SURFACE FUNCTIONALIZED SUBSTRATES AND THEIR INTERACTIONS WITH BIOMOLECULES AND CELLS

CEN LIAN

NATIONAL UNIVERSITY OF SINGAPORE

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CEN LIAN
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2004
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SUMMARY

Polymeric substrates can readily undergo surface modification via graft polymerization with monomers bearing functional groups for further coupling reactions. The grafted polymeric substrates still retain their intrinsic bulk properties with the improvements in their surface characteristics for specific applications. In this thesis, different approaches of surface grafting were developed depending on the system of interest. Further functionalization of the grafted surfaces was carried out either by biomolecular immobilization or post derivatization. The main focus of this thesis is on the subsequent biological assays of such material-based systems with desired functionalities.

Surface modification techniques were developed for the functionalization of electrically conductive polypyrrole (PPY) film with glucose oxidase (GOD) and an electron mediator: viologen moieties. Acrylic acid (AAc) graft copolymerized PPY film was used to covalently immobilize GOD through the formation of amide linkages. Parallel linkage of viologen moieties on the GOD immobilized PPY film was facilitated by the coupling reaction of 4,4’-bipyridine and α,α’-dichloro-p-xylene with the grafted poly(vinyl benzyl chloride) chains on the PPY film surface. The effect of AAc monomer concentration used for grafting on the amount of GOD immobilized as well as on the corresponding film properties was assessed. The investigation of the enzymatic activities of the immobilized GOD was carried out under different temperatures as well as under an extreme condition of oxygen depletion. The effect of the viologen moieties as an electron transfer mediator in the proximity of the GOD-PPY system was thus addressed.
A method was then developed to immobilize hyaluronic acid (HA) through the molecular recognition of the carboxyl groups of HA by the primary amine end groups of the linker molecules introduced on the PPY film via grafting techniques. Subsequent biological assays of the immobilized polysaccharide were carried out. Different systems were employed, from the assay of the specific binding between a protein and HA to a dynamic cellular response mediated by the interaction between HA and cell-surface receptors. A bacteria-based environment was also applied to assess the antifouling properties of the HA-PPY system. A reduction in *Escherichia coli* (*E. coli*) adhesion was observed. However, this did not eradicate the development of a subsequent biofilm from the initially adhered bacteria. Such an observation became the motivation for the subsequent research into antibacterial surface treatments.

Capitalizing on the advantages of versatility and flexibility offered by surface grafting techniques, a promising method was developed for the functionalization of substrates with bactericidal polycationic groups. This method involves the graft copolymerization of polymeric substrates with 4-vinylpyridine (4VP), followed by quaternization of the grafted pyridine groups into pyridinium groups with hexylbromide. The applicability of this method was substantiated by several substrates: poly(ethylene terephthalate) (PET) film, carbohydrate-based cellulosic materials (filter paper and cotton cloth) and poly(vinylidene fluoride) (PVDF) membrane, which showed promising bactericidal activities. The inhibition of biofilm formation of *E. coli* cells was effectively achieved for all the substrates tested. The studies also addressed the issue of bacterial adhesion and the effectiveness of the polycationic groups against multiple species.
**NOMENCLATURE**

<table>
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<tr>
<td>AAc</td>
<td>Acrylic acid</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ATS</td>
<td>3-Aminopropyl triethoxysilane</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HEA</td>
<td>2-Hydroxyethyl acrylate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid buffer</td>
</tr>
<tr>
<td>GOD</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>PPY</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>$R_a$</td>
<td>Average surface root-mean-square roughness</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TSA</td>
<td>Toluene-4-sulfonic acid</td>
</tr>
<tr>
<td>VBC</td>
<td>4-Vinyl benzyl chloride</td>
</tr>
<tr>
<td>4VP</td>
<td>4-Vinylpyridine</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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WSC Water-soluble-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

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CHAPTER 1

INTRODUCTION
 INTRODUCTION

Surface functionalization by modification of polymeric materials has been known for more than half a century. The requirement for a normal material in any particular application always involves both surface and bulk properties. Thus, the development of an effective surface modification method is a focus of research in material science. For example, in bioengineering two purposes are intended with surface modification: one is to endow the material surface biocompatibility and the other to render it with physiological activity. A promising method for achieving this purpose involves the grafting of a polymer surface via coupling reactions of existing polymer chains or the graft copolymerization of monomers (Ikada, 1994), or a combination of these two methods.

The grafted surface can be designed for further functionalization either through coupling reactions with biomacromolecules or post derivatization of the graft chains with chemical agents. Such a design often involves a multidisciplinary approach requiring the efforts of engineers, chemists, physicists and biologists. Recent interest is increasingly focused on biological systems and the interpretation of the interactions between materials and biomolecules as well as cells. It is the emphasis of the present study to achieve the ability to control and monitor biological responses through surface functionalization via coupling with biomolecules and post derivatization on a material based platform.

The choice of substrate is closely related to the desired performance as will be illustrated in the following chapters. For example, a conducting polymer: polypyrrole (PPY) has been used as the substrate for biomolecules immobilization. The study on
electrically conducting polymers has long been established since late 1970s (Shirakawa et al., 1977) with the most thorough investigations carried out on PPY. Both chemical and electrochemical methods can be applied to synthesize PPY with the main interests being on electropolymerization. The properties of the PPY film can be improved by the proper choice of electrolyte, solvent, pH temperature and electrode, and the mechanisms of electropolymerization have also been proposed by many groups. A more detailed description can be then found in Chapter 2, which gives a survey of literature works related to the present work.

Versatile applications of PPY have been developed due to its attractive intrinsic properties, especially its biocompatibility. The ease with which its surface can be further functionalized provides various possibilities for biomolecules immobilization. Factors which influence the performance of such biomolecule-immobilized PPY systems are mainly related to the method employed. In Chapter 3, a surface modification technique was developed for the functionalization of PPY film with glucose oxidase (GOD) and viologen moieties. The PPY film was first graft copolymerized with acrylic acid (AAc) and GOD was then covalently immobilized through the amide linkage formation between the amino groups of the GOD and the carboxyl groups of the grafted AAc polymer chains in the presence of a water-soluble carbodiimide. Viologen moieties could also be attached to the PPY film via graft-copolymerization of vinyl benzyl chloride with the PPY film surface followed by reaction with 4,4’-bipyridine and α,α’-dichloro-p-xylene. X-ray photoelectron spectroscopy (XPS) was used to characterize the PPY films after each surface modification step. Increasing the AAc graft concentration would allow a greater amount of GOD to be immobilized but this would decrease the electrical conductivity of the PPY film. The activity of the immobilized GOD was compared with that of free
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GOD and the kinetic effects were also studied. The immobilized GOD was found to be less sensitive to temperature deactivation as compared to the free GOD. The results showed that the covalent immobilization technique offers advantages over the technique involving the entrapment of GOD in PPY films during electropolymerization. The presence of viologen in the vicinity of the immobilized GOD also enabled the GOD-catalyzed oxidation of glucose to proceed under UV irradiation in the absence of O₂.

The surface modification technique was further extended to the immobilization of a polysaccharide on the PPY substrate. This development which involves hyaluronic acid (HA), a ubiquitous constituent of the extracellular matrix, is described in Chapter 4. HA is of interest since it has potential applications in nerve regeneration and antibacterial adhesion. In this case, the PPY film was first graft copolymerized with 2-hydroxyethyl acrylate (HEA) and subsequently silanized with 3-aminopropyl triethoxysilane (ATS) via Si-O bonding with the hydroxyl groups of the grafted HEA polymer. Water-soluble carbodiimide activated hyaluronic acid was then covalently immobilized through amide linkage formation with the primary amine groups introduced on the PPY film surface via the ATS silanization process. The amount of HA immobilized could be varied by changing the concentration of the primary amine groups on the PPY film surface. The immobilized HA was biologically active as evaluated using protein binding assay and it retained a significant degree of its activity even after 4-day storage in air.

Chapter 5 is devoted to the assessment of the biological activity of the HA functionalized PPY film by means of either in vitro PC12 cell culture or Escherichia coli (E. coli) adhesion test. In PC12 cell culture, the cell attachment was determined by
bicinchoninic acid (BCA) analysis, and the effect of nerve growth factor (NGF) on cell attachment on different substrates was also studied. The cell adhesion kinetics on pristine PPY as well as the PPY after different stages of surface functionalization were assessed. The morphology of the PC12 cells in the presence and absence of NGF was compared using scanning electron microscopy. The investigations on the bacteria adhesion were carried out by immersing the film in the bacteria suspension over a predetermined time followed by scanning electron microscopy (SEM) characterization.

Though HA immobilization on the PPY film surface was shown to reduce bacteria adhesion, it did not prevent the development of subsequent biofilms from the initially adhered bacteria. Biofilm formation is a problem confronted in many biomedical devices and biomaterials, as well as the materials involved in daily life. Bacteria growing within biofilms exhibit significantly different properties from the planktonic ones, especially its high resistance to antibiotics, thus increasing the difficulty in eradicating the biofilms. Hence, surface design in rendering materials with antibacterial characteristics has attracted much attention. Chapter 6 provides a detailed description of a method developed for imparting surfaces with antibacterial properties. The first part this chapter deals with poly(ethylene terephthalate) (PET) films graft copolymerized with 4-vinylpyridine (4VP) and subsequently derivatized with hexylbromide via the quaternization of the grafted pyridine groups into pyridinium groups. The amount of pyridinium groups on the film surface could be controlled by varying the 4VP monomer concentrations used for grafting. The pyridinium groups introduced on the surface of the substrate possess antibacterial properties, as shown by their effect on *E. coli*. The bacteria killing efficiency is very high when the concentration of pyridinium groups on surfaces is 15 nmol/cm\(^2\) or higher. *E. coli*
adhered on the functionalized surfaces are no longer viable when released into an aqueous culture medium.

The second part of Chapter 6 verifies the applicability of this technique to porous materials such as PVDF membranes and carbohydrate-based cellulosic material (filter paper). Since environmentally occurring biofilms often involve a multi-species coexistence system, another cellulosic material (cotton cloth) was also similarly functionalized for tests against biofilm formation of wild type bacteria.

Chapter 7 gives the overall conclusion of the present work and Chapter 8 gives recommendations for further work. Though significant achievements on the surface functionalization methods have been made in this work, there are still many possibilities for enhancing the performance of the functionalized surfaces. For example, the use of conducting polymers in conjunction with its electrical property can enhance the desired functional properties, as in the case of neurite growth. Moreover, studies on the interactions between material surfaces and biological systems also require more specific analytical techniques and pertinent characterization methods.
CHAPTER 2

LITERATURE SURVEY
2.1 Surface Graft Copolymerization

The marked advances in material sciences can be attributed to the development of surface modification techniques or the regulation of interactions of material surfaces with other substances or biological systems (biomolecules, cells etc.). This is also the issue of prime importance in various fields of industrial applications of materials, especially those involving polymeric materials whose performances rely largely upon the properties of the boundaries residing between the bulk polymer and the outer environment. A variety of surface modification techniques have been developed during the past century and Table 2.1 lists some general methods for surface modification (Ikada, 1994).

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<td>Ion implantation</td>
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<tr>
<td>Graft polymerization</td>
</tr>
</tbody>
</table>

Among the above physical, chemical or combination of physical and chemical processes, graft polymerization is the most desirable method as it offers the following advantages over others - easy introduction of graft chains with a controllable density, exact localization of graft chains to the surface with the preservation of the material.
bulk properties. Moreover, as graft polymerization involves the covalent attachment of
graft chains onto a polymer surface, delamination can thus be avoided while the long-
term chemical stability of the introduced chains can be assured (Kato et al., 2003).
There are in principle three methods employed for carrying out grafting on surfaces:
plasma discharge, UV irradiation and ozone methods as well as combinations of two or
more methods.

2.1.1 Plasma discharge method

Plasma is generally composed of highly excited atomic, molecular, ionic and radical
species and typically obtained when gases are excited into energetic states by radio
frequency, microwave, or electrons from a hot filament discharge. In plasma grafting
copolymerization, the substrates are first exposed to the plasma to generate radicals on
the surface. These radicals are formed by inelastic collisions between electrons in the
plasma and the substrate surfaces and can initiate polymerization reactions when the
polymeric substrates are in contact with an aqueous or organic solution, or a vapor of
the monomer (Chu et al., 2002). The close relations between surface modifications of
polymeric materials with plasma treatment are fully demonstrated in a journal Plasmas
and Polymers, first issued in March, 1996.

The plasma technique has been applied to surface modification involving the grafting
of functional groups (Gupta et al. 2001), metallization (Wang et al. 2002),
immobilization of proteins and biological molecules (Hayat et al. 1992), nonfouling
coatings (Pan et al. 2002), antibacterial coatings (Gray et al. 2003), transparent barrier
films (Sobrinho et al. 1999), adhesion promotion (Kuhn et al. 2001),
adsorption/retention of proteins (Janocha et al. 2001), curing (Hofmann et al. 2003)
and so on.
Song et al. (2000) investigated the grafting of poly(ethylene glycol) onto the hydrophobic polysulfone membrane surface by low-temperature plasma techniques to have improved surface hydrophilicity and hemocompatibility while Gupta et al. (2001) carried out the modification of poly(ethylene terephthalate) (PET). The PET films were first argon plasma pretreated, and subsequently exposed to oxygen to create peroxides, followed by graft polymerization with acrylic acid (AAc) monomer. Detailed investigations were performed on the influence of plasma treatment time, plasma power, monomer concentration, temperature, and the presence of Mohr's salt, on the grafting degree. The grafted PET surface was then used to immobilize collagen for biomedical applications, which will be described in detail in the following sections.

The work of Oehr et al. (1999) involves the investigation of the effect of gases used to activate the polymer surface on the degree of grafting of AAc and glycidyl methacrylate monomer. Their work further developed the grafted surfaces for biomedical applications (Oehr et al. 2003). However, in many cases, the plasma treatment was often combined with UV irradiation to facilitate the surface modification process (Ji et al. 2002). Two stages are thus involved in this process with the activation of the substrate via exposure to argon plasma treatment as the first step and followed by the UV-induced polymerization via the contact of the plasma pretreated substrate with functional monomers under UV irradiation. The detailed application of UV irradiation in the field of polymer modifications is described in the following section.

2.1.2 UV irradiation method

Application of UV energy for surface graft polymerization with the aid of a photoinitiator or photosensitizer has been extensively developed (Yang et al., 1996).
Earlier works on surface graft polymerization promoted by UV irradiation can be dated back to those of Wright et al. (1967), Tazuke et al. (1978) and Ogiwara et al. (1981). Their works were performed with UV irradiation under a reduced pressure or in the presence of inert gas. The solvent/carrier used and the type of polymer to be grafted both affect the grafting process. Zhang et al. (1990) further developed a novel process of continuous photoinitiated graft polymerization of acrylamide and AAc onto the surface of a high-density polyethylene tape film which was presoaked in a solution containing monomer and initiator under nitrogen atmosphere. This method has the advantages of easy and continuous operation, short irradiation times, low cost, and no requirements of severe vacuum conditions, and was thus applied to various substrates, such as polypropylene film (Zhang et al. 1991), PET fiber surface (Zhang et al. 1990).

A more recent work concerned with the lamination of polymeric films or sheets by photografting, was carried out by Yang et al. (1997). In their work, hyperbranched macromolecules of large size (10-20 µm) were obtained after initiation with aromatic ketones, and a range of different organic polymeric films were successfully laminated. However, the requirement of the addition of a photosensitizer in the above methods often promotes light-induced degradation or leaching to the surrounding environment if it remains on the sample after treatment. Moreover, an additional difficulty arises from homopolymerization initiated by free radicals formed during irradiation of the monomer. These shortcomings were overcome by Ma et al. (2000) with a two-step method of photo-induced living graft polymerization. In this method, benzophenone abstracts hydrogen from the substrate to generate surface radicals and semipinacol radicals, which combine to form surface photoinitiators in the absence of monomer solutions in the first step. The monomer solutions are then added onto the active substrate, and graft polymerization is thus initiated by the surface initiators under UV
irradiation. As the formation of initiators and graft copolymerization occur independently in a successive manner, the control over the graft density and graft polymer chain length can be achieved successfully.

A parallel development related to the UV irradiation method is the enhancement of the effect of UV irradiation on grafting with the aid of ozone treatment, as shown in the work of Walzak et al. (1995), and Mathieson and Bradley (1996), and so on. Therefore, it is necessary to make a brief introduction of ozone treatment before the efficient usage of this combination is described.

2.1.3 Ozone method

Gatenholm et al. (1997) have exposed isotactic polypropylene to ozone to oxidize polypropylene surface and form peroxides and hydroperoxides. The subsequent graft copolymerization of 2-hydroxyethyl methacrylate was then carried out on the ozonated samples. Ozone-induced graft polymerization of AAc with cotton linters and wood pulp fiber as substrates was carried out to give pH sensitive cellulose fiber-supported hydrogels (Karlsson et al. 1999). As the combination of ozone with other methods often promotes significant improvements in grafting efficiency and the properties of the grafted surfaces compared with the usage of a single method, the subsequent development gradually shifted to the combination of two or more methods. For example, it has been shown that an ultraviolet-ozone oxidation process is more effective in improving the surface wettability of polyethylene and polyetherketone, compared with the usage of a single method (Mathieson et al., 1995, Mathieson et al., 1996). Foerch et al. (1990) also developed a novel two-step process for surface modification of polyethylene and polystyrene involving the
exposure of the substrate to nitrogen plasma treatment as the first stage and ozone discharge as the second stage.

### 2.2 Further Functionalization of Grafted Surface

The development of various techniques used for surface graft copolymerization as described in the previous section brings a quantum leap in the applications of the normal polymeric substrates. The convenience of introducing functional groups with versatile choices via surface grafting overcomes the shortage confronted by many of the conventional polymeric substrates which possess a non-polar, less reactive surface. A broad range of functional groups such as amine (Jimbo, et al. 1998), hydroxyl (Guan, et al. 2000), carboxylic acid (Lee et al. 1996), sulfonate (Inagaki, et al. 1997), pyridine (Yang, et al. 2001), epoxide (Yu et al. 1999), etc. thus provides possibilities for further functionalization of the grafted surface either through coupling reactions with biomacromolecules (proteins, polysaccharides, and nucleic acids) or post derivatization of the graft chains with certain molecules to endow normal polymeric substrates with desired properties such as antibacterial activities. The biomacromolecules immobilization and antibacterial derivatization of the grafted surface are addressed in detail as follows.

#### 2.2.1 Immobilization of biomolecules

The immobilization of physiologically active biomolecules on conventional synthetic polymers could render surfaces with an ability to interact specifically with biological systems. Macromolecules which have been immobilized include proteins, nucleic acids, and polysaccharides. Among those macromolecules, proteins with specific functionalities or enzymes are the major class which have been immobilized, studied
and employed. Thus, the following discussion on various immobilization methods would focus on enzymes although the same mechanisms can be applied to the immobilization of other macromolecules.

2.2.1.1 Enzyme immobilization

The term “immobilization” connotes confinement to a defined spatial region, which means a protein, polysaccharide or other macromolecule is associated with a support material, either in soluble or insoluble form with restricted mobility. The reasons for the preparation and use of immobilized enzymes lie in the fact that it offers a more convenient handling of enzyme preparations, ease of separation of the enzyme from the product and reusability of the enzyme (Tischer and Wedekind, 1999). The above benefits thus not only simplify enzyme application, support a reliable and efficient reaction technology, but also provide cost advantages which are often the essential target for establishing an enzyme-related process. Due to these expected advantages, the number of applications is increasing substantially, with the earliest man-made usage of binding enzymes onto solid materials tracing back to the 1950s (Hartmeier, 1988). During the past decades, numerous methods of immobilization on a range of various materials have been developed and can be grouped as adsorption, covalent binding or entrapment.

(a) Immobilization methods:

**Adsorption:** this is the simplest method involving a mild procedure of mixing an enzyme with a support under appropriate conditions. Such an immobilization is achieved through weak van der Waals interaction and hydrogen bonding or stronger hydrophobic and ionic links between the enzyme and the substrate. Enzymes
immobilized via this method often retain high activities as little conformation changes occur in the enzyme (Gemeiner, 1992). However, the hydrophilicity of proteins plays an important role in adsorption (Brash et al., 2004). Furthermore, the support is regenerable as the binding is reversible and regeneration can be easily achieved by removal of the deactivated enzyme, followed by reloading with fresh, active catalyst (Piacquadio et al., 1997). However, this is also a drawback since the detachment of the macromolecules may occur, especially when the optimal conditions during operation are significantly different from those used during adsorption. Therefore, covalent coupling may be a better alternative when adsorption cannot satisfy the desired purpose.

**Covalent binding:** this method is based on the covalent attachment of protein or other macromolecules to water-insoluble matrices. This method has been the most thoroughly investigated as well as the most widely used since the strong binding leads to excellent stability of the immobilized enzymes without release into the solution, even in the presence of high ionic strength solutions. However, the selection of conditions for immobilization via this method is more stringent than for other methods and it also involves relatively complicated reaction conditions. Therefore, it is necessary to take some factors into consideration before the specific work is carried out:

1) It is essential that the coupling reaction should only involve those functional groups of the enzyme that are not important for catalysis in order to ensure that the active site of the enzyme is unaffected.

2) The coupling reactions between the proteins and the supports need to be clarified.
3) The suitability of the supports to be functionalized has to be verified.

The versatility in the choices of binding reactions and matrices with functional groups capable of covalent coupling or activation make this method an attractive and applicable one for immobilization. Examples of recent work are those of Kulik et al. (1993), Yasui et al. (1997), Li et al. (1998), Jolivalt et al. (2000), Chen et al. (2000). Efforts have also been made on the improvement in the performance of the immobilized enzyme by introducing a spacer between the substrate surface and the enzyme molecules (Wang and Hsiue, 1993; Itoyama et al., 1994).

**Entrapment**: the entrapment of enzymes within a polymer network is a physical process accomplished by mixing the species to be entrapped with an aqueous solution of the monomer or polymer with subsequent polymerization or gelation. This method can be applied to any kind of enzyme, biocatalyst, even whole organelles and cells of different sizes and properties. It is superior to chemical binding in the preservation of the biological activity of the immobilized species. However, it suffers from serious diffusion limitations (Gemeiner, 1992). An example of a widely applied system for enzyme entrapment is the polymer lattice of a polyacrylamide gel, obtained via either polymerization or cross-linking of acrylamide in the presence of the particular enzyme (Wang et al., 1996; Bu et al., 1998).

**(b) Performance of the immobilized enzymes**

There are three factors which affect the performance of the immobilized enzymes: (i) distribution of substrate, products or hydrogen ions between the enzymes and the macroenvironment and the effect of electrostatic or hydrophobic interactions between the matrix and components of the environment on the distribution; (ii) diffusion
restrictions and mass transfer limitations, e.g. resistance to the transportation of substrates and products between active sites of the immobilized enzyme and the macroenvironment; (iii) conformational changes of the immobilized enzyme and steric hindrance exerted by both the mode of binding and the specific carrier. The performance of the immobilized enzyme is expressed mainly in its functional activity, kinetics and stability (Bailey and Ollis, 1986). Generally, enzymes consist principally of a protein chain folded upon itself to form a compact macromolecular assemblage with a well defined structure. The delicate molecular structure offers two functions: to adsorb the particular substrate molecule onto the complementary surface of the enzyme and to catalyze the conversion of this substrate to a product which is released back to the surrounding solution leaving the enzyme catalyst unaltered (Rosevear et al., 1987). Therefore, parameters which affect significantly the specific structures of the enzyme are described below in detail and they must be carefully monitored during handling and usage in order to preserve enzymatic activities and stabilities.

**pH and buffer:** The electrostatic effect of pH on the activity of the free and immobilized enzymes can be attributed to the fact that enzymes are polyionic molecules with a three-dimensional structure and active site-substrate interaction which involves charged residuals. Normally, the apparent activity of all enzymes shows a classic bell-shaped curve relationship with the pH of the solution in which they are dissolved. Therefore, the pH at which enzymes are immobilized to a support is a critical parameter to be determined for any method used. Moreover, the possibility of the non-uniform distribution of hydrogen ion between the microenvironment of the enzyme and the bulk solution should also be taken into consideration (Trevan, 1980; Gemeiner, 1992). A buffer solution which is capable of stabilizing the pH at a chosen
value even when small amounts of acid or alkali enter the solution can thus help to achieve the good control over the pH of both reaction medium and substrate solution. For a buffer solution to be effective, its components must be at or near the pH where acidic and basic forms of the solute are present in equal amounts (usually expressed as pI or pK), whereas the ability to accommodate changes in hydrogen or hydroxyl ions is largely determined by the concentration of a buffer. The concentration of the buffer is best limited to a level just sufficient to fulfill its task in order to minimize the disruptive effect resulting from the osmotic contribution made by the high molarities of the buffer salts. Furthermore, no chemical interference between the buffer and the enzyme immobilized substrate is acceptable during the operation.

**Temperature:** There are two aspects when considering the effect of temperature on enzymes: the enzyme-catalyzed reactions are subjected to the general laws of thermodynamics in that the rate of reaction increases with temperature as described by the Arrhenius equation. However, the catalytic function falls with temperature. Hence, the temperature at which to operate the enzyme-immobilization and the one at which the catalysis proceeds have to be carefully controlled. Normally, the temperature is kept low during immobilization (often a cold room at 4°C or an ice bath at 0°C) in order to minimize protein denaturation and the operation of the immobilized enzyme system is often kept at a little above the temperature of minimum growth tolerance of the organism from which it was derived (Rosevear et al., 1987).

**Kinetics:** The kinetics of a simple enzyme reaction can be described by the Michaelis-Menton equation ($v = v_{max} \frac{S}{(K_m + S)}$). The detailed description as well as the determination of the specific kinetic constants of a particular enzyme can be found at
Section 3.3. Immobilization of an enzyme, however, alters the apparent speed with which substrate is converted to product when compared with the equivalent free enzyme, due to the change from homogeneous to heterogeneous catalysis as a result of immobilization. The change can be attributed to the diffusion of solutes to the active sites within the matrix, the interactions of the enzyme with the matrix, and partitioning of components between the stationary and mobile phases. The direct effect observed from attachment of an enzyme to a matrix is the change in the conformational flexibility of the protein, whereas the transportation of substrates and products between the mobile and stationary phases is a major determinant of the kinetics in an immobilized biocatalyst matrix, which is also the cause for the increasing activity of immobilized enzymes with decreasing size of the substrate molecule (Gemeiner, 1992).

**Enzyme stability:** In most cases, enzyme durability and stability against denaturation due to temperature, reagents, or other unfavorable conditions increase after being immobilized on the carrier matrix, especially in the case when the binding of the enzyme leads to stabilization against conformational changes in the region of the active sites of the enzyme (Palmer, 1995). However, the stability of the enzyme during the immobilization needs to be distinguished from the stability of the enzyme once immobilized either during storage or operational conditions since the former is the main target to be achieved and the latter is the major advantage from immobilization.

**2.2.1.2 Polysaccharide immobilization**

Polysaccharides are another group which so far has been investigated thoroughly either as carriers (such as cellulose, starch, dextrans, agarose, and the cross-linked derivatives of agarose) for a variety of natural substances or as biologically active species
immobilized on material surfaces to elicit desired receptor-specific responses from particular biological systems. The latter involves mainly the family of glycosaminoglycans, i.e. hyaluronic acid, heparin, chondroitin sulfate and keratin sulfate.

**Hyaluronic acid** (also called hyaluronate, hyaluronan, HA): is a ubiquitous constituent of the extracellular matrix (Kuettner et al., 1986), and the only nonsulfated glycosaminoglycan. With its regular repeating sequenced structure, it has become a well-studied member in the glycosaminoglycan family. Karl Meyer first discovered HA when he worked in the eye clinic of Columbia University (1934) and he also later revealed the chemical structure of the polysaccharide (1958). The high molecular mass, polyanionic character and a slight stiffness in its unbranched polysaccharide chain render HA with unique rheological properties (Balazs et al., 1991). Besides the structural capacity of providing hydration in the extracellular matrix, HA is also involved in a number of complex cell signaling events including migration, attachment, and neuronal sprouting (Laurent and Fraser, 1992). It was also shown that HA responds to the cell signaling events via cell-surface receptors such as RHAMM for HA-mediated motility (Nagy et al., 1995) and CD-44 (Lesley et al., 1993). An important role played by HA is its hydrodynamic properties of the extracellular environment, particularly in embryonic tissues (Toole, 1991). Furthermore, HA also provides structural support for other matrix components as it is essential for the formation of protein aggregates in brain and cartilage and it serves as a lubricant and confers the resilience property on joints as an important constituent of joint fluid (Arslan et al., 1994).
Applications of HA:

(a) Matrix engineering: One application of HA is related to matrix engineering for rheologically augmenting the synovial fluid, or as a vitreous substitute in the eye (Balazs et al., 1991). Its use was further extended to viscosurgery where HA is used as a surgical tool or implant. The more recent work, however, dealt with the application of HA as a surface coating to either improve the blood compatibility of materials, or as biomaterials which physically support tissue growth and stimulate specific cell functions. Albersdörfer and Sackmann (1999) have studied the swelling behavior and viscoelasticity of ultrathin grafted hyaluronic acid films which were covalently coupled to glass substrates. Abdelghani-Jacquin et al. (2001) reported on the biofunctionalization of magnetic beads with gold films which were subsequent coupled with HA, and which can be used for measurement of local viscoelastic properties of cell membranes or cytoplasms through microrheometry. Another work on the synthesis of a composite of the HA and the electrically conducting polymer polypyrrole (PPY) aims to combine inherent biological properties which can specifically trigger desired cellular responses (e.g., angiogenesis) with electrical properties which have been shown to improve the regeneration of several tissues including bone and nerve (Collier, et al., 2000). This composite was obtained by electropolymerization of PPY films on the electrode surface in an aqueous solution containing both pyrrole monomer and HA molecules. The evaluation of the cellular and tissue responses indicated that this kind of biomaterial possessed noncytotoxicity and angiogenic properties, thus making it an attractive candidate for tissue engineering. However, low loading and entrapment of biomolecules without any surface exposure are problems to be overcome. Moreover, the PPY films synthesized by this way have poor mechanical and electrical properties which are desired characteristics for a substrate. Therefore,
efforts in tissue engineering continue to focus on methods for immobilizing polysaccharides which maintains both the biological activities of the polysaccharide and the inherent properties of the substrates.

*(b) HA receptor:* Another application is mediated by the interactions with hyaluronate-binding macromolecules (Arslan et al., 1994). Various HA binding proteins have been identified, though it was not discovered until 1972 that HA specifically interacts with other macromolecules (Hardingham and Muir, 1972). Thorough studies on HA binding proteins were carried out by Hascall and Heinegård (1974) who showed that there is a specific and firm binding between hyaluronan, the N-terminal globular part of the proteoglycan and a link protein. Moreover, the specific interactions of HA with certain cell types can also be attributed to the presence of HA receptors on these cell surfaces. CD44 and RHAMM (receptor for HA mediating motility) are two HA receptors identified by Underhill and Tolle (1979) and Turley et al. (1982), respectively. It is expected that HA-anchored surfaces can enhance the specific interactions with cells that express CD44 or RHAMM in order to manipulate their growth, differentiation or the functionalities of the dynamic cellular systems. Seckel et al. (1995) have applied HA in the nerve regeneration, and a good conduction velocity, high axon counts, and a trend towards quick myelination were observed.

*(c) Anti-fouling:* The interesting possibility of surfaces anchored with the highly hydrophilic polysaccharide for resisting bacteria adhesion is another subject of importance, since anti-fouling resistance is being investigated in several different fields of biomaterial science. As shown by Morra and Cassineli (1999), polystyrene Petri dishes, coated with HA and alginic acid (another natural polysaccharide that exists in many species of seaweed with its main role to regulate the water content) can
greatly reduce bacterial cells (*S. epidermidis* and *E. coli*) adhesion *in vitro* and in several *in vivo* applications. Bacterial infections are a significant problem associated with implant devices which is difficult to eradicate without surgical revision or implant removal. Hence, materials that are less adherent to bacteria would be preferred to minimize infection which is initiated by the bacteria adhesion.

However, the reduction or inhibition in bacterial adhesion cannot solve the problem of bacterial induced infections due to the difficulty in achievement of complete inhibition and the fact that some loosely attached bacteria on material surfaces will have the ability to colonize on the surface and proliferate. Based on such considerations, a material with excellent antibacterial or antimicrobial surface properties would find applications not only in biomaterial fields but also in daily life either from a health-related or environmental point of view.

### 2.2.2 Biofilm inhibition

Bacterial biofilms and its significance is a large field covering different aspects of nature and human life, such as marine science, soil and plant ecology, food industry, and most importantly, the biomedical field as the pathogenesis of infection is initiated from the adhesion of bacteria to human tissue surfaces and implanted biomaterial surfaces (Costerton et al., 1995; Kumar and Anand, 1998).

#### 2.2.2.1 Bacteria adhesion and biofilm formation

Microorganisms have a strong tendency to become associated with surfaces (Costerton et al., 1999), and their preference is to grow on available surfaces rather than in the surrounding aqueous phase like tissue cells growing in *in vitro* culture. Events occurring during the process of stable adhesion of a bacterium involve the transport of
bacteria to the vicinity of the substrate (tissue cells or material surfaces), attachment to
the substrate, and molecular interactions to resist detachment in the presence of any
dislodging force. A two-phase process is often used to describe the adhesion of
bacteria to solid surfaces: an initial instantaneous and reversible physical phase (Phase
1) and a time-dependent and irreversible molecular and cellular phase (Phase 2)
(Marshall, 1985, An et al, 2000). Phase 1 is also known as physicochemical
interactions which has been predicted or interpreted by a common approach applying
colloid concepts (Busscher et al., 1992; Schamhart et al., 1994). The forces governing
the rate of deposition of a charged colloidal particle on a surface involve long-range
van der Waals forces and electrical double layer forces. van der Waals forces are due
to the dipole interactions between molecules in the colloidal particle and molecules on
the substrate surface, and are generally attractive. On the other hand, electrical double
layer forces result from the overlap of counterion clouds near charged surfaces and the
change in free energy as the surfaces are moved closer or farther apart, and are
generally an attractive force for like-charged surfaces and a repulsive force for
oppositely charged surfaces. However, due to the heterogeneous macromolecular
structure of the cell surface, such a theoretical approach for making quantitative
predictions of bacterial attachment is seriously limited. More detailed and realistic
theories that account for the macromolecular structure of the cell surface and the
discrete binding interactions or steric repulsion of the macromolecules in the interface
are still pending. In Phase 2, molecular and cellular interactions between bacterial
surface structures and substratum surfaces become predominant. A firmer adhesion of
bacteria to a surface by the bridging effect of bacterial surface polymeric structures
(capsules, fimbriae, pili, or slime) occurred in this phase.
The complicated process of bacterial adhesion is affected by many factors, including bacterial characteristics, the chemical and physical nature of the target material surface, and compositions of the bacterial suspension medium. It is necessary to acquire a good understanding of the unique behavior of the bacteria species involved, the surface characteristics of the materials, and the relevant environment in order to control the process (Merritt and An, 2000).

The initial attachment of the microorganisms to a substratum surface is often followed by a multi-step process which leads to the formation of a complex, adhering microbial community termed as ‘biofilm’. A biofilm is generally defined as a complex heterogeneous consortium of microorganisms associated with surfaces and interfaces in an exopolysaccharide matrix of microbial and host origin with a spatially organized three-dimensional structure. It has been shown to play an important role both in causing disease and for maintaining health (Bos et al., 1999; Costerton et al., 1995). The ubiquitous distribution of biofilms is illustrated in Figure 2.1 (Jass et al, 2003).

**Figure 2.1** Schematic representation of the diverse environments that man is directly associated with that harbour biofilms.
Regardless of the wide diversity of biofilm structures and architectures, biofilm is composed of a substratum to which the bacteria attach; a conditioning film; the biofilm matrix; and the liquid or gas phase. The biofilm matrix is an important part of the biofilm, containing the microbial cells (2-5% of the total matrix), exopolysaccharide (up to 2%) and water (95-99%). Traces of DNA, RNA, proteins and enzymes reaching levels of approximately 2% in total can often be found in the matrix (Costerton et al., 1995; Sutherland, 2001).

Bacteria growing within biofilms present different properties that significantly distinguish them from the corresponding planktonic ones as shown in Table 2.2 (Jass et al., 2003), namely protection from the extreme conditions in the surrounding environment; differences in phenotypic expression and growth characteristics; cooperation between organisms either in mono or in mixed populations in utilizing nutrients; intercellular communication (Jass et al., 2003).

The formation of biofilms involves events of a continual dynamic sequence, which has generally been divided into four developmental stages: bacterial growth as planktonic cells is the first stage, followed by the transportation to a surface or interface. In the second stage, bacteria interact with a conditioned surface and a monolayer thus forms. The bacteria-surface interaction in this stage occurs in a reversible manner which has been documented by O’Toole and Kolter (1998) with the aid of time-lapse phase-contrast microscopy of *P. aeruginosa* biofilms. The third stage of biofilm formation is the irreversible attachment of bacteria and formation of microcolonies through specific (adhesions) and non-specific interactions (hydrogen bonds, van der Waals forces and hydrophobic interactions) with the surface (Characklis, 1990). The fourth stage involves the dispersal and detachment of bacteria from the mature biofilm. It is the
Chapter 2

characteristics of a mature biofilm that the bacteria eventually return to the planktonic phase (Jass et al, 2003).

Table 2.2 General features and advantages of microbial growth as a biofilm

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
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<tbody>
<tr>
<td>Protection</td>
<td>◦ From host defences and predators</td>
</tr>
<tr>
<td></td>
<td>◦ From antimicrobial agents: slow growth rate; poor penetration; altered phenotype</td>
</tr>
<tr>
<td></td>
<td>◦ From desiccation</td>
</tr>
<tr>
<td></td>
<td>◦ From fluid hydrodynamic and mechanical forces</td>
</tr>
<tr>
<td>Nutrient acquisition</td>
<td>◦ Elevated concentrations of nutrients: surface phenomenon and nutrient trapping</td>
</tr>
<tr>
<td></td>
<td>◦ Microbial and environmental heterogeneity for metabolic cooperation</td>
</tr>
<tr>
<td></td>
<td>◦ Spatial heterogeneity to optimize transport of by-products and increase nutrient influx</td>
</tr>
<tr>
<td>New traits</td>
<td>◦ Phenotypic plasticity-novel gene expression and bacterial phenotype</td>
</tr>
<tr>
<td></td>
<td>◦ Plasmid or genetic transfer between organisms</td>
</tr>
<tr>
<td></td>
<td>◦ Mutation due to selection</td>
</tr>
<tr>
<td>Intercellular</td>
<td>◦ Quorum sensing/density-dependent communication</td>
</tr>
<tr>
<td>communication</td>
<td>◦ Interspecies communication</td>
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</table>

In addition to the intrinsic characteristics of bacteria and material surface, many environmental factors including nutrient sources and local conditions (pH, osmolarity, temperature, oxygen, surface properties and hydrodynamic conditions) can exert great effects on the biofilm formation. These factors can influence which species will be
able to colonize to form biofilms and the maximum biofilm thickness and density (van Loosdrecht et al., 1995; Stoodley, et al., 1999).

2.2.2.2 Strategies for biofilm eradication

In retrospect, the knowledge of microbial biofilms has increased dramatically during the last few years with the notable step being the recognition of ‘biofilm formation’ as a phenomenon distributed widely in both natural and man-made environments, in both the medical and non-medical fields. Such a realization eventually leads to the development of strategies to overcome the problems encountered in biofilm control in vitro. A number of ways including those still under investigation are described below (Jass et al, 2003):

a) Synergism between chemical and physical techniques in concert to control biofilms. Chemical treatments include the application of biocides, surfactants, and agents which are associated with cell membranes (chelating agents and enzymes), while physical treatments (such as ultrasound, electrical current) aim to enhance the effects of chemical agents. Examples can be found in the works of Jass and Lappin-Scott (1996) and Watanakaroon and Stewart (2000) involving the application of electrical currents to strengthen the efficacy of antibiotics, while Rediske et al. (2000) applied ultrasound to enhance the killing ability of aminoglycoside antibiotics against E. coli. The environmental risks posed by the application of biocides and the development of resistance of bacteria need to be taken into consideration.

b) Development of novel antibiotic derivatives with increased antibacterial activities. New antibiotics can be obtained by incorporation of some functional groups into the existing antibiotic to enhance its potency, as works performed by Cho et al.
Novel antiadhesive compounds were also developed to inhibit bacterial binding to surfaces or prevent the expression of bacterial adhesions (Svensson et al., 2001; Ohlsson et al., 2002).

c) Application of probiotic bacteria and fungi to prevent or remove microbial contamination. This approach is described in the publications of Busscher et al. (1997) and Van der Mei et al. (2000).

d) Design of surfaces with potential antibacterial characteristics to reduce and prevent biofilm growth and contamination. This approach is different from the conventional way of impregnating materials with antibiotics. Surface antimicrobial treatment has the advantage of being environmentally friendly and bacteria resistance development is not possible. A promising development in this field is related to the antibacterial effects of the pyridinium-type polymers. Early work on pyridinium-type polymers was reported by Nakagawa et al. (1982) and Kawabata et al. (1988). Both groups focused on the synthesis and investigation of the antimicrobial characteristics of those polymers, while recent works carried out by Li et al. (2000a, 2000b) also dealt with the preparation and characterization of similar type of polymers with different pyridinium groups. These pyridinium polymers have been used to functionalize ordinary material surfaces to impart them with antibacterial activities (Kawabata et al., 1992; Tiller et al., 2001; Tiller, et al., 2002; Lin et al., 2002). Other types of antimicrobial agents such as quaternized polyethylene polyamines (Tashiro, 1991), alkylated polyethyleneimine (Lin et al., 2003), TiO₂ particles (Kwak et al., 2001; Yu et al., 2003) and silver (Gray et al., 2003; Park et al., 2003) have been incorporated onto different material surfaces via various methods.
2.3 Substrates for Enzyme Immobilization, Cell Culture and Biofilm Prevention

2.3.1 Electroconductive polypyrrole

A new era of material science in biological applications has been brought forward since the discovery of conducting electroactive polymers by Shirakawa et al. (1977), whose work achieved the synthesis of continuous and free standing films of conducting PPY with high electrical conductivity (~100 Scm\(^{-1}\)) and excellent air stability using platinum electrodes. The extended \(\pi\)-electron backbone with alternating single and double bonds along the electrically conducting polymer chain is responsible for their unusual electronic properties such as electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity. Such \(\pi\)-conjugated system allows the presence of an overlap of molecular orbitals to allow the formation of delocalized molecular wave function, which is the key requirement for a polymer to be intrinsically electrically conducting (Gerard et al., 2002). Among the numerous conducting polymers (polyacetylene, polythiophene, PPY, polyindole and polyaniline) (Pandey, 1988; Shinohara et al., 1988; Bartlett and Cooper, 1993) investigated to date, PPY is by far the most extensively studied. The monomer, pyrrole, is easily oxidized, water soluble and commercially available, while polypyrrole possesses good redox properties, environmental stability, and high electrical conductivities (Ashwell, 1992). Furthermore, its excellent biocompatibility is a key feature for research interests in its applications in biological systems (Schmidt et al., 1997; Garner et al., 1999).

2.3.1.1 Electrochemical synthesis of polypyrrole
The inherent properties of PPY, which subsequently influence its applications, are highly dependent on the synthesis conditions. Mainly, there are two methods used to synthesize PPY: chemical and electrochemical polymerization. Electrochemical synthesis is rapidly becoming the preferred general method because of its simplicity, reproducibility, and the possibility to obtain PPY with high conductivities. Electrochemical polymerization of conducting polymers is generally carried out by: (1) constant current or galvanostatic; (2) constant potential or potentiostatic; (3) potential scanning/cycling or sweeping methods (Gerard et al., 2002). Normally, the reaction is carried out in a simple electrochemical cell containing a solvent, an electrolyte and the monomer (pyrrole). The generation of a polymeric film on the anode (platinum, aluminium, ITO glass, stainless steel, etc) is then initiated by the flow of an anodic current in some specific electrical conditions. The direct deposition of the conducting polymer onto the electrode surface is of special interest for electrochemical applications, and the film thickness can be flexibly controlled by the electrical charges passed during polymerization. It can also be expected that electrochemical generation of PPY is a fast, easy and clean process since no catalyst is involved (Nalwa, 1997). In principle, factors influencing the electropolymerization process and the properties of the PPY include the nature of electrolyte, solvent, pH, temperature and monomer substitution as well as the electrochemical method (Sadki, et al., 2000).

2.3.1.2 Mechanism of pyrrole polymerization

The elucidation of the mechanism of electropolymerization of PPY is impeded by the rapidity of the polymerization which causes difficulties in determination of the different stages of reaction, and the insolubility of the PPY coupled with its non-crystalline nature which makes it exceedingly difficult for structural characterization.
and analysis of physical properties. Among the different mechanisms proposed by Diaz (Lund and Baizer, 1991), Kim (Kim et al., 1988), Pletcher (Asavapiriyanont, et al., 1984), and Reynold (Qiu and Reynolds, 1992), the one by Diaz and his colleagues is believed to be the most representative and has also been confirmed by Waltman and Bargon (1986) with their theoretical studies (Figure 2.2). According to their proposal, the reaction begins with electron transfer (E) followed by a succession of chemical reactions (C) and electron transfer reactions. The term E(CE)n, as an extension of ECE, is then used to describe all the steps involved in the formation of the film (Lund and Baizer, 1991; Sadki, et al., 2000).

![Figure 2.2 mechanism of electrochemical polymerization of PPY](image)

2.3.1.3 Applications of polypyrrole

The main projected applications either developed or still in the process of investigation include antistatic coating (Stanke et al., 1993; Bhat and Shaikh, 1994), electromagnetic shielding (Kaynak et al., 1994), conductive textiles and fabrics (Gregory et al., 1989; Kuhn et al., 1995), capacitors (Kudoh, 1993), rechargeable batteries (Bhat and Yasmin, 1995), electrochromic displays (Somani, et al., 2000), and drug release systems (Zinger and Miller, 1984; Smyrl and Lien, 1993; Nalwa, 1997). The most significant
application of PPY is in the construction of biosensors, which is triggered by the increasing demand in the fields of diagnostic biotechnology, food and agriculture product processing, health care, medicine and pollution monitoring. The construction of a biosensor always involves the cooperation of a biological sensing element (redox proteins, enzymes, coenzymes) either directly connected to or integrated within a transducer. PPY-based biosensors have long been investigated resulting in significant achievements not only in fabrication methods but also in sensing efficiency and sensitivity (Cosnier, et al., 1998; Becerik and Kadirgan, 2001; Trojanowicz, et al., 1990; Bélanger et al., 1989). Another fascinating area is the study of the electrical stimulation in promoting biological or cellular functioning. Works of importance are the assessment of electrical stimulation to encourage neurite outgrowth or nerve guidance with PPY acting as the platforms. In vivo studies have shown that PPY is not cytotoxic and can be formed into conduits to support the regeneration of damaged peripheral nerves in rats (Schmidt et al., 1997; Garner et al., 1999). Additional nerve guide studies have also proved its biocompatibility and its potential as an effective guidance channel for the regeneration of nervous tissue (Williams and Doherty, 1994).

Since it has been shown that electrical current strengthens the function of antibiotics (Wattanakaroon and Stewart, 2000), the possibility of using such electrical conductive polymers in antimicrobial applications is also a fascinating area.

2.3.2 Polymeric Nonporous and Porous Materials

**Poly(ethylene terephthalate) (PET):** PET is one of the thermoplastic polymers which have found wide application in different fields of biomedical applications. This class of plastics is generally made up of long linear chain molecules which exhibit large scale chain mobility and deformation under shear forces above their softening
temperature, while remaining as a viscoelastic solid at room temperature. The processibility is a key advantage for developing these materials in biomedical applications (Black and Hastings, 1998). PET is made by the condensation reaction of ethylene glycol with either terephthalic acid or dimethyl terephthalate (Zachariades and Porter, 1983). The average molecular weight ranges from 15000 to 20000 and its physical properties are largely determined by the degree of crystallinity, which varies between 30 and 40% depending on the processing conditions. The glass transition temperature, $T_g$ for PET is $86^\circ$C and it exhibits good chemical resistance to acids, alkalis and organic solvents. Additional characteristics include high strength and stiffness, favorable creep characteristics, hard surface capable of being polished and high dimensional stability (Black and Hastings, 1998). Applications of PET in the biomedical field include its use as a template for cell culture (Jain and von Recum, 2003; Bisson et al., 2002), implanted medical devices and prostheses (Brodbeck et al., 2002; Rosch et al., 2003; Quester et al., 2003), or scaffolds (Li et al., 2001).

**Poly(vinylidene fluoride) (PVDF):** PVDF is a hydrophobic polymeric material which has been known since the 1960’s for its excellent mechanical and physicochemical properties. It has good solubility in common solvents such as dimethylacetamide (DMAc), dimethylformamide (DMF) and $N$-methyl-2-pyrrolidone (NMP) and good tolerance to oxidizing agents such as chlorine. PVDF can be formed in many types of molecular and crystal structures depending on the preparation conditions. Wide spread industrial applications and research interests have been developed, especially for PVDF membranes (Seiler and Scheirs, 1998; Stevens, 1999). PVDF membranes are widely applied in microfiltration and ultrafiltration for their excellent processability,
chemical resistance, well-controlled porosity and good thermal property (Seiler and Scheirs, 1998; Stevens, 1999).

**Cellulose:** The cellulose molecule is a linear homopolymer of anhydroglucopyranose units which are linked together through β-(1,4) glucosidic bonds. Materials made from cellulose such as filter paper and cotton cloth, have been widely used not only in scientific research fields but also in daily life. Filter paper is a typical common necessity in technical systems, while cotton cloth is closely related with medical facilities and daily usage. Furthermore, cotton cloth has also been investigated as a matrix to support cell or enzyme immobilization (Albayrak et al., 2002; Kamath et al., 1988) and fermentation studies (Huang and Yang, 1998; Tan and Yang, 2002). The fibrous matrices of cotton cloth with high porosity, open structures and high mechanical strength contribute to its popularity. Its natural abundance makes it inexpensive and it is commercially available in different grades. The above features prompted research interest in cotton cloth in the current work. Similarly investigations were carried out on PET and PVDF due to their wide applications and intrinsic characteristics mentioned above.
CHAPTER 3

SURFACE FUNCTIONALIZATION OF POLYPYRROLE FILM WITH GLUCOSE OXIDASE AND VIOLOGEN
3.1 Introduction

As mentioned in Section 2.3.1, intrinsically conducting polymers with conjugated double bonds have been viewed as attractive advanced materials for electronic devices (Potember et al., 1987), electrochromic displays (Skotheim, 1986), chemical and biochemical sensors (Sadik, 1999), drug release systems, rechargeable batteries (Bidan, 1992) and for modifying electrode surfaces for electrical wiring of biomolecules (Trojanowicz, 1990; Garnier and Youssoufi, 1994). In addition to the advantages polypyrrole (PPY) has over the other conducting polymers as specified in Section 2.3.1, its versatile applicability and the availability of a wide variety of molecular (redox) species which can be covalently linked to the pyrrole group (Usmani and Akmal, 1994), also contribute to the choice of PPY as a substrate for this part of work.

One of the common applications of PPY’s biocompatibility (as compared with other conventional polymeric materials) is its use as a substrate for the immobilization of enzymes for applications in biomaterials (Kobayashi and Ikada, 1991; Okada and Ikada, 1992), bioseparators (Behm et al., 1989), and biosensors (Sadik, 1999). PPY was shown to be biocompatible with nerve tissue (Schmidt et al., 1997; Wang et al., 2004) but it is not blood compatible (Li et al., 2003). Different methods of enzyme immobilization have been reviewed in Section 2.2.1.1., and many studies have focused on the electrochemical entrapment of enzymes in PPY films during their electrogeneration on the electrode surface (Foulds and Lowe, 1986; Umana and Waller, 1986; Edelman and Wang, 1992; Bélanger et al., 1989). This method involves the application of an appropriate potential to the working electrode immersed in aqueous solutions containing both enzyme and monomer molecules. Enzymes present in the immediate vicinity of the electrode surface are thus incorporated into the growing
polymer (Foulds and Lowe, 1986). A supporting electrolyte may or may not be added (Bélanger et al., 1989; Adeloju and Moline, 2001). However, this method is obviously restricted to the use of monomers soluble in aqueous solutions and the ability to electropolymerize at biological pH values. Moreover, it suffers from the problem of low loading and low relative activity of the immobilized enzyme compared with the free one (Cosnier, 1999). Covalent binding thus offers the advantage of the possibility of using optimal conditions for each step since it is a sequential procedure, namely electropolymerization followed by covalent binding. The initial polymerization can be performed under conditions (organic solvents, high potential values for the polymerization process, reactivity of the generated radical monomers) which may be deleterious for biomolecules. Furthermore, compared with the entrapment technique this approach provides better access of the analyte to the immobilized biomolecules and facilitates macromolecular interactions as well as preserving the initial properties of polymer films, such as its conductivity (Cosnier, 1999).

In the present study, the carboxylic groups on PPY surface obtained from surface modification of PPY films via graft copolymerization with acrylic acid (AAc) were used to covalently immobilize an enzyme, glucose oxidase (GOD). In addition, a redox mediator (viologen group) was also introduced on the PPY surface to investigate the interaction of the redox mediator with the enzyme. Since viologen groups are highly water-soluble, systems based on these mediators suffer the inherent drawback of the diffusion of the soluble mediating species away from the electrode surface into the bulk solution. A previous work (Rajagopalan et al., 1994) addressed this problem by crosslinking the enzyme with osmium derivatized poly(N-vinylimidazole) in a hydrogel on glassy C electrodes. In the present work, the viologen group was immobilized on the electrically conductive PPY surface by a surface graft technique
(Ng et al., 2001). The changes in the surface composition after grafting and enzyme immobilization of PPY film were investigated using X-ray photoelectron spectroscopy (XPS). The efficiency of AAc surface graft copolymerization and its subsequent effect on the amount and activity of the immobilized enzyme and the conductivity of the film were studied. The behaviors of the immobilized enzyme at different conditions in the presence and absence of the viologen moieties on the PPY surface were also studied and compared with that of the free enzyme.
3.2 Experimental

Materials and reagents

Pyrrole (99%) was obtained from the Aldrich Chemical Co. and was distilled before use. 4-Vinyl benzyl chloride (VBC), 4,4'-bipyridine, α,α’-dichloro-p-xylene (98%) and acrylic acid (AAc) were also obtained from Aldrich Chemical Co.. Water-soluble-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC) was purchased from Dojindo Chemical Co. and was used as received. Glucose oxidase (GOD) and peroxidase (POD) were purchased from Sigma Chemical Co.. Dulbecco's phosphate buffer solution (PBS) (containing 8000 mg of sodium chloride, 200 mg of anhydrous monopotassium phosphate per liter of water), used for the enzyme immobilization work, was freshly prepared. Bio-Rad dye reagent for protein assay was obtained from BioRad Chemical Co. Toluene-4-sulfonic acid (TSA) was obtained from Fluka. The solvents and other reagents were of analytical grade and were used without further purification.

Electrochemical synthesis of polypyrrole film

Electrochemical polymerization of pyrrole was carried out using an Autolab-PGSTAT30 (Metrohm-Schmidt Ltd). As shown in Figure 3.1, the electrolyte solution of 0.1 M TSA in acetonitrile containing 1vol.% water was purged for 20 min with dry nitrogen to remove dissolved oxygen prior to the addition of the monomer. Pyrrole was then added to the electrolyte solution to achieve a 0.1 M concentration and the solution was subsequently purged with nitrogen for another 5 min. A highly polished stainless steel plate acted as the working electrode (anode) while a platinium wire gauze served as the counter electrode (cathode), and an Ag/AgCl electrode was used as the reference electrode. PPY was electrodeposited on the stainless steel plate by applying a voltage
of 8 V at 0°C under a dry nitrogen atmosphere and the film thickness can be controlled by the charge passed (Choi and Tachikawa, 1990). The electropolymerization was carried out for half an hour to obtain doped PPY films of approximately 50 µm in thickness. After these films were removed from the stainless steel plate, they were washed thoroughly with acetonitrile and water and dried by pumping under reduced pressure. The conductivity of polypyrrole films was measured using a four-point probe (Singaton Co.) (Van der Pauw, 1958). The conductivities of the films were calculated from measurements of the voltage under controlled current applied to the film using the equations derived by Van der Pauw (Van der Pauw, 1958).

**Figure 3.1** Setup for the electrochemical polymerization of PPY film

**Immobilization process**

PPY film strips of about 2 cm × 4 cm were used in all experiments. In the case of graft copolymerization with AAc, each PPY film was immersed in an aqueous AAc solution of a predetermined concentration between 2 and 10 vol.% in a Pyrex glass tube. Degassing of the solutions was achieved by bubbling nitrogen vigorously into the solution in the tubes for 30 min before stoppering and sealing with silicon rubber stoppers. The tubes of AAc solution containing PPY films were then exposed to UV irradiation in a Riko rotary photochemical reactor (RH400–10W) for 40 min at about 25°C. The graft-copolymerized films were finally subjected to washing with doubly-distilled water at 50°C for 50 h to remove the residual homopolymer.
For the case of grafting viologen and AAc groups together on PPY films, similar procedures were applied. However, dioxane was used as the solvent for the VBC and AAc which were mixed together in predetermined ratios. The UV irradiation time was prolonged to 1 h and the subsequent washing was performed in water and dimethylformamide (DMF) alternately. After the initial process of AAc and VBC graft copolymerization, the films were immersed in DMF solution containing 0.024 M 4,4’-bipyridine and the reaction was allowed to proceed in a water bath at a temperature of 60°C for 20 h, followed by reaction in 0.024 M α,α’-dichloro-p-xylene in DMF solution under the same conditions. The reaction of the VBC-graft copolymerized films with 4,4'-bipyridine and α,α’-dichloro-p-xylene results in the formation of viologens (Ng et al., 2001) and is illustrated in Figure 3.2. The films were washed with DMF and doubly-distilled water to remove residual reactants and homopolymers. The viologen and AAc grafted films were then dried by pumping under reduced pressure and stored in the dark until further use.

The covalent immobilization of GOD onto the AAc-graft copolymerized film was facilitated by the activation of the carboxylic groups grafted on the film surface. The COOH groups were preactivated for 1 h with WSC at 4°C in 0.1 M PBS, containing 5 mg/ml of WSC. The films were then transferred to the 0.1 M PBS(+) (pH 7.4, with 0.02 M CaCl₂ added) containing GOD at a concentration of 4 mg/ml. The immobilization was allowed to proceed at 4°C for 24 h. After that, the reversibly bound GOD was desorbed in copious amounts of PBS(+) for 1 h at 25°C (Kulik et al., 1993). The process for the immobilization of GOD is summarized in Figure 3.3, which is the general scheme for the covalent immobilization of proteins or enzymes on the AAc-graft copolymerized polymer substrates (Sano et al., 1993).
Figure 3.2 Schematic representation of (a) graft copolymerization of VBC on polypyrrole film surface, (b) grafting of viologen moieties on VBC-graft copolymerized polypyrrole film.
**Determination of AAc, viologen graft concentration and immobilized GOD concentration**

XPS analysis of the films was made on an AXIS HSi spectrometer (Kratos Analytical Ltd.) using the monochromatized Al Kα X-ray source (1486.6 eV photons) at a constant dwell time of 100 ms and a pass energy of 40 eV. The anode voltage was 15 kV and the anode current was 10 mA. The pressure in the analysis chamber was maintained at 5.0×10⁻⁸ Torr or lower during each measurement. The polymer films were mounted on standard sample studs by means of double-sided adhesive tape. The core-level signals were obtained at a photoelectron take-off angle of 90° (with respect to the sample surface). To compensate for surface charging effect, all core-level
spectra were referenced to the C 1s hydrocarbon peak at 284.6 eV. In spectral deconvolution, the linewidth (full width at half-maximum) of the Gaussian peaks was maintained constant for all components in a particular spectrum. The peak area ratios for the various elements were corrected using experimentally determined instrumental sensitivity factors.

The amount of GOD immobilized on PPY film was determined by the modified dye-interaction methods (Kang et al., 1993; Bonde et al., 1993), using the BioRad protein dye reagent. For the preparation of the dye solution, the Bio-Rad stock dye solution was diluted five times with doubly-distilled water. GOD solution (100 µl) of known concentration was added to 5 ml of the dye solution. The GOD-dye solution was kept for 10 min and centrifuged at 5000 rev/min for 15 min. In the latter process, the GOD-dye complexes were precipitated and the free dye remained in the upper layer. The absorbance of the supernatant at 465 nm was used for the standard calibration. For the quantitative determination of immobilized GOD, the dye solution (5 ml) was added to a test tube and the GOD immobilized PPY film (2cm × 4cm) was immersed into the dye solution. After 3 h of reaction, the film was removed and the absorbance of the dye solution was measured at 465 nm. The amount of GOD immobilized on the surface of PPY film was calculated on the basis of the standard calibration.

**Assay of GOD activity**

The enzyme activity of the free and immobilized GOD was measured using the method reported in the Sigma Technical Bulletin (Sigma Technical Bulletin, 1983). The method is based on the spectrophotometric determination of the amount of hydrogen peroxide formed. The assay mixture (3 ml) contains 0.1 ml of peroxidase (POD) solution (60 units/ml), 2.4 ml of dye buffer solution (0.21 mM of o-dianisidine
solution in 0.05 M acetate buffer, pH=6), and 0.5 ml of β-D(+) -glucose solution (18 wt%). The reaction was initiated by either adding 0.1 ml of free GOD solution to the assay mixture or by dipping the GOD-immobilized PPY film in the assay mixture. The initial rate of absorbance increase at 500 nm was used to evaluate the activities of the free and the covalently bound enzyme.

For the investigation of the involvement of viologen in the GOD-catalyzed oxidation of glucose in the absence of O$_2$ and under UV irradiation, 5 ml β-D(+) -glucose solution (18 wt%) was used as the assay mixture and the activities were calculated by measuring the difference of the concentrations of β-D(+) -glucose solution before and after the dipping of GOD-immobilized viologen-contained PPY film for a predetermined time. The concentrations of β-D(+) -glucose solution were measured by the YSI Model 2700 SELECT Biochemistry Analyzer from YSI Incorporated, (USA). The assay mixture in this case was first bubbled with nitrogen for 30 min before immersing the GOD-immobilized film and sealing the tube with a silicon rubber stopper. After that, the mixture was exposed to near-UV irradiation in a Riko rotary photochemical reactor (RH400-10W) for different periods of time. The reactor was equipped with a 1000 W high-pressure Hg lamp and a water bath which kept the reaction mixture at a constant temperature of 25°C. The effect of temperature in the range of 20~60°C on the activity of free and immobilized GOD was also measured in the time scale of 30 min and compared to that of free GOD. These experiments were also carried out using the YSI Biochemistry Analyzer instead of the spectrophotometric determination of hydrogen peroxide since the former is a more convenient method. A comparison of the results obtained by the two techniques in a number of tests shows that the results obtained are comparable.
3.3 Results and Discussion

Graft copolymerization of AAc

The success of the UV-induced surface graft copolymerization of AAc on the PPY film can be ascertained by comparing the XPS C 1s spectra of the films before and after grafting process. The XPS C 1s core-level spectrum of the pristine PPY indicates a small amount of oxidized C, primarily C-O (286.2 eV) and C=O (287.6 eV) (Figure 3.4(a)). The presence of these species may have resulted from surface oxidation or charge-transfer complexing with oxygen. These species are desirable for the subsequent surface grafting process. The N 1s core-level spectrum of the pristine PPY (Figure 3.4(b)) indicates a predominant peak at 399.4 eV due to the NH species and a high binding energy tail above 400 eV attributable to positively charged nitrogen. The \( N^+/N \) ratio is 0.29, consistent with the doping level of PPY reported in a previous study (Zhang et al., 1996). After AAc graft copolymerization on the PPY film a distinct peak at about 288.7 eV attributable to the carboxylic acid groups (Kang et al., 1992) is observed in the C 1s spectrum (Figure 3.4(c)). The intensity of the N 1s spectrum (Figure 3.4(d)) is significantly reduced due to the coverage of the PPY surface by the AAc copolymer. The loss of the S 2p core-level signal on the wide scan of the PPY film surface after surface-graft copolymerization with AAc indicates that during the graft copolymerization in AAc solution and the subsequent vigorous washing process the toluene sulfonic acid dopant in the original films has been removed. Since the PPY films remain in the doped state with a significant proportion of \( N^+ \), the COO\(^-\) groups of the AAc graft copolymer probably serve as counterions.
Figure 3.4 XPS C 1s and N 1s core-level spectra of (a) and (b) pristine PPY film, (c) and (d) PPY film after UV-induced graft copolymerization with AAc in 10 vol.% AAc monomer aqueous solution; (e) and (f) PPY film graft copolymerized with AAc in 10 vol.% AAc monomer concentration and subsequently immobilized with GOD.
The extent of surface grafting of AAc is estimated from the corrected area ratio of the C 1s peak component at 288.7 eV attributable to the carboxyl group of the AAc polymer, and the total N 1s area due to the PPY film, and is expressed as the [COOH]/[N] ratio. With the increase in AAc monomer concentration, the surface graft concentration of the AAc polymer increases as shown in Figure 3.5. The large increase occurring at monomer concentration higher than 8 vol.% may be due to the high degree of homopolymerization since a similar behavior has also been observed on PTFE substrate when grafted with AAc (Kang et al., 1996).

Figure 3.5 Effect of AAc monomer concentration on the surface graft concentration of the AAc polymer and PPY film conductivity.
Since the AAc-graft copolymer is non-conductive, the increase in the concentration of the AAc copolymer would result in a lower electrical conductivity of the PPY film. However, the decrease in the conductivity of the grafted PPY film is not proportional to the increase in the surface graft concentration as shown in Figure 3.5. In the range of AAc monomer concentrations from 2 vol.% to 8 vol.%, the surface graft concentration does not change much while the conductivity shows a steady decrease. Since the conductivity measured by four-point probe is not the surface conductivity, the observed trend suggests that the AAc copolymer may not exist only on film surface, but a substantial amount of AAc polymer may have also affected the molecular level of the sublayer of the PPY film.

**Grafting of viologen**

In order to graft viologen on the PPY film, the first step is to ensure the graft copolymerization of VBC. The success of the simultaneous graft copolymerization of AAc and VBC is ascertained by the XPS C 1s and Cl 2p spectra of the films after co-polymerization (Figure 3.6). As discussed above, the COOH group in the C 1s spectrum (Figure 3.6(a)) is attributable to the AAc-graft copolymer. The Cl 2p core-level spectrum of the VBC graft-modified film (Figure 3.6(b)) show a spin-orbit split doublet (Cl 2p 3/2 and Cl 2p 1/2) with peaks at 200.2 eV and 201.7 eV attributable to covalent Cl (Moulder et al., 1992; Neoh et al., 1993) of the VBC groups. An indication of the approximate amount of VBC grafted on PPY film can be inferred from the [Cl]/[N] ratio while an indication of the relative amount of AAc and VBC grafted on film can be approximated from the ratio of ([COOH]/[Cl]). The total amount of AAc and VBC is fixed at 20 vol.% in the dioxane solution, and a variation in the relative graft concentrations can be achieved using different AAc/VBC monomer ratios in
solution (Figure 3.7). The results in Figure 3.7 indicate that the ([COOH]/[Cl]) ratio increases almost proportionally with the AAc/VBC ratio in the reaction mixture at low AAc concentrations but at high concentrations of AAc, the increase in the ([COOH]/[Cl]) ratio on the PPY film surface lags behind the increase in monomer ratio.

Figure 3.6 XPS C 1s and Cl 2p core-level spectra of the PPY film (a) and (b) after UV-induced surface graft copolymerization with 10 vol.% AAc and 10 vol.% VBC in dioxane; (c) and (d) after surface grafting with 10 vol.% AAc and 10 vol.% VBC in dioxane, followed by reaction in 0.024 M bipyridine in DMF and 0.024 M α,α’-dichloro-p-xylene in DMF respectively.
Figure 3.7 Effect of AAc/VBC monomer ratio in dioxane on [COOH]/[Cl] ratio (as determined by XPS) on the film surface.

The Cl 2p core-level spectrum of the viologen-grafted films (Figure 3.6(d)) is different from that of the VBC-grafted modified film (Figure 3.6(b)), and can be deconvoluted into two spin-orbit split doublets with binding energies for the Cl 2p$_{3/2}$ peaks at 197.1 and 200.2 eV attributable to ionic (Cl$^-$) and covalent (-Cl) chlorine species, respectively (Moulder et al., 1992; Neoh et al., 1993). An indication of the amount of
viologen grafted on film can be obtained by the ratio of the ionic Cl to the total Cl ([Cl\(^-\)])/[Cl\(_T\)]). A high Cl\(^-\)/Cl\(_T\) ratio would imply that a large proportion of the VBC groups has reacted with the 4,4’-bipyridine and \(\alpha,\alpha’\)-dichloro-p-xylene to form the viologen. It would be highly unlikely for the Cl/Cl\(_T\) to be close to 1 since the end groups of the viologen have a -Cl group. The integrity of the COOH group on the PPY film is preserved as seen from the C 1s spectrum (Figure 3.6(c)) although the [COOH]/[C] ratio has decreased due to the contribution of the viologen to the total C. These COOH groups thus allow for the subsequent covalent immobilization of GOD. The amount of viologen moiety on the PPY film can be expected to increase with the VBC graft concentration. In the subsequent discussion to demonstrate the effects of viologen, the films obtained from a grafting solution of 10 vol.% AAc and 10 vol.% VBC in dioxane were used.

Immobilization of GOD

The XPS C 1s and N 1s core-level spectra before and after GOD immobilization are compared in Figure 3.4(c) and (d), and Figure 3.4(e) and (f) respectively. In Fig 3.4(e), the intensity of the peak at the BE of 288.7 eV attributable to the carboxyl group is largely reduced compared to that of Figure 3.4(c), while the intensity of the peak at the BE of 287.8 eV is considerably enhanced. This is attributed to the formation of the peptide linkages (CONH) and peptide groups of GOD. The N 1s core-level spectrum also confirms the presence of the peptide linkage which gives rise to a strong signal at 399.4 eV. It can be expected that as the AAc graft concentration increases, the amount of GOD immobilized via the covalent coupling between the grafted AAc chains through the WSC intermediate would also increase. This is illustrated in Figure 3.8 where the amount of immobilized GOD per unit surface area of the film as determined
using the protein-dye reagent is plotted against the AAc monomer concentration. At the same time, with the increase in the amount of immobilized GOD the conductivity of the PPY film decreases further (compare Figure 3.8 with Figure 3.4). However, the films still remain quite conductive with the conductivity ranging from 10 to 30 S/cm for the range of conditions tested. This indicates that the film has the potential to be used as an electrode material.

Figure 3.8 Amount of GOD immobilized and film conductivity after GOD immobilization as a function of AAc monomer concentration used in the graft copolymerization process.
For the GOD catalyzed glucose oxidation process, the general reaction mechanism is as follows (Deshpande and Amalnerkar, 1993):

\[
\text{Glucose} + \text{GO(FAD)} \rightarrow \text{gluconolactone} + \text{GO(FADH}_2) \\
\text{GO(FADH}_2) + \text{O}_2 \rightarrow \text{GO (FAD)} + \text{H}_2\text{O}_2
\]

where GO(FAD) and GO(FADH$_2$) represent the oxidized and reduced forms of glucose oxidase, respectively. The activity of the immobilized GOD in Units is defined as the number of $\mu$mols of $\beta$-D-glucose oxidized to D-gluconolactone per minute and the relative activity (RA) is defined as the ratio of the observed surface enzyme activity over the activity obtained from an equivalent amount of the free enzyme. The enzyme activities are plotted against the AAc monomer concentrations in Figure 3.9. It can be seen that the GOD activity increases and then gradually levels off while the relative activities of the immobilized enzyme decrease with increasing AAc monomer concentration (which is equivalent to increasing surface graft concentration of AAc on the PPY substrate). The increase in observed enzyme activity must be associated with the increase in the amount of enzyme immobilized. However, this increase will gradually diminish due to increasing diffusion limitation since a considerable fraction of the enzyme molecules is probably embedded in the grafted AAc polymer layer and becomes less accessible to the analyte. Furthermore, at high GOD concentrations the local pH value may differ from that in the bulk fluid and pH is one of the major factors affecting the degree of dissociation of critical groups within the active sites of the enzyme. Thus, the increase in steric hindrance and the interaction between the active sites of the enzyme and the AAc polymer are potential factors inhibiting the functions of the enzyme.
Figure 3.9 Observed enzymatic activity and relative activity of the covalently immobilized GOD on PPY film surface as a function of AAc monomer concentration used in the graft copolymerization process.

The immobilized enzyme retains only about 20 to 35% of the activity of the equivalent amount of enzyme in the free state (Figure 3.9). This decrease in enzyme activity is a phenomenon commonly observed in enzyme conjugation to polymers and is usually interpreted, either as a minor modification in the enzyme tertiary structure that may be reflected in the distortion of amino acid residues involved in catalysis, or on the basis that the analyte approach to the active site of the enzyme is hindered (Caliceti et al.,
1993). On the other hand, the activity level of the immobilized enzyme also depends on the degree of hydration of the polymer matrix: a highly hydrophilic film surface is advantageous for retaining the activity of the enzyme immobilized on it. The graft copolymerization of AAc increases hydrophilicity of the substrate (Kang et al., 1992), in addition to acting as the linkage between the substrate and enzyme. The results obtained compare very favorably with those obtained in an earlier study on the electrochemical entrapment of GOD in PPY during electropolymerization (Yabuki et al., 1989). In the latter, a maximum activity of about 30 mU/cm² was obtained. In the present work, activities of >100 mU/cm² can be readily achieved (Figure 3.9). Moreover, the GOD is more effectively utilized in the immobilization step in the present work. For example, the enzyme activity of GOD immobilized on the electrode normalized by the activity of the total amount of GOD in the solution used in the immobilization step in terms of units is <0.003% in the earlier study (Yabuki et al., 1989), compared to a value that is two orders of magnitude higher in the present work.

**Kinetic effect of immobilization**

The kinetics of a simple enzyme reaction can be described by the Michaelis-Menton equation where the velocity of the reaction, \( \nu \), at a particular analyte concentration, \( S \), is given by:

\[
\nu = \nu_{\text{max}} \frac{S}{(K_m + S)}
\]

(Rosevear et al., 1987). The constants, \( \nu_{\text{max}} \) and \( K_m \), can be obtained from the slope and intercept of a straight line plot of \( 1/\nu \) versus \( 1/S \) (Lineweaver-Burk plot). Figure 3.10 shows the Lineweaver-Burk plots for glucose oxidation by the free and immobilized GOD. The plot for the free enzyme has better linearity and gives a higher \( \nu_{\text{max}} \) and lower \( K_m \) values compared to those for the immobilized GOD (Table 3.1). These differences can be attributed to the diffusion limitation of the analyte to the active sites within the matrix, the interactions of GOD
molecules with the polymer matrix and the partitioning of components between the stationary and mobile phases (e.g. polymers and solvents ions) (Rosevear et al., 1987; Treven, 1980). The conformational changes of the immobilized enzyme may also have certain effects on the kinetic parameters.

Figure 3.10 Lineweaver-Burk plots for immobilized GOD on AAc graft copolymerized PPY (0.024 mg GOD/cm$^2$), and an equivalent amount of free GOD in pH 7.4 PBS buffer solution at room temperature.
Table 3.1 Kinetic parameters for free and immobilized GOD

<table>
<thead>
<tr>
<th>Properties</th>
<th>$v_{\text{max}}$ (mM/min)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free GOD 0.192 mg GOD in 5 ml assay solution, equivalent to the amount immobilized on the PPY film mentioned below</td>
<td>0.86</td>
<td>51.4</td>
</tr>
<tr>
<td>GOD immobilized on PPY film Film size: 2cm × 4cm Surface graft concentration of AAc ([COOH]/[N]): 1.43 Immobilized GOD: 0.024 mg/cm$^2$</td>
<td>0.53</td>
<td>82.6</td>
</tr>
</tbody>
</table>

Effect of viologen

The effect of viologen on the behavior of the immobilized GOD was tested using the film graft copolymerized with AAc and VBC in a dioxane solution containing 10 vol.% VBC and 10 vol.% AAc. The kinetic behavior of GOD immobilized on this film is compared to that of a film immobilized with a similar amount of GOD without viologen moieties in Table 3.2. A decrease in $v_{\text{max}}$ from 0.53 to 0.33 mM min$^{-1}$ and an increase of $K_m$ from 82.6 mM to 101.8 mM are observed in the case of the film with viologen moieties. The presence of the viologen groups on the film surface may have the following effects: increasing the diffusion resistance, forming a more complex and perhaps a non-favorable partitioning of components, and interacting with other molecules or ions which may be present. All of these factors may complicate the ability of the immobilized GOD to interact with the analyte.
### Table 3.2 Effect of viologen on PPY film properties and behavior of immobilized GOD

<table>
<thead>
<tr>
<th>Film</th>
<th>Monomer solution for graft copolymerization</th>
<th>Amount of GOD immobilized (mg/cm²)</th>
<th>Conductivity (S/cm)</th>
<th>Relative activity of the immobilized GOD (%)</th>
<th>Kinetic parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPY graft copolymerized with AAc</td>
<td>4% AAc in water</td>
<td>0.0241</td>
<td>24.8</td>
<td>34.0</td>
<td>0.53 82.6</td>
</tr>
<tr>
<td>PPY graft copolymerized with AAc &amp; viologen</td>
<td>10% AAc and 10% VBC in dioxane, followed by reaction with bipyridine and α,α'-dichloro-p-xylene</td>
<td>0.0244</td>
<td>11.1</td>
<td>15.1</td>
<td>0.33 101.8</td>
</tr>
</tbody>
</table>
To further study the involvement of the viologen in the activity of the GOD, the enzyme-catalyzed reaction was studied under UV irradiation in the absence of oxygen. From Figure 3.11, it can be seen that the enzyme still retains a certain amount of activity even in the absence of oxygen. In the initial stage, the activity increases very quickly with the increase in the UV irradiation time but the activity levels off after 30 min or so, and with a further increase in the UV irradiation time, the activity gradually decreases. A similar series of experiments were carried out with the film immobilized with almost the same amount of GOD but without any viologen. In this case, no GOD activity can be observed in the absence of O\textsubscript{2} under UV irradiation. Similarly, no activity can be observed with free GOD and the film graft copolymerized with AAc and viologen but without immobilization of GOD under the same experimental conditions. All the above experiments were conducted under almost constant temperature at around 25°C. A feasible explanation for the retention of enzymatic activity of the GOD immobilized on the film with viologen moieties in the absence of O\textsubscript{2} is the participation of the viologen in the reaction. Viologens have been investigated as redox indicators in biological studies (Pang and Abruna, 2000) and they possess one of the lowest (most cathodic) redox potentials of any organic system showing a significant degree of reversibility. For the GOD catalyzed oxidation of glucose, the following mechanism using redox mediators to shuttle electrons between the reduced flavin adenine dinucleotide center of the enzyme (FADH$_2$) and the electrode can be written (Usmani and Akmal, 1994):

\[
\text{Glucose} + \text{GO(FAD)} \rightarrow \text{gluconolactone} + \text{GO(FADH}_2) \\
\text{GO(FADH}_2) + 2\text{M}_{\text{ox}} \rightarrow \text{GO (FAD)} + 2\text{M}_{\text{red}} + 2\text{H}^+ \\
2\text{M}_{\text{red}} \rightarrow 2\text{M}_{\text{ox}} + 2\text{e}^- 
\]
The mediator $M_{\text{ox}}/M_{\text{red}}$ is assumed to be a one-electron couple. In the previous case discussed where $O_2$ is involved in the reaction, the $O_2/H_2O_2$ couple acts as $M_{\text{ox}}/M_{\text{red}}$. An earlier work (Hale et al., 1991) has shown that viologen molecules have sufficiently anodic redox potentials to reoxidize reduced glucose oxidase. Viologen dication groups ($V^{2+}$) can be reduced to the cation radical form ($V^+$) under the irradiation of UV and the results in Figure 3.11 indicate that under photoirradiation viologen may act as the redox mediator $M_{\text{ox}}/M_{\text{red}}$ in the GOD catalyzed oxidation of glucose. The actual mechanism is not known at present.

![Figure 3.11](image.png)

**Figure 3.11** Effect of UV irradiation time on the relative activity of GOD immobilized with viologen moieties on PPY in the absence of $O_2$ in pH 7.4 PBS buffer solution at 25°C.
In tests carried out with an equivalent amount of free and immobilized GOD in the presence of O$_2$ without UV irradiation, the maximum activity is reached after 30 min and thereafter a constant value is maintained. However, in the presence of UV irradiation and O$_2$, a gradual decrease in activity of the free GOD after 30 min is also observed. This suggests that prolonged UV irradiation may have an effect on the GOD structure. Thus, the gradual decrease in GOD activity seen in Figure 3.11 may be for the same reason. However, it is also possible that under extended UV irradiation, the viologen radical cations may be converted to other species (Monk, 1997), which affects the redox couple. Nevertheless, the results show that a PPY film that has been grafted with viologen and immobilized with GOD can serve as an electrode material and the possible pathway of the electron transfer from the glucose analyte to the electrode can be visualized as in Figure 3.12.

![Figure 3.12 Schematic representation of pathway of the electron transfer.](image)
Effect of temperature on the activity of GOD

The effect of temperature on the GOD activity was investigated for films immobilized with GOD with or without viologen moieties and compared with that of the free GOD (Figure 3.13). The free GOD attains a maximum activity at around 30°C while a slightly higher temperature is optimum for the immobilized GOD in both cases. A more critical temperature dependence is observed for the free GOD activity as compared to those of the immobilized GOD. Moreover, it is interesting to notice that at higher temperatures the immobilized GOD retains a higher activity than that of the free GOD. For example, at 70°C, the activity of the immobilized GOD with and without viologen is about 1.67 and 2.47 units/mg respectively, whereas the free GOD has an activity of only 1.57 units/mg. The decrease in activity of free GOD as the temperature increases may be due to conformational changes. The immobilized GOD may be less susceptible to this change because of its covalent bonding to the substrate. In the case of viologen, no structural or chemical change is expected in this temperature range (Kamogawa and Nanasawa, 1993). Hence there is little difference in the effect of temperature on the performance of the GOD immobilized with or without the viologen moieties.
Figure 3.13 Effect of temperature on the relative activity of the free GOD, and immobilized GOD on PPY film in pH 7.4 PBS buffer solution. PPY films are obtained either from graft copolymerization with AAc in 4 vol.% solution, or from graft copolymerization with AAc 10 vol.% and VBC 10 vol.% in dioxane followed by the formation of viologen moieties, respectively.
3.4 Conclusion

Electrochemically synthesized PPY films can readily undergo surface graft copolymerization with AAc and VBC and the graft concentration can be varied by changing the monomer concentrations. Further functionalization of the film is possible via the COOH groups of the AAc which can be used to immobilize GOD, and the VBC polymer which reacts with 4,4′bipyridine and α,α′-dichloro-p-xylene to form viologen moieties on the film surface. A high graft concentration of AAc would lead to more GOD immobilized but the relative activity of the immobilized GOD gradually decreases. The immobilized GOD displays a different kinetic behavior compared with that of the free GOD under the same conditions. The presence of viologen groups further reduces the enzymatic relative activity. However, due to the redox property of the viologen, it can function as an electron mediator. In this case, in the absence of O$_2$ and under the UV irradiation, the enzyme still retains about 15% enzymatic activity of the equivalent amount of the free GOD which works in the presence of O$_2$ but without UV irradiation. On the other hand, free GOD and immobilized GOD in the absence of viologen show no activity in the absence of O$_2$ under UV irradiation. Through the immobilization technique described in this work, the viologen groups on the PPY film do not diffuse away from the film surface into the bulk solution but retain the flexibility and proximity to the enzyme active sites to participate in the reaction. The stability of the immobilized GOD over a wide temperature range also increases the range of applications.
CHAPTER 4

SURFACE FUNCTIONALIZATION OF ELECTRICALLY CONDUCTIVE POLYPYRROLE FILM WITH HYALURONIC ACID
4.1 Introduction

The development of biomaterials that will permit more effective communication with the biomolecular world and extract relevant information from biomolecular systems is the quest of many tissue engineering efforts. Biomaterials have been designed to support tissue growth physically and to elicit desired receptor-specific responses from particular cell types (Ratner, 1996).

The work described in this chapter involves the investigation of a technique for anchoring hyaluronic acid (HA) on the surface of an electrically conductive polypyrrole (PPY) film. The beneficial effects of polypyrrole observed in the nerve study (Williams and Doherty, 1994; Schmidt et al., 1997; Garner, et al., 1999) as described in Section 2.3.1, provides incentive for this investigation. HA is a ubiquitous constituent of the extracellular matrix and the description of its potential applications in wound-healing, tissue regeneration and angiogenesis is given in Section 2.2.1.2. HA contains up to several thousand sugar residues, in a regular repeating sequence of nonsulfated disaccharide units: glucuronic acid $\beta$ (1→3) and N-acetylglucosamine $\beta$ (1→4) linkages as shown in Figure 4.1 (Arslan et al., 1994).

![Figure 4.1 Schematic representation of HA structure: a repeating disaccharide sequence.](image-url)
In the present study, an approach was developed to covalently bind HA to pre-formed conductive PPY films. Hydroxyl groups were first introduced on the PPY film surface by means of an UV-induced surface grafting technique. These groups enhance the chemisorption of silane bearing the amine groups (-NH$_2$), which are capable of reacting with carboxyl groups (COO-) in the HA molecules to form the peptide linkages (CONH) on the PPY film surface. The changes in the surface composition and morphology of the PPY film after the various functionalization steps were investigated using X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). The efficiency of the surface grafting process and its subsequent effect on the amount of amine groups introduced and the HA immobilized were studied. The biological activities of the immobilized HA at different conditions were assayed and the stability of the immobilized HA was also investigated.
4.2 Experimental

Materials and reagents

2-hydroxyethyl acrylate (H₂C=CHCO₂CH₂CH₂OH, denoted as HEA), toluidine blue, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid buffer (HEPES) used for HA immobilization were all from Fluka. 3-aminopropyl triethoxysilane (H₂N(CH₂)₃Si(OH)₃, denoted as ATS) and the solvent, 1,4-dioxane, used for surface graft copolymerization were obtained from Aldrich Chemical Co. Hyaluronic acid (HA) from human umbilical cord, bovine serum albumin (BSA), o-phenylenediamine dihydrochloride (OPD) and polyoxyethylene sorbitan monolaurate (Tween 20) were all from Sigma Chemical Co. Biotinylated hyaluronic acid binding protein (bHABP) from bovine nasal cartilage was purchased from Calbiochem. Avidine-biotin-peroxidase complex (ABC kit; Vectastain kit PK-4000) was a product from Vector Laboratories. Other solvents or reagents involved were the same as those described in Section 3.2.

Electrochemical synthesis of polypyrrole film

The process of electrochemical polymerization of pyrrole was carried out as described in Section 3.2.

Surface functionalization with hyaluronic acid

The process of functionalizing the PPY films with hyaluronic acid basically comprises four steps, as indicated in Figure 4.2. The detailed description is given below.
Figure 4.2 Schematic representation of the mechanism of anchoring HA on surface modified PPY film.
Chapter 4

(a) Surface graft copolymerization with HEA

The PPY films were cut into strips of about 2 cm × 3 cm in size and subjected to argon plasma pretreatment in an Anatech SP100 plasma system, equipped with a cylindrical quartz reactor chamber. The glow discharge was produced at a plasma power of 35 W, an applied oscillator frequency of 40 kHz and an argon pressure of approximately 0.6 Torr. The film was placed between the two electrodes and subjected to glow discharge for 10 seconds. After that, the film was exposed to air for 5 – 10 min to facilitate the formation of surface oxide and peroxide groups before graft copolymerization was carried out (Suzuki et al., 1986; Momose, et al., 1992). An increase in C-O species on the plasma pretreated PPY films was confirmed by XPS analysis (C-O/N = 0.96, compared to 0.61 for the pristine film). Hence, the oxidation occurs at the ring C and not the N heteroatoms of the PPY film. The plasma-pretreated PPY films were immersed in 1,4-dioxane solutions of HEA of a predetermined concentration between 2 and 8 vol.% in a Pyrex glass tube. Degassing of the solutions was achieved by bubbling nitrogen vigorously into the solution in the tubes for 30 min before stoppering and sealing with silicon rubber stoppers. The tubes of HEA solution containing PPY films were then exposed to UV irradiation in a Riko rotary photochemical reactor (RH400–10W) for 20 min at about 25°C. The graft-copolymerized films were finally subjected to washing with copious amounts of acetone to remove the residual monomer and physically adsorbed homopolymer (Wu et al., 2000).

(b) Silanization of PPY film surface (Wu et al., 2000).

After UV-induced graft copolymerization of PPY film surface with HEA, the films were immersed in 1.0 vol.% 1,4-dioxane solution of ATS for 1 min. The films were then washed with copious amounts of the solvent to remove the physically adsorbed
ATS. The chemisorption of ATS was achieved via Si-O bonding (Vargo, et al., 1993) with hydroxyl groups on the PPY film surface.

(c) Preactivation of hyaluronic acid

The anchoring of hyaluronic acid onto the HEA-graft copolymerized and subsequently ATS-silanized films was facilitated by the activation of the carboxylic groups of HA. HA was first dissolved in 10 mM HEPES buffer at a concentration of 1 mg/ml and pH of 7.0. 0.2 M WSC was then added and incubated for 1 h.

(d) Anchoring of hyaluronic acid on PPY Film substrates

After the incubation period of 1 h, the films were immersed in the activated HA solution and further incubated at room temperature for 24 h. After that, the films were washed in deionized water for two days so as to ensure that only covalently bound HA remains on PPY film surface (Abdelghani-Jacquin, et al., 2001).

Determination of hyaluronic acid

The amount of HA immobilized on the film surfaces was determined using the toluidine blue method (Kim et al., 2000; Kang et al., 1996; Ito et al., 1986). 16 mg of toluidine blue was dissolved in 100 ml 0.01 N hydrochloric acid containing 0.2 wt% NaCl. 2 ml of a HA aqueous solution was added to 3 ml of the toluidine blue solution and the mixed solution was agitated vigorously. n-Hexane (3 ml) was then added, and the mixture was shaken well so that the toluidine blue-HA complex was extracted into the organic layer. The unextracted toluidine blue remaining in the aqueous phase was determined by measuring the absorbance at 631 nm and the UV-visible absorption was monitored on a Shimadzu UV-3101 PC scanning spectrophotometer. The linear relationship between the absorbance at 631 nm caused by the residual toluidine blue
and the concentration of HA in the aqueous solution was ascertained and used as a calibration curve. In the determination of the amount of immobilized HA, the toluidine blue solution (3 ml) was mixed with 2 ml of water and the HA-immobilized PPY film (2 × 3 cm²) was immersed in the solution for 30 min. As in the experiments to establish the calibration curve, 3 ml of n-hexane was added and the mixture was shaken well. After the removal of the film, the aqueous layer was sampled and its absorbance at 631 nm was measured. The amount of immobilized HA was then calculated using the previously established calibration curve.

**Film characterization**

XPS analysis of the film surface was made on an AXIS HSi spectrometer (Kratos Analytical Ltd.) as described in Section 3.2.

Surface water contact angles were measured at 25°C and 60% relative humidity by the sessile drop method, using a 3-µl water droplet in a telescopic goniometer (Rame-Hart, Model 100-0-230). The telescope with a magnification power of 23× was equipped with a protractor of 1 deg graduation.

The conductivity of the polypyrrole films was measured by a four-point probe as specified in Section 3.2.

Scanning electron microscopy (SEM) was carried out on a JEOL JSM 5600LV scanning electron microscope. The samples were cleaned with an inert dusting gas and sputter-coated with a thin film of platinum for imaging purposes. Images were taken at 15 kV at a magnification of ×10,000.
**Protein binding assay** (Collier, et al., 2000)

Films of 2 cm × 2 cm in size were attached to a 12-well tissue culture plate and were washed with two 10-min rinses in a washing solution (0.85% NaCl, 0.05% Tween 20). The films were transferred to another well and the blocking solution (3% bovine serum albumin (BSA) in 10 mM phosphate buffered saline (PBS), pH 7.4, 0.9% NaCl) was then added. The films were washed again after 1 h with two 10-min rinses in the washing solution and incubated in bHABP solution (3.4 µg/ml bHABP in blocking solution) at room temperature for 24 h, after which they were rewashed and transferred to another well. 1 ml avidin-biotin-peroxidase complex was then added for 1 h. The films were washed and transferred to another well with 2 ml of OPD substrate added. The substrate reaction was allowed to proceed for 12 min. Then the solutions were transferred to a quartz cell in 75-µl aliquots and quenched with 20 µl of 3 M sulfuric acid. The absorbance of the solutions was then measured at 490 nm with an aliquot of OPD substrate used as reference.
4.3 Results and Discussion

4.3.1 Surface functionalization of PPY films

In Steps (a) and (b) of Figure 4.2, the PPY films were first graft copolymerized with HEA and then silanized with ATS. It is postulated that the grafting of HEA occurs under UV irradiation via reactions with the surface oxide and peroxide groups generated by the plasma pretreatment of the PPY film, resulting in the formation of ether linkages to the PPY surface. The success of the UV-induced surface graft copolymerization of HEA on the PPY film and the subsequent silanization can be ascertained by comparing the XPS spectra of the films before and after each step (Figure 4.3). The XPS C 1s and N 1s core-level spectra of the as-synthesized PPY have already been shown in Section 3.3. The presence of surface grafted HEA polymer can be deduced from the C 1s spectrum of the PPY after the UV-induced graft copolymerization step (Figure 4.3(a)) by the increase in intensity of the C-O peak component and a new peak at 288.5 eV attributable to the COO species as dictated by the chemical structure of HEA. The corresponding N 1s spectrum (Figure 4.3(b)) shows little change except for a small decrease in the \( \text{N}^+ / \text{N} \) ratio to 0.21.

The extent of surface grafting of HEA is estimated from the corrected area ratio of the C 1s peak component at 288.5 eV for the COO species and the total N 1s peak due to the PPY film and is expressed as the \([\text{COO}^-]/[\text{N}]\) ratio. The surface graft concentration of the HEA polymer increases with the respective monomer concentration used for graft copolymerization as shown in Figure 4.4.
Figure 4.3 XPS C 1s and N 1s core-level spectra of (a) and (b) PPY film after UV-induced graft copolymerization with HEA in 5 vol.% HEA monomer in dioxane solution; (c) and (d) XPS C 1s and Si 2p core-level spectra of PPY film after UV-induced surface graft copolymerization with 5 vol.% HEA and subsequent silanization with 1 vol.% ATS in dioxane.
Figure 4.4 Effect of HEA monomer concentration on the surface graft concentration of the HEA polymer and on the amounts of chemisorbed silane.

Figure 4.3(c) and (d) show the C 1s and Si 2p core-level spectra of the PPY film after UV-induced surface graft copolymerization with 5 vol.% HEA and subsequent silanization with 1 vol.% ATS in dioxane. No significant difference can be observed in the C 1s core-level spectra before (Figure 4.3(a)) and after silanization process (Figure 4.3(c)). The appearance of the Si 2p core-level signal after silanization as shown in Figure 4.3(d) indicates that the ATS must have been successfully chemisorbed onto the HEA grafted PPY film surfaces. The effect of increasing the HEA monomer concentration used for graft copolymerization on the amount of chemisorbed silane on
the respective PPY surfaces, expressed as the \([\text{Si}]/([\text{N}]-[\text{Si}])\) ratio, is also shown in Figure 4.4. The \([\text{Si}]/([\text{N}]-[\text{Si}])\) ratio increases with increasing HEA monomer concentration and this phenomenon suggests that the amount of chemisorbed silane depends on the concentration of hydroxyl groups arising from the grafted HEA polymer. However, this trend gradually levels off at higher HEA monomer concentrations. Through the silanization process, primary amine groups are introduced on the PPY (which would serve to couple with the WSC activated HA, as shown in Figure 4.2). Thus, the concentration of the amine groups will vary with the HEA monomer concentration used, in the same manner as the chemisorbed silane, as indicated in Figure 4.4.

### 4.3.2 Immobilization of HA

The presence of HA on the surfaces of the respective PPY films after Step (d) of Figure 4.2 can also be confirmed using XPS analysis. The XPS C 1s and N 1s core-level spectra of the PPY film surface grafted with HEA and then silanized with ATS are shown in Figure 4.5(a) and (b). The corresponding spectra after HA immobilization on these films are shown in Figure 4.5(c) and (d). In Figure 4.5(c), the peak attributable to the C-O species is greatly enhanced while a new peak attributable to the C=O species can be observed. The COO peak is reduced compared with that in Figure 4.5(a). These changes are quite consistent with the chemical structure of HA (Figure 4.1). To further confirm that the changes in the XPS results are due to the surface coverage of HA, the C 1s core-level spectrum of pristine HA shown in Figure 4.5(e) can be compared with Figure 4.5(c). The similarity of the two spectra is obvious.
Figure 4.5 XPS C 1s and N 1s core-level spectra of (a) and (b) PPY film after UV-induced graft copolymerization with HEA in 5 vol.% HEA monomer in dioxane solution and subsequently silanized with 1 vol.% ATS in dioxane; (c) and (d) the PPY film graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane followed by HA immobilization; (e) and (f) pristine HA.
A similar comparison of the N 1s spectra can be made. The N 1s core-level spectrum in Figure 4.5(b) for the PPY film after silanization shows an additional peak at 398.2 eV attributable to =N- species, as compared to the spectrum of the film before ATS silanization (Figure 4.3(b)). The washing process with dioxane after silanization may have caused partial deprotonation of the PPY film itself where some of the N\textsuperscript{+} groups are converted to =N- groups. A reduction in the S 2p core-level signal is also seen in the XPS wide scan indicating the loss of some of the toluene sulfonic acid groups acting as counterions to the N\textsuperscript{+}, which confirms that partial deprotonation of the PPY film has occurred. The N 1s spectrum of the PPY film after HA immobilization (Figure 4.5(d)) shows considerable differences from that in Figure 4.5(b). The peak attributable to the =N- species has almost disappeared while the peak attributable to the NH species is enhanced. These changes are consistent with the structure of HA since it does not have =N- groups, as evident from its N 1s spectrum in Figure 4.5(f). Since the amount of HA immobilized may be less than the probing depth of the XPS technique, the high binding energy tail seen in Figure 4.5(d) may be attributable to the N\textsuperscript{+} groups of the underlying PPY film.

The amount of immobilized HA per unit surface area of the film (as determined using the toluidine blue method) is plotted against the HEA monomer concentration in Figure 4.6. As expected, the amount of HA increases with the HEA monomer concentration used in Step (a) of Figure 4.2. The gradual leveling off of the amount of HA immobilized at higher HEA monomer concentrations as shown in Figure 4.6 is similar to the trend observed for the amount of chemisorbed silane (Figure 4.4). This observation further confirms the direct relationship between the number of primary amine groups on the PPY surface and the amount of HA immobilized. As HA is a
Figure 4.6 Amount of HA anchored on HEA surface-copolymerized PPY films as a function of HEA monomer concentration used in the graft copolymerization process. The solid symbols denote results obtained with the silanized substrate while the open symbol is for the non-silanized substrate.

macromolecule with an average molecular weight of 350-400 kDa (Scott et al., 1990), steric hindrance may also be an inhibiting factor at high levels of immobilization.

It should be mentioned that ‘control experiments’ for HA immobilization were carried out with the as-synthesized PPY film and the PPY film grafted with 6 vol.% HEA without ATS silanization. For the former, the toluidine blue test indicated that there is
no significant HA immobilized on this film. In the case of the latter, the amount of HA immobilized, shown by the open symbol in Figure 4.6, is almost negligible compared with the amount on the corresponding silanized substrate. Therefore, it can be concluded that the amine groups introduced on the PPY film through silanization contribute predominantly to the coupling reaction with the –COOH groups of HA. Another point to note is that in the toluidine blue method for determining the amount of HA, the pH value of the system was found to have a small effect on the interaction between the HA molecule and toluidine blue. The pKₐ value of HA is about 3 (Rueda, et al., 2001) and the pH value of the solution used to obtain the results in Figure 4.6 is about 2.2. Under this condition, it can be expected that the H⁺ in the solution would be mostly from the HCl added to maintain the pH of the solution and the effect of the COOH groups of HA would be negligible.

Since the water retention property of HA is of interest for cosmetic applications (Davies, et al., 1982), the water contact angle of films with different amounts of HA immobilized was measured and the results are shown in Table 4.1. The as-synthesized PPY film without any surface modification is fairly hydrophobic with a water contact angle of about 70° while the HA immobilized PPY films are quite hydrophilic with water contact angles of around 30°. The electrical conductivity of the different films is also shown in Table 4.1. The electrical conductivity of the as-synthesized PPY film is 71 S/cm which is close to the value reported in the literature for similar films (Lee and Chung, 1993). The PPY films retain a relatively high level of conductivity even with a high concentration of HA immobilized. This property suggests further potential for enhancing the biological behavior of the immobilized biomolecules via the application of electrical stimulation through the PPY film. Furthermore, the HA functionalized PPY films still possess good mechanical properties. Mechanical tests (performed on an
Instron 5544 tensile tester at a constant crosshead displacement rate of 25.4 mm/min) showed that for the HA functionalized PPY film (Sample P-6%H-Si-HA in Table 4.1) a maximum load of 11.9 N with an elongation of about 20% was achieved. These values are comparable to those of the as-synthesized PPY film which recorded a maximum load of 13.4 N and an elongation of about 24% under the same conditions. Therefore, the present surface modification technique for HA immobilization on PPY films overcomes the drawback present in the entrapment technique which was reported to result in poorer mechanical properties of the film (Collier, et al., 2000).

<table>
<thead>
<tr>
<th>Film type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HA immobilized (Toluidine blue method) (mg/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Conductivity, σ (S/cm)</th>
<th>Surface wettability: water contact angle, θ (degree)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPY</td>
<td>0</td>
<td>71 ± 1</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>P-2%H-Si-HA</td>
<td>0.0274</td>
<td>68 ± 1</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>P-3%H-Si-HA</td>
<td>0.0301</td>
<td>63 ± 2</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>P-4%H-Si-HA</td>
<td>0.0364</td>
<td>62 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>P-5%H-Si-HA</td>
<td>0.0502</td>
<td>55 ± 1</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>P-6%H-Si-HA</td>
<td>0.0628</td>
<td>44 ± 1</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> PPY denotes as-synthesized PPY film; P-X%H-Si-HA denotes HA immobilized on PPY film which has been surface graft-copolymerized with X vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane.
The change in the surface morphology of the PPY film after HA immobilization can be observed by means of SEM. Figure 4.7 shows the SEM images of the as-synthesized PPY film and PPY film after surface grafting with HEA and subsequently silanized with ATS and PPY film after HA immobilization. The as-synthesized PPY film and the film after surface grafting and silanization are very similar in surface morphology (Figure 4.7(a) and (b)). However, the surface morphology has changed significantly after HA immobilization (Figure 4.7(c)) and spherical nodules of HA are clearly visible on the PPY surface.

4.3.3 Protein binding assay

Protein binding assay was performed to determine whether the HA immobilized on the PPY film is biologically active. Figure 4.8 shows the absorbance of the OPD substrate at 490 nm after interaction with the PPY film functionalized with HA, and those obtained with the other two films without HA. It has been shown that OPD was the most sensitive substrate for the detection of peroxidase and the specificity of this assay has also been firmly established (Varga et al., 2002). Therefore, by observing the UV absorbance at 490 nm, the amount of the peroxidase by way of the avidin-biotin-peroxidase attached to the bHABP (which binds specifically with high affinity to the immobilized HA) can be inferred. Thus, Figure 4.8 confirms the biological activity of the immobilized HA on the PPY film. The protein binding assay of films with different amounts of immobilized HA (as determined by the toluidine blue method) was also carried out and the results are shown in Figure 4.9. The intensity of the absorbance at 490 nm increases with the amount of HA immobilized on film surface but it gradually approaches an asymptotic level. This phenomenon may be due to the conformational changes of HA after immobilization which affects its effectiveness in protein binding.
Figure 4.7 Scanning electron micrographs of different PPY films: (a) as-synthesized PPY film; (b) PPY surface grafted with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane; (c) PPY film graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane followed by HA immobilization.
Figure 4.8 Amount of biologically active HA immobilized on PPY film surface as indicated by protein binding assay. PPY: as-synthesized polypyrrole film; P-H-Si: PPY film graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane; P-H-Si-HA: HA immobilized P-H-Si film; P-H-Si-HA (after 4 days): P-H-Si-HA after storage in air at room temperature for 4 days.
Figure 4.9 Amount of specific protein binding with HA (as measured by the OPD substrate absorbance at 490 nm) as a function of amount of HA immobilized on PPY film.
4.3.4 Stability test

XPS analyses were carried out to assess the changes in the chemical structure of the HA immobilized on PPY film after the films were stored in air and water at room temperature over a period of time. The C 1s spectrum of the HA modified PPY films after storage in air for 2 days (Figure 4.10(a)) shows that the intensities of the peaks at the BE of 287.6 eV and 286.2 eV attributable to the C=O and C-O respectively, are reduced compared with those of the Figure 4.5(c) which is for the freshly prepared film. On the other hand, the COO- component of the film after storage in air shows an increase in intensity. Furthermore, the N 1s core-level spectrum in Figure 4.10(b) shows that the peaks with binding energy above 400 attributable to positively charged nitrogen are also higher in intensity compared to Figure 4.5(d). Increasing the storage time to 4 days (Figure 4.10(c) and (d)) does not result in further significant changes in the C 1s and N 1s spectra beyond those observed after 2 days. These results suggest that the immobilized HA may have experienced a degree of structural degradation or dissociation of the functional groups of the PPY film surface, and these changes occur within the first few days of storage. These changes lead to a higher doping level of the PPY film surface as indicated by the N⁺/N ratio.

Parallel experiments were carried out with the samples stored in water and XPS results for these samples are shown in Figure 4.10(e) to (h). A similar conclusion can be drawn but the degree of degradation or dissociation is more severe for films stored in water. It has been proven for HA in aqueous solutions that some conformational changes take place as a consequence of the break of intramolecular links and the beginning of the ionization process (Rueda et al., 2001). It is possible that some degree of conformational changes has also occurred with the immobilized HA in the present case. The exact mechanism for this degradation is unknown but in the aqueous
Figure 4.10 XPS C 1s and N 1s core-level spectra of HA immobilized PPY film (which has been graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane) after storage in (a) and (b) air at room temperature for 2 days; (c) and (d) air at room temperature for 4 days; (e) and (f) water for 2 days; (g) and (h) water for 4 days.
medium hydrolysis is a possibility (Collier et al, 2000). It can also be seen from Figure 4.10 that the N 1s spectra of the films after storage in water (Figure 4.10(f) and (h)) are quite different from those of the as-synthesized film or the film stored in air. In Figure 4.10(f) and (h), the peak at 398.2 eV attributable to the =N- species is obvious and this peak seems enhanced with the increase in the storage time in water. The presence of the –N= species suggests that the deprotonation of the PPY film has occurred during storage in water.

The toluidine blue method was used to further investigate the stability of the immobilized HA, and the results showed that after storage in air and water for 4 days respectively the amount of immobilized HA on the PPY films (grafted with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS) decreases to 0.044 and 0.038 mg/cm$^2$, compared to 0.050 mg/cm$^2$ for the freshly made film. Therefore, it can be concluded that about 80 to 90% of the HA still remains immobilized on the film surface even after 4 days in air or water.

The biological activity of the immobilized HA after storage in air for four days at room temperature was evaluated by the protein binding assay. As shown in Figure 4.8, the absorbance obtained with a freshly prepared HA immobilized PPY film (graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane) is 0.64 while the sample prepared under same conditions and stored for four days in air has an absorbance of 0.28, which is about 43% of the former. Therefore, the immobilized HA still remains its biological activity even after 4 days storage in air but the biological activity has decreased, consistent with the structural changes indicated by the XPS analysis.
4.4 Conclusion

Electrochemically synthesized PPY films can readily undergo surface graft copolymerization with HEA and the hydroxyl groups arising from the grafted HEA can be used to chemisorb silane so as to introduce the desired primary amine functional groups. Further functionalization of the film is possible via the primary amine groups on the film surface which react with the COOH groups of HA activated by WSC. The HEA graft concentration varies with the monomer concentration and is of key importance in the further functionalization process since it determines the amount of functional groups available for linking the biomolecules. The amount of HA immobilized on PPY film as determined by toluidine blue method ranges from 0.03 to 0.07 mg/cm², depending on the HEA graft concentration on the PPY surface. The HA modified PPY film still retains its electrical conductivity and mechanical strength, with improved surface wettability imparted by the immobilized HA. The immobilized HA is biologically active as evident from its high affinity for the HA binding protein. A certain amount of degradation of the HA is observed after storage in air or water at room temperature, and its activity correspondingly decreases. After 4 days of storage in air, the HA still retains about 40% of its activity. Therefore, electroactive polypyrrole surface functionalized with biologically active HA may find further application in the development of new tissue engineering strategies, wound healing and angiogenesis. The in vitro cell compatibility of such material is assayed in the following chapter.
CHAPTER 5

ASSESSMENT OF BIOLOGICAL RESPONSES OF
HYALURONIC ACID FUNCTIONALIZED
POLYPYRROLE FILM
Chapter 5

5.1 Interactions of Hyaluronic Acid Functionalized Polypyrrole Film with PC12 Cells

5.1.1 Introduction

The ability to monitor biomolecular interactions, manipulate the growth/differentiation and eventually to regulate the functionalities of the dynamic cellular system is perhaps the most important requirement of biomolecular communication (Campbell et al., 1999; Garner et al., 1999; Jiang et al., 2002; Kotwal and Schmidt, 2001; Schmidt, et al., 1997). Therefore, it is of paramount importance to assess the biological functions of a material desired for such ability from a cellular response perspective.

It has been shown in the previous chapter that HA can be successfully immobilized on PPY film surface via a surface modification technique. The immobilized HA is still biologically active as shown by the protein binding assay, and the functionalized PPY film also retains electrical conductivity and mechanical strength after surface functionalization. It has also been shown that in the presence of HA, nerve regeneration and reinnervation show better conduction velocity, higher axon counts and a trend toward earlier myelination (Seckel et al., 1995). Hence, the bioactivity of the PPY-HA system in \textit{in vitro} neural cell culture was also investigated in this work.

PC12, a cell line originally derived from a transplantable rat adrenal pheochromocytoma (Greene and Tischler, 1976), has been used as a model system for neurobiological and neurochemical studies. These cells respond reversibly to nerve growth factor (NGF) and in the process, they become electrically excitable, and increase catecholamine synthesis and synapses formation with the assumption of many characteristics of sympathetic neurons (Greene and Tischler, 1982). Hence, in the present work PC12 was chosen for the evaluation of the application of HA
immobilized PPY in the neurite outgrowth. The cell attachment on PPY films, pristine and surface modified, and the attachment kinetics were investigated. Cell growth in the presence and absence of NGF was also evaluated using scanning electron microscopy (SEM).
5.1.2 Experimental

Materials and reagents

Type I collagen (Vitrogen, 3.0 mg/ml in 0.012 N HCl) was purchased from Cohesion Technologies Inc. 10× PBS (containing 80 g of sodium chloride, 2 g of potassium chloride, 11.5 g of anhydrous disodium phosphate and 2 g of anhydrous monopotassium phosphate in 1 L of distilled water) and its 10× diluted counterpart or 1× PBS, were obtained from Pierce Chem. Co. BCA Protein Assay Reagent Kit was a product from Pierce Biotechnology. Reporter Lysis Buffer was obtained from Promega Corporation. Trypsin-EDTA solution, Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin and heat-inactivated horse serum were all from Sigma Chemical Co. Fetal calf serum was obtained from Moregate BioTech.

Cell culture and cell attachment assay

Rat PC12 cells from ATCC (American Type Culture Collection) were passaged at least once after thawing, and cultured in rat tail collagen-coated tissue culture flasks (Falcon) in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum and 100 U/ml penicillin-streptomycin. The cells were maintained at 37°C in an incubator of a humidified 5% CO₂ atmosphere with the medium replaced every 2~3 days and passaged following detachment with trypsin (0.25% trypsin-EDTA) (Akeson and Warren, 1986; Blackman et al., 1993).

Pristine and modified PPY films were cut into round discs with a diameter ~15 mm and fixed into a 24-well tissue culture plate by silicone adhesive (Applied Silicone Implant Grade). The plate was then sterilized in 70% ethanol for 3 h and washed three times with PBS. The cell attachment on four types of surfaces was assayed: pristine
PPY; PPY film graft-copolymerized with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane (P-5%H-Si); P-5%H-Si with subsequent immobilization of HA (P-5%H-Si-HA); uncoated tissue culture polystyrene (TCPS).

Cells were detached from the tissue culture flasks, collected by centrifugation, and resuspended in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum and 100 U/ml penicillin-streptomycin at a density of $3.3 \times 10^4$ cells/ml as determined by a hemocytometer. 300 µl of the above cell-containing medium was then dispensed on the 24-well tissue culture plate containing different films so as to yield the desired cell density of $1 \times 10^4$ cells/well. The plate was then placed in an incubator with a humidified 5% CO$_2$ atmosphere. The above experiment was also performed under the same conditions with the addition of NGF in the above medium at a concentration of 50 ng/ml. This concentration has been shown to induce maximal neurite outgrowth (Damon et al., 1988).

For the cell attachment assay, the plate was taken out from the incubator after two hours and gently shaken. The medium in each well containing unattached cells was aspirated and transferred to microcentrifuge tubes. The well was washed with 300 µl PBS and the washing solution was combined with the medium in the corresponding tube. The cells in the tubes were collected by centrifugation at 4000 rpm for 10 min and washed once with PBS. The PBS was then removed and 200 µl 1× Reporter Lysis Buffer was then added into each tube. The tubes were then vortexed and subjected to three freeze-thaw cycles. The protein concentration in the lysate was then determined by BCA protein assay (Shihabi and Dyers, 1988; Smith et al., 1985; Wiechelman et al., 1988). The number of attached cells was calculated from the difference in the cell
seeding density and the number of unattached cells determined from the BCA protein assay.

**Cell culture on different surfaces**

The attached PC12 cells on different surfaces were cultured for several days in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum and 100 U/ml penicillin-streptomycin. The medium was replenished every other day. For cells primed with NGF at the time of cell seeding, NGF at 50 ng/ml was also added when the medium was changed.

At the end of the culture period the samples were immediately washed with PBS. An aqueous solution of 3 vol.% glutaraldehyde was added and the sample was stored at 4 °C for 5 h. The glutaraldehyde solution was then removed and the sample was washed with PBS, followed by step dehydration with 25%, 50%, 70%, 95% and 100% ethanol for 10 min each. The sample was then dried for SEM characterization using a JEOL JSM 5600LV scanning electron microscope. For imaging purposes the samples were sputter-coated with a thin film of platinum. The images were taken at either 15 kV or 20 kV at magnifications of ×500 and ×1500.
5.1.3 Results and Discussion

Cell attachment

Figure 5.1 shows the percentage of PC12 cells attached on the pristine PPY, P-5%H-Si, P-5%H-Si-HA and TCPS after two hours’ incubation at 37 °C in the absence of NGF. More than 70% of the cells are attached on TCPS, while the PC12 cells show the least affinity for the P-5%H-Si-HA surface (18%), followed by P-5%H-Si (22%) and PPY (40%). The poor adhesion of the PC12 cells on the HA functionalized PPY surface is similar to that demonstrated by many cell types such as 3T3 BALB cells (Abatangelo et al., 1982; Carbonetto et al., 1983). HA have long been implicated in cell migration as supported by the in vivo observation that the extracellular matrix is enriched in HA during embryonic morphogenesis and during the invasion of fibroblasts in the early stage of tissue repair in the adult organism (Toole et al., 1984). However, there is no unanimous conclusion as to whether those events are HA concentration-dependent or cell-type dependent.

In the presence of NGF, the cell attachment increases substantially for all four surfaces with the P-5%H-Si-HA surface showing the largest increase, from < 20% to about 80%. A previous study (Akeson and Warren, 1986) has also shown that NGF can increase the percentage of PC12 cells adherent on a number of substrates. This increase may bear some relationship with the interaction of HA on the film surface with the HA receptors on cell surfaces, as molecules of the extracellular matrix regulate adhesive interactions and signaling events in a number of cellular processes including growth and motility through binding to their corresponding receptors on the cell surface. It has been verified that the cell surface glycoprotein (CD44) and RHAMM (receptor for HA-mediated motility) can bind hyaluronan, and RHAMM is concentrated at the tips as well as along neurites of PC12 cells upon the treatment with
NGF and is a major neuronal HA receptor (Nagy et al., 1995). Therefore, it is possible that cell binding on the HA functionalized PPY surface in this work involves a HA recognition process. Since it is well established that upon NGF stimulation, PC12 cells differentiate into sympathetic neuron-like cells with neurites, the substantial increase in the cell attachment on the P-5%H-Si-HA surface is most probably mediated by an increase in the expression of RHAMM.

![Figure 5.1](image)

**Figure 5.1** PC12 cell attachment on pristine PPY, silanized and HEA graft-copolymerized PPY film (carried out with 5 vol.% HEA monomer in dioxane solution and subsequent silanization with 1 vol.% ATS in dioxane) (P-5%H-Si), the HA functionalized PPY film (after graft-copolymerized with HEA and subsequently silanized) (P-5%H-Si-HA), and tissue culture polystyrene (TCPS). The attachment was assessed 2 h after cell seeding both with and without 50 ng/ml NGF.
To further study the time sequence of the cell binding events, different sets of experiments were performed by varying the time interval of the attachment assay so as to investigate the adhesion kinetics of PC12 cells. The results of these experiments which were carried out in the presence of NGF are shown in Figure 5.2. The percentages of cell attachment were almost similar for all four substrates in a short term-assay (30 min). However, after a 1 h interval, the difference in cell attachment is very significant. From 30 min to 1 h, the rate of increase in cell adhesion on TCPS is the highest, followed by that of PPY, while P-5%H-Si and P-5%H-Si-HA showed a similar rate of increase in cell adhesion. With further increase in culture time beyond 1 h, the rate of cell attachment slows down significantly, with the exception of the HA functionalized PPY substrate (P-5%H-Si-HA). As mentioned above, the HA recognition process by the cell receptor may have contributed to this phenomenon since the cell attachment on the other substrates appears to be reaching a saturation level after 1 h.

![Figure 5.2 Adhesion kinetics of PC12 cells on the PPY, P-5%H-Si, P-5%H-Si-HA and TCPS substrates. All the assessments were carried out in the presence of 50 ng/ml NGF. Values reported are the average values from four similar samples in four separate experiments.](image)
Morphology of PC12 cultured on different surfaces

After 36 h of culture with NGF, the PC12 cells on the different substrates exhibited different morphologies as shown by the SEM images in Figure 5.3(a) to Figure 5.3(d). Cells on TCPS were more elongated and well separated (Figure 5.3(a)) with irregular polygonal shapes (Figure 5.3(b)) and distinguished processes in certain directions. On the P-5%H-Si-HA surfaces, the cells possessed a rounded morphology (Figure 5.3(c)) and aggregates of round cells were also formed on this surface, as shown in Figure 5.3(d). Since the cells were cultured on these substrates under the same conditions, the possibility that at the time of seeding, the cells on the P-5%H-Si-HA surface were in the form of aggregates can be ruled out since those on TCPS were well distributed without any obvious aggregation. Therefore, the aggregates observed on P-5%H-Si-HA surface most probably resulted from cell migration. Furthermore, the cells on PPY and P-5%H-Si also exhibited the rounded morphology similar to those on P-5%H-Si-HA but without obvious aggregation (results not shown). As been suggested by Turley, HA opens up migration paths and actually promotes locomotion (Turkey, 1989). Hence, HA may have played a role in the aggregation of the cells. However, such suggestions have not been unanimously agreed upon because there are reports that HA actually prevents the aggregation of chondrogenic and SV-3T3 cells (Pessac and Defendi, 1972; Underhill and Dorfman, 1978), whereas it supports the induction of aggregation of lymphoid cell lines (Underhill and Toole, 1979; Wasteson et al., 1973). The form of the HA existing under the experimental conditions as well as its concentration may have contributed to the differences observed.
Figure 5.3 Scanning electron micrographs of PC12 cells after culturing in the presence of NGF added at the same time as cell seeding, after 36 h on TCPS (a) and (b); after 36 h on P-5%H-Si-HA (c) and (d); after 96 h culture on TCPS (e) and (f) and after 96 h on P-5%H-Si-HA surface (g) and (h).
After 4 days of culture under the same conditions (with the medium changed as specified in the Section 5.1.2), the PC12 cells appeared to grow in different ways on different substrates. PC12 cells on TCPS (Figure 5.3(e) and (f)) tended to form a monolayer after 4 days culture with a few small aggregates resulting from the spread of the cell bodies. Most of PC12 cells on TCPS showed confluence with the cell bodies not as distinguished as those observed after 36 h (Figure 5.3(a) and (b)). However, the cells on the P-5%H-Si-HA surface still retained a rounded morphology in the form of aggregates, and the multi-processes after the 4-day culture (as shown in Figure 5.3(g) and (h)) are longer than those on cell bodies after 36 h culture (Figure 5.3(c) and (d)). Since cells on P-5%H-Si-HA surface are likely to remain as aggregates, these neurites are well connected with each other.

From the observation of the morphology of the cells after different time intervals, it appeared that the cells on P-5%H-Si-HA surface formed aggregates first followed by the neurite outgrowth, while cells on TCPS expressed processes quickly and formed a monolayer after several days’ culture. However, the surface of the cell bodies on P-5%H-Si-HA appeared rough and this may be attributed to the many short neurites on the cell surface. It seems that the cells on P-5%H-Si-HA experienced a more dynamic process which is mediated by the covalently immobilized HA molecules. These cells after seeding are likely to form aggregates first followed by the subsequent process expression. Moreover, the aggregate morphology would enhance correlation of cell-cell interactions with cell differentiation, viability and migration as well as subsequent tissue formation. Therefore, it would be desirable to have aggregates for cell function and survival.
Further experiments to observe cell morphology were carried out under the same culture conditions but without NGF added. Figure 5.4(a) to (d) show the results after 4 days of culture. Cell fusion was observed on TCPS (Figure 5.4(a) and (b)) but the cells still formed a layer, though not as well established as that of corresponding cells with NGF added (Figure 5.3(e) and (f)). Furthermore, the processes were poorly expressed since there is no NGF stimulation on its neurite outgrowth. However, the cells on P-5%H-Si-HA surface showed similar morphologies with aggregates and multi-directional processes (Figure 5.4(c) and (d)) as the corresponding case with NGF added (Figure 5.3(g) and (h)). The only difference is the decrease in cell density which is also confirmed by the cell binding assay (Figure 5.1) as mentioned earlier. Thus, the neurites of PC12 cells were also well-grown even without NGF induction.

Additional experiments were also conducted for the cells which were already primed with NGF at a concentration of 50 ng/ml for 4 days before the cell seeding, followed by the continuous addition of NGF when the medium was changed during cell culture. These cells after detachment from the tissue culture flask were seeded on P-5%H-Si-HA and cultured for 6 days and the results are shown in Figure 5.4(e) and (f). These cells, also with multi-directional expressed processes, appeared more homogenously distributed on the surface instead of existing in large aggregates. But these cells should still have good communications with each other as they are distributed like a well-organized net. Since these cells have already expressed processes before they were dispensed on the surface, the expression would likely continue and these neurites would prevent the further migration of cells towards each other when they reach a certain distance between them. From the above results and those from Section 4.3, it is expected that HA can play an important role as an agent which aids nerve growth.
Figure 5.4 Scanning electron micrographs of PC12 cells without NGF after 96 h culture, on TCPS (a) and (b); on P-5%H-Si-HA surface (c) and (d). Micrographs of PC12 cells which were first primed with 50 ng/ml NGF for 96 h before seeding and followed by 6 days of culture on P-5%H-Si-HA surface in the presence of 50 ng/ml NGF (e) and (f).
5.1.4 Conclusion

Hyaluronic acid functionalized PPY film surface has been shown to support PC12 cell culture with no cytotoxic effects. From the PC12 cellular responses, it can be concluded that the immobilized HA still retains biological activity. Cell attachment, as determined by BCA protein assay, shows that the HA functionalized PPY surface exhibited a high cell attachment ratio of about 80%, which is desirable for a biomaterial designed specifically for the application of nerve regeneration and nerve repair. PC12 cells on HA functionalized PPY surface display a different morphology compared with those on TCPS as well as those on pristine PPY, and PPY functionalized with HEA and ATS prior to HA immobilization. HA promotes cell locomotion resulting in aggregate formation on the PPY surface, and the neurite growth of PC12 is enhanced even in the absence of NGF. As most cellular responses to specific biomolecules are concentration sensitive as well as environmental sensitive, further work may focus on the investigation of the optimal relative concentrations of HA and NGF required to achieve the conditions more suitable for medical implantation applications. Moreover, with the choice of PPY as substrate, the application of electrical stimulation coupled with the effects of HA on the neurite outgrowth and other cellular events is a promising subject for further investigation.
5.2 Antibacterial Assay of Hyaluronic Acid Functionalized Electroactive Polymer

5.2.1 Introduction

Our work in Section 5.1 investigates the interaction of the HA functionalized PPY film surface with neural cells, which shows promise for the development of this material in medical implantation application. However, materials for biomedical application are often associated with a serious clinical problem of infections. Therefore, this part of the work was designed to exploit the role of HA surface coating to impart the material surface with fouling resistance. It has been proposed that the hydrophobic and ionic interactions between the surface of biomaterials and bacterial cell membranes play an important role in bacterial adhesion (Hogy et al., 1986). Therefore, the issue of bacterial adhesion is addressed in this work and comparison is also made between the PPY films before and after surface modification with HA. *Escherichia coli* (*E. coli*) was selected for this study as this species is one of those frequently implicated in implant infections (Dobbins et al., 1988; Gristina et al., 1988). Furthermore, the cell viability of the adhered *E. coli* on different film surfaces was also assessed.

5.2.2 Experimental

Materials and reagents

The PPY films were synthesized as described in Section 3.2, and the HA functionalized PPY film (denoted as PPY-HA) was obtained from graft-copolymerization of PPY film with 5 vol.% HEA and subsequently silanized with 1 vol.% ATS in dioxane, followed by HA immobilization as specified in Section 4.2. Biaxially oriented poly(ethylene terephthalate) (PET) films of about 100 µm in thickness were purchased from the Goodfellow Inc. Peptone, yeast extract, agar and
beef extract were purchased from Oxoid. *E. coli* was obtained from American Type Culture Collection (ATCC, # 14948). Phosphate buffer solution (PBS) (containing NaH$_2$PO$_4$ of 4.68 g/l and Na$_2$HPO$_4$ of 8.662 g/l, pH of 7.0) was prepared afresh and sterilized in an autoclave before use.

**Bacteria culture**

*E. coli* was cultivated in 50 ml of a 3.1% yeast-dextrose broth (containing 10 g/l peptone, 8 g/l beef extract, 5 g/l sodium chloride, 5 g/l glucose and 3 g/l yeast extract at a pH of 6.8) (Cunliffe et al., 1999) at 37°C. The bacterial cell concentration was estimated from the optical density at 540 nm on the assumption that an optical density of 1.0 at 540 nm is equivalent to approximately $10^9$ cells per ml (Hogt et al., 1986). Solid growth agar plates were prepared by adding 1.5 wt.% agar in yeast-dextrose broth, autoclaved, poured into a Petri dish, and dried under reduced pressure at room temperature overnight.

**Bacterial adhesion assay**

The bacteria attachment on pristine PPY and PPY-HA surfaces was assayed as follows: The films were cut into strips of 2 cm × 3 cm in size. The *E. coli*-containing broth was centrifuged at 2700 rpm for 10 min, and after the removal of the supernatant, the cells were washed twice with PBS (pH 7.0) and resuspended in PBS at a concentration of $10^7$ cells/ml. The substrates were immersed in this suspension in a sterile Erlenmeyer flask. The flask was then shaken at 200 rpm at 37°C for 2 h. After that, the substrates were removed from the above flask and immediately washed with sterile PBS. Three different experiments were carried out: (i) In the first series, after the substrates were washed with sterile PBS, an aqueous solution of 3 vol.% glutaraldehyde was added.
The substrates were then stored at 4°C for 5 h. The glutaraldehyde solution was then removed and the substrates were washed with PBS, followed by step dehydration with 25%, 50%, 70%, 95% and 100% ethanol for 10 min each. The substrates were then dried for SEM characterization; (ii) In the second series, solid growth agar was immediately added to the substrates after washing with PBS, which were then sealed in Petri dishes and incubated at 37°C for 24 h. After that, these substrates were washed with PBS and prepared for SEM characterization; (iii) In the third series, after the samples were washed with PBS, the moisture on the substrate surface was removed using sterile filter paper. The substrates were transferred to a yeast-dextrose broth. After culturing at 37°C for 24 h, the substrates were removed and the optical density at 540 nm of the broth was then measured. The pristine PET film, representative of the normal polymeric films with wide applications, was also assayed as the control experiments and the corresponding optical density of the broth at 540 nm was then used to normalize those obtained from runs using PPY and PPY-HA.

**Bactericidal effect**

Pristine PPY and PPY-HA films were cut into round discs of diameter ~15 mm and fixed into a 24-well tissue culture plate (TCPS) by silicone adhesive (Applied Silicone Implant Grade). The PET film was also used as one sample for comparison, and was prepared in the same manner as that used for PPY films. Therefore, each plate comprised four samples: PPY; PPY-HA; PET; and TCPS (no sample added and used as a control) and each sample was prepared in triplicate. The *E. coli*-containing broth was centrifuged at 2700 rpm for 10 min, and after the removal of the supernatant, the cells were washed twice with PBS and resuspended in PBS at a concentration of 10^5 cells/ml. 200 µl of the above *E. coli*-containing suspension was then dispensed on the
24-well tissue culture plate containing the four samples. The plates were then placed in an incubator at a constant temperature of 37°C. After predetermined time intervals, a plate was removed from the incubator and 3 × 20 µl samples were taken from each well and spread onto the separate solid growth agar plate and incubated at 37°C for 48 h to determine the number of viable cells in terms of colony forming units (CFU) per ml.
5.2.3 Results and Discussion

The results from the first series of bacterial adhesion assay are shown in Figure 5.5. Figure 5.5 parts a and b show the SEM micrographs of the pristine PPY and PPY-HA after immersion in the *E. coli* cell suspension (of $10^7$ cells/ml) for 2 h, respectively. A few bacteria cell bodies can be observed on the pristine PPY film surface (Figure 5.5(a)), though the bacteria adhesion is not extensive. The number of bacteria adhered on the PPY-HA surface (Figure 5.5 (b)) is further reduced compared with the PPY (Figure 5.5 (a)). As discussed in Section 2.2.2.1, bacterial adhesion on a surface is a complicated process which is governed by many factors: the physicochemical properties of the associated material surface and the bacteria cell wall; the environmental conditions such as the hydrodynamic forces and the components of the surrounding medium (Merritt and An, 2000). Since the environmental conditions were the same and the two substrates differ only in their surface properties, the difference in the number of cells adhering on the two substrates may be due to their difference in surface hydrophilicity (the water contact angle of pristine PPY is $\sim 70^\circ$ and that of PPY-HA is $\sim 30^\circ$ as mentioned in Table 4.1 in Section 4.3.2). It has been shown that polysaccharides exhibit interesting properties in their ability to resist adhesion of biological moieties in the aqueous media mainly due to their hydration forces to prevent non-specific interactions (Morra and Cassineli, 1999). Moreover, one study by Nomura et al. revealed that hydrophobic interactions are important in bacterial adhesion to polymers (1997). Thus, it is possible that PPY-HA surface can reduce bacterial adhesion as a result of the strong interaction of HA with water. The results in the Section 5.1 also showed that PC 12 cell attachment on the PPY-HA surface is decreased compared with that of pristine PPY (from 40% to 18%). It is possible that
the surface hydrophilicity also plays a role in the reduction of PC 12 cell attachment on
the PPY-HA surface.

**Figure 5.5** Scanning electron micrographs of (a) pristine PPY and (b) PPY-HA films after immersion in a PBS suspension of $10^7$ cells/ml *E. coli* for 2 h; (c) pristine PPY and (d) PPY-HA after immersion in a PBS suspension of $10^7$ cells/ml *E. coli* for 2 h and subsequently incubated in solid growth agar for 24 h.
Furthermore, the strong negative charges on the *E. coli* cell surface may also play an important role in the reduction of bacterial adhesion. The main component of the outer membrane of gram-negative bacteria like *E. coli* is lipopolysaccharides whose polysaccharide portion is composed of sugars (O polysaccharides), and the lipid portion is composed of lipid A. As mentioned in Section 4.3.2, the amount of HA present on the PPY-HA surface is 50 µg/cm$^2$, or ~ 130 nmol/cm$^2$ disaccharide units of HA. Therefore, based on the HA structure as shown in Figure 4.1, an electrical repulsion between the *E. coli* cell surface and the PPY-HA surface could be expected.

The opaque nature of the PPY film makes it difficult to accurately assess the small difference observed in SEM figures (Figure 5.5(a) and (b)). Hence, to further illustrate the effect of HA on bacteria adhesion, the substrates after the immersion in the same bacteria suspension were subjected to culture with the solid growth agar (second series of runs) as described in the experimental section. The SEM characterization of these films is shown in Figure 5.5 (c) and (d). Although both surfaces exhibit significant increase in the density of the cell population (compared to Figure 5.5 (a) and (b), respectively), the increase is much more significant for the pristine PPY film surface (Figure 5.5 (c)) as active cell growth and division can be observed with the cells differing in length and the presence of small clusters. Since the number of bacteria cells initially present on the PPY-HA (Figure 5.5 (b)) is less than PPY (Figure 5.5 (a)), the reduced cell population on PPY-HA (Figure 5.5 (d)) is expected. Thus, it can be concluded that the initial reduction in bacterial adhesion could significantly inhibit the bacteria proliferation on the material surface, thus retarding biofilm formation.

The third series of experiments aimed at verifying not only the difference in bacterial adhesion, but also the issue of the cell viability. Figure 5.6 shows the normalized
optical densities of the medium in contact with the pristine PPY and PPY-HA, respectively. The bacterial adhesion on pristine PPY is similar to that on PET and the difference in optical densities between PPY and PPY-HA is again attributed to the difference in the initial amounts of bacteria adhered. These results indicate that bacteria which adhered on the surface remain viable and can be further released from the surface upon culture in the aqueous medium, which is also the phenomenon often observed for a mature biofilm in aqueous environment (Jass et al., 2003).

![Figure 5.6](image_url)

**Figure 5.6** Normalized optical densities at 540 nm of nutrient broth after 24 h in contact with PPY and PPY-HA surfaces with adhered *E. coli*. OD\(_{\text{control}}\) is the optical density obtained using pristine PET as the substrate in the experiment.
Another experiment conducted to elucidate whether the PPY-HA film surface has bactericidal effect on *E. coli* cells involved counting the number of viable cells in contact with the substrates after different periods of time. The results are shown in Figure 5.7. The control experiment without the addition of a substrate is represented as TCPS in this Figure. It can be concluded that similar results were obtained in all four cases. Only a minor reduction in the viable cell concentration with time was observed due to natural apoptosis. Hence, it can be concluded that no bactericidal effect can be expected for the HA functionalized PPY film.

![Figure 5.7](image_url)

**Figure 5.7** Number of viable *E. coli* cells in PBS at 37°C as a function of time in contact with the different substrates. The cell number was determined by surface-spread method.
5.2.4 Conclusion

Hyaluronic acid functionalized PPY film surface has been shown to reduce the extent of *E. coli* adhesion compared with pristine PPY film. However, no bactericidal effect can be observed on the surfaces of either PPY film or HA functionalized PPY film. Hence, a different approach is needed to control biofilm formation. This approach is described in the next chapter and for this work PET instead of PPY was selected as the substrate since it is more representative of polymeric film.
CHAPTER 6

SURFACE FUNCTIONALIZATION OF NONPOROUS AND POROUS SUBSTRATES TO ACHIEVE ANTIBACTERIAL PROPERTIES
6.1 Surface Functionalization Technique for Conferring Antibacterial Properties to Polymeric Film Surface

6.1.1 Introduction

Conferring materials with anti-bacterial surface properties is a fascinating area for research and development in the battle against microbial contamination. The use of conventional disinfectants or antimicrobial agents is restricted by the toxicity of their residues since they are liquids or gases of low molecular weight (Tan et al., 2000). An alternative is the development of polymeric materials with antimicrobial activities and previous studies have shown that cationic polymers with quaternary ammonium groups possess such properties (Ikeda and Tazuke, 1983; Ikeda et al., 1984; Ikeda et al., 1986). Pioneering work on the properties of pyridium-type polymers was carried out by Kawabata et al. (1988; 1992). The application of antimicrobial polymers minimizes the environmental problems accompanying the conventional agents, and enhances the efficiency, selectivity, and lifetime of the antimicrobial agents (Kenawy, 2001; Kenawy et al., 1998; Kenawy et al., 2002). Hence, this approach has good potential for application in the areas related to bioengineering, water treatment, polymeric disinfectants and environmental protection.

One application of the antibacterial pyridium-type polymers is shown by the work of the MIT/Northeastern group which involves the surface treatment of glass and commercial polymers with N-alkylated poly(4-vinylpyridine) groups (Tiller et al., 2001; Tiller et al., 2002; Lin et al., 2002). The treated surfaces were shown to be lethal on contact to both gram-positive and gram-negative bacteria and it was also shown that N-alkyl chains of six carbon units in length is one of the most effective (Tiller et al., 2001). However, their method involves a number of chemical reaction steps, which
increases the difficulty in controlling the concentration of pyridinium groups on the surface. The method was effective for nonporous surfaces and was not applicable to carbohydrate-based materials, such as paper or clothing. For such materials, another group has developed a technique based on lipophilic alkyl chains (Abel, et al., 2002; Cohen et al., 2000).

In this part of the work, a simple two-step technique for functionalizing surfaces with N-hexylbromide poly(4-vinylpyridine) groups is described. This technique has the advantage of being effective for both nonporous and porous materials. Poly(ethylene terephthalate) (PET) was chosen as the polymeric substrate to be modified since its excellent physicochemical properties, such as good mechanical strength, good stability in the presence of body fluids and high radiation resistance make it an excellent candidate in biomaterial applications (Gupta et al., 2002; Uchida et al., 1994).

The approach we developed involves UV-induced surface graft copolymerization of 4-vinylpyridine (4VP), followed by the alkylation of the grafted poly(4-vinylpyridine) with hexylbromide. The manner in which the amount of active groups on the surface of the substrate can be controlled was illustrated and the issues related to the viability and adhesion of the Escherichia coli (E. coli) bacteria on the functionalized surfaces were investigated.
6.1.2 Experimental

Materials and reagents

Poly(ethylene terephthalate) (PET) films are the same as those described in Section 5.2.1. The 4VP monomer of 95% purity, was obtained from Aldrich Chemical Co. and was freshly distilled under reduced pressure before use. Hexylbromide, nitromethane and cetyltrimethylammonium chloride were all from Aldrich. Fluorescein (Na salt) was purchased from Sigma Chemical Co. Peptone, yeast extract, agar and beef extract were purchased from Oxoid. *E. coli* was obtained from American Type Culture Collection (ATCC, # 14948). Phosphate buffer solution (PBS) (containing NaH$_2$PO$_4$ of 4.68 g/l and Na$_2$HPO$_4$ of 8.662 g/l, pH of 7.0) was prepared afresh and sterilized in an autoclave before use.

Surface functionalization of substrates with pyridinium groups

The process of functionalizing the substrates with pyridinium groups is indicated in Figure 6.1. These steps are described in detail below.

(a) Argon plasma pretreatment

The PET film was cut into strips of 2.5 cm × 5 cm in size. The PET film was cleaned with absolute ethanol for 5 min in an ultrasonic water bath. The substrates were then subjected to argon plasma pretreatment in an Anatech SP100 plasma system, equipped with a cylindrical quartz reactor chamber. The glow discharge was produced at a plasma power of 35 W, an applied oscillator frequency of 40 kHz and an argon pressure of approximately 0.6 Torr. The substrate was placed between the two electrodes and each surface was subjected to glow discharge for 30 seconds, which was earlier found to be an optimal condition for the grafting procedure (Ying et al.,
Chapter 6

2003). After plasma pretreatment of both surfaces, the substrate was exposed to air for 5 – 10 minutes to facilitate the formation of surface oxide and peroxide groups before graft copolymerization was carried out (Suzuki, et al., 1986).

![Diagram of surface functionalization process]

**Figure 6.1** Schematic representation of the process of surface functionalization of PET film with pyridinium groups.
(b) *Surface graft copolymerization with 4VP*

The plasma-pretreated substrates were immersed in isopropanol solutions of 4VP of a predetermined concentration between 1 and 20 vol.% in a Pyrex glass tube. Degassing of the solutions was achieved by bubbling nitrogen vigorously into the solution for 30 min before stoppering and sealing the tubes with silicon rubber stoppers. The substrates in the 4VP solution at 25°C were then exposed to UV irradiation in a Riko rotary photochemical reactor (RH400–10W) for 60 min on each surface. The graft-copolymerized substrates were finally subjected to washing with copious amounts of ethanol to remove the residual monomer and physically adsorbed homopolymer (Yang et al., 2001).

(c) *Quaternization of the graft-copolymerized 4VP*

The substrates with graft-copolymerized 4VP were then placed in 30 ml solutions of nitromethane containing 20 vol.% hexylbromide and the reaction mixture was stirred for 48 h at 70°C (Tiller, et al., 2001; Biesalski and Rühe, 1999). At the end of the reaction period, the substrates were thoroughly rinsed with nitromethane to remove the unreacted substances and then dried under reduced pressure. We have confirmed that with such procedures, the pyridinium moieties remaining on the substrate are covalently bonded and there is no significant leaching of these moieties even after the functionalized substrate has been immersed in PBS for 3 h (time and medium are similar to those used for antibacterial assay as described in a later section).

**Surface analysis**

*XPS characterization*
XPS analysis of the film surface was made on an AXIS HSi spectrometer (Kratos Analytical Ltd.) as described in Section 3.2.

The surface morphologies of the PET films after each modification step were characterized using a Nanoscope IIIa atomic force microscope (AFM). All images were obtained in the air using the tapping mode under a constant force (scan size: 10 µm × 10 µm, scan rate: 1.0 Hz).

**Titration of pyridinium groups on PET film surfaces**

The PET films after surface functionalization were immersed in a 1% fluorescein (Na salt) solution in distilled water for 5 min with constant shaking, followed by thorough rinsing with doubly distilled water. The stained film was then placed in an aqueous solution of 0.25 wt.% cetyltrimethylammonium chloride and the mixture was shaken for 10 min to desorb the dye. A 0.1 M aqueous phosphate buffer, pH 8.0, was then added in a ratio of 1 part buffer to 9 parts of cetyltrimethylammonium chloride solution and the absorbance of the resultant solution was measured at 501 nm. The amount of dye bound to pyridinium groups on the film surface was calculated on the basis of a standard calibration. The corresponding pyridinium concentration was then calculated based on the assumption of one dye molecule per seven hexyl-PVP monomeric units (Tiller, et al., 2001).

**Determination of antibacterial characteristics**

*E. coli* was cultivated as described in Section 5.2.2.

For the airborne antibacterial assay, the *E. coli*-containing broth was centrifuged at 2700 rpm for 10 min, and after the removal of the supernatant, the cells were washed
with doubly distilled water and resuspended in doubly distilled water at a concentration of $10^7$ cells per ml. The PET films were then sprayed with the bacterial suspension using a commercial chromatography sprayer. After several minutes of drying in air, the substrates were placed in Petri dishes. The Petri dishes were then sealed with growth agar (0.7% agar in yeast-dextrose broth, autoclaved, and cooled to 37°C) and incubated at 37°C for 24 h (Tiller et al., 2002).

For the waterborne antibacterial assay, the \textit{E. coli}-containing broth was centrifuged at 2700 rpm for 10 min, and after the removal of the supernatant, the cells were washed twice with PBS (pH 7.0) and resuspended in PBS at a concentration of $10^7$ cells/ml. The substrates were immersed in this suspension in a sterile Erlenmeyer flask. The flask was then shaken at 200 rpm at 37°C for 2 h. The substrates were then removed from the above flask and washed three times with sterile PBS and placed in Petri dishes. This was followed by the immediate addition of solid growth agar (1.5% agar in yeast-dextrose broth, autoclaved, poured into a Petri dish, and dried under reduced pressure at room temperature overnight). The Petri dishes were then sealed and incubated at 37°C for 24 h (Tiller et al., 2002).

The substrates after the airborne or waterborne antibacterial assays were characterized by either optical microscopy (using an Olympus BX60) or SEM. The sample fixation and preparation for SEM characterization were conducted in the same manner as that described in Section 5.1.2.

\textbf{Adhesion and viability assay of bacteria on PET substrates}

The PET substrates were immersed in a PBS (pH 7.0) suspension of $10^7$ cells/ml in a sterile Erlenmeyer flask. The flask was then shaken at 200 rpm at 37°C for either 5 min
or 2 h. After that, the substrates were taken out and washed extensively with sterile PBS. Sterilized filter paper was used to suck up the moisture on the substrate surface, after which the substrates were immersed into a yeast-dextrose broth (Li and Shen, 2000). After being cultured at 37°C for 24 h, the substrates were removed and the optical density at 540 nm of the broth was then observed. Control experiments were made with the pristine PET substrate and the optical densities were compared. The substrates before and after culturing in the yeast-dextrose broth were also examined under SEM to assess how the adhesion and viability of the bacteria were affected by the presence of the pyridinium groups.
6.1.3 Results and Discussion

Surface graft copolymerization of PET films with 4VP

The success of the UV-induced surface graft copolymerization of 4VP on the PET film can be ascertained by comparing the XPS spectra of the films before and after the grafting process (Figure 6.2). The XPS C 1s core-level spectrum of the pristine PET film (Figure 6.2(b)) shows a predominant peak component at the binding energy (BE) of 284.6 eV due to the aromatic carbon, and two peak components at the BE of 286.2 eV and 288.6 eV due to singly bonded CO and carboxyl carbon (COO-) respectively. The areas of the three peak components are approximately in a ratio of 3:1:1 which is in good agreement with the chemical structure of PET (Loh et al., 1995). Since there is no nitrogen in the PET structure, no nitrogen component can be detected as shown in Figure 6.2(c). The presence of the surface grafted 4VP polymer after the UV-induced graft copolymerization step can be deduced from the wide scan (Figure 6.2(d)) which shows the presence of the N 1s peak at around 400 eV. The corresponding N 1s spectrum (Figure 6.2(f)) shows an intense peak at the BE of 398.5 eV attributable to the imine moiety (-N=) of the pyridine rings (Yang et al., 2001). The C 1s core-level spectrum after 4VP grafting (Figure 6.2(e)) shows an additional peak at 285.5 eV attributable to the C-N species and a decrease in the intensity of the CO and COO- peak components as compared with Figure 6.2(b). As the surface coverage of the 4VP copolymer increases with increasing 4VP monomer concentration used in the grafting process, the intensity of the peaks assigned to CO and COO- species became increasingly weaker until the peaks are no longer discernable (Figure 6.2(h)). Similarly, as the 4VP surface graft concentration increases, the intensity of the N 1s signal increases relative to the O 1s signal (Figure 6.2(g)).
Figure 6.2 XPS wide scan, C 1s and N 1s core-level spectra of (a), (b) and (c) pristine PET film; (d), (e) and (f) of PET film after UV-induced graft copolymerization with 4VP using 5 vol.% monomer in isopropanol; (g), (h) and (i) of PET film after UV-induced graft copolymerization with 4VP using 20 vol.% monomer in isopropanol.
The concentration of quaternary ammonium groups which have effective antibacterial properties depends on the concentration of the pyridine groups from 4VP graft copolymerized on the surface. The extent of surface grafting of 4VP can be estimated from the sensitivity factor corrected ratio of the total N 1s peak over the total C 1s peak, and expressed as [N]/[C]. The change in the surface graft concentration of the 4VP polymer with the monomer concentration used for graft copolymerization is shown in Table 6.1. It can be seen that the surface [N]/[C] ratio increases with 4VP monomer concentration. At a monomer concentration of 20 vol.%, the ratio of 0.11 is close to the value of 0.14, expected for the 4VP monomeric unit (C\textsubscript{7}H\textsubscript{7}N\textsubscript{1}). This indicates that the surface is almost completely covered by 4VP copolymers to a depth of ~ 8 nm which is the probing depth of the XPS technique for an organic matrix (Briggs, 1998).

**Quaternization of graft-copolymerized 4VP**

The conversion of the N-pyridine rings to pyridinium groups after reaction with hexylbromide were investigated using XPS and fluorescein titration. Figure 6.3 shows the XPS spectra of the surface derivatized PET film which was first grafted with 20 vol.% 4VP and subsequently N-alkylated with hexylbromide. No significant difference can be observed in C 1s core-level spectrum before (Figure 6.2(h)) and after (Figure 6.3(a)) the alkylation process except for the appearance of a small peak component at the BE of 286.2 eV, attributable to the CO species. It is unlikely that this peak is due to the loss of the 4VP graft copolymers from the surface which would result in the exposure of the underlying PET film surface, since PET has equal amounts of CO and COO- species and there was no evidence of COO- species in Figure 6.3(a). Hence, the
Table 6.1 Surface composition of PET after 4VP functionalization and hexylbromide derivatization

<table>
<thead>
<tr>
<th>Substrate</th>
<th>4VP monomer concentration used for surface graft copolymerization (vol.%)</th>
<th>Surface [N]/[C] ratio* after 4VP graft copolymerization</th>
<th>Surface [N⁺]/[C] ratio* after hexylbromide derivatization</th>
<th>Pyridinium concentration ([N⁺])** on modified film surfaces (nmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P-1</td>
<td>1</td>
<td>0.010</td>
<td>0.0049</td>
<td>0.11</td>
</tr>
<tr>
<td>P-2.5</td>
<td>2.5</td>
<td>0.031</td>
<td>0.0098</td>
<td>0.79</td>
</tr>
<tr>
<td>P-5</td>
<td>5</td>
<td>0.053</td>
<td>0.014</td>
<td>2.41</td>
</tr>
<tr>
<td>P-10</td>
<td>10</td>
<td>0.079</td>
<td>0.027</td>
<td>15.0</td>
</tr>
<tr>
<td>P-20</td>
<td>20</td>
<td>0.11</td>
<td>0.032</td>
<td>19.8</td>
</tr>
</tbody>
</table>

* As determined by XPS.

** As determined by fluorescein staining
CO species may be due to oxidation of a trace amount of the carbon species on the surface during the alkylation reaction.

The corresponding N 1s core-level spectrum in Figure 6.3(b) shows an additional peak at a binding energy above 400 eV, attributable to the N\(^+\) groups of the pyridinium ions (Tan et al., 1990). This confirms the derivatization of the –N= groups by hexylbromide. Based on the [N\(^+\)]/[N] ratio, the degree of alkylation of the pyridine rings is around 30 to 40 percent. In the Br 3d core-level spectrum (Figure 6.3(c)) the presence of a doublet (Br 3d\(_{5/2}\) and Br 3d\(_{3/2}\)) at 67.5 eV and 68.6 eV attributable to Br\(^-\) species (Zhao et al., 2000) further confirms the reaction between the 4VP graft copolymer on the PET film surface and hexylbromide. The [N\(^+\)]/[C] ratio of the PET substrate with different extents of 4VP graft copolymer after hexylbromide derivatization is shown in Table 6.1. As expected, with a higher amount of 4VP copolymers present on film surface, the concentration of pyridinium groups is higher.

The quantitative amount of [N\(^+\)] present on the corresponding film surfaces as obtained from the titration method using fluorescein (Na salt) is also shown in Table 6.1. Previous work has shown that this dye only binds to quaternary amino groups but not tertiary or primary ones (Ledbetter and Bowen, 1969). Our control experiments with pristine PET film and PET films with various amounts of 4VP grafted but not derivatized also confirmed that fluorescein (Na salt) only binds to quaternary amino groups. The results in Table 6.1 show that the amount of surface [N\(^+\)] increases by over 2 orders of magnitude as the amount of 4VP monomer concentration used in the grafting process increases from 1 to 10 vol.%. Further increase in monomer concentration beyond this value results in only an incremental increase in [N\(^+\)]. This effect may be due to steric hindrance which limits the approach of the hexylbromide
molecules to the pyridine groups. As will be illustrated below, the antibacterial efficiency of the film is highly dependent on the amount of quaternary ammonium groups on the surface.

Figure 6.3 XPS (a) C 1s, (b) N 1s and (c) Br 3d core-level spectra of PET film after UV-induced graft copolymerization with 4VP in 20 vol.% monomer in isopropanol and subsequently derivatized with 20 vol.% hexylbromide in nitromethane.
Surface morphology of the PET films

Significant differences can be observed in the AFM images of the pristine PET and PET after surface graft-copolymerization with 10 vol.% 4VP (Figure 6.4). The surface roughness ($R_a$) increases from 1.7 nm for PET to 4.3 nm for the 4VP grafted PET. After quaternization, the $R_a$ of the film did not change significantly ($R_a$ of P-10 film = 3.6 nm). The lack of difference in surface morphology after quaternization compared to the significant difference observed after surface graft-copolymerization with 4VP is due to the fact that the 4VP exists on the film surface as a copolymer with long chains whereas hexylbromide is a single molecule. The uniformity of the surface morphology as observed in Figure 6.4 ensures the homogeneity of the surface antibacterial properties.
Figure 6.4 AFM images of (a) pristine PET film, (b) PET film after UV-induced graft-copolymerization with 4VP using 10 vol.% monomer in isopropanol, and (c) P-10 film.
Antibacterial characteristics of functionalized PET

Both airborne and waterborne tests were performed to simulate the natural deposition of *E. coli* on substrates. Three substrates were chosen for these tests: pristine PET film; PET film which was graft copolymerized with 1 vol.% 4VP monomer concentration and subsequently quaternized with hexylbromide (P-1 in Table 6.1); PET film obtained under the same conditions except the monomer concentration used was 10 vol.% (P-10 in Table 6.1). Figure 6.5 shows the optical microscopy results of the PET films after airborne and waterborne antibacterial tests. In Figure 6.5(a), numerous distinguishable bacteria colonies can be observed on the pristine PET surface. The number of colonies on P-1 surface as shown in Figure 6.5(b) is significantly less and this decrease was even more substantial for the P-10 surface (Figure 6.5(c)). Control experiments conducted with PET films grafted with 1 vol.% and 10 vol.% 4VP but without subsequent derivatization gave results similar to that obtained with the pristine PET film. A similar trend showing the effectiveness of the functionalized surfaces in preventing *E. coli* growth can be observed in the waterborne tests (Figure 6.5(d), (e) and (f)). The importance of having a high concentration of [N+] to achieve effective antibacterial activities is thus clearly demonstrated in both the airborne and waterborne tests.

A difference observed between the airborne and waterborne tests is the higher concentration of colonies on the corresponding substrates in the former. This may be due to the fact that in the airborne tests the bacteria were sprayed directly on the different substrates whereas for the waterborne tests, only the bacteria which adhered to the substrates after their removal from the suspension were considered. The issue of whether the surface groups affect the adhesion of the bacteria in the waterborne test would be addressed in greater detail in a later section.
Figure 6.5 Optical micrographs of (a) and (d) pristine PET, (b) and (e) P-1, (c) and (f) P-10 surfaces after exposure to airborne and waterborne *E. coli* respectively, and subsequent incubation in solid growth agar for 24 h.
The waterborne antibacterial assay was also performed on the P-10 film after storage in air for 2 months. The antibacterial effect observed was similar to that of the freshly modified film as shown in Figure 6.5 (f). Hence, the antibacterial properties imparted by this surface modification technique can be preserved for long periods of time.

The SEM micrographs of the pristine PET and P-10 substrates after the above-mentioned airborne test are shown in Figure 6.6. These micrographs show a clear difference between the *E. coli* on the PET and P-10 substrates. In addition to the substantially higher concentration of bacteria on the pristine PET, the bacteria on this surface also show active cell growth and division. A number of the bacteria cells are between 2 to 6 µm in length. In contrast, the *E. coli* on the P-10 substrate (Figure 6.6(b)) is sparsely distributed as single cells of the order of 1 µm in length. There is no evidence of growth or reproduction. The results confirm that normal polymer films are good templates for the proliferation of microorganisms and biofilm formation occurs readily on such polymeric surfaces in contact with bacteria (Costerton et al., 1999; Lewis, 2001). The presence of quaternary ammonium groups attached to a hexyl group at a surface concentration of 15 nmol/cm$^2$ is thus demonstrated to be very effective in preventing such a biofilm formation.

**Adhesion and viability assay of cells adhered on polymeric film surfaces**

This assay was intended to address the issue of whether the surface groups on the modified PET affected the initial adhesion of the bacteria during the waterborne tests. In addition, it would be of interest to elucidate whether cells adhered to the functionalized substrate for a short period of time remained viable when released into a fresh culture medium. In these adhesion and viability tests, the procedures were similar to the procedures for the waterborne test as described earlier except that solid growth
Figure 6.6 Scanning electron micrographs of (a) PET and (b) P-10 films after exposure to airborne *E. coli* and subsequently incubated with solid growth agar for 24 h.
agar was not applied on the substrates. Instead, the substrates with adhered bacteria were either immediately sent for SEM characterization or recultured in 45 ml of yeast-dextrose broth in a sterile Erlenmeyer flask at 37 °C for 24 h with constant shaking. The SEM micrographs of the PET and P-10 substrates after the initial immersion in the cell suspension (of $10^7$ cells/ml) for 2 h are shown in Figure 6.7(a) and (b) respectively. The immersion time of 2 h was chosen to facilitate the metabolic recovery of bacteria from a suspension to a substrate (Arnold and Silvers, 2000). No significant difference in the quantity and distribution of the attached bacteria on the two surfaces can be observed from the SEM micrographs. The microbial adhesion to different substratum surfaces is known to be affected by the various chemical and physicochemical factors of the substrate under the same environmental conditions, such as hydrophobicity or hydrophilicity (Reid et al., 1993), steric hindrance (Kuhl et al., 1994), roughness (Arnold and Bailey, 2000), and the existence of a ‘conditioning layer’ at the substrate surface (Abarzua and Jakubowski, 1995). It is often the collaboration of those factors that contributes to the initial cell attachment process. In the present work, both the PET and P-10 surfaces are quite hydrophobic as the water contact angle of pristine PET is more than 70° and that of P-10 is around 70°, as measured by a telescopic goniometer (Rame-Hart, Model 100-0-230). Furthermore, the root mean square surface roughness of the pristine PET and P-10 surfaces as measured by atomic force microscopy (measurement area of 10 µm) is about 1.67 nm and 3.57 nm respectively. Although it has been reported that the surface hydrophobicity and roughness enhances bacteria attachment (Reid et al., 1993), the differences in hydrophobicity and roughness between the PET and P-10 surfaces are not expected to be sufficient to contribute to a significant difference in bacteria adhesion, and this is confirmed by the SEM micrographs in Figure 6.7(a) and (b).
Figure 6.7 Scanning electron micrographs of (a) pristine PET and (b) P-10 surfaces after exposure to a PBS suspension of $10^7$ cells/ml *E. coli* for 2 h.
Another factor to consider is the negatively charged nature of the bacteria surface. It may be expected that the interaction between the bacteria and the positively charged pyridinium groups on the P-10 surface is stronger than with PET which is an inert substratum. However, it has also been reported that interaction forces include the ever present Lifshitz-van der Waals forces, electrostatic forces, and acid-base interactions (Van Oss, 1995), and all these nonspecific forces act together in cell adhesion or repulsion. Moreover, each interaction force might be constrained by its specific operative distance (Bos et al., 1999). As this assay was carried out in an aqueous suspension with shaking at a high speed (200 rpm), the hydrodynamic forces are also believed to be important. Hence, the association between a microorganism and a substratum surface that contributes most to the microbial adhesive interactions is believed to be a dynamic process which takes some time to achieve equilibrium. In the present work, adhesion assays using the substrates in suspensions of different bacteria concentrations were compared. With a bacteria concentration of $10^9$ cells/ml in the suspension, substantially more bacteria were observed to adhere on the PET and P-10 substrates compared to the results shown in Figure 6.7(a) and (b) which were obtained with a suspension of $10^7$ cells/ml. However, there is still no significant difference between the amounts of adhered bacteria on the two different substrates. Therefore, the concentration of the bacteria adhered on the different substrates is affected more by the concentration in the suspension than the presence of the positively charged pyridinium groups of the concentration used in our experiments.

The SEM micrographs of the pristine PET and P-10 substrates with adhered bacteria after reculturing for 24 h are shown in Figure 6.8(a) and (b). As can be seen from Figure 6.8(a), the surface of the pristine PET substrate was bestrewed with bacteria in the form of big colonies, which may lead to the formation of a biofilm. On the other
Figure 6.8 Scanning electron micrographs of (a) pristine PET and (b) P-10 film surfaces after exposure to a PBS suspension of $10^7$ cells/ml *E. coli* for 2 h and subsequently recultured in yeast-dextrose broth for 24 h.
hand, the bacteria present on the P-10 surface after reculturing (Figure 6.8(b)) remained as single cells in a very sparse distribution, similar to that observed before reculturing (Figure 6.7(b)). The SEM micrographs of both the PET and P-10 surfaces after reculturing (Figure 6.8(a) and (b)) show the presence of a thin film. This film is the result of adsorption of organic matters present in the yeast-dextrose broth and is generally referred to as ‘conditioning film’ (Gristina, 1987).

After the substrates were removed from the nutrient broth, the optical density of the broth was measured at 540 nm to obtain an indication of the bacteria concentration. The results from the experiments using four different substrates: pristine PET, P-1, P-5 and P-10, are compared in Figure 6.9. In this Figure, the optical density obtained from the nutrient broth with the three functionalized PET substrates was normalized to that obtained with the pristine PET film. For the series of experiments carried out with the substrates initially immersed for 2 h in the *E. coli* suspension, the optical densities of the medium with the P-1, P-5 and P-10 substrates are about 70%, 5% and <1% of that obtained with the pristine PET control respectively. Therefore, it can be concluded that the adhered bacteria on the pristine PET substrate not only grows well on the substrate surface, but upon release from the substrate into an aqueous medium, it remains viable and reproduces in the medium. However, the P-10 surface is highly detrimental to the bacteria upon contact and the bacteria cells released into growth medium are no longer viable. The importance of having a sufficiently high concentration of pyridinium groups (~15 nmol/cm²) is also illustrated since the same bacteria released from the P-1 and P-5 substrates continued to multiply in the growth medium.
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Figure 6.9 Normalized optical densities at 540 nm of nutrient broth after 24 h in contact with P-1, P-5 and P-10 surfaces with adhered *E. coli*. The substrates were initially immersed in an *E. coli* suspension of $10^7$ cells/ml for either 2 h or 5 min before transferring to the nutrient broth. OD$_{\text{control}}$ is the optical density obtained using pristine PET as the substrate in the experiment.

To further investigate the effect of time on bacteria adhesion and destruction, the experiments were also carried out for substrates immersed in the same bacteria suspensions for 5 min instead of 2 h. The optical density of the nutrient broth cultured with the pristine PET which was immersed for 5 min in the bacterial suspension was about 30% of the value obtained with a 2 h immersion period (not shown). As a low
optical density is equivalent to a low bacteria concentration in the medium, it is clear a short contact time of 5 min results in less bacteria adhering to the substrate and subsequently transferring to the culture broth. The higher concentration of bacteria adhered on the pristine PET and P-10 substrates after 2 h as compared to 5 min immersion in the bacteria suspension is confirmed by SEM investigation of the substrates after removal from the suspension. The resultant optical densities of the nutrient broth for the series of experiments carried out with the 5 min immersion time are also shown in Figure 6.9. The optical densities in this series are normalized with respect to the corresponding value obtained with the pristine PET as the control. In this case, the optical densities of the medium with the P-1, P-5 and P-10 substrates are about 90%, 70% and 15% respectively of that obtained with the corresponding pristine PET control. From Figure 6.9, it can be seen that for any substrate the normalized optical density obtained from the 5 min immersion experiments is higher than the corresponding value from the 2 h immersion experiments. This observation indicates that not only is the adhesion of bacteria to the substrates time-dependent, but it may also take time for the N-hexyl pyridinium groups to be effective in killing the \textit{E. coli}.

Nevertheless, as shown in Figure 6.9, the P-10 substrate is still very effective in killing \textit{E. coli} on contact even for a period of time as short as 5 min.

The bactericidal ability of the surface-attached polycationic chains have been attributed to the penetration of those polycationic chains into the bacterial membrane resulting in the cell damage and death (Tiller et al., 2001; Lin et al., 2002). The following sequential steps have been proposed for this mechanism: (1) adsorption of positively charged polycations onto the negatively charged cell surfaces, (2) penetration into the cell wall, (3) binding to the cytoplasmic membrane, (4) disruption of the cytoplasmic membrane, (5) release of $\text{K}^+$ ions and constituents of the
cytoplasmic membrane, (6) death of the cell (Tashiro, 2001). The present work further confirms the antibacterial activity of surface-attached polycationic N-hexyl chains. Moreover, the good control of the concentration of the functional groups offered by the present technique meets the requirement of a sufficiently high concentration of the polycationic groups to achieve a high killing efficiency.
6.1.4 Conclusion

Commercial PET films can readily undergo UV-induced surface graft copolymerization with 4VP, and the subsequent alkylation of the grafted 4VP groups to yield the pyridinium groups. The 4VP graft concentration can be controlled by varying the monomer concentration used in the grafting process and is a key factor in determining the amount of surface functional groups available for quaternization. Both airborne and waterborne assays with \textit{E. coli} show that the polycationic chains introduced on the substrate surface via derivatization of the pyridine groups by hexylbromide possess the desired antibacterial activity. The bacteria killing efficiency is dependent on the surface pyridinium concentration and a surface concentration of 15 nmol/cm\textsuperscript{2} on PET has been shown to be highly effective. At this concentration, the \textit{E. coli} bacteria are rapidly killed on contact, and very few cells remain viable even after release into growth medium. The functionalization technique developed in this work offers the flexibility in altering the type and concentration of the surface functional groups as well as the long term stability. The investigation of the applicability of this technique to porous material surfaces is described in the next section.
6.2 Surface Functionalization Technique for Conferring Antibacterial Properties to Porous Material Surfaces

6.2.1 Introduction

Environmentally occurring biofilms with ubiquitous distributions in aquatic environments, on tissues of plants, animals and man, as well as on surfaces of technical systems such as filters and other common porous materials may initiate devastating consequences to public health. The technique developed in Section 6.1 offers advantages of simplicity in processing, good control over the surface concentration of the functional groups, and long term effectiveness. This surface functionalization method circumvents the shortcomings suffered by the conventional method of impregnating materials with antibacterial agents. The latter poses an environmental risk due to the leaching of antibacterial agents into the environment and has short term effectiveness due to the exhaustion of these agents (Nohr and Macdonald, 1994; Medlin, 1997; Buchenska, 1997). The applicability of this technique to different types of porous substrates is described in this section. Since most biofilms involve far more than one species, an investigation of the effectiveness of the functionalized material in systems with coexistence of multi-species was also carried out.

Filter paper was selected as one of the substrates as it is widely used in many technical systems. Commercial PVDF membrane which has been shown to be amenable to surface graft-copolymerization (Hietala et al., 1999), was also selected. There is also interest in cloth due to its extensive usage in everyday life as well as in scientific research (Kawabata and Nishiguchi, 1988).
6.2.2 Experimental

Materials and reagents

Whatman Grade 1 filter paper was chosen as it is the most widely used filter paper in the Whatman range. PVDF membranes with an average pore size of 0.45 µm were obtained from Millipore, US. 100% cotton cloth was commercially available. The other reagents involved in this work were all the same as those specified in Section 6.1.2

Surface functionalization of substrates with pyridinium groups

The procedure for functionalizing filter paper, PVDF membrane and the cotton cloth with pyridinium groups is similar as that described in Section 6.1.2 for PET film. Basically there are three steps involved:

(a) Argon plasma pretreatment. The substrates were cut into strips of 2.5 cm × 5 cm in size and washed with sterilized water for several hours in order to remove residual organic materials (Tan et al., 2000). These strips after drying under dynamic vacuum were then subjected to argon plasma pretreatment as described in Section 6.1.2 in an Anatech SP100 plasma system.

(b) Surface graft copolymerization with 4VP. The plasma-pretreated substrates were immersed in isopropanol solutions containing 10 vol.% of 4VP in a Pyrex glass tube. This concentration was chosen based on the results from the previous work on PET films which showed that the substrates treated under such conditions possess sufficient functional groups to be highly effective in killing E. coli on contact.

(c) Quaternization of the graft-copolymerized 4VP. The substrates with graft-copolymerized 4VP were subjected to the same derivatization reaction as in Section
6.1.2, except that at the end of the reaction period, the porous substrates were immersed in nitromethane with stirring for 2 h to remove the unreacted reagents and then dried under reduced pressure.

**Determination of antibacterial effects on *E. coli***

*E. coli* cells were cultivated as described in Section 5.2.2 and assays of antibacterial activities were carried out as follows:

(a) *Airborne and waterborne antibacterial assays.* These two assays were carried out as described in Section 6.1.2. The substrates after these assays were characterized using scanning electron microscopy (SEM). The sample fixation and preparation for SEM were also similar to those developed for the PET films in Section 6.1.2.

(b) *Evaluation of the viable *E. coli* cell counts.* Only cotton cloth was used in this assay due to its high specific surface area. The *E. coli*-containing broth was centrifuged at 2700 rpm for 10 min, and after the removal of the supernatant, the cells were washed twice with PBS and resuspended in PBS at a concentration of $10^5$ cells/ml. One cotton cloth strip of 2.5 cm $\times$ 5 cm in size was immersed in 50 ml of this suspension in a sterile Erlenmeyer flask shaken at 200 rpm at 37°C. At predetermined times, 0.6 ml samples were taken from the flask and the viable cell counts of the *E. coli* were measured by the surface spread-plate method. In this method, decimal serial dilutions with PBS were made from each initial sample. A drop of 0.02 ml of the diluted sample was then spread onto triplicate solid growth agar plates. After incubation of the plates at 37°C for 48 h, the number of viable cells (colonies) was counted manually and the results after multiplication with the dilution factor were expressed as mean colony forming units (CFU) per ml.
This assay was also carried out for the functionalized cloth before and after weathering. The weathering of the cloth was carried out in a Ci3000 Xenon Weather-Ometer (Atlas Electric Device Co., Chicago, IL). During the test, the weather chamber was maintained at a relative humidity of 60%, and a dry bulb temperature and black panel temperature of 35°C and 63°C, respectively. The simulated solar irradiation was directed at the film’s surface with an intensity of 0.42 W/m² at 340 nm. A water spray was activated for 5 min in each 30 min cycle. After 20 h, the cloth was removed from the chamber and dried under reduced pressure before being subjected to the antibacterial assay.

Wild type antibacterial assays of cotton cloth

Soil was placed in a small crucible with sterile distilled water and thoroughly ground up with a glass rod. The suspension was then centrifuged at 300 rpm to allow the sedimentation of the coarse grains. Three ml of the supernatant was then transferred into a sterile Erlenmeyer flask with yeast-dextrose broth. The flask was shaken at 200 rpm at 30°C for 24 h in an incubator (Reasoner and Geldreich, 1985). Airborne and waterborne antibacterial assays similar to those carried out with E. coli were performed. The same cell concentration of 10⁷ cells/ml was used in these assays with the assumption that similar optical densities of the pure E. coli suspension and the multi-microorganism suspension would yield similar cell concentrations. Antibacterial activity was also measured by pipetting 200-μl aliquots of the wild type microorganisms of concentration of either 10⁷ cells/ml or 10⁹ cells/ml in sterile water directly onto the cotton cloth substrates (of 2 × 2 cm²) followed by the immediate addition of the solid growth agar (the same as that used in the waterborne assay). This test was carried out to assess the effectiveness of the functionalized cloth in preventing
biofilm formation at high concentrations of microorganisms. The microorganisms were cultured for 24 h at 30°C in an incubator and then subjected to the SEM analysis in the same manner as that carried out after the airborne or waterborne assays.
6.2.3 Results and Discussion

Surface functionalization

*Filter paper:* In a manner similar to that described in Sec 6.1.3, XPS analysis of the filter paper (Figure 6.10 and Figure 6.11) confirms the success of the surface functionalization procedures for this substrate. The XPS C 1s core-level spectrum of the pristine filter paper (Figure 6.10(a)) shows a predominant peak component at the binding energy (BE) of 286.2 eV due to singly bonded CO and two peak components at the BE of 284.6 eV and 288.6 eV due to the aromatic carbon and carboxyl carbon (COO-) respectively. As there is no nitrogen in filter paper, no nitrogen component can be detected as shown in Figure 6.10(b). The C 1s core-level spectrum after 4VP grafting (Figure 6.10(c)) shows an additional peak at 285.5 eV attributable to the C-N species of the pyridine ring and a decrease in the relative intensity of the CO peak component as compared with Figure 6.10(a). The presence of the surface grafted 4VP polymer is further confirmed by the intense peak at 398.5 eV attributable to the imine moiety (-N=) of the pyridine rings in the corresponding N 1s spectrum (Figure 6.10(d)) (Yang et al., 2001). The conversion of the N-pyridine rings to pyridinium groups after reaction with hexylbromide was also ascertained using XPS as shown in Figure 6.11. The C 1s core-level spectrum before (Figure 6.10(c)) and after (Figure 6.11(a)) the alkylation process exhibit no significant difference except for a small increase in the intensity of the peak component at 284.6 eV, due to the introduction of the alkyl chains on the surface. The corresponding N 1s core-level spectrum in Figure 6.11(b) shows an additional peak at a binding energy above 400 eV, attributable to the N⁺ groups of the pyridinium ions (Tan et al., 1990), which confirms the derivatization of the –N= groups by hexylbromide. The presence of the Br⁻ species as a result of the derivatization can be seen from the doublet (Br 3d₅/₂ and Br 3d₃/₂) at 67.5 eV and 68.6
eV in the Br 3d core-level spectrum as shown in Figure 6.11(c). From SEM micrographs of the filter paper before and after surface functionalization, it can be concluded that the filter paper did not show significant changes in its morphology and structure during the course of the surface functionalization.

Figure 6.10 XPS C 1s and N 1s core-level spectra of (a) and (b) pristine filter paper; (c) and (d) of filter paper after UV-induced graft copolymerization with 4VP using 10 vol.% monomer in isopropanol.
Figure 6.11 XPS (a) C 1s, (b) N 1s and (c) Br 3d core-level spectra of filter paper after UV-induced graft copolymerization with 4VP in 10 vol.% monomer in isopropanol and subsequently derivatized with 20 vol.% hexylbromide in nitromethane.
**PVDF membrane:** Similarly, XPS analysis was used to verify the success of the surface functionalization procedures, as shown in Figure 6.12 and Figure 6.13. The XPS wide scans of the pristine PVDF membrane, 4VP grafted PVDF membrane, and 4VP grafted followed by hexylbromide derivatized PVDF membrane are shown in Figure 6.12. The appearance of the N (Figure 6.12(b)) and Br signals (Figure 6.12(c)) confirm the success of the corresponding procedures as described in detail for the filter paper. Figure 6.13(a) and (b) shows the N 1s core level spectra of the 4VP grafted PVDF membrane before and after quaternization with hexylbromide, respectively, which confirms the conversion of N-pyridine rings to pyridinium groups. The modification process resulted in only a small decrease (less than 10%) in the mean effective pore size (measured by Coulter Porometer II apparatus).

**Cotton cloth:** The success of the UV-induced surface graft copolymerization of 4VP on the cotton cloth substrate was similarly ascertained by comparing the XPS spectra of the cloth before and after the grafting process (Figure 6.14). Since the cotton cloth was used for more antibacterial assays than the filter paper and the PVDF membrane, a more detailed investigation of the surface composition of the functionalized cotton cloth was carried out. For the cotton cloth, a value of [N]/[C] of 0.10 was obtained with 10 vol.% 4VP monomer concentration. This value is slightly higher than that achievable with PET (0.08) under the same grafting conditions. Since the PET film with [N]/[C] of 0.08 was shown to be effective in preventing *E. coli* colonization, a similar 4VP monomer concentration was adopted for the grafting process for the cotton cloth in the following work.
Figure 6.12 XPS wide scan of (a) pristine PVDF membrane; (b) PVDF after UV-induced graft copolymerization with 4VP using 10 vol.% monomer in isopropanol; and (c) PVDF after UV-induced graft copolymerization with 4VP in 10 vol.% monomer in isopropanol and subsequently derivatized with 20 vol.% hexylbromide in nitromethane, denoted as PVDF-10.
The $[N^+]/[N]$ ratio, which is equivalent to the degree of alkylation of the pyridine rings is around 75%. This value is also higher than that obtained for the PET film (~35%) under the same conditions. The corresponding $[N^+]/[C]$ ratio of the cotton cloth substrate after modification is around 0.065, which is more than 2 times higher than that of the PET film (~0.027). The higher grafting efficiency and higher degree of alkylation may due to the larger specific surface area of cloth.
Figure 6.14 XPS wide scan and N 1s core-level spectra of (a) and (b) pristine cotton cloth; (c) and (d) cotton cloth after UV-induced graft copolymerization with 4VP using 10 vol.% monomer in isopropanol; (e) and (f) cotton cloth after UV-induced graft copolymerization with 4VP in 10 vol.% monomer in isopropanol and subsequent derivatization with 10 vol.% hexylbromide in nitromethane.
Antibacterial assays using *E. coli*

The pristine filter paper, denoted as FP, and the 4VP grafted (using 10 vol.% 4VP monomer) and hexylbromide derivatized filter paper, denoted as FP-10, were assayed with airborne and waterborne antibacterial tests, similar to those carried out for PET films described in Section 6.1.2. The SEM micrographs of the substrates after the airborne and waterborne tests are shown in Figure 6.15. These SEM images are representative of the entire surface of the respective substrates. Figure 6.15(a) shows that after the airborne tests, the *E. coli* was distributed not only on the FP upper surface but also entrapped in the space among the fibers as indicated by the arrows. After the waterborne tests, numerous bacteria could be seen to grow in the interstitial spaces and along the thin fibers of FP (Figure 6.15(b)). More bacteria could be seen to grow on FP after the waterborne test (Figure 6.15(b)) compared with those on FP after the airborne test (Figure 6.15(a)). This phenomenon is in contrast to the results obtained for the pristine PET substrate after the airborne and waterborne tests (Figure 6.5(a) and (d)). The reason lies in the difference between porous and nonporous substrates. Since the interstitial spaces of the filter paper are large enough for bacteria to penetrate into, it can be expected that some bacteria would have penetrated deep into the filter paper during the immobilization procedure of the waterborne test which lasts for 2 h. Therefore, the larger concentration of bacteria entrapped in the porous matrix during the waterborne test before the subsequent incubation with growth agar results in a denser population of bacteria growing on FP as shown in Figure 6.15(b).

In the case of the FP-10 after the airborne test, very few sparsely distributed bacteria cells can be spotted over the entire surface (Figure 6.15(c)). A similar observation was obtained for the FP-10 substrate after the waterborne test (Figure 6.15(d)). In contrast to the clumps of bacteria seen in Figure 6.15(b), only few single cells can be spotted.
among the fibers in Figure 6.15(d). Hence, the results obtained using the functionalized filter paper confirm the applicability of the technique to high surface area porous materials and a similar high efficiency of antibacterial action is obtained for both the porous and nonporous materials.
Figure 6.15 Scanning electron micrographs of (a) and (b) FP after exposure to airborne and waterborne *E. coli* respectively and subsequently incubated with solid growth agar for 24 h; (c) and (d) FP-10 after exposure to airborne and waterborne *E. coli*, respectively, and subsequently incubated with solid growth agar for 24 h.
In the case of PVDF, antibacterial assays using waterborne *E. coli* were also carried out for the pristine PVDF membrane and the membrane after surface graft-copolymerization with 10 vol.% 4VP and subsequently derivatized with hexylbromide (PVDF-10). The SEM results of the substrates after the waterborne *E. coli* assay are shown in Figure 6.16. For the pristine PVDF membrane, numerous *E. coli* cells were seen to be distributed on the upper fibers as well as in the interstitial spaces as indicated by the arrows in Figure 6.16. In contrast, almost no active cell growth and division could be seen on the surface of the PVDF-10 membrane after the assay with waterborne *E. coli* (Figure 6.16). Only a few single cells can be seen over the entire surface. This result further confirms the applicability of the technique for conferring high antibacterial efficiency on porous substrates.

The SEM micrographs of the cotton cloth after airborne and waterborne *E. coli* tests are shown in Figure 6.17. Figure 6.17(a) shows the pristine cloth after the airborne test. The presence of *E. coli* on the surface of the cloth fibers is obvious, and aggregates as well as active cell growth and division can also be observed. A similar phenomenon was observed on the pristine cloth after the waterborne test (Figure 6.17(b)), with numerous bacteria cells in the interstitial spaces and along the fibers of the cloth. These cells originated from the ones which initially adhered reversibly on this neutral surface, and which had remained in the active stage. Since the interstitial spaces of the cotton cloth are larger than the *E. coli* cell bodies, the difference in the waterborne and airborne results was not as obvious as those observed with filter paper. Similarly, for the PVDF membranes, the average pore size is in the same range as the *E. coli* cells. Therefore, among the three porous substrates, the filter paper is the most amendable for cell attachment. The results obtained with the three porous substrates confirm that normal material surfaces are good templates for the proliferation of microorganisms.
and biofilm formation will occur readily on such materials in contact with bacteria (Costerton et al., 1999; Lewis, 2001).

**Figure 6.16** Scanning electron micrographs of (a) the pristine PVDF membrane and (b) the PVDF-10 membrane after exposure to the waterborne *E. coli* and subsequently incubated with solid growth agar for 24 h. The PVDF-10 membrane was prepared via UV-induced graft-copolymerization of 4VP (using 10 vol.% 4VP in isopropanol) on pristine PVDF membrane and subsequently derivatized with 20 vol.% hexylbromide in nitromethane.
Figure 6.17 Scanning electron micrographs of (a) and (b) pristine cotton cloth after exposure to airborne and waterborne *E. coli*, respectively, and subsequently incubated with solid growth agar for 24 h; (c) and (d) functionalized cotton cloth after exposure to airborne and waterborne *E. coli*, respectively, and subsequently incubated with solid growth agar for 24 h.
In the case of the functionalized cloth after the airborne test, some single cells can be spotted over the surface of the cloth fibers (Figure 6.17(c)), but the cell bodies are smaller than those observed in Figure 6.17(a). In addition, aggregation and cell division are greatly reduced on this surface. A similar observation can be made for the functionalized substrate after the waterborne test (Figure 6.17(d)). In contrast to the vivid images of bacteria cells with length ranging from 2 to 6 µm as seen in Figure 6.17(b), most of the cells on the fibers in Figure 6.17(d) are of the order of 1 µm in length.

Most of the cells observed on the functionalized cloth surface (Figure 6.17(c) and 6.17(d)) were probably from the initial deposition of the *E. coli* cells either via the airborne or waterborne mechanisms rather than from cell division. Since this substrate bears a high concentration of surface positive charges, strong electrostatic attraction between the substrate surface and the *E. coli* cells which have a negatively charged surface is expected. This is also the case for the other two porous substrates. Since little cell growth or reproduction can be observed from Figures 6.17(c) and 6.17(d), the bactericidal property of the surface-attached polycationic N-hexyl chains is confirmed.

The bactericidal activity of the functionalized cloth was further supported by a comparison of the number of viable cells in the suspension in contact with either functionalized or pristine cotton cloth (Figure 6.18). With the functionalized cloth (2.5 cm × 5 cm), the viable cell number decreased by more than one magnitude after 1 h, and this reduction was further enhanced with time. After 3 h, more than 99.9% of the cells are no longer viable. Control experiments were also performed without the addition of a substrate and with the addition of the pristine cloth. Without any substrate, and with the pristine cloth, the number of viable cells in the suspension decreased by
<6% and <20%, respectively, after 3 h. This relatively minor reduction may have resulted from natural apoptosis. In addition, in the presence of the pristine cloth, the adhesion of the cells to the cloth may also contribute to a small decrease. Since we have earlier shown that the pyridinium moieties are covalently bonded to the substrate with very little possibility for leaching into the suspension, the results of Figure 6.18 further substantiate the effectiveness of the surface polycationic N-hexyl chains in killing the *E. coli* cells.

![Graph](image)

**Figure 6.18** Number of viable *E. coli* cells in PBS at 37°C as a function of time in contact with the different substrates. The cell number was determined by surface-spread method.
The stability of the surface functional groups and their effective bactericidal property was addressed via exposing the functionalized substrate to simulated weathering conditions. After undergoing the simulated weathering for 20 h, the substrate was again tested in the *E. coli* suspension and the viable cell number as a function of time was monitored. As shown in Figure 6.18, a similar efficiency in killing the *E. coli* cells was achieved with the functionalized cloth after weathering. XPS analysis was also carried out on the functionalized cloth after weathering, and the results show that the \([\text{N}^+]/[\text{C}]\) ratio decreased by less than 5%. Therefore, the cotton cloth functionalized via the present method is not only efficient in killing *E. coli* cells on contact but it can also maintain its effectiveness under weathering conditions, with UV-visible irradiation and water spray. This property enhances the application of this technique for substrates required for outdoor use.

**Wild type antibacterial assays of cotton cloth**

The assessment of the antibacterial activities was extended to the wild type microorganisms since biofilms formed by natural environmental microorganisms always host mixed populations with a very diverse consortium of microorganisms, including protozoan species, fungi and diatoms (Shu et al., 2000). Due to this diversity, the microorganisms are also versatile in their ability to colonize substratum. Therefore, it is necessary for a material which is designed for antibacterial purposes to be assessed in terms of mixed-culture biofilm formation on its surface in addition to the pure culture tests.

The wild type multi-species were isolated from soil as described in Section 6.2.2. Millions of multi-species microorganisms exist in the top few centimeters of the soil
but it is possible that after the cultivation in the growth medium (yeast-dextrose broth) the population may be reduced, since certain species would have specific medium requirements. However, since the yeast-dextrose broth can satisfy the nutritional requirements of many types of bacteria, it is expected that a large portion of the original species can be sustained in this medium. Both airborne and waterborne tests were carried out using the wild multi-species bacteria suspension on the pristine and functionalized substrates. The great difference in the cell distribution and subsequent proliferation on the two substrates can be seen in Figure 6.19. The fibers of the pristine cloth were thickly covered with cells of different morphologies and sizes. Interspecies communications were likely to occur after the initial adhesion on this substrate since most cells were in close contact with each other as shown in Figure 6.19(a) and (b). The formation of aggregates on the pristine cloth after the waterborne test is also observable. As discussed in the earlier section on E. coli assays, the pristine cotton cloth serves as an inert substratum for the bacteria adhesion, which occurs reversibly. However, for the multi-species coexistence system, the adhesion may proceed in a sequential way. Certain species would act as the primary colonizer of the substrate, followed by the second stage where other bacteria recognize and attach to these initial colonizers. This in turn may act as a bridge to facilitate the further recognition by other late colonizers, in the so-called coaggregation phenomenon (Rosan and Lamont, 2000). Unlike the airborne and waterborne tests with E. coli, more aggregation of the bacteria can be observed on the pristine cloth after the waterborne test in this case since more multi-species interactions were involved during the initial two-hour immersion in the bacteria suspension of the waterborne test.
Figure 6.19 Scanning electron micrographs of (a) and (b) pristine cotton cloth, (c) and (d) functionalized cotton cloth, after exposure to airborne and waterborne wild type multi-microorganisms, respectively, and subsequently incubated with solid growth agar for 24 h.
In the case of the functionalized surface subjected to the similar tests, the bacteria on the surface of the fibers are more sparsely distributed (Figure 6.19(c) and (d)). The presence of the cells on the functionalized cloth surface may result from the initial deposition or adsorption, since the functionalized surface has a dense positive charge while most of the microorganisms bear negative charges on their cell surfaces at physiological pH (Hogt et al., 1985). From Figure 6.19, the antibacterial effect of the functionalized cotton cloth on mixed populations of wild microorganisms has also been demonstrated, although it is possible that some bacteria may survive the action of the surface polycationic N-hexyl chains since a diverse community with differences in cell structures and cell walls can possess different resistance to the surface functional groups.

The adhesion and coaggregation phenomenon of the wild microorganisms on the pristine and functionalized substrates was further studied after the initial immersion in the cell suspension (of $10^7$ cells/ml) for 2 h (i.e. like the waterborne test but without the overnight incubation). Aggregates were obviously formed and distributed randomly on the pristine cotton cloth (Figure 6.20(a)), which confirms the above proposed mechanism that coaggregation readily occurred during the initial contact process between the pristine cotton cloth and the microorganisms. As far as the effect of surface wettability on bacteria adhesion is concerned, it has been proposed that bacteria species with hydrophobic cell surface property (determined by cell surface components) prefer those materials with the similar hydrophobicity, while those with a hydrophilic cell surface have a preference for hydrophilic materials (Han et al., 1998). It is possible that the cell suspension from soil contains species with a different surface wettability. Therefore, some bacteria species in the suspension with hydrophilic cell
Figure 6.20 Scanning electron micrographs of (a) pristine cotton cloth and (b) functionalized cotton cloth surfaces after immersion in a PBS suspension of $10^7$ cells/ml of wild type multi-microorganisms for 2 h.
surface property would act as the initial colonizer on this pristine substrate surface which is very hydrophilic. The subsequent adhesion may involve those specific cell-cell interactions, similar to the mechanism proposed for the mix-culture biofilm formation (Shu et al., 2000; Kolenbrander, 2000). The initial adhesion is limited by a certain portion of the species in the suspension and their relative population, which accounts for the relatively sparse distribution of aggregates on this surface. However, such a sparse distribution would develop into a higher coverage of the substrate surface with bacteria colonies in close communication after the subsequent overnight culture (Figure 6.19(b)).

In the case of the functionalized substrate, isolated cell bodies without the obvious formation of aggregates are seen (Figure 6.20(b)). The functionalized surface is expected to be more hydrophobic as a result of surface modification with the alkylated functional groups. The increase in hydrophobicity is confirmed by the visual observation of the interaction of the functionalized cloth with water, as compared to that of the pristine cloth. The increase in hydrophobicity and the high density of positive charges of this surface could favor the adhesion of cells to this substrate. It is likely that the electrostatic attraction between the cell surface and the substrate predominates before the coaggregation occurs. However, since there is no significant increase in the density of the microorganisms after further culture (Figure 6.19(d)) from the initial population (Figure 6.20(b)), the effect of the functional groups on the cloth on the retardation of biofilm formation is illustrated.

Additional tests were designed to introduce a higher concentration of the multi-species bacteria on the substrates. Two hundred µl aliquots containing either $10^7$ cells/ml or $10^9$ cells/ml of the wild type microorganisms were pipetted directly onto the substrates
and cultured for 24 h. A fairly uniform layer of biofilm was formed on the pristine cloth surface (Figure 6.21(a)), and with the increase in the cell density of the initial suspension to $10^9$ cells/ml, the biofilm became much thicker (Figure 6.21(b)). In this case, the substrate surface was almost completely covered by the biofilm layer such that its surface morphology is masked. Since these microorganisms were from the natural environment where there is competition for severely limited nutrients, the presence of rich growth nutrients in this test can be expected to lead to the propagation of their population at a more rapid rate than that of the equivalent cultured species (Shu et al., 2000). The corresponding results with the functionalized substrate are shown in Figure 6.21(c) and (d), respectively. With a bacteria concentration of $10^7$ cells/ml, some small colonies or aggregates of bacteria were formed on the surface of the functionalized cloth. When the concentration is increased to $10^9$ cells/ml, the problem of biofilm formation also became more serious (Figure 6.21(d)). However, a comparison with the situation on the pristine cloth shows that the biofilm development at the surface of the functionalized cloth is severely retarded. Due to the variety in the species involved, some species may have better resistance to the inimical attack of the bactericidal functional groups. Furthermore, since in this test, the bacteria cells were deposited directly on the substrate, the accessibility of the surface functional groups may be limited by the coverage of bacteria of high concentration. Nevertheless, the present technique of surface functionalization of cloth with N-alkyl pyridine groups has been shown to be promising for controlling bacterial contamination and biofilm formation.
Figure 6.21 Scanning electron micrographs of (a) and (b) pristine cotton cloth, (c) and (d) functionalized cotton cloth after deposition of 200-µl aliquots containing $10^7$ cells/ml and $10^9$ cells/ml of wild type microorganisms in sterile water, respectively, and subsequently incubated with solid growth agar for 24 h.
6.2.4 Conclusion

Commercial filter paper, PVDF membrane and cotton cloth can also be readily functionalized with the bactericidal polycationic chains using the technique developed for PET films in Section 6.1. The pyridinium groups introduced on these substrate surfaces exhibited a high bacterial killing efficiency as shown by airborne and waterborne *E. coli* assays. The ease of application to different types of porous substrates is thus illustrated as an additional advantage of this technique. The antibacterial efficiency was further substantiated by an additional test on functionalized cotton cloth which measured the number of viable *E. coli* cells in suspension on contact with the cloth. This property can be preserved even after the cloth is subjected to prolonged weathering under UV irradiation and water spray. The functionalized cloth surface also exhibits effective inhibition of biofilm formation by wild type bacteria which were cultured from soil. The effect was observed even with the inoculation of high concentrations of microorganisms.
CHAPTER 7

CONCLUSION
CONCLUSION

A number of surface modification techniques have been developed to functionalize polymeric substrates and biological assessments of the functionalized systems have been designed to evaluate their performances under various working environments. Firstly, graft copolymerization with AAc was carried out on the electrochemically synthesized PPY films to introduce COOH groups. An enzyme, GOD, was then successfully immobilized on the grafted PPY film surface through covalent binding. Viologen groups were also introduced on the PPY film to act as redox mediators. The graft concentration of AAc was found to be dependent on the monomer concentration used and was a key parameter in determining the amount of GOD immobilized. The immobilized GOD still retained ~ 30% of the activity of the equivalent free enzyme, while the presence of viologen moieties under UV irradiation was demonstrated to facilitate the functioning of the GOD in the absence of oxygen at a relative enzymatic activity of ~ 15%. The ability of GOD to resist temperature-induced denaturization was enhanced upon immobilization via the technique developed in this work.

The surface functionalization technique was then successfully extended to the immobilization of HA on the PPY films. HEA was first grafted and the desired primary amine functional groups were introduced on PPY via silanization of the hydroxyl groups arising from the grafted HEA. Immobilization of HA was then achieved via the coupling reaction between the COOH groups of HA with the amine functional groups. The HEA monomer concentration has a determinative effect on the surface graft concentration of HEA and the amount of HA immobilized. The highest amount of HA on the PPY surface achieved was 0.07 mg/cm². The electrical
conductivity and mechanical strength of the HA modified PPY film was well preserved. Protein binding assays confirmed the biological activity of the immobilized HA and stability tests showed that HA still retains about 40% of its activity even after 4-day storage in air. The bioactivity of the immobilized HA was further assessed from cellular responses of PC 12 cells. A high degree of cell attachment of ~ 80% was achieved with the cells primed with NGF, probably as a result of the high expression of CD44 as a HA receptor on the PC 12 cell surface. Moreover, neurite outgrowth of PC 12 cells was also enhanced on the HA functionalized PPY surface even in the absence of NGF. On the other hand, the HA functionalized PPY film can also reduce *E. coli* bacteria adhesion, though it does not possess bactericidal property to inhibit the subsequent biofilm development.

A strategy to eradicate biofilm formation was then designed based on a surface modification technique to introduce pyridinium groups. Commercial PET films were first surface graft copolymerized with 4VP, which was subsequently alkylated with hexylbromide to form pyridinium groups. The 4VP graft concentration was controlled by varying the monomer concentration which in turn determined the concentration of the polycationic groups, which possess the antibacterial property. Simulated airborne and waterborne assays with *E. coli* ascertained the antibacterial activities of the modified PET surface. A surface concentration of 15 nmol/cm² on PET has been shown to be highly effective on killing the bacteria on contact. The long-term stability of the bactericidal property of the modified PET film is another advantage of this surface grafting technique. This functionalization technique was then extended to porous materials which are polymer based as well as carbohydrate based, i.e. filter
paper, PVDF membrane and cotton cloth. A similar antibacterial property was effectively conferred on these porous materials.

A more detailed antibacterial assay was carried out on the commercial cotton cloth. The presence of the cloth functionalized with pyridinium groups (2.5 cm × 5 cm) in a bacterial suspension (50 ml) of $10^5$ cells/ml resulted in more than 99% reduction in the number of viable cells in 3 h. A wild type and multi-species system which originated from soil was also tested to evaluate the antibacterial activity of the surface polycationic groups against environmentally occurring biofilm formation. The biofilm formation was significantly inhibited on the functionalized cotton cloth compared with the corresponding pristine one. The bactericidal property of the modified cotton cloth can be preserved even after prolonged weathering under UV irradiation and water spray. Hence, the technique developed in this work to confer bactericidal properties on surfaces via immobilized pyridinium groups has been successfully applied to materials of different chemical compositions and morphologies. This method has the advantages of ease of application and good control of the surface concentration of the antibacterial groups.
CHAPTER 8

RECOMMENDATIONS FOR FURTHER STUDY
The present work has demonstrated the application of surface modification to render materials with desired properties for interacting with biological systems. The following are some recommendations for further work:

**Fabrication of Electrically Conductive Polymer Based Biosensors**

As mentioned in Chapter 2, conducting electroactive polymers have been demonstrated to have remarkable applications especially in biosensor constructions. The work described in Chapter 3 has shown that an electron mediator (viologen) and an enzyme (GOD) can be simultaneously incorporated on the surface of the PPY conducting polymer. Thus further efforts may be devoted to the investigation of this system in biosensing applications, especially under conditions devoid of oxygen. The mechanisms of the process should be investigated in greater depth. Furthermore, progress in this area may be combined with developments in microelectrode technologies which have already been established in the electronic nose device to further exploit the design of smaller, more compact and portable sensing systems.

**Investigation of Electrical Stimulation in Combination with Hyaluronic Acid on Neurite Outgrowth**

It has been shown that electrical stimulation promotes the neurite outgrowth, and in vivo experiments have also shown that no negative response on tissue compatibility and transected sciatic nerve regeneration studies has been observed while nerve regeneration was enhanced (Schmidt et al., 1997; Shastri et al., 1996). Thus, as an extension to the work described in Chapter 5, the effect of electrical stimulation on the
in situ PC 12 cell culture on HA functionalized PPY film may be investigated. The investigation should cover the effects of the time interval and the level of current passed with constant monitoring of the behavior of cells.

**Investigation of Effect of Electrical Stimulation on Antibacterial Activity**

As discussed in Chapter 2, strategies for biofilm eradication include the synergism between chemical and physical techniques. The effect of electrical current on the function of biocides has been studied by Wattanakaroon and Stewart (2000) and the results showed the enhancement of the bacteria killing efficiency. Such an effect would be well suited for electrically conductive polymers in concert with the antibacterial surface functionalization technique described in Chapter 6. It is possible that the synergistic actions may bestow the conducting polymers with excellent antimicrobial surface properties.

**Assessment of Antibacterial Activities of Functionalized Surfaces on Different Species**

The present work only involves the antibacterial assay on *E. coli* and wild type multi-species without clear identification of its components. The applicability of antimicrobial surface treatment method may be extended to a wider range of microbes, such as fungi and parasites or even viruses which would be a great challenge. Additional methods should also be developed to assess the antibacterial activities as well as to explore the mechanism involved in the process. The monitoring of
potassium leakage by atomic absorption spectrophotometry could be an indicator of possible damage to the membranes of microorganisms. The potassium ions leak out of the cells very quickly when the membranes are damaged or disrupted. Thus, this test may give further information on the mechanism involved. Furthermore, transmission electron microscopy can also be applied to examine the internal cell organelles of microorganisms in contact with the functionalized material surfaces.
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