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Electrically conducting polymers can noninvasively control the shape and growth of mammalian cells

(polypyrrole/fibronectin/DNA synthesis/tissue engineering/culture substratum)

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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. Such properties might render conducting polymers unique for biological applications. However, the majority of research on conducting polymers has been carried out under nonbiological conditions. We synthesized optically transparent polypyrrole thin films and studied them in environments suitable for protein adsorption and mammalian cell culture. *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, both cell extension and DNA synthesis were inhibited 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 function. These data suggest that electrically conducting polymers may represent a type of culture substrate which could provide a noninvasive means to control the shape and function of adherent cells, independent of any medium alteration.**

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 (1–5). 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) (4, 5). 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 (6). Electrically conducting polymers provide potentially interesting surfaces for cell culture in that their properties (e.g., surface charge, wettability, and conformational and dimensional changes) can be altered reversibly by chemical or electrochemical oxidation or reduction (7, 8). One can imagine a noninvasive 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.

Electrically conducting polymers characteristically have a conjugated backbone with a high degree of π-orbital overlap. Through a process known as "doping," the neutral polymer chain can be oxidized or reduced to become either positively charged (oxidative, or p-type) or negatively charged (reductive, or n-type), respectively, with polarons and bipolarons as the charge carriers for electrical conduction (7). 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 (9). 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 (10). This doping process can be achieved either chemically or electrochemically and is reversible.

Most research on conducting polymers has been conducted under nonbiological conditions. Of these conducting materials, polypyrrole is perhaps the most widely studied polymer due to its chemical and thermal stability, ease of preparation, and electroactivity (11). In fact, polypyrrole has been examined in biological environments for use as biosensors (12), electrodes to obtain electrochemically controlled drug release (13), and substrates which bind proteins (14–16) or DNA (17). However, the interaction of living cells with electrically conducting polymers has remained essentially unexplored.

The objective of the present study was to examine the suitability of conducting polymers for cell culture and the usefulness for controlling cell function. We now show that these polymers represent a 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.

MATERIALS AND METHODS

Synthesis of Polypyrrole. Pyrrole (Kodak) was purified by passage through an activated alumina column until it became colorless. Electrochemical synthesis of polypyrrole (18) was carried out in an electrochemical cell containing an optically transparent indium tin oxide anode (40 Ω per square; Delta Technologies, Stillwater, MN), a platinum mesh counter-electrode, and a saturated calomel reference electrode (SCE). The electrodeposition solution contained 0.1 M pyrrole and 0.1 M tetraethylammonium *p*-toluenesulfonate (Alfa Products, Ward Hill, MA) in acetonitrile with 0.5% (vol/vol) ultrapure water (Milli-Q Reagent Water System; Millipore).

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Abbreviations: FN, fibronectin.

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Films were made potentiostatically at 1.1 V (versus SCE; Pine Instruments AFRDE4 bipotentiostat; Linseis x–y recorder) until about 100 mC/cm^2 was passed.

Polymer Characterization. Cyclic voltammetry experiments were carried out with a Pine Instruments AFRDE4 bipotentiostat and a Linseis x–y recorder. The polypyrrole films were cycled between +0.4 and −1.0 V at 50 mV/sec in serum-free medium [Dulbecco's modified Eagle's medium (DMEM) (wt/vol)/1% bovine serum albumin/20 mM Hepes, pH 7.4]. All potentials were defined relative to a Ag/AgCl reference electrode. Spectroscopic data were obtained with a Hewlett–Packard 8452A diode-array spectrophotometer.

Cell Studies. Bovine aortic endothelial cells (kindly provided by P. D'Amore, Children's Hospital, Boston, MA) were maintained in DMEM supplemented with 10% (vol/vol) calf serum (HyClone). Polypyrrole and control surfaces were assembled in a six-well chamber modeled after the Bionique chamber (19). To analyze effects on cell attachment, cells were plated (15,000 cells per cm^2) in serum-free medium on surfaces precoated with FN (10 μg/cm^2), as previously described (3, 4). 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 (ref. 3; Sigma), and photographed on a Nikon Diaphot microscope under 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 per cm^2) 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; Collaborative Research), high density lipoprotein (10 μg/ml; Cappel), 1% bovine serum albumin, and fibroblast growth factor (5 ng/ml; kindly supplied by Takeda, Osaka) (3). 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 (20). Effects on DNA synthesis were measured by quantitating nuclear incorporation of 5-bromo-2′-deoxyuridine (BrdUrd; Amersham) with a commercially available fluorescence assay (Amersham RPN20) except that rhodamine-conjugated goat IgG directed against mouse IgG Fc (Cappel) was used as the secondary antibody. The potential (−0.25 V) was applied from 15 to 20 hr of culture, the time when these G_0-synchronized cells begin to reenter S phase. BrdUrd (3 μg/ml) was included only during this 5-hr period. Total number of cells and labeled nuclei were counted in four random fields (at ×200; >50 cells per field) by using the phase-contrast and fluorescence capabilities of a Nikon Diaphot inverted microscope. Cell viability was quantitated with an assay (Live/Dead viability/cytotoxicity assay; Molecular Probes) that is based on the cellular incorporation of two fluorophores, calcein acetoxymethyl ester (viable cells) and ethidium homodimer (nonviable cells).

RESULTS

The electrochemical synthesis resulted in formation of uniform films of polypyrrole on indium tin oxide-coated glass substrates. Film thicknesses were estimated from the amount of charge passed during electrodeposition (21) and were ≈0.1 μm. Polypyrrole obtained via electrochemical synthesis was in its oxidized state as a polycation with dopant anions to balance the charge (Fig. 1) and was able to be cycled between its charged and neutral forms electrochemically in culture medium (Fig. 2). The oxidation state of polypyrrole was monitored by UV/visible spectroscopy (Fig. 3). A broad peak near 800 nm associated with the bipolarons (22) 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 (23). 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 (24).

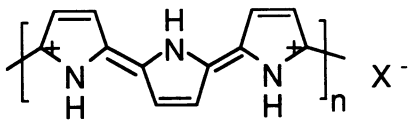

FIG. 1. Structure of polypyrrole in the oxidized state. X^-, dopant anion.

Bovine aortic endothelial cells attached poorly and did not extend on uncoated polypyrrole when cultured in serum-free medium. In contrast, both cell attachment and spreading were observed (Fig. 4*A*) when the oxidized polymer films were precoated with FN, an extracellular matrix molecule that adsorbs to surfaces and mediates binding to specific cell surface integrin receptors (25). However, when cells were plated on FN–polypyrrole films that were converted to their neutral state, the cells attached but they remained round (Fig. 4*B*). Cell rounding was also observed within 1 hr when the electrical potential (−0.5 V) was applied to spread cells cultured for 4 hr on oxidized FN–polypyrrole before reduction (data not shown). 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-coated 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. Yet when polypyrrole that was coated with ^{125}I-labeled FN was reduced by similar means, no significant release of adsorbed protein was observed (data not shown). Cells were not included in these experiments, however, and thus local removal of FN anchors beneath the cells due to cell tractional forces remains a possibility.

Cell shape and growth have been shown to be tightly coupled in many anchorage-dependent cells (1–5). Similarly, we found that cell retraction induced by applying an intermediate electrical potential (−0.25 V) to FN–polypyrrole provided control over cell cycle progression (Fig. 5). Ap-

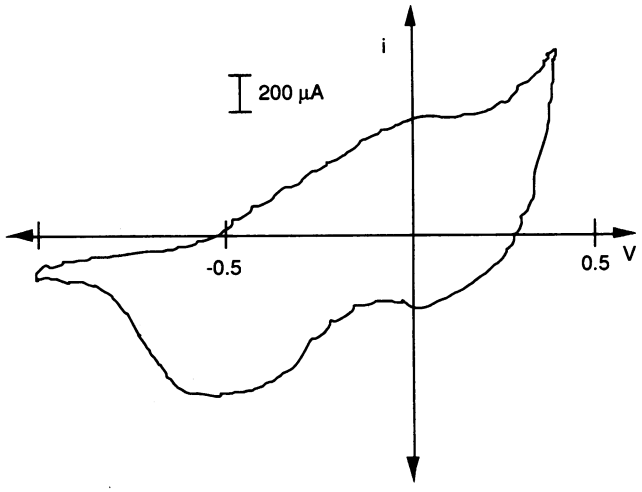

FIG. 2. Cyclic voltammogram of polypyrrole in serum-free culture medium. Scan rate, 50 mV/sec.

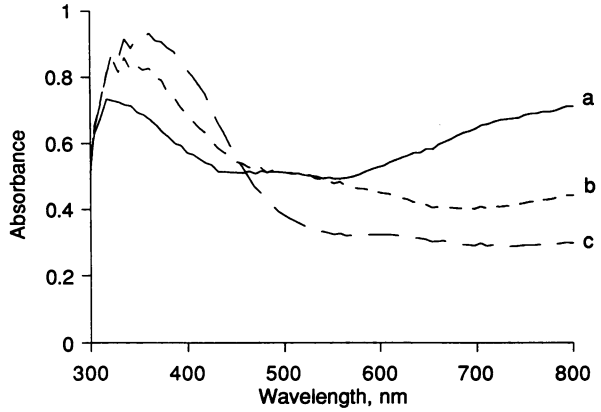

FIG. 3. UV/visible spectra of polypyrrole in its native oxidized state under no potential (spectrum a) and reduced by application of either −0.25 V (spectrum b) or −0.5 V (spectrum c).

proximately 75% of cells cultured in serum-containing medium on FN-coated Petri dishes, indium tin oxide, or oxidized polypyrrole spread normally and entered S phase synchronously 15–20 hr after plating. Applying −0.25 V to indium tin oxide 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. Similar results were obtained when chemically defined, serum-free medium was utilized, except that labeling indices were slightly lower (data not shown). These effects were not due to cell death, since >90% of the cells remained viable on the neutral FN–polypyrrole, as determined by quantitating incorporation of the vital dye calcein acetoxymethylester.

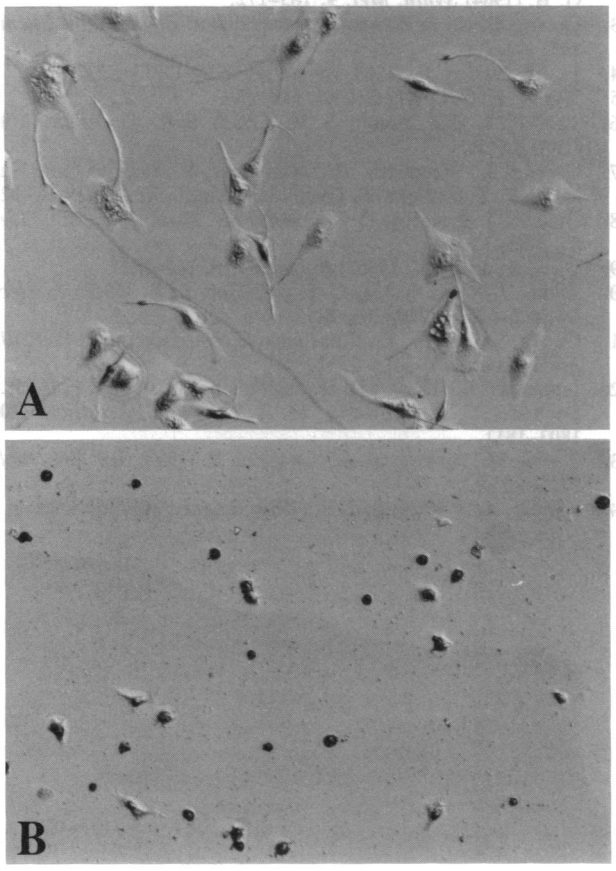

FIG. 4. Photomicrographs of endothelial cells cultured for 4 hr on FN–polypyrrole in either its native oxidized state (*A*) or after reduction by application of −0.5 V for 4 hr (*B*). (×700.)

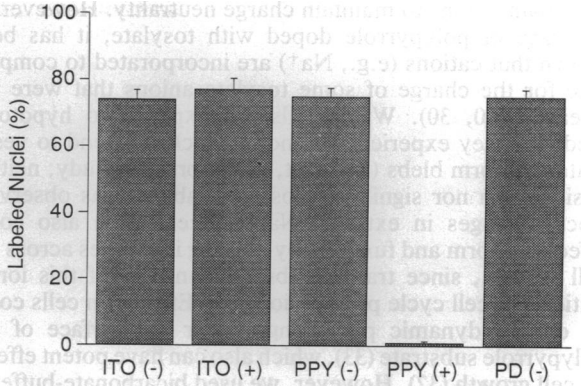

FIG. 5. DNA synthesis in cells cultured in serum-containing medium on various FN-coated substrata in the absence (−) or presence (+) of an applied electrical potential (−0.25 V). Data are presented as mean ± standard error of the mean. ITO, indium tin oxide electrode; PPY, polypyrrole; PD, Petri dish. Cell viability was >90% under all conditions.

DISCUSSION

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 (15) and DNA (17) adsorb more efficiently onto oxidized polypyrrole than onto the neutral polymer. The wettability of a surface is also dependent upon its oxidation state (N. Abbott, personal communication). 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 (26).

Tight coupling between cell shape and growth also has been observed in the past (1–5). 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 we 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 (27). However, when we applied the same electrical potential to cells using FN–indium tin oxide surfaces, cells did not round. Associated changes in electrical fields could also play a role. However, in our case, cells were exposed to a current density of only 20 $\mu A/cm^2$ (as determined by chart recording during polymer reduction), a density which was previously shown not to affect attachment, spreading, or growth of cultured fibroblasts (28). Electrochemically reduced polypyrrole films exhibit a conductivity on the order of 10^{-6} $S \cdot cm^{-1}$ (29), compared with ≈ 1 $S \cdot cm^{-1}$ 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 (10, 30). When cells are exposed to hypotonic medium, they experience osmotic shock and tend to vesiculate and form blebs (31). Yet, in the present study, neither vesiculation nor significant loss of viability was observed. Local changes in external Na^+ concentration 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 (32). Effects on cells could be due to dynamic pH changes near the surface of the polypyrrole substrate (33), which also can have potent effects on cell growth (32). 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 also is the possibility that the polymer changes mechanically (e.g., becomes more malleable), the integrity of the cell's basal adhesions weakens, or 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.

In summary, these data indicate that polypyrrole can potentially be a very important biomaterial, since it is possible to externally change its properties and surface binding characteristics reversibly by using applied electrical potentials. Polypyrroles and other electrically conducting polymers therefore may be especially useful as substrates for both small- and large-scale cell cultures since they provide a noninvasive way to regulate cell form and function. The present study shows 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 (4, 5). Thus, use of conducting polymers may provide a relatively simple and inexpensive means to control cell growth and differentiation noninvasively, without altering medium composition or refeeding. This type of experimental manipulation may be extremely useful for applications in biotechnology and tissue engineering (34). It also provides a way to analyze the fundamental biochemical mechanisms by which cell–substrate interactions regulate cell physiology.

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