CELL ATTACHMENT AND PROTEIN ADSORPTION TO POLYPYRROLE THIN FILMS

Joyce Y. Wong*, Robert S. Langer*, and Donald E. Ingber**
* Dept. of Chemical Engineering, MIT, Cambridge, MA 02139
** Depts. of Surgical Research and Pathology, Children's Hospital and Harvard Medical School, Boston, MA 02115

ABSTRACT

Thin films of polypyrrole were synthesized using both chemical oxidative and electrochemical methods. The resulting oxidized films were characterized by UV/VIS spectroscopy, contact angle and conductivity measurements. *In vitro* studies suggest that extracellular matrix molecules, such as fibronectin, adsorb efficiently onto polypyrrole thin films and that 3T3 Balb/c mouse fibroblasts attach and spread normally on polypyrrole.

INTRODUCTION

Electrically conducting polymers are a relatively new class of materials which are unique in that their properties, e.g. surface charge, wettability, conformational and dimensional changes, can be altered reversibly by oxidation or reduction. These properties make conducting polymers attractive for biological applications: Surface charge density has been shown to affect nerve regeneration [1]; and substrate wettability and charge are important for protein adsorption and cell adhesion [2, 3]. Cell adhesion, in turn, has been shown to regulate both cell growth and differentiation [4, 5].

Currently, the majority of research on conductive polymers examines their behavior under conditions which would be rather stringent for cells: for example, conditions of O_2-free environment, little or no moisture, low pH, and solvents would be harsh for mammalian cells. There have been some studies of polypyrrole in biological environments to be used as biosensors [6]; electrodes to obtain electrochemically controlled release of dopamine [7], glutamic acid [8], and ATP [9]; and as substrates which bind proteins [10, 11] and DNA [12]. However, there is very little work looking at the interaction of cells with electrically conducting polymers. We chose to study polypyrrole because of its relatively good stability and because there already is some work in the literature examining polypyrrole in biological environments. We first wanted to evaluate the oxidized form of polypyrrole. The purpose of this study was to synthesize oxidized polypyrrole on various substrates, to characterize the films and to examine protein adsorption and cell attachment to these films.

MATERIALS AND METHODS

Synthesis of polypyrrole

Chemical synthesis of polypyrrole was carried out based on a method by Gregory[13] to coat polystyrene petri dishes (Falcon, 35 mm) and polystyrene tissue culture dishes (Corning, 35 mm). Pyrrole (Kodak Laboratories) was

passed through an activated alumina column until it became colorless. An aqueous solution of ferric chloride hexahydrate (0.018 *M*, Mallinckrodt), p-toluene sulfonic acid (0.026 *M*, Fluka), and purified pyrrole (0.006 *M*) was added to beakers containing the substrates. After 3 hrs the substrates were removed from the solution, rinsed several times with water, and dried in air at room temperature.

Electrochemical synthesis of polypyrrole were carried out in an electrochemical cell containing an indium tin oxide (ITO) anode (Delta Technologies), platinum mesh counter electrode, and a Ag/Ag^+ reference electrode. Electrodeposition (Pine AFRDE4 bipotentiostat) was performed in a solution of purified pyrrole (0.1 *M*), tetraethylammonium- p - toluene sulfonate (0.1*M*, Alfa), highly pure water (0.5 v/v%) and acetonitrile. Polypyrrole films were made potentiostatically at 0.8 V (vs. Ag/Ag+) until about 150 mC/cm^2 was passed, which corresponded to a thickness ≤ 1 μm.

Polymer Characterization

Sheet resistance of the chemically formed polypyrrole were measured using a four-point probe meter (Four Dimensions, Model 101). Conductivity was calculated from sheet resistance and thickness measurements. Thickness of the electrochemically synthesized films was determined by a Sloan Dektak IIA profilometer. The thickness of the chemically synthesized films were determined from a calibration curve of absorption at 950 nm vs. thickness for the electrochemically formed films. Spectroscopic data were obtained using an Oriel Instaspec model 250 spectrometer. Conductivity of electrochemically synthesized polypyrrole was determined by the van der Pauw method [14] after the film had been removed from the anode.

A Ramé-Hart goniometer was used to measure static contact angles of water on the various samples. A minimum of 10 measurements were made for each sample.

Protein adsorption

Fibronectin (FN) was used as a model protein and its adsorption was quantitated according to a previously reported method [4]. Various amounts of human serum FN (Cappell Laboratories) were mixed with ^{125}I-labeled FN (ICN Radiochemicals) and dissolved in 0.1 M carbonate buffer (pH 9.4). Samples were tested in duplicate in a 12-well chamber, modeled after the Bionique chamber [15]. 100 μl of FN solution was added to the chamber wells and allowed to adsorb for 24 hr at 4°C. Wells were washed twice with Dulbecco's phosphate-buffered saline (Gibco). The samples were removed from the chamber; and the supernatant, washes, and samples were counted in a gamma counter (LKB-Wallace CliniGamma, Model 1272).

Cell culture

Cell attachment of Balb/c 3T3 mouse fibroblasts (ATCC 6587) was performed both in the presence and absence of calf serum. In a typical experiment, a cell suspension in Dulbecco's Modified Eagle Medium (DMEM,

Gibco) supplemented with 10% calf serum (Hyclone), 100 units/ml of penicillin (Gibco), and 100 µg/ml of streptomycin (Gibco) were plated onto dishes at a density of 5×10^4 cells/cm^2.

Table I shows the surfaces used in the cell culture experiments. Each sample was tested in triplicate. The electrochemically synthesized polypyrrole samples were assembled in a cell chamber modeled after the Bionique chamber [15]. The polypyrrole surfaces were sterilized via exposure to either UV-irradiation for 30 min or to ethylene oxide overnight. The tissue culture polystyrene was used as received from the manufacturer. In order to eliminate any stray charges on the polystyrene bacteriologic petri dishes, the dishes were pre-incubated for 1 hour with DMEM containing 1% bovine serum albumin (Sigma). Following a 4 hr incubation time (37°C, 5% CO_2), the cells were carefully washed with phosphate-buffered saline (PBS) to remove unattached cells. The attached cells were then removed by trypsinization. The cells from the wash and trypsinization were counted with a Coulter Counter (Coulter Electronics, Inc., Model Z_F). Attachment was determined by the counts of the trypsinized cells divided by the total counts from the wash and the trypsinized cells.

Table I. Materials for Cell Culture Experiments

Abbreviation	Material
TC	Tissue culture polystyrene (Corning, 35 mm)
PD	Bacteriologic grade polystyrene (Falcon, 35 mm)
PPY/TC	Polypyrrole formed chemically on TC
PPY/PD	Polypyrrole formed chemically on PD
PPY/ITO	Polypyrrole formed electrochemically on indium tin oxide (ITO)

Morphological analysis

Cells were fixed with glutaraldehyde and were observed under a phase-contrast microscope (Nikon TMS, Type 104). Projected cell area was determined by staining the fixed cells with Coomassie blue and using a computerized image analysis system (Zeiss Interactive Digital Analysis System) as previously described[4]. A minimum of 50 randomly selected cells were analyzed for each condition.

RESULTS

Polymer Characterization

Polypyrrole can be synthesized either electrochemically or chemically. Since it was not known which method would produce better materials for cell culture, both methods were examined.

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.

The absorption spectra (Figure 1) 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 [16].

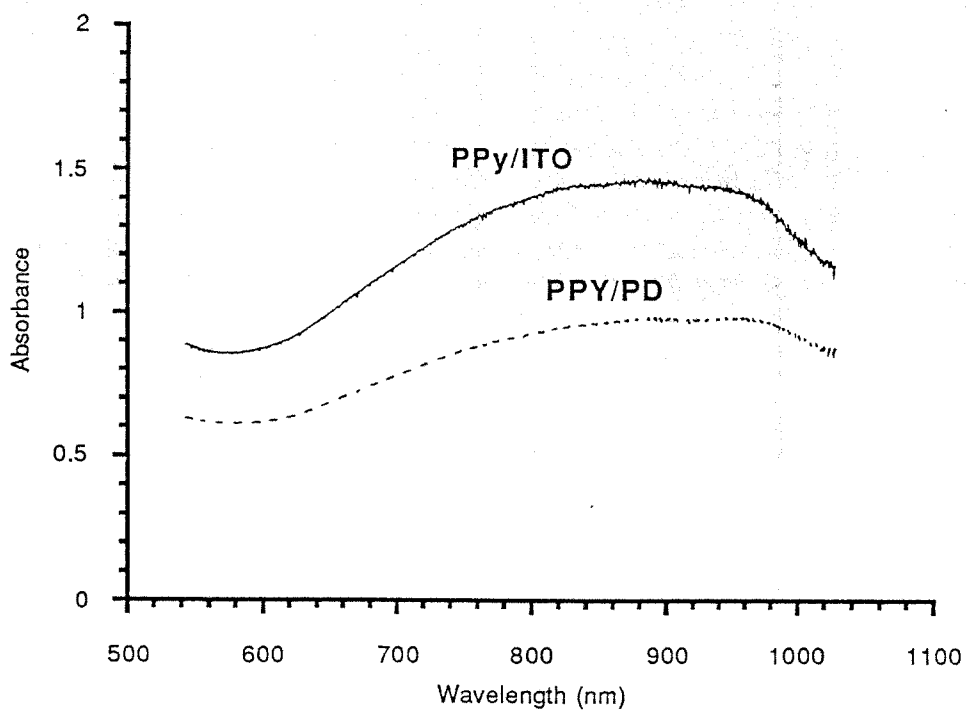

Figure 1. Absorption spectra of polypyrrole thin films formed electrochemically and chemically.

Conductivity measurements (Table II) confirmed that the polypyrrole thin films 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. The differences in wettability between PD and PPY/PD (Table II) indicate that there is a uniform coating of the polypyrrole.

Furthermore, it appears that the properties of polypyrrole, such as wettability, vary depending on the method of synthesis: PPY/ITO is more hydrophobic than PPY/PD or PPY/TC.

Table II. Polymer Characterization

	Conductivity $(\Omega\text{-cm})^{-1}$	Contact angle (°)
PD	10^{-20}	71 ± 1
TC	10^{-20}	53 ± 2
PPY/PD	10	54 ± 7
PPY/TC	10	56 ± 7
PPY/ITO	100	75 ± 3

Protein Adsorption

We wanted to examine the adsorption of fibronectin onto polypyrrole. Fibronectin is a 220 kD glycoprotein which has been well-established as a cell adhesion protein, i.e. it adsorbs to a surface and mediates cell attachment. The results for when a high density of fibronectin (1000 ng/cm^2) was added (Table III) show that fibronectin does adsorb to polypyrrole. The amount adsorbed is in the range necessary for cell attachment and spreading[17]. It is interesting to note that a significantly smaller amount of fibronectin adsorbed to TC than to PD. This may be counterintuitive since cells spread and attach on TC rather than PD surfaces. It has been suggested, however, that the protein binds to hydrophobic surfaces in different conformations compared to hydrophilic surfaces[18]. In order to better evaluate the polypyrrole thin films, we decided to perform cell studies.

Table III. Fibronectin Adsorption

	% Adsorbed	p-value vs. PPY/PD
PD	37 ± 2	0.25
TC	21 ± .1	0.005
PPY/PD,TC	40 ± 2	
PPY/ITO	43 ± 9	0.64

Cell culture

Fibroblasts were chosen because they are anchorage-dependent cells, i.e. they need to attach and spread to a surface in order to survive. Furthermore, they are available as a stable immortal cell line. Cell attachment studies were not performed on the fibronectin-coated surfaces but on the surfaces which had been pre-incubated with either serum-free or serum-containing medium. The serum contained cell adhesion proteins, such as fibronectin, which mediate cell attachment. The tissue culture dishes with medium-containing serum served as the positive control, whereas the petri dishes with serum-free medium served as

the negative control. The cell attachment on TC in the presence of serum (Fig. 2) is greater than 80%, as expected, whereas there is practically no attachment to PD in the absence of serum. Between 75% and 85% of the cells attached to PPY/TC and PPY/PD in the presence of serum, respectively, which is comparable to TC with serum. However, only about 50% of the cells attached to PPY/ITO, which is another indication that the two methods of synthesis yield polypyrrole with different properties. Interestingly, in the absence of serum, about 50% of the cells attached to the polypyrrole thin films, in contrast to only 25% on TC. Furthermore, morphological analysis of cells cultured in the absence of serum indicated that there was no difference in the extent of spreading on PPY/PD, PPY/TC, and TC which ranged from 300-1100 μm^2, in contrast to 100-350 μm^2 for PD.

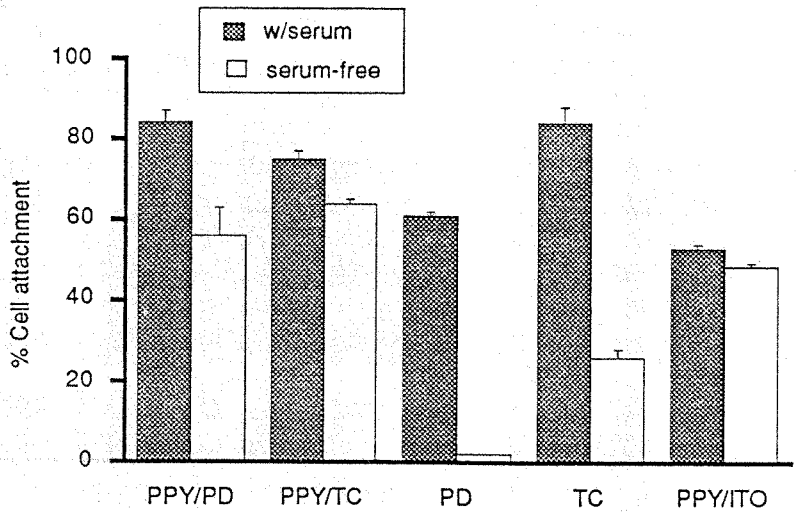

Figure 2. Attachment of 3T3 Balb/c fibroblasts after 4 hrs

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 cell adhesion 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. Future studies will examine cell growth. Much more work is needed to be done, however, looking at areas such as the biocompatibility and toxicity of electrically conducting polymers. Only then will we have a better idea of their limitations and possible applications as biomaterials.

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