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**INVESTIGATION AND DEVELOPMENT OF TECHNIQUES FOR THE
CHARACTERISATION OF THE SYNTHETIC/BIOLOGICAL INTERFACE**

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Doctor of Philosophy

University of Aston in Birmingham

October 1991

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INVESTIGATION AND DEVELOPMENT OF TECHNIQUES FOR THE CHARACTERISATION OF THE SYNTHETIC/BIOLOGICAL INTERFACE

THESIS SUMMARY

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The purpose of this study is to increase our knowledge of the nature of the surface properties of polymeric materials and improve our understanding of how these factors influence the deposition of proteins to form a reactive biological/synthetic interface. A number of surface analytical techniques were identified as being of potential benefit to this investigation and included in a multidisciplinary research program.

Cell adhesion in culture was the primary biological sensor of surface properties, and it showed that the cell response to different materials can be modified by adhesion promoting protein layers; cell adhesion is a protein-mediated event. A range of surface rugosity can be produced on polystyrene, and the results presented here show that surface rugosity does not play a major role in determining a material's cell adhesiveness. Contact angle measurements showed that surface energy (specifically the polar fraction) is important in promoting cell spreading on surfaces. The immunogold labelling technique indicated that there were small, but noticeable differences, between the distribution of proteins on a range of surfaces. This study has shown that surface analysis techniques have different sensitivities in terms of detection limits and depth probed, and these are important in determining the usefulness of the information obtained. The techniques provide information on differing aspects of the biological/synthetic interface, and the consequence of this is that a range of techniques is needed in any full study of such a complex field as the biomaterials area.

Keywords: cell adhesion, biocompatibility, surface properties, surface analysis, polystyrene

**To Jane
and
the boys**

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MISSION STATEMENT

When a synthetic material is placed in a biological environment the surface will be modified by the adsorption of biological molecules in the process known as "interface conversion". The purpose of this study is to increase our knowledge of the nature of the surface properties of polymeric materials and improve our understanding of how these factors influence the deposition of proteins, singly and in complex biological fluids, to form a reactive biological/synthetic interface, using surface analytical techniques. Of particular importance are techniques that will contribute to the understanding of the surface physicochemical properties of polymers and the nature of the biomolecular layer, including the organisation of proteins deposited on polymer surfaces. A number of surface analytical techniques have been identified as being of potential benefit to this investigation of the biological/synthetic interface.

The range of techniques available for surface analysis will be reviewed and their strengths and weaknesses highlighted. Many of them are widely used in the materials science field; those techniques which have been used to study the biological/synthetic interface and have already provided useful information will not be investigated further. Others which have had limited application in this field will be assessed and a program of work embarked upon to investigate their usefulness by presenting the chosen analytical techniques with samples of polymer surfaces with and without deposited biological molecular layers. Our model system is based on the materials used in tissue culture which is widely used by the research community as the routine biological test for the interface conversion process.

CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

INTRODUCTION AND LITERATURE SURVEY

1.1 Interface Conversion

This is a collaborative study to investigate the process of interface conversion and gain an understanding of the mechanisms involved. In interface conversion (Baier, 1982) a surface, when placed in a biological environment, is modified (conditioned) by the adsorption of biological molecules (Andrade, 1985; Baier, 1982; 1984; Baier and Weiss, 1975; Baier et al, 1971; Dutton et al, 1969; Feijen et al, 1979; Horbett, 1982). The protein adsorption begins within milliseconds (Cottonaro et al, 1981; Dutton et al, 1969; Zucker and Vroman, 1969). This process then determines the fate of many biotechnological processes and prosthetic devices, from wound dressings (Lydon, 1986) to contact lens surfaces (Baker and Tighe, 1981; Lydon, 1986; Royce Jr et al, 1982), dental prostheses (Baier, 1984), replacement joints (Baier, 1982) and blood vessels (Baier, 1977; Feijen et al, 1979), and bacterial infection of implants (Barth et al, 1989; Vaudaux et al, 1984) or teeth (Bjorvatn, 1986). This study requires the investigation and characterisation of the surface properties of polymers which are thought to be important in interface conversion (Hogt et al, 1985; Lydon et al, 1985; Ratner, 1982) and the biochemical events which take place at a biological/synthetic interface.

Interface conversion may have two roles and these will depend on the application desired. With contact lenses and blood compatible systems, eg. dialysis membranes and extracorporeal piping, the initial adsorption of blood proteins onto the foreign surface determines the biocompatibility of that material (Baier, 1977; Baier, 1982; Ruckenstein and Gourinanskov, 1984; Vroman et al, 1971). The surfaces need to be either incapable of adsorbing biological molecules and cells (Hoffman, 1974; Royce Jr et al, 1982), attractive to only those molecules which will not promote cell adhesion (Baker and Tighe, 1981), or to allow the retention of the natural protein conformation (Baker and Tighe, 1981; Castillo et al, 1984b; 1985; 1986; Ruckenstein and Gourinanskov, 1984). This is especially important with fibrinogen, the major protein involved in the blood clotting system, which is present at all blood contacting surfaces but induces the blood clot cascade system only when its tertiary structure is

altered from its natural globular conformation. When fibrinogen comes into contact with a biologically incompatible surface the molecule is unwound from its globular structure into a form which can activate platelets (Young et al, 1983: Zucker and Vroman, 1969).

Marine fouling organisms have an economic importance in the maintenance of ships and other engineering materials when water is used as a cooling medium (Baier, 1982: 1984: Daniel et al, 1987). Diatoms form at least part of a community of fouling organisms, which also includes bacteria and algae (Daniel et al, 1987). Minimal bioadhesion is critical to preventing bacterial fouling of heat exchangers and food processing equipment (Baier, 1982: 1984).

Alternatively, the synthetic surface needs to be able to sustain cell contact and allow normal growth and division of cells (Lydon et al, 1985). This is required, for example, in the field of bioreactors where the cells are immobilised onto a support surface and catalyse the reaction using substrates in solution. The product, such as monoclonal antibody or drug compound, is released back into the solution (Facchini et al, 1989). Biomedical implants usually have to be long lasting and therefore become integrated into the body without producing a foreign body reaction (Horbett, 1982). Modern wound dressings materials are designed to optimise physiological function (Lydon, 1990). The cell reaction to materials aimed at these fields can be tested using the culture of anchorage-dependent cell lines as a model (Lydon et al, 1985: Minett et al, 1984: Thomas et al, 1986). Cell and tissue culture is now an important area for the use of materials which will support the attachment and growth of cells in its own right, as a research tool, as a biotechnological process (many biological molecules are produced during batch processes) and as a testing system to replace animal models for assessing the safety and efficacy of pharmaceutical products.

This dual nature to interface conversion is well illustrated with the case of plastic arterial or heart valve inserts which are in place permanently or semi-permanently (Curtis, 1977: Guidoin et al, 1989). On the one hand the prosthesis must be accepted or tolerated by the tissue into which it is implanted, with cells interacting with the implant material and continuing to perform normally. At the same time

the prosthesis must allow the passage of blood without precipitating the adhesion of platelets which is the initial step leading to thrombosis.

A further requirement for the biocompatibility of material is in the biomedical and biosensors area. Here the requirement is to attach specific proteins to surfaces in such a form that they remain active even after dehydration, eg. diagnostic testing kits and immobilised enzyme reactors (Veronese et al, 1989). In the case of these devices the aim is to have a surface which is non-protein adsorptive but to which the protein of interest can be attached, usually covalently. These various requirements for the interaction between biological molecules and devices and materials effectively results in a dual definition of biocompatibility.

Previous work from our laboratories (Baker and Tighe, 1981: Lydon, 1986: Lydon and Clay, 1985: Lydon and Foulger, 1986: Lydon et al, 1986) and in the literature (Andrade, 1985: Baier, 1970: Baier, 1977: Baier, 1982: Baier and Weiss, 1975: Baier et al, 1971: Horbett, 1982: Revel and Wolken, 1973: Taylor, 1970: Vroman et al, 1971: Witkowski and Brighton, 1972: Young et al, 1982) has established the fundamental role of deposited biological molecules on synthetic polymer surfaces in the development of a biologically reactive interface. In order to better understand the process of interface conversion on a synthetic surface it is necessary to be able to analyze the nature, composition and state of the synthetic/biological interface including the surface properties of polymers and the role of deposited proteins and lipids. Our overall aim is to study the key molecular interactions which govern the establishment of a stable biological/synthetic interface. As part of this approach we need to identify and develop methods for *in situ* analysis of adsorbed layers, since the interactions within these layers determine the nature of the interface.

1.2 Adhesion - Physicochemical Basis

Adhesion is the ability of two substances to stick or bond together, and this occurs in numerous situations. Usually the two materials are intended to bond together as is the case when an adhesive is used to glue two materials together. Conversely in tribology, two materials which are in close proximity are separated by a third material which is acting as a lubricant, and the aim in these circumstances is for the materials not to bond together but to move over each other. In the biological field, adhesion of cells to each other and to external supporting matrices is central to the organisation of organs and tissues. However, cell adhesion is a complex event as cells, besides sticking together need also to be able to reorganise in response to specific stimuli. In these circumstances cells must be able to free themselves from the adhesive situation and move to new locations, as occurs in organogenesis (Trinkaus, 1976). This is also the case in wound healing where cells need to reorganise in order to repair the damaged organ or tissue. Cell adhesion tends to be a relatively weak form of adhesion, compared to material adhesion, and is a transient process with adhesions being released and formed continually in a dynamic system (Curtis, 1977).

Cell adhesion differs from most other adhesive events in that it is the result of specific interactions. Cells interact with the external environment, whether other cells, extracellular matrices or prosthetic devices, through intermediary molecules. Therefore, for a cell to react with non-biological materials the prospective material must have a coating of biomolecules, some of which are adhesive for cells. The acquisition of this coating happens rapidly when the synthetic material is brought into contact with blood or plasma, and determines the nature of the reaction of the cells to the material. Cell adhesion is, therefore, a two-stage process, the observable cell adhesion event being the second stage, which is the result of specific molecular interactions with the biomolecule deposition which occurs in the first stage. The initial deposition of biomolecules is determined by the physicochemical relationship of these molecules and their underlying substratum.

The physicochemical details of the phenomenon of adhesion are not fully understood and are difficult

to measure. Thus, in the adhesives technology field adhesion is defined in an empirical way as the mechanical force required to separate two materials (Brewis and Briggs, 1985). In the equally important converse situation of non-adhesion (abhesion), eg. mould release agents, lubricants, plasticisers, adhesion is normally avoided but when contact occurs it is measured as friction. In both instances, however, the close proximity of two materials brings into play intermolecular forces which act across the interface. The strength and nature of these forces determining whether adhesion occurs.

Several theories of adhesion have been propounded. It is relevant to the process of protein deposition to review them here as the forces involved are likely to be similar and the processes would be expected to have common requirements. To the physical chemist, adhesion represents the intermolecular forces acting across an interface. There are four theories of adhesion at present:

1.2.1 Mechanical Theory

According to this theory, the adhesive interlocks around the surface irregularities or pores in the surface of the substrate (Packham, 1983; Sykes, 1983; Wake, 1982). An alternative explanation may be that a rugose surface provides an increased surface area for interaction between the two materials involved (Brewis, 1982; Sykes, 1982; Wake, 1982). On the other hand, stress concentrations due to voids are more likely with a rough surface. So, if roughness is increased by a surface treatment, the reasons for the change in adhesion may be complex.

1.2.2 Adsorption Theory

This theory proposes that adhesive molecules are adsorbed onto the surface of the substrate and are held there by the forces of attraction such as London dispersion forces, van der Waals' forces or hydrogen bonds (Schornhorn, 1978; Wake, 1982). These forces have interfacial strengths far exceeding the real strengths of one or other of the adhering materials (Schornhorn, 1978). Chemical reaction can

sometimes occur at interfaces making desorption unlikely and then separation involves the mechanical breaking of these chemical bonds (Wake, 1982). A consequence of the adsorption theory is that the two materials must be in close contact as van der Waal's forces operate over very small distances. This is thought to explain the need for good wettability of adhesive materials and the use of cleaning pretreatments (Clark et al, 1978) to enable a large number of individual van der Waal's forces to interact. Covalent bonds are involved in some instances which will lead to much better joint strength durability (Brewis, 1982: Kinloch, 1979).

1.2.3 Diffusion Theory

The adhesive molecules diffuse into the substrate which has the effect of eliminating the interface by forming an interwoven network (Voyutskii et al, 1973). However, diffusion is thought to be the result of adhesion and not the cause (Schornhorn, 1978). It certainly is difficult to explain how adhesion to hard materials such as metals or glass can result from diffusion of polymer into such materials and so can apply only to polymeric materials. The evidence supporting this theory suggests that diffusion occurs only when two identical (or similar) polymers are brought together at temperatures above their glass transition temperature, ie. autoadhesion (Brewis, 1982: Wake, 1982).

1.2.4 Electrostatic Theory

Here electrostatic forces are responsible for holding the two materials together arising from the transfer of electrons. Contact electrification has been shown to occur in fine particle adhesion owing to static electrical charges (Wake, 1982). Evidence put forward in support of this theory includes the observation that parts of a broken joint are sometimes charged (Wake, 1982), although, this charge may have been produced during the destruction of the joint (Brewis, 1982).

It is now generally accepted that no single theory or set of forces provides the complete explanation for all adhesive situations, but that elements of each of the above theories may be involved in different situations (Brewis and Briggs, 1985: Schornhorn, 1978: Wake, 1982). The evidence is strongest for the adsorption theory in the adhesives technology field, but mechanical keying and diffusion sometimes plays an important role (Brewis, 1982: Voyutskii et al, 1973). Smooth, non-porous substrates present no opportunity for mechanical keying. Therefore to achieve good adhesion to such substrates the chemical composition must result in good bonding by means of specific interactions (Ashley et al, 1985). In solvent-borne systems the solvent can swell or partially dissolve the polymer and so improve adhesion by interdiffusion. With a different field such as the biomaterials area a very different situation may exist, with contributions from differing mechanisms being important.

The importance of physicochemical surface properties is well recognised in the adhesives industry, where surface analysis techniques have been widely used in studying adhesion problems. The use of surface pretreatments is also well established (Briggs, 1985: Briggs et al, 1980: Clark et al, 1978: Dukes and Kinloch, 1977: Schornhorn, 1978). The modification of polymer surface properties whilst retaining desirable bulk properties has much to commend it (Caldwell and Jackson, 1968: Clark et al, 1978). These treatments are intended to increase the close apposition of two materials by cleaning the surfaces of potential weak boundary layers (Allen and Alsalim, 1977: Brewis, 1982: Clark et al, 1978: Kinloch, 1979: Sykes, 1982), increasing the surface roughness (Brewis, 1982: McGregor and Perrins, 1970: Packham, 1983: Poole and Watts, 1985: Sykes, 1982), possibly also by making the surface porous (Bascom, 1979: Ditchek et al, 1980: Kinloch, 1979: Schornhorn, 1978: Yasuda, 1983) and altering the surface chemistry (Briggs, 1982b: Briggs et al, 1980: Caldwell and Jackson, 1968: Kinloch, 1979: Willis and Zichy, 1978). These are all factors which need to be borne in mind when embarking on a programme of experiments designed to look at a similar phenomenon in a different scientific field such as a study of the events of protein deposition at a biological/synthetic interface, a subject of this thesis.

The surface chemistry of materials is a key factor in defining their adhesive behaviour and involves

a knowledge of the degree of wetting of the material surface, the rate of wetting and the interactions across the interface. Surface pretreatments usually increase the wettability of the surface as a result of changes in the surface chemistry (Briggs et al, 1980), but the significance of this is disputed (Brewis et al, 1978; Schornhorn, 1978). The surface energies of the components will be determined by their chemistries and will affect the degree and rate of wetting (Dukes and Kinloch, 1977). Surface rugosity can also affect the kinetics of wetting (Dukes and Kinloch, 1977) with the rate of spreading on a very rough surface being much greater than on a polished surface. It is obviously important that the components are brought into as close a contact as possible and the viscosity of the adhesive will be a factor in achieving this (Ashley et al, 1985; Brewis, 1982). The surface chemistries affect the degree of contact involved and will also determine the magnitude of the interaction where contact has occurred, ie. whether chemical bonding, hydrogen bonding or van der Waals' forces are involved. Molecules in the surface region of a material are subject to attractive forces from adjacent molecules which result in a net attraction into the bulk phase. The attraction tends to reduce the number of molecules in the surface region and this results in a surface tension. The remaining unsatisfied bonds at the surface result in a surface free energy. The intermolecular attractions which cause surface tension result from a variety of well known intermolecular forces: hydrogen bond, dispersion forces, hydrophobic bond, etc.

Although the exact nature of the forces involved are not known it is clear that some requirements are essential for good adhesion and are likely to be important regardless of the exact nature of the adhesion event (Brewis and Briggs, 1985). These are close contact (ie wetting of the substrate by the mobile phase), absence of weak boundary layers and avoidance of stress concentrations which could lead to disbonding. The last of these properties is not likely to be of importance in interface conversion when one or two molecular layers are present (Baier and Dutton, 1969; Vroman et al, 1971). However, the first two properties should be considered when studying the deposition of proteins (Baier, 1970; Baier and Dutton, 1969; Pedley and Tighe, 1979; Weiss, 1970), although weak boundary layers have been discounted in some polymer adhesion situations (Brewis et al, 1978).

Surface treatments are commonly used in the adhesives field to increase the strength of the adhesive bond (Briggs, 1982b; Briggs et al, 1980; Clark et al, 1978; Schornhorn, 1978). The reasons for this are not clear but may be due to an increased mechanical strength of the polymer in the surface region owing to a greater extent of cross-linking (Brewis et al, 1978; Clark et al, 1978; Schornhorn 1978). A polymer matrix will be restrained in movement by adsorption of polar groups or by London's forces between polymer chains in close contact and is likely to exhibit a much greater tensile strength than the bulk (Bullett and Prosser, 1985). In effect the bonding to the substrate increases the crosslink density of the polymer locally, although surface pretreatment of PTFE can reduce the strength of the surface layers (Dukes and Kinloch, 1977). The introduction of oxygen-containing groups at the surface is also thought to be a key factor (Briggs, 1982b; Briggs et al, 1980; Willis and Zichy, 1978). Surface treatments, both chemical (Curtis et al, 1983; Lydon, 1986; Maroudas 1976) and by plasma glow discharge (Amstein and Hartman, 1975; Hoffman, 1984; Lydon, 1986; Thomas et al, 1986), have been used to render polystyrene adhesive to cells in culture. Because similar forces are involved a study of the effects of these treatments on the biological/synthetic interface would benefit from the knowledge gained in the adhesives technology field. Surface analysis techniques have been used to study the adhesive/solid interface and a consideration of those techniques found to be of greatest benefit is an obvious starting point for the biological field.

1.3 Surface Properties of Interest

Physicochemical properties of the synthetic substrata are an important consideration in interface conversion, at least in the initial stages (Goodman et al, 1989; Hogt et al, 1985; Hoffman, 1984; Horbett, 1982; Lydon et al, 1985; Ruckenstein and Gourinanskov, 1984). The type of forces involved in ordering the biological/synthetic interface is a fertile area for investigation, and may include long-range forces (Eriksson, 1985; Heaysman and Peagram, 1982; King et al, 1979; Taylor, 1970), short-range forces (Kochwa et al, 1967; Weiss, 1970) or both (Bjorvatn, 1986; Cottonaro et al, 1981; Curtis, 1977; Hoffman, 1974; Pashley et al, 1985; Pedley and Tighe, 1979).

Weiss (1970) postulated that cell adhesion involved overcoming electrostatic repulsion by local surface variations at pseudopodia before the short-range forces take over and provide attractive forces between cells and materials. The extension, attachment and contraction of local outgrowths can be regarded as one of the main forms of cellular interaction with their environment (Gershman and Rosen, 1978; Izzard and Lochner, 1980; Vasiliev, 1982). King et al (1979) have shown that the cell-substratum gap distance, but not the gap at focal contacts, varies with the ionic strength of the medium. They postulated from this that long-range electrostatic forces may have two roles; a) provision of an adhesive force prior to focal contact formation, and b) supplementation of focal contact-substratum adhesion in later stages of spreading and during locomotion. Their experiments demonstrated that at least two types of forces are involved in cell adhesion with electrostatic attraction or repulsion being one of them (Curtis, 1964; King et al, 1979; Pethica, 1961). Van der Waals-London forces, which play an important part in colloid stability, are thought to be the other forces involved, although there are several others which may be implicated (Curtis, 1964; Pethica, 1961). The interaction at focal contacts is obviously very different as its gap is not affected by ionic strength. It is at these points on the cell surface where protein-receptor binding to hold the cell to the substratum is taking place, and this requires intimate contact between the adhesion molecules on the substratum with the cell surface receptor molecule. In order to achieve this close contact electrostatic forces must be overcome by other, presumably stronger, short-range forces which can only operate once the electrostatic forces have brought cell surface and the substratum surface within range. Cells will not adhere to surfaces with very high charge density (Curtis, 1977).

Ionic attraction is thought to be involved in blood-prosthesis interaction (Cottonaro et al, 1981). The action of heparin in providing materials coated by it with blood compatibility properties is thought to be due to ionic attraction of protein adsorption in an ordered fashion (Hoffman, 1974; Horbett and Hoffman, 1975; Shiomi et al, 1989). Heparin inhibits the aggregation of platelets when adsorbed to surface but not when dissolved in the blood, and this is thought to be due to the cationic surface produced reducing the activation of factor XII (Feijen et al, 1979). The introduction of amino groups into urethane surfaces by quaternization and by heparinisation suppresses platelet adhesion (Ito et

al, 1989). Sulphonated polystyrenes and perfluorosulphonic acid have pendant sulphonic groups which are thought to confer antithrombogenic properties due to the negative charge of the sulphonic groups (McAuslan et al, 1988).

There is recent evidence that some hydrophobic forces act over larger distances than previously thought (Pashley et al, 1985). This indicates that a range of forces may be available for adsorption including electrostatic (Baier, 1977: Eriksson, 1985: Maroudas, 1973: Maroudas, 1974: Vroman et al, 1971), hydrogen bonding (Bjorvatn, 1986: Maroudas, 1973) and hydrophobic bonding (Bjorvatn, 1986: Cottonaro et al, 1981: Maroudas, 1973: Royce Jr et al, 1982: Vroman et al, 1971). The presence of polar, charged and nonpolar amino acid side chains in proteins provides the opportunity for multiple modes of binding with many different types of surfaces (Horbett, 1982). Hydrogen bonding is thought to be the main force involved in the self-adhesion of corona-treated polyethylene (Briggs, 1982b: Dukes and Kinloch, 1977) and in the action of silane adhesion promoters (Bullett and Prosser, 1985). Adhesion of polymer coatings to metal surfaces is by the secondary bonds or relatively weak forces such as hydrogen bonding and van der Waal's forces (Yasuda, 1983). Consequently, water molecules, which are one of the strongest hydrogen bonding agents, can easily break such existing bonds and result in bond failure. As well as these forces of attraction and/or repulsion, several surface parameters are thought to be important for protein deposition and cell adhesion as they determine the actual forces involved at the interface.

1.4 Surface Chemistry

The chemical composition of the polymer used in the synthetic material is thought to have an effect on interface conversion (Lydon, 1986: Minett et al, 1984: Royce Jr et al, 1982: van der Valk et al, 1983). In practice it is difficult to alter the chemical composition without affecting other physicochemical parameters. An example of this is given using the example of hydrogel poly-hydroxyethyl methacrylate (pHEMA). Substitution of the hydrophilic hydroxyethyl groups with either methyl or ethyl groups (Klebe et al, 1981: Lydon et al, 1985: Minett, 1986) or the introduction of the

styrene moiety (Lydon et al, 1985: Minett, 1986) alters the equilibrium water content (EWC) and, therefore, the hydrophobic/hydrophilic balance. It also has a marked effect on the cell adhesion response (Lydon et al, 1985: Rosen and Schway, 1980: Tanzawa et al, 1980). Similarly, the introduction of polyamine into pHEMA/polyamine copolymers results in increased cell adhesion (Maruyama et al, 1988). Study of the response of platelets to polyurethane surfaces shows that the chemical structures of these materials plays a significant role along with surface energy (Goodman et al, 1989).

Polystyrene is a commonly used material for routine cell and tissue culture yet, in its pure form it is non-adhesive for eukaryotic cells in the presence (Lydon and Clay, 1985: Lydon et al, 1985: Thomas et al: 1986) or absence (Curtis et al, 1983: Grinnell and Feld, 1982: Hughes et al: 1979: Maroudas, 1975: 1976: Minett, 1986) of serum. There are, several methods, however, of rendering polystyrene adhesive to cells. These include chemical treatments with sulphuric (Curtis et al, 1983: Gibson and Bailey, 1980: Klemperer and Knox, 1977: Lydon and Clay, 1985: Lydon et al, 1985: Matsuda and Litt, 1975: Thomas et al, 1986), chromic (Klemperer and Knox, 1977) and chloric (Curtis et al, 1983) acids, exposure to UV light (Curtis et al, 1983: Martin and Rubin, 1974), and plasma glow discharge treatment (Amstein and Hartman, 1975: Lydon, 1986: Minett, 1986: Thomas et al, 1986). These treatments are thought to work by the introduction of hydroxyl groups (Curtis et al, 1983: Thomas et al, 1986) into the surface and not sulphonate groups as was originally suggested (Gibson and Bailey, 1980: Klemperer and Knox, 1977: Maroudas, 1975: 1976: Matsuda and Litt, 1975). Support for this idea is that X-ray photoelectron spectroscopic investigations of plasma-treated polystyrene shows an increase in C-O bonding (Clark et al, 1978: Thomas, 1988). This is unlikely to be the sole reason for the cell response as the hydroxyl-rich hydrogel polyHEMA is non-adhesive for cells (Klebe et al, 1981: Lydon, 1986: Lydon et al, 1985: Maroudas, 1976), while poly-ethyl methacrylate and polymethylmethacrylate contain no hydroxyl groups and yet allow cell adhesion (Klebe et al, 1981: Lydon, 1986: Lydon et al, 1985: Rosen and Schway, 1980). This is supported by X-ray photoelectron spectroscopy studies of argon- and nitrogen-plasma-treated polystyrene which shows there is no increase in surface oxygen after plasma treatment and yet the cell adhesion on these surfaces is

comparable with commercial tissue culture polystyrene (Minett, 1986).

A key factor in the chemical modification of polymer surfaces in the adhesives technology field is the introduction of oxygen in the form of hydroxyl, carbonyl groups (Briggs, 1982b; Bullett and Prosser, 1985; Schornhorn, 1978) and keto groups (Briggs and Kendall, 1979). It has been observed that there is a critical surface concentration above which increased adhesion occurs with printing inks onto plastics (Ashley et al, 1985). Caldwell and Jackson (1968) showed that the adhesion of polycarbonates is increased by oxidation with chromic acid or the addition of amine groups. However, the use of antioxidants does not reduce the adhesion of polyethylene to high energy (ie. metal) surfaces (Evans and Packham, 1979). Oxidative treatment of polymers can be enhanced in production by increasing oxygen availability with ozone (Ashley et al, 1985). If diamine or polyamines are used the polarity of the polycarbonate surface is increased which imparts new surface properties such as wettability, dyeability, improved adhesion and increased conductivity (Caldwell and Jackson, 1968). Amino groups attached by radio frequency plasma have been used to permit binding of heparin to plastic surfaces (Leininger et al, 1966). Oxidative pretreatments are commonly used for increasing adhesive strengths with a range of metals, including aluminium (Cotter, 1977; Kinloch, 1979), titanium (Cotter and Mahoon, 1982; Ditchek et al, 1980; Natan et al, 1982), steel (Allen and Alsalim, 1977; Brewis, 1982; Evans and Packham, 1979; Sykes, 1982), copper and zinc (Evans and Packham, 1979). However, oxidation has been shown not to increase the strength of adhesion of epoxy resin to aluminium (Poole and Watts, 1985). Brittle oxide layers (rust) need removing in normal steels and this can be achieved chemically or mechanically (Sykes, 1982). Corrosion-inhibiting agents have been added to primers for bonding to aluminium which act as oxidising agents to replace oxide layers that become damaged in service (Moloney, 1982).

Chemical treatment of stainless steel is claimed to provide a better adhesive joint than mechanical removal, due mainly to the removal of a weak boundary carbon layer (Allen and Alsalim, 1977). However, this work did not investigate the effects of these pretreatments on the chemistry of the steel surface after treatment. To add to the complexity of the picture, the chemistry of the adhesive is also

an important factor in bond strength, as phenolic-based adhesives give stronger joints than epoxide-based adhesives (Kinloch, 1979). Chemical pretreatments can cause complex changes including removing weak layers, roughening the surface and changing the surface chemistry (Brewis, 1982). Thus identifying the reason behind a change in adhesion will not be straightforward.

Attempts have been made to surface treat polystyrene using inert gas plasma-glow discharge without introducing hydroxyl groups. These have been unsuccessful because the glow discharge-treated surfaces have reacted with atmospheric oxygen on removal from the reactor to form hydroxyl groups at the surface (Thomas et al, 1987). This is a consequence of the numerous reactive species which result from the use of plasma sources including ions, metastables, neutral molecules and free radicals in ground and excited states (Ashley et al, 1985; Clark et al, 1978). Oxidation of free radicals by atmospheric oxygen has also been reported in the inert plasma treatment of polyethylene (Blythe et al, 1978; Briggs, 1982b). In nitrogen discharge treatment a high concentration of nitrogen groups, mainly $-NH_2$, have been detected (Blythe et al, 1978; Courval et al, 1976). Nitrogen is also introduced into corona discharge-treated polymer surfaces (Brewis et al, 1978).

The orientation of the polymer molecules can also have a part to play. Polymer crystallinity can affect cell adhesion with highly crystalline melinex which gives significantly higher levels of cell spreading than other less crystalline grades (Minett, 1986). The chemical structures of segmented polyurethane surfaces plays a significant role in the response of platelets to these materials (Goodman et al, 1989). A mixture of crystalline and amorphous structures is thought to be important for the antithrombogenicity of polymer surfaces (Yui et al, 1988). However, crystallinity has been ruled out as being a factor in the cell response of a range of polymers (Thomas, 1988).

1.5 Wettability

The hydrophilic/hydrophobic nature of the substratum is important in developing a biologically reactive interface, although there is considerable debate on the question of what degree of

hydrophilicity or hydrophobicity is required. Klebe et al (1981) have proposed that hydrophobic surfaces are required for cell adhesion and growth, whilst Maroudas (1974) has shown that fibroblasts will not adhere to a rigid hydrophobic or a yielding hydrophilic surface. The work with polystyrene culture substrata indicate that an increase in wettability increases its susceptibility to cell adhesion (Curtis et al, 1983: Grinnell, 1978: Jansen et al, 1989: Lydon and Clay, 1985: Thomas et al, 1986). Poly(ethylene terephthalate) can also be used as a tissue culture substratum material and is more hydrophilic than unmodified PET. It is this increase in wettability that promotes better cell adhesion on the surface treated tissue culture PET (Van Wachem et al, 1989). Cell adhesion to peptide derivatives with a range of wettabilities has shown a peak level of cell adhesion with a water contact angle of around 70° (Kang et al, 1989). Hydrophobic latex particles have been shown to induce more platelet aggregation than particles with a more hydrophilic surface (Miyamoto et al, 1989). The use of hydrophilic poly(ethylene oxide) spacers to immobilise heparin onto polyurethane surfaces results in increased heparin bioactivity compared to an alkyl spacer (Park et al, 1988). Although part of the action of these spacers is likely to be due to them enabling the heparin molecule mobility to react (Tay et al, 1989). Adsorption of albumin to polyurethane is increased by incorporation of long chain length alkyl groups which is thought to be due to the decreasing polar nature of the polymer surface (Pitt et al, 1986a).

Wetting of surfaces with polymers is an important factor in the production of a strong adhesive joint, and this property is determined by the surface chemistry and surface energy (Briggs et al, 1980: Bullett and Prosser, 1985). Surface treatments for PTFE render the polymer surface more polar with resulting increase in bond strength (Dwight, 1977), whilst the correct balance of polar and non-polar groups is important in the adhesion of printing inks (Ashley et al, 1985). Good adhesion of resist materials to silica substrates depends on good wettability which is determined by the balance of surface energies of the components (Deckert and Peters, 1983).

Protein deposition has been shown to differ on hydrophobic and hydrophilic surfaces (Bentley and Klebe, 1985: Brash and ten Hove, 1989: Grinnell and Feld, 1982: Klebe et al, 1981: Royce Jr et al,

1982: Young et al, 1982), but it is not clear what is the most desirable hydrophilic/hydrophobic balance. Part of the confusion, here, arises from the poor definition of the terms hydrophilicity and hydrophobicity. The work of Lydon and co-workers has attempted this definition by using equilibrium water content (EWC) and surface energy (Lydon et al, 1985). Here, a cell adhesion permissive EWC zone of 2-34% has been identified, and this is related to the polar fraction of the surface free energy (Lydon et al, 1985). Most blood-compatible surfaces have an EWC of 35-60%, in the region which does not support cell adhesion (Minett, 1986). Equilibrium water content has also been a useful indicator in studies with soft contact lenses which use hydrogels where similar requirements to blood-contacting surfaces exist (Corkhill et al, 1987). EWC is related to the balance of hydrophilicity and hydrophobicity in the polymer hydrogel and, in particular, the steric and polar contributions of backbone substituent groups.

1.6 Surface Energy

Surface energy is a useful descriptor of the actual surface chemical identity of polymeric materials (Baier, 1977). With polymers, the gross chemistry is very similar in all cases but the detail differences lead to vastly divergent adhesive properties. Surface energy has long been recognised as an indicator of the physicochemical state of surfaces (Fowkes, 1964: Hoffman, 1974: Zisman, 1963). From thermodynamic considerations it is to be expected that the entropy and free energy of the biological/synthetic interface will be minimised (Feijen et al, 1979: Hoffman, 1974: Norde and Lyklema, 1978: Nyilas et al, 1974: Ratner, 1982: Ruckenstein and Gourinanskov, 1984). This has formed the reasoning behind the "minimal interfacial tension" hypothesis for blood compatibility requirements for biomaterials (Andrade, 1973: Feijen et al, 1979: Hoffman, 1974: Ruckenstein and Gourinanskov, 1984), where the proteins adsorb at the interface in an attempt to reduce the solid-liquid interfacial tension (Baier et al, 1970). When the interfacial tension is high the adsorbed proteins will interact strongly with the solid surface and denature by anchoring at multiple sites (Nyilas et al, 1974). This is the case with fibrinogen when the unfolding of the protein on surfaces of high interfacial tension leads to platelet activation (Morrissey, 1977: Young et al, 1983: Zucker and Vroman, 1969). Unfolding of the molecule

results in the protein's non-polar residues being exposed at the aqueous interface promoting thrombus deposition (Young et al, 1983). On the other hand, if the interfacial tension is low the adsorbed proteins undergo minimal distortion from their native conformation. The surface properties of polymers will also contribute to this as a decrease in interfacial energy can arise by backbone chain reorientation and side-chain relaxations (Hogt et al, 1985).

It is this reasoning that explains the interest in synthetic polymer hydrogels for biomedical applications, including drug delivery systems (Corkhill et al, 1989; Langer and Folkman, 1980; Wang, 1989), replacement blood vessels (Andrade, 1973; Feijen et al, 1979; Hoffman, 1974; Ratner and Hoffman, 1976; Ruckenstein and Gourinanskov, 1984), contact lenses (Baker and Tighe, 1981; Lydon, 1986) and wound dressings (Corkhill et al, 1989). The high water content of hydrogels give them a superficial resemblance to living tissue extracellular matrix (Andrade et al, 1973; Horbett and Hoffman, 1975), and is believed to reduce the tendency of plasma proteins to adsorb, and that when they do adsorb they retain their natural conformation. This is in contrast to adsorption to hydrophobic surfaces where denaturation occurs which is driven by the need to increase hydrophobic bonding (Horbett, 1982; Soderquist and Walton, 1980). It has been shown with polystyrene latices that molecular unfolding of IgG molecules affects their immunogenic properties (Kochwa et al, 1967; Morrissey and Han, 1978). The water in hydrogels is to a large extent structured with the polar groups binding water molecules strongly (Corkhill et al, 1987; Drost-Hansen, 1971; Pedley and Tighe, 1979; Roorda et al, 1988). The proportion of bound to free water determines the oxygen transport and osmotic properties (Pedley and Tighe, 1979), and allows the selective penetration of biomolecules into the gel network (Baker and Tighe, 1981; Refojo and Leong, 1979; Royce Jr et al, 1982; Tanzawa et al, 1980). In contrast, it has been shown that fibrinogen does not penetrate into polyHEMA gel (Horbett and Hoffman, 1975), which suggests that penetration will depend on pore size and the size of molecule as well as the natural attraction or repulsion between them.

Bacterial adhesion is thought to be largely determined by the balance between the surface free energy of the suspending liquid medium, that of the solid substrata and of the bacteria (Barth et al,

1989: Ludwicka et al, 1984). The change in free energy is a parameter for hydrophobic interaction which has been shown to play an important role in microbial attachment (Nesbitt et al, 1982) and in plant cell attachment to polymeric materials (Facchini et al, 1989).

A more practical approach is the "moderate surface energy" hypothesis of Baier et al (1971), who showed that a range of thromboresistant materials have a critical surface tension of 20-30 dynes cm^{-1} . The critical surface tension is an empirical measurement first propounded by Zisman (1963), and is related to the dispersive component of surface energy (Hoffman, 1974; Ito et al, 1989). The kinetics of cell spreading is strongly influenced by the surface energy of the substratum material (Schakenraad et al, 1989). Ruckenstein and Gourinanskov (1984) proposed that both polar and dispersive components need to be in certain ranges for optimal thromboresistance. The two components of surface energy are thought to represent the different molecular forces at the surface (Hamilton, 1972; Owens and Wendt, 1969), and thus may be a more accurate representation than critical surface tension.

For cell and tissue response to biomaterials using cell adhesion models, the polar component of surface energy has been shown to be a more important criterion (Clay et al, 1987; Lydon, 1986; Lydon et al, 1985; Thomas, 1988; van der Valk et al, 1983). The polar component and hydrogen bonding components of surface energy of polyurethanes increase markedly with the addition of amino groups by quaternisation and heparin treatment (Ito et al, 1989). They also increase significantly with protein adsorption to these surfaces, which indicates an important contribution of the polarity and hydrogen bonding properties of adsorbed proteins to the surface property of the substratum material. Polar surface groups, which include oxygen, are thought to be important because of its ability to mediate hydrogen bonding (Thomas, 1988). The polar component of surface energy of both resist and substrate has been found to be of prime importance in determining adhesion of resist/ SiO_2 composites (Deckert and Peters, 1983). Best adhesion is achieved if γ^P of both resist film and substrate is small and this has been suggested as necessary for the majority of adhesive occasions (Rance, 1985).

1.7 Surface Charge

Much of the above has been concerned with electrostatically neutral polymers where ionic interactions are not nearly as significant as hydrophobic and hydrophilic interactions (Royce Jr et al, 1982). However, the electrical state of the surface may be involved in protein deposition and cell adhesion in some circumstances (Corpe et al, 1970: Kochwa et al, 1967: Maroudas, 1975: Morrissey, 1977: Norde and Lyklema, 1978: Todd and Gingell, 1980: Weiss, 1970), since the cell surface is electrically charged (Weiss and Subject, 1974).

Cell spreading has been shown to be a function of the surface charge density (Maroudas, 1976). Cell spreading onto glass is slower and less extensive when a serum layer is present (Lackie, 1982: Maroudas, 1975: Witkowski and Brighton, 1972), indicating that the serum layer masks the electrostatic interactions (Maroudas, 1973). It seems that more time and possibly effort is required by the cells to overcome the electrostatic repulsion of the cell and substratum surface before the short-range forces of attraction that may lead to cell adhesion can be brought into range (Weiss, 1970: Weiss and Subject, 1974). In the extreme case that the electrostatic repulsion is too strong then cell adhesion can be inhibited as the physical gap cannot be bridged. The action of hydroxyl groups at the cell surface may, in part be due to the weak positive charge which they give to the surface (Thomas, 1988). Primaria tissue culture ware contains nitrogen radicals conferring a positive charged surface (Klein-Soyer et al, 1989). Sulphuric groups had been suggested as important for the cell adhesive nature of sulphuric acid treated polystyrene (Maroudas, 1976), but recent evidence has shown that this is not the case due to the negative charge introduced to the surface with this moiety (Thomas, 1988).

The action of heparin in reducing thrombogenesis in vascular prosthesis is due to its electronegative nature (Baker and Tighe, 1981). An electronegative surface is also thought to be important in the induction of bone regrowth using demineralised bone or synthetic hydrogels (Eriksson, 1976: Eriksson, 1985). Electrical stimulation of wound repair has also been reported, although in this

instance it is not clear whether the effect is due to the charge being resident at a surface but is proposed to reduce vascularity of tissue (Reich et al, 1990).

1.8 Surface Topography

Surface topography may be another factor in biocompatibility (Hoffman, 1984: Kowalski, 1982: Lydon and Clay, 1985: Minett, 1986: Thomas et al, 1986), as induced surface roughness has been used in polymeric implants (Kowalski, 1982). Surface rugosity has been suggested as a possible important factor influencing cell adhesion to polymeric substrates in previous studies in our series of investigations (Minett, 1986: Thomas, 1988). It is likely, however, that the cell response varies with the scale of the roughness feature, as Baier (1970) has reported that features less than a micron will be tolerated, which is also supported by the work of Thomas et al (1986). This contrasts with the work on contact guidance where cells will polarise on surfaces with step features or grooves of several microns (Curtis and Varde, 1964: Dunn, 1982), or on tubes (Dunn, 1982) or fibres with a high radius of curvature (Curtis and Varde, 1964: Dunn and Heath, 1976). There has been shown to be more protein deposited onto lathe cut polyHEMA than spin cast pHEMA (Castillo et al, 1984b: 1985).

The use of adhesive islands to support cell growth demonstrates that cells try to conform to the shape of the substratum. Long islands only 3 μ m wide provide effective attachment for cells (O'Neill et al, 1990). The anchorage stimulus is mediated by the cytoskeleton which assembles around the points of attachment to the substratum. The focal contact area is more important for cell growth stimulation than the arrangement or number of the foci (O'Neill et al, 1990). The cytoskeleton restricts the shape cells can take, which limits the radius of curvature of the substratum to which they can conform and upon which they can grow.

Mechanical keying has been proposed as a theory to explain adhesion, where the adhesive interlocks around surface irregularities (Bascom, 1979: Brewis and Briggs, 1985: Sykes, 1982). Packham and coworkers have clearly demonstrated the importance of topography in the adhesion of polyethylene

to anodic aluminium (Packham et al, 1974), and with polyethylene-copper joints (Evans and Packham, 1979). Good electroplate adhesion on polypropylene is achieved by treatment with an etching acid. Maximum peel strength coincides with maximum surface roughness, suggesting that mechanical keying is necessary to get good electroplate adhesion (McGregor and Perrins, 1970).

Treatment methods for aluminium which produce thicker but porous layers have also been shown to lead to better micromechanical anchoring which results in higher bond stability (Brockman, 1983). Poole and Watts (1985) showed that the adhesion of epoxy resin to aluminium is not influenced by the surface chemistry but is increased by grit blasting to produce a roughened surface. Surface oxide thickness and, more importantly, surface micro-roughness are important parameters for obtaining a good durable bond on titanium and aluminium (Chen et al, 1978; Ditchek et al, 1980). Phosphate treatment of steels can produce either thin coatings which enhance bond strength, or thick, coarsely crystalline layers which produce poor adhesion (Sykes, 1982). It is, therefore, important to identify the degree of roughness required for best adhesion. The nature of the surface profile rather than its amplitude is thought to be important (Sykes, 1982). Surface morphology is thought to influence the orientation of the polymer molecules during adsorption. This orientation influences the subsequent crosslinking mechanisms and thereby the adhesive molecular structure and the bond strength (Brockman, 1983).

The action of grit blasting as a pretreatment for adhesive bonding to metals works in various important ways by cleaning the surface, roughening the surface to allow mechanical keying to work, and by increasing the potential bonding area (Brewis, 1982; Bullett and Prosser, 1985; Trawinski et al, 1984). Sand-blasted surfaces of aluminium bonded with epoxy resin have greater fracture strengths than polished aluminium, and the fracture is cohesive in the resin close to the interface and replicates the aluminium surface (Mulville and Vaishnov, 1975). Surface pretreatments for steel which increase the rugosity of the surface produce increased adhesive bonding, supporting the idea that three-dimensional interlocking with the applied adhesive is crucial to good bond durability to steel surfaces (Trawinski et al, 1984). It has been shown that without significant change in topography, subsequent

adhesion of deposited metal can be enhanced or diminished (McGregor and Perrins, 1970) by appropriate treatment of the etched polymer surface, demonstrating that multiple factors are important. Two related surface preparations for aluminium produce drastically different bond strengths although they appear to have very similar micromorphologies (Knock and Locke, 1983). It is likely that bond strength depends on differing factors when rough or smooth surfaces are involved. In the case of smooth surface morphologies, the bond strength depends on chemical interaction. With rough surface morphologies, the bond strength is also influenced by mechanical interlocking (Ahearn et al, 1983).

With metals, the presence of fluoride ions leads to much smoother surfaces and is associated with inferior joint strength, although the change in topography is thought to be the dominant factor (Chen et al, 1978). Chemically etched metal surfaces have increased roughness which is correlated with increased adhesive strength (Allen and Alsalim, 1977; Cotter and Mahoon, 1982). It does seem that improved adhesion is not simply due to mechanical keying as failure occurs within the polymer (Allen and Alsalim, 1977; Kinloch, 1979; Mulville and Vaishnov, 1975; Packham, 1983). The distance of the plane of fracture from the interface varies with the strength of the adhesive bond, weaker bonds failing near to the interface (Allen and Alsalim, 1977).

1.9 Mechanical Properties

Physical weakness of the substratum is important as cells exerting too strong a tractive force will "tear up" the substratum, thus effectively preventing locomotion (Harris, 1982; Vasiliev and Gelfand, 1977). This criterion will depend on the cells involved (Lackie, 1982) as cells with weak attachment to the substratum can move over a surface which would be disrupted by cells which could exert a strong force on the substratum. Some polymers express high levels of cell spreading but support relatively few cells, while other substrates may show good cell attachment but lower levels of cell spreading than might be expected (Minett, 1986). This varying strength of attachment is obviously due to the interaction of the cells with the substratum which differs depending on cell type and material.

Cells which are in a stable environment can be induced to migrate during wound healing, organogenesis and tumour metastasis. In these instances cell attachment for the substratum is lessened in favour of the ability to migrate to a new location by some unknown signal. The change which occurs is likely to be in the cells' adhesion strength as a response to other transmembrane signalling event rather than by a change in the structure of the extracellular matrix. For example, cells which are induced to produce elevated levels of hyaluronic acid show increased motility.

Lyman and Knutson (1980) have shown that the mechanical properties of vascular prostheses are important, with the artificial material requiring strength and elasticity. Similarly, collagen-coated (Guidoin et al, 1989) and albumin-coated (Merhi et al, 1989; Rumisek et al, 1986) arterial prostheses rely on polyester textile backings for mechanical strength, but need to be made impervious without provoking abnormal tissue reaction. PVC has good mechanical characteristics for biomedical use, although, it needs to be complexed with hydrogels and heparin to give it suitable blood compatibility (Akashi et al, 1989; Tanzawa et al, 1980). Hydrogels are often used as coatings on biomaterials with a more mechanically stable substrate (Corkhill et al, 1989).

1.10 Protein Deposition

Protein layers alter the synthetic surface in such a way as to allow cellular interactions to take place (Baier and Weiss, 1975; Lydon et al, 1985; Ruckenstein and Gourinanskov, 1984; Young et al, 1982). Several proteins may be involved so as to present the correct micro-environment (Gendreau et al, 1982; Turley et al, 1985). This will entail those proteins which are involved in cell adhesion to be present (Holmes, 1967; Hynes and Yamada, 1982; Yamada and Olden, 1978) in an exposed position and in the correct conformation (Grinnell and Feld, 1981; Grinnell and Phan, 1983; Hoffman, 1974; Iwamoto et al, 1982; Ruckenstein and Gourinanskov, 1984) such that the cells can interact with the synthetic surface. Protein adsorption on a hydrophobic surface will involve conformational changes to optimize the various bonding interactions between the protein's hydrophobic and hydrophilic sites with the surface and water phases, respectively (Horbett, 1982). Protein adsorption to hydrophobic

surfaces is characterised by three stages; an initial period when adsorption is reversible, a second phase when slow conformational changes take place to give irreversible adsorption, and finally denatured material is slowly desorbed (Soderquist and Walton, 1980).

Different enthalpy changes show fundamental differences in the adsorption of proteins onto surfaces with varying composition and surface energy characteristics (Chiu et al, 1978). More fibrinogen is adsorbed onto glass than isotropic carbon. Grinnell and coworkers have demonstrated, using antibody labelling (Grinnell and Feld, 1981; Grinnell and Feld, 1982; Grinnell and Phan, 1983), that fibronectin assumes different conformations on hydrophobic or hydrophilic surfaces. At low concentrations, more fibronectin was shown to deposit on to bacteriological grade plastic than tissue culture plastic, but the adsorbed material was biologically less active on bacteriological grade plastic (Grinnell and Feld, 1981). These changes could affect the recognition by the cell surface receptor, and this has been confirmed by others using Total Internal Reflection Fluorescence (Iwamoto et al, 1985). FTIR spectroscopy studies have shown that more fibronectin was adsorbed onto hydrophobic surfaces, and the extent of spectral changes was greater than on hydrophilic surfaces, indicating greater conformational change (Pitt et al, 1987).

Similar conformational changes on hydrophilic/hydrophobic surfaces have been demonstrated for prothrombin (Chuang and Andrade, 1985), insulin (Iwamoto et al, 1982), albumin (Castillo et al, 1984b), lysozyme (Castillo et al, 1985) and mucin (Castillo et al, 1986). The change of configuration on adsorption is a kinetic phenomenon where the rate is determined by the chemical composition, water content and surface roughness of hydrogel substrates (Castillo et al, 1984b). Haptoglobin has been shown to have differing adsorption and desorption kinetics to hydrophilic or hydrophobic silica, and this is thought to involve conformational changes (Lowe et al, 1985). Royce Jr et al (1982) showed that individual proteins have different affinities for hydrophobic or hydrophilic surfaces and that this may involve conformational changes as well as surface packing densities. Several proteins have been demonstrated to have varying adsorption properties to the surfaces of different polymers (Bentley and Klebe, 1985; Grinnell and Feld, 1981; Klebe et al, 1981). It seems likely that these events

are influenced by the surface properties of the underlying substrata.

Competition effects between proteins for adsorption to surfaces have been demonstrated with albumin and serum inhibiting fibronectin adsorption (Eberhart et al, 1982: Horbett and Hoffman, 1975: Vaudaux et al 1984b) and fibrinogen adsorption (Horbett, 1982: Lee et al, 1974). Fibrinogen has been shown to displace albumin (Gendreau et al, 1982), and competition has been demonstrated between albumin and γ -globulin (Cheng et al, 1985: Horbett and Hoffman, 1975), and between albumin and haemoglobin (Horbett, 1982). The adsorption of fibrinogen from plasma or blood exhibits a maximum at dilutions of about 100:1, and this has been attributed to competition from other proteins (Brash and ten Hove, 1989). The adsorption of fibrinogen to cuprophane from buffer results in monolayer amounts, whilst there is complete absence of adsorption from plasma (Brash and ten Hove, 1989), and the adsorption of prothrombin is maximal in buffer and minimal in plasma (Chuang and Mitra, 1984). Experiments on the competitive adsorption of fibrinogen and albumin to silicone rubber have shown that albumin adsorbs first followed by fibrinogen at a rate slower than for fibrinogen alone (Lok et al, 1983). The continued adsorption of fibrinogen could be due to different preferred sites for BSA and fibrinogen adsorption or to multilayer adsorption.

Cooperative effects have also been observed with very small amounts of albumin enhancing the adsorption to polystyrene of fibronectin in an active conformation as detected by antibody labelling (Grinnell and Feld, 1981: Grinnell and Feld, 1982). Fibrinogen is preferentially adsorbed to a wider range of material surfaces when deposited from blood than compared to non-competitive conditions from buffer solutions. (Chiu et al, 1978). The binding of fibronectin to polymer surfaces is non-specific except when the surface is treated with collagen, when specific binding to the collagen occurs (Klebe et al, 1981). Fibronectin has been found to complex with a range of biomolecules, such as collagen (Doillon et al, 1985: Hedman et al, 1982: Klebe et al, 1981: Turley et al, 1985: Vaudaux et al, 1984b), fibrin (Grinnell, 1978: Grinnell and Phan, 1983: Young et al, 1982) and proteoglycans (Hayman et al, 1982b: Hedman et al, 1982), and this is thought to be an important factor in *in vivo* reactions of cells with extracellular matrices (Grinnell, 1978). Studies with artificial collagen matrices show that proteins

and proteoglycans (collagen, chondroitin sulphate, chondroitin sulphate proteoglycan, hyaluronic acid and fibronectin) interact with each other to facilitate their incorporation into the gel matrix (Doillon et al, 1985; Turley et al, 1985). For example, chondroitin sulphate and fibronectin assist in the retention of hyaluronate in collagen gels (Turley et al, 1985). The presence of other plasma proteins can also change functional aspects, with thrombin showing increased amidase and clotting activity in the presence of albumin, fibrinogen and heparin (Chuang et al, 1980).

Ascorbic acid has been shown to increase the adsorption of proteins onto polycarbonate surfaces when exposed to blood. Whilst more proteins are present on the material surface thrombus formation is markedly reduced (Chandy and Sharma, 1985). It seems that the adsorption of albumin is increased in the presence of increased levels of vitamin C (ascorbic acid), when compared with that of fibrinogen and γ -globulin from an equal amount of protein mixture. Conversely, steroid hormones have been shown to be active at the blood-polymer interface in reducing the surface albumin concentration and increasing the surface fibrinogen concentration (Sharma and Chandy, 1989). Albumin has been used to coat fabric vascular grafts, and improves the short-term blood compatibility of these in *in vitro* tests (Kottke-Marchant, et al, 1989). Baier et al (1970) concluded that blood compatibility of blood vessel linings was due to an intrinsically low surface energy lipoprotein lining. This was found to be mimicked in alloy heart valve parts owing to the presence of a layer of organic contaminants from a hydrocarbon-based final polishing compound. High-density lipoprotein have been shown to preferentially adsorb to vascular prostheses which may adversely affect their biological function (Jayakumari et al, 1989).

Cell interaction with surfaces requires the presence of proteins, but other biological components, eg. lipids (Baker and Tighe, 1981; Galdston and Shah, 1970; Holmes, 1967; Jayakumari, et al, 1989; Owen et al, 1982; Thomas et al, 1987), or inorganic (Baier, 1982; Baker and Tighe, 1981), or ionic material (Baker and Tighe, 1981; Grinnell, 1978; Klebe et al, 1981; Maroudas, 1976; Weiss, 1970) particularly calcium (Cottonaro et al, 1981; Daniel et al, 1987; Galdston and Shah, 1970; Owen et al, 1982; Pethica, 1961; Thomas, 1988; Yui et al, 1989), may be necessary to precondition the surface in order

to render it suitable for protein deposition.

Calcium is thought to alter the hydrophilic/hydrophobic balance by interacting specifically with hydrated protein surfaces and causing them to dehydrate and undergo conformational changes (Baier, 1970). Calcium is important in maintaining normal intercellular contacts (Ringer, 1980; Loewenstein and Rose, 1978), cellular motility and contractility (Bromstrom and Wolff, 1981; Kart et al, 1980; Korn, 1982) and is required for cell spreading (Edwards et al, 1987). The leaching of cations from metal alloys may promote bacterial adhesion and growth (Brown and Williams, 1985). Phospholipids are thought to be the surfactant present in the alveolar lining layer and confer surface properties that are essential for normal function (Scarpelli and Colacicco, 1970). Lipids have been proven to be very important in contact lens spoilage (Hart et al, 1986). The involvement of lipids with cell adhesion is not clear as pure lipids have been shown to reduce cell adhesion (Cavenee et al, 1981; Demel and de Krupff, 1976; Ivanova and Margolis, 1973) whilst they can mediate cell adhesion (Huang, 1978; Thomas, 1988; Weigel et al, 1978), presumably by interaction with other components of the interfacial layer. Depth profiling of the biological/synthetic interface is an important approach to show whether a preconditioning step occurs and determine its nature.

1.11 Cell Culture

The use of anchorage-dependent cells in culture (Abercrombie, 1982; Grinnell, 1978; Heaysman and Pegrum, 1982) is an integral part of our investigations into the biocompatibility of synthetic polymers (Lydon, 1986; Lydon et al, 1985; Minett et al, 1984). The cell culture model is a convenient means of testing synthetic materials and relating their surface properties to cellular response (Bentley and Klebe, 1985; Lydon and Clay, 1985; Lydon et al, 1985; Thomas et al, 1986). An *in vitro* testing model allows more immediate and less ambiguous control of the cellular environment in which to study cell adhesion, spreading and motility (Trinkaus, 1976). Proteins bind tightly and nonspecifically in low amounts to plastic tissue culture dishes. Cells can be plated into such dishes and their response examined for attachment and the morphological effects of the adsorbed proteins. The activity and

specificity of adhesion molecules can be examined in such a model system. An understanding of the mechanisms involved in cell adhesion using model substrata and cell culture is an important approach in the study of cell/matrix and cell-cell interactions *in vivo*.

Work from our laboratories has been performed primarily using BHK fibroblasts as the model cell culture system, using short incubation times. Most cells will synthesize their own attachment proteins with time, but in short incubations the stimulation of the attachment of many trypsinized fibroblasts can be investigated. The long term survival of many cells, however, is a different situation and many cells such as hepatocytes (Sattler et al, 1978), corneal endothelial cells (Gospodarowicz et al, 1979), breast epithelial cells (Wicha et al, 1979), epidermal cells (Murray et al, 1979) and some fibroblasts (Gey et al, 1974), are maintained in a viable state longer on collagenous substrate than on plastic. Serum is a required medium supplement for routine, long-term cell and tissue culture. Much experimental work has been done in the presence (Lydon and Clay, 1985: Lydon et al, 1985: Thomas et al: 1986) or absence (Curtis et al, 1983: Grinnell and Feld, 1982: Hughes et al: 1979: Maroudas, 1975: Maroudas, 1976) of serum.

Established cell lines, such as baby hamster kidney fibroblasts, are commonly used cell types owing to their multivalent cell attachment mechanisms (Couchman et al, 1983: Kleinman et al, 1981). However, we, and workers in other laboratories, have used other cell types such as hepatocytes (Barnes and Reing, 1985: Hughes et al, 1979: Gjessing and Seglen, 1980: Neumeier and Reutter, 1985: Sattler et al, 1978), epithelial cells (Barnes and Sato, 1979: Jansen et al, 1989: Terranova et al, 1980: Wicha et al, 1979), neurones (Carbonetto et al, 1982: Doherty et al, 1988), carcinoma cells (Baier and Weiss, 1975: Barnes and Sato, 1980: Hayman et al, 1985a: Knusden et al, 1988), macrophages (Maroudas, 1976), monocytes (Miller et al, 1989), and endothelial cells (Hayman et al, 1985a: McAuslan et al, 1988: van Wachem et al, 1989). The study of the response of platelets *in vitro* is used when blood compatibility is the desired objective (Goodman et al, 1989: Ito et al, 1989).

1.12 Cell Adhesion and Protein Deposition

Most cells in culture will adhere to and spread on tissue culture plastic whilst others require a proteinaceous substrata such as collagen or fibrin (Elsdale and Bard, 1972). Foetal calf serum (FCS) is of particular importance in cell and tissue culture as it is a required supplement for the culture of anchorage-dependent cell lines (Barnes et al, 1980: Holmes, 1967: Lydon et al, 1985: Neumeier and Reutter, 1985). It is, however, a complex substance with numerous constituents (Barnes and Sato, 1980: Manabe et al, 1987: Rockwell et al, 1980), some of which have been isolated and shown to have the ability to promote the adhesion of cells to surfaces (Grinnell et al, 1977). These proteins include fibronectin (Barnes and Sato, 1979: Grinnell, 1978: Hynes and Yamada, 1982: Klebe et al, 1981: Neumeier and Reutter, 1985: Yamada and Olden, 1978) and vitronectin (Barnes and Sato, 1979: Barnes et al, 1980: Hayman et al, 1985b: Holmes, 1967: Lydon and Foulger, 1987: Neumeier and Reutter, 1985: Pytella et al, 1985).

The cell-adhesion properties of serum were originally attributed to fibronectin, but the mechanisms of serum- and fibronectin-mediated adhesion have been shown to differ considerably (Neumeier and Reutter, 1985). Serum depleted of fibronectin is capable of promoting the adhesion and spreading of cells (Knox and Griffiths, 1980: Neumeier and Reutter, 1985: Thom et al, 1979). Fibronectin is, according to some reports, not present in the serum layer deposited from culture medium containing 10% foetal calf serum (Curtis et al, 1983: Grinnell and Feld, 1981: Grinnell and Feld, 1982) or is present in very small quantities (Horbett and Hoffman, 1975). In contrast, fibronectin is reported to adsorb to glass or plastic substratum in greater amounts from serum than from a solution of plasma fibronectin (Haas and Culp, 1982). The biological activity of adsorbed fibronectin also is not related to the amount of adsorbed fibronectin and is surface dependent (Grinnell and Phan, 1983). However, its functional importance may start when it is complexed with other proteins of the extracellular matrix such as collagen (Doillon et al, 1985: Hedman et al, 1982: Klebe et al, 1981: Turley et al, 1985), or fibrin (Grinnell, 1978: Young et al, 1982). Its function may be to retard cell locomotion by promoting strong cell adhesion (Couchman and Rees, 1979) and thus to guide cell migration in embryonic tissue

(Geuskens et al, 1986: Nakatsuji et al, 1985: Trejdosiewicz et al, 1981), and in wound healing (Doillon et al, 1985: Kleinman et al: 1981). Fibronectin mediates cell migration in some cell lines using the same receptor which mediates cell attachment in other cell lines, although, migration is controlled by unidentified factors (Straus et al, 1989). The loss of adhesion properties is thought to be an important factor in erythroid differentiation (Patel and Lodish, 1984). It may also act as an opsonic factor to remove particulate material from circulation (Grinnell and Phan, 1983: Ryden et al, 1983).

Vitronectin has been known for some time to have adhesion-promoting properties (Barnes and Silnutzer, 1983: Barnes et al, 1980: Hayman et al, 1982a: Holmes, 1967: Stenn, 1981), and has been known by several names (serum spreading factor, α -1 serum protein, epibolin). It has been demonstrated to be present at cell surfaces and in tissues (Hayman et al. 1983). A cell surface-associated adhesive factor (un-named) was identified by Hattori et al (1984) and has a similar molecular weight to vitronectin. It has been shown that cells synthesise and release into the medium both fibronectin and vitronectin but will respond only to the latter (Barnes and Reing, 1985). A cell surface receptor for vitronectin is a vital requirement for a cell adhesion-promoting protein, and has been identified (Pytella et al, 1985). This receptor recognises the same sequence of amino acids as occurs in fibronectin, the Arg-Gly-Asp sequence (Hayman et al, 1985a: Pierschbacher and Ruoslahti, 1984: Pytella et al, 1985), but it is separate from the fibronectin receptor (Pytella et al, 1985: Ruoslahti and Pierschbacher, 1987). This indicates that vitronectin and fibronectin are recognized by cells through similar mechanisms. This sequence is also found in trigramin, a peptide from snake venom, which is a potent inhibitor of platelet binding and platelet aggregation, and demonstrates that cell adhesion may have a common origin but with many detail differences in specificity (Knudsen et al, 1988). This Arg-Gly-Asp sequence is thought to be common to many proteins (fibronectin, vitronectin, fibrinogen, collagens, thrombospondin and von Willebrand factor), and is suggested as a protein recognition factor in cells (Nandan et al, 1988: Pierschbacher and Ruoslahti, 1984: Ruoslahti and Pierschbacher, 1987: Tuszynski et al, 1989).

Fibronectin and vitronectin cause cell spreading via different mechanisms (Barnes et al, 1983: Knox

and Griffiths, 1980; Neumeier and Reutter, 1985). Vitronectin has been shown to be the more quantitatively significant spreading factor for a number of cell lines (Knox and Griffiths, 1979; Knox and Griffiths, 1980). Fibronectin causes spreading in the absence of protein synthesis, whilst vitronectin requires protein synthesis, and the kinetics of spreading are also different (Knox and Griffiths, 1980; Neumeier and Reutter, 1985; Stenn, 1981). Vitronectin seems, therefore, to have a better claim (Hayman et al, 1985b; Lydon and Foulger, 1987; Neumeier and Reutter, 1985) to being the prime factor in cell adhesion to tissue culture plasticware and prosthetic polymeric materials. Other constituents of serum can also be used in cell adhesion studies, eg. albumin can be used as a non-stimulatory control (Eberhart et al, 1982; Gendreau et al, 1982; Royce Jr et al, 1982; Young et al, 1982).

The identification of two cell-adhesion promoting proteins from serum, and the fact that they complex with other extracellular constituents (Armstrong, 1982; Doillon et al, 1985; Eberhart et al, 1982; Grinnell and Phan, 1983; Hynes and Yamada, 1982; Klebe et al, 1981; Turley et al, 1985; Yamada and Olden, 1978), indicates that the process of cell adhesion to synthetic substrata is complex. The situation *in vivo* would be expected to be more complicated due to the large variety of cell-matrix and cell-cell interactions that occur in tissues (Aberchrombie, 1982; Grinnell, 1978; Hall et al, 1988; Heaysman and Peagram, 1982; Taylor, 1970). Cultured neurons become attached to pHEMA surfaces, but few grow unless fibronectin, collagen or nerve growth factor is incorporated into the hydrogel (Carbonetto et al, 1982). The adsorption of fibronectin in a natural conformation can be enhanced by small amounts of albumin (Grinnell and Feld, 1982). Proteins have been shown to interact in artificial collagen matrices and help or compete with each other to bind (Doillon et al, 1985; Turley et al, 1985). In a study using protein solutions deposited singly, sequentially or competitively, the platelet response and thrombus deposition was strongly influenced by the sequence of protein adsorption with the first protein deposited providing the determinant factor (Pitt et al, 1986b). This complexity shows that single protein deposition studies are an invaluable step in the interpretation of studies using the cell culture model.

The ability to form stable contacts with their neighbours, with extracellular matrix and solid substrata is one of the fundamental properties of eukaryotic cells, and takes place through restricted, specialised sites along the plasma membrane (Abercrombie, 1982; Odin and Obrink, 1988). The formation of stable cell-cell contacts is a highly selective process depending on the tissue specificity of the participating cells (Vasiliev and Gelfand, 1977). The cell-cell and cell-substrate contact areas are related as they are associated with actin-containing microfilament bundles at their cytoplasmic faces (Abercrombie et al, 1971; Geiger, 1981; Hall et al, 1988; Heaysman and Pegrum, 1982; Wehland et al, 1979). The membrane components that mediate cell attachment have not all been identified but include vinculin and talin which are involved in organising the membrane-microfilament association and possibly focal contacts (Bozyczko et al, 1989; Geiger, 1981; 1982; Horwitz et al, 1985). Integrins are complexes of integral membrane proteins that appear to function as a dual receptor for both intracellular cytoskeleton and extracellular matrix components (Bozyczko et al, 1989; Hall et al, 1988; Ruoslahti and Pierschbacher, 1987; Tuszynski et al, 1989). They participate in several different associations between the cytoskeleton and the extracellular matrix.

1.13 Cell Adhesion and Extracellular Matrix

Most cells *in vivo* are in contact with an extracellular matrix. This interaction is responsible for cell proliferation and differentiation (Kleinman et al, 1981; Odin and Obrink, 1988), and influences cell motility (Trinkaus, 1976), embryonic induction (Trinkaus, 1976), phenotypic properties and maintenance of organ morphology (Odin and Obrink, 1988). Recent progress in understanding the molecular events involved in cell-substratum adhesion demonstrated that adhesion *in vitro* resembles growth conditions *in vivo*. This is the result of the purification and extensive characterization of specific adhesion-mediating factors (Hall et al, 1988; Yamada, 1983). Fibronectin and vitronectin have been identified as mediating cell adhesion to synthetic substrata when applied in isolated form (Neumeier and Reutter, 1985), but other proteins are also involved *in vivo* such as laminin and chondronectin (Yamada and Olden, 1978).

Collagen substrates enhance the growth (Gospodarowicz and Ill, 1980; Wicha et al, 1979) as well as differentiation (Gospodarowicz et al, 1979) of many cells in culture above that observed with other substrates such as plastic and glass (Elsdale and Bard, 1972; Gospodarowicz et al, 1979; Kleinman et al, 1981; Murray et al, 1978; Wicha et al, 1978). Many cultured cells are thought not to bind directly to the collagen substrate or to the plastic substratum (Grinnell, 1978; Hayman et al, 1985a; Hook et al, 1977; Terranova et al, 1980). Instead, extracellular glycoproteins bind the cells to the substrate. Experiments with fibronectin fragments indicate that cell attachment involves only binding to cell-surface integrin and proteoglycan, rather than the mediation of a collagen/fibronectin/proteoglycan matrix secreted and deposited by fibroblasts (Hall et al, 1988). The components of the extracellular matrices of fibroblasts, chondrocytes and epithelial cells differ with the cell type, and they require separate attachment proteins to provide additional specificity to the interaction of the cell with its matrix (Kleinman et al, 1981). This specificity is an important, integral part of organogenesis, although the recognition mechanisms are thought to be related (Hayman et al, 1985a; Pierschbacher and Ruoslahti, 1984; Pytella et al, 1985). Alterations in cell-substratum interactions are observed in differentiating cells and after spontaneous transformation of cells or exposure to oncogenic agents (Hynes, 1976; Vaheri et al, 1978). Tumour promoting phorbol esters have been shown to enhance the attachment of fibroblasts to laminin and type IV collagen, but has little or no effect on attachment to fibronectin and type I collagen (Kato et al, 1988). Cell spreading in collagen gels appears to use a different mechanism from cell spreading on collagen coated dishes, and this will depend on the topographical organisation of the collagen (Grinnell et al, 1989). The organisation of collagen gels influences the growth of fibroblasts and their response to growth factors (Nakagawa et al, 1989).

Fibronectin is a large glycoprotein which is implicated in a wide variety of cellular properties, particularly those involving the interactions of cells with extracellular material (Hynes and Yamada, 1982; Klebe et al, 1981; Kleinman et al, 1981; Yamada and Olden, 1978). These are cell adhesion, morphology, migration, differentiation and cytoskeletal organisation. Fibronectin has a complex molecular structure consisting of multiple specific binding sites which allows the molecule to participate in numerous complex biological phenomena. It is involved with cell adhesion, and possess

binding sites to both cells, collagen and proteoglycans, and is also involved in the blood clotting process, possessing sites for fibrin, factor XIII and heparin (Hall et al, 1988: Yamada, 1983). It has two forms; soluble plasma fibronectin and cellular fibronectin which appears as a fibrillar extracellular protein. The fibrils can also contain collagens and proteoglycans (Kleinman et al, 1981: Yamada et al, 1980). It is possible that interactions between fibronectin and other matrix molecules such as glycosaminoglycans, are important in the formation of these fibrils (Hayman et al, 1982b: Hedman et al, 1982: Laterra et al, 1983: Yamada et al, 1980). Heparin and chondroitin sulphate proteoglycans are thought to be integral components of extracellular matrix, and the association with the fibronectin-procollagen matrix is stabilised by multiple molecular interactions (Hedman et al, 1982: Laterra et al, 1983). A heparin sulphate-rich proteoglycan has been localised at the cell surface where it is thought to be involved in anchoring cells directly to the extracellular matrix (Rapraeger and Bernfield, 1985). Glycosaminoglycans have been implicated in starting cell movement during the early stages of development (Trinkaus, 1976). They are involved with cell-cell interactions and are thought to be responsible for the specificity of cell attachment reactions (Schubert and LaCorbiere, 1980). Plasma fibronectin has been reported to mediate the adhesion of cells to substrata (Grinnell, 1978: Hynes and Yamada, 1982: Klebe et al, 1981: Neumeier and Reutter, 1985: Yamada and Olden, 1978), although more recent studies have suggested that other serum proteins may be more important (Hayman et al, 1985b: Lydon and Foulger, 1987: Neumeier and Reutter, 1985). Plasma fibronectin stimulates macrophages to engulf particles coated with gelatin (Kleinman et al, 1981) as well as bacteria (Ryden et al, 1983: Saba and Jaffe, 1980).

Laminin is a cell adhesive protein with a specific function to anchor epithelial cells to basement membrane (Terranova et al, 1980). It is a large protein of two subunits which contain binding regions specific for type IV collagen and epithelial cells. It seems to function by the combined action of independent cell-, collagen- and heparin-binding domains on each molecule. Fibroblasts have been shown to possess a laminin receptor which is distinct from the fibronectin receptor (Couchman et al, 1983). Hepatocytes also possess receptors for laminin which is thought to be involved in liver regeneration. Cells from normal liver attach poorly to surfaces coated with laminin whilst cells

obtained from regenerating liver were shown to attach efficiently to surfaces coated with either fibronectin or laminin (Carlsson et al, 1981). Nerve cells have also been shown to respond to laminin as nerve growth factor-dependent neurite outgrowth is greatly enhanced by substratum bound laminin (Doherty et al, 1988).

Chondronectin has a similar role for mediating the adhesion of chondrocytes to type II collagen, and displays minimal activity for fibroblasts (Yamada, 1983). Vitronectin is a glycoprotein family with reported molecular weights of 65,000 and 75,000 (Barnes and Silnutzer, 1983), which are immunologically related (Barnes et al, 1983). It is found at cell surfaces, as a constituent of extracellular matrices and in blood, amniotic fluid and urine (Barnes and Reing, 1985; Hattori et al, 1984). Cells can also interact directly with several types of collagen and with hyaluronic acid by means of plasma membrane binding sites, apparently without the need for an intermediary molecule. It is likely that cells can interact with extracellular matrix by multiple mechanisms even within the same cell (Gjessing and Seglen, 1980; Kleinman et al, 1981; Yamada, 1983). Hepatocytes have been shown to interact directly with fibronectin, vitronectin, collagen and laminin (Neumeier and Reutter, 1985; Rubin et al, 1981). PC12 nerve cells have been demonstrated to possess receptors for laminin and various collagens but not fibronectin (Doherty et al, 1988).

1.14 Cell Adhesion on Biomaterials

Cell attachment and spreading of cultured cells have been used as a practical test for the biocompatibility of potential biomaterials (Bentley and Klebe, 1985; Lydon, 1986). Fibroblast spreading was used to evaluate potential bioactive glasses, which showed that the initial events were independent of the bioglass composition (Häkkinen et al, 1988). Fibroblast outgrowth from rat tendon tissue has been used as an *in vitro* model for assessing the biocompatibility of Kevlar fibres used as reinforcement in ligament and tendon tissue repair (Zimmerman and Gordon, 1988). Model substrates have been prepared with differing surface properties, particularly wettability, which are then tested in cell culture to assess their effect on cell attachment and growth (Kang et al, 1989).

Immobilisation of heparin onto surfaces has been used to render biomaterials blood compatible (Akashi et al, 1989; Hoffman, 1974; Horbett and Hoffman, 1975; Ito et al, 1989; Park et al, 1988; Shiomi et al, 1989). Hydrogels are used in areas of blood compatibility as cells tend not to adhere due to the high water content. Hydrogel coatings for hydrophobic silicone rubber results in less thrombus build-up than the native silicone surface (Seifert and Greer, 1985). The nonthrombogenic activity of endothelium is accomplished in part by prostoglandin-I₂. Prostoglandin-I₂ and heparin have been used, crosslinked to polyacrylamide hydrogels, to prevent the aggregation of platelets to PVC (Akashi et al, 1989). The mechanism is thought to be due to the release of the heparin from the hydrogel network (Akashi et al, 1989). Vitamin C has also been shown to prolong blood clotting time by reducing the number of platelets adhering to the surface (Chandy and Sharma, 1983). This effect is thought to be, in part, due to the vitamin C complexing with calcium ions, which are essential for the clotting process (Chandy and Sharma, 1985).

Cell growth on hydrogels can be promoted by the incorporation of extracellular matrix proteins (Carbonetto et al, 1982). No significant cell growth occurs on polyHEMA, whereas cell growth shows a dose-response curve expressing maximal cell growth against collagen concentration (Civerchia-Perez et al, 1980). Endothelial cells have been used as linings for vascular prostheses to promote blood compatibility to PTFE grafts (Kadletz et al, 1987) and Dacron fabric grafts (Eskin et al, 1978). A composite material comprising PTFE and perfluorosulphonic acid has been proposed as a vascular graft material allowing the attachment of endothelial cells to ensure good blood compatibility properties (McAuslan et al, 1988). Successful implantation of dialysis catheters is associated with infiltration by collagen fibrils overlain by layered connective tissue composed of fibroblasts and collagen fibre bundles (Gregory et al, 1985). Collagen coatings have been used to allow successful implantation of fabric arterial prostheses, although not all these coatings have been successful (Guidoin et al, 1989; Marois et al, 1989).

1.15 Cell Adhesion and Surface Energy

Previous work by our group and others has led to an overview of the relationships between chemical group expression (Curtis et al, 1983: Gibson and Bailey, 1980: Klemperer and Knox, 1977: Maroudas, 1973: Thomas et al, 1986), surface free energy (fractional polarity) (Clay et al, 1987: Lydon et al, 1985: Thomas et al, 1986: van der Valk et al, 1983) and cell adhesion. The surface energy of the substratum is clearly important in triggering interface conversion (Clay et al, 1987: Lydon et al, 1985: van der Valk et al, 1983), but is not the sole criterion since the expression of chemical groups at the surface does have a part to play (Curtis et al, 1983: Lydon et al, 1985: Minett et al, 1984: Thomas et al, 1986). The process of molecular deposition at a synthetic/biological interface is likely to involve similar forces to those occurring in conventional adhesion events (Baier, 1970: Hoffman, 1974: Vroman et al, 1971: Weiss, 1970).

1.16 Bacterial Adhesion

The adhesion of many other cells types, especially bacteria, shows many similarities to the adhesion of mammalian cells. Initial attachment in all instances appears to be determined by the physicochemical balance of the substratum material surface and the adhering cell, but after that differences occur. Mammalian cells use protein adhesion molecules with very specific recognition parameters such that different cell types can recognise each other and their environment which is an essential prerequisite for organogenesis and differentiation. Protein adhesion is also a mechanism thought to be used by some, but certainly not by all, bacteria. Many cell types use exopolysaccharide materials for attachment purposes which is a mechanism not used by mammalian cells. Thus, a study of cell adhesion using mammalian culture cells will be applicable in many ways to the attachment of other cell types, particularly in the role played by the physicochemical properties of the polymeric substratum material.

Bacterial adhesion to a surface is thought to be an essential step in the infection process (Costerton

et al, 1978: Fujioka-Hirai et al, 1986: Miedzobrodzki et al, 1989: Peters et al, 1981). Bacterial slimes are thought to be polysaccharide in nature, although inorganic "hold-fast" materials may also be used (Corpe, 1970: Costerton et al, 1978). Staphylococcal strains which are good producers of exopolysaccharide show greater colonisation of implants than strains which are poor producers of exopolysaccharide (Barth et al, 1989). Slime mediated adherence is thought to be a critical factor in the pathogenesis of *Staphylococcus epidermis* infections of medical devices (Christensen et al, 1982), and in mastitis for pathogenic *Staphylococcus aureus* (Johne et al, 1989). However, exopolysaccharides seem to have little impact on the adhesion of *Staphylococcus aureus* to biomaterial surfaces (Falcieri et al, 1987), although their importance may be in masking surface proteins (Johne et al, 1989). The adhesion of bacteria to surfaces may be protein mediated in some circumstances (Bar-Ness and Rosenberg, 1989: Costerton et al, 1978: Corpe, 1970: Scott et al, 1989). The formation of pellicle on teeth is a precursor to bacterial colonisation of dentin (Bjorvatn, 1986: Gibbons and van Honte, 1980), and is thought to consist primarily of glycoproteins and is essentially free of bacteria. The proteinaceous film deposited *in vivo* is thought to facilitate the chemical interactions with exopolysaccharide (Barth et al, 1989), although the chemical interactions with biomaterials are probably non-specific (Christensen, 1987).

Implanted foreign bodies are considerably more prone to infection than routine surgical procedures, and are less susceptible to treatment by antibiotics (Barth et al, 1989: Christensen, 1987: Gristina, 1987: Vaudaux et al, 1984b). The bacterial exopolysaccharide produced by *Staphylococcus epidermis* appears to offer an effective protection against host defences (Barth et al, 1989: Brown and Williams, 1985: Christensen, 1987: Gray et al, 1984: Johnson et al, 1986: Peters et al, 1981). Fibronectin has been shown to promote bacterial adhesion to implanted/explanted coverslips (Vaudaux et al, 1984a), and is thought to have an opsonic role *in vivo* (Lanser and Saba, 1981), although this is now in dispute (van der Water et al, 1983: Verburgh et al, 1981). A fibronectin-binding protein has been identified in *Staphylococcus aureus* (Espersen and Clemmensen, 1982: Maxe et al, 1986: Ryden et al, 1983), but other proteins may be involved including collagen, immunoglobulin, fibrinogen or C3b, a component of complement (Miedzobrodzki et al, 1989: Ryden et al, 1983: Verburgh et al, 1981). S.

aureus has also been shown to possess cell surface receptors for collagen (Speziale et al, 1986; Voytek et al, 1988). Bacteria have been shown to specifically attach to endothelial cells and fibronectin coated surfaces, which suggests that the ability to attach to these substrates may reflect the propensity to invade and colonise vascular tissues (Vercellotti et al, 1984).

The bacterial colonisation on metal implant materials has been shown to differ in terms of species and bacterial types, with *Staphylococcus aureus* predominating. The colonisation found on polymeric implants is dominated by coagulase-negative *Staphylococcus epidermis* (Barth et al, 1989). This effect was determined *in vitro* and *in vivo* with PMMA, polyethylene and titanium. Non-enzymatic degradation of nominally stable polymers is thought to result in depolymerisation products which stimulate production of exopolysaccharides which cement bacteria together (Phua et al, 1987). Bacterial adhesion is largely determined by the balance between the surface free energy of the medium, of the solid substrata and of the bacteria (Barth et al, 1989; Ludwicka et al, 1984). Hydrophobic interaction has been shown to play an important role in microbial attachment (Bar-Ness and Rosenberg, 1989; Nesbitt et al, 1982), and can be blocked by the use of surfactants (Humphries et al, 1986). In *Serratia marcescens*, cell surface hydrophobicity is thought to be due to the involvement of a 70 kDa protein (Bar-Ness and Rosenberg, 1989).

A major characteristic of many fouling organisms, whether bacteria, algae or diatoms, is the ability to become permanently attached to surfaces. Attachment is invariably associated with the extracellular mucilaginous substances which are almost exclusively polysaccharide in composition (Daniel et al, 1987). The presence of proteins has been shown to impair the adhesion of marine bacteria to polystyrene (Fletcher, 1976). The adhesion of plant cells to polymers for use in bioreactors is thought to be similar to bacterial cells, with initial attachment due to physicochemical interactions and firm retention due to the secretion of extracellular proteinaceous and polysaccharide adhesive materials (Facchini et al, 1989; Robins et al: 1986).

CHAPTER 2

REVIEW OF SURFACE ANALYSIS TECHNIQUES

REVIEW OF SURFACE ANALYSIS TECHNIQUES

2.1 Introduction

In order to better understand the process of protein deposition and interface conversion of a synthetic surface, it is necessary to be able to analyze the nature, composition and state of the synthetic/biological interface. To this end we have begun a series of studies of polymer surface properties in order to gain a better understanding of the interplay between various factors involved in promoting the deposition of biological molecules. Our overall aim is to study the key molecular interactions which govern the establishment of a stable biological/synthetic interface. As part of this approach we have identified several methods for the *in situ* analysis of polymer surface properties and of adsorbed layers, as the interactions within these layers determine the nature of the interface.

Most of these instrumental techniques have been developed by materials scientists and will need to be adapted for application to biological samples. These surface analytical techniques will be investigated to assess their usefulness, practicality, and the information they can provide. This can be achieved partly by studying the theoretical aspects of the techniques, especially the mechanisms involved in signal formation and the levels of detection and sensitivity, to assess the information obtained and its potential usefulness. From a practical aspect the techniques can be tested by presenting them with model and real samples of deposited biological layers on polymer surfaces.

There are several extensive reviews, although none is very recent, of these techniques for the technical aspect (Larrabee, 1977), the application to polymeric materials (Briggs, 1982a; Lichtman, 1975), and for the biomaterials implications (Ratner, 1982; Sakurai et al, 1980). It is pertinent, however, to review the techniques considering the information obtainable, the potential application to our studies and their obvious limitations. Those techniques which are identified as potentially proving useful will then be investigated further in order to see if they can aid in elucidating the problems of the biological/synthetic interface.

Traditionally, techniques are separated into microscopic and spectroscopic techniques. Such a distinction is rapidly becoming more difficult as some spectroscopic techniques have been developed to give such high degrees of spatial resolution that they should be considered microscopic (Briggs, 1985). There is an important distinction to be made between those techniques which can produce an image and those which cannot, due to spatial inhomogeneities. Non-imaging techniques, therefore, have a built-in oversimplification which can lead to misinterpretation of the information they produce.

It is usually the case in the adhesion technology field that adhesion problems are complex and are unlikely to be solved by the application of a single analytical technique (Briggs, 1982a). This is also likely to be the case when studying the interaction of biomolecules with synthetic materials. Combination of techniques is not considered in detail here, but is routinely favoured and enables complementary information to be accumulated, which is invaluable in elucidating the overall picture. Technique combination is becoming the norm amongst the ultra-high vacuum techniques as a combination excitation and/or detection capabilities can provide many types of surface or subsurface information. This also saves money by the use of a single vacuum system. A common combination of techniques is to use X-ray photoelectron spectroscopy for surface chemical characterisation and scanning electron microscopy for surface morphological study (Briggs, 1985; Dwight, 1977). Together they form the most cost effective approach to adhesion problem solving and both are widely available.

2.2 Microscopy

2.2.1 Electron Microscopy

Scanning electron microscopy (SEM) is an electron-probe imaging technique which can be used to give topographical and compositional views of surfaces. It is by far the most powerful tool for obtaining surface topographical information. It works by focusing an electron beam into a fine probe (typically 5-100nm) which is scanned in raster fashion over the sample. When an electron beam strikes a solid object it undergoes a wide variety of interactions with the sample leading to the emission of electrons, X-ray and light photons (Figure 2.1). With the scanning electron microscope,

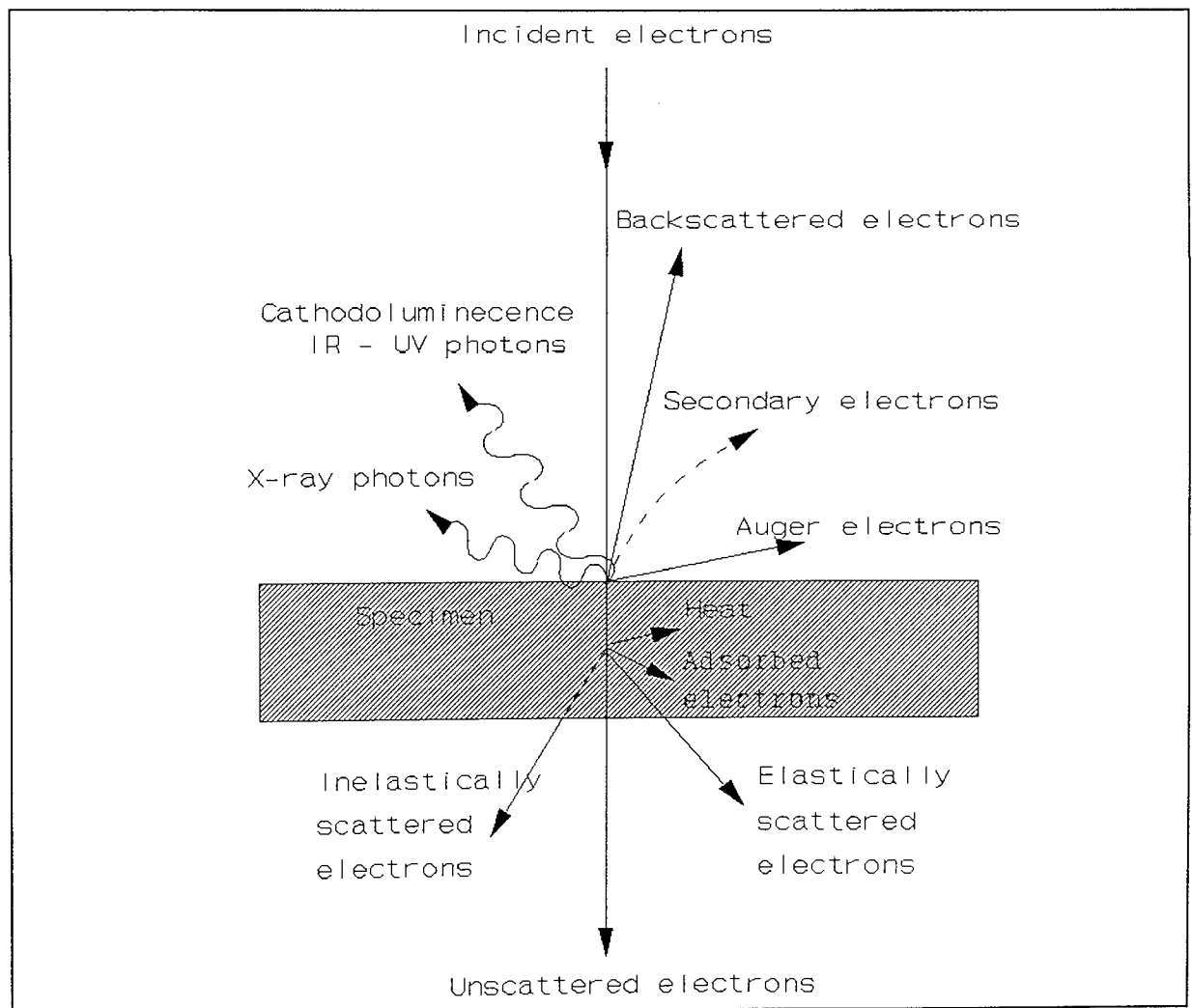


Figure 2.1 Multiple signals are generated in the electron microscope by the interaction of the beam with the sample

the sample is effectively infinitely thick so that no electrons will transmit through. The commonest used signal in the SEM is the secondarily excited electrons which have a low energy and only leave the sample, and can be detected, when they are generated near the surface. The contrast of the image is strongly influenced by the surface topography of the sample. This explains the pseudo-three dimensional images which are normally associated with the scanning electron microscope.

In this way the scanning electron microscope can provide an insight into the topographical nature of biomaterials and cell culture substrata (Baier, 1977: Baier et al, 1984: Kowalski, 1982: Lydon and Clay, 1985: Minett et al, 1984: Thomas et al, 1986: Veronese et al, 1989: Yasin et al, 1989). It can also be used to study the reaction of tissue to implanted materials, such as the contact area of artificial lenses and suture materials (Figueras et al, 1986: Jongebloed et al, 1986), and intrauterine contraceptive devices (Khan and Wilkinson, 1985). SEM has been used to study the status of Hydromer linings coated on polyurethane, and has shown problems with damage and defects to the coating which compromise its intended role (Bylock et al, 1986). In a study of the implantation failure of dialysis catheters, SEM was able to show that failure was associated with continual bleeding (Gregory et al, 1985). SEM is commonly used to assess blood compatible biomaterials by detecting the presence and amount of thrombus formation (Eskin et al, 1978: Marois et al, 1989: Seifert and Greer, 1985: Therrien et al, 1989), or by studying the interaction of platelets or leucocytes with biomaterials (Kottke-Marchant et al, 1989: Miyamoto et al, 1989: Okkema et al, 1989; Park et al, 1986b: Pitt et al, 1986b). SEM examination has also shown a degradation of polyurethane vascular prostheses after long term implantation (Therrien et al, 1989). It is widely used in dental research to examine dentine and enamel surfaces, and to study pellicle and plaque formation (Baier, 1982: 1984: Bjorvatn, 1986: Gibbons and van Honte, 1980), and implants (Kawakami et al, 1987). SEM has demonstrated that infected intravenous catheters are heavily colonised with bacteria, and that the organisms are closely packed and cemented in place by an amorphous substance, which may play a protective role against host defence mechanisms (Brown and Williams, 1985: Peters et al, 1981).

The scanning electron microscope can also be used, because of its large magnification range and

its ability to collect numerous signals, to investigate the relationship between cell behaviour and the underlying substratum (Baier, 1977: Goodman et al, 1989: Hughes et al, 1979: Humalda et al, 1986: Lydon and Clay, 1985: Maruyama et al, 1988: Mattson, 1985: Miller et al, 1989: Schakenraad et al, 1989). The rate and morphology, as seen by SEM, of fibroblast outgrowth from rat tendon tissue has been used as an *in vitro* test of the biocompatibility of kevlar fibres (Zimmerman and Gordon, 1988). The arrangement of proteins can be investigated using immunogold techniques (Murthy et al, 1987: Park et al, 1986a: 1986b: Pitt et al, 1986b), and has also been used to investigate cell surface-associated proteins in cells (Geuskens et al, 1986: Hattori et al, 1984: Trejdosiewicz et al, 1981) and tissues (Nakatsuji et al, 1985: Trejdosiewicz et al, 1981). SEM has also been used to study cell behaviour and morphology in culture in response to material surfaces (Gershman and Rosen, 1978: Miller et al, 1989: Schakenraad, et al, 1989: Tanzawa et al, 1980: van Wachem et al, 1989: Vasiliev and Gelfand, 1977). This includes the process of cell spreading (Vasiliev and Gelfand, 1977), the cell spreading patterns on deposited protein layers (Hughes et al, 1979: Lydon and Clay, 1985) and mechanisms of cell movement (Trinkaus, 1976: Vasiliev and Gelfand, 1977). The morphology of monocytes on biomaterial surfaces has been shown to be indicative of their state of activation (Miller et al, 1989).

The theory that mechanical keying is important in increasing the strength of adhesive bonding is well supported by scanning electron microscope characterisation of the surface topography of metals after pretreatments (Chen et al, 1978: Cotter and Mahoon, 1982: Evans and Packham, 1979: Mulville and Vaisnav, 1975: Packham, 1983). In the pretreatment of steel, surface coatings have been studied by SEM, and a link between coating microcrystallinity and adhesion strength demonstrated (Trawinski et al, 1984). A fibrillar structure of oxide whiskers have been demonstrated by SEM, and are thought to be necessary for good adhesive bonding to aluminium (Chen et al, 1978: Evans and Packham, 1979), copper (Dwight, 1977) and titanium (Ditchek et al, 1980). Porous surface oxide layers are seen by SEM which enable interlocking to contribute to a strong adhesive joint with titanium (Ditchek et al, 1980) and aluminium (Venables et al, 1978). SEM has also been used to demonstrate the converse situation where smooth surfaces are associated with increased adhesive strength showing that

mechanisms other than mechanical keying are involved (Dukes and Kinloch, 1977). SEM has been used to determine the locus of failure of adhesive joints (Knock and Locke, 1983).

Transmission electron microscopy (TEM) is not primarily a surface technique as samples must be of the order of 100nm thick. Applying sectioning techniques, it can be used for a more detailed study of the molecular events of cell attachment and cell spreading, and of the intracellular processes involved (Abercrombie, 1982: Heaysman, and Peagram, 1982: Revel and Wolken, 1973: Taylor, 1970: van Wachem et al, 1989: Vasiliev and Gelfand, 1977). TEM has also been used to study extracellular matrix and artificial collagen matrices (Elsdale and Bard, 1972). Focal contacts have been studied in sectioned culture cells, as has their relationship with the cytoskeleton (Abercrombie et al, 1971: Vasiliev and Gelfand, 1977). The points of cell-cell contacts, desmosomes and gap junctions, were discovered during the study of sections of tissue (Curtis, 1977: Trinkaus, 1976). The events of thrombus formation have been studied by examining sectioned blood-foreign material interactions (Dutton et al, 1969: Fumagalli et al, 1987). Thrombin has been localised on the surface of platelets using high resolution electron microscopic autoradiography (Tollefsen et al, 1974). TEM has been used to demonstrate that cells grown on collagen hydrogels have similar ultrastructure to those grown on tissue culture substrata (Toselli et al, 1983). Investigations into the nature of bacterial adhesion phenomena have also relied on sectioned TEM materials (Costerton et al, 1978: Falcieri et al, 1987: Johne et al, 1989: Scott et al, 1989: Vercellotti et al, 1984).

High-voltage electron microscopy (HVEM) is a development of transmission electron microscopy and has been used to study cytoskeletal organisation in platelets without sectioning by allowing examination of whole cells and correlating this with scanning electron microscope examination (Goodman et al, 1989). It has also been used to study the cytoskeleton of fibroblasts in the study of adhesion contacts (Heath and Dunn, 1978). Surface detail can be studied using replica techniques, in the transmission electron microscope whereby a thin carbon/platinum replica is produced (Revel and Wolken, 1973). The resolution offered by transmission electron microscopy of sectioned or negatively stained material is of obvious benefit in the study of the detail events and mechanisms of

bacterial adhesion (Costerton et al, 1978: Gibbons and van Honte, 1980: Willcox et al, 1989).

2.2.2 Electron Probe Microanalysis

Electron probe X-ray microanalysis (EPMA) is a scanning electron microscope technique in which the X-rays generated in the sample by the electron beam are detected. Analysis of the X-ray energies provides elemental information of the samples which can include synthetic surfaces and deposited biological layers (Chandler, 1979). It has some restrictions in the biological field owing to its poor sensitivity to elements of low atomic number as are abundant in biochemical molecules (Cheetham et al, 1984).

When electrons strike an object X-rays are produced by the ionisation events which take place within the atoms making up that material (Figure 2.2). Primary electrons from the beam interact with the

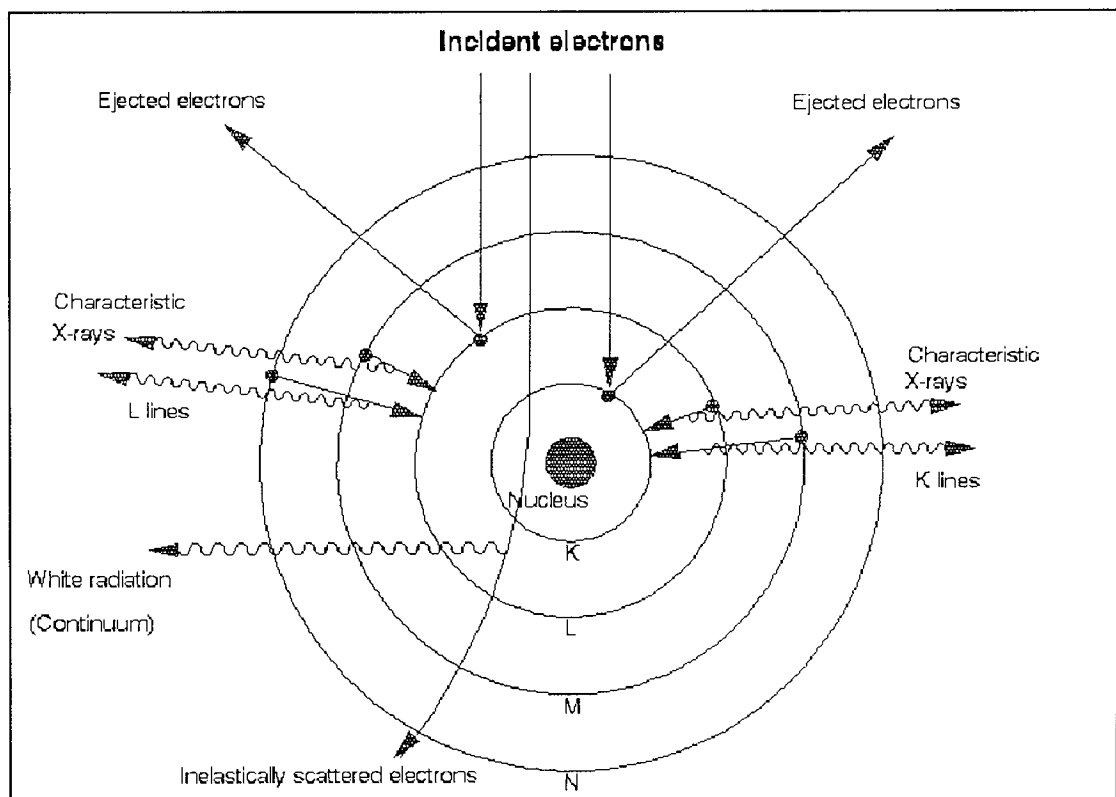


Figure 2.2 Ionisation events following the impact of a primary beam electron with an atom showing generation of X-rays

sample atoms by ejecting an inner shell electron from the atom, and outer shell electrons lose energy

to occupy the vacancy created in the inner shell. This energy loss can take one of two forms; either by the emission of a low energy Auger electron, as in Auger electron spectroscopy, or by the emission of X-ray photons, which are detected radiation in X-ray microanalysis. In both cases the energy of the emitted radiation is specific for the element concerned, being equal to the energy difference between the electron shells involved in the relaxation process. In practice, vacancies may be created in any of the electron orbitals contained within that atom. Thus, elements can be identified by determining the energy at which peaks occur in the X-ray spectrum. Measurement of the height of a given peak above the background level provides an estimate of the elements concentration, and quantitative data is established by comparing this value with that obtained from a standard containing a known concentration of the element.

By using a scanned electron probe and detecting the emitted X-ray spectra by X-ray microanalysis, an elemental map can be produced from either thin sections of the sample or from the top surface of bulk material. As at least two electron orbitals are required for the relaxation process to result in the generation of X-rays, then elements such as hydrogen and helium can never be detected by this technique; and lithium and beryllium have X-ray energies which are so low that they are absorbed in the specimen and detector. The generation efficiency of X-rays is strongly dependent on atomic number which is a problem with biological X-ray microanalysis since the elements most commonly found in biological molecules (carbon, nitrogen and oxygen) are difficult to detect and require expensive windowless detectors. Quantitative analysis is, in practice, limited to elements with atomic numbers of 11 (sodium) or higher, when a high degree of accuracy is achievable (Newbury et al, 1986). Proteins do contain elements such as phosphorus and sulphur which are detectable by the commonly available energy dispersive spectrometers. X-ray microanalysis has been used to demonstrate the calcification of intrauterine devices with long term use (Khan and Wilkinson, 1985) and the patency of dental implants (Kawakami et al, 1987).

Spatial resolution:	x/y:	1.5-5nm (SEM)
		1nm (TEM)
	z:	1-5nm
Information:	Topography	
	Morphology	
	Elemental distribution (X-ray microanalysis)	
Sensitivity (EPMA):		10^{-16} g 10^3 ppm

2.2.3 Light Microscopy

Interference microscopy has been used along with TEM to show that there are two classes of cell adhesive sites (Curtis, 1964: Geiger, 1979: Izzard and Lochner, 1976: 1980). Focal contacts are where the apparent distance between cells and the substrate is 10-15nm and produce interference-reflection dark images (Couchman and Rees, 1978: Segel et al, 1983: Wehland et al, 1979). Close contacts are somewhat less firm attachments (30nm distance between cell and substrate) that produce grey interference-reflection images (Izzard and Lochner, 1976: 1980). These focal contacts are seen to have a radial arrangement near the cell periphery when cells undergo spreading (Grinnell, 1978: Vasiliev and Gelfand, 1977). In the initial stages of cell spreading the ventral cell membrane forms a nearly uniform belt of close contact at the periphery, with radial patches of focal contact being detected subsequently (Segel et al, 1983). When the cell starts to move and acquires a polar shape the periodicity is lost, and the typical pattern of contact sites is obtained (Geiger, 1982: Heath and Dunn, 1978: Heaysman and Pegrum, 1982: Izzard and Lochner, 1976: 1980: Wehland et al, 1979).

Immunofluorescence light microscopy has also been used to study cell focal contacts by labelling the proteins involved, notably vincullin, integrin or the intracellular microfilaments (Bozyczko et al, 1989: Couchman and Rees, 1979: Couchman et al, 1983: Geiger, 1979: 1981: 1982: Wehland et al, 1979). It has also been used to study the distribution of adhesion-promoting proteins from extracellular matrix (Couchman et al, 1983: Hayman et al, 1982a: 1983: Hedman et al, 1982: O'Shea and Dixit, 1988). O'Neill et al (1990) used fluorescently labelled vinculin to measure the area of focal contacts and determine their distribution on cells grown on adhesive islands of varying shapes and sizes. They showed that the total area of focal contacts was more important than their arrangement.

Harris (1973) has used time-lapse cinemicrography and micromanipulation to localise cell adhesions and to study cell adhesion behaviour. The light microscope has been extensively used in cell biology, and of particular relevance to the study of cell adhesion has been the study of cell movement (Trinkaus, 1976) and cell growth (Carbonetto et al, 1982: Civerchia-Perez et al, 1980: Curtis et al,

1983). In the biomaterials field the light microscope is commonly used to study thrombus formation on foreign material surfaces (Dutton et al, 1969; Marois et al, 1989). Fluorescence microscopy has been used to identify, by immunological techniques, that the deposits on contact lenses are composed, at least in part, of normal tear proteins (Gudmundsson, et al, 1985). Fluorescence microscopy has also been used to show that proteins such as albumin and lysozyme can penetrate into hydrogel matrices (Refojo and Leong, 1979).

2.2.4 Confocal Light Microscopy

The resolving power of the light microscope is controlled primarily by the wavelength of the illumination used and the numerical aperture of the objective.

$$R = \frac{\lambda}{2NA \cdot k}$$

where λ = wavelength of illumination

NA = numerical aperture

k = constant depending on the coherence of illumination

of

The numerical aperture is in turn defined by:

$$NA = \eta \sin \theta$$

where η is the refractive index of the medium of the space between the specimen and the objective lens

θ is the half-angular aperture of the objective.

Thus, the resolution is optimised if coherent illumination of a short wavelength is used with an objective of large numerical aperture in a highly refractive index medium. With an oil immersion lens of NA 1.4 working with monochromatic blue light, a resolving power of 150nm is possible. In the confocal light microscope the diffraction limitations to resolving power do not apply, with the result that an improvement in resolution by a factor of up to 2 is possible.

In the confocal scanning light microscope, source and detector apertures are located in conjugate focal planes. In this way, the detector is preferentially exposed to light emanating from the focal region in the specimen. Contrast is improved by the rejection of light from out-of-focus planes. By reducing the field of view to a small spot the field of interest must be viewed by scanning the spot of light in some way. This is done either by moving the specimen in X and Y, by scanning the optical system or by scanning the focused light by using galvanic mirrors. The lateral resolution and the thickness of the optical section are dictated by the properties of the spot of light that is imaged at the detector, which is controlled by three factors:

The wavelength of light; the shorter this is the smaller the spot

The Numerical Aperture (NA) of the objective lens; higher NA for a smaller spot size

The size of the confocal aperture, which determines the amount of information reaching the detector.

The increased power of the technique is considerable and largely due to the improved contrast images available. The advantages of the technique have been considerably well explored by biologists in tissue studies using fluorescent staining techniques (Boyde et al, 1990; Dixon and Benham, 1988; White et al, 1987). However, it has seen use by surface scientists in the semiconductor field. Its potential for studying the biological/synthetic interface is considerable but yet to be developed, which will no doubt be rectified with the increasing availability of the instruments.

Spatial resolution:	x/y:	150-200nm
	z:	10nm
Information:	Morphology	
	Composition	

2.3 Surface Energy Measurements

Contact angle measurements allow the surface energy of a substance to be measured and separated into the dispersive and polar fractions which are thought to have different roles to play in interface conversion (Lydon et al, 1985; van der Valk et al, 1983). Contact angle measurements are very sensitive to the first few atomic layers of the surface. The wetting of one substance with another can be described in terms of thermodynamic parameters, such as surface and interfacial free energy, which characterise the interacting materials. These surface properties are determined by the surface chemistries, and the use of contact angle measurements are important in developing an understanding of the nature and strength of interfacial interactions (Rance, 1982). With careful interpretation contact angles can also provide information about the morphology of solid surfaces, surface roughness, crystallinity and orientation (Rance, 1985).

2.3.1 Surface Energy

The surface chemistry of materials is a key factor in defining their adhesive behaviour, and involves a knowledge of the degree of wetting of the material surface, the rate of wetting and the interactions across the interface. The surface energies of the components will be determined by their chemistries and will affect the degree and rate of wetting. It is obviously important that the components are brought into as close a contact as possible. Thus, a substrate that is wetted by the mobile phase is of paramount importance, and leads to concern about the surface energies which are given by Young's equation for the surface energy of solids:

$$\gamma_{sl} = \gamma_{sv} + \gamma_{lv}$$

The surface chemistries affect the degree of contact involved, and also determine the magnitude of the interaction where contact has occurred, ie. whether chemical bonding, hydrogen bonding or van

der Waals' forces are involved. Whether or not surface contaminants can be displaced will also depend on the chemistry of the adhesive.

Molecules in the surface region of a material are subject to attractive forces from adjacent molecules which result in a net attraction into the bulk phase. The attraction tends to reduce the number of molecules in the surface region and this results in a surface tension. The remaining unsatisfied bonds at the surface result in a surface free energy.

The intermolecular attractions which cause surface tension result from a variety of well known intermolecular forces: hydrogen bond, dispersion forces, hydrophobic bond, etc. The London dispersion forces arise from the interaction of fluctuating electronic dipoles with induced dipoles in neighbouring non-polar atoms and molecules. Debye forces are caused by a molecule with a permanent dipole inducing a dipole in a neighbouring molecule by polarisation, and Keesom forces arise between molecules with permanent dipoles. The London forces are ubiquitous and account for a major part, if not all, of the strength of polymers such as polyethylene. These forces act over only short distances, typically 0.5-10nm and certainly no greater than 20nm (Rance, 1982).

The surface free energy is a sum of its component contributions from the different intermolecular forces at the surface (Fowkes, 1964). Thus, the surface free energy of water could be written:

$$\gamma_M = \gamma_I^p + \gamma_I^d$$

where p and d refer to the hydrogen bonding (polar) and dispersion force components.

This, however, is considering the ideal situation of a true surface in a vacuum; a more common, and relevant, occurrence is the interaction of two substances across an interface. Let us consider an interface between two liquids which interact solely through dispersion forces (Fowkes, 1964). The interface is composed of two adjacent interfacial regions. The interfacial tension will be the sum of the various tensions between these regions.

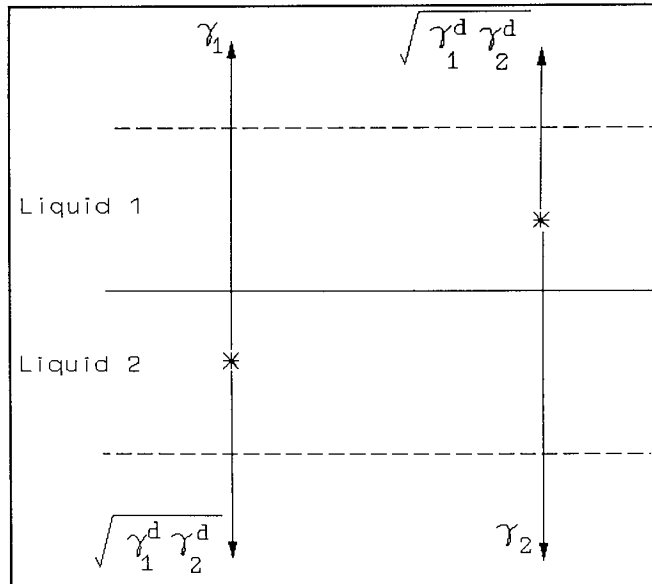


Figure 2.3 Forces acting at an interface

The forces acting on the molecules at an interface are shown in Figure 2.3. The bulk liquid 1 produces a surface tension γ_1 on molecules in its own interface region. However, there will also be an attraction for these molecules from those of liquid 2. The effect of the interfacial attraction can be predicted by the geometric mean of the dispersion components of the surface tension of the two liquids:

$$\sqrt{\gamma_1^d \gamma_2^d}$$

Thus the tensions in the interfacial region of liquids 1 and 2 are given by:

$$\gamma_1 - \sqrt{\gamma_1^d \gamma_2^d}$$

and:

$$\gamma_2 - \sqrt{\gamma_1^d \gamma_2^d}$$

Therefore, the interfacial tension (γ_{12}) is the sum of the tensions in those two layers:

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2\sqrt{\gamma_1 \gamma_2}$$

As the forces involved between two substances are universal, the same considerations will apply to the interaction of two solids and between a liquid and a solid. In this way we are able to consider the surface energy of solids, and this can be measured by reference to the known surface tension of liquids.

2.3.2 Surface Energy of Solids

The extent to which a liquid interacts with a solid (Figure 2.4) is characterised by the contact angle which the liquid, L, makes on a plane surface, S.

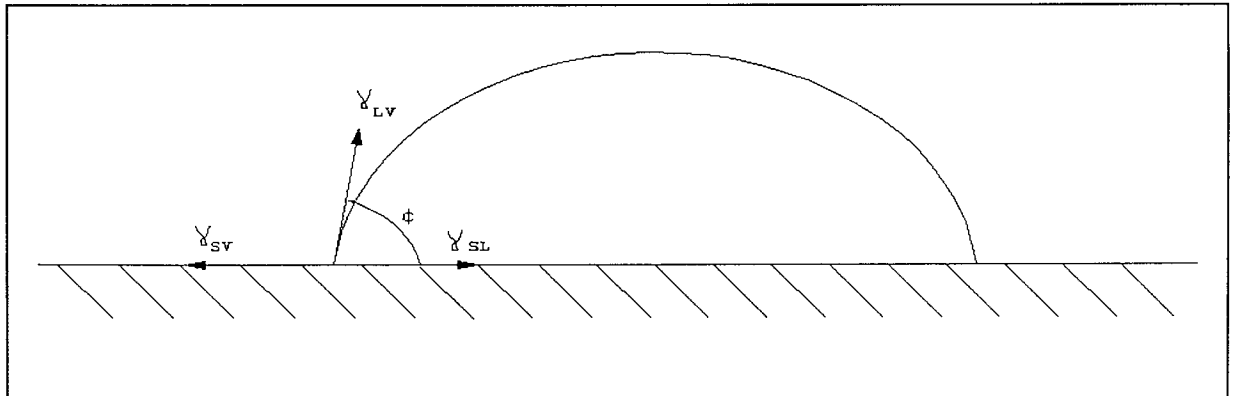


Figure 2.4 Schematic diagram of a liquid drop on a surface and the forces acting at the point of contact

The liquid contact angle is given by:

$$\gamma_L \cos \theta = \gamma_S - \gamma_{LS} - \pi_e$$

where π_e is the equilibrium film pressure of adsorbed vapour on the solid surface. This can be combined with Fowkes equation for interfacial tension:

$$\gamma_L \cos \Theta = -\gamma_L + 2\sqrt{\gamma_L \gamma_S^d} - \pi_e$$

But when $\gamma_L > \gamma_S$, $\pi_e = 0$.

This will be the case with low energy polymers where the liquid will have a finite contact angle, which is evidence that the liquid has a higher energy than the polymer surface and therefore π_e should be zero. From this, therefore, the contact angle of a liquid on a surface is (Fowkes, 1964):

$$\cos \Theta = -1 + 2\sqrt{\gamma_S^d} \frac{\sqrt{\gamma_L^d}}{\gamma_L}$$

From this, Zisman and coworkers (Zisman, 1963) have plotted $\cos \Theta$ versus γ_L for a homologous series of liquids and the value of γ_L at the intercept where $\cos \Theta = 1$ is termed the critical surface tension for wetting (γ_C), and is where the interfacial tension is zero.

The above has just considered the case where the only or major contributory forces are dispersion forces, but a more general form of the contact angle equation is given by the work of Owens and Wendt (1969) which takes account of both the polar and dispersion forces:

$$1 + \cos \Theta = 2\sqrt{\gamma_S^d} \frac{\sqrt{\gamma_L^d}}{\gamma_L} + 2\sqrt{\gamma_S^p} \frac{\sqrt{\gamma_L^p}}{\gamma_L}$$

By measuring the contact angle (Θ) of two different liquids against a solid, simultaneous equations are obtained which can be solved for γ_S^d and γ_S^p . Thus, the components of the surface energy due to various forces can be approximated and the sum of those components yields a reasonable approximation of the total surface energy. The use of the more general subscript s instead of sv assumes that the vapour pressure of the solid is negligible (Owens and Wendt, 1969).

From this theoretical consideration of surface energy the method of Owens and Wendt for calculating the surface free energy of solids has been developed. It depends on the measurement of water and methyl iodide contact angles, and resolves the surface free energy into two components, polar and dispersion. The simplicity of the method and the ability to separate the two surface energy components make it a useful tool in any study where surface interactions are important. Since γ_S^d and γ_S^p are sensitive to the surface chemistry, the method should also be useful as a semi-quantitative measure of surface composition and any changes to this.

Surfaces are often classified according to whether they are high energy or low energy surfaces. Generally, high energy surfaces are those which have a surface free energy $> 100\text{mJm}^{-2}$, and include inorganic solids glasses and metals. On the other hand, low energy surfaces have a surface free energy $< 100\text{mJm}^{-2}$, and include all organic liquids, waxes and polymeric solids. This provides an immediate distinction between polymers and metals, and they can be expected to interact differently with liquids with which they are brought into contact.

With fluorocarbon polymers a large increase in surface free energy is obtained by etching and is associated with the resultant increase in adhesion (Dukes and Kinloch, 1977; Dwight, 1977). Acid etching of polyolefin sometimes results in increased adhesion of smooth surfaces without concomitant increased surface roughness. In these cases the increased adhesive strength is due to increases in surface polarity (Dukes and Kinloch, 1977). Contact angle studies, in conjunction with XPS, has shown that discharge treatment of PET leads to the production of large amounts of low MW oxidised material which rapidly migrate into the bulk (Ashley et al, 1985). Surface pretreatments are responsible for an increase in surface energy of polymers which is associated with increased adhesion, and these are seen to deteriorate with time (Ashley et al, 1985; Briggs et al, 1980). Contact angle measurements have been used to show the importance of the polar component in determining the adhesion of resist coatings to silica substrates (Deckert and Peters, 1983).

Contact angle measurements can be performed in one of several ways. The commonest method used

is the equilibrium sessile drop method in which a single drop is placed on a flat surface and the contact angle measured with a travelling microscope using a goniometer eyepiece (Rance, 1985: Thomas et al, 1986). Fibres can also be investigated using contact angles by touching the fibres to a liquid surface (Rance, 1985). Classically, contact angles have been measured with a sessile drop on dry surfaces (Baier and Dutton, 1969: Owens and Wendt, 1969: Thomas et al, 1986). The results from these measurement can be presented as surface energy values with the critical surface tension parameter commonly used for biomaterials (Baier et al, 1970: 1971: 1984: Ito et al, 1989).

Alternatively, the method of Owens and Wendt (1969) provides a separation of the polar and dispersive components of surface energy. This is useful for investigating the role of the physicochemical state of the material surface and how this is altered with the deposition of biological layers (Clay et al, 1987: Ito et al, 1989). This latter finding is further proof that the intrinsic surface properties of a material can be investigated only if the surface is clean at a molecular level (Rance, 1985), which is very difficult to avoid with high energy surfaces due to adsorption of atmospheric water.

Contact angle measurements can also be performed using an inverted gas or octane droplet (Hamilton, 1972). This potentially provides a measurement in a more natural environment as it avoids the effect of looking at dehydrated surfaces, and has been used to demonstrate the increasing hydrophilic nature of polyurethanes with increasing polydimethylsiloxane segments incorporated (Okkema et al, 1989). Alternatively, the contact angle can be measured indirectly from macrophotographs by measuring the height and diameter of the drop, and calculating the contact angle (Deckert and Peters, 1983). This method requires that the drop is sufficiently small in size ($1\ \mu\text{l}$) so that any distortion due to gravity is negligible (Rance, 1982). Contact angle hysteresis is the difference between the advancing and receding contact angles and can occur under certain conditions; the most important are surface roughness and surface heterogeneity (Rance, 1982). The advancing angle is sensitive to the low energy domains and the receding angle to the high energy domains in heterogenous materials. Contact angle hysteresis can also be caused by the mobility of

surface polymeric chains and their side groups (Hogt et al, 1985). It has demonstrated increased water content and surface mobility of charged moieties which dominate at the surface in an aqueous environment. Contact angle hysteresis has been used to demonstrate the increase in polar sites at the surface of sulphuric acid treated polystyrene (Matsuda and Litt, 1975).

Spatial resolution:	x/y:	several mm
	z:	1 molecular layer
Information:	Surface free energy	

2.4 Biochemical Labelling

Radiolabel experiments are a common method of establishing the extent of adsorption of proteins to a surfaces (Andrade, 1985: Chiu et al, 1978: Chuang et al, 1978). The usual (ie. commonest) label is iodine (I^{125}) which can be used to study proteins if pure preparations are available. This is essential as I^{125} is a non-discriminatory label. If these precautions are taken into account, then radiolabels can be used as a quantitative measurement of protein adsorption (Andrade, 1985). Alternatively, it can be used in a highly specific manner using immunochemical techniques when the labelled protein is a radiolabelled IgG molecule directed at a deposited protein (Chuang, 1984: Grinnell and Feld, 1982). Radioactive antibody assay can be used to quantify the amount of protein adsorbed, and fibronectin deposition has been studied using both ^{125}I -labelled fibronectin (Haas and Culp, 1982) and an indirect radiolabelled antibody technique (Grinnell and Phan, 1983). Fibrinogen adsorption has been measured using radiolabelled protein (Brash and ten Hove, 1989: Chiu et al, 1978: Lee et al, 1974), as has albumin and γ -globulin deposition (Lee et al, 1974). Immunochemical detection by radiolabelled antibodies have been used to demonstrate the occurrence of conformational changes (Bentley and Klebe, 1985: Chuang and Andrade, 1985: Grinnell and Feld, 1981: Grinnell and Feld, 1982).

One potential problem of radiolabelling experiments is that the iodine label is large and may alter the conformation of the protein which it is used to label (Andrade, 1985). ^{125}I -labelled fibronectin has been shown to have altered biological activity (Grinnell and Feld, 1982). This is a problem with the technique which is overcome using an indirect radio-labelling technique such as indirect antibody labelling (Grinnell and Feld, 1982, Iwamoto et al, 1985).

Immunochemical labelling techniques can also be used as a microscopical localisation technique. Immunofluorescence microscopy has been used to study cell attachment by labelling the proteins involved or the intracellular microfilaments (Bozyczko et al, 1989: Couchman and Rees, 1979: Couchman et al, 1983: Geiger, 1979: 1981: 1982: Wehland et al, 1979). It has also been used to study the distribution of adhesion promoting proteins from extracellular matrix (Couchman et al, 1983:

Hayman et al, 1982a: 1983: Hedman et al, 1982: O'Shea and Dixit, 1988). In the biomaterials field immunofluorescence microscopy has been used to identify that the deposits on contact lenses are composed of normal tear proteins (Gudmundsson, et al, 1985). The arrangement of proteins can be investigated in the scanning electron microscope using immunogold techniques (Murthy et al, 1987: Park et al, 1986a: 1986b: Pitt et al, 1986b). Colloidal gold immunocytochemistry has been used to investigate cell surface associated proteins in cells (Geuskens et al, 1986: Hattori et al, 1984: Trejdosiewicz et al, 1981) and tissues (Nakatsuji et al, 1985: Trejdosiewicz et al, 1981).

Spatial resolution:	x/y:	10-50mm
	z:	~0.5nm
Information:	Molecular distribution	
Sensitivity:	Molecular	

2.5 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) is often referred to by its old name of Electron Spectroscopy for Chemical Analysis (ESCA) which is rather misleading as several electron spectroscopic techniques are now available. X-ray photoelectron spectroscopy uses X-ray photons to ionise inner shell electrons, the resultant relaxation results in the emission of a low energy electron which carries away excess energy from the ionisation event, and is shown diagrammatically in Figure 2.5. The energy of

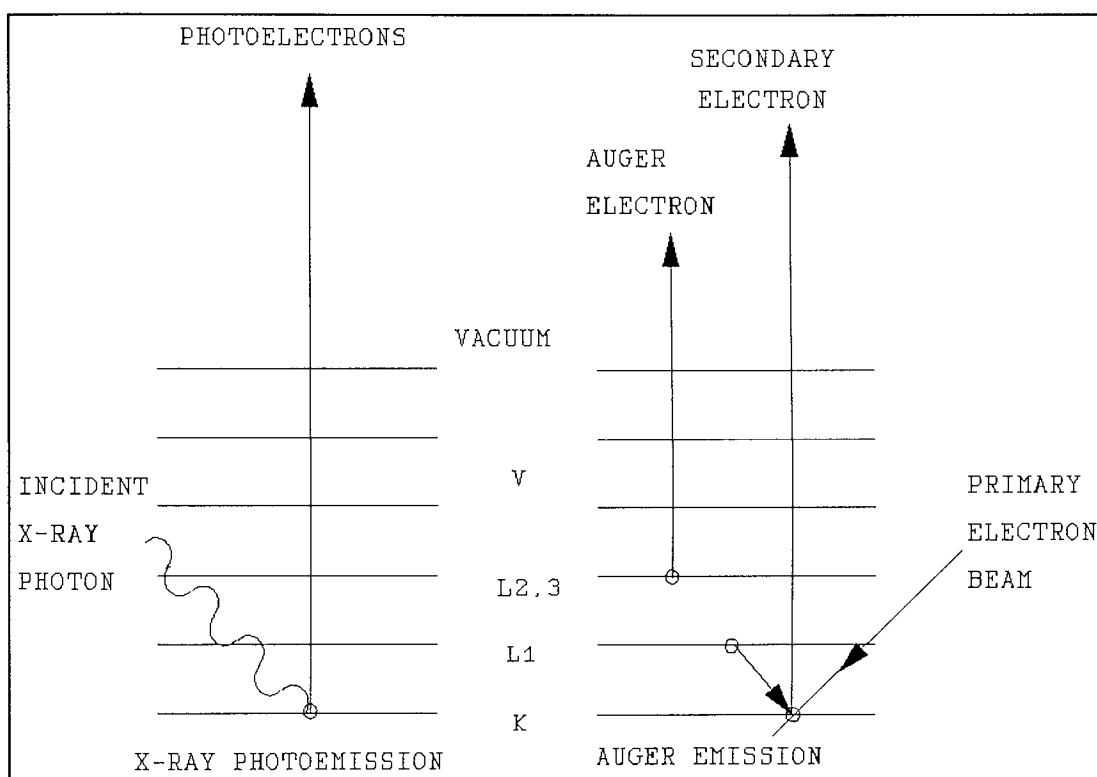


Figure 2.5 Ionisation events when X-ray photons interact with inner shell electrons

the emitted electron is characteristic of the atom from which it has escaped, and is not influenced by the energy of the ionising beam. The resulting signal contains mainly elemental information about the sample (Briggs, 1982a: Janssen and Venebles, 1979: Ratner, 1982: Riggs and Parker, 1975). Chemical information about the local environment of the atom ionised can be obtained from the Auger electron component of the signal (Briggs, 1982a: Ratner, 1982).

As the signal resulting from the technique consists of low energy electrons, the equipment relies on

ultra-high vacuum (UHV) technology. The photoelectrons emitted are analyzed in terms of kinetic energy (E_K) to give an energy spectrum. Three types of peaks are seen superimposed on a secondary electron background. Core level peaks, eg. C1s, are due to photoelectrons emitted from the inner (core) orbitals of the atoms present. The binding energies (E_B) of these electrons are obtained from the Einstein relation:

$$E_B = h\nu - E_K - \phi$$

(where $h\nu$ is the X-ray energy, the simple work function)

The binding energy is highly characteristic and allows identification of all elements except hydrogen. The peak intensities are proportional to the number of atoms sampled and, thus, atomic compositions can be calculated. Detection limits are typically 0.2% (Briggs, 1982a).

The much weaker series of peaks at very low binding energies, eg. F2s, is due to photoelectron emission from the valence band (molecular orbitals). The other intense peaks, eg. F KLL, are due to Auger electrons. These result from a process in a core-ionised atom whereby a second electron from a higher orbital drops into the vacancy caused by photoelectron emission, and the energy released is transferred to a third, weakly bound electron (see Figure 2.5). This Auger electron leaves the atom with kinetic energy given by:

$$E_K = E_A - E_B - E_C - \phi$$

(where A, B and C are the photoionised level, the level of the second electron and the level of the Auger electron respectively: ϕ is the work function).

The energies of the Auger electrons are also element specific, but not all elements produce strong Auger signals under typical X-ray photoelectron spectroscopy conditions.

X-ray photoelectron spectroscopy is surface sensitive technique because, although the exciting X-rays

penetrate some microns into the sample, the ejected electrons can only be distinguished if they escape without losing energy. Those that experience significant energy losses contribute to the signal as background radiation. Peak electrons have mean free paths of $\sim 0.5 - 3\text{nm}$, and are clearly a function of the particular peak under scrutiny. The commonest spectra obtained by XPS are known as wide or survey scans since they allow immediate element identification. Narrow scans can also be taken using higher energy resolution to allow accurate binding energies to be measured. Small variations in peak positions and shape occur for a given core level of an element in different chemical states. These are known as chemical shifts and enables some structural information to be obtained, although this can be ambiguous if surface charging effects occur. Auger peaks also exhibit shifts which can provide additional structural information.

To regard X-ray photoelectron spectroscopy simply as a surface analysis technique can be misleading (Walls and Christie, 1982). Although XPS derives its usefulness from the intrinsic surface sensitivity of the emitted electrons, it can also be used to determine the composition of much deeper layers. This is normally achieved by the controlled erosion of the surface by ion bombardment or by tapering the surface by some mechanical means, usually cratering techniques (Walls and Christie, 1982). In this way, composition-depth profiles can be obtained which provide a powerful means for analysis of thin films, surface coatings and interfaces. The provision of a composition-depth profile through the bond itself can be used to characterise the bond chemically, and the results can be correlated to bond performance.

X-ray photoelectron spectroscopy is extensively used for studying surface chemistry in the adhesives technology field (Briggs, 1982b; Briggs et al, 1980; Clark et al, 1978; Everhart and Reilley, 1981; Kinloch, 1979; Ranby, 1978; Schornhorn, 1978). It has proved decisive in investigating the reasons for the poor adhesive properties of many plastic surfaces, and the role of surface pretreatments used to overcome these fundamental problems (Blythe et al, 1978; Briggs, 1982a). The surface sensitivity is an important factor in determining the presence of weak boundary layers and its removal by treatments (Brewis et al, 1978; Rance, 1985). A key factor in the chemical modification of polymer

surfaces is the introduction of oxygen in the form of hydroxyl and carbonyl groups, as shown by XPS studies (Briggs, 1982b; Schornhorn, 1978). XPS has also shown that there is a critical surface concentration above which increased adhesion occurs with printing inks onto plastics (Ashley et al, 1985). In contrast, XPS has been used to show that corona treatment of polyethylene surfaces in a nitrogen atmosphere result in chemical reaction of nitrogen with the polymer surface (Courval et al, 1976).

The heat sealability of polypropylene is reduced by the presence of silicone in the surface, as shown by XPS (Ashley et al. 1985). XPS showed that PTFE surfaces are defluorinated after treatment to increase adhesion, with oxygen now present (Dwight, 1977). XPS can be used to show the location of the fracture plane in joint failure and the presence of surface contaminants, usually carbon (Dwight, 1977; Kinloch, 1979). It has shown the presence of trace amounts of elements at metal surfaces which are thought to act as contaminants acting to reduce bond strength (Chen et al, 1978; Sykes, 1982). The chemical composition of pretreated aluminium surfaces was studied by XPS, but no significant correlations between surface chemical composition and durability of adhesive joint were identified (Poole and Watts, 1985).

X-ray photoelectron spectroscopy has also been used to determine the surface chemistry of biomaterials (Therrian et al, 1989; Thomas et al, 1986). The hard and soft segments of polyurethane vascular prostheses, which are thought to be responsible for the biocompatibility of polyurethane, can be determined in virgin samples along with surface contaminants (Therrian et al, 1989). Implanted prostheses show increased levels of sulphur and nitrogen due to adsorbed proteins, and these mask the surface elements found on the virgin polyurethane surfaces (Therrian et al, 1989). XPS has also shown that non-polar polydimethylsiloxane segments incorporated into polyether polyurethanes were preferentially oriented at the surface (Okkema et al, 1989). The excellent depth resolution of the technique has been used to demonstrate the complete or incomplete coverage of a surface by a deposited protein film (Andrade, 1985; Paynter and Ratner, 1985).

Spatial resolution:	x/y:	1-5mm
	z:	~1nm
Information:	Elemental and chemical	
Sensitivity:		10^{-19} g 10ppm

2.6 Auger Electron Spectroscopy

Auger electron spectroscopy (AES) is similar to X-ray photoelectron spectroscopy except that the exciting beam is composed of electrons, and the information available from the signal is provided by the low energy Auger electrons (Chang, 1974; Janssen and Venebles, 1979; Ratner, 1982; Walls and Christie, 1982). The excitation process has already been described in the section on XPS (see Figure 2.5). Since Auger electrons have well defined energies, they appear as relatively small steps on the secondary electron background, and by recording the differential spectrum (dN/dE vs E_K) the background is suppressed and the Auger electron signal appears as distinct peaks (Walls and Christie, 1982). Peak positions are element characteristic allowing qualitative analysis of all elements except hydrogen and helium since they possess insufficient energy levels to allow Auger transitions to occur. It is particularly sensitive to light elements as the emission efficiency is inversely proportional to atomic number.

Quantification is more difficult than for X-ray photoelectron spectroscopy, but it can be achieved; peak intensity is related to composition, but in a more complex way (Briggs, 1982a). Structural information is inherent in Auger electron spectroscopy, but has been little exploited. Chemical shifts, as in XPS, occur, and line-shapes also vary with chemical state because Auger transitions frequently involve a valence electron.

A further development of Auger electron spectroscopy, which does not have an equivalent in X-ray photoelectron spectroscopy, is scanning Auger microscopy (SAM) in which the exciting electron beam is focused into a fine probe, which is then scanned across an area of the sample, as in scanning electron microscopy. If the electron energy analyzer is tuned to a specific peak energy then this enables elemental distribution maps to be displayed (Jansen and Venebles, 1979; MacDonald, 1971). Thus, by simultaneously collecting the secondary electron signal, surface topography and composition can be directly related. Very high resolution scanning Auger microscope images can be obtained at slow scan rates by digitally stepping of the electron beam and pulse counting both the

peak and background intensities.

Both X-ray photoelectron spectroscopy and Auger electron spectroscopy are extremely sensitive surface techniques as the low energy electrons have a very short mean free path and, therefore, only those which arise in the top few atomic layers of the surface are able to escape with their original energy and be detected (Walls and Christie, 1982). The low energy of the electrons emitted during excitation in these techniques also means that ultra-high vacuum apparatus must be used. As with XPS, AES can be used for depth profile studies by using ion beam sputtering to erode the surface, or by cratering techniques (Briggs, 1982a; Walls and Christie, 1982).

Auger electron spectroscopy, however, has not found ready use in the study of polymers owing to the destructive nature of the high beam currents used (Briggs, 1982a; Ratner, 1982; Riggs and Parker, 1975; Schornhorn, 1978; Walls and Christie, 1982). It is ideally suited to analysis of metal surfaces where it has been used to study the oxidative state of surfaces following oxidative pretreatments (Cotter and Mahoon, 1982; Sun et al, 1978). It has also shown the presence of trace amounts of elements at metal surfaces which are thought to act as contaminants acting to reduce bond strength (Chen et al, 1978; Ditchek et al, 1980; Kinloch, 1979; Natan et al, 1982; Sykes, 1982). AES can also be used in conjunction with ion sputtering to allow depth profiles to be performed. This has enabled thickness measurements of oxide layers on titanium, and also the variation of important contaminants such as carbon and fluorine (Natan et al, 1982). Depth profiling has been used to show variations in the thickness of magnesium-rich contaminant layers in aluminium alloys which adversely affects bond durability (Sun et al, 1978).

Spatial resolution:	x/y:	30nm
	z:	0.3nm
Information:	Elemental (mainly)	
Sensitivity:		10^{-19} g 10ppm

2.7 Mass Spectrometry

Mass spectrometry requires a sample in a volatile form, but can be performed on solids by etching the surface using either ion or atom beams, and analyzing the ejected molecules or ions. It is by its nature, therefore, a destructive technique, although there are two very different degrees to this. Secondary ion mass spectrometry (SIMS) is a well established technique which can be used for depth profiling, and for distribution mapping when a focused, scanned ion beam is employed (McHugh, 1975; Ratner, 1982). FAB-MS (fast atom bombardment mass spectrometry) is a development of SIMS in which a neutral beam is used to etch away the sample surface. With FAB-MS the species detected are atoms or molecules compared to a mixture of atoms, molecules and ions with SIMS, and so is a less harsh technique.

Spectra come in two forms which are independent of the exciting ion beam which is usually positively charged (eg. Ar^+ , O_2^+ , Ga^+ , Cs^+), positive and negative. Most of the ejected fragments are neutral, but a small proportion are either positively or negatively charged. These are extracted into a mass spectrometer to give separate positive and negative secondary-ion mass spectra, each consisting of peaks due to elemental species (eg. Mg^+ , O^-) and cluster ions (eg. MgO^+ , $\text{C}_6\text{H}_5\text{O}^+$).

In "static" SIMS the ion beam current density is sufficiently low for material removal to be negligible within the time for spectral acquisition (Briggs, 1982a; Brown and Vickerman, 1984). Here the emphasis is on maximising the molecular information depth to the region of 1nm. It is a basic tenet of SIMS that the ion fragments have structures closely related to the atomic arrangement when that fragment was part of the original surface (Briggs, 1982a). In "dynamic" SIMS the ion beam current density is deliberately high so that a high sputtering rate is achieved. The mass spectrometer is tuned to a given mass value, and the peak intensity is monitored as sputtering proceeds, giving a concentration depth profile. Secondary ion mass spectrometry can be a very sensitive technique, with detection as low as 0.1% typical for the static mode, and <1ppm routinely achieved in the dynamic mode. Variations of ion yield from one material to another (matrix effects) are extreme, and this must

be taken into account in depth profiling experiments (Briggs, 1982a).

The surface sensitivity of secondary ion mass spectrometry has been useful in showing that the removal of surface contamination by cleaning regimes improves adhesion performance (Rance, 1985). SIMS has demonstrated the existence of chemical bonds in silane primer-coated surfaces by the detection of FeSi containing ions (Kinloch, 1979).

Spatial resolution:	x/y:	1 μ m (SIMS) 1mm (FAB-MS)
	z:	1nm
Information:	Chemical and elemental	
Sensitivity:	1-5 ppb	

2.8 Goniophotometry

Goniophotometry is a light scattering technique which is used to investigate the micro-roughness of a surface (Tighe, 1978). It is ideally suited to the study of paints where one of the most important properties of a painted surface is the extent of gloss finish which is apparent (Tighe, 1978). Our perception of a gloss surface is a function of the amount of light which is reflected by that surface without scattering the incident light in multiple directions, and is due to the smoothness of the surface. Whilst the extent of gloss finish of a surface is readily apparent to observers, a proper study of glossiness requires an objective assessment of the shininess of a surface which can be measured using the goniophotometer.

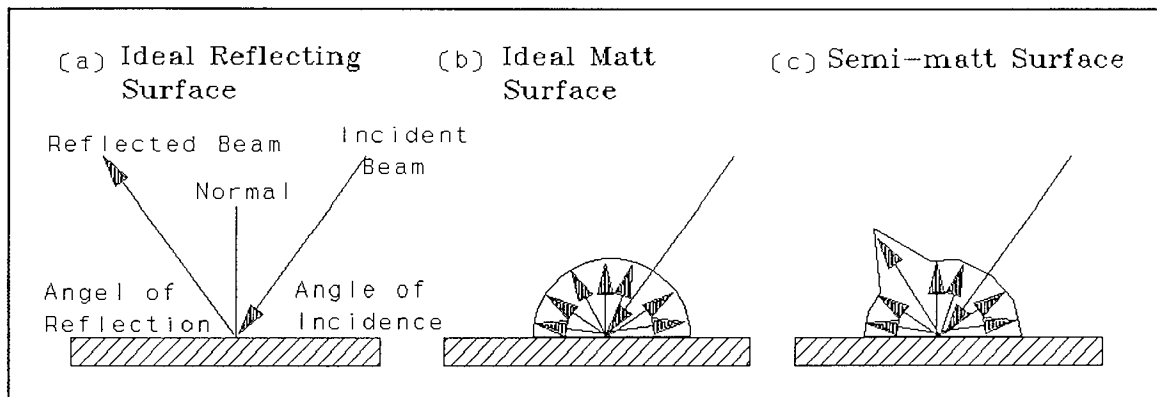


Figure 2.6 Reflectance phenomena

The goniophotometer can be used to give a measure of the roughness of a surface by showing how light is reflected by it. An ideal smooth surface reflects a light beam incident upon it in a conventional fashion of angle of reflection equal to angle of incidence, as shown in Figure 2.6a. An ideal matt surface will differ from this by providing total light scattering of any incident beam so that the reflected light is scattered in all directions from the surface in equal intensities (Figure 2.6b). In contrast a semi-matt surface will reflect light with a principle reflection at the expected angle of reflection (specular intensity) but with a considerable intensity to the diffuse reflected light (Figure 2.6c). Hence, the way that a surface reflects light can be used as a measure of its roughness.

Gloss is not solely influenced by the surface rugosity of a surface, although that is the major contributory factor (Tighe, 1978). Material differences mean that gloss factors cannot be treated in absolute terms. At each point of reflection from a surface there will be an intensity loss due to absorption of light energy by the material instead of total reflection occurring. These various absorption losses present a limitation on comparison of surface rugosity between materials by assessing the way light is reflected from a surface. However, when the material is kept constant, then goniophotometry is an ideal means of comparing the effects of treatments on the surface rugosity of that material.

2.8.1 The Technique

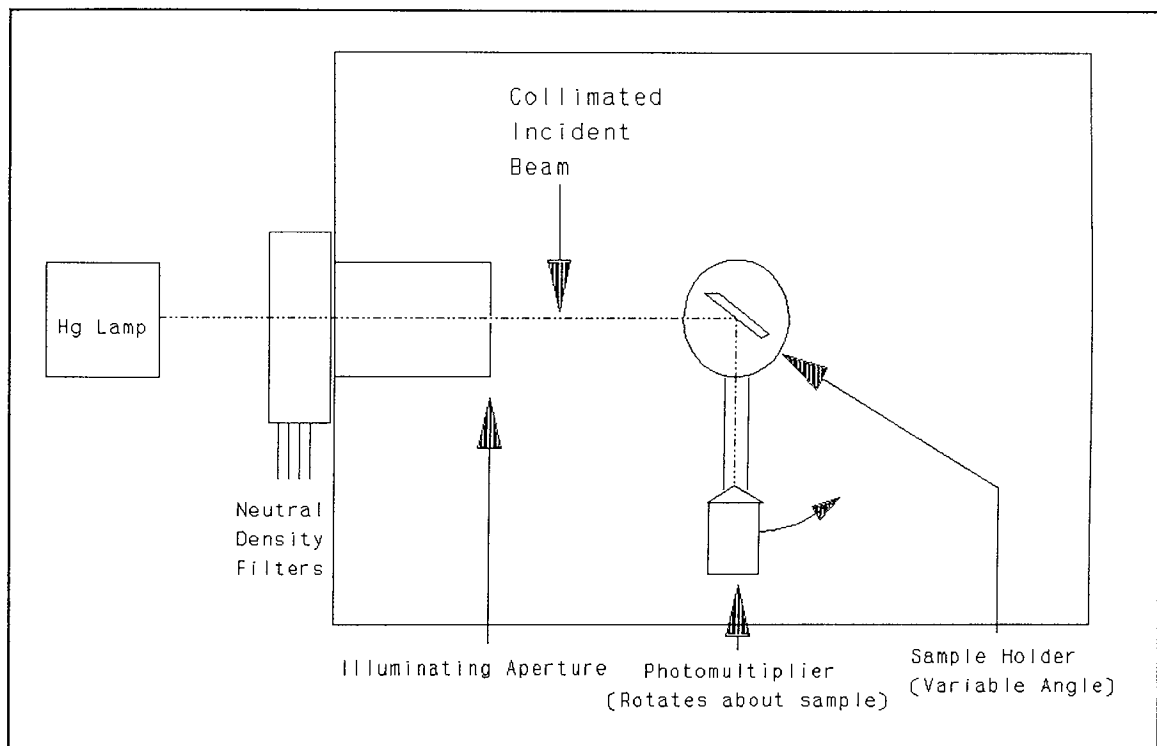


Figure 2.7 The essential layout of a goniophotometer

The goniophotometer is essentially a light-tight box in which a collimated beam of light is directed onto the surface in question, the reflected light is captured and measured by a travelling photomultiplier. The output is displayed as the intensity of light detected as a function of the angle subtended by the reflected light from the surface. A diagrammatic representation of a typical goniophotometer as used in the experiments described in Chapter 5 is shown in Figure 2.7. It is a

modified Brice-Phoenix light-scattering photometer in which the light from the mercury lamp passes through a set of neutral density filters (and also through a wavelength filter, if desired) into a collimating tube, which has a slit aperture. The width of the slit aperture may be varied to suit the sample which is mounted in the centre of the stage at an angle of 45° to the incident beam, although this can also be altered if desired. The reflected, transmitted or scattered light from the sample is measured with the travelling photomultiplier, and with the output recorded on a chart recorder, or more commonly nowadays, on a micro-computer. The photomultiplier is motor-driven through 270° around the sample.

The data from a goniophotometer is usually represented as a trace of the light intensity versus the angle of reflection, with curves from reflective surfaces having a sharp peak at the appropriate angle

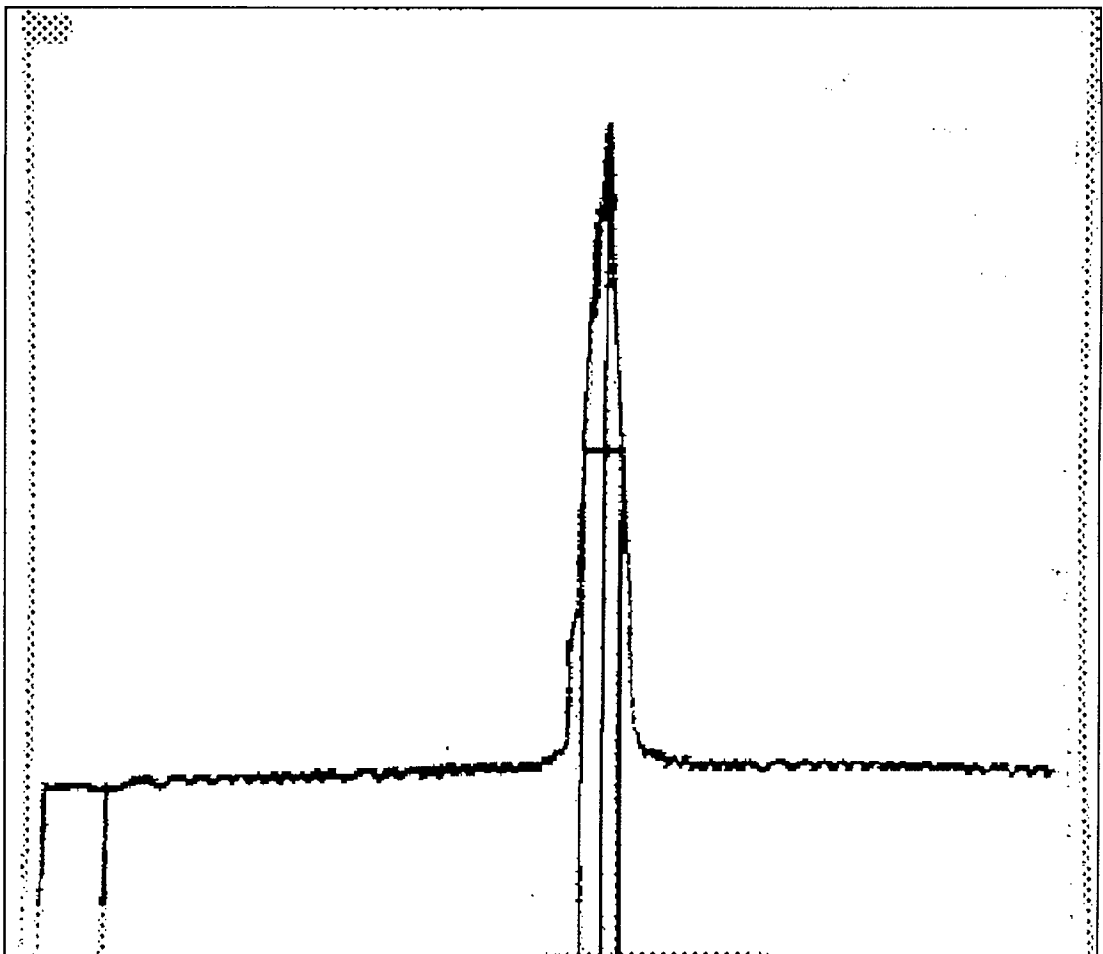


Figure 2.8 Typical goniophotometer trace from a highly reflective surface; a clean glass microscope slide

from the incident beam: an example is given in Figure 2.8. The simplest value from this trace is the Specular Intensity (I_S), which is the height of the peak, whilst the baseline reading is the Diffuse Reflectance (I_D). Another important factor in assessing glossiness is the shape of the peak with reflective surfaces giving tall, narrow peaks, and matt or semi-matt surfaces having broad short peaks. One useful way of indicating this peak shape is using the peak width at half height ($W_{1/2}$). In the case of an ideal mirror surface the peak width will be determined by the beam width.

By combining these values the sharpness of the peak can be taken into account, and the surface finish of a sample can be represented by a "gloss factor".

$$GF = \frac{I_S - I_D}{W_{1/2}}$$

The magnitude of the gloss factor depends on the angle of incidence chosen (usually 45°) and the intensity of the incident light beam. It has proved to be invaluable in monitoring the change in reflection properties of a surface coating with aging or deterioration studies. A linear relationship between $\log GF$ and the average height of roughness irregularities (AHRI) has been noted.

Goniophotometry is a light scattering technique which is limited in resolution by the wavelength of the light used, which is in the visible spectrum in appearance studies. It is, however, a non-destructive technique and can provide useful information on the surface roughness of biomaterials. It has been used to study the increase in surface roughness which occurs when biodegradable polymers breakdown (Yasin et al, 1989).

Spatial resolution:	x/y:	several mm
	z:	1 μ m
Information:	Topographical	

2.9 Infra-red Spectroscopy

For the qualitative analysis of organic systems in general, infra-red spectroscopy is paramount. The infra-red spectrum from a compound is due to the absorption of vibrational frequencies, and provides an identification fingerprint. Libraries of characteristic IR spectra exist, including those of polymers, although the identification of particular functional groups can be equally important (Briggs, 1982a). The specificity of these data is dependent upon which vibration is considered; some groups vibrate at frequencies with little variation due to the molecular context, others can be assigned only to a range of frequencies (Briggs, 1982a).

Infra-red spectroscopy has improved in sensitivity with the advent of powerful microcomputers, by applying Fourier transform techniques when it is known as FTIR spectroscopy (Briggs, 1982a: Gendreau et al, 1982). In Fourier transform Infra-red, an interferometer replaces the monochromator which enables spectra-containing information on the intensity of each frequency to be collected simultaneously. This enables multiple scans to be collected and added together to increase the signal-to-noise ratio which results in improved sensitivity. With this improvement in sensitivity the range of applications for infra-red spectroscopy now encompasses reflection and microscopical techniques. Studying the reflected signal means that FTIR spectroscopy can be used to investigate surfaces or interfaces: this is known as attenuated total reflection (ATR) FTIR spectroscopy (Gendreau et al, 1982: Ratner, 1982: Ranby, 1978), or multiple internal reflection spectroscopy (MIR) spectroscopy (Briggs, 1982a). The principle is shown diagrammatically in Figure 2.9. The reflection

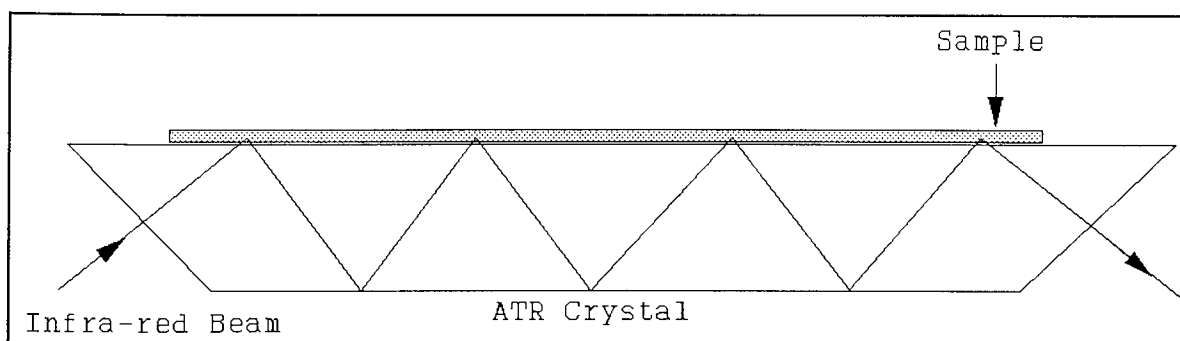


Figure 2.9 Diagram showing the principle of the Attenuated Total Reflection technique

crystal is usually germanium, and during each internal reflection of the beam at the interface between the sample and the crystal some penetration into the sample occurs leading to selective absorption of the infra-red beam energy. The effective penetration depth of the radiation (the sampling depth) is given by (Harrick, 1967):

$$d_p = \frac{\lambda_0}{2\pi n_1 \left[\sin^2\theta - \left(\frac{n_2}{n_1} \right)^2 \right]^{\frac{1}{2}}}$$

(where θ is the angle of incidence of the IR beam, n_1 and n_2 are the refractive indices of the crystal and the sample respectively, and λ_0 is the exciting wavelength).

Despite the typical sampling depth of $\sim 1\mu\text{m}$, the spectrum contains information on the composition of the sample surface, and in favourable cases, ie. strong absorption bands, this can be from the first few nm. Depth profiling can be performed by altering the angle of incidence of the reflected beam (Jakobsen, 1979).

Attenuated total reflection-FTIR spectroscopy has been used to investigate polymer surfaces (Briggs, 1982b; Castillo et al, 1984a; Ranby, 1978; Schornhorn, 1978; Willis and Zichy, 1978) and protein monolayers adsorbed at an interface (Castillo et al, 1986; Gendreau et al, 1982; Jakobsen, 1979; Ratner, 1982). It has been used to characterise polyurethane biomaterial coated with heparin (Barbucci and Magnani, 1989). A particular strength of IR spectroscopy is its sensitivity to polymer morphology (crystallinity, orientation, chain conformation, etc). With one-way drawn polypropylene film, MIR and polarised radiation showed that there was a strong degree of machine direction orientation present (Willis and Zichy, 1978). Chloroacetic acid etching of PET film is a pretreatment used to improve adhesion. ATR-FTIR was used to demonstrate a change in molecular conformation and crystallinity of the PET on etching (Willis and Zichy, 1978).

As the technique uses infra-red radiation, it is non-destructive and has been used to follow the adsorption of blood proteins onto plastic surfaces (Baier and Dutton, 1969; Kellner and Gotzinger, 1984; Jakobsen, 1979; Seifert and Greer, 1985), and the adsorption of single proteins from solution such as fibrinogen (Baier and Dutton, 1969; Baier et al, 1971; Pitt et al, 1986b), albumin (Pitt and Cooper, 1986; Pitt et al, 1986a) and serum (Baier and Weiss, 1975). It has been used to show differences in the chemical groups distributed on protein layers deposited on polycarbonate surfaces in the presence and absence of vitamin C (Chandy and Sharma, 1985). FTIR studies have shown that polyethylene adsorbs proteins rich in carbohydrates, such as glycoproteins, whilst heparin-treated polyethylene adsorbs proteins low in carbohydrates, such as albumin (Jakobsen, 1979).

The fraction of the carbonyl groups in proteins adsorbed to silica surfaces that are in contact with the silica has been used to detect conformational changes using infra-red spectroscopy (Horbett, 1982). ATR-FTIR spectroscopy has been used to demonstrate conformational changes occurring on the adsorption of proteins to hydrogel surfaces (Castillo et al, 1984b; 1985; 1986). The study of protein deposition in a flow cell indicates that proteins adsorb in two layers, the first is rapid irreversible binding (Kellner and Gotzinger, 1984). Adsorption of fibronectin has been shown to be greater on hydrophobic surfaces than on hydrophilic surfaces by ATR-FTIR (Pitt et al, 1987). In these studies the extent of spectral changes, compared to solution fibronectin, were greater on hydrophobic surfaces, indicating greater conformational change. The spectra of the protein which adsorbs first appear to have more extensive spectral changes than protein adsorbing at later stages.

ATR-FTIR has been used to determine adsorption isotherms for polyelectrolyte macromolecules from moderately concentrated solutions upon an ionic surface (Belton and Stupp, 1983). It can also examine the structure of the adsorbed layer, thereby lending insight into the nature of the adhesive interaction. Neutralisation of polyacid with NaOH causes the amount of polymer adsorbed to decrease. This is due to a decrease in molecular aggregate migration in the more concentrated solutions, whilst in more dilute solutions conformational changes are also involved.

Spatial resolution:	x/y:	several mm (ATR-FTIR) 10 μ m (FTIR microspectrometry)
	z:	1 μ m
Information:		Chemical and conformational
Sensitivity:		Very variable depending on conditions

2.10 Total Internal Reflection Fluorescence

Total internal reflection fluorescence (TIRF) is an optical technique which can be used to study protein adsorption by measuring the natural fluorescence induced by an intense laser beam using UV radiation (Hlady et al, 1985; van Wagenen et al, 1982) or visible wavelengths (Cheng et al, 1985). The technique relies on total internal reflection occurring within the prismatic detecting element. A portion of the incident beam will penetrate into the medium of lower refractive index, giving rise to an evanescent wave. This wave interacts with any proteins at the interface to an extent which decreases exponentially with distance, so that photon interaction is limited to the immediate vicinity of the surface (Watkins and Robertson, 1977). The system allows real-time measurements of protein adsorption which may be under flowing conditions (Watkins and Robertson, 1977).

The fluorescence relies on the presence of amino acids with aromatic rings, principally tryptophan (Horbett, 1982; Iwamoto et al, 1982; 1985; Hlady et al, 1985; van Wagenen et al, 1982) or tyrosine residues (Iwamoto et al, 1982; Hlady et al, 1985). Tryptophan residues are often buried in the protein structure and thus provide a natural marker for changes in protein structure at interfaces (Horbett, 1982). Alternatively an extrinsic fluorochrome can be used (Cheng et al, 1985; Iwamoto et al, 1982). The fluorescence signal can be calibrated enabling quantification of protein adsorption at solid-liquid interfaces (Rockhold et al, 1983).

TIRF has been used to demonstrate adsorption differences of haptoglobin (Lowe et al, 1985), insulin (Iwamoto et al, 1982) and fibronectin (Iwamoto et al, 1985) onto hydrophobic or hydrophilic surfaces. Detail study of the fluorescence from adsorbed fibronectin layers reveals a 5nm red shift in the emission maximum which suggests a change in protein conformation and/or microenvironment (Iwamoto et al, 1985). Competitive adsorption of albumin and fibrinogen has been followed using TIRF (Lok et al, 1983).

A related technique is laser induced photobleaching which has been used to study protein mobility

in bacterial cell membranes. It should be practicable to use this technique to study the mobility of protein on biomaterial surfaces which may well differ on surfaces of differing surface physicochemical properties. It would be expected that some surfaces will attract proteins strongly and restrict the proteins freedom to move, either laterally or rotationally, whilst others bind the proteins only weakly allowing them relatively large degrees of freedom of movement.

Spatial resolution:	x/y:	several mm (TIRF) < 1 μ m (laser bleaching)
	z:	1 μ m
Information:		Chemical and conformational
Sensitivity:		Very variable depending on conditions

SUMMARY OF TECHNIQUES

Technique	Spatial Resolution		Type of Information	Sensitivity
	x/y	z		
Electron Microscopy	1.5-5nm (SEM) 1nm (TEM)	1-5nm	Topography Morphology	Not applicable
Electron Probe Microanalysis	5µm	5µm	Elemental ID & distribution	10 ⁻¹⁶ g 10 ³ ppm
Light Microscopy	200nm	10nm	Morphology	N/A
Contact Angle Measurements	several mm	1 molecular layer	Surface energy	N/A
Biochemical labelling	10-50nm	~0.5nm	Molecular distribution	Molecular
X-ray photoelectron spectroscopy	1-5mm	~1nm	Elemental and chemical	10 ⁻¹⁹ g 10ppm
Auger electron spectroscopy	30nm	0.3nm	Elemental	10 ⁻¹⁹ g 10ppm
Mass spectrometry	1µm (SIMS) 1mm (FAB-MS)	1nm	Chemical and elemental	1-5ppb
Goniophotometry	several mm	1µm	Topography	N/A
Infra red spectroscopy	10µm	1µm	Chemical and conformational	Variable
Total internal reflection fluorescence	several mm	1µm	Chemical and conformational	Variable

OBJECTIVES AND SCOPE OF INVESTIGATION

- 1). To investigate, and where necessary, develop methods for the *in situ* analysis of the surface properties of polymers and adsorbed biomolecular layers, since the interactions within these layers determine the nature of the interface.
- 2). To investigate the fundamental role of deposited biological molecules on synthetic surfaces in the development of a biologically reactive interface.
- 3). To identify and characterise the surface properties of polymers which are important in the deposition of biological molecules.

The approach to this investigation will entail the use of both physicochemical and biological probes. Cell adhesion in culture is used as the primary biological sensor of surface properties and changes. The range of techniques will also cover physical and chemical methods of investigation which will allow correlations to be made between biological activity and surface properties and interactions.

Surface analysis techniques will be assessed and a program of work embarked upon to investigate their usefulness by presenting the chosen techniques with samples of polymer surfaces, with and without, deposited biological molecular layers.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

It is usual for experimental work, particularly in the biological field, to be performed using model systems in which the number and nature of the variables are controlled. Our model system is based on the tissue culture technique, which is a widely used biological test for the occurrence or effectiveness of the interface conversion process. Generally, cell activity is studied in monolayer culture conditions which is not usually found in tissues where three-dimensional lattices are the norm for individual cells or for cell groups, except for certain spreading epithelia (Trinkaus, 1976). Nevertheless, cellular activity can probably best be investigated in artificial cell culture.

Permanent cell lines are well characterised and readily available but have altered morphology compared to progenitor primary cultures which should be taken into account (Vasiliev and Gelfand, 1977). Fibroblasts are a common cell type for studying cell activities in culture as they are capable of attachment to numerous substrata, and exist in culture in two main morphological states, spherical and polarised. The spherical state is characteristic of cells not attached to solid substrata, and when they come into contact with a suitable substratum they are gradually transformed into polarised cells (Vasiliev and Gelfand, 1977). This transformation usually passes through an intermediate state; that of the radially spread cell. These transitions are reversible and can be repeated many times.

The normal substratum material for cell and tissue culture is polystyrene which is normally non-adhesive for cells but can be rendered adhesive by surface treatment. In this way we can have the same substratum material in two forms which produce two very different cell responses. Bacteriological grade polystyrene plasticware is essentially untreated polystyrene which will not support the adhesion and growth of eukaryotic cells. Commercial tissue culture plasticware is surface treated polystyrene which will allow anchorage-dependent cells to grow and proliferate.

Glass and siliconised glass are also two materials which are chemically very similar to each other, but markedly different from the carbonaceous polymers represented by polystyrene. This change of

chemistry extends the model to silicon-based materials which can also have a range of cell adhesive properties, with untreated glass being cell adhesive whilst siliconised glass is not. In this way we have a set of four basic substratum materials for our model, which give a range of physical parameters (especially polarity) and with diverse chemical nature, and to which other materials may be added for particular experiments when necessary.

In a similar way the choice of deposited proteins is chosen carefully to allow the study of proteins with varying cell responses. In any consideration of interface conversion the obvious protein layer of interest is foetal calf serum which is a required media supplement for cell and tissue culture. It is, however, a complex mixture of proteins and other biomolecules such as lipids, and supplies growth factors, hormones and cell adhesion-promoting proteins to allow the growth and proliferation of cells. Two cell adhesion-promoting proteins from serum are of major importance in interface conversion, fibronectin and vitronectin. These seem to have different functions *in vivo*, and by studying their deposition and action on the biological system we can aid the interpretation of the mechanism of action of media serum supplement. The capability of forming stable contacts with their neighbours, with extracellular matrix and solid substrata is one of the fundamental properties of eukaryotic cells, so that proteins involved in this process must be included in any model for the study of interface conversion.

Other proteins which are not involved in the adhesion of cells can also be used in a model as controls to check that the effects on the deposition of the adhesion-promoting proteins is physiologically relevant. Albumin is a protein which has little influence directly on cells, nor does it have any adhesion-promoting properties. In fact, it is often used to block cell adhesion when precoated onto dishes and to block protein deposition onto free surface sites by occupying those surface sites. Immunoglobulins were, at one time, suspected of being involved in the adhesion of cells to materials because they bind to the membrane surfaces of cells. They are proteins which are functionally active on cells and act through cell surface receptors as adhesion molecules would, but they produce very different effects upon the cells. Commercially they are important since they are widely used in medical

diagnostic testing kits (eg. pregnancy testing) where they are immobilised onto surfaces.

3.1 Substratum Materials

The materials were purchased commercially except where detailed:

1). Tissue culture plastic (Corning,), Bacteriological grade plastic (Nunc), Blue Star Washed Micro Slides (Chance Propper Ltd., UK).

2). Spun coated polystyrene with a range of defined molecular weights, polyhydroxyethylmethacrylate (pHEMA), polyethylmethacrylate (pEMA), 50/50 pEMA/pHEMA were manufactured in the laboratories of the Speciality Materials Group, Aston University.

3). Siliconised glass was prepared by treating Blue Star Washed Micro Slides (Chance Propper Ltd., UK) with Repelcote (2% dimethyldichlorosilane in 1,1,1 trichloroethane: BDH)

3.2 Proteins

1). Bovine serum albumin (crystallized and lyophilized, essentially globulin free), Human Immunoglobulin G (IgG) from Sigma, Dorset, UK; Human vitronectin (Calbiochem, California); Foetal calf serum (Flow Labs, UK)

2). Partially purified bovine vitronectin was isolated from foetal calf serum according to the method of Lydon and Foulger (1986); details in Appendix 1.

3). Affinity purified rat fibronectin was kindly donated by Dr W T Gibson, Unilever Research, Colworth House. It had been isolated from rat tails by the preparation technique of Vuento and Vaheri (1979), which is described briefly in Appendix 1.

3.3 Antibodies

1). Anti-bovine serum albumin, Anti-Human IgG (Sigma); Anti-Human vitronectin (Calbiochem)

2). Affinity purified anti-rat fibronectin was kindly donated by Dr W T Gibson, Unilever Research, Colworth House. It had been prepared by the method described in Appendix 1.

3.4 Cell Culture Materials

Baby hamster kidney (BHK) fibroblasts were a gift from Mrs C.A. Foulger, Unilever Research, Colworth House, Bedfordshire. The original cell line had been purchased from Flow laboratories, Irvine, Scotland.

The following culture media materials were also purchased from Flow;

Dulbecco's Modified Eagles Medium (DMEM)

L-glutamine

foetal calf serum

phosphate buffered saline (PBS: Dulbecco's formulation without Ca²⁺ and Mg²⁺)

trypsin-EDTA

penicillin/streptomycin mixture

3.5 Sample Preparation

3.5.1 Preparation of Substratum Materials

Areas of substrate material were cut to size (the exact size was dependent upon the experiment and the particular requirements of, and the limitations imposed by the technique used). They were washed in 1% Tween 20 and rinsed extensively with distilled water to remove the detergent and finally with phosphate buffered saline (PBS).

3.5.2 Surface Modification

Bacteriological-grade and tissue culture petri dishes (35mm diameter) were treated with concentrated (98%) sulphuric acid (Fisons, AR grade) for 10 minutes at room temperature. For comparison of treated and untreated surfaces, the dishes were tilted slightly and acid applied until approximately one half of the dish was covered by the acid. Following sulphuric acid treatment, the petri dishes were rinsed thoroughly with distilled water and finally with culture medium.

Spun coated polystyrene samples, as prepared in the Speciality Materials Group laboratories, Aston University, were exposed to nitrogen plasmas in a Nanotech plastech 250. Discharge conditions were 150 kV under a vacuum of 10^{-2} torr for 5 minutes.

3.5.3 Deposition of Protein Layers

The cleaned material surfaces were placed in sterile siliconised glass petri dishes (treated with Repelcote, 2% dimethyldichlorosilane in 1,1,1 trichloroethane: BDH) and incubated with foetal calf serum or one of the following proteins in solution in PBS⁻ for 1 hour at 37°C before proceeding with the technical investigation:

10% foetal calf serum in PBS⁻

1% bovine serum albumin in PBS⁻

5µg/ml pure vitronectin in PBS⁻

5µg/ml pure fibronectin in PBS⁻

1mg/ml pure IgG in PBS⁻

These solutions were designed to be used and deposited at near physiological conditions and amounts.

3.5.4 Cell Culture

BHK fibroblasts were routinely maintained in Dulbecco's Modified Eagles Medium supplemented with L-glutamine and 10% foetal calf serum in 50 cm² tissue culture flasks. Cells were transferred by detaching them using trypsin-EDTA, resuspending in fresh media, which neutralises the trypsin, and reseeding in fresh tissue culture flasks at a density of 0.25-0.5x10⁶ cells/flask. The cells were incubated at 37°C under a 10% CO₂ atmosphere for 2-3 days, when the cells had grown to confluence. A cell growth curve was obtained so as to characterise the growth of the cells under normal conditions. This enables cells which are actively growing to be used in the cell spreading experiments; the cell growth curve is described in Appendix 2.

For cell adhesion experiments, cells in the exponential phase of growth were detached using

trypsin/EDTA and resuspended in culture medium containing penicillin and streptomycin. The cells were cultured in duplicate with and without 10% foetal calf serum supplementing the medium. The cell numbers were counted using a Coulter counter and the cell suspension inoculated at 10^6 cells/ml into each siliconised glass petri dish; 20 ml of medium was added (ensuring that the substrate materials remained submerged) and the cells incubated at 37°C for 6 hours under a 10% CO₂ atmosphere.

Cell spreading experiments were conducted over 6 hours when the cells were still in the lag phase of growth. Cell counts represent, therefore, the number of cells attached, and are not confused by cell multiplication.

3.5.5 Preparation for Light Microscopy

The samples were washed briefly with PBS⁻ before being fixed with 2% glutaraldehyde in PBS⁻ for 30 minutes. Cells were counted using a Leitz Ortholux light microscope, covering 10 fields per 1mm². The results were initially tabulated as the number of cells attached (meaning attached whether spread or not), and the number of cells which once attached have also been able to spread, per unit area. From this the percentage of cells which had spread was calculated. The number of cells which had attached and spread on the various surfaces were also normalised against the number of cells attaching and spreading on tissue culture plastic, which is the positive cell adhesion control for these experiments.

3.5.6 Preparation for Scanning Electron Microscopy

The samples were washed briefly with PBS⁻ before being fixed with 2% glutaraldehyde in PBS⁻ for 30 minutes. The samples were dehydrated through a graded series of ethanol-water mixtures (50%, 70%, 90%, 100% x3) for 15 minutes each. The samples were transferred to a Polaron E3100 II critical point drying apparatus and dried from liquid CO₂ using the critical point method (Anderson, 1951). The

samples were mounted on aluminium specimen stubs using Uhu glue and coated with 10nm of gold in a Polaron E5100 "Cool" Sputter coating unit at 1kV and 20mA (Echlin et al, 1982). The cells were examined in a Philips SEM525M scanning electron microscope at 15kV accelerating voltage, and photographed onto Kodak T-Max400 roll film.

3.5.7 Preparation for X-ray Microanalysis

The samples were prepared as for SEM examination with the exception of the type of specimen coating used. After mounting on stubs or on copper microscope specimen holders they were lightly coated with carbon by evaporation in an Edwards 306 evaporation unit and examined in a JEOL 100CX TEMSCAN at 20kV accelerating voltage. Energy dispersive X-ray spectra were collected for 10 minutes using a Link 860 series 2 energy dispersive X-ray microanalyser and the results shown as energy spectra.

3.5.8 Preparation for Backscattered Electron Imaging

The samples were prepared as for X-ray microanalysis except that the carbon coating was deposited using a Polaron Carbon Evaporation Head attached to the Polaron E5100 Sputter Coating Unit. They were examined in a Philips SEM525M scanning electron microscope fitted with a KE Developments backscattered electron detector at 25kV accelerating voltage, and photographed onto Polaroid film.

3.6 Contact Angle Measurements

The surfaces were blotted dry with tissues ready for the contact angle measurements. Contact angles for distilled water and methyl iodide were measured using a travelling microscope with a rotating eyepiece calibrated in degrees, with the left and right contact angles being paired and averaged. Surface energies (γ_S^d and γ_S^p) were calculated according to the method of Owens and Wendt from the formula:

$$1 + \cos\theta = 2\sqrt{\gamma_S^d \frac{\sqrt{\gamma_L^d}}{\gamma_L}} + 2\sqrt{\gamma_S^p \frac{\sqrt{\gamma_L^p}}{\gamma_L}}$$

3.7 Goniophotometer Measurements

Care was taken to mount the samples vertically and flush with the front of the sample holder, and the sample mounted at 45° to the incident beam. The samples were backed with a flat sheet of metal painted with an even matt black coating to reduce reflections from the back surface of the transparent samples. The instrument was calibrated using a clean microscope slide before running the experimental samples.

The goniophotometer traces were recorded before protein deposition and after the proteins had been deposited at room temperature for 1 hour, using a BBC Master microcomputer.

CHAPTER 4

CELL ADHESION TO PROTEIN COATED SURFACES

CELL ADHESION TO PROTEIN COATED SURFACES

4.1 Introduction

In any investigation into the role and mechanisms of "interface conversion" on the fate of polymeric materials in a biological environment, the response of a biological probe is obviously important. The cell culture model is fundamental to our investigations (Minett, 1986; Thomas, 1988) and it is, therefore, the obvious choice for the first method of testing a range of biomaterials and biomolecules. Whilst it is not practical to cover the whole range of surface properties in detail, the range of materials used in the study was chosen to express a variety of cell responses due to different surface physicochemical properties. The cell response will be determined by these experiments and will lead to further examination of these surfaces and deposited layers by other techniques.

The cell culture technique is a convenient means of testing synthetic materials and relating their surface properties to cellular response (Lydon et al, 1985; Thomas et al, 1986). An *in vitro* testing model allows more immediate and less ambiguous control of the cellular environment in which to study cell adhesion, spreading and motility (Trinkaus, 1976), compared to an *in vivo* test. The activity and specificity of adhesion molecules can be examined in such a model system. An understanding of the mechanisms involved in cell adhesion using model substrata and cell culture is important in the study of cell/matrix and cell-cell interactions *in vivo* and will aid the development of improved biomaterials.

Established cell lines, such as baby hamster kidney fibroblasts, are commonly used cell types owing to their multivalent cell attachment mechanisms (Couchman et al, 1983; Kleinman et al, 1981). Most cells in culture will adhere to and spread on tissue culture plastic whilst others require a proteinaceous substrata such as collagen or fibrin (Elsdale and Bard, 1972). The identification of two cell-adhesion promoting proteins from serum, and the fact that they complex with other extracellular constituents (Doillon et al, 1985; Grinnell and Phan, 1983; Hynes and Yamada, 1982; Turley et al,

1985) indicates that the process of cell adhesion to synthetic substrata is complex. The situation *in vivo* would be expected to be more complicated due to the large variety of cell-matrix and cell-cell interactions that occur in tissues (Abercrombie, 1982: Grinnell, 1978: Hall et al, 1988: Heaysman and Peagram, 1982: Taylor, 1970). The use of two of the main cell adhesion-promoting proteins, vitronectin and fibronectin, are likely to be of major importance in interface conversion. They seem to have different functions *in vivo*, and by studying their deposition and action on the biological system we can aid the interpretation of the mechanism of action of media serum supplement.

4.2 Results

The cell adhesion results are summarised in Tables 4.1 to 4.5. The cell response is expressed as the number of cells which have attached, the number of cells which have also spread, and the percentage of cells which had spread was calculated. From these the amount of attachment and spreading was normalized to the attachment and spreading of cells grown on tissue culture plastic, which was taken as the 100% reference point.

Table 4.1 presents the results of cell spreading on albumin coated surfaces. When albumin is

Table 4.1 Cell response to albumin coated surfaces

Sample Surface	±FCS	Number Attached	Number Spread	%Spread	Attached Normalised (%FCS TC)	Spread Normalised (%FCS TC)	Notes
Bacteriological polystyrene	+	19	1	5.2	2.5	0.1	
Tissue Culture polystyrene	-	63	3	4.8	8.2	0.4	
"	+	252	153	60.7	32.7	20.9	Few well spread cells
"	-	75	0	0	9.7	0	
Glass	+	345	129	37.4	44.7	17.6	Few well spread cells
"	-	139	2	1.4	18.0	0.3	
Siliconised Glass	+	118	20	16.9	15.3	2.7	No well spread cells
"	-	142	0	0	18.4	0	

deposited onto the various surfaces cells did not adhere at all in serum free conditions confirming that albumin has no adhesion-promoting properties. In serum supplemented conditions, the fibroblast cells adhered and spread on tissue culture plastic and glass. These are the same conditions for cell adhesion when no pretreated protein layer is present as occurs under routine cell culture conditions. BHK fibroblasts cells will attach to most surfaces when immunoglobulin is present as a pretreated layer but will only go through the full adhesion and spreading stages on tissue culture plastic and glass when foetal calf serum is used as media supplement. The pattern for cell adhesion for immunoglobulin coated surfaces is shown in Table 4.2, and is seen to be similar to that of albumin coated surfaces.

Table 4.2 Cell response to immunoglobulin coated surfaces

Sample Material	±FCS	Number Attached	Number Spread	%Spread	Attached Normalised (%FCS TC)	Spread Normalised (%FCS TC)	Notes
Bacteriological polystyrene	+	178	38	21	23.1	5.2	
	-	44	0	0	5.7	0	
Tissue culture polystyrene	+	499	445	89.2	64.7	60.9	
	-	30	0	0	3.9	0	
Glass	+	391	332	84.9	50.7	45.2	
	-	22	0	2.9	0	0	
Siliconised Glass	+	115	27	23.5	14.9	3.7	No well spread cells
	-	49	2	4.1	6.4	0.3	No well spread cells

When fibronectin (Table 4.3) or vitronectin (Table 4.4) is present as a deposited protein layer then cells will attach and spread on all surfaces. The results show that these cell-adhesion-stimulatory protein layers promoted the attachment and spreading of cells on all materials irrespective of the nature of that surface.

Table 4.3 Cell response to fibronectin coated surfaces

Sample	±FCS	Number Attached	Number Spread	%Spread	Attached Normalised (%FCS TC)	Spread Normalised (%FCS TC)	Notes
Bacteriological polystyrene	+	576	451	78.3	74.7	61.7	Few well spread cells
"	-	645	307	47.6	83.7	42.0	Few well spread cells
Tissue Culture polystyrene	+	680	640	94.1	88.2	87.6	
	-	530	457	86.3	68.7	62.5	
Glass	+	530	370	69.8	68.7	50.6	Well spread cells
"	-	495	255	51.5	64.2	34.9	Well spread cells
Siliconised Glass	+	537	450	83.7	69.6	61.6	
	-	462	297	64.3	59.9	40.6	

Table 4.4 Cell response to vitronectin coated surfaces

Sample	±FCS	Number Attached	Number Spread	%Spread	Attached Normalised (%FCS TC)	Spread Normalised (%FCS TC)	Notes
Bacteriological polystyrene	+	552	405	75.4	71.6	55.4	
	-	270	126	46.7	35.0	17.2	
Tissue Culture polystyrene	+	760	600	86.8	98.6	82.1	
	-	580	477	82.1	75.2	65.3	
Glass	+	648	533	82.2	84.0	72.9	Well spread cells
"	-	558	378	67.7	72.4	51.7	Few well spread cells
Siliconised Glass	+	597	516	86.4	77.4	70.6	
	-	399	348	87.2	51.8	47.6	No well spread cells

Table 4.5 shows the cell response for cells when foetal calf serum is deposited onto material surfaces prior to culture. From this it is seen that cells spread on tissue culture plastic and glass when foetal calf serum was present as a pretreated layer in serum-free conditions or when used as a medium supplement routine tissue culture conditions. Cells did not adhere to the hydrophobic surfaces of bacteriological grade plastic and siliconised glass in the presence of deposited serum layers. Foetal calf serum appears to be capable of changing the cell adhesive behaviour of materials coated with non-adhesive protein layers such as albumin and immunoglobulin (Tables 4.1 and 4.2). The spreading behaviour of BHK fibroblasts on all deposited protein layers appeared to be better when serum was present as a medium supplement.

Table 4.5 Cell spreading on foetal calf serum deposited on various surfaces.

Sample	±FCS	Number Attached	Number Spread	%Spread	Attached Normalised (%FCS TC)	Spread Normalised (%FCS TC)	Notes
Bacteriological polystyrene	+	101	10	10.0	13.1	1.4	
Tissue Culture polystyrene	-	78	29	21.7	10.1	4.0	
"	+	771	731	94.8	100	100	Well spread cells
"	-	612	585	95.6	79.4	80.0	Well spread cells
Glass	+	725	653	90.0	94.0	89.3	Well spread cells
"	-	438	408	93.2	56.8	55.8	Well spread cells
Siliconised Glass	+	99	41	41.1	12.8	5.6	No well spread cells
"	-	145	46	31.7	18.8	6.3	No well spread cells

4.3 Discussion

The results of this cell culture experiment confirm the earlier work from our laboratories and others, that cells will grow on certain adhesive surfaces in the presence of serum in the medium, whereas non-adhesive surfaces do not support cell adhesion in the presence of serum (Figures 4.1 and 4.2). Cells can be stimulated to adhere to normally non-adhesive surfaces when those materials have been pretreated with a deposited layer of an adhesion-promoting protein such as vitronectin or fibronectin. Albumin, as expected, has no adhesion promoting properties. Immunoglobulin results in cell attachment to the substratum, but little cell spreading occurs except when serum is in the medium on tissue culture plastic or glass, ie. normally adhesive combinations.

An interesting finding is that serum in the medium as a supplement, can alter the cell response to deposited proteins which do not have an adhesion-stimulatory effect such as albumin or IgG. The cell response in these circumstances has the same relationships to that seen in the control situation with serum supplemented medium, although the number of cells is modulated (reduced) compared to the normal situation, when a precoating layer of albumin is present (Figures 4.3 and 4.4). This finding, that a media supplement of serum can over-ride the expected effects of albumin had been noticed previously (Gjessing & Seglen, 1980). Schakenraad et al (1989) have also reported cell spreading on albumin coated surfaces, but as they were using serum supplemented medium their results are due to the same effect. Serum from the medium must be either displacing the deposited albumin or IgG layers or forming additional layers on top of the previously deposited protein layer. The cells then respond to these layers which will stimulate cell attachment and spreading. It shows that cell adhesion experiments need to be conducted with and without serum supplementing the medium in order for the full effects of a protein layer on cell adhesion to be assessed.

Cell growth on normally non-adhesive materials when pretreated with vitronectin or fibronectin suggests that the cell response, when using single protein layers, is dominated by the cell adhesion stimulatory property of the protein and, in serum-free conditions, is largely independent of the

substrate material properties. When serum is used, either with pretreated surfaces in serum-free conditions or with serum supplemented medium, the cell response is influenced by the surface properties (eg. wettability) of the substratum material.

The adhesion-promoting proteins will induce spreading of cells on non-adhesive surfaces. This is presumably not as efficient as for serum supplemented growth on the normally adhesive surfaces as cells on fibronectin- and vitronectin-treated surfaces appear rather spindly (Figures 4.5 and 4.6). They appear to spread better when serum is present in the medium as a supplement. This suggests that the cells require other hormones and growth factors which are to be found in serum for optimum growth (Rockwell et al, 1980). This effect of more complete spreading has also been noticed for endothelial cells grown on fibronectin coated poly(ethylene terephthalate) surfaces (van Wachem et al, 1989). Also, it has been reported that cells show greater spreading on protein coated substrata than on bare surfaces when in serum supplemented medium (Schakenraad et al, 1989).

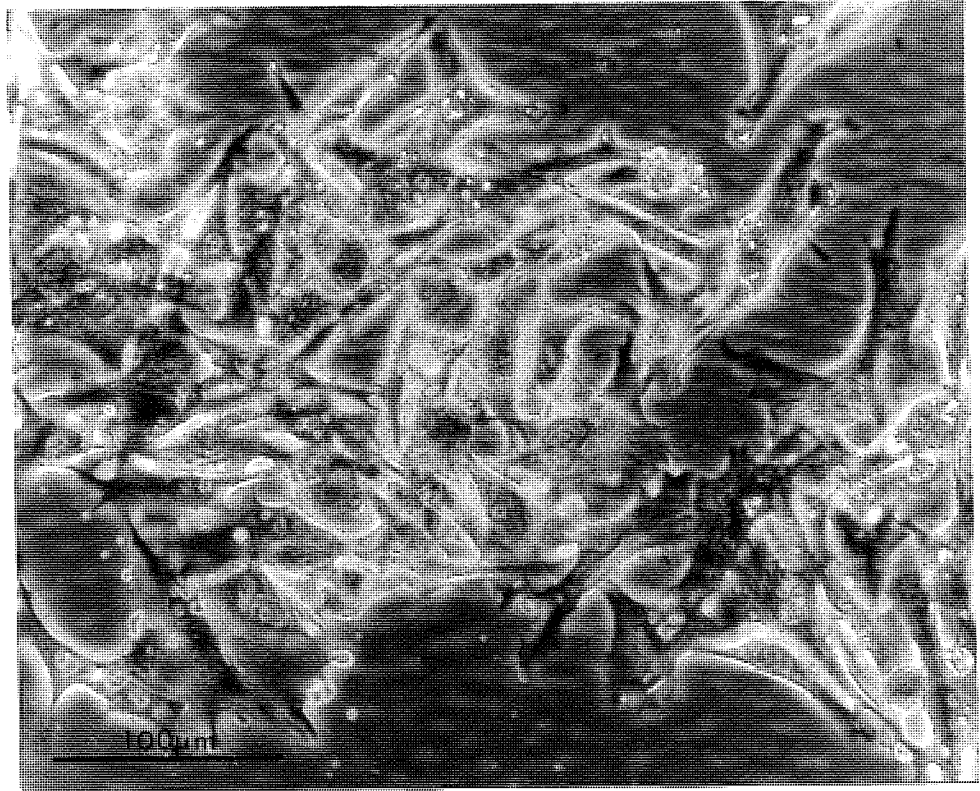


Figure 4.1 Cell adhesion to foetal calf serum coated tissue culture plastic.

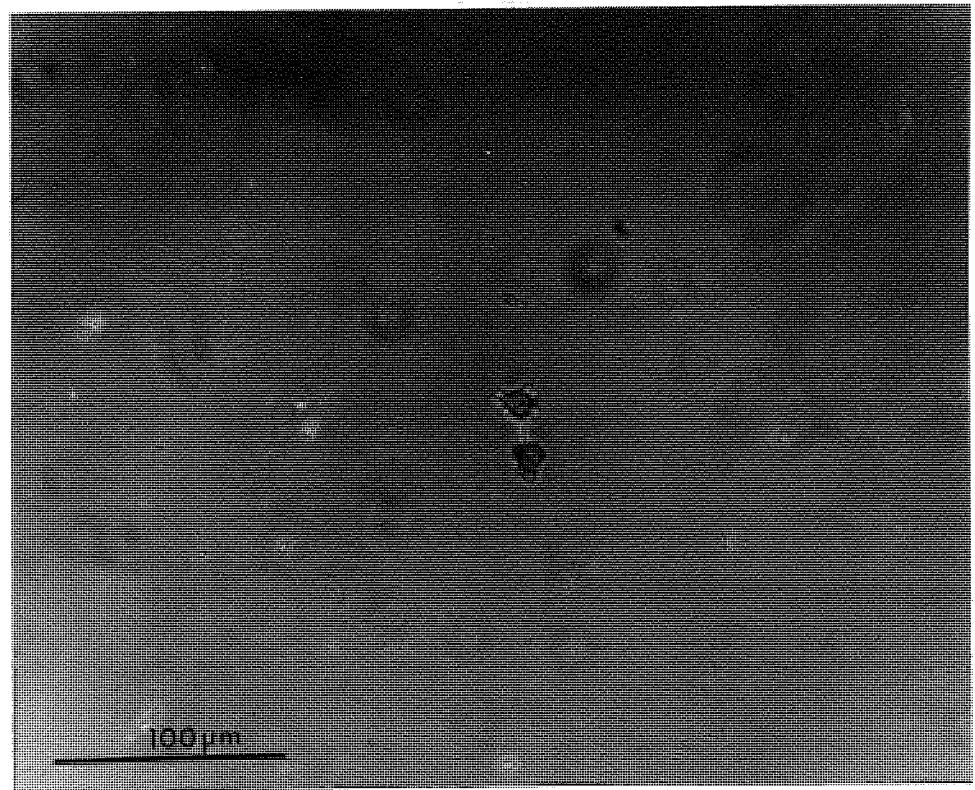


Figure 4.2 Cells seeded on foetal calf serum coated bacteriological grade plastic show virtually no cells attached or spread

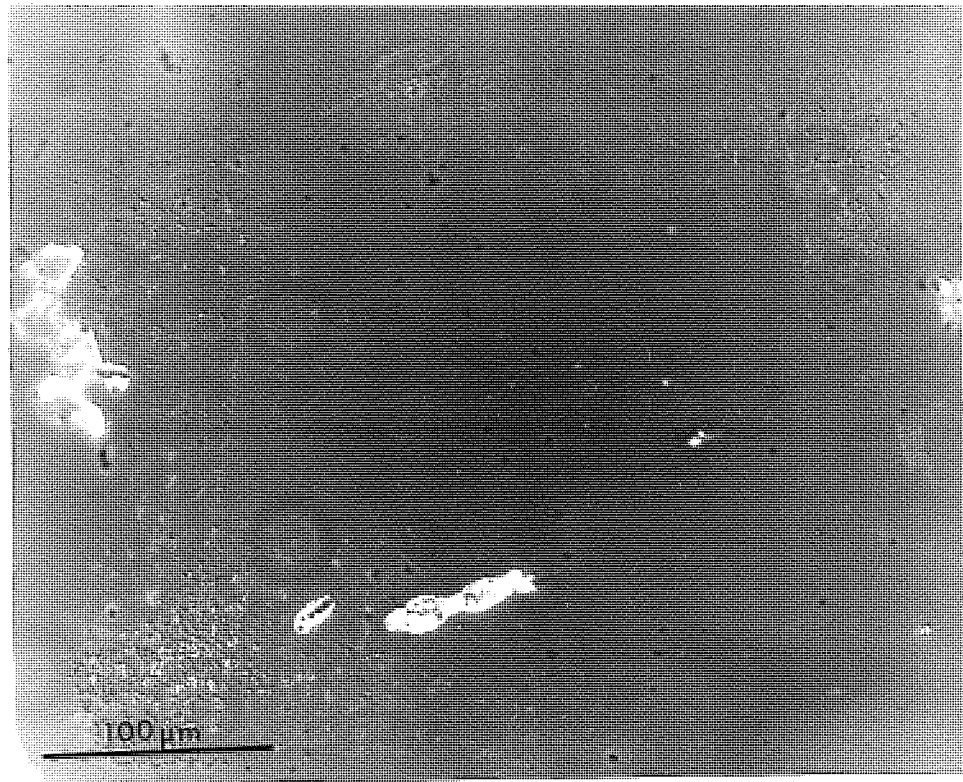


Figure 4.3 No cells are seen spreading on albumin coated tissue culture plastic when serum-free culture conditions are used.

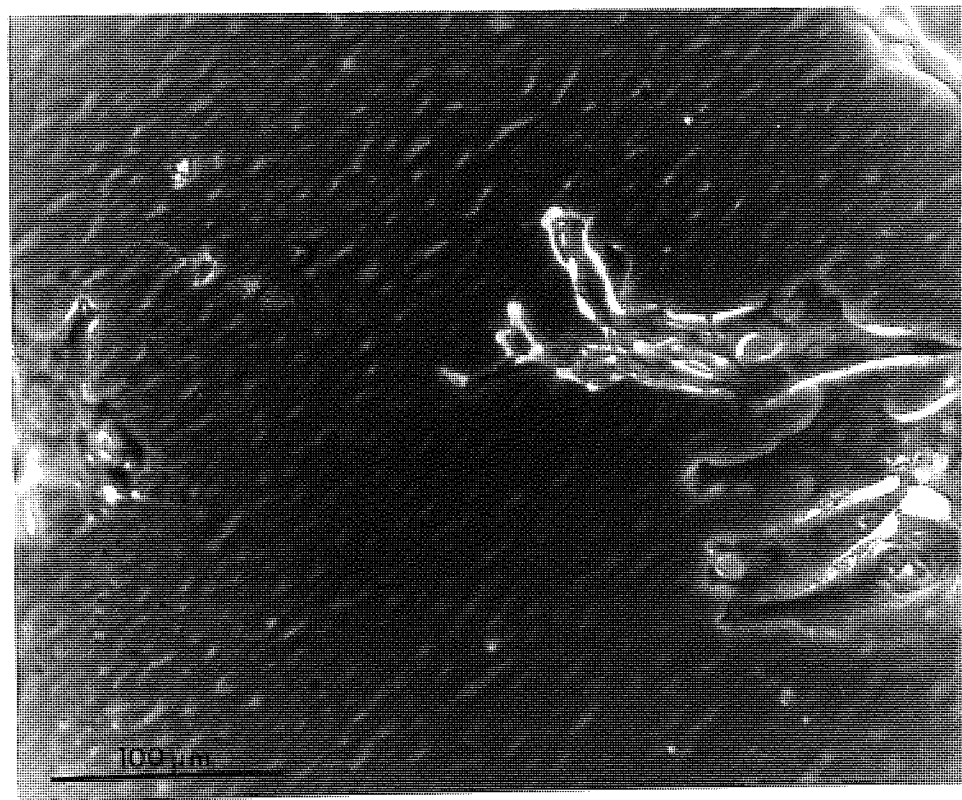


Figure 4.4 The cell response to albumin coated tissue culture plastic when serum supplement is present is similar to the response seen with serum precoated tissue culture plastic.

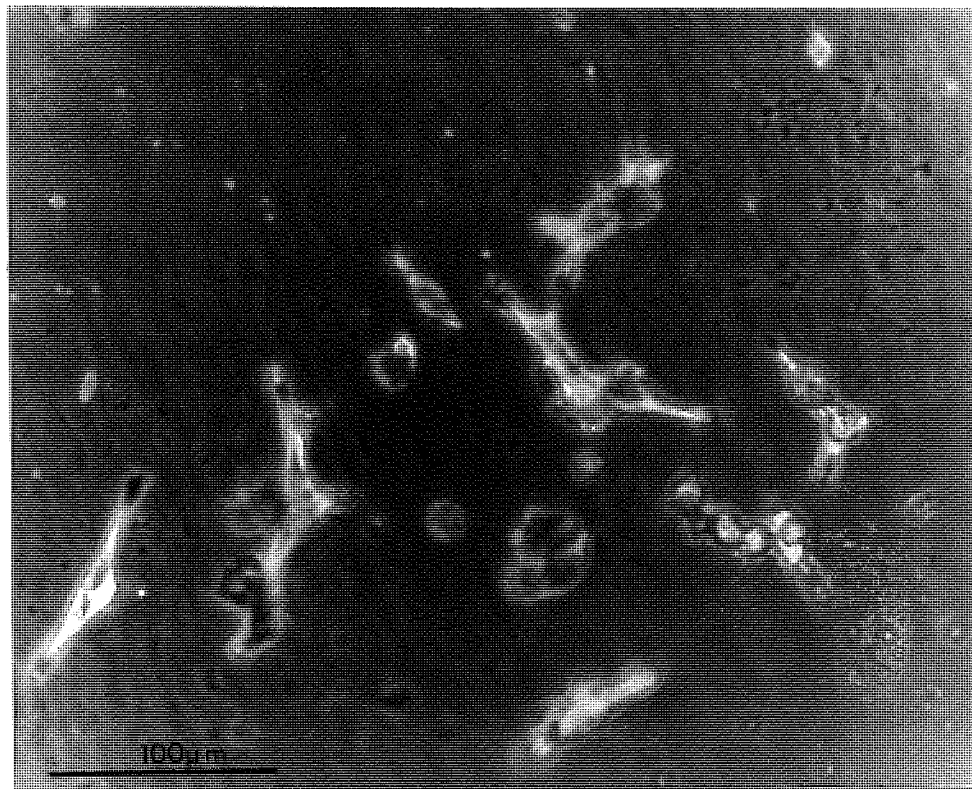


Figure 4.5 Cell spreading on fibronectin coated bacteriological grade plastic in serum-free conditions reveals predominantly bipolar cells rather than well spread cells.

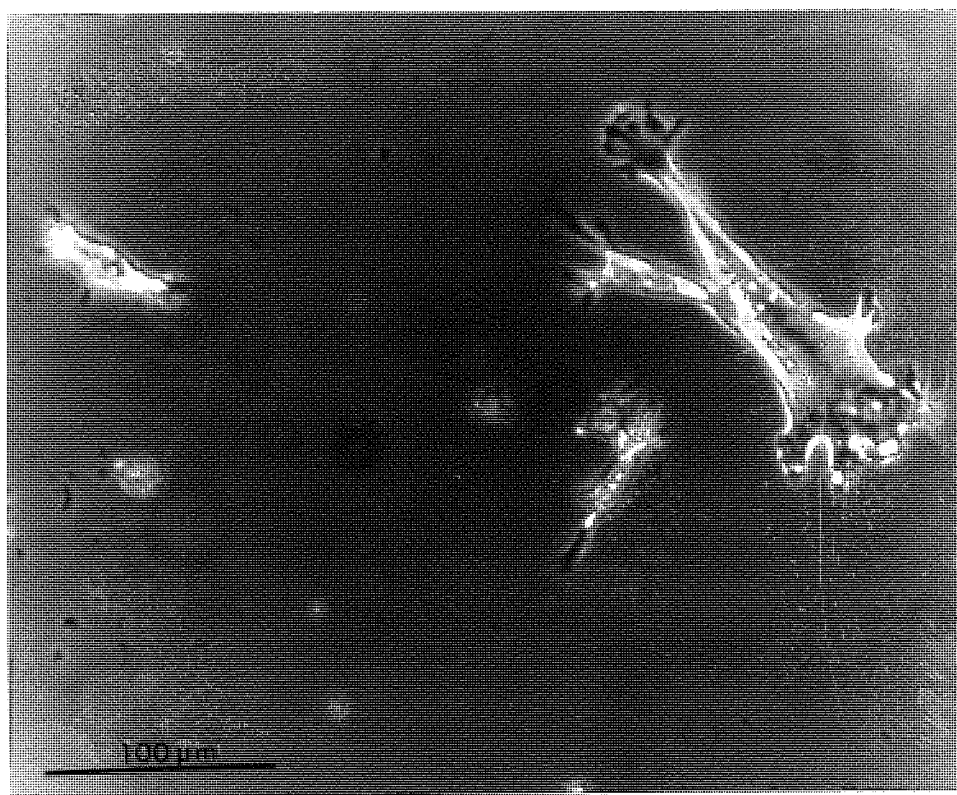


Figure 4.6 Cells on a normally adhesive surface, glass, tend to be spindly when an adhesion-promoting protein (vitronectin) is deposited and cells grown in serum-free conditions.

4.4 Conclusions

Adhesion-promoting proteins will induce cells to spread, even on surfaces which are not normally attractive to them when in normal culture conditions. Vitronectin and fibronectin were demonstrated to be cell adhesion-promoting proteins, whilst IgG and albumin were not. The presence of serum in the culture medium had a beneficial effect even with adhesion-promoting protein layers, presumably due to the presence of other growth factors. Single protein layers, even though they may promote cell spreading appear to produce inferior spreading when compared to that produced by a layer derived from foetal calf serum.

Cells spread onto surfaces pretreated with "non-adhesive" proteins if serum was used as a culture media supplement. This indicates that either these protein layers are replaced by the correct proteins from serum or that the new protein layers deposited from serum are laid on top of the first layer. The replacement or superimposition of single protein layers with a serum layer could also help to explain the improved cell spreading response of the adhesion-promoting proteins when foetal calf serum is present in the culture medium. Cells obviously need other factors besides adhesion-promoting factors for optimal spreading and growth.

Cell attachment and spreading, in serum-free conditions, is dominated by the cell adhesion stimulatory property of the protein and is largely independent of the substrate material properties. The response of cells, in serum-supplemented media, is much more influenced by the surface properties or chemistry of the underlying material. In these cases, the substratum material surface physicochemical properties must influence the deposition of the serum layer in a way which will determine the cell adhesion response of the material/protein combination. When serum is the sole deposited layer, whether from solution or by pretreatment of the substrate material, the physicochemical nature of the substrate material must determine the exact nature and composition of the biomolecules in the deposited serum layer. This in turn determines whether cells are or are not able to attach and spread on that surface.

Further work is necessary to investigate the exact physical and chemical surface properties which are important in the process of interface conversion using a multitude of surface analytical probes. The cell culture model is of key importance in determining the exact nature of the process of interface conversion from the biological perspective. Previous work from our group has suggested that surface rugosity may be a factor in cell adhesion (Minnett, 1986; Thomas, 1988). In order to study this aspect there are two techniques which can be used to characterise the surface roughness of materials, goniophotometry and scanning electron microscopy. As goniophotometry is capable of quantifying the degree of surface rugosity encountered, this technique is the first choice for investigation in our aim to probe the mechanisms of "interface conversion".

CHAPTER 5

GONIOPHOTOMETER MEASUREMENTS OF PROTEIN COATED SURFACES

GONIOPHOTOMETER MEASUREMENTS OF PROTEIN COATED SURFACES

5.1 Introduction

The cell culture experiments have shown that the cell response to different materials can be modified by adhesion promoting protein layers. Previous work from our group suggested that surface rugosity may be a factor in cell adhesion to polymeric materials (Minnett, 1986; Thomas, 1988). The varying cell response to the range of substratum materials tested here could be explained by the surface roughness. The action of the adhesion promoting proteins may be due to changes in surface rugosity brought about by the deposition onto polymeric materials of protein or serum films which are only a few nanometres thick. As the bulk of the material is not changed by the deposition process, any variations in glossiness detected will reflect changes in surface rugosity produced by the deposition of the protein layers. The surface rugosity of polymeric materials and deposited protein layers needs to be characterised in order to provide answers to these points.

Goniophotometry is a technique which offers a potential means of characterising and quantifying any differences in surface roughness of the polymeric materials which may explain the observed cell response. The changes in surface rugosity brought about by deposited protein or serum layers can be measured in an attempt to assess the influence of the various deposited layers on the cell adhesive properties of the test materials. In these experiments the intention was to look at the surface rugosity of the test materials, and the deposited protein and serum layers and relate these to the results of the cell experiments.

5.2 Results

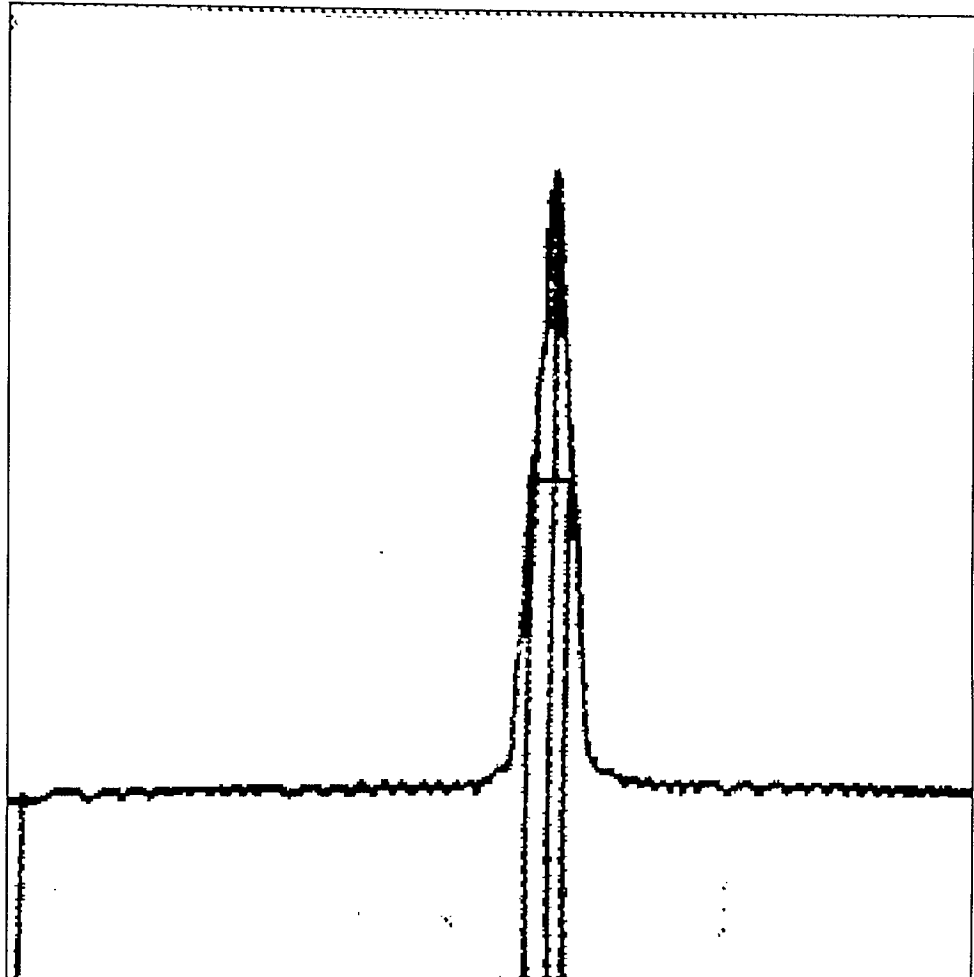


Figure 5.1 A typical trace from the experimental samples, in this case a glass microscope slide which has had a foetal calf serum layer deposited

A typical goniophotometer trace from these samples is shown in Figure 5.1 where the sample is a clean microscope slide treated with foetal calf serum. It shows a sharp, narrow peak which is typical of a highly reflective surface. It needs to be compared to the untreated glass microscope slide which is shown in Figure 2.8 (page 90) as an example of a highly reflective surface; the profile can be seen to be very similar in size and shape. All the samples, including those with a deposited protein layer, produced traces which appeared to the eye to be identical to that shown here in Figure 5.1.

Table 5.1 Combined results of goniophotometer measurements on deposited protein layers on surfaces

	Is	Id	HPW	GF
Glass	513.4	124.8	2	194.3
Glass + BSA	554.1	125.6	2	214.2
Glass	555.8	125.6	1.8	239
Glass + IgG	580.3	126.8	2	226.7
Glass	583.4	125.3	2	229.1
Glass + FCS	523.9	127.9	2.2	180.0
Glass	544.1	125.3	2.4	174.5
Glass + Fn	485.3	126.8	1.9	188.7
Siliconised Glass	512.7	122.5	2.2	177.4
Siliconised Glass + BSA	535.3	127.4	1.8	226.6
Siliconised Glass	519.3	126	2.1	187.3
Siliconised Glass + IgG	566	127.4	2.1	208.9
Siliconised Glass	544.8	123.4	2	210.7
Siliconised Glass + FCS	502.7	127.7	2	187.5
Siliconised Glass	528.1	125.6	2	201.2
Siliconised Glass + Fn	502.9	123.1	2.1	180.9
Bacteriological polystyrene	573.9	126	2.2	203.6
Bacteriological polystyrene + BSA	568	122.9	2.3	193.5
Bacteriological polystyrene	650.1	127.7	2.1	248.8
Bacteriological polystyrene + IgG	594.4	127.4	2.3	203
Bacteriological polystyrene	613.4	125.3	2.4	174.5
Bacteriological polystyrene + FCS	408.1	123.7	2.6	109.4
Bacteriological polystyrene	599.6	127.7	2.3	205.2
Bacteriological polystyrene + Fn	432.4	122.9	2.3	134.6
Tissue Culture polystyrene	586.8	123.4	1.8	257.4
Tissue Culture polystyrene + BSA	563.9	127.2	1.8	242.6
Tissue Culture polystyrene	608.7	122.5	1.9	255.9
Tissue Culture polystyrene + IgG	626.6	128	1.8	277
Tissue Culture polystyrene	611.8	128	2	241.9
Tissue Culture polystyrene + FCS	630.1	125.3	1.6	315.5
Tissue Culture polystyrene	605.9	127.9	1.9	251.6
Tissue Culture polystyrene + Fn	1149.7	385.9	1.9	402.0

The main results are presented in Table 5.1 and show the specular intensity (Is), diffuse reflectance (Id), peak width at half height (HPW) and gloss factor (GF), which is given as:

$$GF = \frac{I_s - I_D}{W_{\frac{1}{2}}}$$

The results of these experiments are also expressed graphically in Figures 5.2 to 5.5 which are histogram plots of the various materials before and after the deposition of the protein layers of albumin, IgG, fibronectin and foetal calf serum. The histograms show the specular intensity, diffuse reflectance and gloss factor for all these samples.

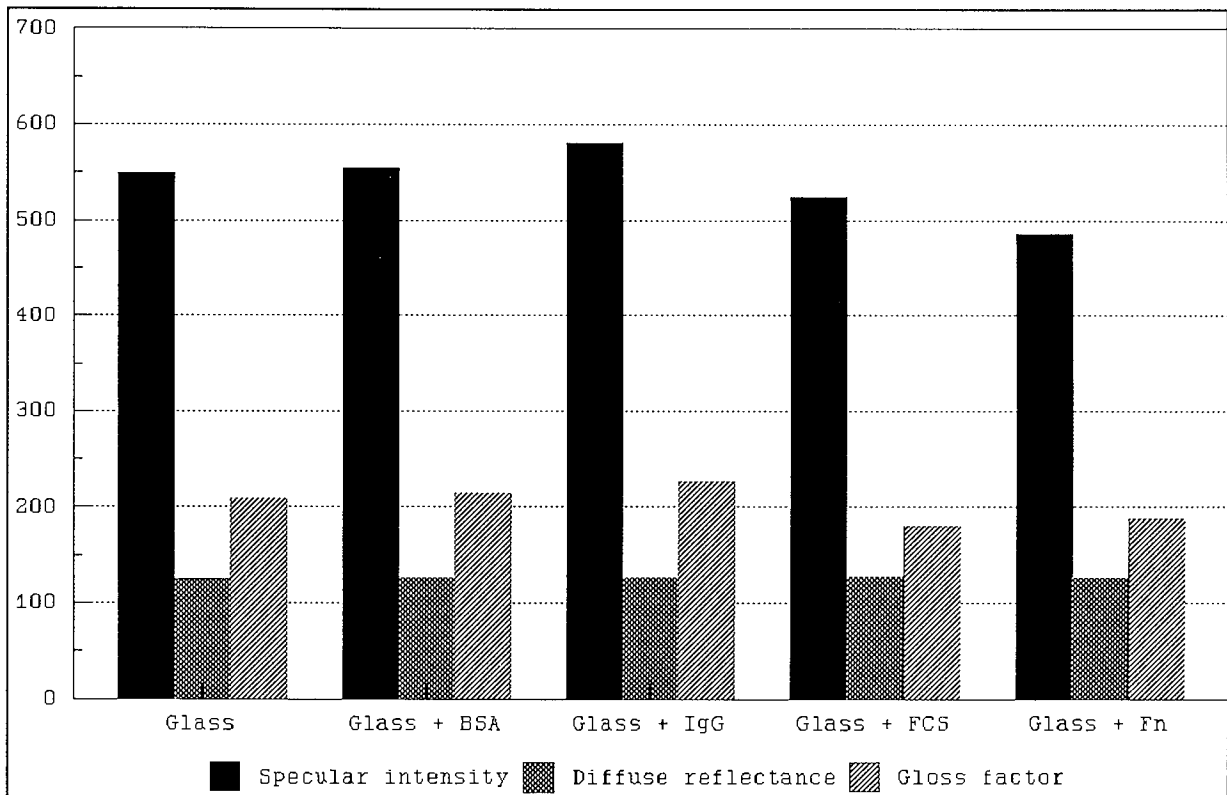


Figure 5.2 Histogram of goniophotometer data for protein and serum layers deposited onto glass

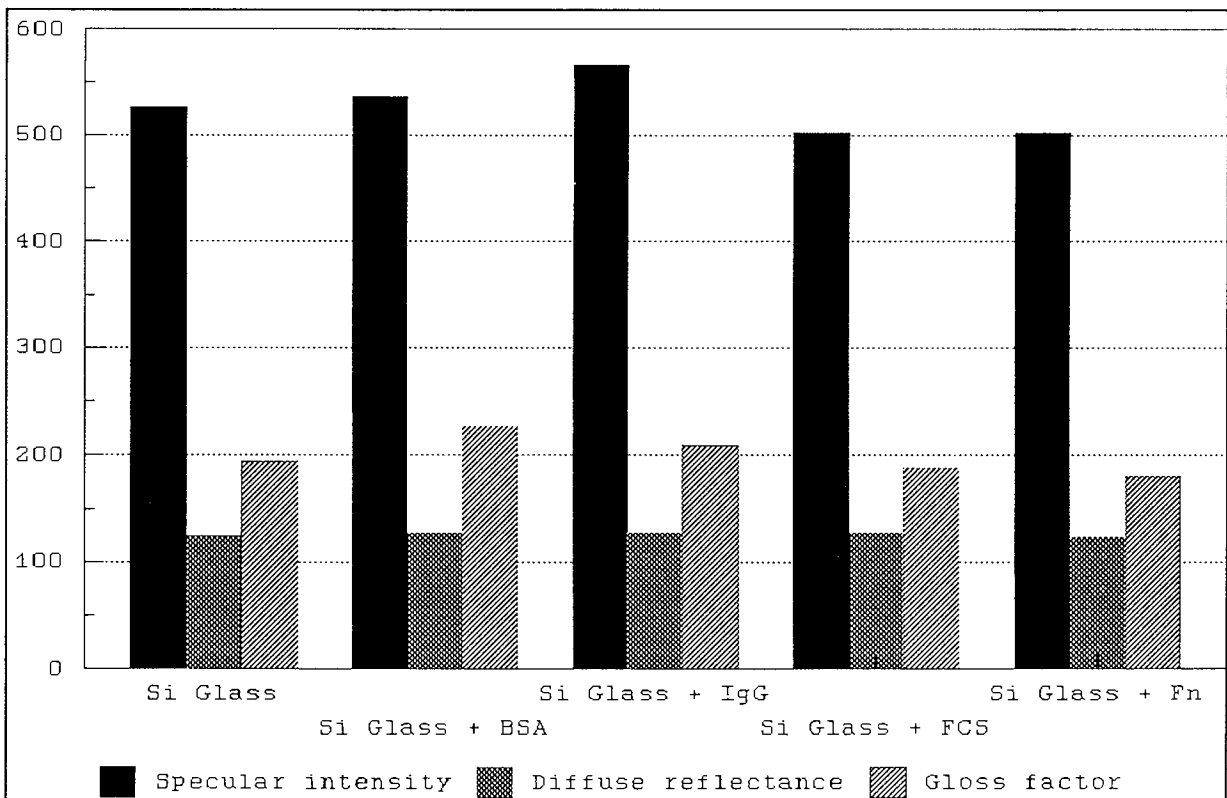


Figure 5.3 Histogram of goniophotometer data for protein and serum layers deposited onto siliconised glass

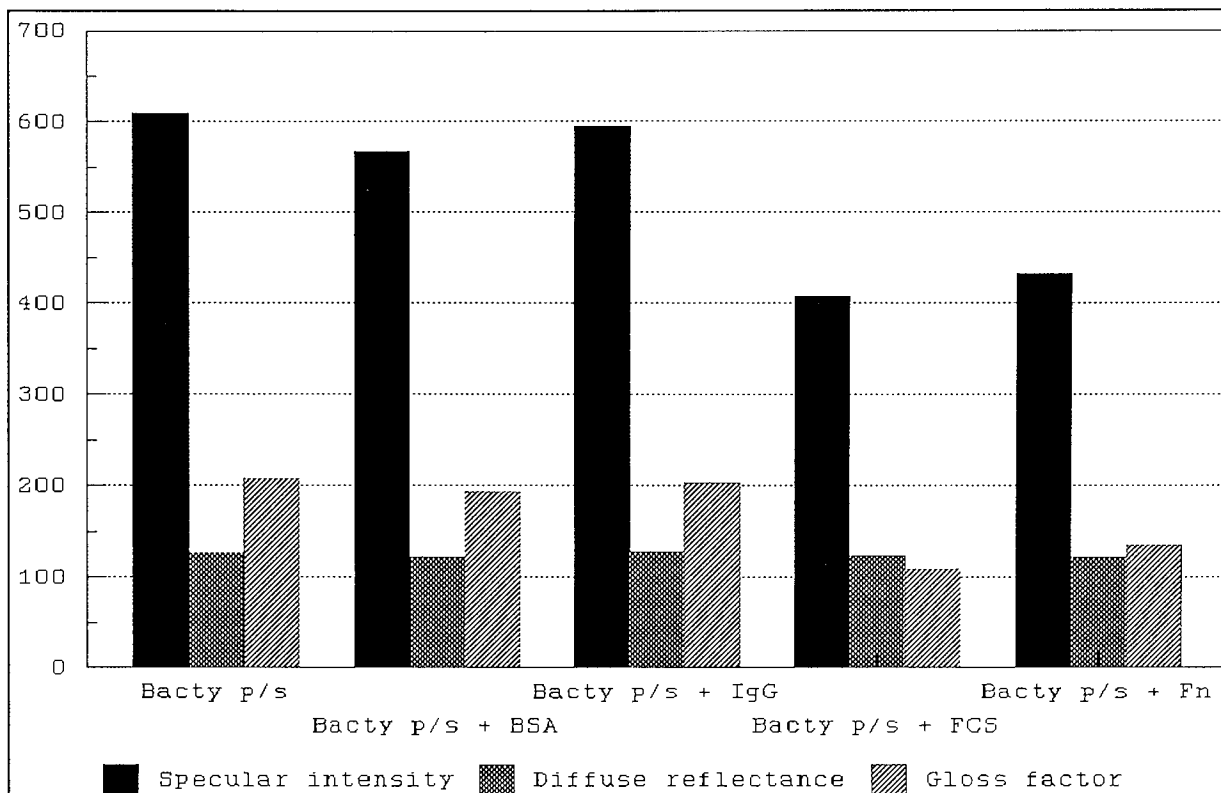


Figure 5.4 Histogram of goniophotometer data for protein and serum layers deposited onto bacteriological grade plastic.

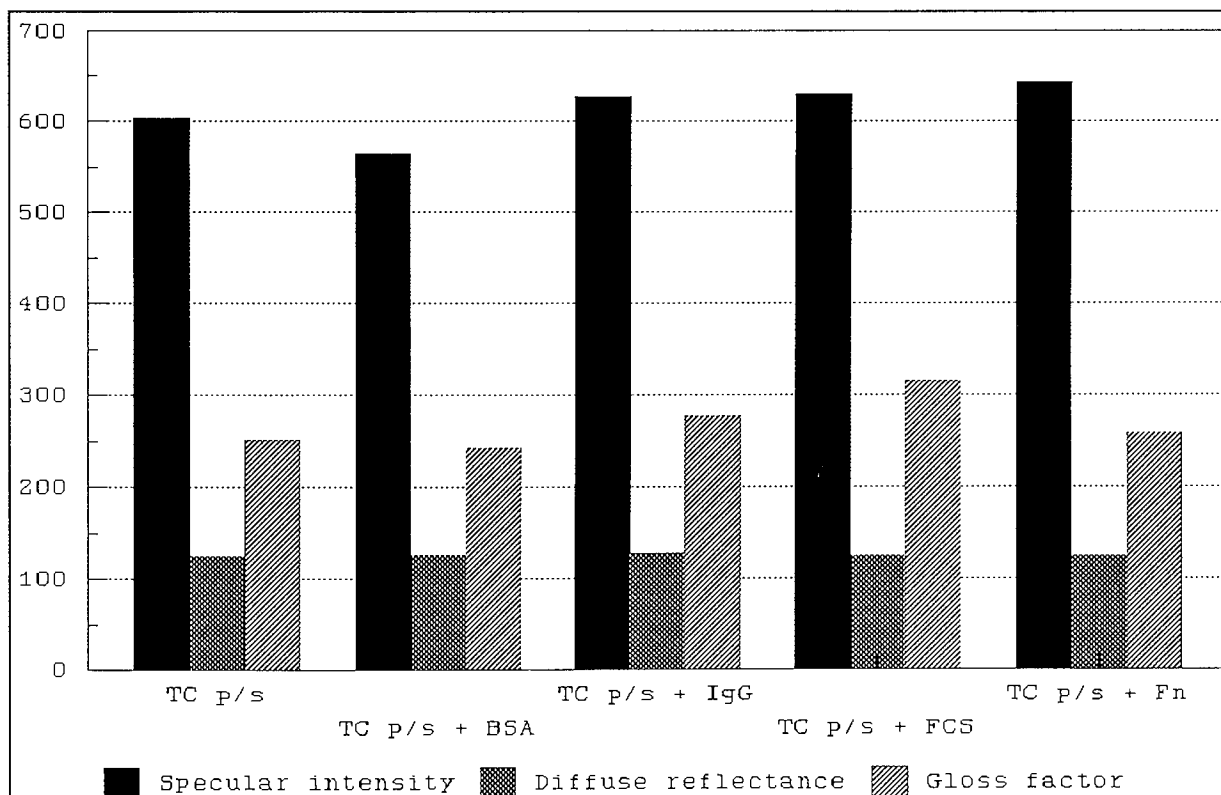


Figure 5.5 Histogram of goniophotometer data for protein and serum layers deposited onto tissue culture grade plastic.

5.3 Discussion

The goniophotometer traces are all very similar to each other and have sharp, narrow peaks which are typical of very smooth, highly reflective surfaces. Variation of I_S on the deposition of proteins is of the order of $\pm 10\%$. As a contrast a goniophotometer trace from a very roughened surface is shown in Figure 5.6. This is a goniophotometer trace from a sample which has a scale of roughness features which are much greater than the polymer and deposited protein surfaces investigated here.

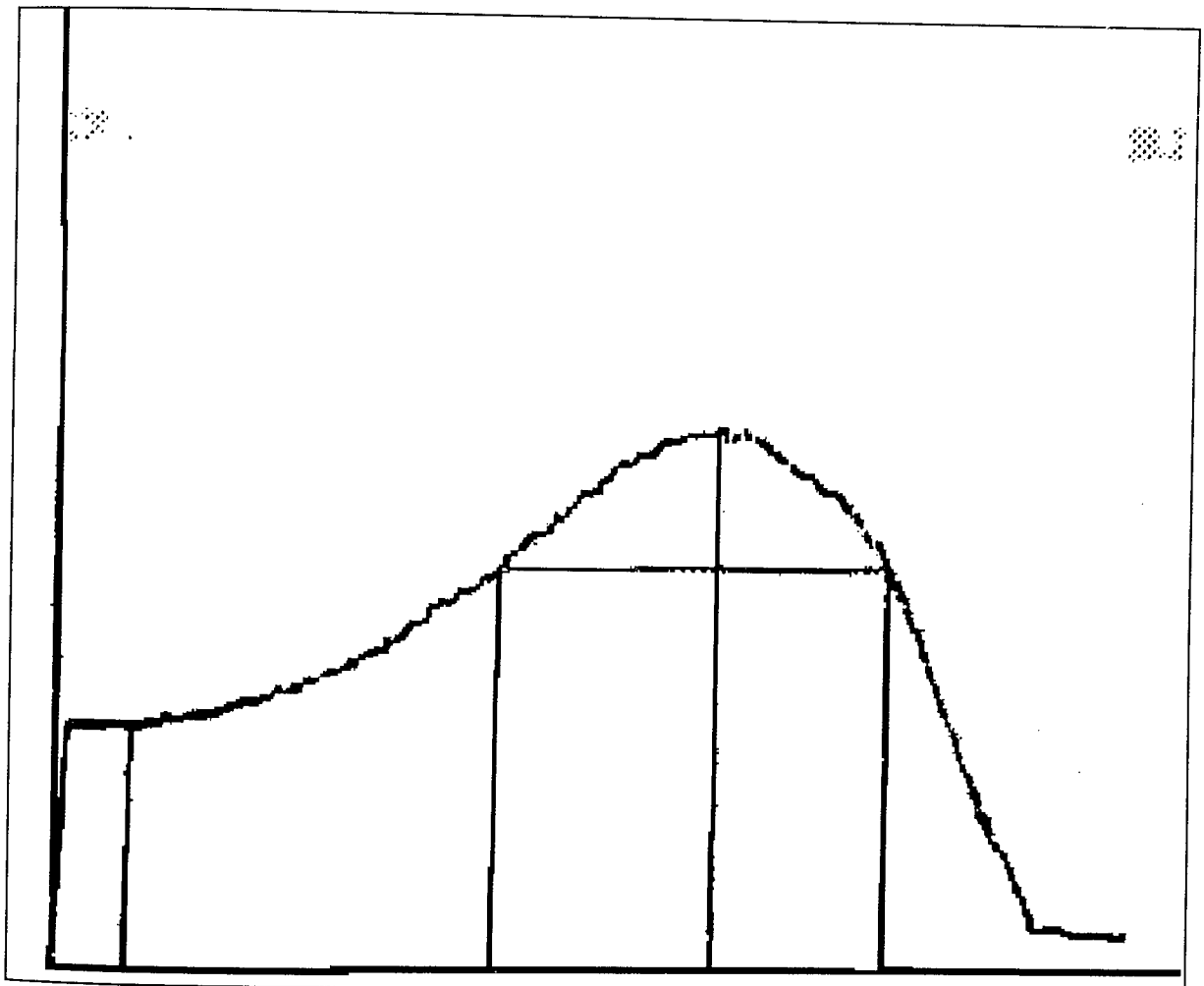


Figure 5.6 A typical goniophotometer trace from a rugose surface

There appear to be no trends here. The deposited protein layers make the material surfaces both more and less shiny with no correlation with the adhesive nature of that surface or deposited protein layer when cells are cultured on the various materials. The results show no significant differences between the various surfaces in terms of rugosity. All surfaces can be classed as highly reflective surfaces with little change seen in diffuse reflectance readings (I_D) and the peak width ($W_{1/2}$). Dip coating is a 'low tech' way of coating surfaces, and variations in the coatings produced could reflect differences seen in the goniophotometer readings obtained.

The limitation of the technique revealed in these experiments is that any surface irregularities present, either before or after protein deposition, are below the wavelength of light. Thus, the variations in I_S , with no discernable changes in I_D and $W_{1/2}$, are due to very small differences in surface rugosity. These differences can be in two forms, increasing/decreasing size of surface imperfections and differences in the population density of the surface features. Had the surface imperfections been larger, and either approaching or larger than the wavelength of the light beam, then peak broadening would have occurred, as occurred in Figure 5.6.

5.4 Conclusion

It can be concluded that the surface features are 'micro' in size in that they are below the wavelength of the light used, ie. $<0.5\mu\text{m}$. Thus, any possible relationship between cell adhesion and surface rugosity must be investigated using a technique which can probe the surface irregularities with a greater resolution. This can be done by a number of techniques such as scanning electron microscopy or profilometry. Both of these have a resolution which is not dependent on the wavelength of light as is the case with goniophotometry. However, the goniophotometer is an excellent non-invasive technique which gives a measure of surface roughness. The exploitation of these factors was the object of these experiments where the same surface was measured before and after the deposition of a protein layer.

With the scanning electron microscope the surface to be investigated usually has to be prepared to enable it to be examined, for SEM samples must be both free from volatile material and electrically conductive. Either of these requirements run the risk that the sample will be altered in the course of preparing it for examination, although a considerable amount of work has been expended in the field of scanning electron microscopy to ensure that samples are prepared without introducing artifacts (Clay et al, 1986; Wollweber et al, 1981). However, the fact that the sample needs to be prepared does prevent true before and after experiments from being performed on the same samples, which was one of the objects of the goniophotometer experiments. The advantage of the SEM is that it can detect and resolve surface details much smaller than the wavelength of light. It has been used previously by workers in our laboratories to investigate the role of surface roughness in biological/synthetic interface reactions (Minett, 1986; Thomas, 1988). This work suggested that surface roughness was possibly an important factor influencing cell adhesion to polymeric substrata.

Profilometry is another means of measuring surface roughness which is well established. It was originally developed for looking at hard metallographic surfaces, but will be of limited use in investigating polymeric materials as the stylus is likely to damage the surface it is probing and so will

not give a true representation of the surface roughness. The technique has, however, been adapted for investigating soft biological material such as skin surface topography by the use of replica techniques. Here the skin surface is replicated using some setting material, which can be through either a liquid solidifying or a resin or rubber polymerising. Profilometry can then be performed on the solidified replica, or a second replica, provided that it is made from a material which is sufficiently hard. The replica technique has also been adapted for use in the low power light microscope or the scanning electron microscope as a non-invasive means of examining skin surface topography by the cosmetics and healthcare industry (Clay and Cherry, 1990).

A recent development of profilometry which works in a fundamentally different way is the scanning tunnelling microscope. This technique uses a scanned stylus to image surface topography by the modulation of the tunnelling current present between the stylus and the sample. It has proved capable of very high resolution in the z direction (at the atomic scale), and it is possible for the instrument to work under ambient atmospheric conditions and even under liquids. It is, however, a new technique with the equipment just now becoming available, and it has not been possible to use it in this series of experiments. Its properties make it worthy of further investigation as a technique for the investigation of the biological/synthetic interface. In the absence of the availability of this exciting new technique it was decided to carry out further SEM investigations into the relationship of surface rugosity and cell adhesion.

CHAPTER 6

SURFACE RUGOSITY AND CELL ADHESION

SURFACE RUGOSITY AND CELL ADHESION

6.1 Introduction

Previous work in our series of investigations has suggested an important role for surface rugosity as a factor in cell adhesion to polymeric materials (Minett, 1986; Thomas, 1988). The goniophotometer experiments were unable to detect any relatively large scale surface roughness features which may have been present. To this end the experiments failed to answer the questions on whether the varying cell response to the range of substratum materials tested in chapter 4 could be explained by the surface roughness of the substratum materials or by the action of the adhesion promoting proteins changing the surface rugosity. The surface roughness seen by previous workers on plasma-etched polystyrene had been in the order of 100nm in size which would be too small to be detected by goniophotometry. The scanning electron microscope has been used previously by our group and others to probe the relationship between substratum topography and cell response. However, it had not been possible to separate the effects of surface energy from surface rugosity in those experiments.

Polystyrene is an important material for use as a model synthetic substratum, as in its pure form it is non-adhesive to cultured cells (Curtis et al, 1983; Grinnell and Feld, 1982; Hughes et al: 1979; Lydon et al, 1985; Thomas et al, 1986). However, it is widely used in a modified form, as tissue culture plastic, for cell culture of anchorage-dependent cells (Stoker et al, 1968), after being surface treated, probably by corona discharge. Commercial tissue culture plastic is considerably more hydrophilic than pure polystyrene (Minett, 1986; Thomas et al, 1986), which is represented by bacteriological-grade plasticware. Polystyrene can be rendered adhesive to cells by a variety of treatments which increase its relative hydrophilicity (wettability) (Grinnell, 1978). These include plasma glow discharge treatment (Amstein and Hartman, 1975; Lydon, 1986; Minett, 1986; Thomas et al, 1986) and brief treatment with concentrated sulphuric acid (Curtis et al, 1983; Gibson and Bailey, 1980; Klemperer and Knox, 1977; Lydon and Clay, 1985; Lydon et al, 1985; Matsuda and Litt, 1975; Thomas et al,

1986), chromic acid (Klemperer and Knox, 1977), chloric acid (Curtis et al, 1983), and by exposure to UV light (Curtis et al, 1983; Martin and Rubin, 1974). These treatments are thought to work by the introduction of hydroxyl groups into the polymer surface (Curtis et al, 1983; Thomas et al, 1986). However, XPS studies of argon- and nitrogen-plasma treated polystyrene shows there to be no increase in surface oxygen after plasma treatment, and yet the cell adhesion on these surfaces is comparable with commercial tissue culture polystyrene (Minett, 1986). An increase in surface rugosity was seen and suggested as a possible explanation for the cell response on these surfaces.

We have recently discovered that a high molecular weight polystyrene has a very slight degree of surface rugosity, similar to argon plasma-etched polystyrene seen previously (Minett, 1986). This represents an opportunity to test whether surface rugosity has a role to play in cell adhesion by comparing surfaces with similar levels of surface rugosity and different wettabilities. We have found that the serum layer deposited onto the surface of materials does have different appearances depending upon the material. An attempt was made to correlate the surface appearance of the deposited serum layer with the surface rugosity of the substratum material.

6.2 Polystyrene Molecular Weight and Surface Rugosity

The surfaces of the low molecular weight (3770 Da) polystyrene were found to be smooth before and after etching when viewed at all magnifications (Figure 6.1). The medium molecular weight (180,000Da) polystyrene has a smooth surface appearance in its native form, but is slightly roughened after plasma etching (at x20,000 magnification: Figure 6.2). The high molecular weight (3×10^6 Da) polystyrene surface has a similar degree of rugosity, both before and after glow discharge treatment (Figure 6.3), to the etched 185,000 Da polystyrene (Figure 6.2).

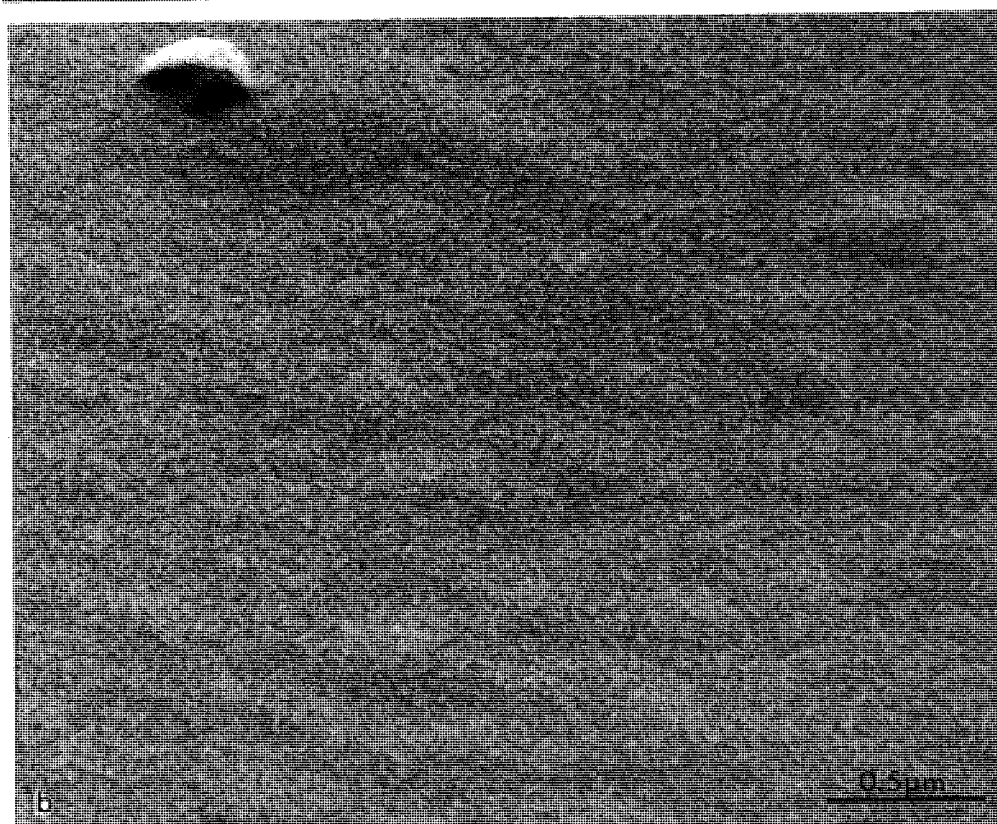


Figure 6.1 SEM micrographs of the low molecular weight (3770 Da) polystyrene show the surfaces to be smooth before (a) and after (b) etching when viewed at all magnifications.

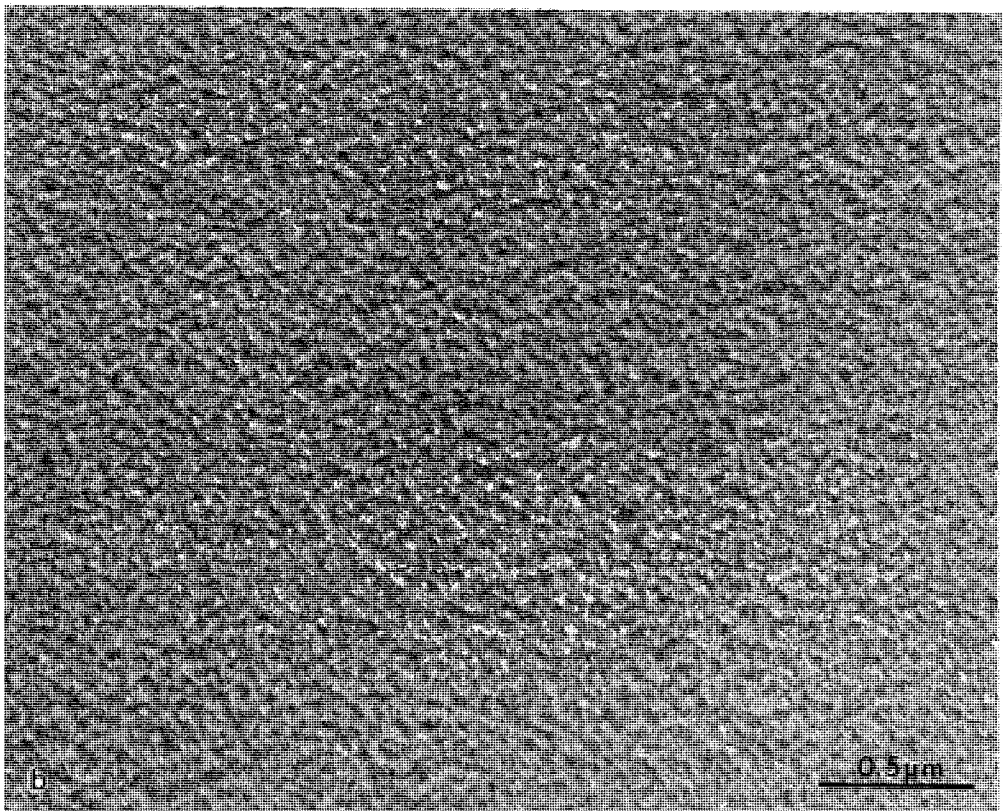
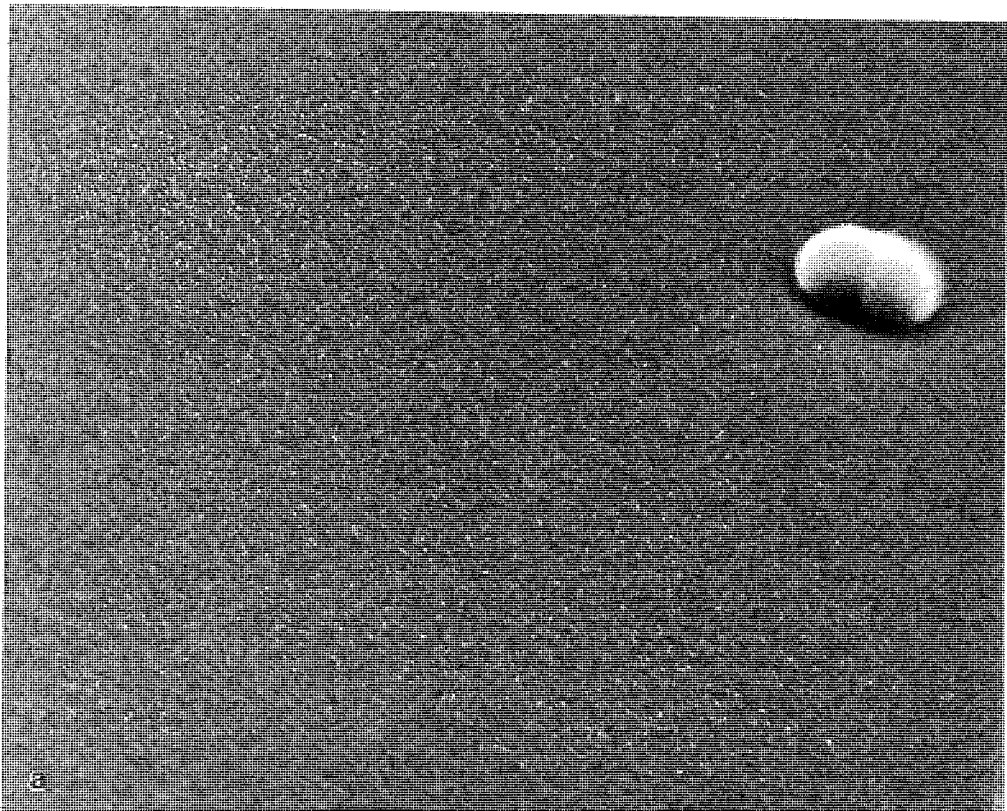


Figure 6.2 The 185,000 Da polystyrene is seen in the SEM to be smooth before etching (a) but with a small degree of surface roughness after etching (b).

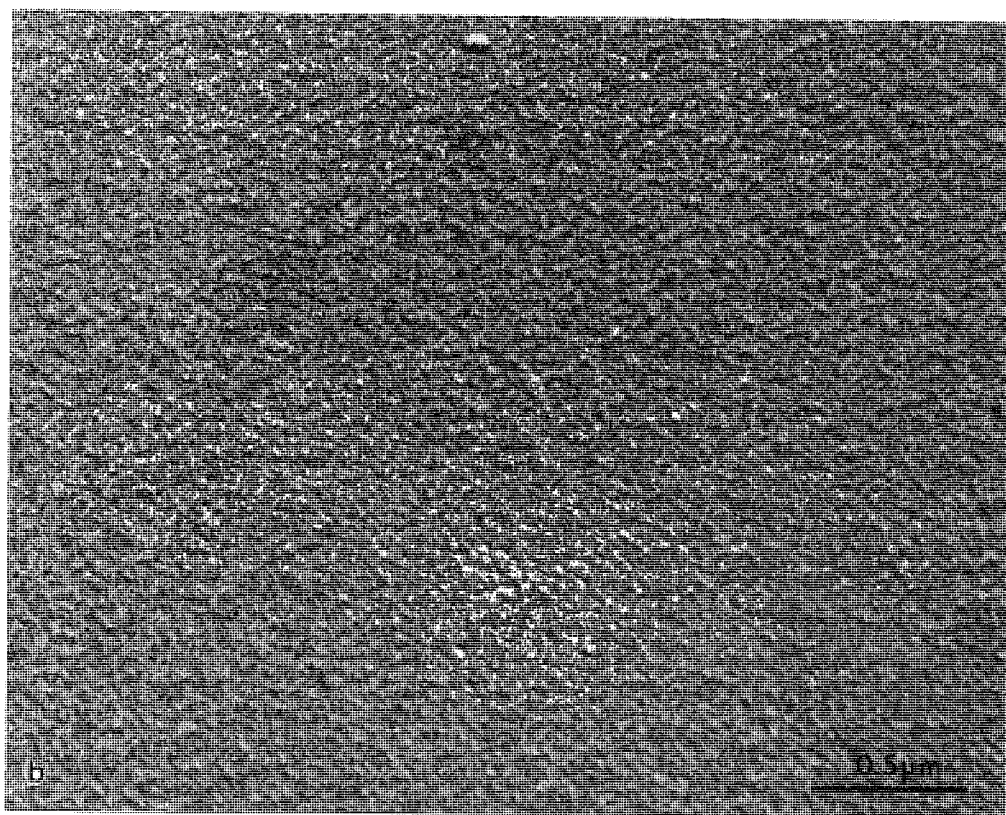
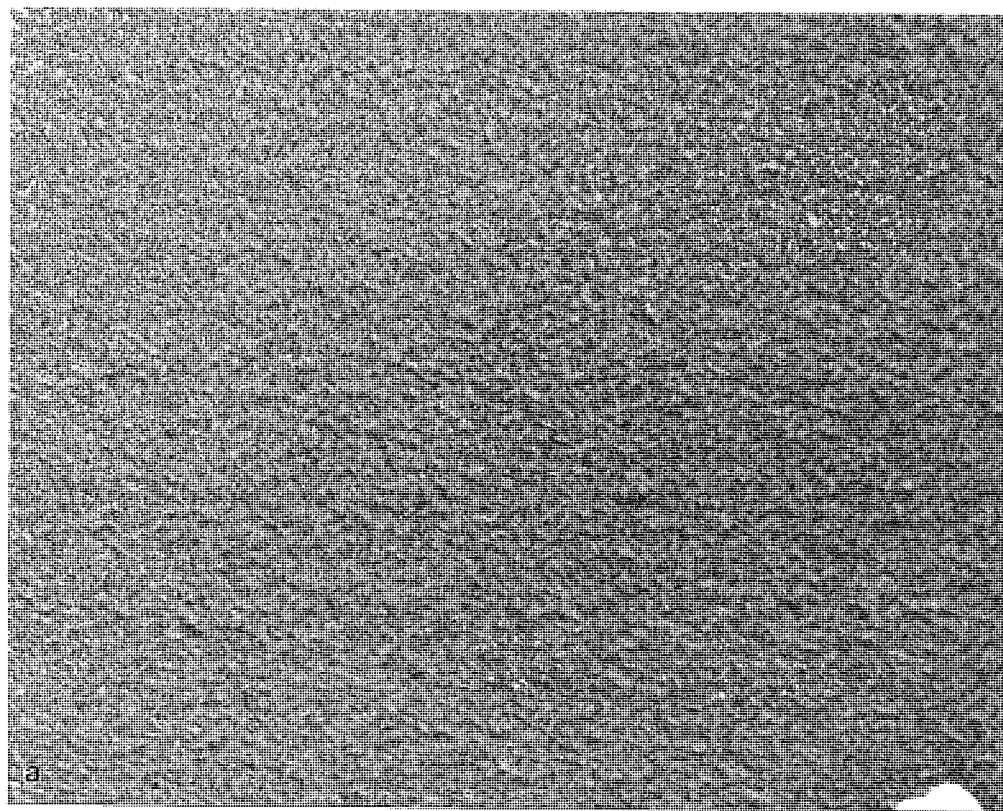


Figure 6.3 SEM micrographs of the high molecular weight (3×10^6 Da) show that the surface is rough both before (a) and after (b) plasma treatment.

BHK fibroblasts were found to adhere to the plasma-etched polystyrene surfaces irrespective of the molecular weight, or whether the surface was seen to have a surface texture at high magnification in the SEM (Figure 6.4). No cell adhesion was observed on untreated polystyrene of all molecular weights. The low molecular weight (3770 Da) polystyrene had a lower water contact angle than the untreated higher molecular weight polystyrene. However, all the etched polystyrene samples had identical, very low water contact angles. The cell response and water contact angle results are summarised in Table 6.1. From these, it is seen that the low molecular weight polystyrene is slightly

Table 6.1 Effects of molecular weight and plasma etching on the wettability, surface appearance and cell adhesion of polystyrene

MW	Etched	Water Contact Angle	Rugose	Cell Adhesion
3770	-	65	-	-
3770	+	18	-	+++
185,000	-	80	-	-
185,000	+	17	+	+++
3×10^6	-	83	+	-
3×10^6	+	17	+	+++

more wettable than the higher molecular weight polystyrenes. This will be due to the increased number of polar groups present at the free ends of the molecule due to the shorter chain length. The high molecular weight polystyrene has the same degree of surface rugosity, in both etched and untreated states, as the plasma-etched medium molecular weight polystyrene, but cells will only adhere to the etched polystyrene samples. Cells were also observed to adhere to the etched low molecular weight polystyrene although this shows no surface rugosity even at high magnifications in the SEM. Cells would adhere to polystyrene that had been plasma treated to make its surface more wettable. However, the small degree of hydrophilicity found with the short chain-length low molecular polystyrene when untreated is not sufficient to render it adhesive to cells.

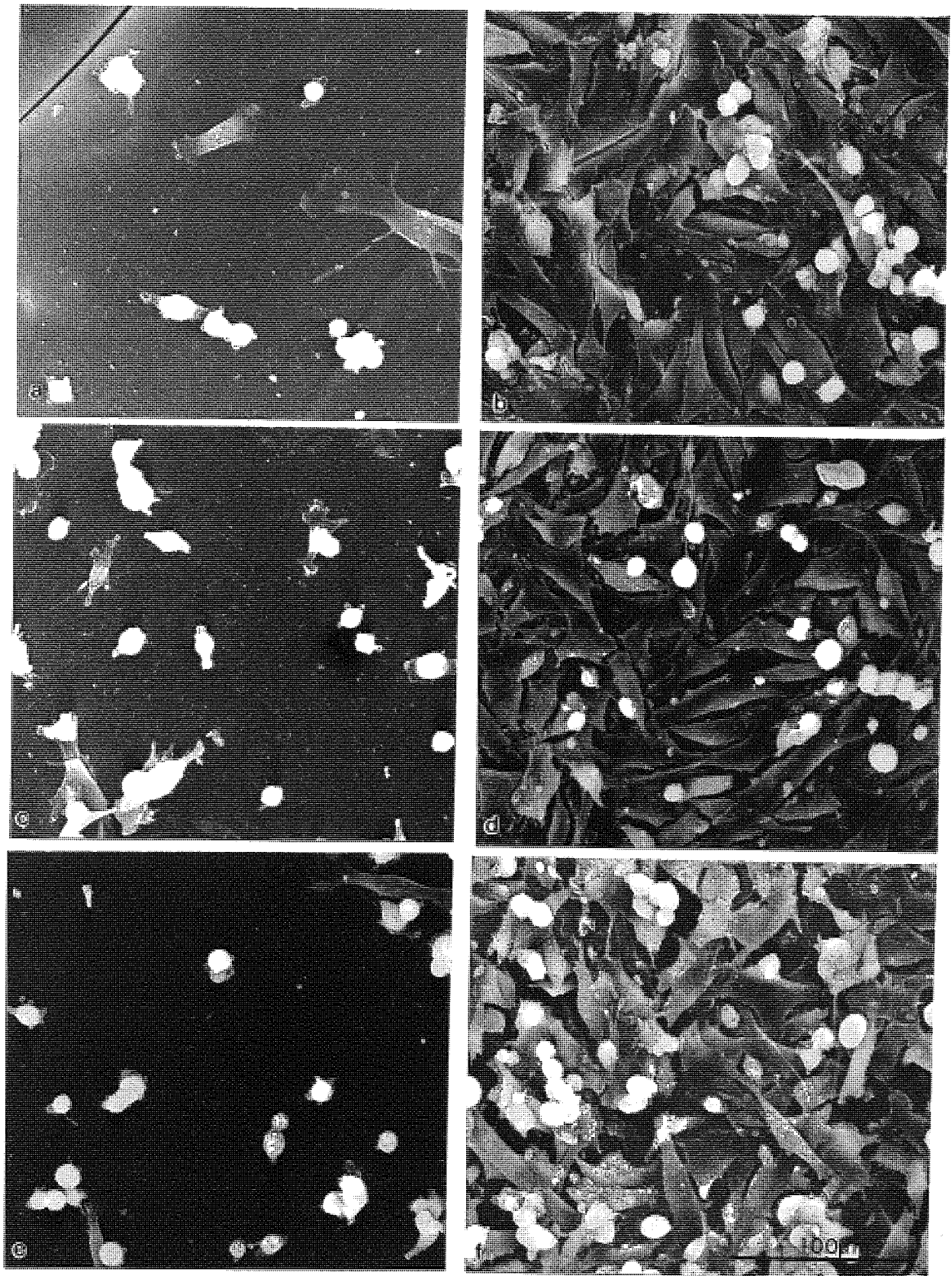


Figure 6.4 Micrographs showing the cell response to the different molecular weight polystyrene surfaces both before and after plasma treatment.

3770 Da polystyrene	a) untreated	b) plasma glow discharge treated
185,000 Da polystyrene	c) untreated	d) plasma glow discharge treated
3×10^6 Da polystyrene	e) untreated	f) plasma glow discharge treated

It had been observed previously that glow discharge treated polystyrene surfaces were roughened (Thomas et al, 1986). This led to the idea that a Wenzel effect might be operating, whereby a rugose surface is more wettable than might be assumed from its chemical structure alone (Wenzel, 1936). However, the fact that sulphuric acid treated polystyrene has been seen in the SEM to be smooth surfaced (Lydon and Clay, 1985; Thomas et al, 1986) contradicts the idea of surface rugosity influencing wettability and cell adhesion.

The results presented here show that cells will adhere to only those polystyrene surfaces which have a reasonable degree of hydrophilicity, ie. plasma glow discharge treated polystyrene. The low molecular weight polystyrene was expected to be slightly more wettable than the other native polystyrenes owing to the shorter monomer chain lengths resulting in greater numbers of the polar end groups. This was found to be the case; the water contact angle is 15 degrees less than with the higher molecular weight polystyrenes (Table 6.1). However, plasma etching the surface of the 3770Da polystyrene increased the wettability and the cell adhesion considerably, without influencing the surface rugosity. Conversely, the high molecular weight (3×10^6 Da) polystyrene possessed a rugose surface both before and after plasma-etching, and yet the wettability and the cell adhesion increase only following plasma glow discharge treatment.

This would seem to rule out microrugosity as being important in cell adhesion to synthetic surfaces, although macrorugosity can allow cells to adhere to an otherwise non-adhesive surface, as seen when cells are found adhering to scratches in polystyrene bacteriological grade dishes (Heath and Dunn, 1972). It may be that the surface of the polymeric material is not the surface which is relevant to the cells, but rather the protein coated surface is more important. This can be tested by observing the surfaces of materials with and without deposited protein layers.

6.3 Sulphuric Acid-Treated Polystyrene

Sulphuric acid treatment of polystyrene is a commonly used laboratory method of increasing relative hydrophilicity (wettability) in order that cells will adhere and grow (Maroudas, 1975: 1976: Klemperer and Knox, 1977: Gingell and Vince, 1982: Curtis et al, 1983). Examination of fibroblasts seeded onto bacteriological grade plastic petri dishes treated with sulphuric acid shows that cells will attach and spread (Figure 6.5) in a similar manner to cells on tissue culture plastic, which is glow discharge treated polystyrene. Cells on untreated bacteriological grade polystyrene are seen to attach very rarely and never spread (Figure 6.6).

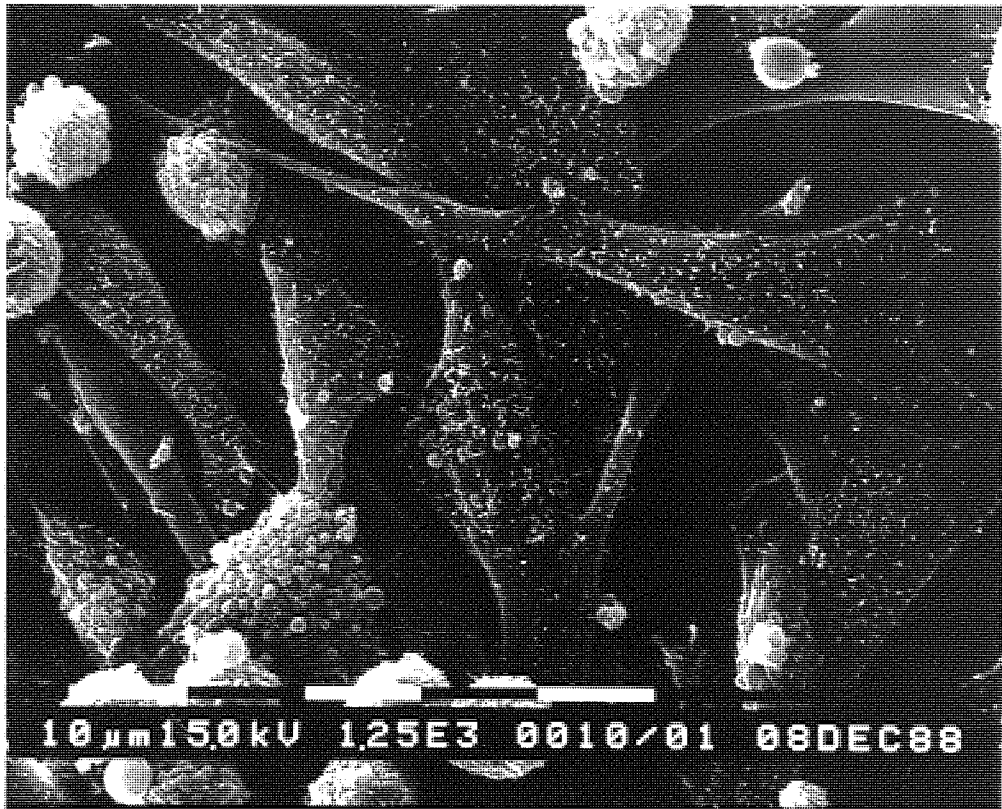


Figure 6.5 Cells attach and spread on sulphuric acid treated bacteriological grade polystyrene petri dishes

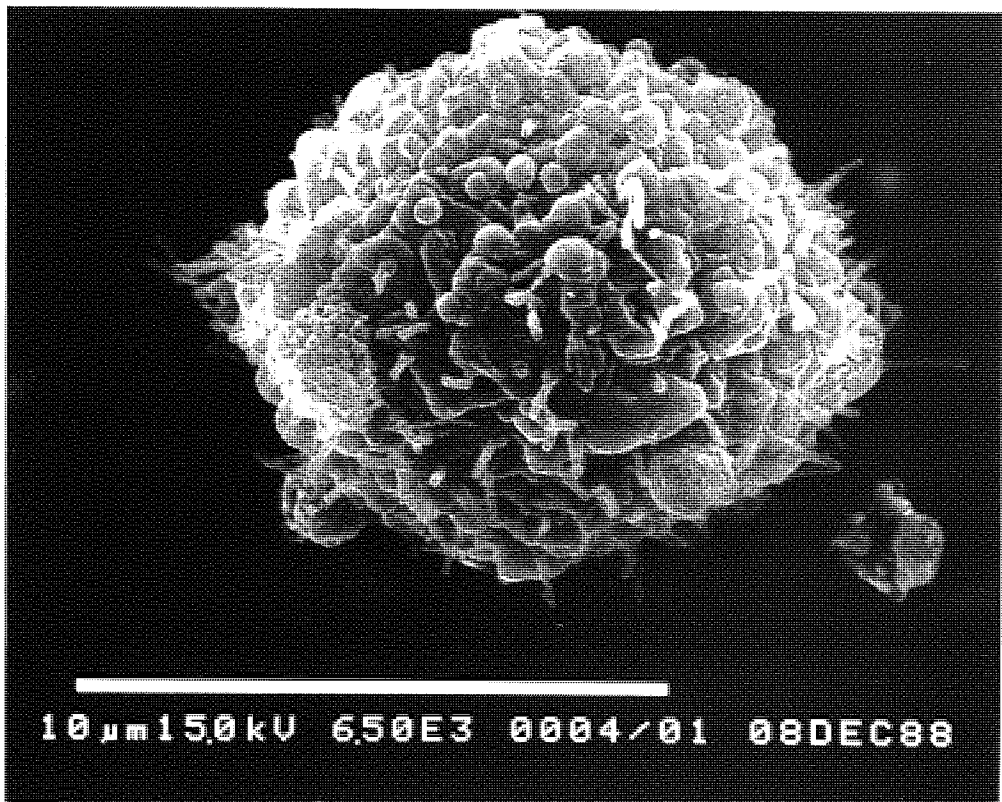


Figure 6.6 When cells can be found on untreated bacteriological grade plastic dishes they are always rounded and never spread.

Treatment of half the surface area of a bacteriological petri dish with sulphuric acid results in a dish which is hydrophilic over only the treated half. Examination of such dishes gives a graphic demonstration of the differential adhesion of cells to wettable and non-wettable polystyrene. The treated side of the dish is readily wettable and, in cell culture, fibroblasts settle preferentially onto this surface (Figure 6.7). Cells are obviously reluctant to cross the boundary between the hydrophilic and hydrophobic halves of the dish (Figure 6.8). At high magnification the cells at this boundary are seen to align themselves along the boundary line (Figure 6.9).

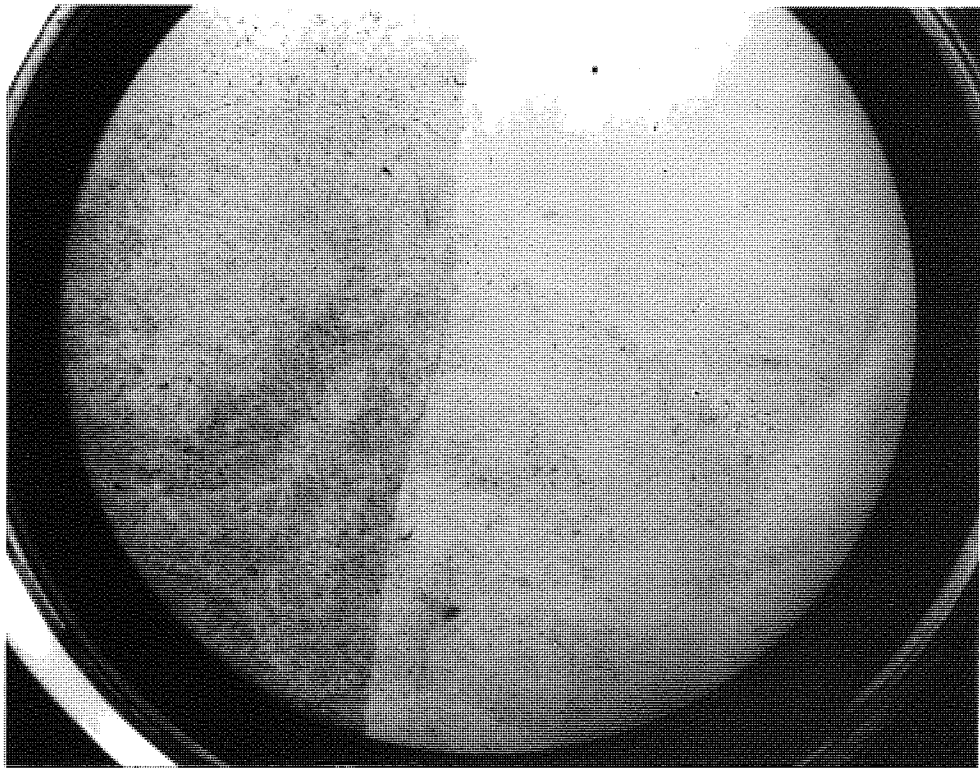


Figure 6.7 Low power light micrograph of BHK fibroblasts on bacteriological grade petri dish half treated with sulphuric acid after fixation for SEM examination which stains the cells

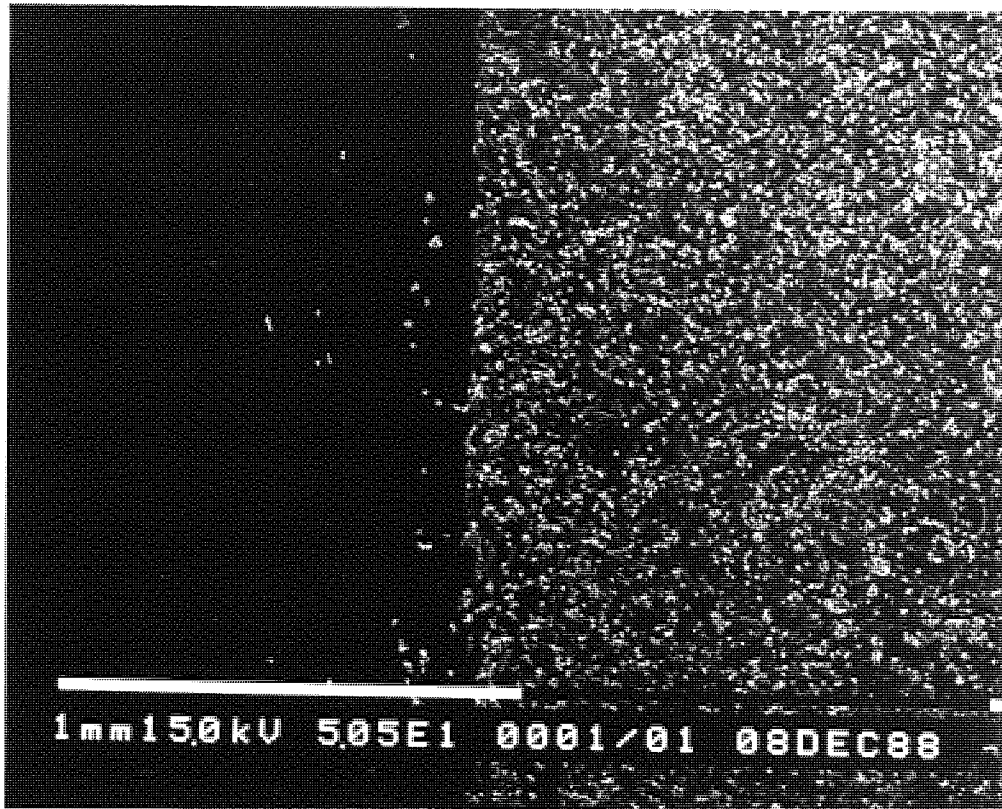


Figure 6.8 Low power scanning electron micrograph shows that the hydrophilic/hydrophobic boundary is sharply defined by the cells

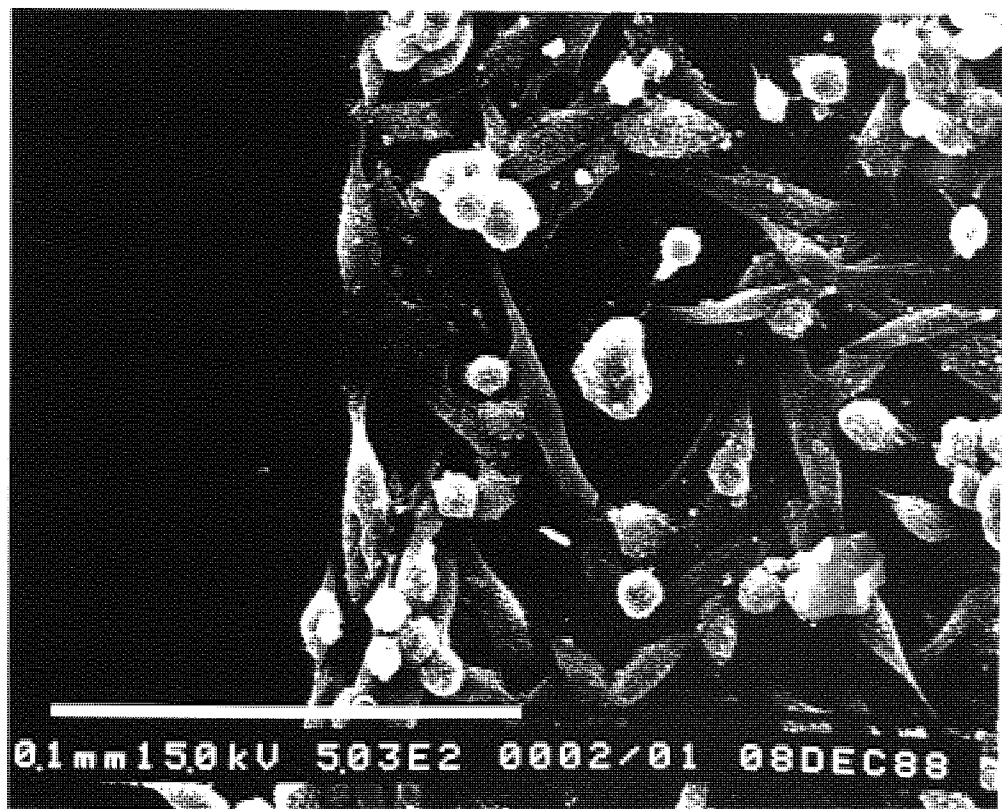


Figure 6.9 At higher magnification in the SEM, the fibroblasts are seen to align themselves along the hydrophilic/hydrophobic boundary.

6.4 Substratum Topography

Examination of the surfaces of the materials used as substrata for cell culture are generally found to be smooth. This is the case for tissue culture polystyrene, sulphuric acid treated polystyrene and glass, all of which will support the adhesion and growth of cells (Figure 6.10). Substrata which are non-adhesive to cells, untreated bacteriological grade plastic and siliconised glass, show very variable surface topography, with bacteriological grade polystyrene being smooth but the siliconised glass has a roughened appearance (Figure 6.10). In this respect the untreated bacteriological grade plastic has a very similar topography to tissue culture plastic and sulphuric acid treated polystyrene, although the wettability and cell adhesion properties are very different.

Oliver and Mason (1977) showed that surface features with a step height of only 50nm could influence the contact angles of liquids on metal surfaces. The scale of the topographical features seen here on polystyrene surfaces is considerably smaller than this. Although we were not able to measure any microscopic changes in contact angle, the macroscopic contact angles for rough and smooth polystyrene surfaces were identical to the limits of experimental error. This would indicate that surface topographical features of this dimension does not affect the wettability of surfaces.

One effect of such rugose surfaces could be to increase the surface area available for protein adsorption which might increase the ability of cells to adhere. This is not supported by the fact that etched low molecular weight polystyrene is smooth (Figure 6.1), and yet supports cell adhesion to the same degree as the higher molecular weight polystyrenes with rugose, etched surfaces (Figures 6.2 and 6.3). Plasma glow discharge treatment renders polystyrene of various molecular weights wettable and cell adhesive because of the introduction of surface oxygen (Thomas et al, 1986), and not due to any increased topographical features.

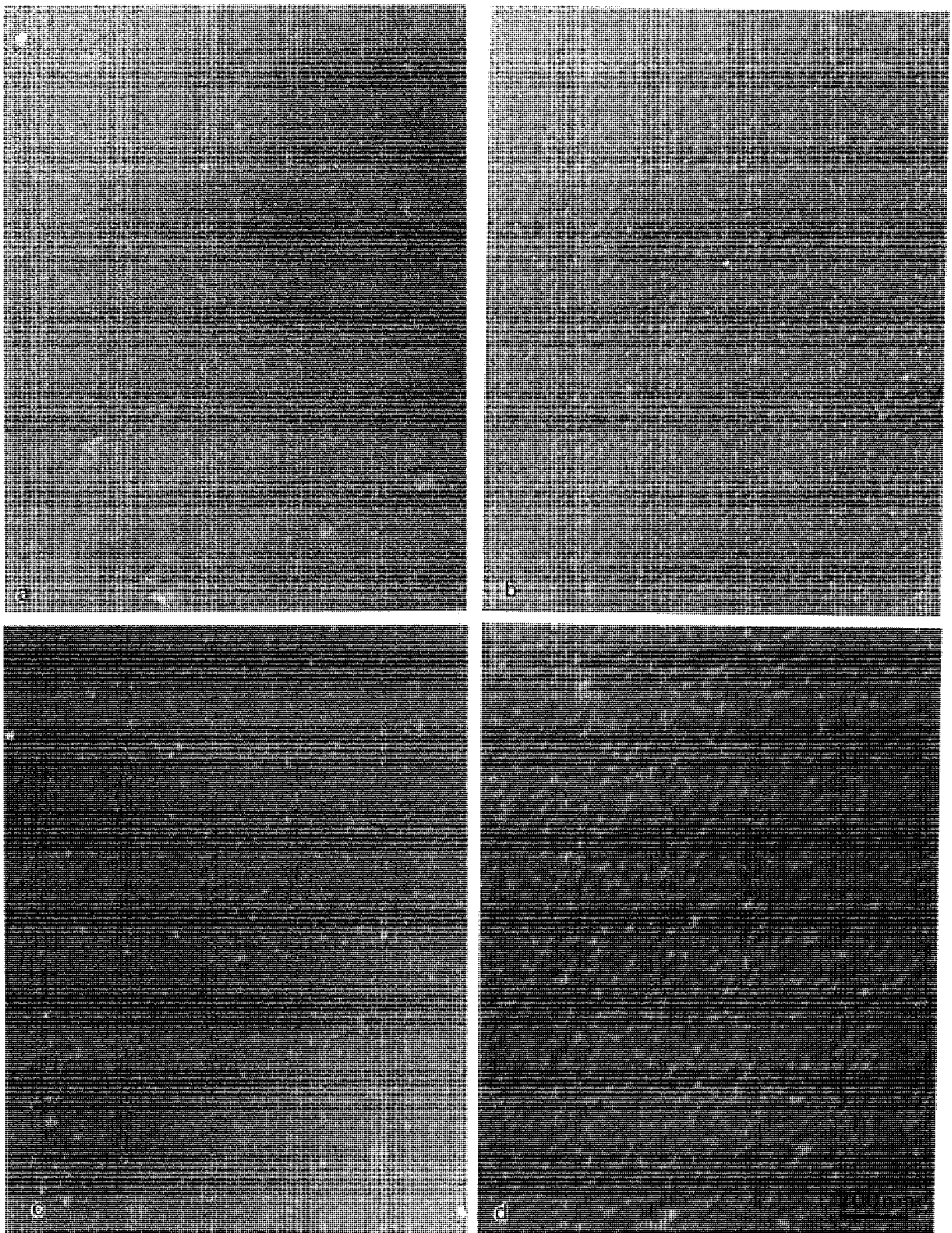


Figure 6.10 Surface appearance of a) tissue culture polystyrene, b) bacteriological grade plastic, c) glass and d) siliconised glass as seen at high magnification in the SEM

6.5 The Serum Layer

Surface features seen on the substratum are not necessarily reflected in the appearance of the serum layer surface structure, as can be seen by comparing Figures 6.10, 6.11 and 6.12. Tissue culture plastic is smoother than bacteriological grade polystyrene, but the serum layers show more topography on tissue culture plastic and is cell adhesive. However, siliconised glass has a surface structure which is larger, and this is reflected in the structure seen when the serum layer is present. It might have been expected that the proteins of the serum would smooth out the surface rather as snow obliterates fine detail, but this is not the case.

In contrast, the serum layer on acid-modified bacteriological grade plastic is composed of fine ridges and furrows which lie in one direction over the entire surface of the dish whilst the acid-treated polystyrene surface is seen to be smooth at the same magnification (Figure 6.12). These corrugations have a periodicity of 100-200nm. Samples in which only half the surface area has been acid-treated also show ridging of the serum layer, although this is often less apparent on the untreated area. We have no obvious explanation for these phenomena, although incomplete or sub-optimal modification of the surface chemistry (Maroudas, 1975) might lead to differential adsorption of serum components across the sample area. In one instance we found the serum layer to be ridged on untreated bacteriological plastic in a small area where some cells had settled. We cannot say whether this area of the plastic surface was physicochemically similar to acid-treated substrata, or whether the cells themselves induced the ridging effect.

It occurred to us that local heating effects produced on dilution of concentrated sulphuric acid during the rinsing process following surface treatment might be causing structural artifacts in the polystyrene which results in the serum ridging effects. In control experiments, pieces of bacteriological-grade plastic were immersed in boiling distilled water, rinsed and used as culture substrata. Little cell adhesion is observed on these surfaces and the adsorbed serum layer is perfectly smooth at similar high magnifications.

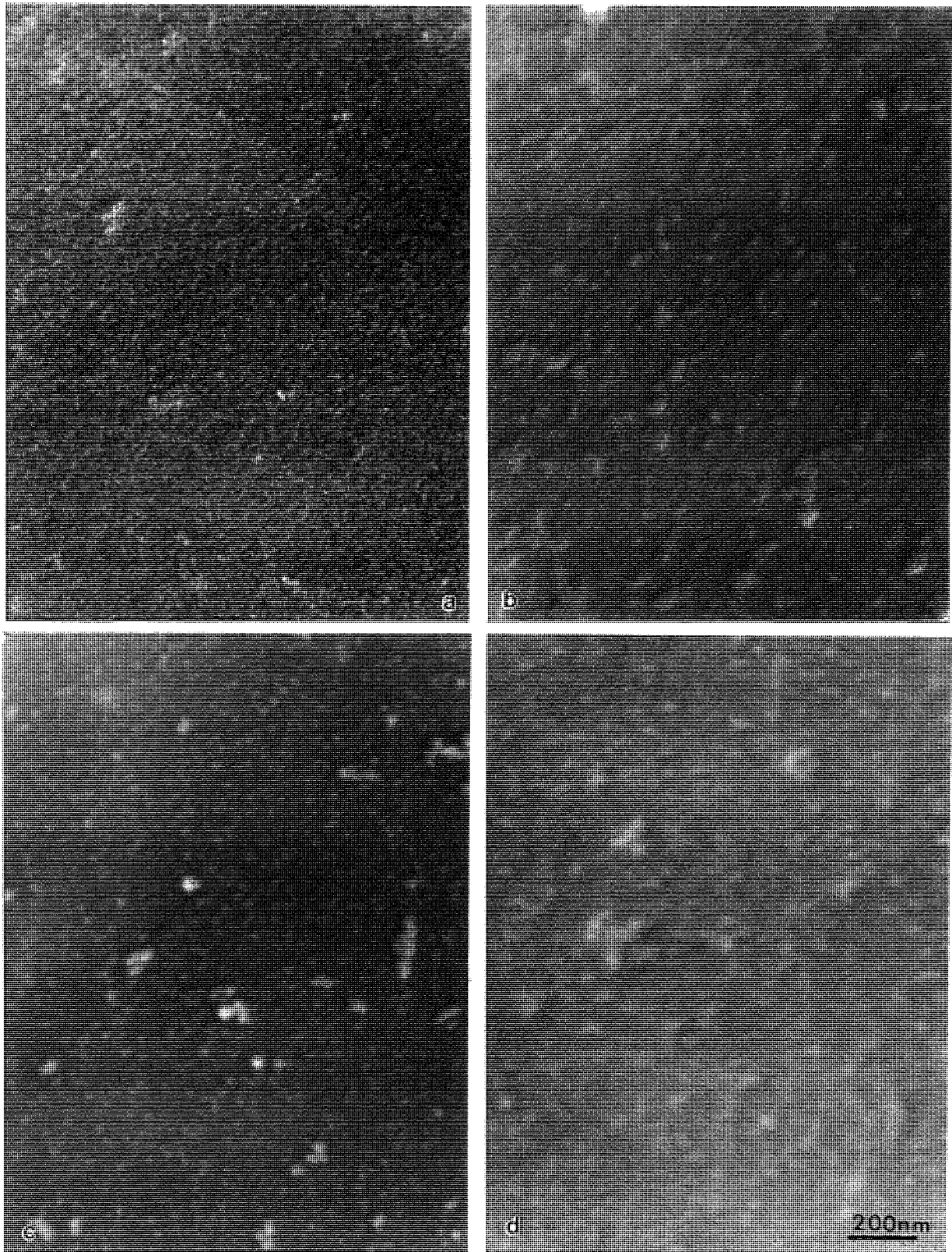


Figure 6.11 Surface topographies of serum layers deposited onto a range of materials: a) tissue culture plastic b) bacteriological plastic, c) glass and d) siliconised glass

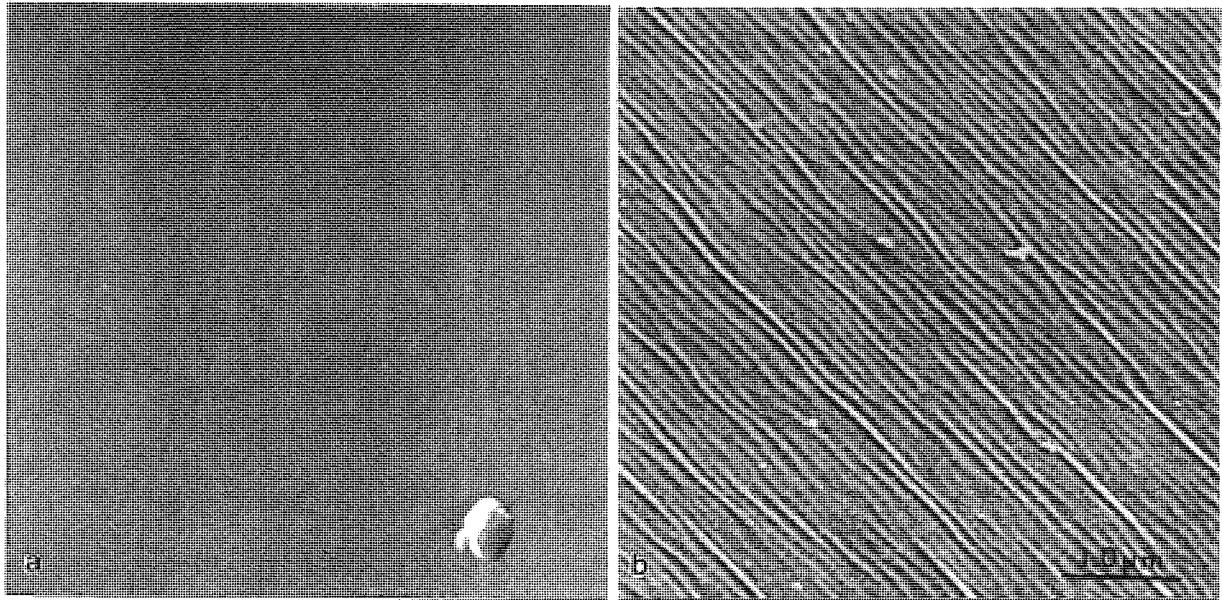


Figure 6.12 The surface of sulphuric acid treated polystyrene is seen to be smooth (a) but the serum layer deposited on this surface is composed of fine ridges and furrows (b)

SEM of the surfaces of substrata before coating with protein layers confirm the results seen with the varying molecular weight polystyrene samples that surface features of this order are not important for cell adhesion. It is necessary to have the correct surface energy or wettability, ie. it is the hydrophilic/hydrophobic balance which is the determining factor.

6.6 Cell Traction

High magnification examination of the fibroblasts growing on acid-treated polystyrene, reveal that the cells interact with the adsorbed serum layer, leading to distortions in the direction of the corrugations and to minute tears in the serum layer. These distortions are the result of the cells exerting tractive forces upon the serum layer, which suggests that study of such effects on the serum layer might prove useful in analysis of the forces exerted by culture cells during the spreading or locomotory processes. Consideration of a motile cell in culture illustrates this point (Figure 6.13). This cell represents a morphology typical of a migrating fibroblast (Abercrombie, 1982), with a large fan-shaped leading edge and an extended tail which will eventually retract as the cell moves forward.

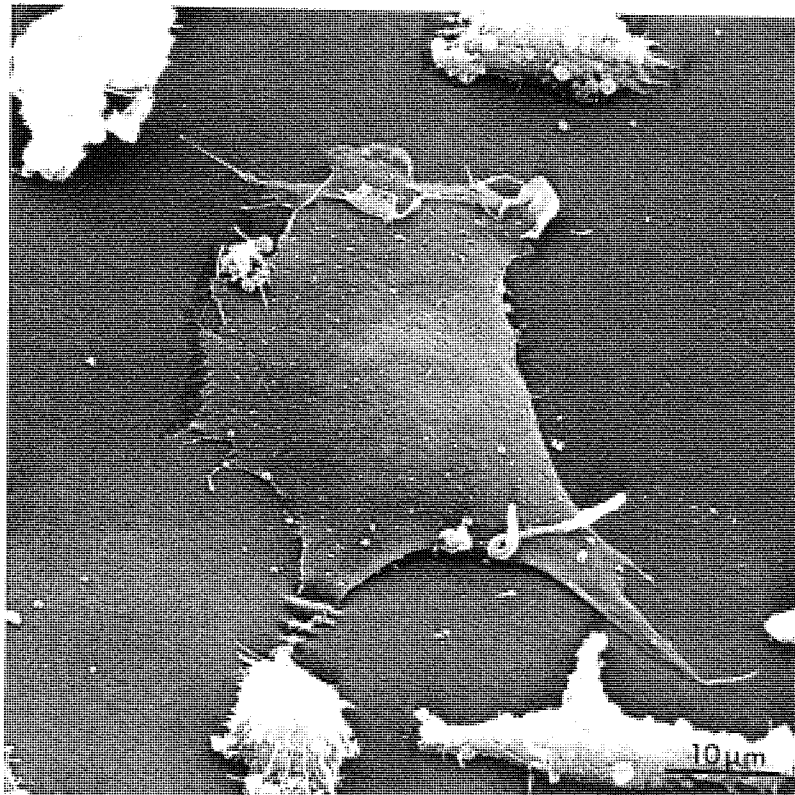


Figure 6.13 Motile BHK fibroblast on sulphuric acid treated bacteriological grade plastic.

High magnification examination of this cell shows that there are several interesting features of the adjacent serum layer. Tears in the serum layer are seen at both the leading and trailing edges, suggesting that the cell is exerting contraction forces upon the serum layer. A similar effect has been reported previously, where sheets of protein were torn off hydrophobic surfaces by blood platelets. (Scarborough et al, 1969). A pseudopodial extension is seen to "grip" the serum layer compressing and distorting the corrugations (Figure 6.14).

Cellular behaviour *in vivo* has been suggested to be controlled to a large extent by relative contractile strengths of different cell types (Harris, 1982). However, few model systems exist to examine this hypothesis. Harris has studied cell traction and locomotion using plasma clots and silicone rubber sheets (Harris, 1980: 1982). The finding that the serum layer is ridged presents us with another possible approach to the study of cell traction and locomotion. In this way the SEM can be used to study the interaction between cells and the substratum.

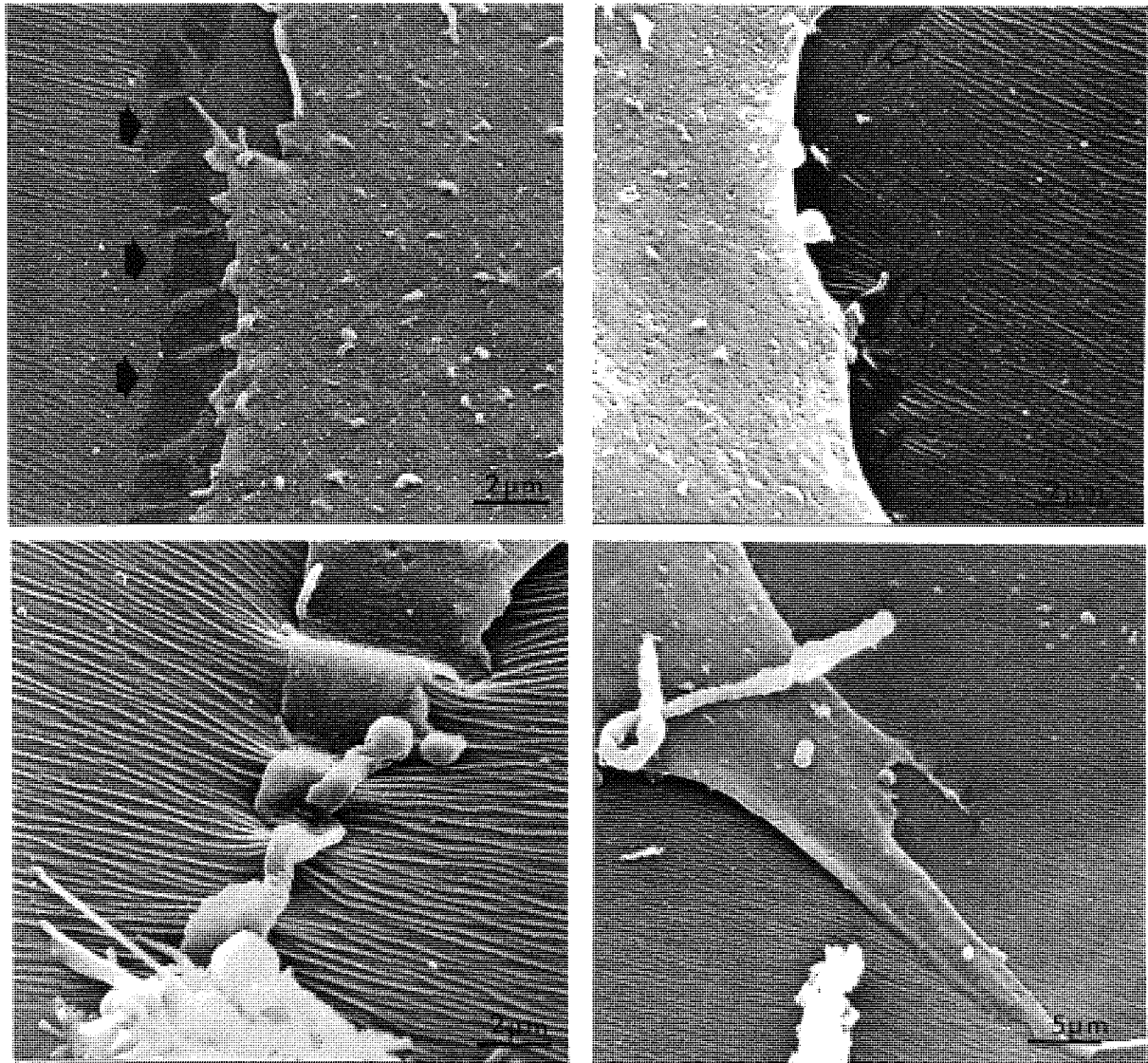


Figure 6.14 At high magnification, the cell shown in Figure 6.13 shows tears (arrows), flattened serum layer and distortions in the serum corrugations

6.7 Conclusion

In this chapter we have concentrated largely on polystyrene because it can be rendered cell adhesive by plasma glow discharge treatment and sulphuric acid treatment, and yet shows differing surface rugosity. Low molecular weight polystyrene is slightly more wettable than polystyrenes of higher molecular weight, but supports cell adhesion only after glow discharge treatment. The surface, as seen in the SEM at high magnification, is found to be smooth in both cases. This is supported by the observation that sulphuric acid etched polystyrene surfaces are smooth and support cell adhesion. Unetched high molecular weight polystyrene has a rugose surface but does not support cell adhesion. After plasma etching the surface rugosity is of a similar scale, and cells readily adhere to this surface. These findings rule out microrugosity as being important in cell adhesion to polystyrene surfaces.

Sulphuric acid treated bacteriological grade polystyrene is smooth, but the serum layer on top is ridged, and cells adhere and grow on this surface. Conversely, the serum layer on top of siliconised glass is roughened to a similar degree as the native surface, but is not adhesive to cells. These observations show that rugosity of either the substratum surface or the deposited serum layer is not a deciding factor in determining whether cells will adhere to, and grow on that surface.

Goniophotometry is capable of providing objective measurements of the surface roughness of materials. However, it was not capable of differentiating between the surface appearance of the materials under test. The two types of glass, native glass and siliconised glass, were determined to be equally smooth by goniophotometry, but using SEM these two surfaces were shown to have very different surface rugosities. The native glass is seen to be as smooth as the resolution of the technique allows, the siliconised glass has a roughened surface with features which might contribute to the cell adhesive property of the material. With polystyrene, varying surface appearances can be produced by a range of surface treatments. These can all be clearly investigated by the resolution offered by SEM, and the results presented here show that surface rugosity does not play a major role in determining a material's cell adhesiveness.

Previous work in our series of investigations has shown that surface energy is important in determining the cell response to that material (Minett, 1986; Thomas, 1988). Work on the surface chemistry of materials using XPS has indicated that the presence of surface oxygen is important in promoting cell adhesion to that surface (Curtis et al, 1983; Thomas, 1988). Surface oxygen may not be the ultimate determinant as it is not found in argon- or nitrogen-plasma treated polystyrene (Minett, 1986), where other polar groups, such as amino groups, may play a part (Brewis et al, 1978). It is appropriate at this point to investigate the surface energy characteristics of cell adhesive and non-adhesive surfaces to determine the importance of this physicochemical property. Of particular importance is the question of whether the cells can sense and respond to the surface energy of the material directly.

CHAPTER 7

SURFACE ENERGY MEASUREMENTS OF PROTEIN LAYERS DEPOSITED ON POLYMERS

SURFACE ENERGY MEASUREMENTS OF PROTEIN LAYERS DEPOSITED ON POLYMERS

7.1 Introduction

In the last chapter we demonstrated that surface rugosity was not important in determining the cell adhesiveness of materials, but that substratum wettability was a factor as had been reported previously, both by our group and others. This has led to an overview of the relationships between chemical group expression (Curtis et al, 1983; Gibson and Bailey, 1980; Klemperer and Knox, 1977; Maroudas, 1976; Thomas et al, 1986), surface free energy (fractional polarity) (Lydon et al, 1985; Thomas et al, 1986; van der Valk et al, 1983), and cell adhesion. The surface energy of the substratum is clearly important in triggering interface conversion (Lydon et al, 1985; van der Valk et al, 1983), but is not the sole criterion as the expression of chemical groups at the surface may have a part to play (Curtis et al, 1983; Thomas et al, 1986).

The previous experiments eliminated surface rugosity as a factor influencing the adhesion of cells to polymeric materials. This work also highlighted the importance of surface energy in cell adhesion, so that further investigation into the nature of action of surface energy in determining the cell adhesive property of materials is a potentially valuable line of investigation. This entails investigations of the physicochemical nature of materials rather than their physical surface rugosity properties, which have been under investigation so far. To this end, we set out to investigate the part played by the surface energy characteristics of surfaces. Of particular importance is to determine whether cells are able to detect and respond to the surface energy of the substratum material directly, or whether they respond to the surface energy of the deposited conditioning layers on the surface of the substratum (Baier et al, 1970; van der Valk et al, 1983).

As part of this programme, we carried out surface energy measurements (by the sessile drop contact angle method) on deposited layers on synthetic surfaces, which either support or do not support cell adhesion in the presence of foetal calf serum supplement (Lydon et al, 1985). Serum is of particular

importance as it is a required supplement for the culture of anchorage-dependent cell lines (Barnes et al, 1980; Lydon et al, 1985; Neumeier and Reutter, 1985). It is a complex substance, however, with numerous constituents, some of which have been isolated and shown to promote cell adhesion to surfaces; these include fibronectin (Barnes and Sato, 1979; Grinnell, 1978; Hynes and Yamada, 1982; Klebe et al, 1981; Neumeier and Reutter, 1985; Yamada and Olden, 1978) and vitronectin (Barnes et al, 1980; Hayman et al, 1985b; Holmes, 1967; Lydon and Foulger, 1987; Neumeier and Reutter, 1985). Other constituents of serum can also be used in cell adhesion studies, eg. albumin can be used as a non-adhesion stimulatory control (Chandy and Sharma, 1985; Eberhart et al, 1982; Gendreau et al, 1982; Horbett and Hoffman, 1975).

We selected five polymers for these studies, and assessed dry contact angles under five different conditions: no protein, FCS, BSA, fibronectin, and vitronectin. Polymers were selected to encompass a range of polarity from hydrophobic to hydrophillic, and which were adhesive (tissue culture plastic, pEMA, pEMA/pHEMA) and non-adhesive (pHEMA, bacteriological grade plastic) for cells under routine cell culture conditions in serum-supplemented medium (Lydon et al, 1985).

7.2 Results

Table 7.1 shows the surface energies of the bare polymer surfaces and demonstrates that the hydrophilicity/hydrophobicity of the surface is best represented by the polar component, γ^p . The addition of serum or protein layers shifts the

Table 7.1 Surface energies of the bare surfaces

Polymer Surfaces	Surface Energies (dynes cm ⁻¹)		
	γ^p	γ^d	γ^t
pHEMA	23.7	32.1	55.8
pEMA	10.8	38.3	49.1
50/50 pEMA/pHEMA	19.8	31.8	51.5
Tissue Culture p/s	15.2	36.9	52.1
Bacteriological p/s	1.5	45.5	47.0

fractional polarity of the surfaces to a more hydrophillic state as is shown in Figure 7.1. It can be seen from this graph that the underlying surface energy is still expressed to an extent. The cell adhesion properties of these surfaces with deposited protein layers is shown in Table 7.2. From this table it can

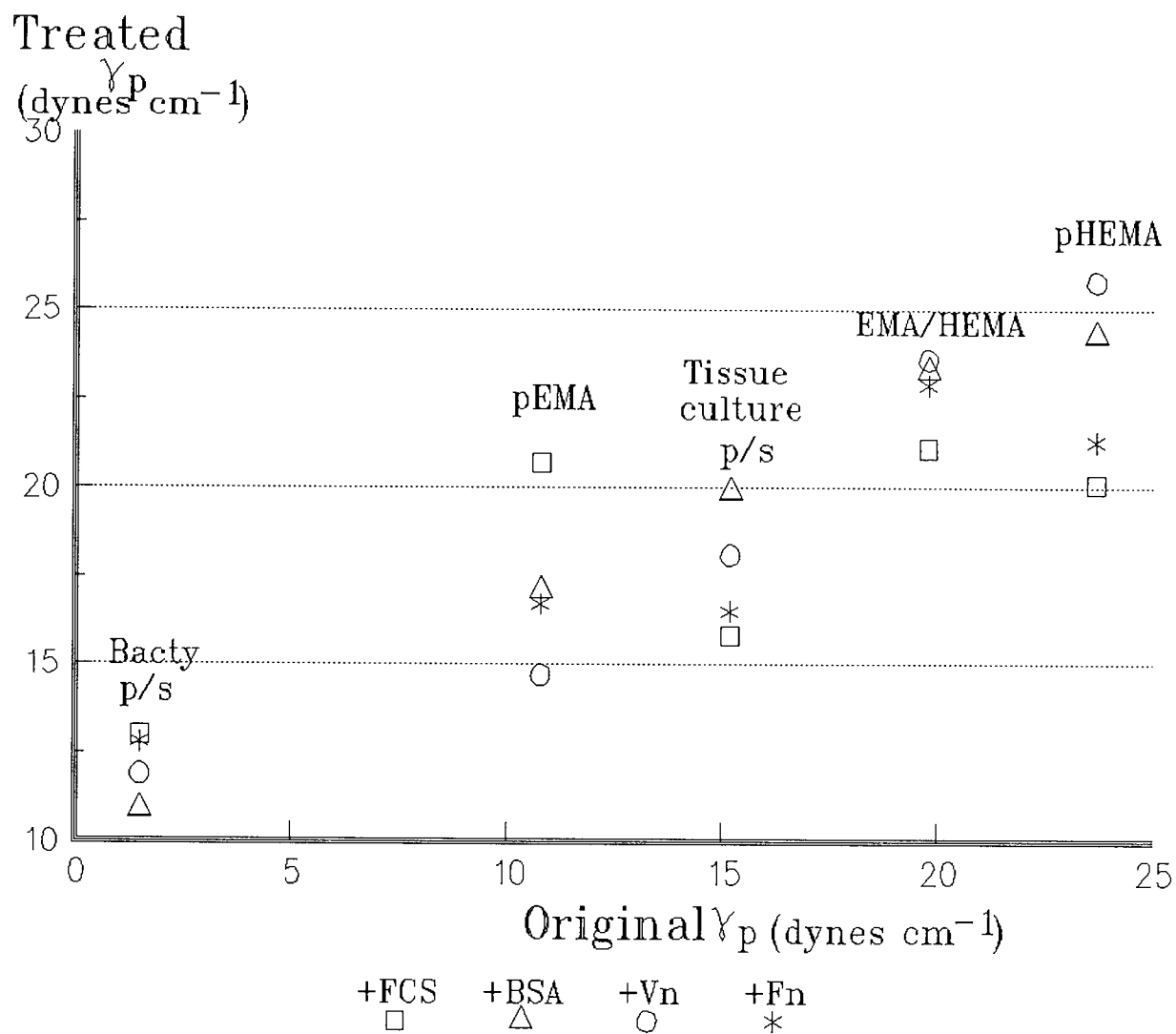


Figure 7.1 Surface energies (polar fraction) of protein treated polymers compared to the surface energies (polar fraction) of the bare material surfaces

Table 7.2 Cell response to polymers and deposited protein layers

Polymer Surfaces	Serum in Medium	Cell adhesion on deposited layers		
		+FCS	+BSA	+Fn
pHEMA	+	-	-	++
"	-	-	-	+
pEMA	+	+	-	++
"	-	+	-	+
EMA/HEMA	+	+	-	+
"	-	+	-	+
Tissue Culture polystyrene	+	+++	+++	+++
"	-	++	-	+
Bacteriological polystyrene	+	-	-	++
"	-	-	-	+

be seen that cells will adhere and spread best on tissue culture plastic irrespective of the deposited serum layer. Cells attach and spread on other surfaces only when fibronectin has previously been deposited onto that surface. There is some cell attachment on polyEMA, either on its own or complexed with polyHEMA, when foetal calf serum is present as a deposited layer. Cells on fibronectin layers deposited on otherwise non-adherent surfaces are usually very spindly shaped cells.

7.3 Discussion

Our surface energy results show that the underlying surface energy (particularly the polar fraction) is still represented, even when deposited serum or protein layers are present (see Figure 7.1). However, fractional polarities of surfaces coated with adhesive and non-adhesive proteins are very similar to one another. The resulting values do not bear any obvious relationship to one another, or to the cell response to the surface in serum supplemented media conditions. For example, tissue culture plastic is adhesive to cells in serum-free medium in the presence of a deposited layer of foetal calf serum, but pEMA with deposited BSA is not adhesive, and yet the fractional polarity values are very similar. Conversely, pEMA with FCS deposited onto its surface is adhesive for fibroblasts, whilst tissue culture plastic with BSA on its surface is non-adhesive, although again, the fractional polarities are very similar. Moreover, tissue culture plastic coated with vitronectin or fibronectin have very similar fractional polarities to tissue culture plastic with BSA (non-adhesive) and are adhesive to cells.

Similarly the other surfaces and the deposited layers show little correlation in their fractional polarities and cell adhesion capabilities. This suggests that the energy of the surface, once coated with a deposited layer of protein, is of no interest or relevance to the cells in deciding whether they will attach to and spread on that surface.

When serum is present in the medium the cell response to a surface is enhanced compared to serum-free medium. This is no doubt due to the increased availability of growth factors and other signalling molecules in the medium, which will enhance a cell's ability to spread onto a surface (Rockwell et al,

1980). These growth factors are presumably not present in the deposited layer in the same amounts, due to competition from the cell adhesive proteins in foetal calf serum. In the case of tissue culture plastic coated with BSA, the cell response is as good as for FCS, indicating that the FCS in the medium can compete with, and replace, the preadsorbed BSA, or can be adsorbed on top of the albumin layer.

The interaction of biological molecules with polymer surfaces is a complex process, therefore, as non-adhesive proteins like albumin can render a surface which is normally adhesive in the presence of FCS non-adhesive to cells. The converse of this also operates, with the adhesion-promoting proteins vitronectin and fibronectin rendering a surface (eg. bacteriological grade plastic), which is non-adhesive in the presence of FCS, adhesive to fibroblasts. This indicates that cell adhesion is a protein-mediated event, but that "interface conversion" depends on the composition and nature of the deposited protein layers.

The cell response to a single adhesion-promoting protein, like fibronectin, is strong attachment but rather incomplete spreading. Cells on fibronectin rarely spread to give a typical fried egg appearance or kite-shaped motile cell, and this suggests that the events of "interface conversion" are complex and are not due solely to the deposition from serum of a single adhesion promoting protein.

7.4 Conclusion

The energy of the surface, once coated with a deposited layer of protein, appears to be of no interest or relevance to the cells in deciding whether they will attach and spread on that surface: cell adhesion is a protein mediated event. Surface energy (specifically the polar fraction) is important in reducing the interfacial tension with aqueous solutions with the consequence that the correct deposition of proteins onto a polymeric surface occurs. It is the nature of this deposited protein layer that determines the cell response to that surface, and this will no doubt rely on which proteins are deposited, and on the conformation and orientation of the proteins within the layer.

It is our working hypothesis that in a non-adhesive surface energy zone "interface conversion" does not proceed correctly and thus prevents cell interaction with the synthetic surface. However, in the non-physiological situation, as with deposited single protein layers, cell adhesion may occur. In these cases, the system cannot be a true test for biocompatibility as it does not predict the behaviour of the material in a physiological environment as represented by mixed, multiple protein components like serum.

The consequence of this is to add to the wealth of evidence in support for a fundamental role for surface energy in determining the biological fate of the polymeric materials . There is a need now to investigate the protein layer deposited on the polymer surface, in order to discover more about the organisation of the conditioning protein layer. Attention will now turn to techniques which may accomplish this. One physical technique which has been used for investigation of biological tissue is X-ray microanalysis, and this will be used to probe the deposited protein layers in an attempt to determine more about the nature of this layer.

CHAPTER 8

X-RAY MICROANALYSIS OF BIOMATERIAL SURFACES

8.1 X-ray Microanalysis of Protein Layers Deposited onto Synthetic Surfaces

8.1.1 Introduction

The work using the SEM showed that the material surfaces possess surface roughness differences which are small but noticeable, but are not of interest to the cells. These studies have shown that the protein layers deposited upon the material influence the surface energy presented to the cells. The surface energy experiments showed that the surface chemistry and surface energy are important in organising the deposited protein layers. These last experiments showed that physical analytical techniques are capable of detecting and characterising biomolecular layers on polymer surfaces to a limited extent. Further investigation into the nature of the deposited layer requires a technique which will provide more information about that biological part of the interface.

Electron probe X-ray microanalysis (EPMA) is a scanning electron microscope technique in which the X-rays generated in the sample by the electron beam are detected. Analysis of the X-ray energies provides elemental information of the samples, which can include synthetic surfaces and biological tissue samples (Chandler, 1979). It has some restrictions in the biological field, owing to its poor sensitivity to elements of low atomic number that are abundant in biochemical molecules (Cheetham et al, 1984). Proteins contain elements such as phosphorus and sulphur which are detectable by the commonly available energy dispersive spectrometers. X-ray microanalysis should be able to detect proteins which have been deposited from solution onto the surface of polymeric materials, and may be able to distinguish between different layers. One advantage of the technique is that, as it uses a scanned electron probe, it can give distribution information on a microscopic scale which could be important to the functioning of these deposited layers in interface conversion.

8.1.2 Results

All the plastic spectra showed the same background spectra with a very small copper $K\alpha$ peak present at 8050eV due to X-ray emission from the sample holder which has been irradiated with scattered primary beam electrons. These spectra showed no difference between all four samples (the two types of plastic before and after coating with foetal calf serum). The examples shown in Figure 8.1 are from bacteriological grade plastic, untreated and with a serum layer deposited.

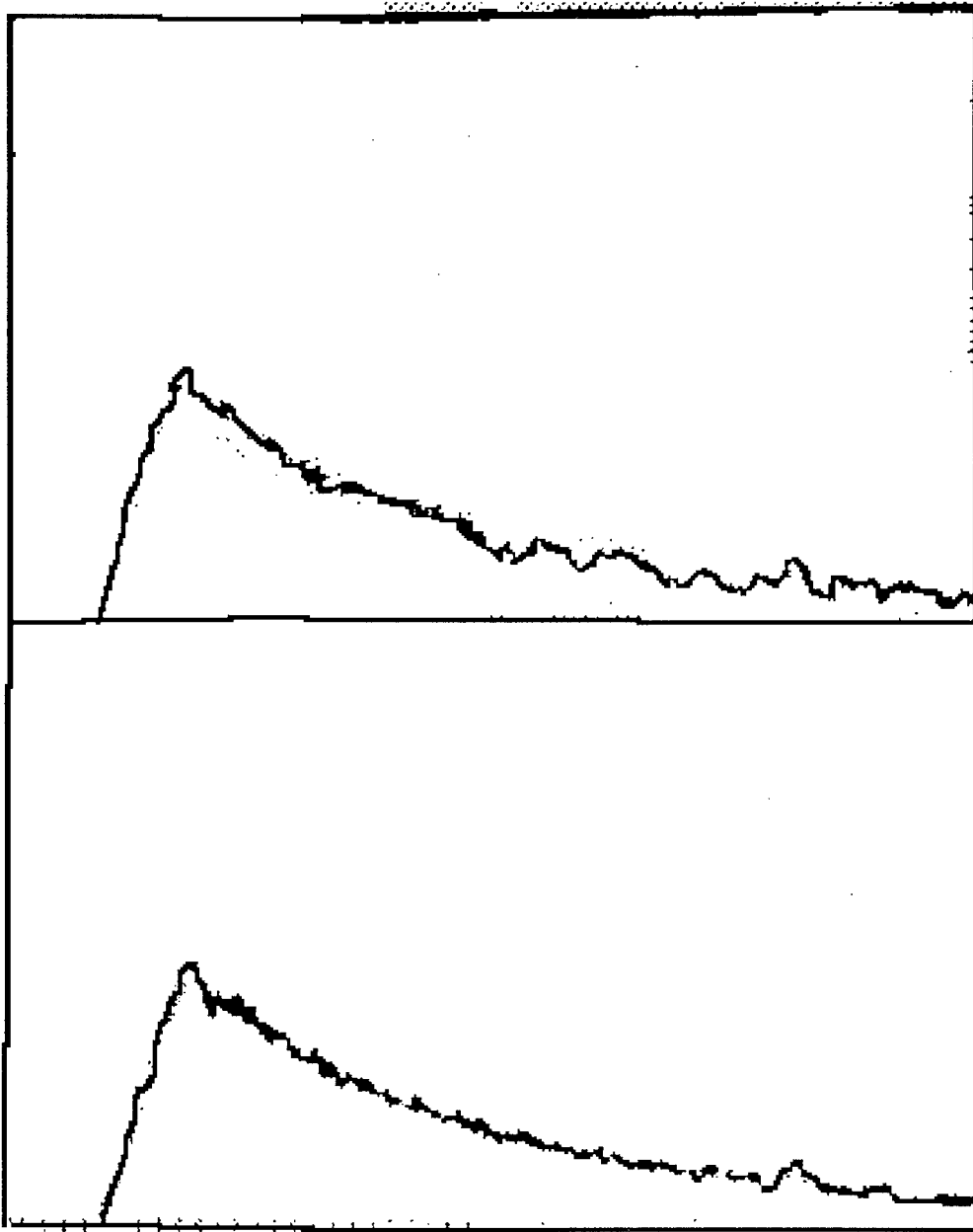


Figure 8.1 X-ray spectrum from bacteriological grade plastic (a) untreated and (b) incubated with 10% FCS in PBS. Only background X-ray signal is seen.

The count rate when acquiring these spectra was low (approximately 250 cps) in comparison to a count rate of approximately 1100 cps for the glass samples, where strong signals were generated. Here the spectra were dominated by the very large silicon peak, with strong sodium and calcium peaks also present (Figure 8.2). Minor peaks for magnesium, aluminium and potassium were also

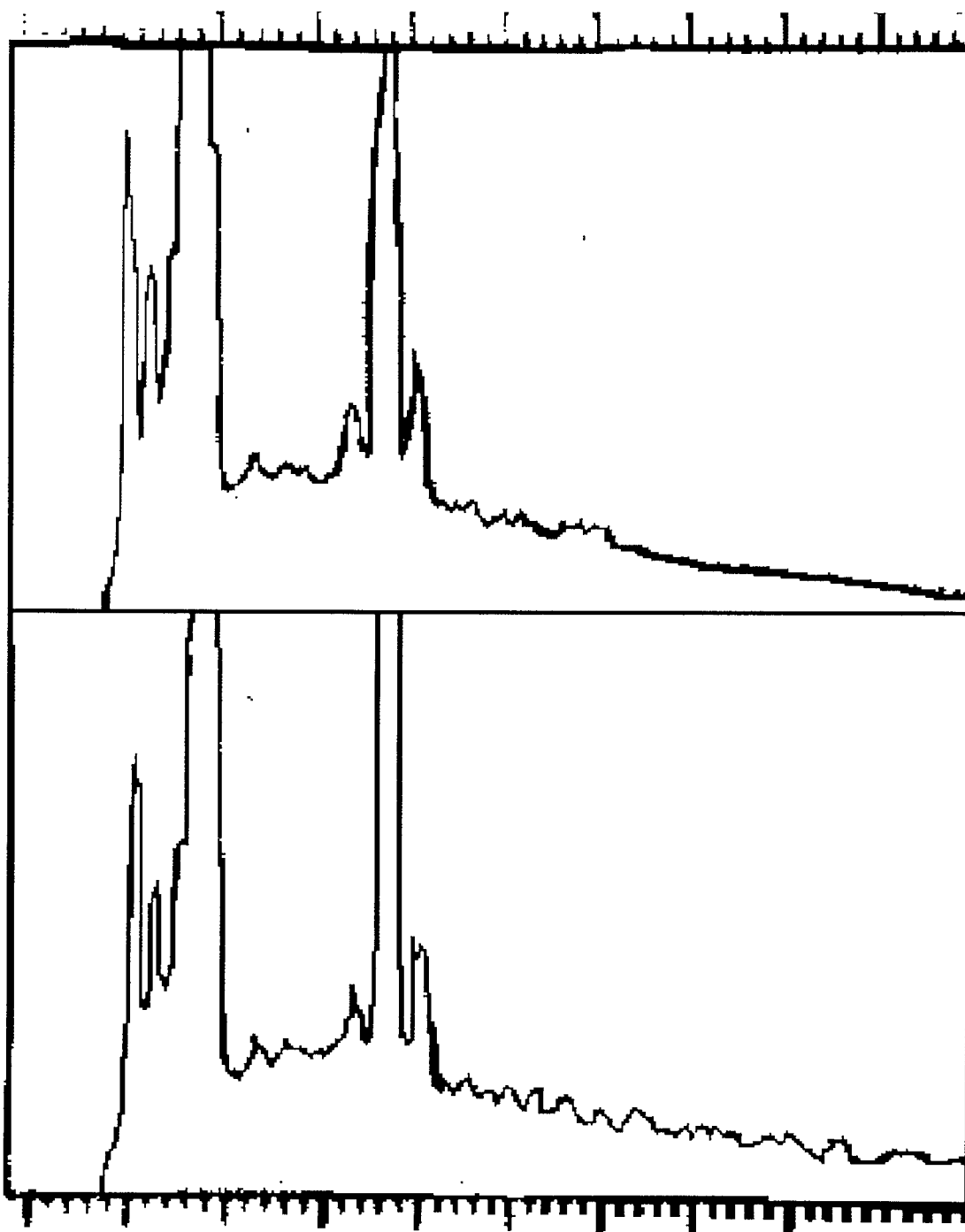


Figure 8.2 X-ray spectrum from (a) untreated glass and (b) serum coated glass, with major peaks for silicon and calcium. Minor peaks for Na, Mg, Al, and K are also seen.

seen; the aluminium peak was almost lost in the shoulder of the silicon peak. A small chlorine peak was observed in the siliconised glass samples due to the dichloromethyl silane.

No peaks were seen that could be attributed to the adsorbed proteins of the serum layer in any of the samples (see for example Figures 8.1 and 8.2), although it is claimed that this can be achieved. This is probably because the quantity of protein deposited was too small for the technique to detect, which has a sensitivity of approximately 0.1%.

8.2 Determination of Thickness of Layers by X-ray Microanalysis

Examination of samples with physiologically relevant deposited serum layers failed to detect the protein content of this deposited layer on polystyrene or glass. This would be due to the serum layer being too thin to give an adequate signal for the sensitivity of the technique. In the case of the glass substrates, the spectra show the elements expected for glass, and indicate that the signal had not been influenced or suffered very little attenuation by the serum layer.

This suggests a further experiment where a glass substrate is coated with varying thickness of a polymer to determine the thickness required to prevent the glass X-ray signal from showing through. This experiment describes the X-ray microanalysis of varying thicknesses of a clear polyurethane varnish drawn down onto glass.

8.2.1 Materials and Method

The substrate used is a glass microscope slide, as used previously. A clear polyurethane varnish was drawn down onto the microscope slide using calibrated knife edges to a range of thicknesses. The samples were then prepared for, and examined by, X-ray microanalysis as described in the Material and Methods chapter (Chapter 3).

8.2.2 Results

Spectra showing the attenuation of the X-ray signal from the glass substrate by the various thicknesses of polymer layers are shown in Figure 8.3. Here, the spectra for uncoated glass is compared with 5 μm thick film, where the signal is still detected but the silicon peak is approximately a tenth of the height and the calcium peak has virtually disappeared, and a 9 μm film where the silicon peak has been totally lost. By comparing the net integral counts under the silicon and calcium peaks, it is seen that the signal from the glass is moderated evenly across the energy spectrum, and a graph

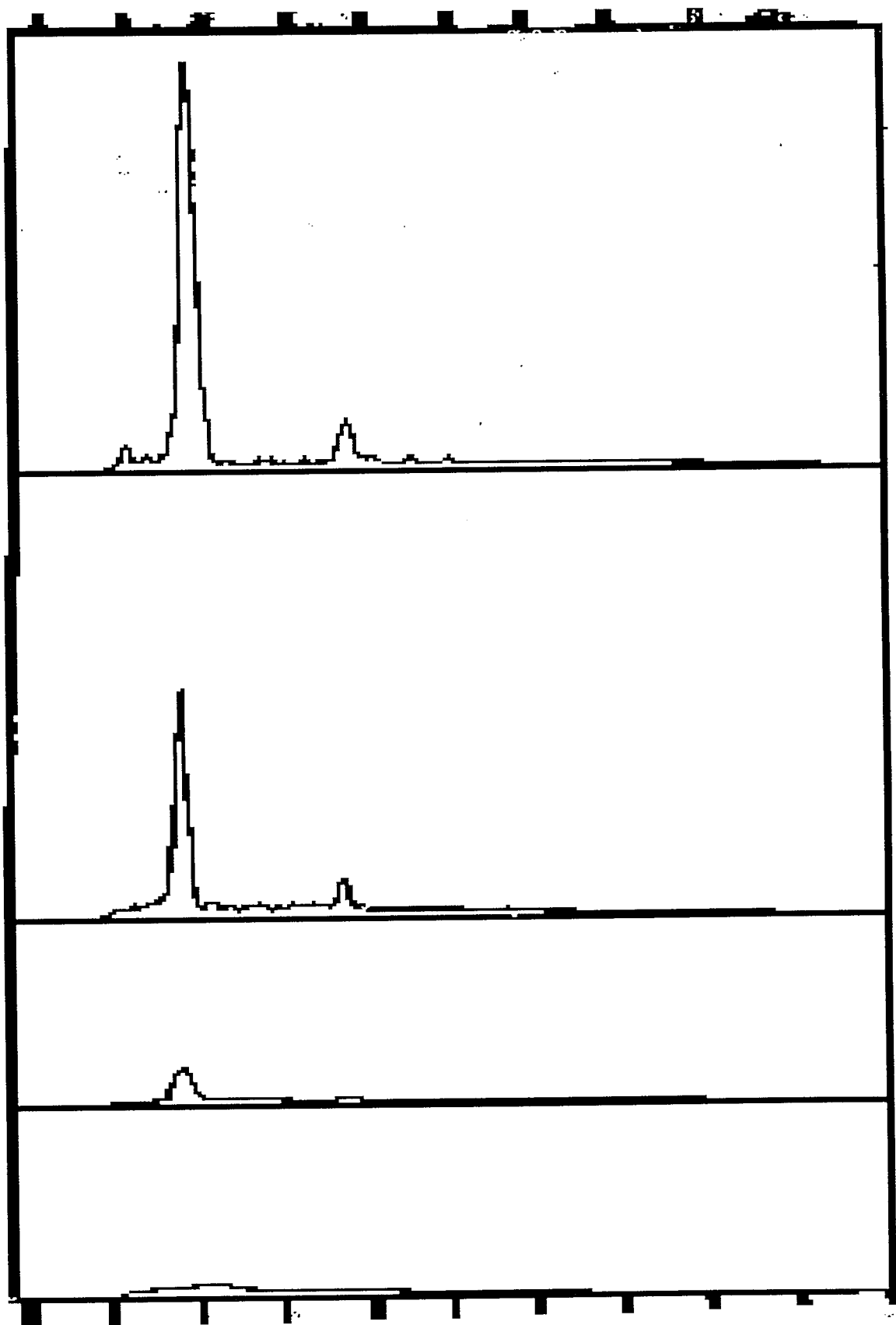


Figure 8.3 Spectra from a) uncoated glass, b) 2.5 μm film, c) 5 μm film and d) 9 μm film show how the silicon peak from the spectra is attenuated by varying thicknesses of polyurethane films

of the signal net intensity can be plotted against the film thickness (Figure 8.4). By extrapolation of this graph it is possible to obtain a measure of the penetration depth of the technique where the graph crosses the x-axis. This is seen to occur at a film thickness of $5.5\mu\text{m}$ (Figure 8.4).

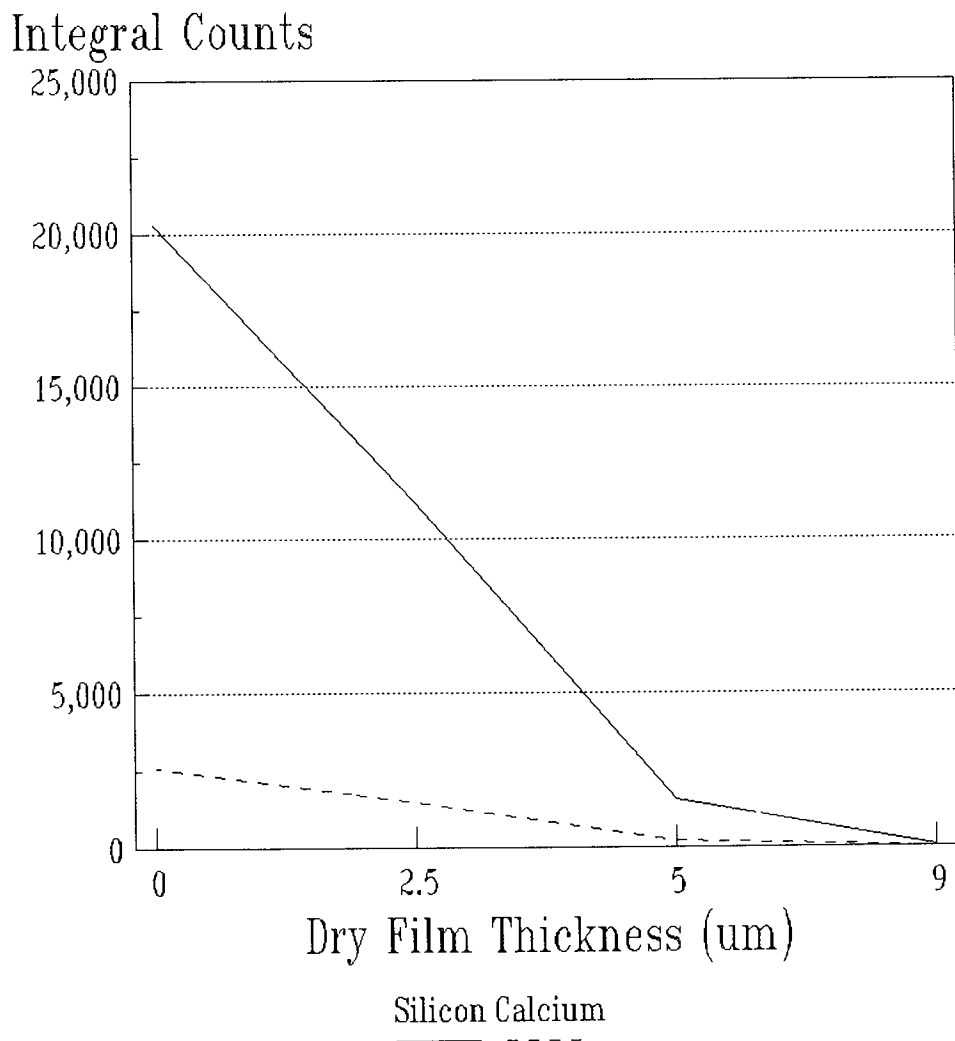


Figure 8.4 Graph of the integral counts under the silicon and calcium peaks versus the polyurethane film thickness.

8.2.3 Discussion and Conclusions

The signal from the underlying glass substratum is seen to be attenuated in a progressive manner in proportion to the thickness of the applied polyurethane film. When very thin films are used ($< 1\mu\text{m}$), the signal attenuation is negligible, and the signal is totally dominated in this technique by the underlying substratum rather than the film of interest.

By a similar argument process it can be ascertained that the penetration depth of the technique (or the analytical volume) is very large in proportion to the required analytical volume. These results show that the technique is sensitive only to film thicknesses of the order of $1\text{-}5\mu\text{m}$. This is an order of magnitude greater than for the cell response testing. Minett's work (1986), using spin coated polyHEMA films, showed that cell adhesion onto tissue culture polystyrene could be inhibited by film thicknesses of approximately $0.5\mu\text{m}$. My work with the surface energy experiments shows that a 50nm thick protein layer will alter the surface energy characteristics of materials, and also alter the cell response to that material. Hence, the biological model and surface energy measurements are far more sensitive than X-ray microanalysis, even though the latter gives very good XY spatial resolution of information.

An alternative approach might be to label the glycoproteins of the serum layer with a lectin (eg. Con A) which had been equilibrated with manganese which ought to be detected and localised. Alternatively, other labelling techniques could be employed, specifically the use of antibody labelling. Scanning electron microscopy is a useful technique because of its large magnification range and its ability to collect numerous signals. By labelling antibodies with colloidal gold particles, use can be made of the backscattered electron signal to localise the deposited protein by detecting the high atomic number gold particles. The next chapter describes the use of this technique to investigate the spatial arrangement of the deposited layers, and determine how this varies on the various surfaces under examination.

CHAPTER 9

ANTIBODY LABELLING OF PROTEINS DEPOSITED ONTO SYNTHETIC SURFACES

ANTIBODY LABELLING OF PROTEINS DEPOSITED ONTO SYNTHETIC SURFACES

9.1 Introduction

In recent years there has been a growth of interest in the application of scanning electron microscopy (SEM) to immunocytochemical studies. Scanning electron microscopy is an electron-probe imaging technique which can be used to give topographical and compositional views of surfaces. One of the advantages of the scanning electron microscope is that a number of signals are generated from the impact of the electron-probe in the sample. These signals include the secondary excited electrons which are used in the traditional topographical SEM images, backscattered electrons, X-ray photons, UV photons and Auger electrons as well as absorbed electrons.

Auger electrons are low energy electrons which can be used to provide chemical information of sample surfaces. They are, however, only produced at low efficiency levels with the result that, in order to obtain practical signal to noise ratios, high beam currents, in the order of 10^{-7} A, have to be used. At this level of beam current most polymers are not stable and, therefore, cannot be analyzed using Auger Electron Spectroscopy (see Chapter 2.6).

Cathodoluminescence detects the UV photon emission of materials, and requires that the sample is a naturally scintillating material, as is routinely used with geological samples; alternatively, extrinsic fluorochromes can be used to label the sample. This is done extensively in light microscopy as antibody-labelling fluorescence microscopy, and can be mimicked in the SEM using cathodoluminescence detectors. As with Auger electron spectroscopy signal levels are low. Bleaching of the fluorochromes by the exciting beam, which also occurs with fluorescence microscopy, is even more severe in cathodoluminescence owing to the high beam currents necessary to overcome signal deficiencies. These two problems restrict the practical use of the technique.

X-ray microanalysis is an established SEM technique which provides compositional information on

a microscopic scale. Its use in biological areas tends to be a little limited owing to the poor signals generated from low atomic number elements which predominate in these samples. This results from the mechanism of electron capture favouring the generation of X-rays with increasing atomic number of the element being bombarded by the electron beam. The technique was considered in more detail in Chapter 8 on X-ray microanalysis of protein coated surfaces, where an attempt to detect the sulphur content of the protein amino acids was unsuccessful.

This leaves us with the two most commonly used signals in the scanning electron microscope, which are secondarily excited and backscattered electrons. These two techniques can be used to obtain considerable information about the topography and composition of surfaces. Secondary electrons have a low energy and leave the sample surface to be detected only when they are generated near the surface. This explains the pseudo-three dimensional topographical images which are normally associated with the scanning electron microscope. Backscattered electrons arise from deeper in the sample, and hence are not as influenced by the sample surface topography. Both secondary and backscattered electron production are influenced by the atomic number of the sample, and this enables compositional information to be obtained. Because secondary electrons are also influenced by the surface topography, backscattered electrons are more useful providers of this compositional information. In practice, the two techniques are better used together to give a more complete "picture" of samples, with the topography being revealed by the secondary electron signal, and the atomic number composition by the backscattered electron signal.

Backscattered electron imaging is routinely used in metallurgy and mineralogy to provide information on the composition of the sample where large atomic number differences are involved. This is not the case with protein molecules, which are all of similar average atomic numbers. Positive staining of features as occurs in light and transmission electron microscopy is not possible with the SEM. Antibody labelling has been used extensively with fluorescent dyes in light microscopy, and attention has allowed the technology to switch to scanning electron microscopy using various labels. Cathodoluminescence detection of fluorescent probes has not proved successful as outlined above.

Morphological markers have been used using a probe that is easily recognisable in secondary electron images. The problem with this is that the marker has to be physically quite large (<100nm) to be identified, the most successful of these markers have been bacteriophages or haemocyanin molecules. Colloidal gold particles have been used as a morphological marker as they can be made in a range of well defined sizes. However, a better solution is to take advantage of the high atomic number of gold to increase backscattered electron production to enable the particle to be detected. This method of detection is preferred over secondary electron imaging owing to the reduced effect of topography on the backscattered electron signal. The backscattered electron imaging mode is the most often used means of detecting the gold label, and it is often used in conjunction with the secondary electron image to provide an image of the surface which is useful in localising the labelled molecule.

Colloidal gold was first used as a cytochemical marker in transmission electron microscopy in 1971, (Faulk and Taylor, 1971) and for scanning electron microscopy in 1975 (Horisberger et al, 1975). It has emerged, by virtue of its particular properties, as the most commonly used marker in immunoelectron microscopy. A variety of particulate, enzymatic and emissive markers has been explored for use in the SEM as visualisation tags for target molecules before being rejected. Colloidal gold has a number of features which make it attractive and effective as a marker. It is non-toxic and can easily and inexpensively be prepared in a range of well defined particle sizes from 1nm to 150nm diameter by chemical reduction of gold chloride. Methods of colloidal gold production are designed to achieve monodisperse gold sols, and homogeneity of particle size and shape. In addition, colloidal gold is capable of forming active and stable complexes with a wide variety of biological substances to provide the gold probes, so forming a flexible labelling system. Gold probes are now widely available in a number of forms, in terms of particle size and gold-ligand complexes. Colloidal gold probes can be stored for long periods, 12 to 15 months at 4°C, and their stability is largely determined by that of the protein complexed with the gold particle.

The high density of the particles and their recognisable spherical appearance are useful

characteristics which enable gold particles to be distinguished and quantified by simple counting procedures. A further advantage is the facility to carry out multiple labelling of several target molecules in one preparation, using gold particles of different sizes (de Harven et al, 1990). As a consequence, gold particles on labelled preparations can be readily distinguished, imaged and quantified by appropriate scanning electron microscopy techniques.

9.2 Immunolabelling Technique

Techniques for the immunolabelling of biological specimens all follow the same basic strategy irrespective of the type of microscopy to be used. Furthermore, the success of all immunocytochemical procedures is subject to a range of similar variable conditions. Different aspects of this labelling technology have been comprehensively discussed in a number of publications (Albrecht and Hodges, 1988; Beesley, 1989; Polak and Varndell, 1984). The antibody labelling technique most successfully used is the indirect labelling technique. Here the target antigen is first labelled with an antibody specific for that protein which has been raised in rabbits. The gold label is then attached to this rabbit antibody using a complex of colloidal gold particle and a second antibody to rabbit IgG. This enables a standard gold labelled antibody to be used for all individual experiments with just the primary antibody changed as it imparts the desired specificity.

Alternately the direct labelling technique uses the colloidal gold particles complexed with the specific antibody directed at the target antigen. The drawback of this technique is that the gold probe has to be custom built for each molecule under investigation. A related technique is to use Con A to label the carbohydrate portion of glycoprotein molecules present in single or mixed protein layers as this can also be complexed with colloidal gold particles.

The nature of the antibody can have an influence on the amount of information obtained from the labelling technique. Polyclonal antibodies have multiple specificities for the single antigen, whilst monoclonal antibodies have a greater specificity of action. One of the restrictions of using polyclonal antibodies, as in these experiments, is that they are not sensitive to specific portions of the target molecule. With this in mind, these experiments will only be capable of revealing distribution information and cannot probe for conformation changes in the deposited proteins.

9.2.1 Immunolabelling Method

The substratum material is incubated at 37°C for 1 hour with the protein solution in phosphate buffered saline (PBS). The protein solution is removed and the sample washed twice with PBS. 3% ovalbumin in PBS is added, for 30 minutes at room temperature, to block any free sites at the material surface. This prevents any non-specific adsorption of the antibody molecule at free substratum sites, and is followed by two washes with PBS. The deposited protein layer and the blocking ovalbumin is prefixed with 1% formaldehyde solution (in PBS) for 30 minutes at room temperature. After washing again with PBS, the primary antibody is reacted with the deposited layer for 1 hour at room temperature. The primary antibody will be a specific antibody for the protein of interest, a control is always included consecutively, using an antibody which will not react with the target protein or any other protein which might be expected to be present. After washing again with PBS the gold-labelled second antibody is reacted with the sample for 30 minutes at room temperature, at the end of which the sample is washed with PBS.

9.2.2 SEM Examination

The samples are examined in the SEM (JEOL JSM 820, Philips SEM 525M) using the backscattered electron detector in atomic number contrast mode. This is done using 25kV accelerating voltage, and a beam current of at least 0.2nA. This needs to be performed using as small a spot size as possible, and this is achieved by using high gun bias and filament emission. As very small colloidal gold particles are used, the samples will need to be searched and photographed at high magnification, typically x20,000, with the gain of the backscattered amplifier at a high setting.

9.3 Immunogold Labelling of Protein Coated Surfaces

Much of the scientific effort using this technique has been to obtain information about the expression and distribution of cell surface antigens and receptors (Geuskens et al, 1986: Hattori et al, 1984: Nakatsuji et al, 1985: Trejdosiewicz et al, 1981). The potential of the technique is also being used to examine the spatial organisation of the cytoskeleton, of chromosomes and of extracellular matrices. Of importance in the structure and function of cells and tissues is the organisation of the extracellular matrix. A study of how cells interact with the extracellular matrix will be concerned with the organisation and distribution of cell adhesion molecules. These are the same molecules which are of prime importance in the events of "interface conversion" which are responsible for the success or failure of prosthetic devices etc (Baier, 1982: 1984: Baker and Tighe, 1981: Lydon, 1986).

The essence of these experiments is to use colloidal gold labelled antibodies to study the distribution of proteins deposited on a range of surfaces which are both adhesive and non-adhesive to cells. The proteins may be used individually, or they may be detected in a multicomponent system such as foetal calf serum, which is used routinely in cell culture as a medium supplement. It should also be possible to consider the distribution of proteins when cells are growing, or attempting to grow, on culture substrata.

One problem, however, is the detection limit of the technique, although recent developments in backscattered detector technology has provided considerable improvement. 15nm gold particles have been used routinely at Unilever Research Laboratories, Colworth in transmission electron microscopy immunocytochemistry, and can just be detected by scanning electron microscopy using a high efficiency BEI detector. It was found not to be possible to detect these gold particle using X-ray microanalysis (previous unpublished work). They can be readily detected using secondary electron imaging, but the images are not very clear owing to the influence of the topography of the serum or protein layer (Figure 9.1). Small surface features, such as contaminating virus particles, protein aggregates and cellular debris, can be of marker size and shape, and prove difficult to distinguish

from single or clumped gold particles, thereby leading to misinterpretations.

As backscattered electron imaging is only slightly influenced by surface topography, it would seem to be the best signal to use to detect colloidal gold probes. Previous work in the literature, however, has often used the secondary electron signal (Park et al, 1986a: Pitt et al, 1986b). A simple comparison of the information provided by the two imaging techniques serves to point out the practical advantages of using the backscattered electron image (BEI), which is now becoming recognised as the best way of localising gold probes (Hodges and Carr, 1990: Hodges et al, 1988). In Figure 9.1 the same sample of a colloidal gold labelled fibronectin layer on tissue culture plastic is shown imaged by both secondary electrons (Figure 9.1a) and backscattered electrons (Figure 9.1b). It can be seen from these pictures that the colloidal gold particles appear as small bright spots distributed over the surface. With the backscattered electron image, the gold particles are the only features contributing to the signal, and so they appear very bright against a black background. With the secondary electron image the gold probes are superimposed on a complex image of the deposited protein layer. This results in a slightly confusing picture. It can be seen from this that backscattered electrons provides a simpler image which enables the distribution of colloidal gold particles, and therefore of the protein antigen, to be more readily discerned.

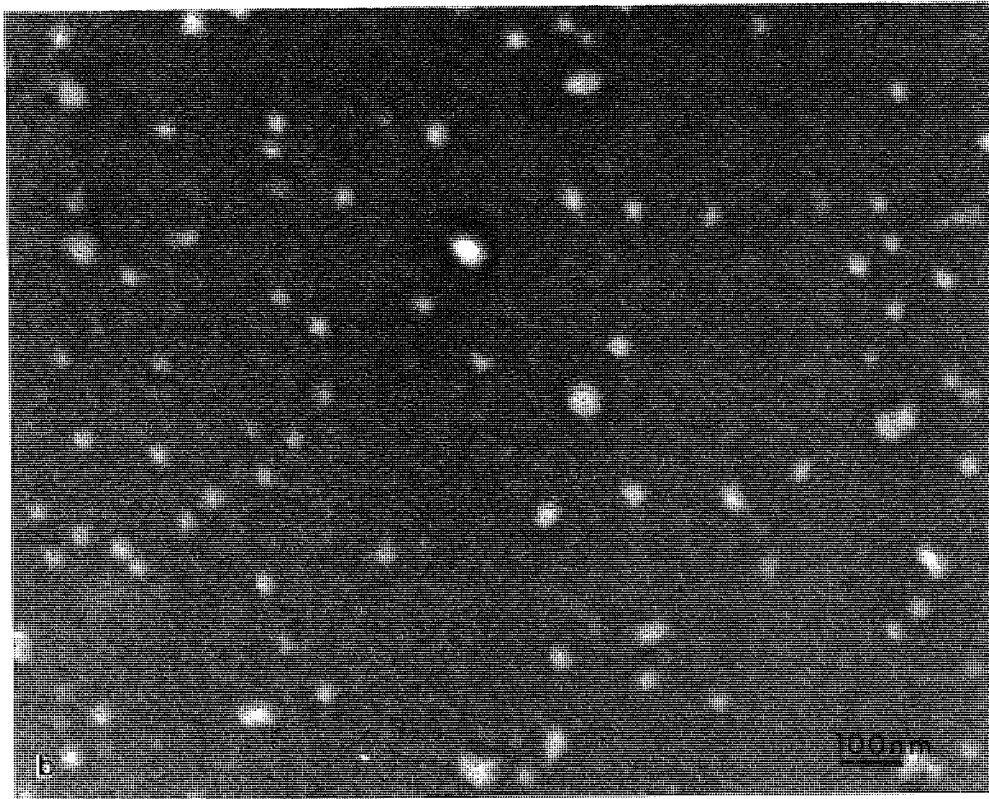
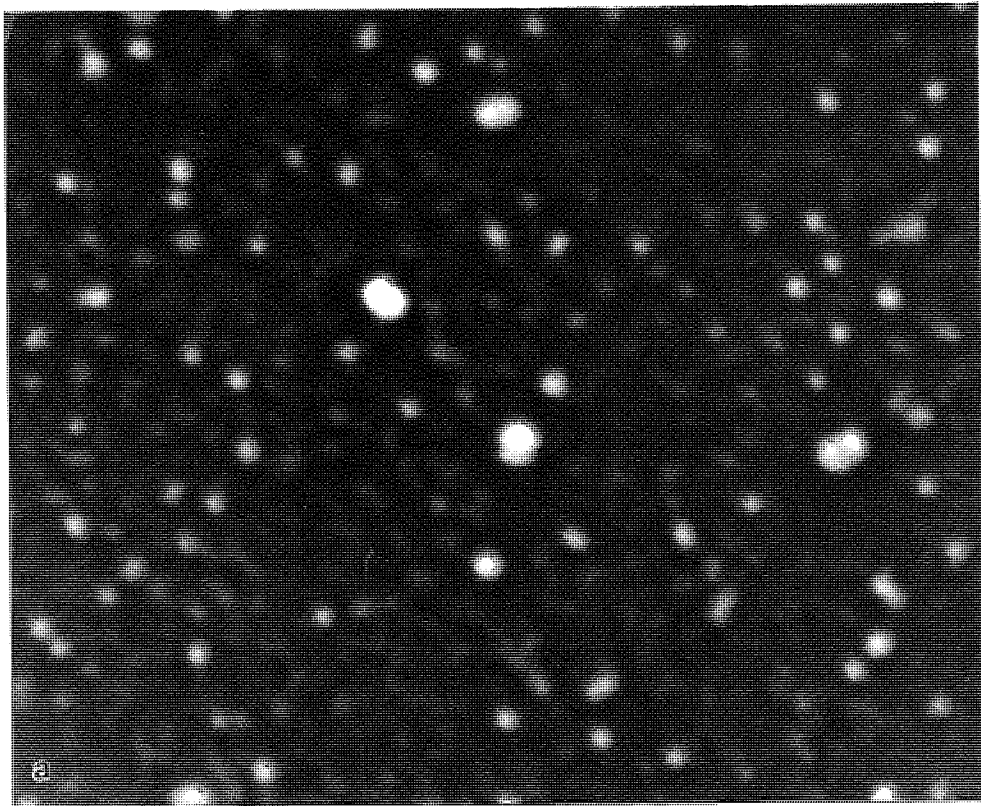


Figure 9.1 Comparison of (a) secondary electron imaging and (b) backscattered electron imaging of gold labelled protein layer (fibronectin on tissue culture polystyrene)

The use of smaller gold probes would be desirable for reasons of steric hinderance, which explains the regular use of 5nm gold particles in transmission electron microscope immunocytochemistry. However, they are very difficult to detect in the SEM, although this has been achieved (de Harven and Soligo, 1989; Walter and Mueller, 1986). It can be seen from the backscattered electron pictures in Figure 9.1 that the noise component of this signal is quite high. This is because the backscattered electron signal amplifier has to be set at a high level in order to detect the relatively small 15nm gold. This signal-to-noise problem is the limiting factor in the use of smaller gold particle sizes. An alternative is to use larger colloidal gold probes which would be easier to detect. This would enable images with less noise component to be produced and should allow lower magnifications to be used with the consequent benefit that larger areas of sample can be examined. The images illustrated in Figure 9.2 show that these points are justified, but it also illustrates the main drawback of the use of large gold probes. In Figure 9.2b the gold particle size is 30nm, which is twice the size of the particle used in Figures 9.1 and 9.2a. From this it can be seen that there are fewer of the larger gold particles per area, although the conditions for deposition of protein were the same in both instances, which means that there should be the same number of target protein molecules per area.

Larger probes are known to produce insufficient immunolabelling, and this is confirmed here (Beesley, 1989; Hodges and Carr, 1990). The number of colloidal gold particles deposited on a group of antigen molecules will decrease with increasing size of the gold. This is the result of two factors. Steric hinderance between the larger gold probes does not permit a high labelling density to be achieved. Furthermore, each large gold particle possesses a high number of antibody molecules and can, therefore, attach to a number of antigen binding sites if they are clustered together. It, therefore, requires fewer large probes than small ones to saturate the same number of clustered antigen molecules. It can be concluded from this that it is desirable to use as small a gold probe as practical to image. In this way any inhomogeneities in the distribution of the target antigen will be detected more readily and at greater resolution.

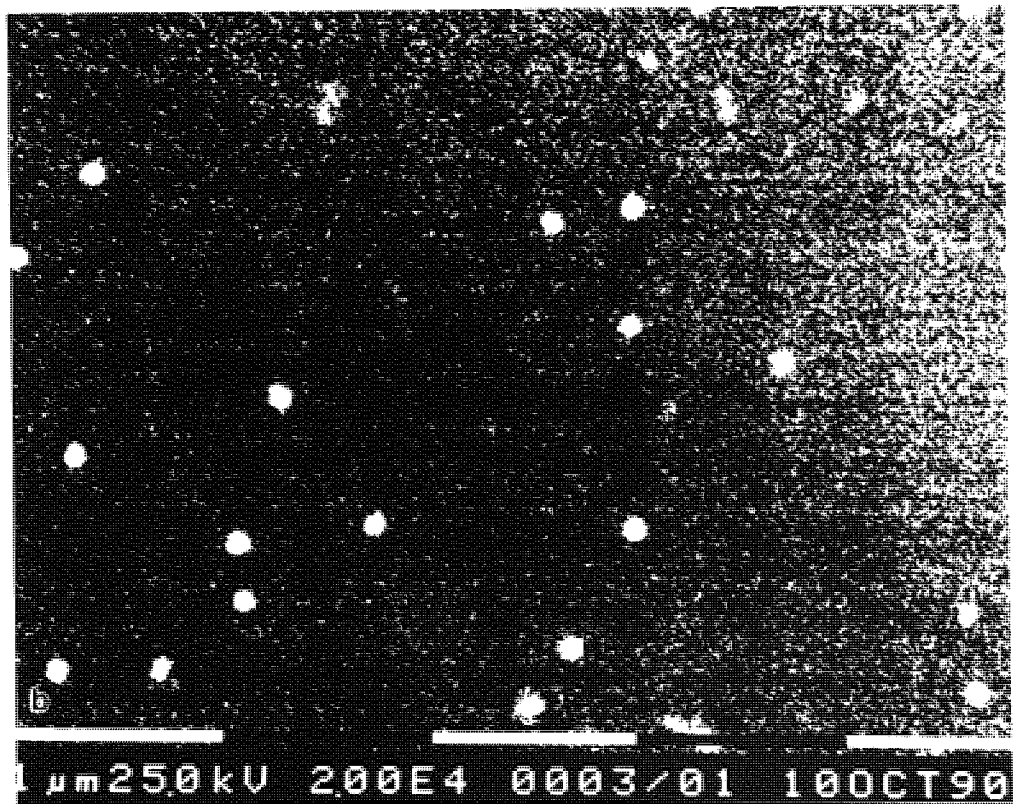
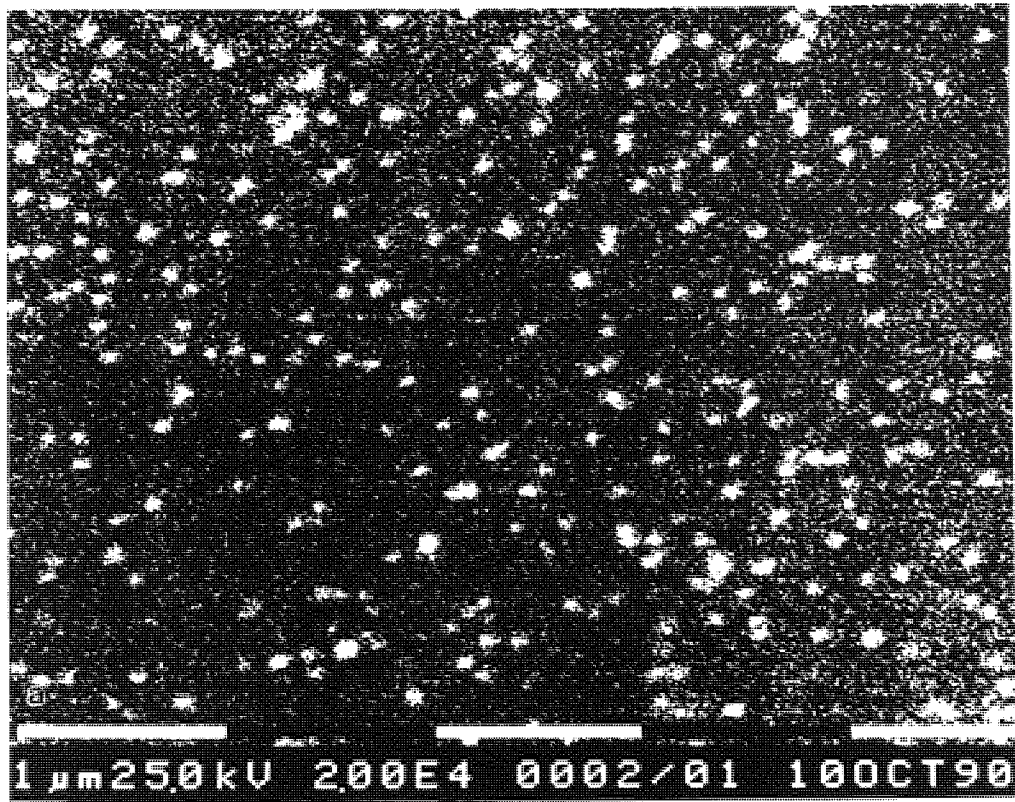


Figure 9.2 A comparison of the use of different sized colloidal gold particles. Immunogold labelled fibronectin layer using (a) 15nm and (b) 30nm colloidal gold

9.4 Technique Development

The various deposited protein layers are probed with the specific antibody for that target antigen, and a control antibody which has no cross-reactivity to that antigen is also used. The concentration of the antibody is decided by a dilution series experiment, and the control antibody is used at the same dilution as the primary antibody. Conventionally, with polyclonal antibodies the labelling is performed at low dilutions (1:50), which is the starting point for the dilution series experiment shown in Figure 9.3. One of the problems experienced in all the images seen in the early stages of these experiments is that the background, or control, labelling is quite high. One way to reduce the level of this non-specific labelling is to use the antibodies, both specific and control antibodies, in more dilute solutions. It can be seen from the images in Figure 9.3 that the 1-in-50 level is actually quite good because further dilution reduces the specific labelling markedly. However, the level of non-specific labelling is unaffected by any reduction in the concentration of the primary, control antibody. This is unacceptable, and so an alternative way of reducing the background labelling needs to be investigated. One way of doing this is to reduce the concentration of secondary, gold-labelled so that fewer gold probe complexes are available to react with free surface binding sites.

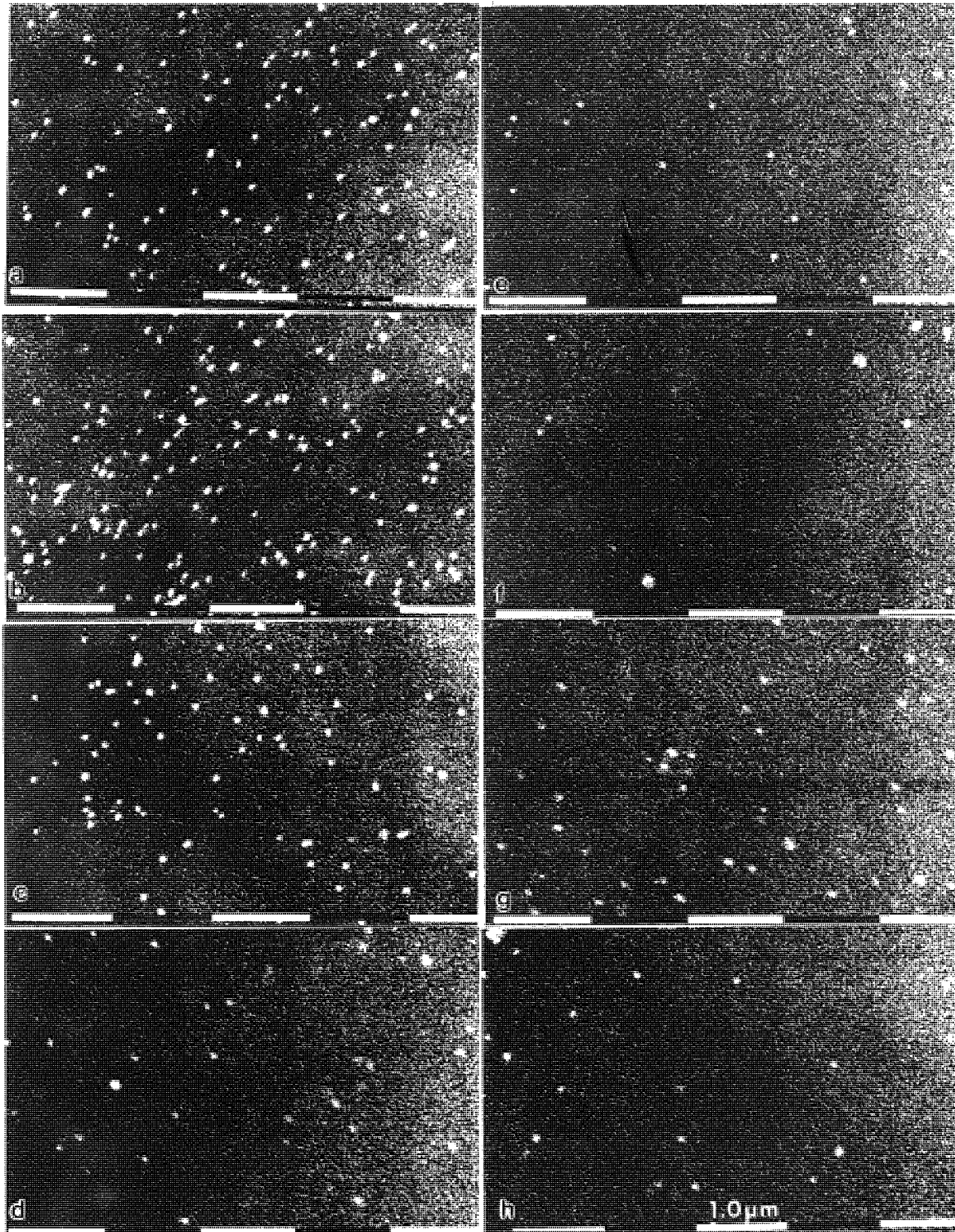


Figure 9.3 Dilution series against fibronectin coated tissue culture polystyrene where the concentration of the anti-fibronectin antibody is varied.

a) 8 μ g/ml anti-fibronectin	e) 7.8 μ g/ml anti-IgG
b) 4 μ g/ml anti-fibronectin	f) 3.9 μ g/ml anti-IgG
c) 0.8 μ g/ml anti-fibronectin	g) 0.78 μ g/ml anti-IgG
d) 0.4 μ g/ml anti-fibronectin	h) 0.39 μ g/ml anti-IgG

A similar series of dilution experiments was performed, but varying the concentration of the secondary, gold-labelled antibody. From this the non-specific background labelling was unaffected by varying the concentration of this antibody. However, the specific antibody labelling was noticeably reduced by the use of lower gold-labelled, secondary antibody (Figure 9.4). This indicates that any reduction of the gold-labelled antibody is restricting the ability of the gold probe to saturate all the available primary antibody molecules which have attached to deposited antigen molecules on the material surface. As this was unable to influence the background labelling, an alternative is to include blocking and prefixation steps before reacting the sample with the primary antibody.

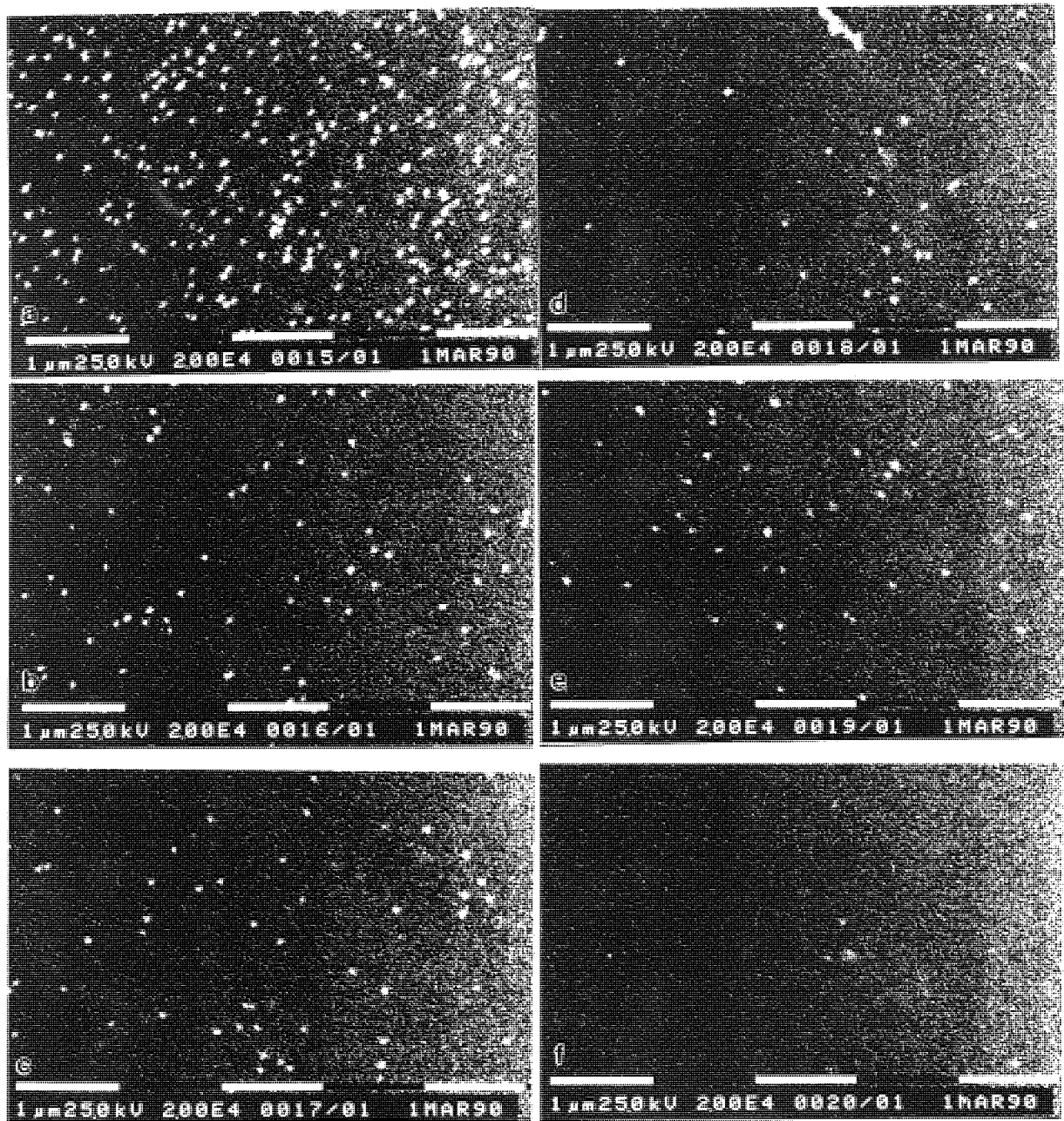


Figure 9.4 Dilution series against fibronectin coated tissue culture polystyrene where the concentration of the secondary, gold-labelled antibody is varied:

a) 1/50 colloidal gold IgG (specific)	d) 1/50 colloidal gold IgG (control)
b) 1/100 colloidal gold IgG (specific)	e) 1/100 colloidal gold IgG (control)
c) 1/200 colloidal gold IgG (specific)	f) 1/200 colloidal gold IgG (control)

Prefixation of specimens is important to prevent surface structural changes in cells and tissues during the labelling period, although this is not relevant in this series of experiments. It is also necessary to prevent clustering and lateral mobility (ie redistribution) of target molecules. Fixation conditions for immunocytochemistry remain a matter of compromise. Formaldehyde, freshly prepared from paraformaldehyde, provides a rapidly penetrating fixative, although glutaraldehyde is a better cross-linking agent. Glutaraldehyde is a larger molecule and, therefore, it is slower to penetrate into the sample. It also possesses two aldehyde groups so that any free aldehyde groups which have not reacted with the sample must be blocked by extensive rinsing with buffer supplemented with a blocking agent such as glycine or ammonium chloride. If this is not done, then non-specific cross-linking with amino groups of the primary antibody molecule will occur.

A common blocking step involves conditioning the sample before beginning the labelling procedure in order to prevent non-specific binding of antibody molecules with free binding sites on the sample surface. This blocking step can be any non-reactive protein, and those commonly used include ovalbumin, bovine serum albumin or defatted milk powder in buffer.

In an attempt to reduce the background staining observed in the earlier experiments it was decided to include both a prefixation and a blocking step in a single experiment, where these steps were included or excluded from the labelling regimen. The results of this are shown in Figure 9.5, where fibronectin layers deposited on tissue culture plastic have been challenged with specific and control antibodies. Figures 9.5a and 9.5b show the increased staining which occurs when no blocking or fixation steps are used. When these steps are included, the staining in both specific antibody (Figure 9.5c) and control antibody (Figure 9.5d) is seen to be reduced.

The main benefit of this is to reduce the background staining on the control antibody labelled samples, which has an obvious subjective benefit. However, the level of primary antibody staining is also reduced by the prefixation and blocking steps.

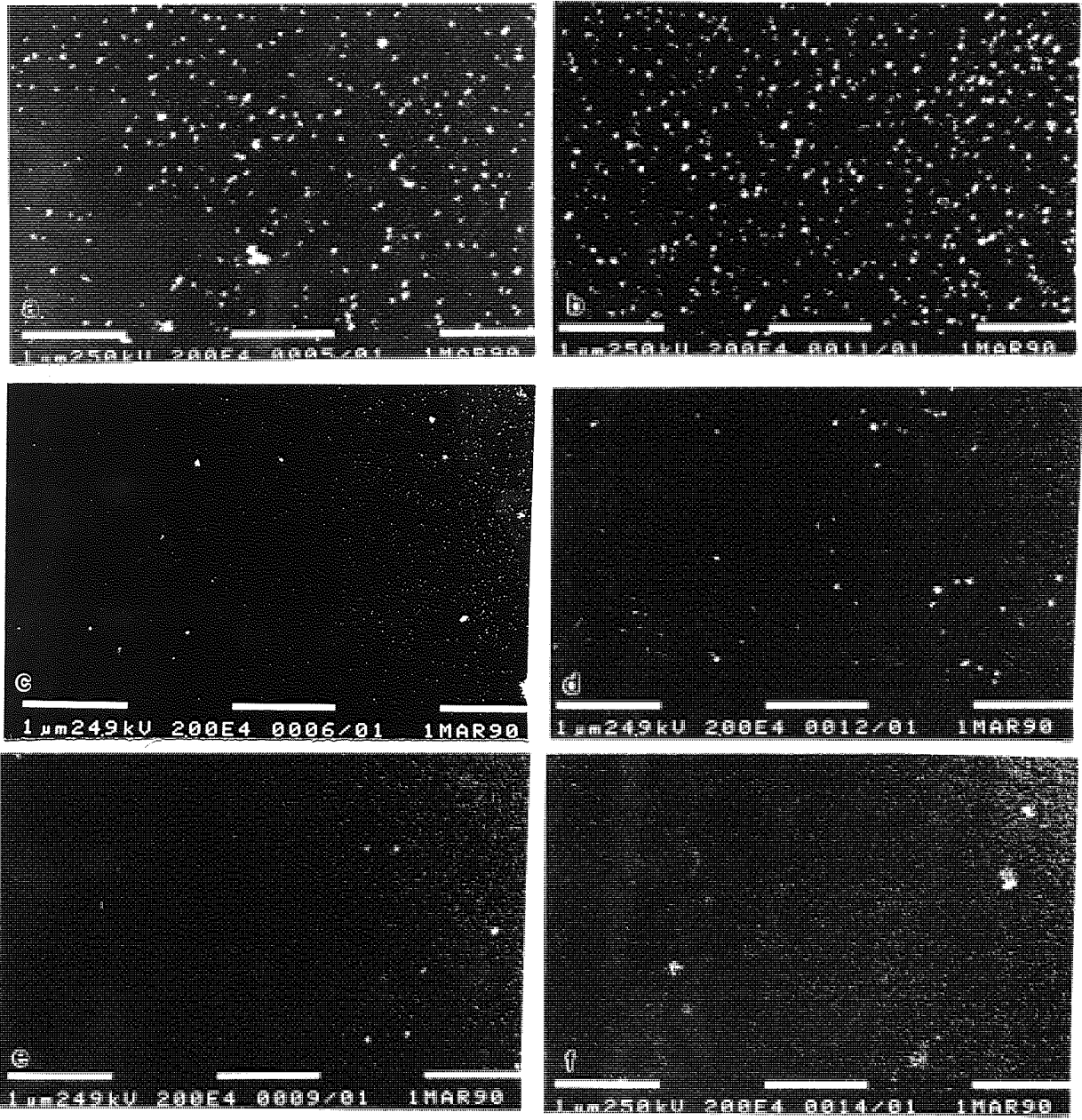


FIGURE 9.5 Comparison of immunogold labelled fibronectin coated surfaces with (a,b) and without (c,d) prefixation and blocking steps: specific antibody (a,c) and control antibody (b,d)

To get a more objective idea of the efficiency of these steps, image analysis was performed on these and similar pictures to count the number of gold particles present. When the amount of staining is compared using image analysis, the ratio of specific to background labelling is constant, whether or not prefixation and blocking steps are included in the immunolabelling methodology (Figure 9.6). Whilst the background labelling appears, subjectively, to be quite high, the image analysis results show that the background labelling is less than 10% of the specific labelling which is acceptable. The level of background labelling is virtually unaffected by reducing the concentration of the primary antibody. This would suggest that there is a finite level of this background labelling in absolute terms.

As the inclusion of prefixation and blocking steps did not alter the ratio of background-to-specific labelling, it seems that there is no need to include these steps. However, because the subjective appearance of the controls is improved by including these additional steps, it was decided to include them. The steps used were to treat the samples with 1% formaldehyde in phosphate buffered saline for 30 minutes followed, after washing with buffer, with a blocking step of 3% ovalbumin in phosphate buffered saline before the primary antibody step.

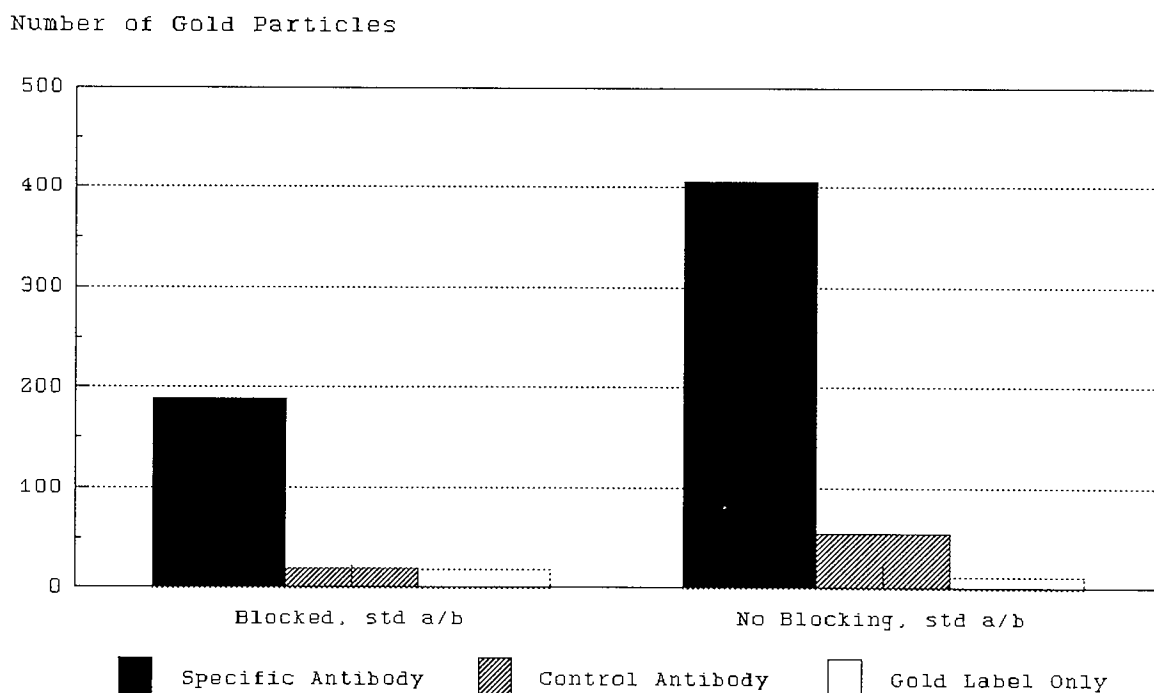


Figure 9.6 Histograms comparing the labelling with and without prefixation and blocking steps

9.5 Distribution of Fibronectin on Material Surfaces

The distribution of fibronectin on the range of materials is shown in Figure 9.7. From these images it appears that there is more labelling on the hydrophobic surfaces of bacteriological grade polystyrene and siliconised glass. Grinnell and coworkers have demonstrated, using radiolabelled antibody labelling (Grinnell and Feld, 1981; Grinnell and Feld, 1982; Grinnell and Phan, 1983), that more fibronectin was deposited on to bacteriological grade plastic than tissue culture plastic, at low concentrations. However, the adsorbed material was biologically less active on bacteriological grade plastic (Grinnell and Feld, 1981). The biological activity of adsorbed fibronectin is also not related to the amount of adsorbed fibronectin, and is surface dependent (Grinnell and Phan, 1983). They deduced from this that fibronectin assumes different conformations on hydrophobic or hydrophilic surfaces.

The results presented here show greater fibronectin labelling on bacteriological grade polystyrene, which needs to be compared to the results presented in Chapter 4 on the cell adhesive response to these surfaces. From these, it is seen that, whilst cells attach and spread onto fibronectin coated bacteriological grade polystyrene, the number of cells and the amount of cell spreading is less than occurs on fibronectin coated tissue culture plastic. The cell response to all of the fibronectin coated surfaces is considerably less than occurs when cells are grown on foetal calf serum deposited onto tissue culture plastic.

The control antibody labelled samples show a relatively small amount of background staining, although this is heaviest on the two polystyrene samples. It could be that this indicates that the plastic substrata are more liable to attract non-specific protein adsorption. However, control experiments in which the primary antibody is omitted, show no deposition of colloidal gold IgG which would conflict this idea.

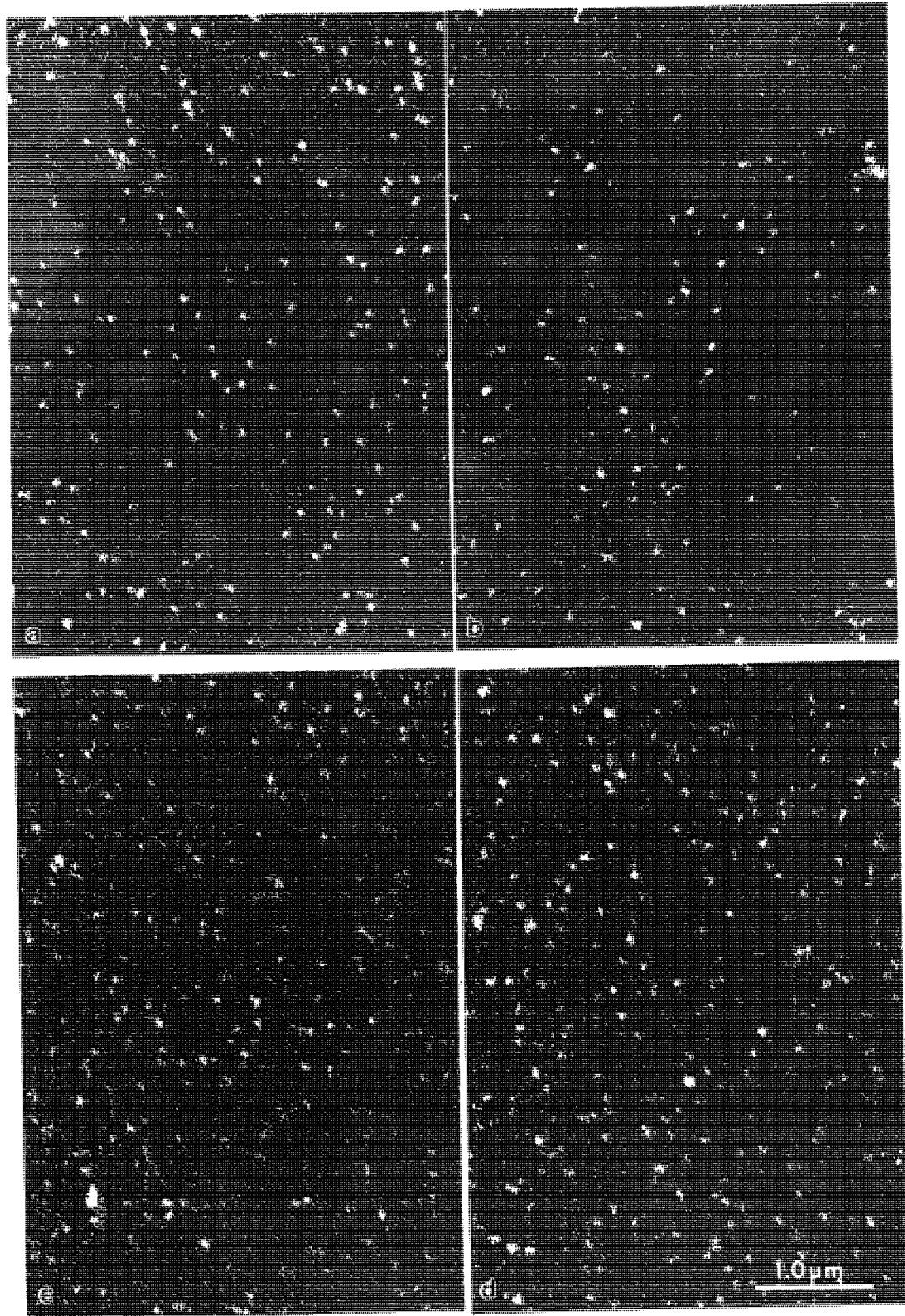


Figure 9.7 Immunogold labelling showing the distribution of fibronectin on a) bacteriological grade polystyrene, b) tissue culture polystyrene, c) glass, d) siliconised glass

9.6 Distribution of Human Immunoglobulin on Material Surfaces

The distribution of immunoglobulin on the various material surfaces is shown in Figure 9.8. With these samples there is very little difference in the labelling of most of the samples except for bacteriological grade polystyrene, which is markedly less than the others.

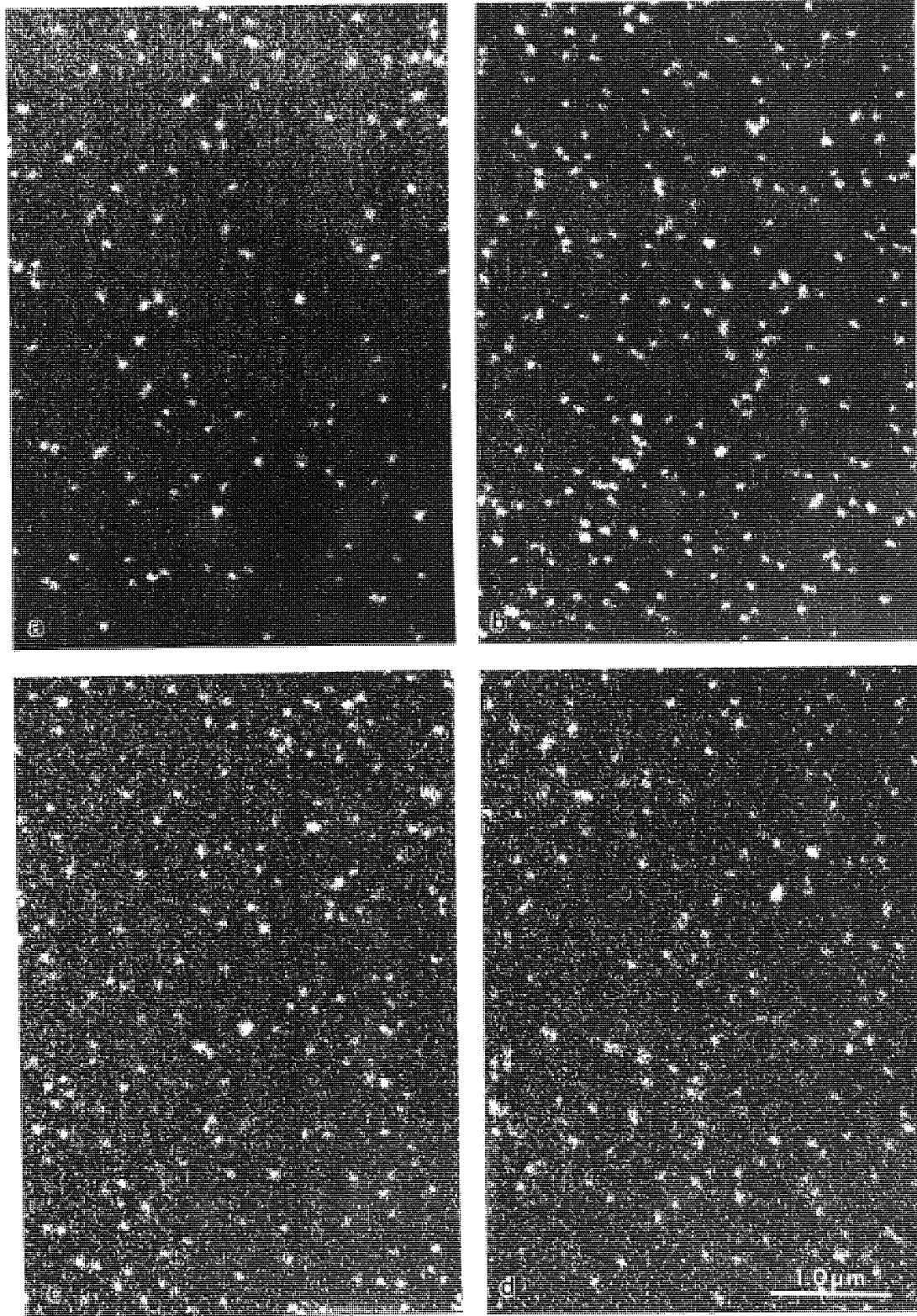


Figure 9.8 Immunogold labelling showing the distribution of human immunoglobulin (IgG) on a) bacteriological grade polystyrene, b) tissue culture polystyrene, c) glass, d) siliconised glass

9.7 Distribution of Vitronectin on Material Surfaces

The distribution of vitronectin on the various material surfaces is shown in Figure 9.9. The most obvious feature demonstrated by these pictures is that there is far less protein present on these materials. There is some variation between the amount deposited on each of them, with more present on the hydrophillic surfaces. This is in contrast to the distribution of fibronectin. The two hydrophillic surfaces are the ones which under normal cell culture conditions will support cell adhesion. However, the differences in amount of vitronectin adsorbed onto the differing surfaces is only small.

When this distribution is compared to the cell response to vitronectin layers adsorbed onto the various surfaces, it is seen that the cell response varies more than might be suggested by the distribution differences revealed here. There are approximately twice as many cells attaching and spreading on the hydrophillic surfaces than on the hydrophobic surfaces, when serum is not supplementing the culture medium. This difference in adhesion is considerably less, but still noticeable, when the medium is supplemented with foetal calf serum. This cell response does correlate with the increased labelling of vitronectin seen on these surfaces, and suggests that small increases in the amount of vitronectin adsorbed has a marked effect on the cell adhesiveness of the surface.

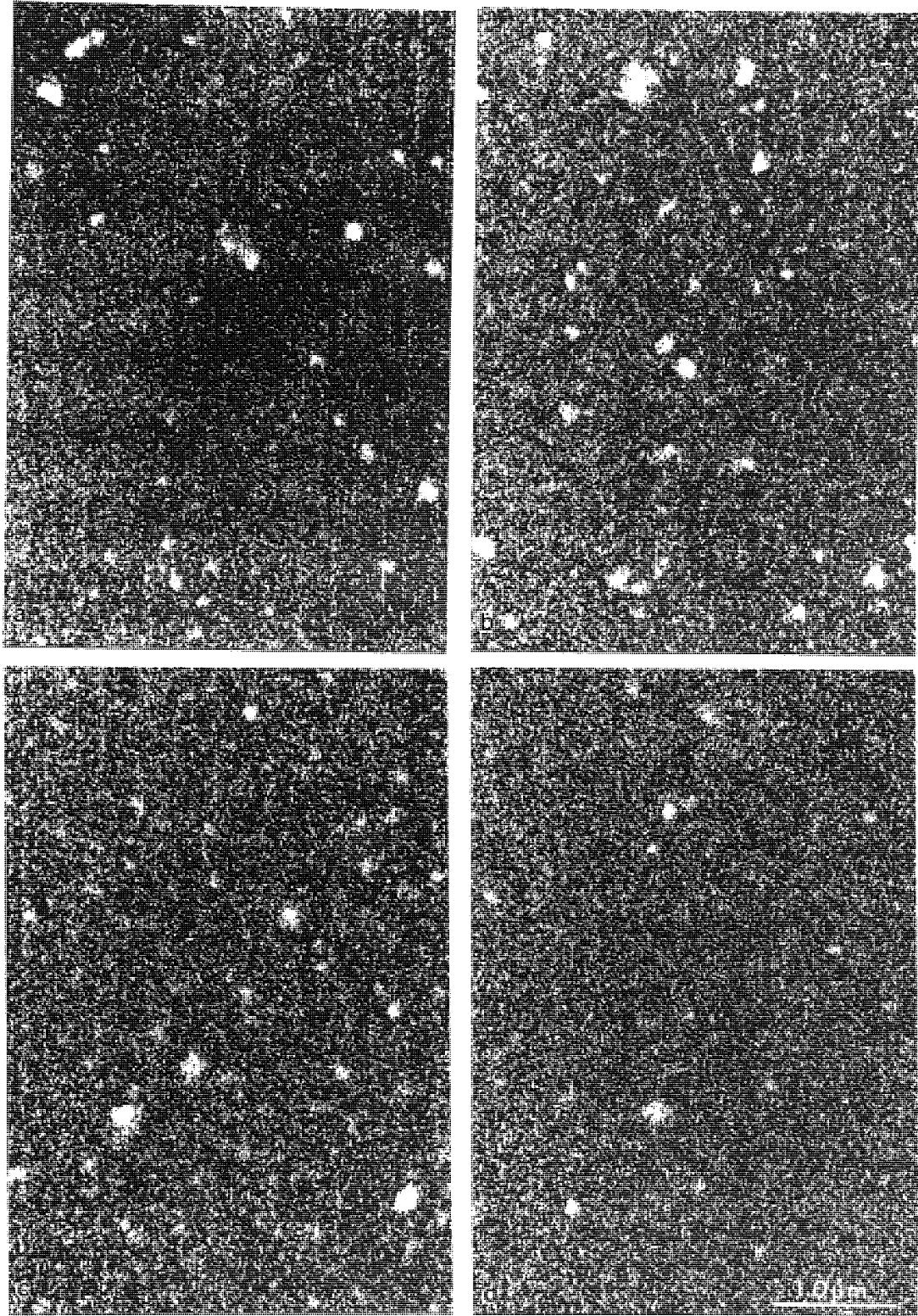


Figure 9.9 Immunogold labelling showing the distribution of vitronectin on a) bacteriological grade polystyrene, b) tissue culture polystyrene, c) glass, d) siliconised glass

9.8 Discussion and Conclusion

These labelling experiments have indicated that there is a difference between the distribution of deposited proteins on a range of surfaces. This difference is not great, but is noticeable. It is of interest to note that these experiments indicate that there is more labelling of fibronectin present on the two hydrophobic surfaces which are non-adhesive for cells under normal conditions for cell culture. This could indicate that the number of deposited adhesion-promoting proteins is not an important factor in determining the biological fate of materials. Conversely, the labelling of vitronectin is heavier on the hydrophilic surfaces, ie. those normally adhesive under standard cell culture conditions with serum supplement present.

When these results are compared with the cell response shown in Chapter 4, then it is seen that the labelling of vitronectin tends to correlate with level of cell attachment and spreading. With fibronectin the cell response does not vary significantly between fibronectin deposited on the various surfaces. However, there is more labelling of fibronectin on the hydrophobic surfaces.

Other deposited protein layers also show differences in the labelling distribution between materials with a range of surface properties. When IgG is deposited on glass the amount of labelling is the same on both types of glass which would indicate that this protein is not greatly influenced by the differences between the various surface properties of the materials under test. As IgG is not normally a surface active protein, then this fact is not too surprising.

Initial results from the labelling experiments using single protein solutions suggest that there are differences between the distribution of the deposited protein on various surfaces. However, the distribution of the protein over the surface under investigation does appear to be fairly uniform, and I have not seen the patterns reported in the paper by Murthy et al (1987). The amount and distribution of deposited adhesion-promoting proteins does not correlate well with the cell response to these surfaces. This is partly a problem with the use of single protein solutions in the cell culture model, as

these are not physiological conditions. Time constraints have meant that colloidal gold labelling experiments on the individual components of a deposited serum layer were not possible. They are the obvious next step for application of the technique. The experiments here do, however, show the importance of using several techniques to study protein deposition as the nature of the deposited protein layer must be analysed in conjunction with its functional properties, as given by the cell adhesion in culture.

The results presented here show that the technique is able to provide considerable information on the distribution of deposited proteins. Relatively small differences between surface densities are detectable, and these need to be related to other surface properties. The technique has the potential for providing conformational information as well using monoclonal antibodies, although this was not possible in the experiments described here. The surface coverage can be quantified using image analysis, and so the main advantage of the technique is that it enables changes in microscopic adsorption to be identified due to the high resolution of the technique.

CHAPTER 10 GLOBAL DISCUSSION AND CONCLUSIONS

10.1 Global Discussion and Conclusions

In our investigation into the role and mechanisms of "interface conversion" on the fate of polymeric materials in a biological environment, the use of a range of investigating surface analytical probes is obviously important. The purpose of this study has been to use such techniques to increase our knowledge of the nature of the polymer surface properties, and of their influence on the deposition of proteins to form a reactive biological/synthetic interface. The range of techniques available for surface analysis has been reviewed, and their strengths and weaknesses highlighted. Selected techniques were chosen and included in a program of work to investigate their usefulness for studying interface conversion in our model system based on the materials used in tissue culture.

This investigation has entailed the use of physical, chemical and biological probes in order to gain a more complete picture than would be obtained by the use of a single technique in isolation. Cell adhesion in culture is well established as the primary biological sensor of surface properties in studies of the biological/synthetic interface. As it is well established, it was necessary to include studies using this technique as a means of defining the biological response of the various materials used in our experimental model so that later results can be placed in perspective. Physical and chemical methods of investigation were used to allow correlations to be made between biological activity and surface properties and interactions.

The cell culture experiments have shown that the cell response to different materials can be modified by adhesion promoting protein layers. Adhesion promoting proteins (vitronectin and fibronectin) will induce cells to spread, even on surfaces which are not normally attractive to them when in normal culture conditions. The presence of a large surface concentration of a cell-adhesive protein on a surface promotes cell adhesion to that surface, contrary to the normal, physiological situation which is expressed when culture is performed with serum supplemented medium.

The results of this cell culture experiment confirm the earlier work from our laboratories and others, that

cells will grow on certain adhesive surfaces in the presence of serum in the medium, whereas non-adhesive surfaces do not support cell adhesion in the presence of serum (Bentley and Klebe, 1985: Curtis et al, 1983: Grinnell, 1978: Lydon, 1986: Minett, 1986: Thomas, 1988). Cell attachment and spreading, in serum-free conditions, is dominated by the cell adhesion stimulatory property of the deposited protein layer, and is largely independent of the substrate material properties. The response of cells, in serum-supplemented media, is much more influenced by the surface properties or chemistry of the underlying material. When serum is the sole deposited layer, whether from solution or by pretreatment of the substrate material, the physicochemical nature of the substrate material must determine the exact nature and composition of the biomolecules in the deposited serum layer, and this in turn determines whether cells are or are not able to attach and spread on that surface. These results show that the use of serum-free culture conditions, or the use of deposited single protein solutions have limited usefulness any investigation into the biocompatibility of materials.

An interesting finding is that cells will spread onto surfaces pretreated with "non-adhesive" proteins, such as albumin or immunoglobulin, if serum is used as a culture media supplement. This will be because either these "non-adhesive" protein layers are replaced by the correct proteins from serum, or the new protein layers deposited from serum are laid on top of the first layer. The replacement or superimposition of single protein layers with a serum layer could also help to explain the improved cell spreading response of the spreading promoting proteins when foetal calf serum is present in the culture medium. Cells obviously need other factors besides mere adhesion promoting factors for optimal spreading and growth. Serum in the culture medium has a beneficial effect on the extent of spreading, even with adhesion-promoting protein layers, presumably due to the presence of other growth factors (Rockwell et al, 1980). This effect of more complete spreading on adhesion-stimulatory protein layers has been noticed before (Schakenraad et al, 1989: van Wachem et al, 1989). These results suggest that cell adhesion experiments need to be conducted with and without serum supplementing the medium in order for the full effects of a protein layer on cell adhesion to be assessed.

Previous work from our group has suggested that surface rugosity may be a factor in cell adhesion to

polymeric materials (Minnett, 1986; Thomas, 1988). Goniophotometry is an excellent non-invasive and non-destructive technique for assessing surface roughness, which was not able to detect the very fine surface features which were revealed by scanning electron microscope investigation. The two types of glass, native glass and siliconised glass, were shown to be equally smooth by goniophotometry, but using scanning electron microscopy these two surfaces were shown to have very different surface rugosities. With polystyrene, varying surface appearances can be produced by a range of surface treatments. These can all be clearly investigated by the resolution offered by the scanning electron microscope, and the results presented here show that surface rugosity does not play a major role in determining a material's cell adhesiveness.

Low molecular weight polystyrene was seen, at high magnification in the SEM, to be smooth both before and after glow discharge treatment, but cells only adhered to the plasma etched surface. Sulphuric acid etched polystyrene and tissue culture plastic surfaces are smooth and support cell adhesion, whereas bacteriological grade polystyrene is also smooth but does not support cell adhesion. Unetched high molecular weight polystyrene has a rugose surface but does not support cell adhesion. After plasma etching the surface rugosity is of a similar scale and cells readily adhere to this surface. These findings taken together rule out microrugosity as being important in cell adhesion to polystyrene surfaces. On the other hand, macrorugosity has been shown to induce cells to adhere to an otherwise non-adhesive surface when cells are found adhering to scratches in polystyrene bacteriological grade dishes (Heath and Dunn, 1972).

It is possible that the surface of the polymeric material is not the surface which is relevant to the cells, but rather the protein coated surface is more important. The cell culture experiments showed that cell adhesion was a protein specific event, their action may be to produce a rough surface which is attractive to cells. Observation of the surfaces of materials with and without deposited protein layers showed that the surface rugosity of these layers was also not significant to the cells. Generally, the deposited protein or serum layer reflects the underlying substratum surface, although, the surface features seen on the substratum are not necessarily reflected in the appearance of the serum or protein layer surface

structure. The cell adhesion property appears very variable, depending on the material and protein layer in question. For example, sulphuric acid treated bacteriological grade polystyrene is smooth, but the serum layer on top is ridged, and cells adhere and grow on this surface. Conversely, the serum layer on top of siliconised glass is roughened to a similar degree as the native surface but is not adhesive to cells. These observations show that rugosity of either the substratum surface or the deposited serum layer is not a deciding factor in determining whether cells will adhere to and grow on that surface.

Having eliminated surface rugosity as a factor influencing cell adhesion, it is necessary to consider what factor could be influencing the biological response. The results from the surface rugosity investigation show that cells will adhere only to those polystyrene surfaces which have a reasonable degree of hydrophilicity after plasma glow discharge treated polystyrene. The low molecular weight polystyrene was more wettable than the other native polystyrenes owing to the shorter monomer chain lengths, resulting in greater numbers of the polar end groups at the surface. However, this surface is not adhesive to cells. Plasma etching the surface of the low and high molecular weight polystyrenes increases the wettability considerably, and renders these materials cell adhesive without influencing the surface rugosity.

Plasma discharge treatment of polystyrene is known to alter the wettability of the polystyrene surface (Curtis et al, 1983; Grinnell, 1978; Jansen et al, 1989; Thomas et al, 1986). The surface rugosity part of this work also suggested an important role for surface energy in cell adhesion. Investigations using contact angle measurements were performed to study the nature of action of surface energy in determining the cell adhesive property of materials. This study showed that the energy of the surface, once coated with a deposited layer of protein, appears to be of no interest or relevance to the cells in deciding whether they will attach and spread on that surface. This is in agreement with the cell culture experiment that cell adhesion is a protein mediated event. Surface energy (specifically the polar fraction) is important in promoting cell spreading on surfaces. The action of this must be for the polar fraction of surface energy to influence the correct deposition of proteins onto a polymeric surface. It is the nature of this deposited protein layer that then determines the cell response to that surface, and this will no

doubt rely on which proteins are deposited, and on the conformation and orientation of the proteins within the layer.

It is our working hypothesis that in a non-adhesive surface energy zone "interface conversion" does not proceed correctly, and thus prevents cell interaction with the synthetic surface. However, in the non-physiological situation, as with deposited single protein layers, cell adhesion may occur. In these cases, the system cannot be a true test for biocompatibility as it does not predict the behaviour of the material in a physiological environment as represented by mixed, multiple biomolecule systems like serum. The consequence of this is to add to the wealth of evidence in support for a fundamental role for surface energy in determining the biological fate of the polymeric materials.

An overview of the relationships between chemical group expression (Curtis et al, 1983; Gibson and Bailey, 1980; Klemperer and Knox, 1977; Maroudas, 1976; Thomas et al, 1986), surface free energy (fractional polarity) (Lydon et al, 1985; Thomas et al, 1986; van der Valk et al, 1983) and cell adhesion had been suggested by our work, and in the literature. The surface energy of the substratum is clearly important in triggering interface conversion (Lydon et al, 1985; van der Valk et al, 1983), but is not the sole criterion, as the expression of chemical groups at the surface may have a part to play (Curtis et al, 1983; Thomas et al, 1986). Work on the surface chemistry of materials using X-ray photoelectron spectroscopy has indicated that the presence of surface oxygen is important in promoting cell adhesion to that surface (Curtis et al, 1983; Thomas, 1988). Surface oxygen may not be the ultimate determinant, as it is not found in argon- or nitrogen-plasma treated polystyrene (Minett, 1986), where other polar groups, such as amino groups, may play a part (Brewis et al, 1978). The use of X-ray photoelectron spectroscopy and secondary ion mass spectrometry need to be used to more clearly characterise which chemical groups are important in promoting a bioreactive interface. Hydroxyl or sulphonate groups have been implicated previously as being necessary for cell adhesion (Curtis et al, 1983; Maroudas, 1976), although, it would seem that several groups may be involved. The action of these chemical groups is likely to be due to the changes they bring about in the surface energetic state of the material surface, which appears from the results of these experiments and those in the literature to be the key determinant

of a cell adhesive surface.

Having focused extensive effort on the material surface properties, attention in this investigation turned to an attempt to determine the nature of the protein layer deposited on the polymer surface in order to discover more about the organisation of the conditioning protein layer. X-ray microanalysis was unable to detect deposited serum layers polystyrene or glass surfaces. This is due to the sensitivity of the technique being inadequate to allow detection of the deposited serum layer, which is too thin. In the case of the glass substrates, the spectra show the elemental spectrum expected for glass, and indicate that the signal had not been influenced or suffered very little attenuation by the thin serum layer. In a further experiment, in which glass was coated with varying thicknesses of a polymer, the signal from the underlying glass substratum was seen to be attenuated in a progressive manner in proportion to the thickness of the applied polyurethane film. From this it was seen that the penetration depth of the technique (or the analytical volume) is very large in proportion to the required analytical volume, and highlights a major inadequacy of the technique.

These results show that the technique is only sensitive to film thicknesses of the order of 1-5 μ m, which is an order of magnitude bigger than that for several other surface analytical techniques, such as X-ray photoelectron spectroscopy, contact angle measurements and cell culture testing. Minett's work (1986), using spin coated polyHEMA films, showed that cell adhesion onto tissue culture polystyrene could be inhibited by film thicknesses of approximately 0.5 μ m. My work with the surface energy experiments shows that a 20nm thick protein layer, as is deposited during interface conversion, will alter the surface energy characteristics of materials, and also alter the cell response to that material. Hence the biological model, and surface energy measurements are far more sensitive than X-ray microanalysis, even though the latter will give very good XY spatial resolution of information.

The failure to determine any detail of the protein layer with the technique of X-ray microanalysis, which has proved so useful in several fields, leads to the need to use other means of analyzing the nature, composition and state of the synthetic/biological interface. There are two techniques with the potential

to further an investigation into the nature of the biological side of the interface which is set up during interface conversion; these are FTIR spectroscopy and biochemical labelling. FTIR spectroscopy is an important new development of an established technique which is non-destructive, and has been used to follow the adsorption of proteins from blood onto plastic surfaces (Baier and Dutton, 1969: Kellner and Gotzinger, 1984: Jakobsen, 1979: Seifert and Greer, 1985), and the adsorption of single proteins from solution (Baier and Dutton, 1969: Baier et al, 1971: Pitt et al, 1986a: Pitt and Cooper, 1986). It has also been used to determine conformational changes when spectral changes of adsorbed proteins are compared to spectra from solution proteins. However, the technique is only now becoming reasonably available with the increasing availability of the instrumentation, and so the technique is becoming established as providing useful chemical information in numerous fields, especially in the field of polymer chemistry.

A brief attempt with an FTIR instrument to detect a protein layer on polystyrene proved unsuccessful and pointed to the problems of the technique, which needed more time and access to equipment than was available. Especially important with using the attenuated total reflection technique is the need to ensure intimate surface contact. As the facilities were not available for an extensive investigation, attention was given to a new biochemical labelling technique which would utilise instrumentation already well used in this series of investigations, namely scanning electron microscopy immunocytochemistry, using colloidal gold markers. Immunocytochemical techniques enable biochemical labelling in a highly specific manner, and by using a scanned electron probe, a distribution map can be produced from either thin sections of the sample or from the top surface of bulk material. The technique has been used to investigate cell surface-associated proteins in cells and tissues, and in extracellular matrix (Geuskens et al, 1986: Hattori et al, 1984: Nakatsuji et al, 1985: Trejdosiewicz et al, 1981). Its use to study the arrangement of proteins on material surfaces has also been investigated (Murthy et al, 1987: Park et al, 1986a: 1986b: Pitt et al, 1986b).

Colloidal gold markers have emerged, by virtue of their particular properties, as the most commonly used marker in immunoelectron microscopy. Colloidal gold is capable of forming active and stable

complexes with a wide variety of biological substances to provide the gold probes, so forming a flexible labelling system. Gold probes are now widely available in a number of forms, in terms of particle size and gold-ligand complexes. The high density of the particles and their recognisable spherical appearance are useful characteristics which enable gold particles to be distinguished and quantified by simple counting procedures. A further advantage is the facility to carry out multiple labelling of several target molecules in one preparation, using gold particles of different sizes (de Harven et al, 1990). As a consequence, gold particles on labelled preparations can be readily distinguished, imaged and quantified.

When examining the biological/synthetic interface surface imaging using scanning electron microscopical techniques are necessary. One of the problems with the technique in the SEM is the resolution offered compared to transmission electron microscopy immunocytochemistry, where the use of 5nm gold particles is routine. The use of smaller gold probes is desirable for reasons of steric hinderance; they are very difficult to detect in the SEM, however, although this has been achieved using state of the art field emission instruments (de Harven and Soligo, 1989; Walter and Mueller, 1986). A compromise was attempted here using 15nm gold probes, where signal-to-noise is the limiting factor in the use of smaller gold particle sizes. An alternative is to use larger colloidal gold probes which would be easier to detect. This would enable images with less noise component to be produced, and should allow the use of lower magnifications with the consequent benefit that larger areas of sample can be examined. The images illustrated in Figure 9.2 show that these points are justified, but it also illustrates the main drawback of the use of large gold probes. From this it can be seen that there are fewer of the larger gold particles per area, although the conditions for deposition of protein was the same in both instances, which means that there should be the same number of target protein molecules per area.

Larger probes are known to produce insufficient immunolabelling, and this is confirmed here (Beesley, 1989; Hodges and Carr, 1990). The number of colloidal gold particles deposited on a group of antigen molecules will decrease with increasing size of the gold. This is the result of two factors. Steric hinderance between the larger gold probes does not permit a high labelling density to be achieved.

Furthermore, each large gold particle possesses a high number of antibody molecules and can, therefore, attach to a number of antigen binding sites if they are clustered together. Consequently, it requires fewer large probes than small ones to saturate the same number of clustered antigen molecules. It can be concluded from this that it is desirable to use as small a gold probe as practical to image. In this way any inhomogeneities in the distribution of the target antigen will be detected more readily, and at greater resolution.

Previous work in the biomaterials literature has used the secondary electron signal to study the distribution of proteins on material surfaces (Park et al, 1986a; Pitt et al, 1986b). A simple comparison of the information provided by the two common SEM imaging modes serves to point out the practical advantages of using the backscattered electron image, which is now becoming recognised as the best way of localising gold probes (Hodges and Carr, 1990; Hodges et al, 1988). With the backscattered electron image, the gold particles are the only features contributing to the signal, and so they appear very bright against a black background. With the secondary electron image, the gold probes are superimposed on a complex image of the deposited protein layer. This results in a slightly confusing picture. Small surface features, such as contaminating virus particles, protein aggregates and cellular debris, can be of marker size and shape, and prove difficult to distinguish from single or clumped gold particles, thereby leading to misinterpretations. It can be seen from this that backscattered electrons provides a simpler image which enables the distribution of colloidal gold particles, and therefore of the protein antigen, to be more readily discerned.

The immunogold labelling technique is able to provide considerable information on the distribution of deposited proteins. Relatively small differences between surface densities are detectable, and can be related to other surface properties. The surface coverage can be quantified using image analysis, and so the main advantage of the technique is that it enables changes in microscopic adsorption to be identified due to the high resolution of the technique. The experiments described here have indicated that there is a small, but noticeable difference, between the distribution of fibronectin on a range of surfaces. It is of interest to note that these experiments indicate that there is more labelling present on

the two hydrophobic surfaces which are non-adhesive for cells under normal conditions for cell culture. This could indicate that the number of deposited adhesion-promoting proteins is not an important factor in determining the biological fate of materials. Other deposited protein layers also show differences in the labelling distribution between materials with a range of surface properties. With immunoglobulin on glass, the amount of labelling is the same on both types of glass indicating that this protein is not greatly influenced by the differences between the various surface properties of the materials under test. As IgG is not normally a surface active protein this fact is not too surprising and indicates that variations in surface properties are more relevant to molecules which act at surfaces.

The potential of this technique has not been fully realised here, but the detail which is possible with the technique has been demonstrated. It is likely that further investigations using immunolabelling in conjunction with other surface analytical techniques and biological probes will be at the forefront of research into the biomaterials field. Techniques for analyzing and characterising the material surfaces are well established in the materials science fields, and some of them, eg. X-ray photoelectron spectroscopy, have provided a useful insight into the surface properties of biomaterials.

X-ray photoelectron spectroscopy has been used to show that the presence of polar groups, like carbonyl, hydroxyl or amine groups, at material surfaces are important for altering the material surface energy characteristics of polymers. This makes the polymer surfaces more hydrophilic and receptive to the deposition of proteins from solution in a suitable orientation and state. Hydrophobic surfaces are known to promote denaturation of proteins, which will render them inactive, and so a suitable hydrophobic/hydrophilic balance is required. The use of contact angle measurements can establish the surface energetic state of surfaces which is important to the correct deposition of biological molecules. The importance of the polar fraction of surface energy has been well demonstrated here, and is able to give a clear guide as to the ultimate biological fate of polymers. The biological probe, culture cells, are of great importance in any study in the biomaterials field. They can also be used in conjunction with the biochemical technique of immunological staining to further study the process of interface conversion.

10.2 Concluding Remarks

The techniques investigated here have confirmed one factor which was indicated in the technical review of surface analysis techniques. This is that the depth resolution is very variable, and that this can often be a deciding factor in the usefulness of the information obtained. The techniques have different sensitivities also in terms of the detectable limits, and provide information on differing aspects of the biological/synthetic interface. The consequence of this is that a range of techniques is needed in any full study of such a complex field as exists in the biomaterials area.

The review of techniques indicated that they have different sensitivities and provide information on differing aspects of the materials under investigation; the experimental studies performed here have also shown this clearly. For example, scanning electron microscopy is capable of showing surface detail in good resolution (a few nanometres), whilst X-ray microanalysis involves a loss of resolution, from the same instrument, to the level of several microns. However, by collecting a different signal it is capable of providing elemental information on the samples under test in terms of both quantitative and distribution detail. The biological probe, cell culture, and surface energy measurements have proved to be very sensitive techniques, capable of detecting minute differences between surfaces and deposited protein layers, whereas X-ray microanalysis was not able to detect the presence of the deposited proteins.

Interface conversion is responsible for the correct deposition of proteins from a complex physiologically relevant solution like serum. The series of experiments detailed here show that physicochemical properties are responsible for determining protein deposition, including the protein layer composition, orientation and conformational state. Prime amongst these surface properties is the polar fraction of surface energy. This protein layer then determines the cell response, which is the ultimate goal in determining the biological fate of potentially biocompatible materials. The organisation of the deposited biomolecular layers can be investigated using biological and biochemical probes in order to determine the features which are necessary for the success of the interface conversion process.

This review and investigation of techniques has underlined the need for a multidisciplinary approach to the complex field of biomaterials science. A considerable number of investigations in the literature have provided useful information to the fund of knowledge, but often the results of these can be confusing and even conflicting. This is possibly due to cell biologists not appreciating the full implications of changes in polymer surface chemistry and, conversely, materials scientists may not have fully understood the complexities of biological tests. The success and failure of the individual surface analytical techniques to answer the questions they had been challenged with in characterising surface physicochemical properties of synthetic materials and deposited biological molecular layers demonstrates the need for an integrated research program. This will involve using a range of techniques in providing a more complete picture of the biological/synthetic interface.

10.3 Suggestions for Future Work

This series of experiments has been able to demonstrate the usefulness, or otherwise, of a number of surface analytical techniques. Some techniques are already well established, while there is a number of new techniques which it has not been possible to include here. X-ray photoelectron spectroscopy and secondary ion mass spectrometry have already been shown to be useful method for investigating polymer surfaces. SIMS is beginning to be used for the study of the biomaterials interface, although the main problem here is the difficulty of preparing fragile biological samples for such ultra-high vacuum techniques. The usefulness of other techniques, such as FTIR spectroscopy and confocal light microscopy, need to be investigated since they are potentially capable of adding to the range of techniques which provide useful information to research workers in this field. A start has been made on developing immunolabelling techniques for use with deposited protein layers on polymer surfaces, and this needs to be taken further.

The large mass range of detected species now available with time-of-flight mass spectrometers enables small biological molecules, such as peptides, to be investigated. Polypeptides can now be custom manufactured. SIMS could be used to study the interaction of peptides, which have defined structures and properties, with a range of polymer surfaces. The peptides used, could be designed to present a range of polar-hydrophobic and acid-base amino acids which would be expected to have varying responses to surfaces with a range of surface chemistry and surface energy.

FTIR spectroscopy is well established for qualitative analysis of organic systems in general, and provides an identification fingerprint. Libraries of characteristic IR spectra exist, including those of polymers, although the identification of particular functional groups can be equally important. The improvement in sensitivity concomitant with the new instruments has resulted in an increase in the range of applications, which now encompasses reflection and microscopical techniques. Previous work in the literature indicates that the use of reflection sampling and microscopical techniques will provide considerable information on the chemical groups at the biological/synthetic interface and the orientation and

conformation of the biological molecules present. FTIR will be included in future studies of biomaterials by our group.

The confocal light microscope is a new development of the light microscope which has considerably increased power, largely due to the improved contrast images available. The advantages of the technique have been considerably well explored by tissue studies using fluorescent staining techniques. Its potential for studying the biological/synthetic interface is considerable, but this area is still to be developed, and will no doubt be rectified with the increasing availability of the instruments.

The logical extensions from this series of experiments will need to concentrate on trying to determine the nature of the biological side of the biomaterials interface. In earlier experiments on the adhesion of cells to surfaces with a range of physicochemical properties, it was seen that albumin will not promote the adhesion of cells to otherwise cell adhesive surfaces when serum is absent from the medium. However, when cells were exposed to albumin-coated surfaces when serum was present in the medium, cells were able to attach and spread if the surface had the correct surface energy and surface chemistry. This could be for one of two possible reasons - either that the albumin layer is replaced by one from the foetal calf serum which contains cell adhesion promoting proteins, or the albumin is overlain by a serum layer.

A further series of experiments could help shed more light on this area. Competition experiments where non-cell adhesion promoting proteins and adhesion promoting proteins are deposited onto surfaces are the way to investigate this phenomenon further. There is some evidence that fibronectin is more effective when deposited with albumin than when deposited on its own (Grinnell and Feld, 1981: 1982). These experiments need to try the effects of sequential deposition of proteins, and deposition from multiple protein solutions. Cells grown on single protein layers, such as fibronectin or vitronectin, have a more spindly morphology than that normally seen on foetal calf serum coated surfaces. So both studying cells and the distribution of adhesion promoting proteins when such a single protein layer is overlain with serum, could be as worthwhile as looking at serum overlaying albumin.

Initial results from the labelling experiments using single protein solutions suggest that there are differences between the distribution of the deposited protein on various surfaces. An interesting experiment would be to antibody label proteins on surfaces with cells present, and by removing the cells and seeing if the cell adhesion promoting proteins are grouped in any way. The adhesion molecules can be labelled in a serum layer, or in single protein layers, to see how their distribution has been altered by the growth and locomotion of the cells. Another extension would be to look at the distribution of cell surface receptors for fibronectin or vitronectin on cells that have been cultured on a variety of substrata with a range of adhesive or non-adhesive properties. It may also be possible to look at the orientation of proteins on surfaces by challenging the protein layers with antibodies directed at specific parts of the deposited molecules.

An obvious extension of the immunolabelling technique which deserves attention is to use smaller gold probes, but using silver enhancement techniques, as used routinely in light microscopy, and adapting them for use in scanning electron microscope. In silver enhancement, the gold-labelled specimens are incubated in a physical developer containing silver ions and a reducing substance. In this medium, concentric shells of metallic silver are formed around the gold particles, which act as nucleating agents. This increases the diameter of the gold probe, and thereby improves their visibility in the SEM. The degree of enhancement provided by the silver staining can be adjusted by altering the development time. By using this technique it should be possible to utilise smaller gold probes than used here, including, and especially, the new 1nm gold particles which have recently become available commercially. This will have an obvious benefit for labelling efficiency, as the gold particles will no longer be the determinant of the size of the antibody-gold complex. Also, the 1nm gold particles have only one antibody molecule conjugated, which means that each gold particle will, therefore, represent a single antigen molecule.

APPENDIX 1

PROTEIN AND ANTIBODY PREPARATION

A1.1 Vitronectin Preparation

Vitronectin has had several names, but is now known by its affinity for glass which is used as the first step in the purification process and itself leads to a reasonable pure protein which is functionally identical to pure vitronectin. It was prepared from foetal calf serum according to the method of Lydon and Foulger (1986).

A water-tight column filled with glass beads was prepared. The beads were washed with 0.1% Tween, followed by an acid wash in a nitric and hydrochloric acid mixture, and a prolonged distilled water wash. The column was packed and equilibrated with 150-200ml 0.6M sodium bicarbonate (pH8.0).

The foetal calf serum was added to the column and run through with the equilibration buffer until the brown band of serum had run down the column. The proteins which had bound to the column were eluted off using buffers of progressively higher pH's, and collect using a fraction collector. Figure A1 shows that two protein peaks elute from the column. Figure A2 is a photograph of an SDS gel showing that Peak Two has the vitronectin bands at approximately 60K and 80K daltons. Figure 3 shows BHK

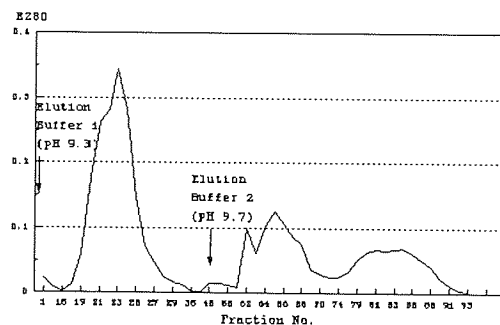


Figure A.1 Fractions collected from the glass column show two peaks of protein eluting off the column.

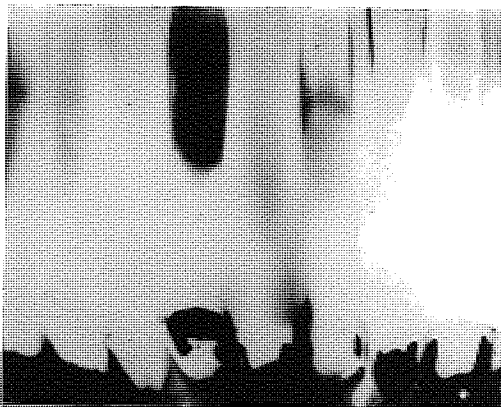


Figure A.2 SDS-PAGE of peak two proteins

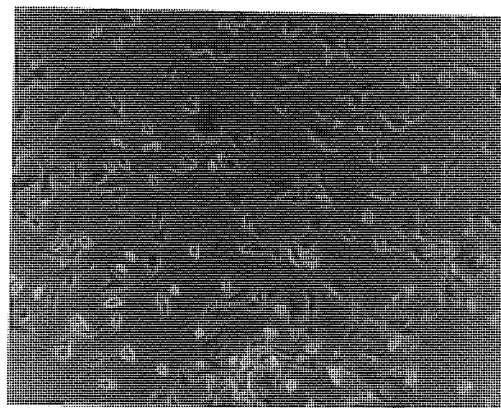


Figure A.3 BHK fibroblasts adhering to partially purified vitronectin coated plastic

fibroblasts adhering to bacteriological grade plastic which has been treated with a solution of Peak Two proteins.

The pure human vitronectin obtained from Calbiochem had been isolated in a similar manner and then purified by an affinity column, concanavalin A-Sepharose (Barnes and Silnutzer, 1983).

A1.2 Fibronectin Preparation

Rat plasma fibronectin was prepared using the technique of Vuento and Vaheri (1979), which is described briefly here. Rat plasma was barium sulphate precipitated (10mg/ml), centrifuged at 3,000 rpm for 20 minutes and repeated. The plasma was then passed through a Sepharose 4B column and a gelatin-Sepharose column which were linked in series. The plasma was equilibrated in 50mM Tris buffer at pH 7.5, 5mM benzamide and 0.02% sodium azide. This chromatography step, and all subsequent ones, were performed at room temperature. The gelatin-Sepharose column was eluted stepwise with 1M sodium chloride, 0.2M arginine and 1.0M arginine in the Tris buffer. Fibronectin is eluted from the column with the 1M arginine, and this fraction was dialysed at 4°C into 20mM Tris at pH 7.5, 20mM Na₂HPO₄, 5mM benzamide, 0.02% sodium azide.

The fibronectin was then concentrated using DEAE sephacel equilibrated in the dialysis buffer (20mM Tris at pH 7.5, 20mM Na₂HPO₄, 5mM benzamide, 0.02% sodium azide). Bound material was eluted stepwise with 0.1M sodium chloride and 0.5M sodium chloride in the Tris phosphate buffer. The fibronectin eluted in the 0.5M sodium chloride fraction was stored at -80°C.

A1.2.1 Affinity Purification

Rat fibronectin prepared as above was bound to cyanogen bromide activated-Sepharose as described in Affinity Chromatography - Principles and Methods (Pharmacia LKB Biotechnology). The rat fibronectin-Sepharose was equilibrated into phosphate buffered saline which did not contain calcium and

magnesium divalent cations (PBS⁻). Anti-rat fibronectin was loaded onto the column and washed with PBS⁻. Bound material was eluted from the Sepharose with 4M magnesium chloride at pH 6.0 and dialysed against PBS⁻ at 4°C overnight.

A1.3 Preparation of Rat Polyclonal Antibodies to Rat Fibronectin

Antiserum was produced in a female New Zealand White rabbit with immunization by subcutaneous injection at multiple sites on the back using antigen in complete Freund's Adjuvant (Difco, UK). Rat fibronectin was prepared from serum after treatment with barium sulphate by chromatography on Sepharose as described above. Primary immunization was made with 620µg and secondary with 440µg 5 weeks later. Blood was taken from the marginal ear vein, and a pool (16-PI) was made from serum obtained 12, 17 and 21 weeks after secondary immunization.

The anti-rat fibronectin was affinity purified using a Sepharose column as described earlier for the purification of fibronectin.

APPENDIX 2

CELL GROWTH CURVE

CELL GROWTH CURVE

BHK cells were seeded at three different densities:

7×10^5 cells/flask

7×10^4 cells/flask

7×10^3 cells/flask

Small (25cm^2) flasks were used for this experiment and three replicates of each cell dilution were prepared. The cells were grown for eight days with cell counts taken every day, using a Coulter counter. The growth curve is shown in Figure A.4. The doubling time is seen to vary between 6 and 19 hours and depended on the seeding density. The lag phase before cell growth was longest for the more dilute cell seeding densities. The lag phase lasted for 18-22 hours and is the period while the cells are still attaching and spreading on the substrate.

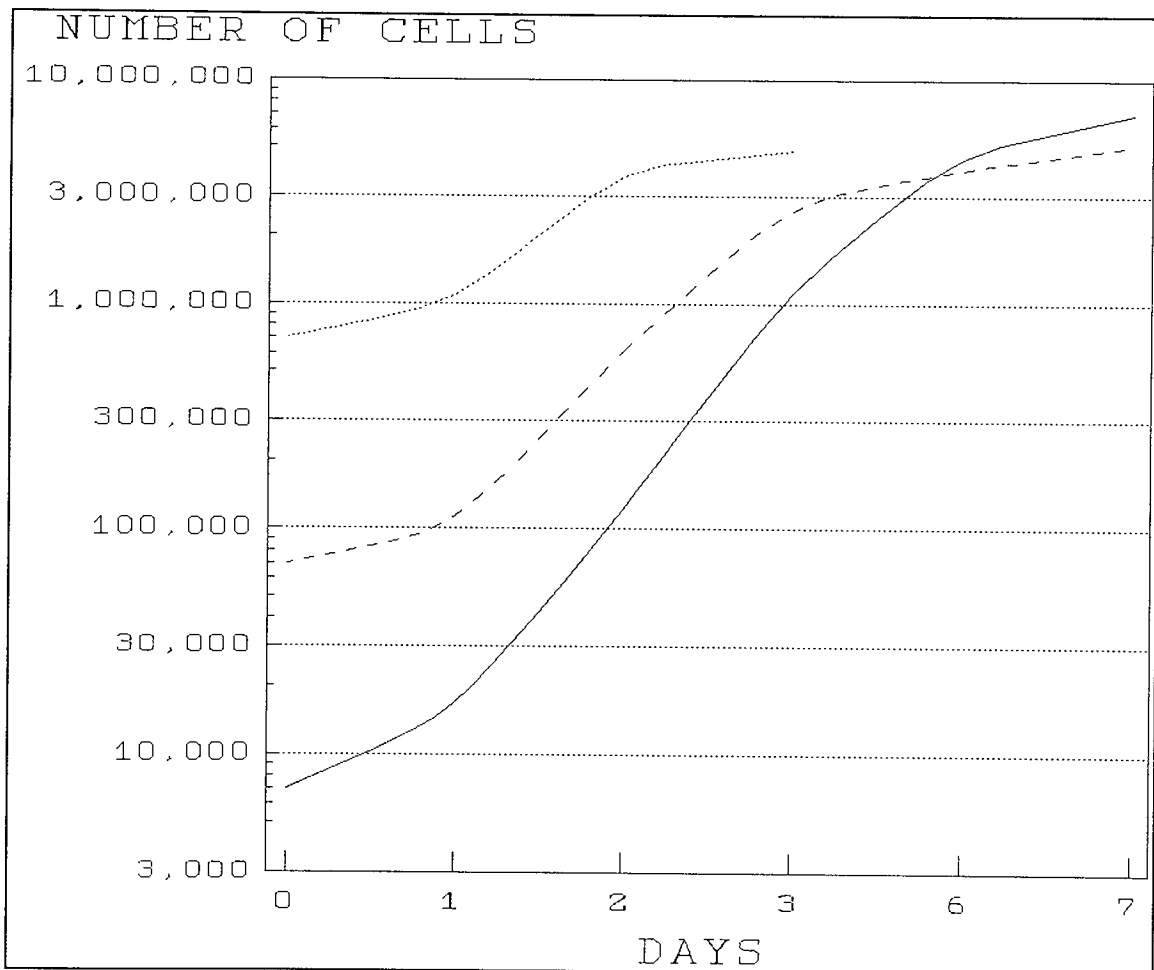


Figure A.4 Growth curve for BHK fibroblasts.

APPENDIX 3: STATISTICAL ANALYSIS OF DATA

Cell Adhesion Experiment Tables: Chapter 4

The figures quoted in these tables are the total number of cells counted over 10 fields. As they are not the mean values of these 10 fields it is not appropriate to quote standard deviations for the data presented in these tables.

Goniophotometer Experimental Data: Chapter 5

In this experiment all data recorded are single values owing to the experimental procedure so it is not possible to treat the data statistically. In this experiment, the non-destructive nature of the goniophotometer technique allows the surface roughness of samples to be monitored before and after treatment. The inability of the technique to pinpoint any differences between samples or treatments mitigated against the need for performing repeat experiments.

Surface Energy Data

In an attempt to minimise measuring errors in the contact angle measurements used to calculate the surface energy values, the left and right contact angles of the liquid drop were paired. Three pairs of contact angles were measured and the means of these were used in the surface energy calculation. The contact angle measurements had a typical standard deviation of 2-3 which would result in the surface energy data quoted having an accuracy of ± 1 . As a consequence of this the surface energy data quoted was rounded to the first decimal place instead of the two decimal places given from the surface energy program.

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