ELECTRICALLY CONDUCTING POLYMERS FOR
NON-INVASIVE CONTROL OF MAMMALIAN CELL BEHAVIOR

by

Joyce Yun-Wei Wong


Submitted to the Department of Materials Science and Engineering
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at the

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Signature of Author ............................................................... Department of Materials Science and Engineering

Robert Langer
Germeshausen Professor of Chemical and Biomedical Engineering
Thesis Supervisor

Accepted by ................................................................. Carl V. Thompson III
Professor of Electronic Materials
Chair, Departmental Committee on Graduate Students

Massachusetts Institute
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ABSTRACT

Electrically conducting polymers are novel in that their surface properties, including charge-density and wettability, can be reversibly changed with an applied electrical potential. Since the nature of interactions of proteins and cells with surfaces are influenced by the physicochemical properties of the underlying surface, electrically conducting polymers can potentially have unique biological applications. However, to date, the majority of research on conducting polymers has been carried out under non-biological conditions.

Optically transparent thin films of polypyrrole were synthesized using chemical oxidative and electrochemical techniques. The formation of thin films of polypyrrole was confirmed by UV/VIS spectroscopy, contact angle and electrical conductivity measurements. Polypyrrole was able to be switched between its native charged, oxidized state and its neutral state via an applied electrical potential in environments suitable for protein adsorption and mammalian cell culture. This was determined by measuring the UV/visible spectrum of polypyrrole during potential application. The neutral form of polypyrrole is unstable in aqueous environments and it was necessary to hold the polymer in its neutral state via an applied electrical potential below 0 V (vs. Ag/AgCl).

In vitro studies demonstrated that extracellular matrix molecules, such as fibronectin, adsorb efficiently onto polypyrrole thin films and support cell attachment under serum-free conditions. When aortic endothelial cells were cultured on fibronectin-coated polypyrrole (oxidized) in either chemically-defined medium or the presence of serum, cells spread normally and synthesized DNA. In contrast, when the polymer was switched to its neutral state by applying an electrical potential (-0.25 V vs. Ag/AgCl), both cell extension and DNA synthesis were inhibited (> 98%) without affecting cell viability. Application of a similar electrical potential to cells cultured on indium tin oxide surfaces had no effect on cell shape or DNA synthesis. These data suggest that electrically conducting polymers may represent a type of culture substrate which could provide a non-invasive means to control the shape and function of adherent cells, independent of any medium alteration.

Thesis Supervisor: Professor Robert Langer
Title: Germeshausen Professor of Chemical and Biomedical Engineering
Dedication

To my parents, Wen-Kuei and Shaio-wen Wong
and to T.C.
Acknowledgments

First, and foremost, I would like to thank my advisor, Bob Langer, for his support and constant encouragement. I feel extremely lucky to have had an advisor whom I felt at ease with whether the subject was about science or the best restaurants in town. To this day, I am still amazed by his unbelievable energy, enthusiasm, and ability to motivate people.

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# Table of Contents

Title Page ......................................................................................................... 1

Abstract ............................................................................................................ 2

Acknowledgments ............................................................................................ 4

Table of Contents ............................................................................................. 6

List of Illustrations and Figures ..................................................................... 9

List of Tables .................................................................................................... 12

Chapter 1: Introduction ................................................................................ 13
  1.1 Motivation ......................................................................................... 13
  1.2 Objectives ....................................................................................... 14
  1.3 Outline of thesis ............................................................................. 15
  1.4 Citations ........................................................................................... 16

Chapter 2: Background ................................................................................. 17
  2.1 Electrically Conducting Polymers ............................................. 17
  2.2 Cell-Surface Interactions ........................................................... 25
  2.3 Biological Applications of Electrically Conducting Polymers ............................................................................. 26
  2.4 Summary ....................................................................................... 27
  2.5 Citations ........................................................................................ 29

Chapter 3: Selection of Conducting Polymer ............................................... 37
  3.1 Selection of Conducting Polymer ............................................... 37
  3.2 Experimental .............................................................................. 39
    3.2.1 Materials and Equipment ............................................. 39
    3.2.2 Methods and Procedures .............................................. 40
    3.2.2.1 Polyaniline Synthesis ......................................... 40
    3.2.2.2 Polypyrrole Synthesis ........................................ 41
    3.2.2.3 Electrical Properties and Stability in Biological Environments .................................... 41
    3.2.2.4 Toxicity Study .................................................... 42
  3.3 Results and Discussion ..................................................................... 45
    3.3.1 Polyaniline ..................................................................... 45
    3.3.2 Polypyrrole .................................................................... 46
  3.4 Conclusions ................................................................................. 50
  3.5 Citations ...................................................................................... 51

Chapter 4: Polymer Synthesis and Characterization .................................... 53
  4.1 Introduction ................................................................................ 53
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2.2.1</td>
<td>Measurement of pH and Temperature as a Function of Potential</td>
<td>117</td>
</tr>
<tr>
<td>7.2.2.2</td>
<td>Contact Angle as a Function of Potential</td>
<td>118</td>
</tr>
<tr>
<td>7.2.2.3</td>
<td>Fibronectin Release</td>
<td>120</td>
</tr>
<tr>
<td>7.2.2.4</td>
<td>Conditioned Media</td>
<td>120</td>
</tr>
<tr>
<td>7.2.2.5</td>
<td>Stability of Polypyrrole as a Function of Potential</td>
<td>121</td>
</tr>
<tr>
<td>7.2.2.6</td>
<td>Reversibility</td>
<td>121</td>
</tr>
<tr>
<td>7.3</td>
<td>Results and Discussion</td>
<td>122</td>
</tr>
<tr>
<td>7.4</td>
<td>Conclusions</td>
<td>132</td>
</tr>
<tr>
<td>7.5</td>
<td>Citations</td>
<td>133</td>
</tr>
</tbody>
</table>

Chapter 8: Future Directions ................................................................. 134
List of Illustrations and Figures

Figure 2.1. Conjugated polymer with delocalized electrons ...................... 18
Figure 2.2. Doping of polyacetylene .......................................................... 20
Figure 2.3. Conductivity Chart ................................................................. 23
Figure 3.1. Experimental setup for conductivity measurements ............ 44
Figure 3.2. Conductivity of polypyrrole doped with tosylate as a function of time .......................................................... 47
Figure 3.3. % Cell viability of 3T3 Balb/c fibroblasts exposed to polypyrrole .......................................................... 49
Figure 4.1. Structure of polypyrrole in the oxidized state ...................... 59
Figure 4.2. Contact angle vs. thickness for polypyrrole thin films made electrochemically .......................................................... 62
Figure 4.3. Absorption spectra of polypyrrole thin films formed electrochemically and chemically .......................................................... 64
Figure 4.4. Absorption spectra of polypyrrole in its oxidized and reduced states .......................................................... 66
Figure 4.5. Absorption spectra of reduced polypyrrole before and after exposure to water and air .......................................................... 67
Figure 5.1. Schematic of chamber used for protein adsorption studies .......................................................... 75
Figure 5.2. % Adsorption of fibronectin to various substrates at 1, 10, 100, and 1000 ng/cm2 coating densities .......................................................... 80
Figure 5.3. Quanitation of amount of fibronectin adsorbed (fmol/cm2) onto various substrates as a function of amount of fibronectin added .......................................................... 81
Figure 5.4. Attachment of bovine aortic endothelial cells after 4 hour incubation in the absence of calf serum .......................................................... 84
Figure 5.5. Bovine aortic endothelial cells after 4 hr incubation on (a) PPy/chem with FN, (b) PPy/chem without FN, (c) TC with FN, (d) TC without FN. .............................................................. 85

Figure 5.6. Bovine aortic endothelial cells after 4 hr incubation on (a) PPy/ITO with FN, (b) PPy/ITO without FN, (c) ITO with FN, (d) ITO without FN. ............................................................... 86

Figure 5.7. Bovine aortic endothelial cells after 4 hr incubation on (a) PD with FN, (b) PD without FN. .............................................................. 87

Figure 5.8. Distribution of projected cell area on polypyrrole formed chemically as measured by image analysis system. ......................... 88

Figure 5.9. Distribution of projected cell area on polypyrrole formed electrochemically. ................................................................. 89

Figure 5.10. Distribution of projected cell area on TC. ......................... 90

Figure 5.11. Distribution of projected cell area on PD. ......................... 91

Figure 5.12. Distribution of projected cell area on ITO. ....................... 92

Figure 6.1. Cyclic voltammogram of polypyrrole in serum-free DMEM culture medium. ............................................................... 101

Figure 6.2. UV/VIS spectra of polypyrrole in its native oxidized state under no potential (a) and reduced by application of either -0.25 V (b) or -0.5 V (c) ................................................................. 102

Figure 6.3. Photomicrographs of endothelial cells cultured for 4 hr on FN-polypyrrole in either its native oxidized state (a) or after reduction by application of -0.5 V for 4 h (b) (x 900). ..... 104

Figure 6.4. DNA synthesis in cells cultured in serum-containing medium on different FN-coated substrata in the absence (-) or presence (+) of an applied electrical potential (-0.25 V) ........................................................................ 105

Figure 7.1. Experimental set-up for determination of contact angle on polypyrrole (PPy). ................................................................. 119

Figure 7.2 pH of unbuffered saline on polypyrrole held in its neutral state at -0.25 V measured as a function of time. ......................... 124

Figure 7.3 pH of DMEM with 20 mM Hepes on polypyrrole held in its neutral state at -0.25 V measured as a function of time. ....... 125
Figure 7.4  Temperature of DMEM with 20 mM Hepes on polypyrrole held in its neutral state at -0.25 V measured as a function of time. ........................................................................................ 126

Figure 7.5  Temperature of unbuffered saline on polypyrrole held in its neutral state at -0.25 V measured as a function of time .............................................................................................. 127

Figure 7.6  UV/Visible spectra of polypyrrole as a function of oxidation state. ................................................................................................................................. 129
List of Tables

Table 3.1. Stability and processing status of representative conducting polymers ......................................................... 38

Table 4.1. Conductivity and contact angle values of films of polypyrrole ........................................................................... 61

Table 7.1. % FN release calculated as the fraction of total amount of fibronectin added ............................................................. 131
Chapter 1: Introduction

1.1 Motivation

The importance of the in vitro culture of mammalian cells has become increasingly evident with the increased demand for products such as vaccines, hormones, monoclonal antibodies, lymphokines, and therapeutic enzymes. Mammalian cell culture is also used to expand a cell population from a patient for grafting purposes in tissue engineering (Langer and Vacanti, 1993). Thus, the need for efficient methods of large scale animal cell culture has led to many efforts to control cell growth or maintain differentiated function. Since most mammalian cells are anchorage-dependent (i.e. attachment to a substrate is required for cell survival or growth) the cellular response will depend on the interactions between cell and substratum. In many cases, there is an additional protein layer that is adsorbed to the substratum. From a vast number of studies (Barngrover, 1986) examining cells and proteins on various artificial substrata, common characteristics of substrata which have been found to be important are (1) surface roughness, (2) charge density, and (3) wettability. Moreover, cell shape has been shown to be linked to its function: differentiated when rounded and growing when extended (Folkman and Moscona, 1978; Ingber, 1990; Mooney et al., 1992). Thus, if one could control cell shape by manipulating the characteristics of the substrate, it follows that cell function could be controlled as well.

Electrically conducting polymers are a class of polymers which are unique in that their surface properties such as charge-density and
morphology can be altered depending on the oxidation state of the polymer. The details of this process is described in Chapter 2. Since the surface properties of electrically conducting can be controlled with an applied electrical potential, the starting hypothesis for this thesis was that cells would behave differently on conducting polymers depending on its oxidation state.

1.2 Objectives

The goal of this thesis has been to develop a system to noninvasively manipulate cell shape and hence function by controlling the interaction between cells and proteins with conducting polymers. In order to reach this objective, several steps have been completed.

(1) A polymer system was developed such that reversible cycling between conducting and insulating states was possible in physiological media and in the presence of oxygen.

(2) The polymer system in its charged state was evaluated for its protein binding capability and its ability to support cell attachment and spreading.

(3) Cell shape and growth as a function of polymer oxidation state were examined.

(4) Studies were carried out to determine the mechanism of cell shape/growth control.
1.3 Outline of thesis

This thesis is organized into eight chapters. A general background to electrically conducting polymers and cell-substrate interactions is presented in Chapter 2. Studies examining different conducting polymer systems selection of the model system are given in Chapter 3. Polymer synthesis and characterization is presented in Chapter 4. The results of protein adsorption and cell attachment to the polymer system is given in Chapter 5. Chapter 6 details the results of cell shape and growth control experiments. Studies conducted to determine the mechanism for cell shape and growth control are detailed in Chapter 7. Finally, in Chapter 8, the key conclusions are summarized and future recommendations are presented. Citations are presented at the end of each chapter.
1.4 Citations


Chapter 2: Background

2.1 Electrically Conducting Polymers

Electrically conducting polymers are a relatively new class of materials. Before their discovery over 20 years ago, polymers were thought to be purely insulating materials. In general, a polymer has the capability to be electrically conducting if it has a conjugated backbone. The generation and propagation of charge carriers occurs via the delocalized electrons (π-orbital overlap) of the backbone or pendant groups (Figure 2.1). These new materials caused quite a bit of excitement when they were first discovered: it was thought that by simply transferring over established processing methods, used in conventional plastics, would result in new and rather exotic applications. Unfortunately, this assumption was found to be incorrect since early attempts resulted in materials that were insoluble, infusible, brittle, and unstable in air (Kanatzidis, 1990).

Two major factors helped change this situation dramatically. The first was the demonstration in the early 1970s that direct polymerization of acetylene yielded strong, self-supporting films of polyacetylene (Shirakawa et al., 1977). However, this polymer was found to be a poor semiconductor, and it was not until the second big breakthrough in 1978 that it was discovered that partial oxidation or reduction transformed a polymer into its conductive form (Chiang et al., 1978). This process is called “doping”, because it is somewhat related to the doping process used in semiconductors. During the doping process, mobile charge carriers are generated from a neutral chain (Figure 2.2).
Figure 2.1. Conjugated polymer with delocalized electrons
The neutral polymer chain can be oxidized or reduced to become either positively (oxidative, or p-type) or negatively charged (reductive, or n-type) respectively, with polarons and bipolarons as the charge carriers for electrical conduction (Kanatzidis, 1990). The conductive form of the polymer contains counterions which serve to maintain charge neutrality but do not affect the oxidation level of the polymer. The dopant ion does influence, however, both the structural properties and the electroactivities (switching between conductive and insulative states) of the polymer (Diaz and Bargon, 1986). When the polymer is switched between the conductive and insulating states, the dopant ions diffuse in and out of the polymer, or in some cases the dopant anion remains and cations diffuse in (Iseki et al., 1991). This doping process can be achieved either chemically or electrochemically and is reversible.

In more detail, the neutral polymer chain (Figure 2.2a) is oxidized with the removal of electrons to form radical cations (Figure 2.2b). The radical ions are delocalized over a portion of the backbone, creating a structural defect known as a polaron which contains both spin and positive charge. Two polarons can then diffuse together and combine spins to form a bond, leaving the bipolaronic species (Figure 2.2c). The positive charges on the polymer backbone act as charge carriers in electrical conduction. Conduction can occur either along segments of the conjugated polymer chain, or charges can hop between chains. The degree of oxidation controls the number of charges created which, in turn, controls the bulk electrical conductivity of the material. Note that counterions (A\(^-\) in Figure 2.2), also known as dopant ions, stabilize the positive charge.
Figure 2.2. Doping of polyacetylene
Significant progress in the area of processing conducting polymers has been made in the past ten years. The development of conducting polymers has been reviewed extensively elsewhere (Kanatzidis, 1990; Naarmann, 1990; Reynolds, 1988; Wnek, 1986). Several approaches have been used to overcome the insolubility problem: soluble precursor polymer route; in-situ polymerization; and synthesis of new derivatives and self-doped monomers (Yue and Epstein, 1990). Using these methods, conducting polymers can now be processed either chemically or electrochemically into films, fibers, coatings, and gels.

Free-standing films of conducting polymers, however, often are brittle, inflexible, and have poor mechanical properties. In order to improve toughness and flexibility, composite films of conducting polymers and support materials such as poly(vinyl alcohol) (Chen and Fang, 1991), polyurethane (Pei and Bi, 1989), and even filter paper (Bocchi et al., 1987) have been prepared. These composites, prepared electrochemically or chemically, combine electrical properties of the conducting polymer with the mechanical properties of the substrate.

Areas of research in conducting polymers during the past 20 years have largely concentrated on exploring different routes of polymerization and characterizing electrochemical properties, but there have also been studies examining mechanical properties, morphology, spectroscopy, surface charge, and crystal structure. However, the major obstacle preventing realization of practical applications is the instability of conductive polymers. It is surprising that despite the obvious importance of stability in any potential application of conducting polymers, there have been remarkably few
systematic studies examining stability (Wang and Rubner, 1990). Moreover, few systematic studies, if any, of the effect of dopant content and plasticization by residual solvent on the mechanical properties of conducting polymers have been conducted. Polypyrrole is one of the few conducting polymers which have been subjected to systematic studies of mechanical properties, and even here the data is insufficient (Billingham and Calvert, 1989).

While there are some obstacles such as stability which still must be overcome, electrically conducting polymers do offer advantages over other materials and justifies further research of these materials. For example, conducting polymers are also known as synthetic metals due to their high intrinsic electronic conductivities (Figure 2.3) (Blythe, 1979).

In addition to high electrical conductivity, the attractive properties of conductive polymers include the capability to control charge-density through the extent of oxidation and reduction; the ability to cycle between conductive and insulating states; derivatization of the polymer; and variation of the dopant ion. Like other synthetic polymers, the properties and quality of conductive polymers depend on a large number of reaction conditions: monomer, synthesis procedure, temperature, concentration of reactants, pressure, additives, impurities, red-ox, environment, and solvent. In addition, the properties depend on factors such as molecular weight, polydispersity, chain branching, crystallinity, and morphology (Reynolds, 1988).
Figure 2.3. Conductivity Chart
Probably the most interesting property relevant to biological systems is the capability of the polymer to switch between charged and neutral states. As discussed above, conducting polymers undergo reversible dimensional and conformational changes during this switching process. Alternative methods to effect large, reversible dimensional changes of polymers can also be achieved through pH changes (Katchalsky and Eisenberg, 1950), electric field (Irie, 1986; Shiga et al., 1989), and photoirradiation (Irie, 1986; Mamada et al., 1991). These studies, however, are limited to non-conducting, or insulating, polymers. Furthermore, since this thesis is concerned with mammalian cell culture which requires the pH to remain essentially at 7.4, it is undesirable to subject the system to changes in pH.
2.2 Cell-Surface Interactions

The study of interactions of cells and proteins with artificial surfaces is important since most mammalian cells are anchorage-dependent and must attach to a surface in order to grow and proliferate. Theories proposed to explain how cells attach to surfaces can be divided into two categories: (1) physicochemical factors and (2) specific involvement of a biological molecule. Physicochemical factors found to affect cell attachment include wettability (Dekker et al., 1991; Horbett et al., 1985; Kang et al., 1989; Matsuda et al., 1990), surface charge (Hattori et al., 1985; van Wachem et al., 1987), (Valentini et al., 1992) surface composition (Dulcey et al., 1991; Stenger et al., 1992), and surface morphology (Ranieri et al., 1993; Ricci et al., 1991). Specific receptor-ligand interactions include a class of molecules, the extracellular matrix molecules (ECM), which are recognized by receptors on the surface of the cell. Examples of ECMs are fibronectin, laminin, and vitronectin.

Two forces thought to be involved in the interaction between cells and their underlying substrate are electrostatic between charged surfaces and van der Waals forces between ions and dipoles. While cells have a net negative charge provided by glycosaminoglycans such as heparan sulfate and sialic acid-containing proteoglycans at the surface, cells attach to both negatively and positively charged surfaces (van Wachem et al., 1987). The situation becomes more complicated since in many instances the cells are not attached directly to the surface, but to an adsorbed layer of proteins approximately 10 nm thick (Jauregui, 1987). The adsorption of these proteins to materials have been shown to depend on the hydrophobicity of the
surface (Matsuda et al., 1990) and thus the physicochemical characteristics of the surface will affect cell behavior. Thus, in designing appropriate substrata to control cell behavior in vitro, one can manipulate the surface charge, hydrophilicity/hydrophobicity, physical and chemical anisotropy, and/or substrate contractility (Jauregui, 1987).

2.3 Biological Applications of Electrically Conducting Polymers

Conducting polymers offer several appealing properties for biological applications. First, application of an electrical potential can change reversibly the surface properties of the conducting polymer via oxidation and reduction under proper conditions: the surface can be manipulated such that its charge-density changes under an applied electric field. This is important because charge-density and electric fields have been shown to affect nerve regeneration (Valentini et al., 1989). Second, exposure of the polymer to oxidizing or reducing potentials causes dopant ions to diffuse into or out of the conducting polymer matrix, respectively. When a conducting polymer is switched from its conducting to insulating state, the polymer undergoes a change in conformation. This is supported by data from cyclic voltammograms (Heinze et al., 1987) which show the shift between anodic and cathodic peaks upon reduction and oxidation. In addition to conformational changes, large dimensional changes may occur upon electrochemical doping and dedoping (Baughman et al., 1990). Thus, cells attached to these polymers may experience mechanical stresses as well. Mechanical compression has been shown to result in fluid flow, pressure gradients, streaming potentials and currents in cartilage. Static mechanical
compression on chondrocytes has been shown to cause a dose-dependent
decrease in sulfate incorporation (Sah and Grodzinsky, 1989).

Currently, the majority of research on conductive polymers examines
their behavior under conditions which would be rather stringent for cells: for
example, low to zero oxygen concentration, little or no moisture, low pH, and
solvents which would be harsh to cells. It is not obvious that conductive
polymers will behave the same way under physiological conditions as they do
in environments for applications such as rechargeable batteries. Some
research groups (Boyle et al., 1989; Shinohara et al., 1985; Shinohara et al.,
1989) have examined the cycling capacity of the conducting polymer
polypyrrole in saline and other buffer solutions. Polypyrrole is perhaps the
most widely studied polymer due to its chemical and thermal stability, ease
of preparation, and electroactivity (Street, 1986). In fact, polypyrrole has
been examined in biological environments for use as biosensors (Umana and
Waller, 1986), electrodes to obtain electrochemically controlled drug release
(Miller, 1988), and substrates which bind proteins (Prezyna et al., 1991;
Smith and Knowles, 1991; Wallace and Lin, 1988) or DNA (Minehan et al.,
1991). However, the interaction of living cells with electrically conducting
polymers has remained essentially unexplored.

2.4 Summary

Once the interactions between electrically conducting polymers and
cells are better understood, a wide range of applications can be envisioned.
This class of polymers is attractive in that the surface charge can be
reversibly altered using an applied electrical field. For example, if
electrically conducting polymers can be shown to switch cellular function
between growth and differentiation or reversibly control the adhesion of cells, they could be used as materials for tissue regeneration, wound-healing, or bioreactors for up-scale production of cell products (proteins, hormones, etc.).
2.5 Citations


3.1 Selection of Conducting Polymer

As discussed in the previous chapter, a great deal of research has been carried out in efforts to exploit the unique redox properties of conducting polymers. Judging from the current status of conducting polymers (Table 3.1), polyaniline, polythiophene, and polypyrrole appear to be the best candidates in terms of stability and processibility. While polythiophene is relatively stable in air, it is very sensitive to water, and its conductivity falls rapidly over a period of a few hours (Billingham and Calvert, 1989). In addition, although poly (3-alkyl thiophene) can be formed into a gel (Yoshino et al., 1989), its reversible swelling and shrinking properties have only been observed in solvents such as ethanol and chloroform, which are clearly not favorable conditions for mammalian cells. Thus it appears that the conducting polymers which show the most promise for use in biological systems are polyaniline and polypyrrole. Furthermore, as discussed in Section 2.3, polypyrrole has been studied in biological environments, although not with mammalian cells.

Since it was not known a priori which conducting polymer would be better suited as a substratum for cell culture, both polyaniline and polypyrrole were studied.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conductivity (S/cm)</th>
<th>Stability (doped state)</th>
<th>Processing Possibilities</th>
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<tr>
<td>Polyacetylene</td>
<td>$10^3 - 10^5$</td>
<td>poor</td>
<td>limited</td>
</tr>
<tr>
<td>Polyphenylene</td>
<td>1000</td>
<td>poor</td>
<td>limited</td>
</tr>
<tr>
<td>Poly (phenylenesulfide)</td>
<td>100</td>
<td>poor</td>
<td>excellent</td>
</tr>
<tr>
<td>Poly (phenylene vinylene)</td>
<td>1000</td>
<td>poor</td>
<td>limited</td>
</tr>
<tr>
<td>Polypyrrole</td>
<td>100</td>
<td>good</td>
<td>good</td>
</tr>
<tr>
<td>Polythiophene</td>
<td>100</td>
<td>good</td>
<td>excellent</td>
</tr>
<tr>
<td>Polyaniline</td>
<td>10</td>
<td>good</td>
<td>good</td>
</tr>
</tbody>
</table>

Table 3.1. Stability and processing status of representative conducting polymers (Rubner, 1992).
3.2 Experimental

3.2.1 Materials and Equipment

Aniline, nitric acid, sodium hydroxide, hydrochloric acid, and gold wire (0.25 mm diameter) were purchased from Aldrich (Milwaukee, WI). Ultrapure water was obtained from a Millipore Milli-Q Reagent Water System (Bedford, MA). Ammonium persulfate was purchased from Mallinckrodt (Chesterfield, MO). Pyrrole was purchased from Kodak Laboratory Chemicals (Rochester, NY). Tetraethylammonium - p - toluene sulfonate was purchased from Alfa Products (Ward Hill, MA). Stainless steel was purchased from Eastern Stainless Corp. (Baltimore, MD). Spectral grade acetonitrile was purchased from EM Science (Gibbstown, NJ). Conductive graphite paint (Electrodag 112) was purchased from Acheson (Port Huron, MI). Ethylene oxide sterilizing gas ampules were purchased from H.W. Andersen Products, Inc. (Chapel Hill, NC). Dulbecco’s Modified Eagle Medium (DMEM), Dulbecco’s phosphate buffered saline without additives (DPBS), calf serum, penicillin, streptomycin, l-glutamine, and trypsin were purchased from Gibco BRL (Grand Island, NY). Balb/c 3T3 fibroblasts (6587) were purchased from the American Type Culture Collection (Rockville, MD). Trypan blue was purchased from Sigma (St. Louis, MO). Tissue culture dishes were purchased from either Falcon (Becton Dickinson & Co., Franklin Lakes, NJ) or Corning (Corning, NY).

Substrates were cleaned in an ultrasonic cleaner purchased from Fisher Scientific (Springfield, NJ). Galvanostatic synthesis was performed using a Keithley 224 programmable current source and a 614 electrometer (Solon, OH). Incubation for stability and tissue culture studies were carried
out in a 37 °C, 5% CO₂ incubator purchased from Forma Scientific (Marietta, OH). Film thicknesses were determined using a Sloan Dektak IIA profilometer (Veeco Instruments, Inc., Santa Barbara, CA).

3.2.2 Methods and Procedures

3.2.2.1 Polyaniline Synthesis

In situ polymerization of aniline on standard glass slide substrates was carried out according to a previously reported method (Wei et al., 1989) with some modification. Standard microscopic glass slides (1” x 3”) were ultrasonically cleaned for 15 min in a solution of water / nitric acid (50 / 50 vol/vol) previously warmed for 15 min; rinsed in ultrapure water; ultrasonically cleaned in a solution warmed for 15 min of 0.2% vol/vol NaOH/H₂O; rinsed again in ultrapure water; and dried in an oven for one hour at 70°C. In a 50 ml beaker, 1 ml of aniline was dissolved in 25 ml of 1 M HCl and placed in a 4°C water bath. In a separate 50 ml beaker, 3.2 g of ammonium persulfate was dissolved in 25 ml of 1 M HCl and placed in a 4°C water bath. The ammonium persulfate solution was slowly added to the aniline solution, and the ultrasonically cleaned glass slides were placed in the mixture for 8 min. The resulting film was then rinsed with ultrapure water. Film thickness was determined using a Sloan Dektak IIA profilometer. At least three different regions of the film were scored with a razor blade. The depth-sensitive stylus was then gently dragged over a 1 mm section surrounding the scored area on the sample surface, and the depth profile was recorded. A minimum of three measurements were taken for each scored area on the sample.
3.2.2.2 **Polypyrrole Synthesis**

Polypyrrole films were synthesized electrochemically according to a previously reported method (Wynne and Street, 1985) which results in polypyrrole in its charged (doped) state. A solution of 0.3 M pyrrole, 0.15 M tetraethylammonium-p-toluene sulfonate, and ultrapure water (0.5 vol/vol %) were added to a 1500 ml beaker which served as the electrochemical cell. Acetonitrile was added to make the total volume of the solution 1200 ml. The anode and cathode (stainless steel, 0.05 cm x 10 cm x 12.7 cm) were separated by a distance of 5 cm and were connected to a Keithley 224 programmable current source with a current density of 0.54 mA/cm². The solution covered approximately 10 cm x 8 cm. The films were prepared without precautions to exclude air, but the cell was covered with aluminum foil to minimize loss of solvent. After six hours, the anode was removed and rinsed with acetonitrile. Film thicknesses were measured with a micrometer by measuring the difference in thickness between the plain stainless steel and areas in which the polypyrrole film was attached. After drying overnight, polypyrrole was carefully removed from the electrode by scraping with a razor blade.

3.2.2.3 **Electrical Properties and Stability in Biological Environments**

A four point probe method (van der Pauw, 1958) was used to determine the electrical conductivity of the films. Equation 3.1 shows the relation used to determine resistivity. The conductivity, \( \sigma \), is the reciprocal of resistivity, \( \rho \).

\[
\rho = \frac{\pi d}{\ln 2} \left( \frac{R_{AB-CD} + R_{BC-DA}}{2} \right) f \left( \frac{R_{AB-CD}}{R_{BC-DA}} \right) \tag{3.1}
\]
where, \( d \) is the thickness of the film, \( R_{AB-CD} \) and \( R_{BC-DA} \) are the resistances, and \( f \) is a function described by van der Pauw (van der Pauw, 1958).

Electrodag 112 and gold wire were used to establish electrical contact with the films. Constant current was generated from a Keithley 224 programmable current source, and the voltage was recorded using a Keithley 614 electrometer (Figure 3.1). Five measurements of current-voltage values in each configuration were made for each sample. Conductivity was recorded periodically up to a month after the initial measurements. In addition, electrical conductivity of polypyrrole (PPy) in various environments was tested. Square samples were cut with a razor blade; adhered to glass slides with double-sided tape; and placed in petri dishes. To determine whether tissue culture conditions would alter the polymer, conductivity was measured before and after ethylene oxide sterilization (24 hr), UV irradiation (30 min on each side of film), and exposure to Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (overnight incubation at 37°C) were measured. In the case of DMEM exposure, measurements were taken after the sample had dried.

3.2.2.4 Toxicity Study

Balb/c 3T3 fibroblasts were maintained in DMEM supplemented with 10% calf serum, 100 units/ml penicillin, l-glutamine (1 mM) and 100 µg/ml streptomycin. Films of polypyrrole (\( \approx 1.5 \) cm x 1.5 cm) were sterilized by UV irradiation for 30 minutes (2400 µJoules) on each side. 5 ml of DMEM supplemented with 10% calf serum was added to individual wells of 6-well tissue culture plates either in the presence or absence of PPy samples and
incubated overnight at 37°C. Confluent monolayers of cells were plated onto a separate 6-well tissue culture plate and incubated at 37°C and 5% CO₂ for 48 hr. After 24 hr incubation of PPy in DMEM, media from the confluent monolayer of cells were replaced with media from either the PPy-containing or normal media. The cells were then incubated overnight. Each condition was tested in triplicate.

The trypan blue exclusion assay was used to determine cell viability. Cells were trypsinized from the plates, and DMEM with 10% calf serum was added to stop the action of the trypsin. Trypan blue (20 vol%) was added to an aliquot of the cell suspension. Live cells exclude the trypan blue, but nuclei of dead cells appear blue. Live and dead cells were counted in a hemocytometer. % Cell viability was determined by the dividing the number of live cells by the total number of cells.
Figure 3.1. Experimental setup for conductivity measurements
The two configurations correspond to the resistances found in Equation 3.1.
3.3 Results and Discussion

3.3.1 Polyaniline

The in situ synthesis of aniline gave transparent films of polyaniline (PAn) of 1000 Å thickness which were an intense blue-greenish color. The conductivity ranged from $10^{-1}$ to $3 \times 10^{-1}$ S/cm. This is less than the value reported in the literature ($\approx 5$ S/cm) (MacDiarmid et al., 1987).

Because the films are transparent, PAn-coated glass slides could easily be observed under a phase-contrast microscope. One important observation was that the films turned blue, in contrast to their initial green color, after exposure to water. Further tests confirmed that the films immediately turned blue when exposed to DMEM, phosphate buffered saline (PBS, Gibco BRL), and ultrapure water, indicating that the polymer reverts to its neutral, insulating state when exposed to these solutions. Reports indicate that the conductivity of PAn is dependent on pH. Since the mechanism of doping polyaniline is protonic, its conductivity, $\sigma$, is strongly dependent on the pH of the solution. It has been shown that polyaniline exists as an insulator ($\sigma \approx 10^{-10}$ ohm$^{-1}$ cm$^{-1}$) at pH=7.0 and that its conductivity increases as the pH is decreased until it is converted to a metal ($\sigma \approx 5$ ohm$^{-1}$ cm$^{-1}$) at a pH of 1.0 (MacDiarmid et al., 1987; Salaneck et al., 1987). This finding is also a possible explanation as to why the conductivity of the initial PAn sample was low: rinsing the film with Millipore water may have decreased its conductivity.

Thus, although polyaniline is an attractive candidate for biological applications because it can be synthesized in aqueous solution, it will exist as an insulator at a pH of 7.4 which limits its use for biological applications.
which involve mammalian cells. For systems which can withstand a lower pH, however, polyaniline is still a viable material. Since this thesis is concerned with mammalian systems, polyaniline will not be considered further.

3.3.2 Polypyrrole

The polypyrrole films produced electrochemically were 7.5 cm x 10 cm and approximately 45 μm in thickness. The thickness actually varied from 45 to 60 μm throughout the film. This may have been due to the surface of the anode, but it has been reported that films prepared electrochemically are not as homogeneous as those prepared chemically (Wang and Rubner, 1990). The films were somewhat brittle but would not tear unless they were under firm tension. Unlike the PAn-coated glass slides, PPy was dark green, almost black, and definitely not transparent. The electrical conductivity of PPy was very stable in air (Figure 3.2). The conductivity of polypyrrole after exposure to ethylene oxide treatment, UV irradiation, and DMEM with calf serum was essentially unchanged. Ethylene oxide and UV irradiation are common methods of sterilization of artificial surfaces before use in tissue culture. Note in particular that the electrical conductivity of polypyrrole seems to be quite stable when exposed to DMEM, unlike polyaniline. In other words, polypyrrole remains in its charged state after exposure to cell culture medium.
Figure 3.2 Conductivity of polypyrrole doped with tosylate as a function of time
Fibroblasts were chosen because they are anchorage-dependent cells and are available as a stable immortal cell line which does not undergo transformation. This contrasts with primary cell cultures in which cells such as hepatocytes must be freshly isolated before each experiment. The major disadvantage of a primary culture is that isolations give heterogeneous cultures and thus, variation between experiments. Polypyrrole was found to be nontoxic to fibroblasts (Figure 3.3). There was no significant difference between the viability of the control and the cells exposed to the medium (DMEM) with polymer. This suggests that there are no toxic agents leaching from the polymer.
Figure 3.3  % Cell viability of 3T3 Balb/c fibroblasts exposed to polypyrrole

Data represents two studies, each condition performed in triplicate. Data is expressed as average and standard deviation.
3.4 Conclusions

Polyaniline cannot be used as a conductor or be switched from insulator to conductor in biological applications which require pH's above 4. Polypyrrole, on the other hand, appears to have stable electrical conductivity when exposed to DMEM at pH ≈ 7.4 and 37°C. Furthermore, no toxic products seem to form. There are, however, potential problems with polypyrrole. For example, polypyrrole, once oxidized and especially in the presence of O₂, tends to remain in the oxidized state and is very difficult to bring back to the reduced state (Diaz and Bargon, 1986; Slater and Watt, 1989; Yue and Epstein, 1990). Since one of the objectives of this thesis is to develop a system which will be able to cycle between conducting and insulating states under physiological conditions, this thesis will take the approach of keeping the polymer as close to its reduced state as possible (Wernet and Wegner, 1987). This can be achieved by controlling the degree of oxidation either electrochemically, by limiting the current density during polymerization, or chemically, by using appropriate amounts of oxidant. Film thickness and method of synthesis needs to be optimized and is the subject of the next chapter. More importantly, however, the cycling behavior of polypyrrole under physiological conditions must be investigated in order to determine whether or not it can be used successfully in biological applications.
3.5 Citations


Chapter 4: Polymer Synthesis and Characterization

4.1 Introduction

Polypyrrole can be synthesized either electrochemically (Street et al., 1982), chemically (Machida et al., 1989) in either aqueous or non-aqueous conditions, or more recently by radio frequency (RF) plasma polymerization (Cherian and Radhakrishnan, 1992). While the majority of the research on polypyrrole has concentrated on films grown by electrochemical methods (Armes, 1987; Street, 1986), it is more desirable to use chemical methods since it would be more economical for large scale production (Shukla et al., 1992). While there have not been as many studies on chemically-prepared polypyrrole, it is known that the polypyrrole prepared chemically tends to yield fine polypyrrole powders, whereas the electrochemical method is limited by the size, shape and nature of the electrode involved (Ruckenstein and Chen, 1991). Since, it is not known which method produces films with the best properties for this thesis, both the electrochemical and chemical methods were studied.

4.2 Experimental

4.2.1 Materials and Equipment

Polystyrene bacteriologic grade petri dishes were purchased from Falcon (Becton Dickinson & Co., Franklin Lakes, NJ) and tissue culture grade polystyrene petri dishes were purchased from Corning (Corning, NY). Pyrrole was purchased from Kodak Laboratories (Rochester, NY). Activated alumina, and ferric chloride hexahydrate were purchased from Mallinckrodt
Toluene sulfonic acid was purchased from Fluka (Ronkonkoma, NY). Indium tin oxide-coated glass slides were purchased from Delta Technologies (Stillwater, MN). Platinum mesh (80 mesh), silver wire (2.0 mm diameter), ferrocene, and lithium perchlorate were purchased from Aldrich (Milwaukee, WI). Hexane, dichloromethane, methanol, acetone, spectral grade acetonitrile and tetrahydrofuran were purchased from EM Science (Gibbstown, NJ). Saturated calomel electrode was purchased from Fisher Scientific (Springfield, NJ). Ultrapure water used is described in Section 3.2.1. Tetraethylammonium-p-toluene sulfonate was purchased from Alfa Products (Ward Hill, MA).

Indium tin oxide substrates were cleaned before electrodeposition in an ultrasonic cleaner purchased from Fisher Scientific (Springfield, NJ). Electrodeposition was carried out using a Pine Instruments AFRDE4 bipotentiostat (Grove City, PA) and recorded on a Linseis xy recorder (Princeton Junction, NJ). Sheet resistance was measured using a four-point probe meter (Four Dimensions, Model 101). Film thicknesses were determined using a Sloan Dektak IIA profilometer as described in Section 3.2.2.1. UV/visible spectroscopic data were obtained using an Oriel Instaspec Model 250 spectrometer. A Ramé-Hart goniometer was used to measure static sessile drop contact angles.

4.2.2 Methods and Procedures

4.2.2.1 Chemical Oxidative Synthesis

Chemical synthesis of polypyrrole was carried out based on a method by Gregory (Gregory et al., 1989) which was modified in order to form
uniform coatings on substrates while maintaining reasonable transparency (Rubner, 1992). The substrates used were 35 mm polystyrene petri dishes and 35 mm polystyrene tissue culture dishes, and indium tin oxide (ITO). Pyrrole was passed through an activated alumina column, consisting of a standard 9 " pasteur pipette with glass wool and packed with activated alumina, until it became colorless. An aqueous solution of ferric chloride hexahydrate (0.018 M), p-toluene sulfonic acid (0.026 M), and purified pyrrole (0.006 M) was added to beakers containing the substrates. The color of the solution turned from light yellow to dark green / black during the synthesis of polypyrrole. After two hours, the substrates were removed from the solution, rinsed several times with water, and dried in air at room temperature.

4.2.2.2 Electrochemical Synthesis

Electrochemical synthesis of polypyrrole was carried out in an electrochemical cell containing an optically-transparent indium tin oxide (ITO) anode, platinum mesh counter electrode, and a pseudo Ag wire reference electrode. The reference electrode was tested in a solution of ferrocene (0.010 M) and lithium perchlorate (0.1 M) in acetonitrile and was found to be very close to SCE (saturated calomel electrode) with a difference of -0.07 V. Indium tin oxide substrates were ultrasonically cleaned in hexane, dichlormethane, acetone, and methanol (15 min each) and dried under a stream of argon gas. Pyrrole was purified by passage through an activated alumina column until it became colorless. Electrodeposition (Pine AFRDE4 bipotentiostat) was performed in a solution of purified pyrrole (0.1 M), tetraethylammonium- p - toluene sulfonate (0.1M), ultrapure water (0.5 vol
and acetonitrile. Before each deposition, the solution was purged with argon gas. Polypyrrole films were made potentiostatically at 0.8 V or 1.1 V (vs. Ag wire or SCE) until about 80 mC/cm² was passed, which corresponded to a thickness of about 0.5 μm (Wernet and Wegner, 1987).

4.2.2.3 Electrical Properties

Sheet resistance of the chemically formed polypyrrole was measured using a four-point probe meter (Four Dimensions, Model 101). The thickness of the chemically synthesized films was estimated from a calibration curve of absorption at 950 nm vs. thickness for the electrochemically formed films. Conductivity of electrochemically synthesized polypyrrole was determined by the van der Pauw method (van der Pauw, 1958) as described in Section 3.2.2.3. The films floated off the substrates after being immersed in water for about 30 min. A standard microscopic glass slide was then placed underneath the floating film, carefully smoothed out with tweezers, and dried overnight. After the film had dried, it was firmly adhered to the glass surface.

4.2.2.4 Contact Angle Measurements

A goniometer (Ramé-Hart, Mountain Lakes, NJ) was used to measure static contact angles of ultrapure water on the various samples. A minimum of 10 measurements were made for each sample.
4.2.2.5 Reduction of Polypyrrole

Thin films of polypyrrole formed electrochemically were reduced for a few minutes at a potential of -1.0 V vs. Ag wire (0.07V vs. SCE) in 0.1 M tosylate in CH$_3$CN.

Chemical reduction of polypyrrole can be achieved by exposing the doped polymer to a solution of sodium naphthalene in tetrahydrofuran (Smith and Knowles, 1991). The same substrates used to prepare polypyrrole via the chemical oxidative method were first added to 100 ml beakers containing 40 ml of tetrahydrofuran to determine whether these conditions were feasible to reduce films of polypyrrole on these substrates.

4.3 Results and Discussion

4.3.1 Polymer Synthesis

Chemical synthesis

The substrates for the chemically synthesized polypyrrole were tissue culture (TC) and bacteriologic grade (PD) polystyrene dishes. The former have been glow discharge-treated, rendering them hydrophilic; the latter are untreated and hence are hydrophobic. These surfaces were chosen with the cell studies in mind. A TC dish is considered to be standard in mammalian cell culture, whereas a PD dish is a surface which is non-adhesive for mammalian cells. We wanted to examine how the cell interaction would change after a thin film of polypyrrole was deposited onto the surfaces.

Since the polystyrene dishes have a very high surface roughness, it was not possible to measure the thickness using profilometry. The thickness of the polypyrrole coating made via the chemical oxidative method was
estimated to be about $700 \pm 50$ Å from a calibration curve of 950 nm absorbances vs. actual thicknesses of polypyrrole on standard microscopic glass slides (Rosner, 1992). Polypyrrole films did not form on indium tin oxide. Polypyrrole did form on the back side of the substrates since only one side of the glass slide substrate is coated with indium tin oxide. The harsh oxidizing conditions of ferric chloride degrade the indium tin oxide layer such that the polypyrrole film cannot adhere to its surface (Asturias, 1992).

**Electrochemical synthesis**

Indium tin oxide (ITO) was chosen as the substrate for electrochemical synthesis of polypyrrole for its optical transparency. Current densities obtained ranged from 0.7 to 1.0 mA/cm². Film thickness were on the order of 0.4 to 0.6 µm.

4.3.2 **Electrical Properties and Contact Angle**

Conductivity measurements (Table 4.1) confirmed that the polypyrrole thin films synthesized from both methods were in the oxidized state. An increase of 21 orders of magnitude in conductivity was observed after the polypyrrole was chemically deposited onto the TC and PD surfaces. In its oxidized state, polypyrrole exists as a polycation with dopant anions to balance the charge (Figure 4.1).
Figure 4.1. Structure of polypyrrole in the oxidized state

$X^-$ indicates dopant anion. In this thesis, $X$ is tosylate.
The differences in wettability between the bare surfaces and after they have been coated with polypyrrole is another indication that there is a uniform coating of the polypyrrole. Note the close agreement between the values of contact angle for the films formed from both the chemical and electrochemical methods. Polypyrrole appears to have an intermediate wettability compared to tissue culture and bacteriologic grade polystyrene.

The contact angles of the polypyrrole films formed electrochemically were observed to depend on the thickness of the film. Figure 4.2 shows that below a thickness of 5000Å, the contact angle varies on the thickness. All of the samples used for protein adsorption and cell studies were thus at least 5000Å thick.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conductivity (Ω-cm)^{-1}</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>&lt; 10^{-10}</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>TC</td>
<td>&lt; 10^{-10}</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>PPY/PD</td>
<td>30*</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>PPY/TC</td>
<td>30*</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>PPY/ITO</td>
<td>1#</td>
<td>73 ± 5</td>
</tr>
<tr>
<td>ITO</td>
<td>800**</td>
<td>55 ± 6</td>
</tr>
</tbody>
</table>

* calculated from sheet resistance measurement and a thickness of 700Å  
#van der Pauw method  
**calculated from sheet resistance (40Ω/sq) and thickness (3000Å) given by manufacturer

PD is bacteriologic grade polystyrene petri dish; TC is tissue culture grade polystyrene dish; PPY/PD and PPY/TC are polypyrrole formed chemically on PD and TC, respectively; PPY/ITO is polypyrrole formed electrochemically on indium tin oxide-coated glass; ITO is indium tin oxide-coated glass.

Table 4.1. Conductivity and contact angle values of films of polypyrrole  
Contact angle data is expressed as average and standard error of the mean from three separate experiments and at least ten measurements per sample.
Figure 4.2. Contact angle vs. thickness for polypyrrole thin films made electrochemically.
4.3.3 UV/VIS Spectroscopy

The absorption spectra (Figure 4.3) show a peak centered around 950 nm which confirms that both the electrochemically and chemically formed polypyrrole are in their oxidized states. These spectra are in agreement with other studies examining the optical properties of oxidized polypyrrole (Diaz and Kanazawa, 1983).

Effect of reduction on absorption spectra

During the electrochemical reduction of polypyrrole, the current dropped from 5.0 to 0 mA, with a concomitant color change from brown/grey to yellow. It took a few minutes for the entire film to become reduced, but continued application of the potential caused parts of the film to turn a greenish color. This is most probably evidence of reoxidation of the film which was accompanied by a slight increase in the current. There are three main points which should be noted when comparing the absorption spectra for polypyrrole in the oxidized and reduced state (Figure 4.4). First, the curves for the reduced polymer exhibit a peak near 450 nm which has been attributed to the reduced polymer (Street, 1986) and this peak is absent in the oxidized polymer. Second, the peak centered around 500 nm is characteristic of the oxidized polymer. Note that this peak is not present in either of the reduced films. Finally, the broad peak near 800 nm has been attributed to the bipolarons present in the film (Blackwood and Josowicz, 1991). The film which was reduced at -0.75 V appears to be slightly oxidized. This indicates that not only is the oxidation state dependent on the potential.
Figure 4.3. Absorption spectra of polypyrrole thin films formed electrochemically (a) and chemically (b).
at which the film is reduced, as Blackwood and coworkers have shown (Blackwood and Josowicz, 1991), but also that the reduced neutral polypyrrole is very unstable in air and will spontaneously oxidize after the reduction potential is removed (Li and Qian, 1989). This was confirmed in a stability study of reduced polypyrrole exposed to water and air (Figure 4.5).

There is a rather large increase in the peak near 800nm (Figure 4.5), indicating that the film has undergone re-oxidation. The blue-shift (i.e. shift to a shorter wavelength) of the peak near 450 nm has been reported to occur when a film goes from its neutral to oxidized state (Street, 1986). Thus, the reduced form of polypyrrole is not very stable.

Chemical reduction of the polypyrrole thin films was attempted, which involves exposing polypyrrole to a solution of sodium naphthalene in tetrahydrofuran (THF) (Smith and Knowles, 1991). Since polystyrene dishes which were coated with polypyrrole were found to dissolve in THF, it was not possible to chemically reduce polypyrrole. Furthermore, even if it were possible to chemically reduce polypyrrole formed on a surface which would withstand exposure to THF (for example glass), it would reoxidize rather quickly, as discussed above.

Thus, when the reduced film is exposed to air or water, it will reoxidize rather quickly, as evidenced by the UV/VIS spectra. On the other hand, Minehan and others (Minehan et al., 1991) were able to see differences in DNA adsorption to reduced and oxidized polypyrrole formed electrochemically. However, their film thicknesses were on the order of 50 to 100 μm, and their experimental time scale for DNA adsorption was only 16 minutes. Mammalian cell culture, however, requires an experimental time
Figure 4.4. Absorption spectra of polypyrrole in its oxidized and reduced states
(-O- oxidized; - ■ - reduced @ -0.7V; - □ - oxidized; - - - - - - reduced @ -0.75V)
Figure 4.5. Absorption spectra of reduced polypyrrole before and after exposure to water and air
(---○--- before; ---○--- 3 days later and exposure to water)
scale more on the order of hours to days. One alternative is to try to hold the film at the appropriate potential. It has been shown that when the reduced form of polypyrrole is placed under applied current "the colors are retained for periods of hours" (DePaoli et al., 1990). The results of this study is presented in Chapter 6.

4.4 Conclusions

Thin films of polypyrrole were synthesized using two different methods: chemical oxidative and electrochemical deposition. From conductivity measurements, polypyrrole was found to exist in its oxidized state. Contact angle measurements and UV/VIS spectroscopy further confirmed that uniform, thin films of polypyrrole were formed. Attempts to reduce polypyrrole to its neutral state indicated that polypyrrole is unstable in its neutral state. However, it may be possible to hold polypyrrole in its neutral state via electrochemical methods. Nevertheless, the oxidized polycationic form of polypyrrole needs to be evaluated as a substrate for mammalian cell culture. This is the subject of the next chapter.
4.5 Citations


Chapter 5: Cell Attachment and Protein Adsorption Studies

5.1 Introduction

Although there have been some studies of polypyrrole in aqueous environments examining the interaction with proteins (Prezyna et al., 1991; Smith and Knowles, 1991) and DNA (Minehan et al., 1991), there has been very little work looking at the interaction of cells with electrically conducting polymers. The purpose of this study was to synthesize oxidized polypyrrole on various substrates and to examine protein adsorption and cell attachment to these films. Thin films of polypyrrole synthesized using both chemical oxidative and electrochemical methods were evaluated.

5.2 Experimental

5.2.1 Materials and Equipment

Human serum fibronectin was purchased from Cappel (Durham, NC). Sodium carbonate and sodium bicarbonate were purchased from Mallinckrodt (Chesterfield, MO). Glacial acetic acid was purchased from Aldrich (Milwaukee, WI). ¹²⁵I-labeled fibronectin was purchased from ICN Radiochemicals (Costa Mesa, CA). Teflon sheets were purchased from AIN Plastics (Norwood, MA). O-rings were purchased from Ace Glass (Vineland, NJ). RBS cleaning solution was purchased from Pierce (Rockford, IL). 96-well tissue-culture treated and non-treated plates were purchased from Corning (Corning, NY). Bovine aortic endothelial cells were kindly provided by Dr. Patricia D'Amore (Children's Hospital, Boston, MA). Cell culture media and supplements were detailed in Section 3.2.1. Calf serum was
purchased from HyClone (Logan, UT). Bovine serum albumin was purchased from ICN Biomedicals (Irvine, CA). Coomassie brilliant blue was purchased from Biorad (Hercules, CA). Methylene blue and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO). Glutaraldehyde was purchased from Polysciences (Warrington, PA). Methanol and ethanol purchased from EM Science (Gibbstown, NJ).

The activity of $^{125}\text{I}$-labeled fibronectin was counted in a gamma counter purchased from LKB-Wallace (CliniGamma, Model 1272). Protein adsorption and cell culture chambers were machined by Sylvester Szczepanowski. Centrifugation of cells was carried out using a Clinical centrifuge purchased from International Equipment Company (Needham Heights, MA). Cells were photographed using a Nikon Diaphot phase-contrast microscope. Quantitation of cell attachment and morphological analysis was carried out using a computerized image analysis system consisting of a KP-M1 video camera and VM-920 monitor purchased from Hitachi (Tokyo, Japan); a Hund Wilovert S phase-contrast microscope (Wetzlar, Germany); a Macintosh IIsi computer with a Scion LG-3 frame grabber (Frederick, MD); and Image 1.49 software (National Institutes of Health, Bethesda, MD). A micrometer disk (10 mm x 10 mm) inserted into the phase-contrast microscope used to quantitate cell attachment was purchased from Klarmann Rulings (Manchester, NH).
5.2.2 Methods and Procedures

5.2.2.1 Protein Adsorption Studies

Fibronectin (FN) was used as a model protein and its adsorption was quantitated according to a previously reported method (Ingber, 1990) with conditions which maximize the adsorption efficiency. Human serum FN were mixed with trace amounts of $^{125}$I-labeled FN (4.58 $\mu$Ci/$\mu$g) and dissolved in carbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH to 9.4 with glacial acetic acid) to achieve fibronectin densities of 1, 10, 100, and 1000 ng/cm$^2$. Samples were tested in duplicate in a twelve well chamber (Figure 5.1), modeled after the Bionique chamber (Gabridge, 1981). The chamber was constructed from a block of Teflon, and twelve holes (0.92 cm diameter) were drilled to form wells for the experiments. Each well had a groove along its bottom edge for an o-ring ($\frac{3}{8}$" x $\frac{1}{16}$" x $\frac{3}{32}$") to prevent leakage.

The upper surface of the Teflon plate was machined to accommodate a standard cover of a six-well tissue culture plate. Twelve holes were drilled into the aluminum base plate, and an inset was added to facilitate viewing on the microscope. The chamber was completely flat after assembly and could be cleaned by soaking in RBS cleaning solution and sterilized by soaking in ethanol overnight.

Polypyrrole and indium tin oxide substrates were used as prepared. Polystyrene dishes (PD and TC) were cut with scissors to fit inside the chamber. When the samples were assembled into the chamber, the exposed surface substrate area was 0.665 cm$^2$. 100 $\mu$l of FN-carbonate buffer solution was added to each of the chamber wells and allowed to adsorb for 24 hr at
Figure 5.1. Schematic of chamber used for protein adsorption studies. View is representative of assembly of substrates. Once the teflon plate is fastened to the aluminum plate with screws, the chamber is flipped over and ready for use.
100 μl of the same solution was added to three vials and counted on a gamma counter to determine the initial activity of the fibronectin-carbonate buffer solution. After incubation, the solution containing non-adsorbed protein was collected, and the wells were washed twice with PBS. The chamber was then disassembled, and the activities of the supernatant, washes, samples and o-rings were counted. % FN adsorbed was calculated by subtracting the combined activities from the supernatant and washes from the total activity and dividing by the total activity.

5.2.2.2 Cell Culture

Bovine aortic endothelial cells were maintained in DMEM supplemented with 10% calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cell attachment in the absence of calf serum was performed both in the presence and absence of fibronectin.

Each substrate type was tested in triplicate. The electrochemically synthesized polypyrrole and bare indium tin oxide samples were used as prepared and were assembled in a 12 well chamber (Figure 5.1). The exposed surface area of the substrate in each well was 0.665 cm². 96-well tissue-culture treated and nontreated plates were used as received from the manufacturer. The wells which contained fibronectin were coated with a density of 1000 ng/cm² as described in Section 5.2.2.1 except radioactive protein was not added. After incubation of fibronectin, the surfaces were washed twice with PBS and once with DMEM containing 1% bovine serum albumin. In order to prevent non-specific binding, the surfaces were incubated (37°C, 5% CO₂) for 15 to 25 minutes with DMEM without serum.
containing 1% bovine serum albumin. Confluent monolayers of cells were
dissociated by brief exposure to trypsin (1-2 min), and the action of the
trypsin was stopped with a solution of soybean trypsin inhibitor in DMEM
(1 mg/ml). In order to remove the trypsin, the cell suspension was then
centrifuged (210 G, 5 min) and the supernatant was removed, and the cells
were resuspended in serum-free medium consisting of DMEM with penicillin
(100 units/ml), streptomycin (100 µg/ml), l-glutamine (1 mM) and 1% bovine
serum albumin. The cells were centrifuged one more time, supernatant
removed, and resuspended in serum-free medium. Cells were plated (2 x 10^4
cells/cm^2) for 4 hr (37°C, 5% CO_2). After incubation, the cells were carefully
washed with phosphate-buffered saline (PBS) to remove unattached cells.
Cells were fixed by adding 1 vol% glutaraldehyde for at least 30 min. The
wells were then washed twice with cold PBS and twice with cold methanol
and allowed to dry overnight. Cells were stained with either methylene blue
(2 g in 50/50 vol% methanol/water) or a solution of Coomassie brilliant blue
(0.5 g) in 40 ml methanol, 10 ml glacial acetic acid, and 100 ml deionized
water.

5.2.2.3 Quantitation of Cell Attachment and Morphological Analysis

Cell attachment was quantitated by counting cells in randomly
selected areas under a phase-contrast microscope. Cells were counted in an
area of 0.25 mm^2, and over 200 cells were counted for each condition.
Projected cell area was determined using a computerized image analysis
system as previously described (Ingber, 1990). Cells were observed with a
video camera connected to a phase-contrast microscope. The image was
inputed into a monitor and captured with a Macintosh IIsi computer containing a frame grabber. A minimum of 50 randomly selected cells were analyzed for each condition, and cell projected area was determined using Image 1.49 software.

5.3 Results and Discussion

5.3.1 Protein Adsorption

Fibronectin is a well-characterized glycoprotein which, when adsorbed to a surface, plays a major role in mediating cell attachment. It consists of 2 250 kD polypeptide chains. Calculations using 600 and 25 Å for the length and width of fibronectin, respectively, (Stryer, 1988), indicate that coatings of 500 ng/cm² would be saturating. Note that this calculation assumes that the protein chains are able to lie side by side. This most likely is not the case because of interactions between the molecules, and the saturation density is probably lower than 500 ng/cm². In general, the coating (Figure 5.2) efficiency decreases as the concentration of added fibronectin increases. This can be expected since once a monolayer of fibronectin is adsorbed, no more protein can be adsorbed. The coating efficiencies are a little lower than expected (Mooney, 1992). A possible reason why the coating densities do not reach a value > 75% may be that the FN-carbonate buffer solution is exposed to several surfaces (the substrate, the o-ring, and the teflon plate) and the these surfaces most likely do not have the same efficiency of fibronectin adsorption. Thus, the coating efficiency reflects the interaction of the protein with three different surfaces.
More important, however, is the determination of the amount of fibronectin that can be adsorbed to polypyrrole surfaces (Figure 5.3) using this method. The amount adsorbed when 1000 ng/cm\textsuperscript{2} fibronectin is added is in the range necessary for cell attachment and spreading (Horbett and Schway, 1988), thus the cell attachment and spreading experiments in this study use the fibronectin coating density of 1000 ng/cm\textsuperscript{2}. 
Figure 5.2. % Adsorption of fibronectin to various substrates at 1, 10, 100, and 1000 ng/cm² coating densities.

Data shown is average and standard error of the mean from five separate experiments.

○ is Polypyrrole formed electrochemically onto indium tin oxide; □ is indium tin oxide; △ is polypyrrole formed chemically onto polystyrene; × is petri dish; ▲ is tissue culture dish.
Figure 5.3. Quantitation of amount of fibronectin adsorbed (fmol/cm²) onto various substrates as a function of amount of fibronectin added.

Data is expressed as average and standard error of the mean.

Data is from the same experiments as in Figure 5.2, but calculated as amount of fibronectin adsorbed. □ is polypyrrole formed chemically onto polystyrene; □ is polypyrrole formed electrochemically onto indium tin oxide; □ is petri dish; □ is tissue culture dish; □ is indium tin oxide.
5.3.2 Cell Culture

Bovine aortic endothelial cells are cells which line the walls of arteries. These cells were chosen because they are anchorage-dependent cells and previous studies have shown that the shape of these cells are closely linked to DNA synthesis and growth (Folkman and Moscona, 1978). The amount of cells attached to the tissue culture treated and the indium tin oxide surfaces is significantly less compared to the other surfaces (Figure 5.4). This is not unusual because contrary to conditions under which tissue culture treated surfaces are considered a standard substrate in cell culture, these studies were performed in the absence of calf serum. Since TC and ITO are considerably more hydrophilic than PPY or the PD (see Table 4.1), it appears that in the absence of serum, hydrophobic surfaces coated with fibronectin are better substrates for attachment of bovine aortic endothelial cells. On all substrates, however, cells were found to attach preferentially on fibronectin-coated surfaces. While cells did attach to noncoated surfaces, the cells appeared to be more spread on the surfaces coated with FN (Figures 5.5, 5.6, 5.7), which was verified by measuring the projected cell area (Figures 5.8 to 5.12).

In the absence of a fibronectin coating, there is no significant cell attachment on the PD or ITO. However, a significant fraction of cells (20-30%) attach to non-FN-coated polypyrrole and tissue culture treated surfaces. This may be due to electrostatic interactions between the cell and substrate. Polypyrrole is a polycation with negative charges (dopant ions) dispersed throughout the polymer. Tissue culture dishes are also charged since the
surfaces are treated by γ-irradiation or with an electric arc to render a charged, wettable surface (Freshney, 1987).
Figure 5.4. Attachment of bovine aortic endothelial cells after 4 hour incubation in the absence of calf serum.

Data is expressed as average and standard error of the mean from three separate experiments. For each experiment, samples were tested in triplicate.

(■ with fn coating (1000 ng/cm²); □ without fibronectin coating)
Figure 5.5. Bovine aortic endothelial cells after 4 hr incubation on (a) PPy/chem with FN, (b) PPy/chem without FN, (c) TC with FN, (d) TC without FN.

Cells were fixed with glutaraldehyde and stained with methylene blue. FN coatings were 1000 ng/cm². The cells on FN-coated surfaces are extended and spindly shaped whereas cells on noncoated surfaces remain round. (x900)
Figure 5.6. Bovine aortic endothelial cells after 4 hr incubation on (a) PPy/ITO with FN, (b) PPy/ITO without FN, (c) ITO with FN, (d) ITO without FN.

See Fig 5.5 for details. (x900)
Figure 5.7. Bovine aortic endothelial cells after 4 hr incubation on (a) PD with FN, (b) PD without FN.
See Fig 5.5 for details. (x900)
Figure 5.8. Distribution of projected cell area on polypyrrole formed chemically as measured by image analysis system. Data is from a representative experiment. At least 50 cells were counted for each condition. Top panel is on FN-coated PPy and bottom is on noncoated PPy; average area and standard deviation are 328 ± 198 and 287 ± 150, respectively.
Figure 5.9. Distribution of projected cell area on polypyrrole formed electrochemically. Top panel is on FN-coated and bottom is on noncoated; average area and standard deviation are 934 ± 462 and 104 ± 40, respectively.
Figure 5.10. Distribution of projected cell area on TC. Top panel is on FN-coated and bottom is on noncoated; average area and standard deviation are 645 ± 360 and 246 ± 84, respectively.
Figure 5.11. Distribution of projected cell area on PD. Top panel is on FN-coated and bottom is on noncoated; average area and standard deviation are $1200 \pm 600$ and $266 \pm 155$, respectively.
Figure 5.12. Distribution of projected cell area on ITO. Top panel is on FN-coated and bottom is on noncoated; average area and standard deviation are 853 ± 450 and 141 ± 73, respectively.
5.4 Conclusions

In this study, we have demonstrated that proteins adsorb to thin films of oxidized polypyrrole and that cells exhibit a normal response, such as attachment and spreading, when cultured on polypyrrole. There is most likely not a cell receptor for polypyrrole. Instead, there is probably an electrostatic interaction between the extracellular matrix proteins and polypyrrole, and in turn, the cells are attached to the proteins. It is encouraging that these polymers do support cell attachment and thus, they may support cell function. The next chapter examines the effect of changing the oxidation state of the polymer on the cell behavior.
5.5 Citations


Mooney, D. J. (1992) “Control of hepatocyte morphology and function by the extracellular matrix,” PhD, Massachusetts Institute of Technology, 49.


Chapter 6: Control of Cell Shape and Cell Growth

6.1 Introduction

Growth and function of cultured cells is commonly controlled by addition of medium supplements, including serum, defined growth factors, and soluble hormones. However, interactions between cells and their culture substrate are also critical for regulation of their growth and function. For example, most mammalian cells are anchorage-dependent and thus, must attach and extend on a surface in order to proliferate (Ben-Ze'ev et al., 1980; Folkman and Moscona, 1978; Ingber, 1990; Ingber and Folkman, 1989; Mooney et al., 1992). Furthermore, the same cells will remain quiescent and differentiate in the identical growth factor-containing medium, if cell spreading is prevented by altering interactions between cells and substrate-adsorbed extracellular matrix proteins, such as fibronectin (FN) (Ingber and Folkman, 1989; Mooney et al., 1992). Thus, if one could modulate the surface properties of the culture substrate, it may be possible to control the shape and function of the cells as well.

Past analysis of various culture substrata has revealed that surface charge density, wettability, and morphology are important for control of cell attachment, metabolism, and function (Barngrover, 1986). Electrically conducting polymers provide potentially interesting surfaces for cell culture in that their properties (e.g., surface charge, wettability, conformational and dimensional changes) can be altered reversibly by chemical or electrochemical oxidation or reduction (Kanatzidis, 1990; Street and Clarke, 1981). One can imagine a non-invasive method in which cell function could
be controlled on a single material whose surface properties can be changed by an externally applied electrochemical potential, independent of any medium alteration.

The objective of the present study was to examine the suitability of conducting polymers for cell culture and the usefulness for controlling cell function. These polymers represent a novel class of “active” culture substrata since their electroactivity provides a way to reversibly change their oxidation state, alter cell-substrate interactions, and hence manipulate cell growth and form.

6.2 Experimental

6.2.1 Materials & Equipment

Materials for synthesis of polypyrrole are described in Section 4.2.1. Cell culture-related media, buffers, and supplements are described in Section 3.2.1. Hepes buffer was purchased from Gibco BRL (Grand Island, NY). Ag/AgCl reference electrodes were purchased from Bioanalytical Systems (West Lafayette, IN). Transferrin was purchased from Collaborative Research (Waltham, MA). Human serum fibronectin, high density lipoprotein and rhodamine-conjugated goat IgG directed against mouse IgG Fc fragment were purchased from Cappel (Durham, NC). Fibroblast growth factor (FGF) was kindly supplied by Takeda Chemical Industries, Ltd. (Osaka, Japan). A commercially available DNA synthesis fluorescence assay (RPN20) was purchased from Amersham (Arlington Heights, IL). Live/Dead Viability/Cytotoxicity assay was purchased from Molecular Probes (Eugene, OR).
Cyclic voltammetry experiments were carried out using a Pine Instruments AFRDE4 bipotentiostat and a Linseis xy recorder. Spectroscopic data were obtained using an HP 8452 Diode Array Spectrophotometer. Cells were photographed on a Nikon Diaphot microscope using Hoffman optics.

6.2.2 Methods and Procedures

Thin films of polypyrrole were formed electrochemically as described in section 4.4.2.

6.2.2.1 Polymer Characterization

Cyclic voltammetry experiments were carried out using a Pine Instruments AFRDE4 bipotentiostat and a Linseis X-Y recorder. The electrolyte was Dulbecco's modified Eagle Medium supplemented with 1% bovine serum albumin, penicillin (100 units/ml), streptomycin (100 μg/ml), and 20 mM HEPES buffer, pH 7.4. All potentials were defined relative to a Ag/AgCl reference electrode. UV/VIS spectroelectrochemistry measurements were carried out by monitoring the UV/VIS spectrum of polypyrrole as a function of oxidation state which was applied using the same equipment for cyclic voltammetry.

6.2.2.2 Cell Studies

Bovine aortic endothelial cells (kindly provided by Dr. P. D'Amore, Children's Hospital, Boston, MA) were maintained in DMEM supplemented with 10% calf serum. Polypyrrole and control surfaces were assembled in a 6-well chamber described in Section 5.2.2. To analyze effects on cell attachment, cells were plated (15,000 cells/cm²) in serum-free medium on
surfaces precoated with FN (10 μg/cm²), as previously described in Chapter 5 (Ingber, 1990; Mooney et al., 1992). Cells were allowed to attach for 10 min before a potential of -0.5 V was applied. Cells were glutaraldehyde-fixed 4 hr later, stained with Coomassie Brilliant Blue (Ingber, 1990), and photographed on a Nikon Diaphot microscope using Hoffman optics.

To analyze effects on cell growth, serum-starved (0.4% calf serum for 2 days) cell monolayers were trypsinized and plated (15,000 cells/cm²) on FN-polypyrrole or similarly coated petri dishes in DMEM with 10% calf serum or in a chemically defined medium, consisting of DMEM supplemented with transferrin (5 μg/ml), high density lipoprotein (10 μg/ml), 1% BSA, and FGF (5 ng/ml) (Ingber, 1990). For these experiments, we chose to use - 0.25 V rather than - 0.5 V because cell lysis was observed in a previous study when - 0.6 V was applied to indium trioxide surfaces (Shinohara et al., 1989). Effects on DNA synthesis were measured by quantitating nuclear incorporation of 5-bromo-2'-deoxyuridine (BrdUrd) using a commercially-available fluorescence assay except that rhodamine-conjugated goat IgG directed against mouse IgG Fc was used as the secondary antibody. The potential (-0.25 V) was applied from 15 to 20 hr of culture, the time when these Go-synchronized cells begin to reenter S phase. BrdUrd (3 μg/ml) was included only during this 5 hr time period. Total number of cells and labeled nuclei were counted in 4 random fields (at 200x; > 50 cells per field) using the phase contrast and fluorescence capabilities of a Nikon Diaphot inverted microscope. Cell viability was quantitated using the Molecular Probes Live/Dead viability assay that is based on the cellular incorporation of two fluorophores, calcein
acetoxymethylester (viable cells) and ethidium homodimer (nonviable cells). Projected cell area was determined as described in Section 5.2.2.3.

6.3 Results and Discussion

Polypyrrole obtained via electrochemical synthesis was able to be cycled between its charged and neutral forms electrochemically in culture medium (Fig. 6.1). The broad peaks near 0 V and -0.5 V correspond to the polymer switching between its charged and neutral states, respectively. Since these peaks are broad, there is actually a range of potentials at which the polymer undergoes oxidation and reduction. The oxidation state of polypyrrole was monitored using UV/VIS spectroscopy (Fig. 6.2). A broad peak near 800 nm associated with the bipolarons (Scott et al., 1984) was present in the oxidized polymer. Application of -0.5 V switched polypyrrole to its neutral state, as indicated by the disappearance of the peak near 800 nm and the appearance of a separate peak near 370 nm. This latter peak has been previously shown to be characteristic of the neutral polymer (Genies and Pernaut, 1985). At -0.25 V, the spectrum fell between those of the oxidized and neutral states. The polymer spectra stabilized within 30 sec after the potential was applied. When the reduction potential was removed, the neutral polymer reverted completely to its oxidized state within 30 sec. This phenomenon has been observed by others as well (Li and Qian, 1989). Thus, by keeping the potential constant at -0.25 V or -0.5 V, the polymer can remain in its neutral state.
Figure 6.1. Cyclic voltammogram of polypyrrole in serum-free DMEM culture medium.

Polypyrrole is switched between its charged and neutral states when cycled between +0.4 V and -1.0 V. Broad peaks indicate that there is a range of potentials corresponding to varying levels of oxidation and reduction. Scan rate: 50 mV/s.
Figure 6.2. UV/VIS spectra of polypyrrole in its native oxidized state under no potential (a) and reduced by application of either -0.25 V (b) or -0.5 V (c).
Bovine aortic endothelial cells attached poorly and did not extend on uncoated polypyrrole when cultured in serum-free medium (see Section 5.3.2). In contrast, both cell attachment and spreading were observed (Fig. 6.3a) when the oxidized polymer films were pre-coated with FN, an extracellular matrix molecule that adsorbs to surfaces and mediates binding to specific cell surface integrin receptors (Hynes, 1987). However, when cells were plated on FN-polypyrrole films that were converted to their neutral state, the cells attached but they remained round (Fig. 6.3b). Cell rounding was not a result of cell injury since the viability of round cells on neutral FN-polypyrrole was similar to that on FN-petri dishes (91± 2% versus 99± 1%, respectively). Thus, the observed effects on cell shape appeared to result from some process associated with polypyrrole reduction. Cell retraction and rounding could be due to release of substrate-adsorbed FN attachment molecules (i.e. detachment of cell anchors) following application of an electrical potential. This is discussed in the next chapter.
Figure 6.3. Photomicrographs of endothelial cells cultured for 4 hr on FN-polypyrrole in either its native oxidized state (a) or after reduction by application of -0.5 V for 4 h (b) (x 900).
Figure 6.4. DNA synthesis in cells cultured in serum-containing medium on different FN-coated substrata in the absence (-) or presence (+) of an applied electrical potential (-0.25 V).

Data are presented as mean ± standard error of the mean. ITO, indium tin oxide electrode; PPy, polypyrrole; PD, petri dish. Cell viability was > 90% under all conditions.
Cell shape and growth have been shown to be tightly coupled in many anchorage-dependent cells (Ben-Ze'ev et al., 1980; Folkman and Moscona, 1978; Ingber, 1990; Ingber and Folkman, 1989; Mooney et al., 1992). Similarly, cell retraction was found to be induced by applying an intermediate electrical potential (-0.25 V) to FN-polypyrrole provided control over cell cycle progression (Fig. 6.4). Projected cell area on polypyrrole in the absence and presence of -0.25 V ranged from 417 to 3239 µm² and 358 to 1920 µm², respectively. Approximately 75% of cells cultured in serum-containing medium on FN-coated petri dishes, ITO, or oxidized polypyrrole spread normally and entered S phase synchronously between 15 to 20 hr after plating. Applying -0.25 V to ITO had little effect on either cell growth or form. In contrast, few cells (<2%) synthesized DNA when the same electrical potential was used to switch the FN-polypyrrole to its neutral state and promote cell retraction. These effects were not due to cell death since greater than 90% of the cells remained viable on the neutral FN-polypyrrole, as determined by quantitating incorporation of the vital dye, calcein acetoxy-methylester.

It is not surprising that cells adherent to FN-polypyrrole exhibited different behaviors depending on the polymer oxidation state. Previous work has shown that proteins (Smith and Knowles, 1991) and DNA (Minehan et al., 1991) adsorb more efficiently onto oxidized polypyrrole compared to the neutral polymer. The wettability of a surface is also dependent upon its oxidation state (Abbott and Whitesides, 1994). In addition, scanning tunneling microscopy studies of surfaces under electrical potential control
reveal that the morphology of the surface differs depending on the surface charge (Tao and Lindsay, 1992).

Tight coupling between cell shape and growth also has been observed in the past (Ben-Ze'ev et al., 1980; Folkman and Moscona, 1978; Ingber, 1990; Ingber and Folkman, 1989; Mooney et al., 1992). However, it was not obvious that the alteration of the oxidation state of a culture substrate could provide control over either of these complex cell behaviors. Nevertheless, this is what was observed: converting polypyrrole to its neutral state resulted in prevention of cell spreading and associated inhibition of DNA synthesis, even though neither the composition of the medium nor the cell plating density was altered. The mechanism by which altering the oxidation state of polypyrrole changes its ability to support cell extension and growth is unknown. One possibility is that this effect results from associated changes in electric potential. For example, others have been able to promote protein production by applying + 0.2 to 0.6 V to tumor cells plated on platinum and indium trioxide surfaces (Kojima et al., 1992). However, when the same electrical potential was applied to cells using FN-ITO surfaces, cells did not round. Associated changes in electrical fields could also play a role. However, in our case, cells were only exposed to a current density of 20 μA/cm² (as determined by chart recording during polymer reduction), a density which was previously shown not to affect cell attachment, spreading or growth of cultured fibroblasts (Giaever and Keese, 1984). Importantly, electrochemically reduced polypyrrole films exhibit a conductivity on the order of 10⁻⁶ S cm⁻¹ (Qian and Qiu, 1987), compared to approximately 1 S cm⁻¹ for cell culture medium. The cells also did not form a continuous (insulating)
monolayer in this study. Thus, it is likely that any electrical current that was generated in these experiments primarily acted to reduce the polymer film, rather than the cells.

Another possible explanation for why cells rounded up relates to the mechanism by which polypyrrole is reduced. During reduction, the oxidized (polycation) polypyrrole is converted to its neutral form with the concomitant discharge of dopant anions to maintain charge neutrality. However, in the case of polypyrrole doped with tosylate, it has been shown that cations (e.g., Na\(^+\)) are incorporated to compensate for the charge of some tosylate anions that were not released (Iseki et al., 1991; Zhou et al., 1987). When cells are exposed to hypotonic media, they experience osmotic shock and tend to vesiculate and form blebs (Cohen et al., 1982). Yet, in the present study, neither vesiculation nor significant loss of viability was observed. Local changes in external [Na\(^+\)] also could affect cell form and function by altering ion fluxes across the cell surface since transmembrane transport of this ion is critical for cell cycle progression (Ingber et al., 1990). Effects on cells could be due to dynamic pH changes near the surface of the polypyrrole substrate (Yaoita et al., 1989) which also can have potent effects on cell growth (Ingber et al., 1990). However, we used bicarbonate-buffered medium that was also supplemented with HEPES in the present study and we did not observe macroscopic pH changes. There is also the possibility that the polymer changes mechanically (e.g., becomes more malleable) (Baughman et al., 1990), the integrity of the cell's basal adhesions weakens, or that a small portion of immobilized FN that is cell surface bound and under mechanical
tension (due to cell tractional forces) detaches when the polymer is reduced. These issues are discussed in more detail in the next chapter.

6.5 Conclusions

In summary, these data indicate that polypyrrole can potentially be a very important and unique biomaterial since it is possible to externally change its properties and surface-binding characteristics reversibly using applied electrical potentials. Polypyrrole and other electrically conducting polymers therefore may be especially useful as substrates for both small and large-scale cell cultures since they provide a novel and non-invasive way to regulate cell form and function. In the present study, it was shown that cell growth (entry into S phase and initiation of DNA synthesis) can be modulated using this approach. Inhibition of growth by preventing cell spreading has been previously shown to be accompanied by a concomitant increase in tissue-specific functions and enhanced secretion of specialized cell products (Ingber and Folkman, 1989; Mooney et al., 1992). Thus, use of conducting polymers may provide a relatively simple and inexpensive means to control cell growth and differentiation non-invasively, without altering medium composition or refeeding. This type of experimental manipulation may be extremely useful for applications in biotechnology and tissue engineering (Langer and Vacanti, 1993). It also provides a new approach to analyze the fundamental biochemical mechanisms by which cell-substratum interactions regulate cell physiology.

The oxidation state of polypyrrole can be controlled via an externally applied electrical potential in cell culture medium. Thus, polypyrrole can be
switched between its charged and neutral forms simply by applying an external potential. Furthermore, it was possible to control cell shape by controlling the oxidation state of the polymer. Since cell function has been shown to be closely related to cell shape, these materials have the potential to be surfaces on which function can be controlled non-invasively.
6.6 Citations


Chapter 7: Elucidation of the Mechanism for Control of Cell Shape/Growth

7.1 Introduction

As discussed in the previous chapter, the mechanism by which altering the oxidation state of polypyrrole changes its ability to support cell extension and growth is unknown. Possible mechanisms are changes of pH or temperature at the surface of polypyrrole while it is being held in its neutral state. Furthermore, physiochemical changes in the surface may occur which would alter the wettability of the surface, or there could be release of fibronectin or other substrate-associated proteins which would affect the cell behavior. While the above list is not an exhaustive one, this chapter attempts to understand what changes are occurring when polypyrrole is changed between its neutral and charged states.

7.2 Experimental

7.2.1 Materials & Equipment

Cell culture-related media, buffers, and supplements are described in Sections 3.2.1. and 6.2.1. Unbuffered saline (0.9% sodium chloride, irrigation) was purchased from Abbott Labs (Chicago, IL). $^{125}I$-labeled FN and the equipment used to count its activity is described in Section 5.2.1. Hexapeptide GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) was purchased from Peninsula Laboratories (Belmont, CA). Platinum and silver wires were purchased from Aldrich.

Temperature was maintained with an air curtain incubator (Model 279) purchased from Orion Research (Boston, MA) and an inflatable glove
chamber (GLOVE BAG™) purchased from Cole Parmer (Niles, IL). 7% air /CO₂ was purchased from Northeast Airgas (Nashua, NH) and was regulated with a flowmeter purchased from Cole Parmer. pH was monitored with a micro-combination glass pH electrode (MI-410) purchased from Microelectrodes (Londonderry, NH) and a digital pH meter (DpH) purchased from Beckman (Fullerton, CA). A three-dimensional micromanipulator (Model MN-153) was purchased from Narishige (Tokyo, Japan). Temperature was monitored using a temperature probe and temperature monitor (Model TH-8 Thermalert) purchased from Physitemp Instruments (Clifton, NJ). Cyclic voltammetry was performed as described in Section 6.2.1. All potentials are defined versus Ag/AgCl unless indicated otherwise. Contact angles were measured using a goniometer purchased from Ramé-Hart (Mountain Lakes, NJ). Images of contact angle were captured by fitting the eyepiece of the goniometer with an adapter machined from Delrin AF® purchased from Alltech Plastics (Boston, MA) and recording the image with a video camera (Hitachi, Tokyo, Japan) and time-lapse recorder/player purchased from Panasonic (Secaucus, NJ). The images were analyzed as described in Section 5.2.1.

7.2.2 Methods & Procedures

7.2.2.1 Measurement of pH and Temperature as a Function of Potential

Experimental conditions were similar to the cell experiments except fibronectin and cells were not added. Polypyrrole and indium tin oxide surfaces were assembled into six-well chambers which were similar to the twelve-well chambers (see Figure 5.1). Each well of the six-well chamber contained 3 ml of either DMEM supplemented with penicillin and
streptomycin and 20 mM Hepes or unbuffered saline. Temperature was maintained at 37°C with an air curtain incubator and an inflatable glove chamber which was placed over the entire system. The chamber was kept under a continuous 7% humidified air / CO₂ flow in order to simulate the environment inside the incubator (McKenna and Wang, 1989). Changes in pH near the surface of the polypyrrole films and bare indium tin oxide surfaces were monitored with a micro-combination glass pH electrode and a pH meter. Surface pH was monitored at -0.5, -0.25, 0 V and under no potential. A three-dimensional micromanipulator was used to position the pH probe at various positions relative to the working electrode surface, counter and reference electrodes. Simultaneously, the temperature of the solution was monitored using a temperature probe connected to a temperature monitor.

7.2.2.2 Contact Angle as a Function of Potential

The sessile drop contact angles of DMEM with 20 mM Hepes (approximately 10 µl delivered with a pipette) were measured using a goniometer. The contact angles were measured at -0.25 V, 0 V, and under no potential application (Figure 7.1). Silver wire served as the reference electrode and was carefully wrapped around a standard Pasteur pipette. Platinum wire served as the counter electrode and was inserted into the Pasteur pipette to avoid contact with the reference electrode. The pipette was then attached to a three-dimensional manipulator and carefully lowered into the drop. The substrates were attached to the goniometer stage with double-sided tape. The entire experimental set-up was covered with an inflatable
Figure 7.1. Experimental set-up for determination of contact angle on polypyrrole (PPy).

WE, working electrode; RE, reference electrode; CE, counter electrode. Figure adapted from (Abbott and Whitesides, 1994). Video camera, inputed into a monitor, and captured with a time-lapse recorder/player. A minimum of three randomly selected areas of the substrate were analyzed. Contact angles were determined using a computer containing a frame grabber, and Image 1.49 software.
glove bag, and steam was generated inside the system to prevent evaporation of the water drop. The current-potential curve was recorded, and a system consisting of a plastic adapter connecting the goniometer eyepiece to the video camera was used to record the images of the sessile drops. The contact angles were determined using Image 1.49 software.

7.2.2.3 Fibronectin Release

Fibronectin release was measured both in the presence and absence of bovine aortic endothelial cells. To determine whether fibronectin was being released during reduction of the polypyrrole films, trace amounts of \( ^{125} \text{I-} \)labeled FN (4.58 \( \mu \)Ci/\( \mu \)g) were included in the coating solution. FN coating and cell plating was carried out as described in Section 6.2.3 except defined media consisting of DMEM supplemented with transferrin (5 \( \mu \)g/ml), high density lipoprotein (10 \( \mu \)g/ml), and 1% bovine serum albumin was used when cells were present. In the experiments when no cells were added, DMEM with 20 mM Hepes was used. In the experiments in which cells were used, after the potential (-0.25 V) was applied from 15 to 20 hr of culture, the wells were washed with PBS and soluble GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) was added (10 \( \mu \)g/ml) in order to remove the cells from the surface. Control surfaces were FN-coated films of polypyrrole in the oxidized state. All washes and supernatants were collected and counted in a gamma counter.

7.2.2.4 Conditioned Media

To determine whether toxic products were being formed during reduction of polypyrrole, media from polypyrrole surfaces which were reduced for 4 hr were transferred to cells which were first allowed to attach and
spread for 4 hr on FN-polypyrrole and FN-petri dishes. Quiescent (serum-starved — 0.4% calf serum for two days) confluent monolayers of bovine aortic endothelial cells were plated (15,000 cells / cm²) in serum-containing (10% calf serum) medium on FN-polypyrrole and FN-petri dish surfaces as described in Section 6.2.2.2. Cells were allowed to attach and spread for 4 hr. During this 4 hr time period, FN-polypyrrole surfaces without cells were reduced (- 0.25 V) in DMEM supplemented with 10% calf serum. The conditioned media was then transferred to cells cultured (4 hr) on FN-polypyrrole and FN-petri dish surfaces. As a control, media not exposed to polypyrrole reduction was transferred as well. Viability of cells was determined by the LIVE/DEAD assay as described in Section 6.2.2.2.

7.2.2.5 Stability of Polypyrrole as a Function of Potential

Spectroelectrochemistry of polypyrrole as a function of potential and time was carried out as described in Section 6.2.2.1.

7.2.2.6 Reversibility

To determine if cells which rounded on reduced FN-polypyrrole surfaces are able to reattach to FN-coated surfaces, after the cells rounded up (15 to 20 hr of culture, see Section 6.3), cells were removed with soluble GRGDSP (10 μg/ml, dissolved in DMEM) and plated onto FN-coated petri dishes in DMEM with 10% calf serum.
7.3 Results and Discussion

The pH was monitored while polypyrrole was held at -0.25 V because others have found that the pH near a polypyrrole electrode changes drastically when the potential is shifted from -0.6 V to various potentials up to 0.6 V (Shinohara et al., 1989) in unbuffered saline. We also found drastic changes in the pH of unbuffered saline near the surface of polypyrrole when it was held at -0.25 V (Figure 7.2). This is expected since the solution has no buffering capacity. Furthermore, saline (which consists mainly of chloride ions) may have had stronger effects on the system in which Shinohara and others studied since the dopant ion in their polypyrrole films were chloride ions. On the other hand, the pH remained essentially constant near 7.4 when polypyrrole was held at -0.25 V in DMEM with 20 mM Hepes (Figure 7.3). Furthermore, the pH did not vary with position. Using the micromanipulator, it was possible to move the pH probe near all three electrodes: working, counter and reference. In all cases, the pH remained near 7.4. The pH was monitored until it stabilized. Although the pH was only monitored for 15 minutes (compared to the 5 hr experiment for the cell studies), the pH measured at the end of the 5 hr experiment was found to be 7.4. Furthermore, Shinohara (Shinohara et al., 1989) observed drastic pH changes to occur within 15 min of application of electrical potential. Thus, the changes observed in cell behavior (see Chapter 6) when polypyrrole was converted to its neutral state were most likely not due to surface pH changes. This does not rule out, however, changes in intracellular pH since the flow of cations or anions into the polymer film may still affect the cell and would not be detected by measuring the pH.
Temperature was found to remain essentially constant at 37 °C (Figure 7.4) in DMEM with 20 mM Hepes. There was a slight increase in the temperature of unbuffered saline (Figure 7.5) to about 38 °C but this was not significant.
Figure 7.2 pH of unbuffered saline on polypyrrole held in its neutral state at -0.25 V measured as a function of time.
Figure 7.3 pH of DMEM with 20 mM Hepes on polypyrrole held in its neutral state at -0.25 V measured as a function of time.
Figure 7.4  Temperature of DMEM with 20 mM Hepes on polypyrrole held in its neutral state at -0.25 V measured as a function of time.

After the temperature had equilibrated (zero time point), the temperature of the solution remained close to 37°C.
Figure 7.5 Temperature of unbuffered saline on polypyrrole held in its neutral state at -0.25 V measured as a function of time.
There was essentially no difference in contact angle between polypyrrole surfaces in the presence or absence of -0.25 V potential application. This is most likely due to the roughness of the polymer film and the chemical heterogeneities within the film. Although potential-dependent wettability has been observed in self-assembled monolayers containing ferrocene at the surface (Abbott and Whitesides, 1994), polypyrrole is not a single electroactive species and the system is actually more complex since there are counterions present to balance the overall charge. Furthermore, the structures of polypyrrole both in its oxidized and neutral state are both rather hydrophobic due to the hydrocarbon chains present.

To investigate whether polymer degradation was occurring, the UV/visible spectra were monitored during the reduction of polypyrrole to its neutral state (Figure 7.6). -0.5 V was chosen since this is the reduction of polypyrrole/tosylate as determined by the cyclic voltammogram (see Section 6.3). Spectra were collected at time 0, 1, 10, 20, 35, 50, and 65 min, but only spectra at 0, 10, 35, and 65 min are shown to illustrate the trend. As discussed in Section 6.3, when the polymer is reduced the peak near 800 nm which is associated with the oxidized polymer disappears, and peaks near 370 nm and 580 nm appear. There does not appear to be degradation or reoxidation of the polymer film since the broad peak near 800 nm does not increase during the reduction process.

However, we still wanted to see if toxic products were being formed during the reduction process. To that end, we transferred cell culture media after 5 hr reduction to monolayers of cells cultured on FN-coated surfaces, let the cells incubate overnight and determined cell viability with a standard
Figure 7.6  UV/Visible spectra of polypyrrole as a function of oxidation state.

Oxidized state under no potential ——; and held at - 0.5 V for — 10 min; — 35 min; and —— 65 min. Note the absence of the rise near 800 nm in the curves in which the polymer is reduced, and the rise of the peaks at 370 nm and 580 nm associated with the neutral polymer as the polymer is being reduced.
fluorescence assay in which live and dead cells appear green and red, respectively. When the “conditioned media” from reduction was transferred to monolayers of cells grown on FN-coated petri dishes or oxidized polypyrrole surfaces, there was greater than 90 % viability. Similarly, nonconditioned media, or media which was not used to reduced the polymer gave greater than 90 % viability as well. Thus, it does not appear that toxic products are being formed during the reduction process.

In order to determine whether or not fibronectin was being released during reduction of polypyrrole, trace amounts of 125I-labeled fibronectin was added to the coating solution, and the potential applied (-0.25 V). In the absence of cells, it was found that application of -0.25 V does not cause an increase of fibronectin release. 2.5% ± 0.1 and 2.2% ± 0.1 of the total fibronectin added was released for application of -0.25 V and no potential, respectively. The data is based on two separate experiments with conditions repeated in triplicate. For the experiments in which cells were plated, the cells were first allowed to attach and spread for 15 hours, and the potential was then applied from 15 to 20 hr of culture. Cells were removed with GRGDSP (Hayman et al., 1985) and were counted with the gamma counter to see if fibronectin was being pulled off the surface by the cell. Note that the fibronectin added yielded a multilayer coating since a large percentage of the fibronectin (between 80 - 95 %) did not adsorb to the surfaces (Table 7.1) Furthermore, applying a potential did not increase FN release when cells were present, and it did not appear as though fibronectin was being pulled of the surface by the cell retraction since the amount of fibronectin which was associated with the cell was negligible.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Petri Dish</th>
<th>PPy no potential</th>
<th>PPy -0.25 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant/ washes before cells added</td>
<td>95.1 ± 0.7</td>
<td>82.5 ± 1.9</td>
<td>81.7 ± 2.3</td>
</tr>
<tr>
<td>Supernatant / washes after 15-20 h treatment</td>
<td>4.8 ± 0.6</td>
<td>9.4 ± 2.0</td>
<td>9.2 ± 1.5</td>
</tr>
<tr>
<td>Supernatant / washes after GRGDSP added</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Polymer film</td>
<td>N/A</td>
<td>5.8 ± 0.5</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>O-ring</td>
<td>N/A</td>
<td>1.9 ± 0.3</td>
<td>2.2 ± 0.5</td>
</tr>
</tbody>
</table>

Table 7.1. % FN release calculated as the fraction of total amount of fibronectin added.

Data represents two separate experiments with each condition repeated in duplicate. Data is average and standard error of the mean.
To answer the question if cells could reversibly reattach to FN-coated surfaces after they had been forced to round up by potential application, we allowed synchronized cells to attach for 15 hours, applied -0.25 V from 15 to 20 hr, trypsinized the cells to remove them from the surface, and replated the cells onto FN-coated petri dishes. Only about 5% of the cells were able to reattach. Since this study showed that pH changes and temperature changes were negligible, and that fibronectin was not being released from the surface during reduction, it remains a possibility that the ion concentrations are being altered which may affect the intracellular pH. Changes in ion concentration during reduction of polypyrrole may be important since cell receptors which recognize extracellular matrix molecules such as fibronectin require divalent cations such as Ca$^{2+}$ or Mg$^{2+}$ for binding (Springer et al., 1987).

### 7.4 Conclusions

In summary, the changes in cell behavior observed when converting polypyrrole from its charged to neutral state is not due to changes in surface pH, temperature, or release of fibronectin. Furthermore, it appears that there is not degradation of the film, and the products which may form during reduction are not toxic since when conditioned media transferred to monolayers of growing cells did not alter cell viability. Further work must be done in order to determine the mechanism of cell shape and growth control on electrically conducting polymers.
7.5 Citations


Chapter 8: Future Directions

The goal of this research was to develop a system to noninvasively control cell shape and hence function by controlling the interaction between cells and proteins with conducting polymers. This goal has been reached by developing a system in which polypyrrole could be switched between its charged and neutral states in biological media and in the presence of oxygen. This study showed that proteins adsorb to polypyrrole efficiently and promote cell attachment and spreading. Furthermore, by controlling the oxidation state of the polymer, it was shown that cell shape and entry into S-phase could be controlled. While the mechanism is still unknown, this study has ruled out the possibilities of changes in surface pH, temperature, and release of fibronectin. Furthermore, products which may form during the reduction process are not toxic to monolayers of cells grown in vitro.

An area for future research, therefore, lies in determination of the mechanism of cell retraction as a function of polymer oxidation state. It would be useful to investigate intracellular signaling during the reduction process. For example, is calcium being depleted from extracellular matrix molecules like fibronectin? Or is the phosphorylation pathway being blocked? Furthermore, cell membrane potentials may be altered as well. In order to answer these questions, it is necessary to take a closer look at the cell biology aspect of this process. Along those lines, it would be interesting to see if other cellular functions are being altered as a function of potential. In this study, only DNA synthesis was examined. In order to address these issues, it may be worthwhile to investigate other cell lines as well.
A second area of future work is to alter the material properties of the conducting polymer. In this study, polypyrrole with tosylate as the dopant anion was studied. An obvious way to change the properties of polypyrrole would be to change the dopant anion since it has been shown to affect many of the material properties of the host conducting polymer such as electrochemical properties, morphology, and mechanical properties. It is possible that changing the dopant anion would also change the interactions with proteins and cells as well. It would also be desirable to form these materials into three-dimensional shapes rather than be limited to thin films. Currently, polypyrrole composites have successfully been synthesized. Thus, it may be possible to use polypyrrole to coat surfaces of other materials in order to take advantage of both materials.

Finally, one would not like to be limited to *in vitro* biological applications of these polymers. Ultimately, it would desirable to use these materials *in vivo*, and although it appears that polypyrrole is nontoxic to cells, these tests were performed *in vitro* and *in vivo* tests really would need to be done before the full potential of these materials can be realized.