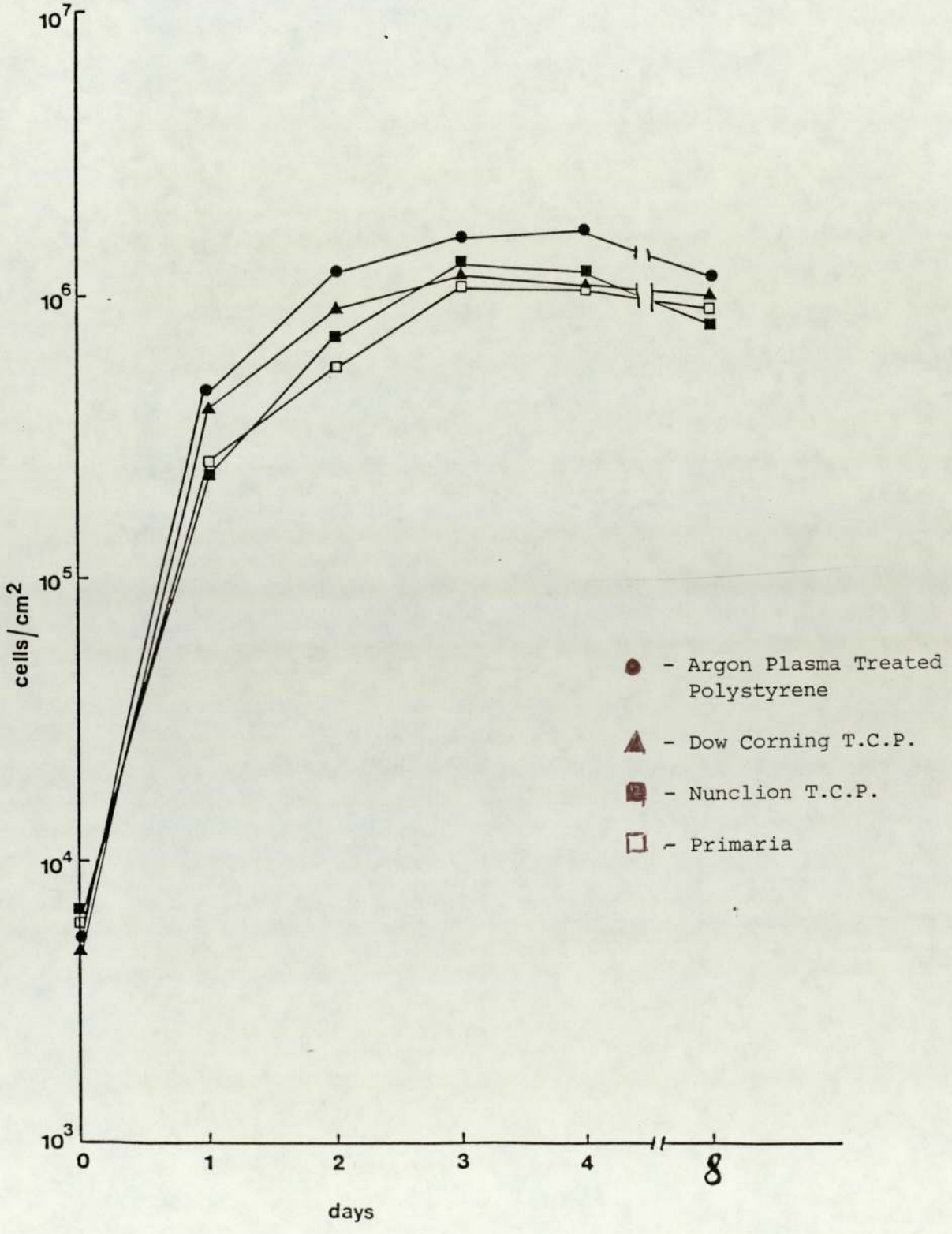
Nunc* = Nunclion T.C.P.

Argon* = Argon plasma treated polystyrene.

Bact* = Bacteriological grade plastic.

Figure 5.5

BHK fibroblast growth on commercial tissue culture surfaces and Argon treated polystyrene



5.8 Discussion

These findings further illustrate a point made previously (ref 14-17) that polystyrene, in the adsence of surface treatment supports only very limited levels of cell adhesion and no cell spreading. Therefore the treatment of polystyrene in order to render the substrate suitable for cell adhesion provides a very useful model system for the determination of factors of importance in cell substratum interactions (ref 57.79). Acid treatment is well established as a method by which to achieve cell growth on polystyrene. However despite several studies the mechanism by which this occurs has not been satisfactorily explained for reasons detailed in the introduction to this chapter.

Corona discharge (gas plasma) treatment of polystyrene is now used for the production of tissue culture plastic, which is utilized daily by biologists during routine cell culture. Possibly due to limited liason between polymer chemists and cell biologists the potential of plasma chemistry as a technique for investigating cell adhesion requirements has not been fully exploited.

Pure polystyrene surfaces were treated with both oxidizing and non oxidizing (N_2+Ar) gas plasmas. Examination of the resultant surface by XPS showed that only oxygen plasma treatment of polystyrene significantly increased the oxygen component within the surface chemistry of polystyrene. Both nitrogen and argon plasma treated surfaces showed only a small 1s2 oxygen peak, which results from a slight oxidation during

the extrusion of the polystyrene and is also present on untreated polystyrene surfaces.

Surface energetic analysis of treated and untreated polystyrene surfaces illustrates that plasma treated surfaces are characterized by a large increase in γ_p when compared to untreated polystyrene surfaces. Both γ_p and γ_d values record for plasma treated surfaces are essentially identical to those for commercial T.C.P. materials while untreated polystyrene is similar to both commercial bacteriological grade materials.

The increase in wettability of the nitrogen and argon plasma treated polystyrene surfaces (without commercial modifications) can be explained by the work of Wenzel (ref 300). This describes the effect of surface rugosity formed from features of more that 500 A° upon surface wettability. A roughness factor (ref 300) is used where:-

$$R = \frac{\cos \theta'}{\cos \theta}$$

θ' = observed contact angle

θ = expected contact angle

S.E.M. studies revealed that untreated polystyrene surfaces were smooth and complete but that all plasma treated surfaces studied showed rugosity formed from features of approximately 1000 - 2000 A° in size. Which would therefore contribute to the observed wettability of these surfaces. This system of substrata encompasses two separate mechanisms for increasing

wettability of polystyrene surfaces. Firstly through chemical modification by oxidation with an oxygen plasma, with some contribution from the rugosity formed during the plasma process. Secondly,mainly through surface rugosity with a small degree of surface oxidation only in the case of surface treatment with argon and nitrogen plasmas.

If surface group modification was required for cell adhesion then only oxygen plasma treated surfaces would be expected to produce cell adhesion and growth. If however only the modification of surface energetic parameters to within a favorable range was required then all plasma treated surfaces would promote cell adhesion and growth.

Cell spreading assays performed on these surfaces revealed that all plasma treated surfaces support a degree of cell spreading which is equivalent to commercial T.C.P. both in terms of percentage attachment and cell morphology. Furthermore an eight day cell growth study shows that argon plasma treated polystyrene gives identical cell growth characteristics to those of commercial tissue culture plastic materials.

Cell spreading assays were also performed in S.F.M., P.B.S.(-) and P.B.S.(+) (eg negatively and positively charged buffer solutions). Under these conditions it is not usual to observe constant or concerted cell spreading even on T.C.P. Interestingly a comparison of argon plasma treated polystyrene and T.C.P. under these conditions showed that in the presence of S.F.M. and P.B.S.(+) the two substrates support similar levels of cell attachment. However in P.B.S.(-) commercial T.C.P.supported the attachment of twice as many cells as argon treated polystyrene. This may signify a difference in the

cell-T.C.P. and cell - argon treated polystyrene,interactions interms of electostatics. However in view of the high level of cell adhesion recorded on untreated polystyrene control surfaces in this particular experiment, this result may simply represent a variation in biochemical expression and hence attachment within a cell population. This is not a particularly unusual phenomenon under these non growth culture conditions.

Cell spreading and growth activity of fibroblasts were also measured on primaria surfaces. A new commercial T.C.P. proposed to inhibit the growth of fibroblasts by the presence of amine groups. This material was proven to support cell spreading and growth to the same extent as both commercial T.C.P. and plasma treated polystyrene

Overall therefore it seems that the fine rugosity produced by plasma treatment (only visible at 20,000 times magnification) is capable of increasing the apparent p of the surface energy and hence the apparent wettability of the substrate. This effect would seem to dominate cell adhesion behaviour. Cells adhere and spread on both oxygen and argon plasma treated polystyrene which have widely differing surface chemistry but similar (apparent) surface energetics therefore it would seem that cell adhesion can be promoted by more than one mechanism providing that the apparent wettability of the substrate and hence the interfacial tension between substrate and contacting media is within certain, yet to be defined, limits. In conclusion therefore the role of hyroxyl groups in promoting cell attachment would seem to represent a secondary mechanism to that of surface energetics. The inhibitory role proposed for amine groups would seem to have little or no basis in fact.

Chapter Six

Cell adhesion studies on an extended range of hydrophilic and hydrophobic substrata

6.1 Introduction

The polymeric substrata so far studied have expressed only a part of the range of physico-chemical properties offered by synthetic polymer chemistry. This was necessary in order to construct a copolymer series in which structural control was systematically exercised. With the hope that principles could be established which could be used to explain and predict cell adhesion on a wide range of substrate materials. The object at this point in the research program was, therefore, to broaden the range of substrate materials studied to encompass not only purpose synthesized polymers but also commercially available materials of widely differing chemical structure and physico-chemical properties.

At this stage it was decided to consider substrate materials particularly in terms of a wide ranging scale of hydrophilicity. This could be expressed in terms of E.W.C. for hydrophilic materials and to some extent by polarity in the case of more hydrophobic materials.Equilibrium water content (E.W.C.) is a useful measure of the hydrophilicity and polarity of hydrogel polymers. Such a property however is obviously of limited significance when considering non hydrogel materials (materials with an EWC of less than 20%) as surface water is capable of contributing equally well to the interfacial behaviour of these materials. Two particularly interesting groups of materials remain to be investigated as regards their cell adhesion properties.

The first of these is the range of hydrophobic polymers which express a surface polarity intermediate to that of polystyrene and poly(EMA) (ie polymers expressing intermediate hydrophobic character)

Some evidence exists in the literature as to the characteristics of these materials in cell culture (ref 44,68) but this is scattered and poorly correlated. P.T.F.E. (polytetrafluoroethylene) is reported to be adhesive for fibroblasts by some groups of workers (ref 53,254-6) but often show only low levels of adhesion. Conversely polyethylene terephthalate (Melinex) is well established to be adhesive for cells and is routinely used for particular electron microscopy techniques where the flexibility of the material is an advantage over conventional tissue culture plastic. Many polymers in this range are commercially manufactured on a large scale (eg melinex, polystyrene, polymethylmethacrylate polypropylene). Such materials therefore would be in plentiful supply for use in medical or technological applications should cell adhesion studies highlight any interesting or useful properties.

The second range of materials of interest in terms of cell adhesion are polymers which are considerably more hydrophilic than poly(HEMA). These polymers range in EWC from 40% to over 80% with correspondingly high polarities.

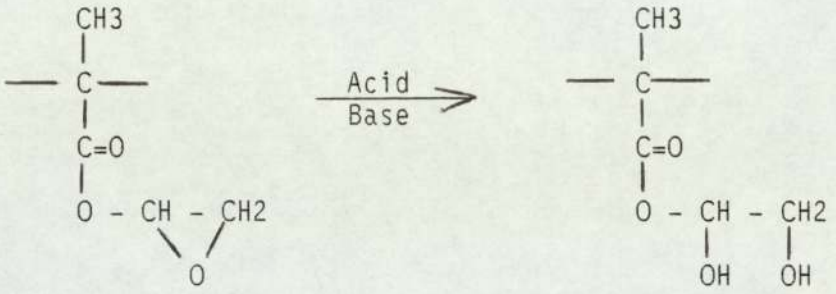
6.2 Choice of substrate materials

Several commercially available polymers were chosen to extend the range of hydrophobic materials beyond polystyrene.

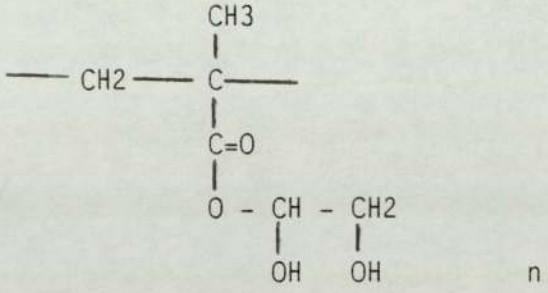
Included in this range are P.T.F.E., poly(4methlypent-1-ene) (known as T.P.X.), polyethylene and polypropylene which all are less polar than polystyrene. All these polymers were studied in the crystalline state as prepared films. Attempts to produce spun coatings of amorphous polypropylene were unsuccessful in the time available. Other commercially available materials were selected to extend this range of hydrophobic materials to overlap the poly(HEMA/EMA) copolymer series interms of hydrophobicity. Polyethylene terephthalate and cellulose triacetate/diacetate express considerable polarity and hence are considerably closer to commercial tissue culture plastic than P.T.F.E. or polystyrene in terms of surface energetic parameters.

Some polymers in this range were custom synthesized. Polymethyl methacrylate is not usually supplied in a pure form commercially and so was synthesized, this polymer is marginally more polar than poly(EMA) and hence would be expected to provide a good substrate for cell adhesion. Also custom synthesized was a range of poly(glycidyl methacrylate/EMA) copolymers. The properties of such a copolymer series is coincident with the more hydrophobic regions of the poly(EMA/HEMA) copolymer series but have an E.W.C. of less than 3%.

A further range of substrates based on glycidyl methacrylate were prepared by surface treatment of the homopolymer with acid or base (HCl or NaOH) to effect a ring opening reaction as follows :-



By varying the time of exposure to the reagent, a range of surfaces with differing wettabilities, polarities and hydroxyl contents can be produced. However as the penetration of these reagents into the polymer bulk is very limited the physical properties of the material are essentially unchanged. For comparison purposes the homo polymer of poly(glyceryl methacrylate) was synthesized. This structure of this polymer is shown below:-



This polymer therefore has a homogeneous structure identical to the surface of glycidyl methacrylate after treatment with a ring opening reagent.

Few hydrophilic materials are available commercially, and it was therefore necessary to synthesise a range of substrate materials which would represent this domain of polymer chemistry.

Polyacrylamide, poly N-methylolacrylamide and diacetone acrylamide were prepared by bulk polymerization. These polymers express E.W.C. of between 60 and 90% and equivalent polarities. Poly(2-hydroxyethyl methacrylate) and poly(hydroxypropoylacrylate) were of considerable interest and they represent materials of similar structure to poly(HEMA) but are rendered slightly more hydrophilic by the replacement of the methacrylate moeties with acrylate groups. Furthermore such materials are very similar in terms of E.W.C. to N. methylol acrylamide but very different in chemical structure.

In order for a polymer to form a high water content hydrogel its monomer structure must incorporate highly polar groups which will bind large quantities of water, (ie the hydroxyl groups present in hydroxymethacrylates.) Sulphonate groups can bind large quantities of water and when incorporated into a synthetic polymer structure could produce materials with E.W.C. of over 90%. Sulphonate groups are of particular relevance to cell adhesion studies, as for several years many researchers (ref 57) believed these groups to be responsible for cell adhesion on acid treated polystyrene surfaces.

Although the recent findings of Curtis et al (ref 253) have proven this not to be the case it seems clear that sulphonate groups are important in cell/cell and cell/substrate interactions. Biomolecules known to be important in cell adhesion (particularly heparan sulphate) are known to be highly sulphonated and blocking these groups has significant effects on the biological activity of such molecules.

The use of sulphonated monomers namely 3 sulphopropyl methacrylate. (SPM) and 3 sulphopropylacrylate (SPA) enables this moiety to be incorporated into otherwise conventional methacrylate and acrylate polymers. As a methacrylate SPM is structurally similar to HEMA. The difference between the two monomers being that SPM has the addition of one alkyl group and the subsitution of the sulphonate group in place of the hydroxyl. Therefore not only does polystyrene represent an interesting substratum for cell adhesion studies, but copolymerization with HEMA gives a range of copolymers where a gradual substitution of the hydroxyl group by the sulphate group is achieved. Associated with this graded chemical change is also a graded rise in the polarity and water content of the copolymer series. Poly(SPA) forms a substrate marginally more hydrophilic than poly(SPM). Overall this range of substrata encompasses a wide range of chemical composition and physicochemical diversity.

Results

Results obtained for both cell adhesion and spreading are presented in table 6.1 and 6.2. Of these two parameters cell spreading is by far the most important as it is of direct relevance to cell viability. The findings presented in table 6.1 and 6.2 are further divided broadly on the basis of substrate hydrophilicity. Cell adhesion on hydrophobic materials is summarized in Table 6.1. Generally the series of poly(EMA/GMA)(ethylmethacrylate/glycidylmethacrylate) copolymers are adhesive for cells. As expected all grades of melinex support high levels of cell adhesion as does polybutyl teraphthalate which would be expected to be marginally less polar due to the presence of the larger butyl chain. Some differences are observed between differing grades of melinex. Good consistency was observed between clear T.E.M. grade used at Unilever and a similarly clear axially orientated grade obtained directly from I.C.I. Cellulose triacetate and diacetate both allow good adhesion and spreading as do poly(EMA) and particularly polymethylmethacrylate which in terms of there structure would all be expected to have similar polarity to that of polyethelyene teraphthalate.

One of the more interesting observations is that T.P.X. poly propylene and poly ethylene all show high levels of cell adhesion and spreading. P.T.F.E. shows good adhesion levels but spreading is reduced, however spreading is still significantly above control levels on polystyrene.

The results shown in figure 6.2 indicate that this sample of polyglycidylmethacrylate was not adhesive for BHK or MRC's fibroblasts. As samples of this polymer made by other workers were found to be comprehensively adhesive in later experiments there would seem at first sight to be some anomaly. It is clear however that short surface treatments to effect a partial ring opening reaction renders the surface universally adhesive were as extended treatment results in a non adhesive surface.

It is also shown that poly(acrylamide) poly(SPM) and poly(SPA) are universally adhesive whereas poly(N methylol acrylamide) is universally non adhesive. In addition increasing the level of SPM in a series of poly(SPM/HEMA) copolymers produces a sudden onset of cell attachment when an 80% level of SPM is attained, prior to this little cell adhesion is observed. It appears therefore that the factors affecting cell adhesion in more polar polymers produce some discontinuity in the polarity-adhesiveness relationship.

Table 6.1

BHK Fibroblast adhesion on hydrophobic materials.

Substrate Material		Number of Cell as % of control	% of cell population spread
Glass Control		100	78
Poly(EMA/GMA) Ratio	copolymers 20/80	53	49
	30/70	99	84
	40/60	121	78
	50/50	128	80
	60/40	144	75
	70/30	45	58
	80/20	92	83
Poly(GMA)		91	78
T.C.P.		75	90
Melinex (Unilever TEM*)		127	98
S grade		116	51
377 grade		43	83
O grade		93	97
226 grade		69	59

Table 6.1 Continued

Cell adhesion results for BHK fibroblasts on hydrophobic materials

Substrate Material	Number of Cell an % of control	% cells population spread
Poly (Butyl teraphthalate)	78	89
Poly (ethylmethacrylate)	84	81
Poly methylmethacrylate	136	96
Cellulose triacetate	76	68
Cellulose diacetate	84	59
T.P.X.	86	91
Poly propylene	78	83
Poly ethylene	72	68
P.T.F.E.	78	39
Polystyrene	No Cell Attachment	

GMA = glycidylmethacrylate.
EMA = ethlymethacrylate.
TPX = poly(4-methlypent-1-ene)
PTFE = polytetrafluoroethylene
* = Clear melinex used as a T.E.M. cell culture substrate

Table 6.2

Cell adhesion on hydrophilic materials

Table 6.2 a

BHK cell adhesion on hydroxyl containing polymers

Substrate Material	Number of Cells as % control	% of Cell Population Spread	Cell Morphlogy
T.C.P. Control	100	86	fully spread
Polyhydroxybutyl methacrylate	91	94	fully spread
Polyhydroxypropyl methacrylate	88	92	fully spread
Polyhydroxyethyl acrylate			
Polyhydroxypropyl acrylate		No Cell Adhesion	
Poly N Methylol acrylamide			

Table 6.2b

Cell adhesion on glycidyl methacrylate/ring opened glycidyl methacrylate and glycerol methacrylate (various cell lines)

	% of cell population in spread morphology			
Cell Line	BHK	MRCS	16C	HEP2
Polyglycidyl methacrylate	0	0	90	90
Polyglycidyl methacrylate. 5 min surface treatment with 1 molar HCl.	84	92	91	92
Polyglycidyl methacrylate. 5 min surface treatment with 1 molar NaOH.	90	93	71	84
Polyglycidyl methacrylate. 90 min surface treatment with 1 molar HCl.	0	0	0	0
Polyglycerol methacrylate	No Cells		52	-

Table 6.2c

Cell adhesion on Acrylamide polymers (various cell lines)

	MRCS	BHK	HEP2	16C	REF
Polyacrylamide	90	91	91	90	92
Poly N.Methylol Acrylamide	No Cells	0	No Cells	0	0
Polydiacetone Acrylamide	-	69	-	-	-

Table 6.2d

Cell adhesion on poly(SPM), poly(SPA) and poly(SPM/HEMA) various cell lines

	% of cell population in spread morphology					
% SPM in copolymer	CEFlo	16C	REF	H2	BHK	MRCS
0	No Cell Adhesion.					
20	0	-	0	0	0	0
40	0	-	0	0	0	0
60	0	-	0	0	0	0
80	77	86	40	23	201	91
100	88	96	81	40	80	80
SPA	-	30	57	31	50	79

Discussion

Several interesting points are immediately apparent from the data presented in this chapter.

Firstly all hydrophobic materials except polystyrene are adhesive for cells. Secondly moderately hydrophilic hydrogels which have an E.W.C. of up to 60% do not support any cell adhesion. Some polymers of greater hydrophilic character do support cell attachment but not all of the latter support full cell spreading. Further points of interest are the high levels of cell adhesion and spreading on poly(SPM) and on glycidyl methacrylate. The latter is also of interest because prolonged surface treatment with acid or base reduces cell adhesion to zero but moderate treatments render surfaces highly adhesive.

Polystyrene therefore, seems to occupy a unique position among non-hydrogel materials in being non-adhesive for fibroblasts in culture. This may seem to represent something of an anomaly. It would be expected for substrata more hydrophilic in nature than polystyrene to be adhesive for cells, as the adhesive properties of polyteraphthalate, polyethlymethacrylate and tissue culture plastic are well established. However four substrata tested, namely poly-4-methylpent-1-ene(TPX), polytetrafluoroethylene(P.T.F.E.), poly(propylene) and poly(ethylene) are less polar than polystyrene ,but allow high levels of cell adhesion and spreading. The answer to this may lie in the consideration of the polymer structure involved,

polystyrene is the only one of these materials to be tested in the amorphous state, the other materials being in crystalline form. It may be that the expression of a regular crystalline array at the surface may promote cell adhesion though microscopic areas of surface polarization, such interactions may not be possible with an amorphous polymer.

Cell adhesion results from the differing grades of melinex provides some additional evidence of a role for polymer crystallinity. The optically clear, highly crystalline O grade melinex and TEM grade melinex (also crystalline) gives significantly higher levels of cell spreading than other less crystalline grades (eg S,377,226). Further study is required to clarify this point. It seems relevant at this point to once more state that commercial bacteriological plastic, which is also non cell adhesive is polystyrene with a silicone coating to mask hydroxyl groups formed during the moulding process. This oxidation would (and does) cause cell adhesion.

No cell attachment is observed on poly(N-methylol acrylamide) poly(HPA), poly(HEA) or poly(glycerol methacrylate). These polymers all have an E.W.C. of approximately 60% despite widely differing chemical structure. In view of the established non-adhesiveness of poly(HEMA) and the fact that poly(acrylamide) and poly(diacetone acryamide)(E.W.C. of 90% and 70% respectively) both support cell spreading. It would seem that hydrogel polymers of between 40% and 60% water content do not suport cell adhesion. Such parameters will be further discussed in a later chapter.

High levels of cell spreading are observed for most cell lines on poly(SPM), poly(SPA) and poly(SPM/HEMA) copolymers which contain more than 60% SPM. It could be reasoned that such results represent a role for sulphonate groups. However in view of the high cell adhesion achieved in poly(acrylamide) and other hydrophilic polymers it seems that adhesion is promoted by the high E.W.C. and consequent high polarity of such materials. A point to consider here is that although a SPM/HEMA copolymer of 60/40 composition would be expected to have a water content considerably in excess of 60% (ie in a cell adhesive zone) no cell adhesion is observed. An explanation for this may require the investigation of water structuring phenomena or possibly mechanical characteristics. Indeed poly(HEMA/SPM) hydrogels have little mechanical integrity and this may increase the threshold level of polarity or E.W.C. at which cell spreading is evident. Indeed the mechanical properties of high water content hydrogel substrates in general may explain why some polymers express high levels of cell spreading but support relatively few cells (eg acrylamide). While other substrates may show good cell attachment but lower levels of cell spreading than might be expected.

Moderate acid (or base) surface treatment of glycidyl methacrylate gives high levels of cell adhesion whereas prolonged treatment renders the surface non adhesive. Such findings represent another case were increasing hydrophilicity results in transitions in cell adhesion behaviour. Similar differences between samples of polyglycidyl methacrylate (ie differences in X link density or degree of hydrolysis) may

account for variations in cell adhesion behaviour recorded on these substrates. Clearly these findings confirm the range for adhesive substrates already defined(ie polyHEMA/STY or poly HEMA/EMA) and also confirm the non adhesive behaviour of poly(glycerol methacrylate).

A further point is that all poly(EMA/GMA) copolymers and cellulose triacetate and diacetate represent adhesive surfaces for fibroblasts. These polymers have fractional polarities which are coincident with the poly(HEMA/EMA) copolymer described in chapter 4 while having a very different chemical composition. This seems to be another part to a substantial argument for important of physicochemical considerations and particularly fractional polarity as parameters for the assessment of the cell adhesive behaviour of a polymer surface. The relevant importance of such factors as E.W.C., fractional polarity and other factors will be examined in the final discussion (chapter 7) in an attempt to draw all the results presented in this and previous chapters together to form some comprehensive basis for understanding cell-substrate behaviour.

Chapter Seven

Concluding Discussion

7.1 Concluding discussion

Initial investigation of cell spreading on poly(HEMA) coatings demonstrated not only the impossibility of using such substrates for cell adhesion control; but also established the non adhesive nature of poly(HEMA). This was significant in view of the widely held belief that hydrophilic surfaces were generally more suitable for cell adhesion (ref 53,63,273). Such opinions were mainly based on comparison of cell attachment to native and surface oxidized polystyrene (ie commercial tissue culture plastic). Although such surface treatments produced a substantially more hydrophilic surface than the native material it was clear that a true hydrogel (E.W.C. 40%) such as poly(HEMA) represented a further extension of this hydrophilic range. Limited evidence existed therefore that although extremely hydrophobic materials and some hydrophilic polymers were non adhesive for cells, synthetic polymers with intermediate properties seemed to be adhesive for cells. This apparent phenomena offered the possibility of cell spreading modulation as well as the elucidation of cell adhesion requirements.

It was decided to investigate this area using three homopolymers of known structure and physicochemical nature, which have already been widely used in studies of cell adhesion : poly(HEMA) poly(EMA) and polystyrene. Poly(HEMA) as a moderately hydrophilic hydrogel containing one hydroxyl group per monomer unit. Poly(EMA) and polystyrene on the other hand are both hydrophobic polymers containing no hydroxyl groups. There are

important differences however between these two homopolymers. Polystyrene is an extremely hydrophobic material in which any polar contribution can only be derived from asymmetry of the aromatic ring about the polymer backbone. Poly(EMA) by comparison is only moderately hydrophobic due to significant polar contribution from the ester linkage. While poly(HEMA) and polystyrene were known to be non adhesive for cells in their pure forms, there were indications in the literature (ref 270,258) that poly(EMA) presented a much more adhesive substratum.

Several cell lines were tested for adhesion on a wide range of both poly(HEMA/EMA) and poly(HEMA/STY) copolymers . These studies confirmed the non adhesive nature of both poly(HEMA) and polystyrene while emphasising the adhesiveness of poly(EMA). For all cell lines tested both poly(HEMA/EMA) and poly(HEMA/STY) copolymers containing up to 92% and 96% HEMA respectively are, however capable of supporting a large fraction of the cell population in a fully spread morphology.

In view of the very different cell adhesion properties of polystyrene and poly(EMA) homopolymers, it would seem that their role in promoting cell adhesion when copolymerized with HEMA is not a function of their intrinsic cell adhesion characteristics. Rather it is clear that some modulation of the physical and/or chemical properties of poly(HEMA) by copolymerization with hydrophobic polymers brings the substratum into an "adhesive zone" within which conditions are favourable for cell culture.

With the establishment of this adhesive domain for cells

it seems reasonable to investigate possible mechanisms and substrata characteristics which might determine such a zone. By demonstrating that materials of very different chemical structure (eg poly(EMA), poly(HEMA)/STY etc) could support cell adhesion these findings suggest that the role of specific chemical groups was secondary to that of some physicochemical phenomenon. This was in conflict with other work in the literature which outlined a specific role for sulphonate (ref 63), hydroxyl (ref 253) or amine moeties. It seemed therefore that further research should be directed towards the definition of any role for surface energetics in cell adhesion.

A system in which the surface energetics of the substrate could be changed without alteration of other factors would obviously be useful. To this end the effect of inert gas plasmas on polystyrene surfaces was investigated. It was demonstrated that exposure of the polymer surface to an inert gas plasma causes no change in the surface chemistry. However, surface etching of the polymer produces a small degree of surface rugosity, which gives a marked increase in the wettability of these surfaces. Polystyrene treated with either nitrogen or argon plasma proved capable of supporting high levels of cell spreading and rapid cell growth. Such observations are obviously strong evidence of an overriding role for surface energetic parameters in determining the suitability of a synthetic substrata for cell culture.

The studies were then extended to encompass a wider range of both purpose synthesized and commercially available polymeric materials. The intention here was to investigate a

wide range of physical and chemical phenomena and observe their effect on cell behaviour. Despite the obvious differences in chemical structure, morphology and crystallinity that exist within the range of materials studied, they are collected together in a single table (table 7.1) for comparative purposes.

A broad attempt to arrange the polymers in order of decreasing E.W.C. (or decreasing polarity of non hydrogels) reveals an interesting transition in cell adhesion behaviour; of the materials studied, no polymer with an E.W.C. of 35 to 60% supports cell attachment. Indeed it is interesting that although the proportions of HEMA:sty and HEMA:EMA critical for cell adhesion are different, each represents a similar E.W.C. of approximately 34%. In addition all hydrogels in the E.W.C. range of 2 - 35% support cell adhesion (table 7.1). Although polymers listed in table 7.1 having a E.W.C. of less than 20% cannot be considered as true hydrogels (ie water swollen networks) they express water near the polymer surface which thus contributes to interfacial behaviour. These polymers must therefore be realistically considered as extending the observed continum of behaviour.

Between 60 and 70% E.W.C. some polymers support limited attachment which is not accompanied by subsequent cell spreading. Similarly cell adhesion on hydrogels in the 70 - 90% E.W.C. region is not straightforward. Most typically a high percentage of cells attach to these polymers but only a small proportion adopt a fully spread morphology, a notable exception to this being 3 sulphopropyl methacrylate. It seems that these observations reflect a more complex set of possibilities for the

Table 7.1

BHK cell adhesion related to fractional and EWC of synthetic polymer substrata.

Substratum	EWC %	Fractional polarity	Cell adhesion (6h)
Poly(SPA)	90+	-	+
Poly(SPM)	90+	-	+
Polyacrylamide	90	0.90	+
Poly-N-N-dimethyl acrylamide	70	0.80	+
Diacetone acrylamide copolymer	70	0.80	+
Poly(HEMA/SPM)	80	-	+
Polyglycerol methacrylate	60	-	-
PolyHEA	60	0.60	-
PolyHPA	50	0.60	-
Poly-N-methylol acrylamide	50	0.60	-
PolyHEMA	40	0.55	-
Glow discharge treated polystyrene	-	0.49-0.51	+
PolyHEMA/EMA(90/100)	33	0.47	+
PolyHEMA/STY(90/100)	30	0.47	+
PolyHPMA	23	0.47	+
PolyHBMA	20	0.40	+
Commercial tissue culture plastic	-	0.32	+
PolyHEMA/EMA(50/50)	12	0.30	+
PolyHEMA/STY(50/50)	8	0.30	+
Cellulose triacetate/diacetate	10	0.18-0.28	+
Polyglycidyl methacrylate	5	0.25	+
Melinex (polyethylene terephthalate)	-	0.22	+
PolyHEMA/STY(10/90)	2	0.12	+
PolyMMA	2	0.17	+
PolyEMA	2	0.14	+
Polystyrene	-	0.05	-
Commercial bacteriological grade plastic	-	0.05	-
PTFE	-	0.02	+
TPX	-	0.02	+
Polyethylene	-	0.02	+
Polypropylene	-	0.02	+

EWC: equilibrium water content; MEM: modified Eagle's medium; EMA: ethylmethacrylate; HEMA: 2-hydroxyethyl methacrylate; HPMA: hydroxypropyl methacrylate; HBMA: hydroxybutyl methacrylate: HEA: hydroxyethyl acrylate; HPA: hydroxypropyl acrylate; MMA: methyl methacrylate; STY: styrene; PTFE: polytetrafluoroethylene; TPX: poly-4-methyl pent-1-ene.

structure (including water structuring) of high water content gels. Indeed the inherent mechanical instability of these hydrogels may tend to inhibit the spreading of cell types exerting high tensile stress (ref 267).

The E.W.C. is essentially a bulk property and is therefore of limited value in determining the cell adhesion properties of hydrophobic materials; where surface bound water and physicochemical properties may be a factor in interfacial behaviour. In order to probe and unify these properties, a parameter which could express the relative hydrophilicity or polarity of a wide range of materials was required. An attempt was made to investigate this by analysis of the polar (γ_p) and dispersive (γ_d) components of the surface free energy.

The fractional polarity is easily calculated from these parameters:-

$$\text{Fractional Polarity} = \left(\frac{\gamma_p}{\gamma_p + \gamma_d} \right)$$

This property has been established as an important factor in wettability and adhesion (ref 301-2). Fractional polarities calculated from primary experimental results are incorporated into table 7.1.

Fractional polarity is profoundly influenced by the E.W.C. particulary in the case of high water content gels, but in the case of more hydrophobic materials the combined effect of surface chemistry and surface bound water becomes overriding.

It seems therefore that fractional polarity measurments may represent a useful unification of the physicochemical forces which contribute to the suitability of substrates for cell culture.

Considered in terms of fractional polarity commercial tissue culture plastic and glow discharge treated polystyrene, both of which support cell adhesion, are contained in the range of materials characterized by an E.W.C. of 2 - 34% (which can now be defined by fractional polarities of 0.14 and 0.51). Pure polystyrene (or bacteriological plastic) which has a very low fractional polarity falls below this region and does not support cell attachment. For more hydrophilic polymers the situation is much the same as that described by E.W.C., but with the non cell adhesion window defined by fractional polarities of 0.55 and 0.60.

This analysis represents the first attempt to describe the cell adhesive requirements over a comprehensive range of substrates. Although fractional polarity provides a guide to the adhesiveness of substrates for cell these results demonstrate that no single physical or chemical property is sufficient for the prediction of cell adhesion over such a broad range of materials.

Substratum adhesiveness for cells can be modulated in a number of ways. In the case of poly(HEMA) cell adhesion can be induced by replacement of hydroxyl moeties with a hydrogen atom, introduction of styrene or by increasing length of the alkyl chain.

In the case of polystyrene, adhesiveness can be produced

by treatment with, sulphuric (ref 63), Chloric (ref 79) or chromic acids (ref 253), glow discharge treatment or introduction of hydroxyl groups by copolymerization. The one common effect of these manipulations is to locate the substratum in a zone of fractional polarity within which all materials so far tested support full cell adhesion and spreading. (table 7.1) Van der Valk et al (ref 303) have also recently shown a significant relationship between the polar component of surface free energy and fibroblast adhesion on a small number of relatively hydrophobic polymers. Their findings over this limited range of polymers are in good agreement with our observations.

Cell adhesion on extremely hydrophobic substrates is not straight forward however, and requires further investigation. Although polystyrene (fractional polarity 0.05) is non adhesive, several less polar materials exist (T.P.X., P.T.F.E., Polypropylene, polyethylene) allow cell attachment. Cell attachment on materials of such low polarity is indeed surprising in view of Grinnels conclusions (ref 273) but has been noted previously (ref 254-6). A possible explanation for these results may lie in a consideration of the crystallinity of these materials. Atactic polystyrene is the only amorphous hydrophobic polymer to be studied, P.T.F.E., T.P.X., polypropylene and polyethlyene were all crystalline in nature. Interestingly cells will not adhere to other amorphous surfaces such as paraffin wax (ref 59). Further more cell adhesion has been shown to be sensitive to the structure of agarose substrates (ref 304). For non polar

polymers it seems likely therefore that crystalline/amorphous balance is a determining factor in cell adhesion.

For poorly hydrophilic substrates in the first adhesive zone (2 - 34% E.W.C.), Chemical composition and E.W.C. seem to be powerful factors. Clearly there is also a possible but as yet unproven role for water structuring properties within this domain. It would seem likely that the same properties are relevant to the non adhesive range of polymers with E.W.C. of between 35 and 60%, but with increasing importance attached to E.W.C. and water binding properties.

The incompletely adhesive behaviour of extremely hydrophilic polymers maybe due to water structuring phenomenum, or hence chemical group expression, however, mechanical properties may have an important role in cell adhesion to these inherently weak polymers. It is however interesting that sulphonated polymers show good adhesion properties particulary in view of the water structuring properties of negatively charged sulphonate moeties.

It seems therefore that a model for cell adhesion based on the polar fraction of the surface energy represents a reasonable approach which can summarize the effect of several factors involved in promoting or inhibiting cell adhesion in vitro. However, such a model will require refinement particulary in view of the behaviour of non polar materials and possibly extremely hydrophilic materials.

Can such a model for cell adhesion requirements explain the conflicts which exist within the literature? Generally, confusion regarding the chemical and physicochemical properties

necessary for cell adhesion has resulted from the utilization of restricted or incomplete ranges of polymer substrates. For example the initial development of the contact adhesion model (ref 63-73) was largely based on the premise that hydrophilic materials were superior to hydrophobic substances for cell adhesion. This hypothesis was based on the cell adhesion characteristics of only two substrates, native and acid treated polystyrene. Clearly the results of these early studies (ref 53-63) are in complete agreement with the model proposed here. A few early workers did experiment with other surfaces confirming the adhesiveness of P.T.F.E. and some other low polarity materials (ref 53,254-6). Generally the substrates tested were in a range bounded by P.T.F.E. and T.C.P. It was generally concluded that the more hydrophilic substrates supported higher levels of cell adhesion. The recent findings of Van de Valk (ref 303) confirmed these recent observations and stressed the importance of the polar component of the surface free energy. These studies have not attempted to explain the striking non-adhesiveness of polystyrene but the reported results are generally in good agreement with the findings in this thesis.

Several workers have confirmed the non adhesiveness of poly(HEMA) (ref 274-279). Most of these subsequently used the entrapment of adhesion active biochemicals, such as fibronectin or collagen to promote cell adhesion. This approach has proved useful in electron microscopy and may have other applications but is of limited value in determining parameters appertaining to cell adhesion on synthetic substrates. In one instance poly(HEMA) was blended with PVA in order to promote cell

adhesion, these blends would clearly fall within the poorly hydrophilic adhesive zone.

The same study (ref 258) goes on to conclude that hydrophobic materials represent superior cell culture substrates. While clearly this is an over simplification, such a conclusion emphasizes the generally adhesive nature of many hydrophobic materials (MMA, EMA, P.T.F.E. etc). Polystyrene is the obvious exception to this conclusion and in fact the results of this previous work outlined the non-adhesiveness of this material and are in good agreement with the findings and model for cell adhesive substrates presented here.

Grinnel has demonstrated that fibronectin adsorbs in different conformations on native and oxidized polystyrene and only the latter is suitable for cell adhesion (ref 273). Furthermore Poly(EMA) (ref 270) and poly(MMA) (ref 258) have been shown to be cell adhesive whereas poly(MMA/NVP) copolymers (EWC 50 - 60%) are not suitable culture substrates (ref 260,304). Both of these observations are in accordance with the proposed model based on work reported here.

The findings of this work are in however striking conflict with several claims in the literature related to the modification of polystyrene to produce a cell culture substrata. In these papers specific roles are proposed for sulphonate groups (ref 63), surface negative charge (ref 57,63,79) or hydroxyl groups (ref 253) in the promotion of cell adhesion. The proposed role for sulphonate groups and the resulting role for surface negative charge has been largely discredited by XPS studies of surface oxidized polystyrene. These have shown that

treatment with sulphuric, chloric or chromic acids give rise to hydroxyl and carboxyl groups, but to only insignificant levels of sulphonation. The same is also true after glow discharge or ozone treatments (ref 20-22,253).

On the basis of these findings Curtis (ref 253) has proposed a specific role for hydroxyl groups in the mediation of cell adhesion to synthetic (and some biological) surfaces. Several finding both in the literature and this report are difficult to reconcile with such a hypothesis :-

(a) The well documented (ref 73,74,258,270) adhesive behaviour of hydrophobic materials which do not contain hydroxyl groups eg poly(EMA) (ref 258,270) poly(MMA) (ref 258), poly(PVA) (ref 258), melinex (ref 261), P.T.F.E. (ref 53,254,256) etc

(b) Poly(HEMA), poly(HEA) and poly(HPA) have one hydroxyl group per monomer unit and are non adhesive, poly(HBMA) and poly(HPMA) have the same hydroxyl content but are adhesive for cells.

(c) Differing levels of hydroxylation are present in poly(HEMA/EMA) and poly(HEMA/STY) copolymers which allow cell spreading.

(d) The adhesion and growth of cells on argon plasma treated polystyrene.

In view of these considerations it is evident that chemical specificity of the type proposed does not provide a reasonable basis for the interpretation of cell substratum adhesiveness.

Furthermore results obtained for cell adhesion in the Curtis work (ref 253) are commensurate with a model based on fractional polarity. Hydroxyl group blocking reagents which inhibit cell adhesion also give surface energy properties similar to those of native polystyrene.

7.2 Cell Adhesion mechanisms

Although the findings within this report cannot be directly applied to the biochemical mechanisms of cell adhesion, areas where research into the absorbed proteins could be most fruitful are emphasized. For example, investigation of protein adsorption and extracellular complexes before, during and after cell culture on polymers which represent the boundaries of adhesive zones might reveal differences which highlight adhesive factors or complexes. It has already been shown that polystyrene and T.C.P. (ref 273) show different fibronectin adsorption characteristics; whereas the glycoprotein appears to be unable to attach to poly(HEMA) (ref 258). Studies of protein adsorption could use existing antibody (monoclonal or polyclonal) and immunofluoresence techniques (ref 221-232). Alternatively relatively recent physical techniques such as fourier transform infarred spectroscopy (ref 306), total Intrinsic internal reflection fluorescence spectroscopy (ref 307) or X.P.S. (ref 299) could be employed.

7.3 Relevance of Cell adhesion to Biomaterials Studies

Quite clearly no direct link has been established between cell culture results and biomaterial testing in vivo. It is also clear that there is no one set of properties which render any material universally biocompatible. It has been observed however, (ref 290) that the biomaterials field is unusual, if not unique in that the use of synthetic implants has preceded research into the required properties for such devices. It is also well established that although in vivo studies have been very useful in observing longer term responses to implants, little attention has been paid to the short term trigger events occuring at the surface of an implant (ref 307). Most workers in this field accept that these initial events lead to the acquisition of a protein/glycoprotein/lipid layer from the surrounding biological fluids (ref 290). Baier (ref 307) has coined the term "interface conversion" for this universal phenomena which has applications in industrial and marine fouling as well as biomedical studies. It is important that in vitro tests for protein adsorption can be related to longer term in vivo results in order to develop an understanding of short term interface conversion phenomena, which are intrinsically difficult to study in vivo. At the present time such a link is not established; it is possible that cell adhesion studies could prove useful in this area.

There is little doubt that the physicochemical properties of the synthetic material are important in determining initial protein adsorption (ref 290,307) and hence the long term biological responses to implants.

The pattern of cell adhesion outlined in these studies shows significant similarities to established phenomena in vivo.

Particular attention has focused on blood compatibility and tissue integration. The observations of many groups have resulted in the formulation of various hypotheses based upon physicochemical considerations to explain interfacial properties of these "biomaterials". The most well known of these theories are the "moderate surface energy" model of Baier (ref 308), the "minimum interfacial tension" hypothesis proposed by Andrade (ref 309) and the later modifications of these ideas (ref 310).

In the same way that all polymers examined with EWC between 2 and 34% support cell adhesion, no polymer in the range 35-60% EWC supports cell attachment (table 7.1). This pattern of adhesiveness bears a remarkable resemblance to theories of blood compatibility indeed the most blood compatible materials lie in the non-cell adhesion window described by an EWC of between 35% and 60%. It is interesting to consider the surface and interfacial energetics of polymers in and adjoining this non cell adhesive window. Surface energetics of high water content polymers (eg. over 80% - cell adhesive) are dominated by the characteristics of the water with in the hydrogel. Cell culture media although largely composed of water, is similar to blood plasma and tear fluid, in that the presence of high molecular weight biochemical species act as natural surfactants. In tear fluid this role is played by mucin, and the surface tension of tears is reduced to 48 dynes/cm ^{2} whereas the surface tension of water is 72 dynes/cm ^{2}.

Therefore highly swollen gels have a surface energy

greater than that of the contacting fluid when in cell culture. Whereas hydrophobic polymers clearly a surface energy lower than the surface tension of culture medium. On this basis the non cell adhesive window represents the area of least interfacial tension with full serum culture media. It would seem therefore that the underlying phenomena behind the cell adhesion characteristics of a wide range of substrate materials is one of interfacial tension.

In tissue integration studies the relevance of cell culture seems even more striking. Baier (ref 307) and others (ref 290) have shown that implants with low surface energy form a fiberous encapsulating layer close to the implant with relatively little adhesion of surrounding fibroblasts and little tissue organization. Implants with "high surface energy" (these are often produced by glow discharge and undoubtedly would fit within the poorly hydrophilic adhesive zone) show no scar inducing capsuale formation and substantial tissue adhesion (fibroblasts) over the entire implant with significantly enhanced cellular organization around the wound site. It may be possible that the use of fibroblast adhesive substrates established in cell culture could form the basis for advanced wound dressing and implant materials. These could be based on either adhesive synthetic materials or intrinsically non adhesive hydrogels with adhesion active species incorporated within the polymer network (ref 274-279).

Despite the fact that these studies have been performed using largely uncharged materials under normal culture

conditions, whereas surfaces in vivo are likely to be charged, complex, dynamic and the environment more complicated, it seems that a systematic investigation into the role of synthetic culture substrata in determining cell adhesion is a potentially powerful approach. This may allow a further understanding of interface conversion and biocompatibility of synthetic devices in vivo. This initial correlation between EWC ,fractional polarity and cell adhesion behaviour in culture represents an important first step.

7.4 suggestions for further work

1. Study the nature of the transition from the nonadhesive window to cell adhesive hydrophilic materials. As this appears to be a different type of transition to that into the hydrophobic adhesive zone.

2. Study the effect of substrate charge on cell adhesion phenomena. To date no published work has adequately separated the effects of surface charge from those of increasing hydrophilicity etc. Further, nearly all charged substrates studied have physicochemical properties coincident with cell adhesive zones. The study of cell adhesion on charged polymers which are within the non adhesive window in terms of surface energetic properties could prove useful. Several polymers systems could be used including:- poly(HEMA/EMA/methacrylic acid) where the methacrylic acid (MAA) content is between 5% and 50%. In conjunction with this a similar series of poly(HEMA/STY/MAA) copolymers could be used. It may also be possible to utilize poly(MMA/SPM) or poly(MMA/SPA) copolymers for this perpose.

3. To separate the affects of surface oxidation and rugosity upon cell adhesion by producing a range of substrates with defined surface chemistry but expressing various degrees of surface rugosity.

4. Investigate the effect of polymer crystallinity upon cell adhesion, utilizing substrates of amorphous polypropylene and polyethylene, as well as other polymers which express varying degrees of axial orientation.

5. To investigate protein and lipid adsorption on substrata of differing cell adhesiveness, in an attempt to unravel the complex biochemical mechanisms involved in cell adhesion in vitro and interface conversion in vivo.

APPENDIX 1

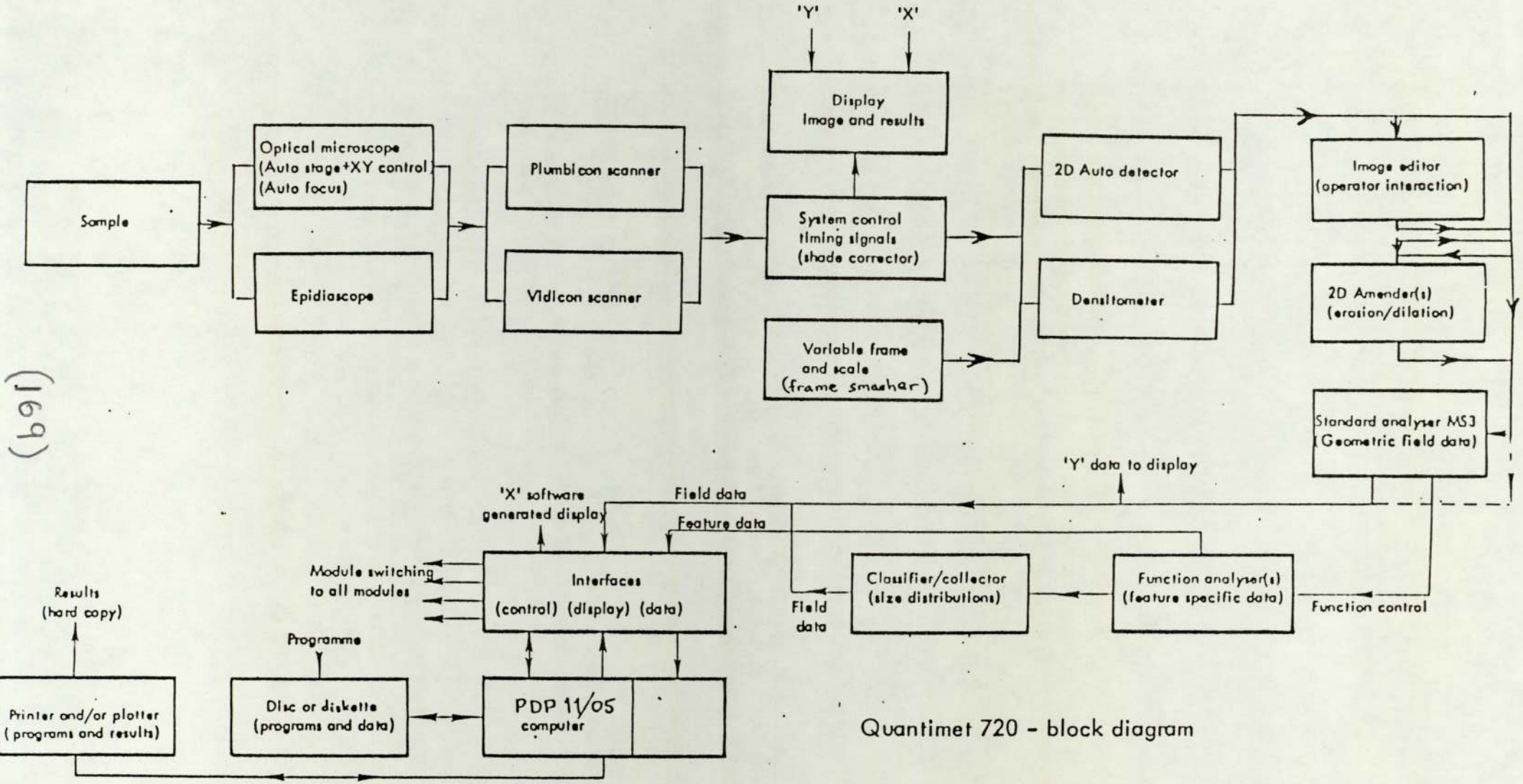
Relationship of polymer solution concentration and spin speed to measured coating thickness.

Polymer concentration (% W/V)	Spin speed (rpm)	Coating thickness (um)
40	1150	5.0
40	3500	4.0
30	1000	3.0
20	3500	1.2
20	2150	1.4
20	1750	1.5
20	1400	1.65
20	1150	2.0
15	3500	0.7
15	2150	0.8
15	1150	1.2
10	3500	0.3
10	1150	0.5
5	1150	0.18
5	3500	0.15
3.5	3500	0.04
3.5	2300	0.05
3.5	2000	0.7
3.5	1750	0.085
3.5	1150	0.14
2	1150	0.025
1	3500	0.01

APPENDIX 2

1) Flow diagram of quantimet 720, Page 169

2) Representation of ferets diameter, Page 170



Quantimet 720 - block diagram

(0±1)

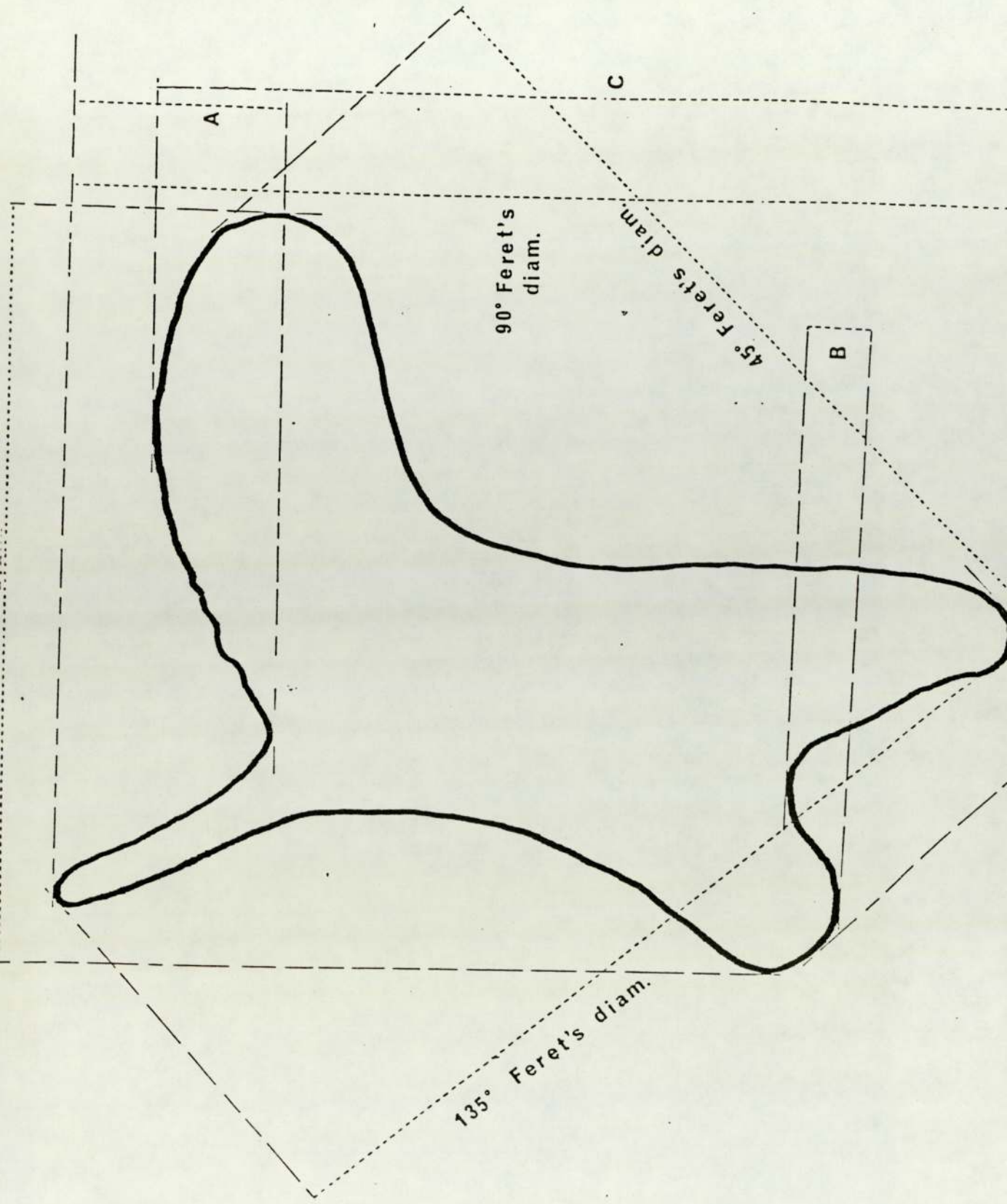
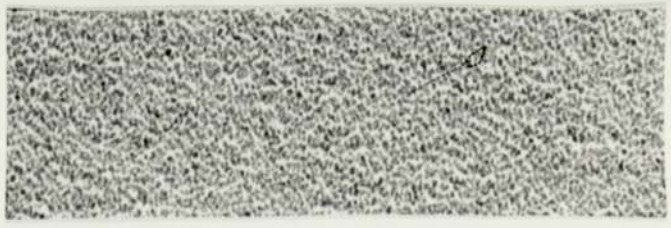


Fig. 2.11

APPENDIX 3

T.E.M. investigation of non-swollen

poly(HEMA) evaporation coatings

Poly(HEMA) concentration (F + M units)	Electron micograph (x 200,000)
1 x 10*-3	
2 x 10*-3	
5 x 10*-3	
1 x 10*-2	
2 x 10*-2	

APPENDIX 4

Cell Counting Data

Cell counts were obtained by manually counting cell populations using light microscopy. Magnification was standardized at 400 x and typically 200-400 cells could be counted in each field. Typically each result here is obtained by the counting of four to six fields. Results are expressed as percentage of the cell population in a spread morphology which was determined from counting of both rounded and spread cells. Morphology was checked by comparision with stock cultures.

Substrate material: Bacteriological plastic

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	17	12	14	16	18	10	12	-	14
16C	7	10	8	9	11	10	8	-	9
BHK	14	16	6	3	16	16	13	-	12
REF	3	8	10	16	1	3	6	-	7
MRC5	16	20	19	14	17	16	19	-	17
HEP2	2	3	1	6	7	5	4	-	4

Substrate material: Poly(10 HEMA/90 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	80	73	69	65	72	70	67	72	71
16C	76	82	83	89	78	79	92	79	82
BHK	76	70	71	73	83	76	68	75	74
REF	90	78	85	87	80	79	89	85	84
MRC5	92	94	91	89	95	92	92	91	92
HEP2	72	71	65	67	66	69	72	71	69

Substrate material: Poly(20 HEMA/80 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	No Results Obtained								
16C	96	95	93	87	91	92	90	-	82
BHK	78	92	94	86	83	82	85	-	86
REF	69	78	77	82	66	74	72	-	74
MRC5	82	87	79	84	82	84	84	-	84
HEP2	76	68	66	72	74	70	71	-	71

Substrate material: Poly(30 HEMA/70 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	96	90	91	93	89	92	93	92	92
16C	89	82	91	90	86	81	82	87	86
BHK	86	89	81	78	76	83	82	81	82
REF	91	85	87	89	96	93	94	93	91
MRC5	85	93	87	91	89	89	87	91	89
HEP2	86	77	75	80	81	78	77	79	79

Substrate material: Poly(40 HEMA/60 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	82	84	87	88	87	86	84	90	86
16C	84	82	83	86	85	80	81	-	83
BHK	76	82	81	80	76	80	81	76	79
REF	84	82	88	89	90	84	85	84	86
MRC5	91	93	84	94	96	95	90	93	92
HEP2	76	74	94	78	86	84	80	84	82

Substrate material: Poly(50 HEMA/50 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	80	71	64	68	67	67	68	67	69
16C	71	72	70	74	70	70	69	72	71
BHK	76	75	73	73	72	70	76	77	74
REF	83	80	87	86	82	81	89	74	83
MRC5	94	91	87	92	86	91	88	90	90
HEP2	71	74	58	59	67	60	57	58	63

Substrate material: Poly(60 HEMA/40 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	No Results Obtained								
16C	84	78	79	76	82	79	80	82	80
BHK	91	95	97	83	86	95	92	90	91
REF	80	67	68	66	64	69	71	67	67
MRC5	96	98	97	95	94	95	96	-	96
HEP2	82	86	84	81	84	81	83	-	83

Substrate material: Poly(70 HEMA/30 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	76	75	81	82	77	76	79	78	78
16C	86	87	80	75	80	81	79	80	81
BHK	84	82	81	80	86	84	87	88	84
REF	73	72	75	75	75	74	75	73	74
MRC5	95	90	92	91	89	88	92	91	91
HEP2	89	87	88	95	97	90	93	89	91

Substrate material: Poly(80 HEMA/20 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	91	96	93	90	89	95	90	92	92
16C	No Results Obtained								
BHK	91	80	79	81	81	82	80	82	82
REF	79	69	83	81	82	79	83	76	79
MRC5	85	96	93	91	98	90	90	-	92
HEP2	68	67	66	66	64	69	83	-	69

Substrate material: Poly(90 HEMA/10 STY

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	89	88	81	78	87	90	89	-	86
16C	98	92	94	93	96	96	96	-	95
BHK	78	73	73	70	69	75	73	-	73
REF	96	98	93	93	95	96	94	-	95
MRC5	91	89	97	97	90	92	94	92	93
HEP2	75	68	62	69	67	70	67	67	68

Substrate material: Poly(EMA)

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	79	80	74	82	70	75	78	78	77
16C	73	79	72	74	77	76	78	74	75
BHK	74	77	68	64	63	78	71	69	70
REF	72	73	70	78	76	66	67	76	78
MRC5	76	72	84	69	71	91	80	79	78
HEP2	74	79	69	80	59	62	73	74	70

Substrate material: Poly(10 HEMA/90 EMA

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	84	80	79	86	90	79	71	79	81
16C	76	75	79	80	81	83	75	75	78
BHK	72	78	69	67	72	71	74	73	72
REF	74	71	79	80	75	73	76	80	76
MRC5	80	81	87	79	80	84	79	86	82
HEP2	82	74	63	71	67	69	68	65	70

Substrate material: Poly(20 HEMA/ 80 EMA)

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	90	96	94	87	92	84	91	93	91
16C	78	79	76	82	74	79	83	73	78
BHK	63	66	67	42	56	54	42	43	54
REF	86	89	84	91	85	86	91	92	88
MRC5	76	70	74	75	70	79	76	74	74
HEP2	87	94	92	86	80	96	92	93	90

Substrate material: Poly(30 HEMA/70 EMA

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	96	92	97	84	96	96	93	90	93
16C	91	94	82	83	84	89	80	85	86
BHK	63	55	64	54	56	65	56	59	59
REF	87	84	83	79	80	89	72	88	83
MRC5	79	76	71	82	81	69	83	83	78
HEP2	89	93	92	84	86	91	81	80	87

Substrate material: Poly(40 HEMA/ 60 EMA)

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	86	88	79	85	89	91	89	87	87
16C	76	75	77	79	70	69	72	74	74
BHK	70	72	79	69	77	73	75	77	74
REF	82	84	87	80	79	78	81	83	82
MRC5	76	79	74	72	79	72	84	80	77
HEP2	84	87	82	86	89	77	82	93	85

Substrate material: Poly(50 HEMA/50 EMA

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	84	82	79	76	87	84	80	76	81
16C	72	74	69	68	74	79	63	-	71
BHK	96	90	90	93	91	95	89	92	92
REF	84	79	75	74	76	82	74	71	77
MRC5	79	76	71	75	82	74	78	90	78
HEP2	74	82	76	81	89	71	70	-	79

Substrate material: Poly(60 HEMA/40 EMA)

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	89	94	88	96	81	94	93	85	90
16C	79	75	70	76	81	70	79	77	76
BHK	85	84	92	92	95	96	95	97	92
REF	79	66	67	65	66	64	72	65	68
MRC5	94	89	90	96	94	86	85	88	89
HEP2	71	61	60	59	62	66	62	63	63

Substrate material: Poly(70 HEMA/30 EMA

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	97	92	94	93	98	98	96	100	96
16C	82	71	73	77	79	74	79	65	75
BHK	91	82	81	83	80	79	87	89	84
REF	76	74	78	81	83	74	90	76	79
MRC5	89	82	87	83	94	84	81	80	85
HEP2	No Results Obtained								

Substrate material: Poly(80 HEMA/20 EMA)

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	96	98	100	95	99	93	95	100	97
16C	74	76	80	81	87	69	77	80	78
BHK	74	92	91	96	90	89	91	89	89
REF	79	84	87	84	76	78	85	82	82
MRC5	81	91	92	80	79	79	91	95	86
HEP2	No Results Obtained								

Substrate material: Poly(90 HEMA/10 EMA

Cell line	Experiment Number 1	2	3	4	5	6	7	8	Mean value
CEF1	96	97	100	95	94	90	96	92	95
16C	94	97	98	99	92	91	93	96	95
BHK	84	93	92	94	96	91	90	88	91
REF	81	89	86	91	81	84	83	86	85
MRC5	95	84	97	92	87	86	88	91	90
HEP2	74	60	61	68	72	71	69	69	68

BHK cell spreading on hydrophobic polymers

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Poly(20 EMA/80 GMA)		
1	63	54
2	50	41
3	46	52
Mean Value	53	48
Poly(30 EMA/70 GMA)		
1	104	94
2	95	80
3	98	78
Mean Value	99	84
Poly(40 EMA/60 GMA)		
1	130	91
2	140	62
3	93	75
Mean Value	121	78
Poly(50 EMA/50 GMA)		
1	124	89
2	115	86
3	106	65
Mean Value	115	80
Poly(60 EMA/40 GMA)		
1	130	81
2	150	70
3	152	74
Mean Value	144	75

BHK cell spreading on hydrophobic polymers continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Poly(70 EMA/30 GMA)		
1	51	64
2	40	50
3	44	60
Mean Value	45	58
Poly(80 EMA/20 GMA)		
1	94	90
2	98	80
3	84	79
Mean Value	92	83
Poly(GMA)		
1	94	91
2	85	71
3	94	72
Mean Value	91	78
Melinex (T.E.M. grade)		
1	133	100
2	125	97
3	123	97
Mean Value	127	98
Melinex S grade		
1	120	60
2	113	40
3	117	53
Mean Value	116	51

BHK cell spreading on hydrophobic polymers continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Melinex 377 grade		
1	45	89
2	40	76
3	44	84
Mean Value	43	83
Melinex O grade		
1	100	100
2	90	96
3	89	95
Mean Value	93	97
Melinex 226 grade		
1	73	58
2	65	64
3	69	56
Mean Value	69	59
Poly(butylterathalate)		
1	74	87
2	86	87
3	74	93
Mean Value	78	89
Poly(EMA)		
1	85	79
2	83	81
3	84	83
Mean Value	84	81

BHK cell spreading on hydrophobic polymers continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Poly(MMA)		
1	142	98
2	132	100
3	134	90
Mean Value	136	96
Cellulose Triacetate		
1	72	65
2	74	72
3	82	67
Mean Value	76	68
Cellulose Diacetate		
1	89	56
2	92	59
3	71	62
Mean Value	84	59
T.P.X.		
1	75	88
2	94	89
3	89	96
Mean Value	86	91
Polypropylene		
1	79	86
2	71	83
3	84	80
Mean Value	78	83

BHK cell spreading on hydrophobic polymers continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Polyethylene		
1	79	72
2	69	66
3	68	66
Mean Value	72	68
P.T.F.E.		
1	71	43
2	84	37
3	79	37
Mean Value	78	39

BHK cell spreading on hydroxy polymers

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
Poly(HBMA)		
1	88	89
2	94	96
3	91	97
Mean Value	91	94
Poly(HPMA)		
1	96	94
2	84	90
3	84	92
Mean Value	88	92

BHK cell spreading on polydiacetone acrylamide

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
1	77	58
2	71	74
3	74	75
Mean Value	74	69

BHK cell spreading on plasma treated polystyrene

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
a) Oxygen plasma treated surfaces		
Treatment Time 20 seconds		
1	99	78
2	94	91
3	95	92
Mean Value	96	90
Treatment Time 30 seconds		
1	96	99
2	104	96
3	106	93
Mean Value	102	96
Treatment Time 1 minute		
1	107	85
2	107	82
3	98	94
Mean Value	104	87
Treatment Time 2 minutes		
1	94	92
2	92	88
3	99	96
Mean Value	95	92
Treatment Time 10 minutes		
1	101	90
2	98	92
3	101	97
Mean Value	100	93

BHK cell spreading on plasma treated polystyrene continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
a) Nitrogen plasma treated surfaces		
Treatment Time 20 seconds		
1	85	92
2	82	92
3	94	98
Mean Value	87	94
Treatment Time 30 seconds		
1	98	72
2	104	91
3	107	89
Mean Value	103	84
Treatment Time 1 minute		
1	95	86
2	85	83
3	87	94
Mean Value	89	91
Treatment Time 2 minutes		
1	94	91
2	95	98
3	81	90
Mean Value	90	93
Treatment Time 10 minutes		
1	102	93
2	100	80
3	110	97
Mean Value	104	90

BHK cell spreading on plasma treated polystyrene continued

Experiment No:	No: of cells adhered as % of control	% of cell population in spread morphology
a) Argon plasma treated surfaces		
Treatment Time 20 seconds		
1	92	80
2	82	99
3	96	94
Mean Value	90	91
Treatment Time 30 seconds		
1	86	86
2	94	85
3	96	99
Mean Value	92	90
Treatment Time 1 minute		
1	90	91
2	106	76
3	107	79
Mean Value	101	82
Treatment Time 2 minutes		
1	103	85
2	100	95
3	106	99
Mean Value	103	93
Treatment Time 10 minutes		
1	91	93
2	109	96
3	106	84
Mean Value	102	91

Cell spreading on polyacrylamide

Cell line	Experiment Number 1	2	3	4	5	Mean value
16C	96	88	87	94	85	90
BHK	97	89	90	90	89	91
REF	97	91	91	89	92	92
MRC5	94	88	87	89	82	90
HEP2	90	91	94	90	88	91

Cell spreading on poly(SPA)

Cell line	Experiment Number 1	2	3	4	5	Mean value
16C	36	29	29	28	28	30
BHK	62	48	47	46	49	50
REF	62	60	54	55	54	57
MRC5	76	75	80	83	81	79
HEP2	28	27	33	34	33	31

Cell spreading on (20 HEMA / 80 SPM)

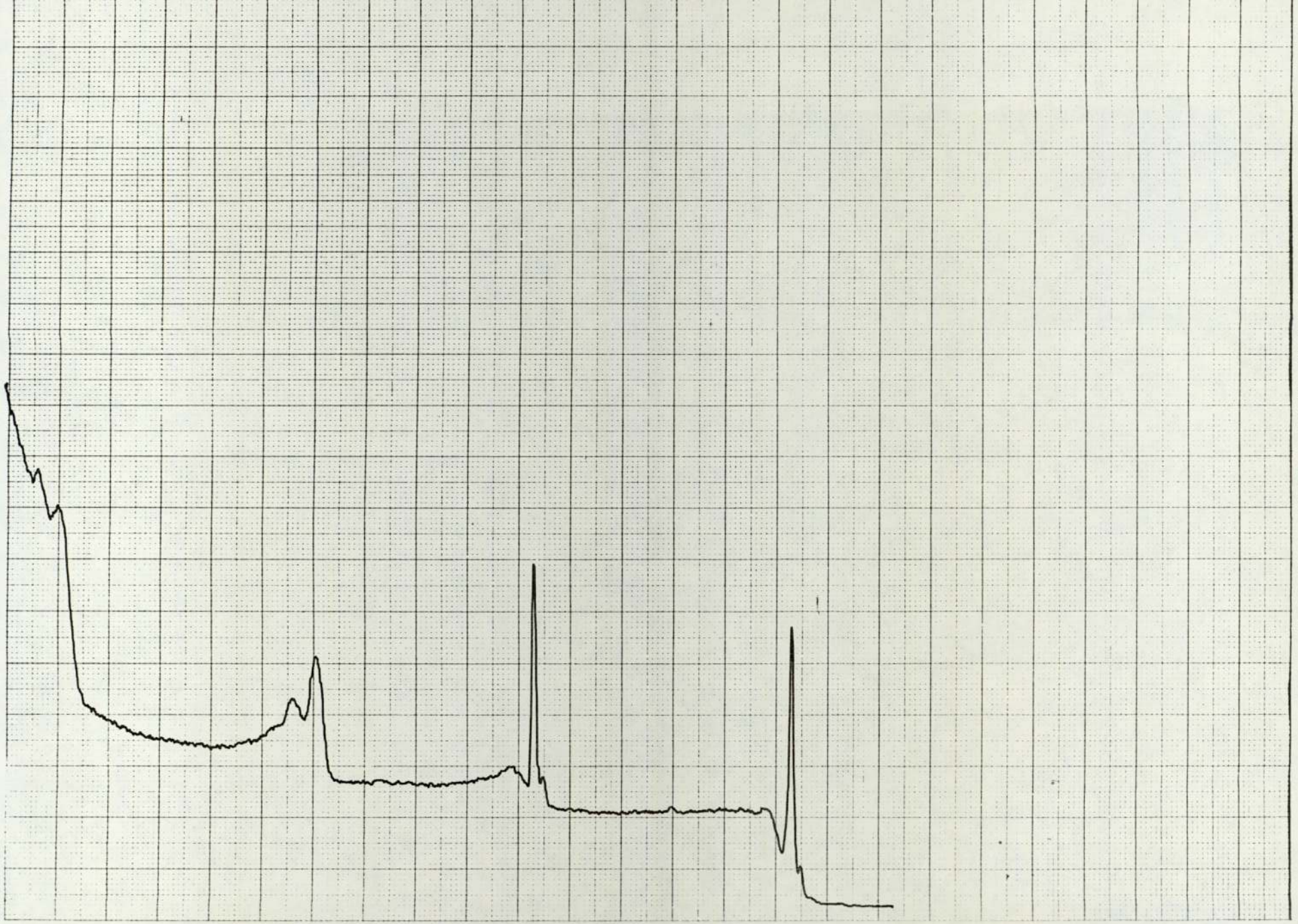
Cell line	Experiment Number 1	2	3	4	5	Mean value
16C	85	84	90	85	86	86
BHK	18	24	19	19	20	20
REF	36	39	48	29	48	40
MRC5	84	89	96	99	87	91
HEP2	04	06	41	30	34	23

Cell spreading on poly(SPM)

Cell line	Experiment Number 1	2	3	4	5	Mean value
16C	94	96	97	97	96	96
BHK	87	80	78	78	77	80
REF	87	89	76	74	79	81
MRC5	69	82	83	85	81	80
HEP2	41	40	39	34	46	40

APPENDIX 5

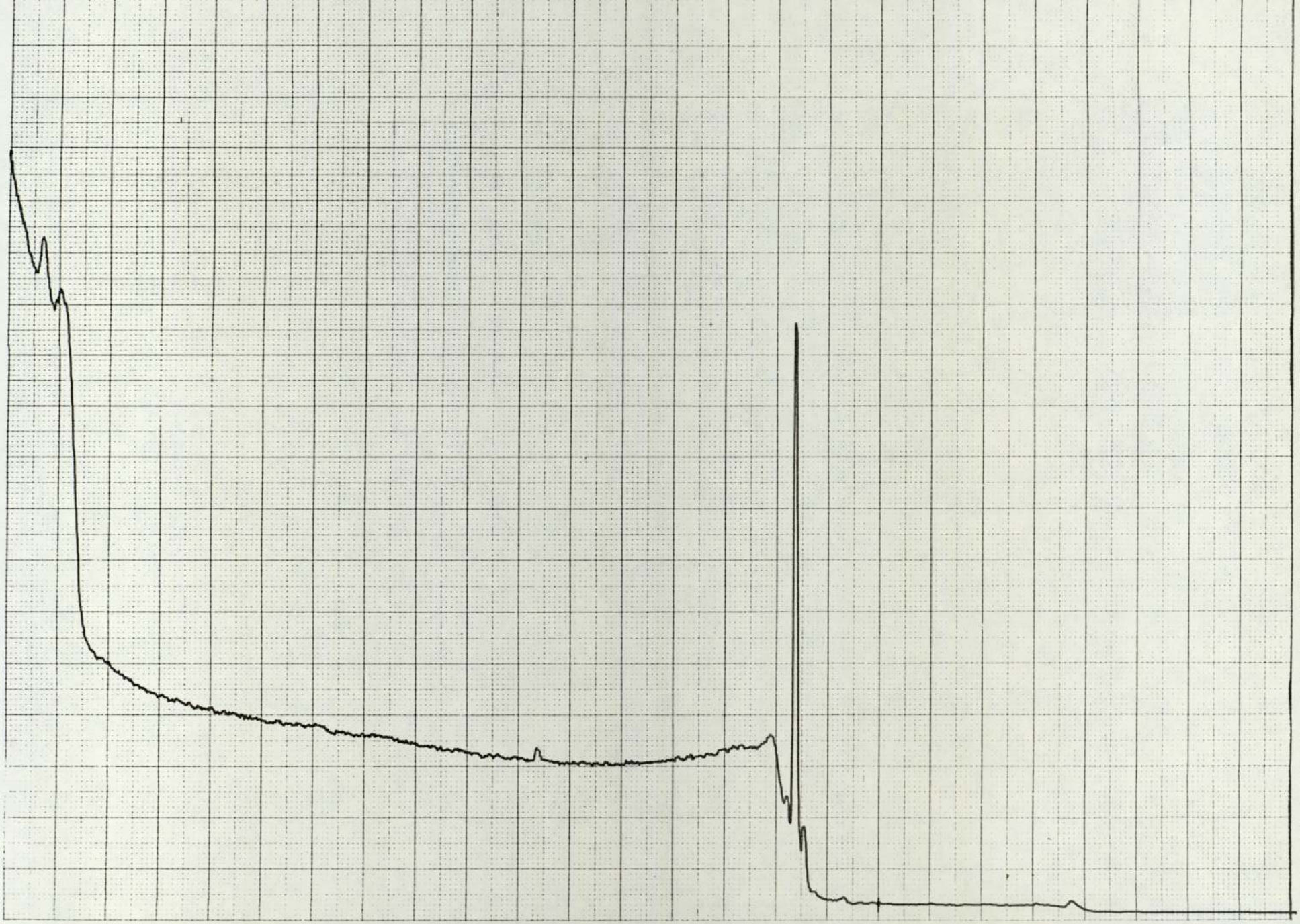
X.P.S. Data from analysis of plasma treated and native polystyrene surfaces.



ASTON AUGER/X.P
ANALYSIS

DATE
SPECTRUM N°
SAMPLE N°
PREPARATION
ETCH ⊘
ETCH VOLTS
TECHNIQUE
PRESSURE
SOURCE
VOLTAGE
SPOT SIZE
CURRENT
MODE
RESOLUTION
MAG.
SCAN
KE/BE
START 200
SCAN WIDTH 1250
SCAN TIME
SENSITIVITY 10^5
T.C.
% BACKOFF
MULT. VOLTS
PREAMP MODE
MOD.
LOCK-IN
MANIPULATOR
X
Y
Z
θ
COMMENTS

O_2



ASTON AUGER/X.P.S.
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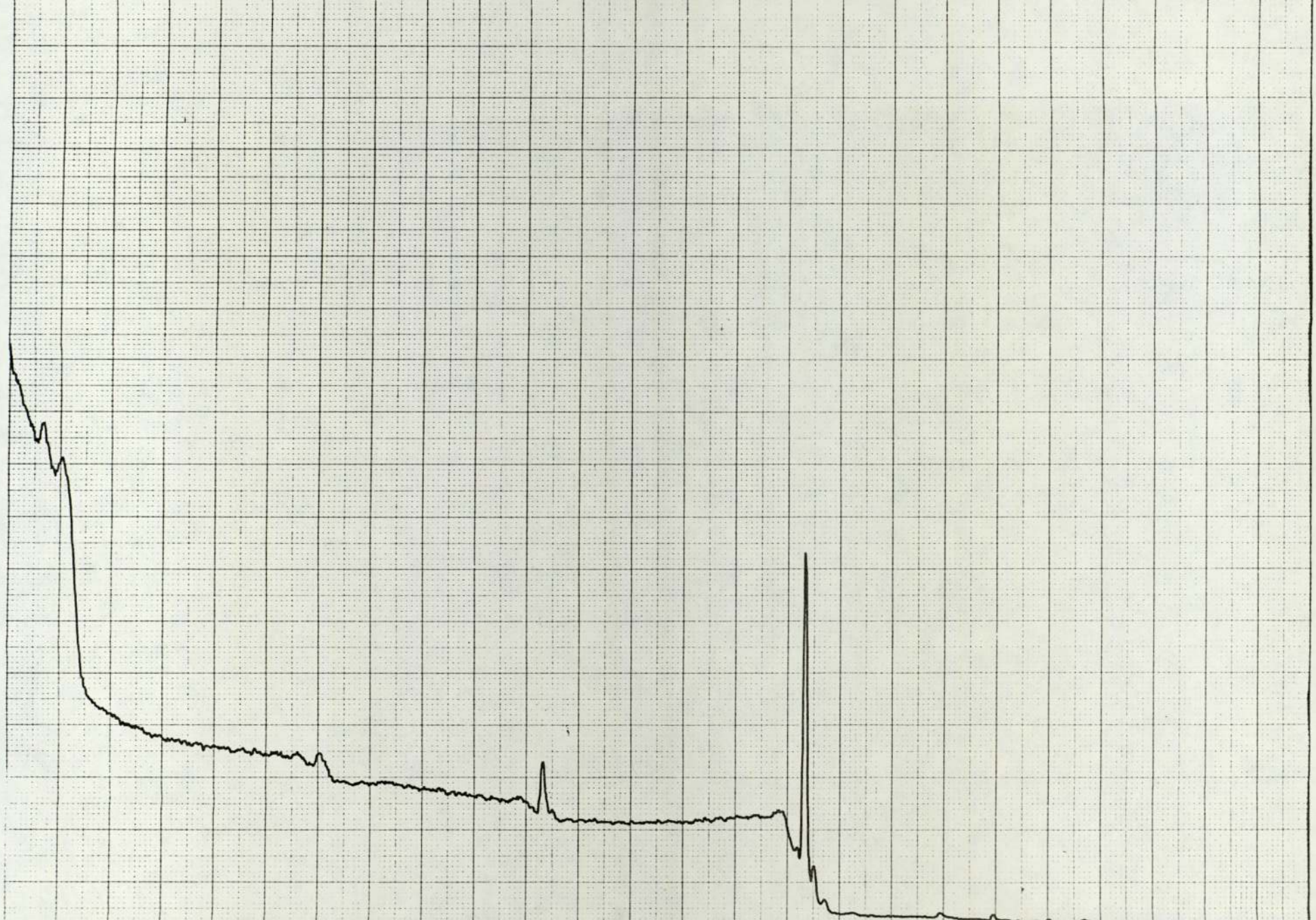

ASTON AUGER/X.P
ANALYSIS

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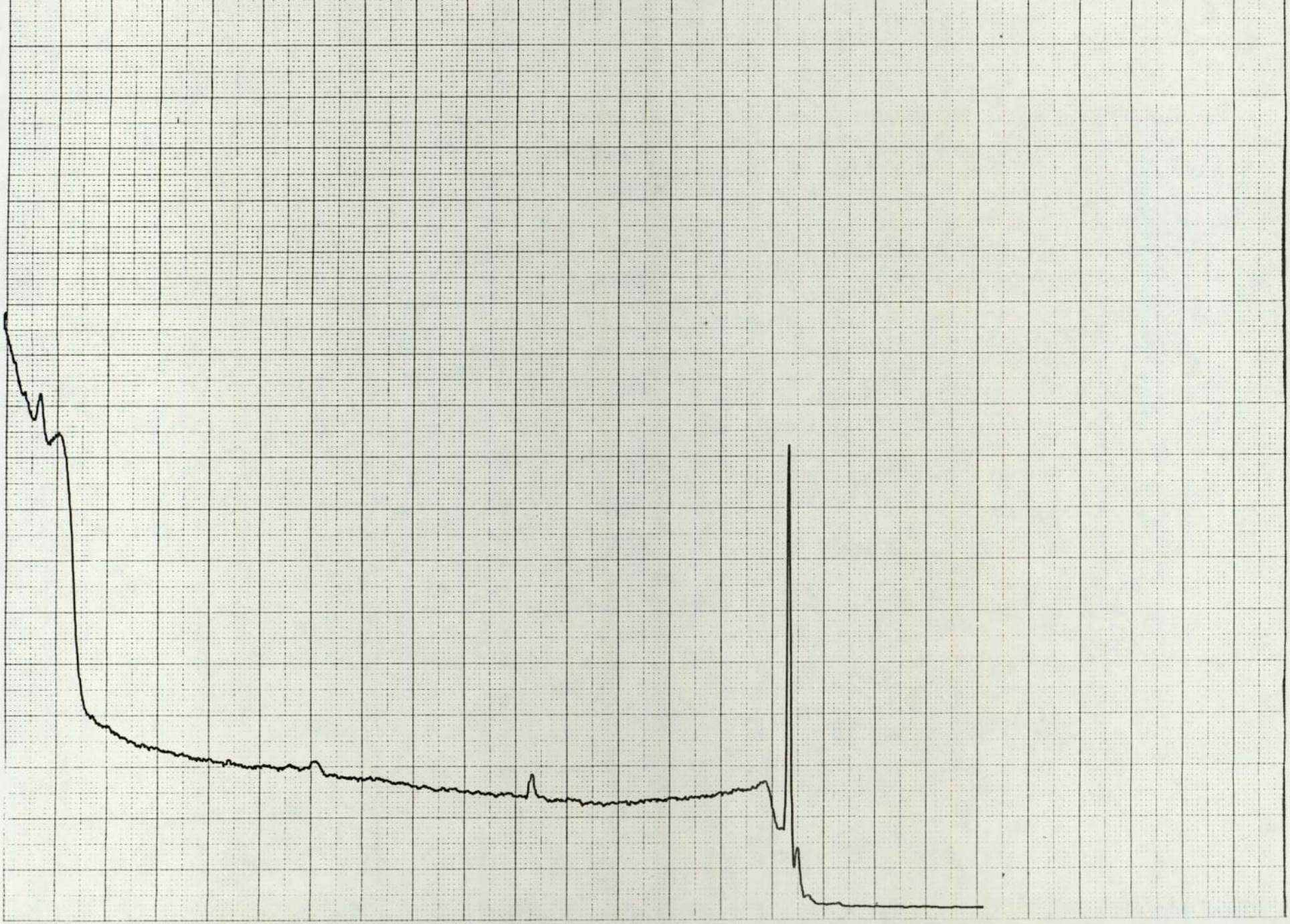

ASTON AUGER/X.P.S.
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