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Carboxy-Endcapped Conductive Polypyrrole: Biomimetic Conducting Polymer for Cell Scaffolds and Electrodes

Joo-Woon Lee[†,‖,*,⊥], **Francisco Serna**[†,⊥], and **Christine E. Schmidt**[†,‡,§,*]

[†]Biomedical Engineering Department, The University of Texas at Austin, Austin, Texas 78712

[‡]Chemical Engineering Department, The University of Texas at Austin, Austin, Texas 78712

[§]Texas Materials Institute, The University of Texas at Austin, Austin, Texas 78712

[‖]Division of Liberal Arts and Sciences, Chungju National University, Chungju, Chungbuk 380-702, Korea

Abstract

Numerous regenerating tissues respond favorably to electrical stimulation, creating a need for a bioactive conducting platform for tissue engineering applications. The drive for biosensors and electrode coatings further requires control of the surface properties of promising conductive materials such as polypyrrole. Here we present carboxy-endcapped polypyrrole (PPy-α-COOH), a unique bioactive conducting polymer with a carboxylic acid layer, composed of a polypyrrole (PPy) surface modified with pyrrole-α-carboxylic acid (Py-α-COOH). This unique structure is simple to produce, provides a stable bioactive surface via covalent bonds, and preserves bulk properties such as electrical conductivity and mechanical integrity. The chemical structure of this polymer composite was characterized by angle-resolved X-ray photoelectron spectroscopy (XPS), which demonstrated the presence of carboxylic acid functionality on the top surface of conductive PPy. A four-point probe test was used to verify the similar conductivity of PPy-α-COOH compared to that of standard PPy. To demonstrate the potential to influence cellular activity, the carboxylic acid monolayer surface was grafted with the cell-adhesive Arg-Gly-Asp (RGD) motif. Human umbilical vein endothelial cells (HUVECs) cultured on RGD-modified PPy-α-COOH demonstrated significantly higher adhesion and spreading than on the negative controls PPy-α-COOH and unmodified PPy.

Introduction

Numerous tissues including bone,[1a] cartilage,[1b] skin,[1c] spinal nerves,[1d] and peripheral nerves[1e] respond favorably to electric fields, motivating the development of conductive, biocompatible biomaterials. Polypyrrole (PPy) is a conductive, biocompatible[2] polymer with the potential to bring the benefits of conductivity into tissue-engineered constructs. PPy features include high conductivity, dopant-mediated tunable physical properties, environmental stability to air and water, and the potential for electroactive copolymers.[3]

Surface modification with biomolecules is a typical strategy to improve the cellular response to conventional biomaterials.[4] Biomaterial surfaces strongly influence the immune response and matrix erosion rate, provide a site for cell adhesion, direct cell migration, and can trigger cell differentiation.[5] The ability to present biomolecules at the surface capable of guiding cell

*To whom correspondence should be addressed. E-mail: E-mail: jwoonlee@chungju.ac.kr (J.-W.L.); E-mail: schmidt@che.utexas.edu (C.E.S.).
⊥These authors contributed equally to this work.

activity is paramount to a successful biomaterial. Thus, physicochemical approaches to biologically tailor the PPy surface to promote specific receptor-mediated events in cells is highly desired to further enhance the performance of PPy in bioelectronics and tissue engineering.[6]

Previous attempts to create bioactive conducting polymers have required complicated screening strategies based on noncovalent specific interactions or have compromised intrinsic bulk properties such as the electrical conductivity and mechanical integrity.[6a–d] The noncovalent linker molecule reported by Sanghvi et al. involves surface modification using a unique peptide sequence (T59) that binds directly to chloride-doped polypyrrole (PPyCl).[6a] T59 was selected from a combinatorial peptide phage display library using biopanning techniques and was successfully demonstrated as a bifunctional linker by chemically conjugating a cell-adhesion-promoting sequence, Arg-Gly-Asp (RGD) motif, at the *C* terminus. Surface modification through noncovalent linker peptides preserves bulk properties, but the selection of these peptides requires a complex screening process and is less robust than covalent modification.[6a]

Another approach to modifying PPy with biomolecules has been explored by incorporating anionic polymers as dopants.[6b–d] Dopant ions are typically incorporated into the PPy matrix during electrochemical polymerization in order to maintain charge neutrality.[3a,b] Song et al. have functionalized the PPy surface by using the carboxylic acid-containing polyglutamatic acid as a dopant.[6b] Exposed carboxylic acid groups from the anionic polymeric dopant were then used to couple proteins and peptides at the surface. One drawback of this approach is that glutamic acid is a neurotransmitter that potentially presents problems with excitotoxicity.[6b] Similar reported examples include the incorporation of angiogenic hyaluronic acid[6c] and anti-thrombic heparin[6d] as bioactive dopants in the PPy matrixes; however, they were found to compromise both the mechanical integrity and conductivity. Thus, surface modification through the dopant ion is often inappropriate.

In a chemical approach, we reported on the potential of carboxylic acid-functionalized PPy, poly(*N*-(2-carboxyethyl)-pyrrole) (P(Py-*N*-COOH)), substituted at the *N* position of the monomeric pyrrole backbone.[6e] This P(Py-*N*-COOH), when modified with a peptide containing the cell-adhesive RGD motif, was preferentially found to promote cell adhesion and spreading. However, the conductivity of P(Py-*N*-COOH) is 4 orders of magnitude lower than unmodified conductive PPy, which would potentially limit the utility of this semiconductive PPy derivative as a bioactive platform for other biomedical applications.

Here we present an advanced surface-functionalization strategy to append a monolayer of the carboxylic acid functionality on the PPy surface without sacrificing PPy bulk properties such as conductivity and mechanical integrity. Surface functionalization occurred by electrochemically blocking (coupling) the α terminus of unmodified PPy with pyrrole-α-carboxylic acid (Py-α-COOH), resulting in the carboxy-endcapped PPy composite, denoted as PPy-α-COOH. RGD-*grafted*-PPy-α-COOH demonstrated superior cell adhesion and spreading compared with ungrafted controls; no significant change in conductivity compared to that of PPy was measured. A schematic of the coupling reaction of PPy-α-COOH and the chemical conjugation of an RGD peptide is presented in Figure 1.

Experimental Section

Materials

Pyrrole-α-carboxylic acid (Py-α-COOH, Aldrich), sodium chloride (NaCl, Aldrich), 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC, Fluka), *N*-hydroxysulfosuccinimide (NHSS, Sigma), GRGDSP peptide (Anaspec), human umbilical vein endothelial cells (HUVECs,

Cambrex), endothelial cell medium-ECM (Cambrex), and MTS reagent (Promega) were used as received. Pyrrole (Sigma) monomer was purified by passing it through a column of activated basic alumina prior to polymerization. Indium tin oxide (ITO) conductive borosilicate glass slides with typical resistance of 30–60 Ω/square (Delta Technologies, Ltd.) were sequentially cleaned in acetone, methanol, isopropyl alcohol, and distilled deionized (DDI) water for 5 min each with ultrasonication. Slides were then dipped in a 1.0 N NaOH solution for 30 min, washed with copious amounts of DDI water, and dried under vacuum.

Polymer Preparation

PPy (**1**) films were electrochemically polymerized[6e] using a bipotentiostat (AFRDE5, Pine Instrument) in a three-electrode cell: a platinum (Pt) mesh served as the counter electrode, a saturated calomel electrode (SCE) served as the reference electrode, and an ITO slide served as the working electrode. The aqueous monomer solution (200 mL) containing 0.1 M pyrrole and 0.1 M NaCl was purged with N_2 for 10 min to prevent oxidation of the monomer prior to polymerization. The films were deposited at an offset voltage of 720 mV onto the ITO electrode. The film thickness was monitored and controlled by integrating the passage of current.

Films of **1** on ITO slides were then electrochemically surface-modified to create carboxy-endcapped PPy (PPy-α-COOH) (**2**).[7] **1** was placed in a solution containing 200 mL acetonitrile (ACN), 0.1 M Py-α-COOH, and 0.1 M $LiClO_4$. An equivalent three-electrode setup was used for the electrochemical reaction; coupling between the α terminus of **1** and Py-α-COOH was ensured by setting the offset voltage at 2.0 V, which is above the oxidation potential of Py-α-COOH (oxidation potential (E_p): 1380 mV_{obsd}, 1501 mV_{calcd}).[7a] Coupling was allowed to occur for 15 min. Poly(*N*-(2-carboxyethyl)-pyrrole) (P(Py-*N*-COOH)) was prepared as previously described.[6e]

Polymer Surface Characterization

The chemical composition of the polymer surfaces was determined using a Physical Electronics (PHI) 5700 X-ray photoelectron spectrometer (XPS) equipped with an Al monochromatic source (Al Kα energy of 1486.6 eV) and a hemispherical analyzer. The energy resolution was 1.0 eV for survey spectra and 0.1 eV for high-resolution (HR) spectra. Binding energies were calibrated by setting the *C*–C/*C*–H_x component in the C 1s envelope at 284.6 eV. HR angle-dependent acquisitions with a pass energy of 11.75 eV were acquired at takeoff angles of 15 and 45° and led to sampling depths of ~2.7 and 7.3 nm for the C1s envelope, respectively.[8] The doping levels before and after surface functionalization were reproducibly (n = 3) determined from the Cl/N ratio of HR Cl 2p and N 1s core-level XPS spectra measured at a 45° takeoff angle. Conductivity at ambient temperature was measured (n = 3) with a conventional four-point probe placed on the polymer films: current and voltage measurements from the four-point probe were used with correction factors for thickness and geometry to determine the sheet conductivity.[9] The film thickness was measured to be 230–250 nm using a profilometer (Alpha Step 200, Tencor Instruments).

Surface Modification with RGD

A cell-adhesive GRGDSP was immobilized onto the surface of **2** films in 10 mM PBS at pH 7.4 as follows:[6e] A solution of 173 μM EDC and 15 μM NHSS was reacted with the **2** film for 3 h at ambient temperature to activate the carboxylic acid groups on the surface in a stable intermediate form of α-sulfosuccinimidyl ester PPy. The primary amine (H_2N−) of the *N* terminus of the GRGDSP peptide (1 mg/mL) was then chemically conjugated with the activated film surface for 4 h, leading to the formation of RGD-*grafted*-PPy-α-COOH (**3**).

Cell Adhesion and Viability

To practically validate both the RGD-*grafting* reaction and the bioactivity of **3**, cell adhesion studies were performed and quantitatively and qualitatively analyzed.[6e] HUVECs were seeded in 1.5 cm^2 wells with 1 mL of ECM media at a density of 30 000 cells/cm^2 and cultured for 1 h in the absence of serum on three surfaces: RGD-grafted **3**, ungrafted **2**, and unmodified **1**. The colorimetric MTS cell proliferation assay was performed to quantify the extent of cell adhesion.[10] Following 1 h of incubation in serum-free media, surfaces were rinsed three times by gently shearing 1 mL of 10 mM PBS over the film surface to remove unattached and loosely attached cells.[6e] PBS was aspirated, and new medium containing 2% fetal bovine serum (FBS) and 20% MTS reagent was added to each well and allowed to incubate at 37 °C for 1, 2, and 3 h time points. Cell viability was measured by removing 100 μL of media from the wells and measuring the absorbance at 490 nm using a fluorescence microplate reader (Synnergy HT, BioTek). At least three samples were averaged to calculate each time point. Images were taken with an optical microscope (Olympus IX-70) immediately following media removal for the 3 h time point.

Results and Discussion

The electrochemical synthesis of **2** in Figure 1 was accomplished on the **1**-coated ITO/glass working electrode. Coupling of pyrrole-α-carboxylic acid was performed above the known oxidative potential (E_p: 1380 mV)[7a] as described in the Experimental Section. Oxidative electrochemical polymerization of pyrrole (Py) is favorable to the α–α linear linkages between Py monomers, which confers a higher degree of conductivity than the α–β and β–β linkages that lead to branching, disruption in planarity, and the resulting disruption of the π-electron conjugation.[3] The monolayered carboxy-endcapped PPy (**2**) was formed through the favorable coupling reaction[7] between the α terminus of PPy (**1**) and the α end of pyrrole-α-carboxylic acid (Py-α-COOH), resulting in a more highly conductive conjugated bond structure.

The presence of the monolayer carboxylic acid (−COOH) functionality at the surface of **2** was investigated through the depth-resolved scans from an angle-dependent XPS study. Photoelectron emission values at takeoff angles of 15 and 45° were used for **2** and the control **1** surface analysis. No additional elements beyond C 1s, N 1s, O 1s, and Cl 2p were detected in the survey spectra (not shown), so no adjustment was made in the analysis. The HRC 1s envelope of XPS at a 15° takeoff angle provides evidence for the presence of carboxylic acid (−COOH) groups on the surface of **2**. The appearance of a new C 1s component[11] centered at 288.8 eV in the PPy-α-COOH trace acquired at a 15° takeoff angle (solid black line in Figure 2) is small, as would be expected from typical monolayer coverage. In angle-resolved XPS, a 15° takeoff angle provides information on chemical composition that is closer to the top surface than a 45° takeoff angle.[8] For this reason, the new C 1s component associated with the carboxylic acid (−COOH) functionality is observed only in the spectrum of the functionalized surface (**2**) at a 15° takeoff angle. The binding energy of the C 1s *C*–C/*C*–H_x component centered at 284.6 eV aligns in both the **2** and the unmodified **1** surfaces at both 15 and 45° takeoff angles in Figure 2. The presence of carboxylic acid (−COOH) groups on the surface of **2** was readily determined by comparing each of the XPS C 1s envelopes at 15 and 45° takeoff angles; no carboxylic acid (−COOH) groups were detected at the control unmodified **1** surface. Further evidence of the coupling reaction was confirmed from preferential cell proliferation on the RGD-grafted PPy-α-COOH surface and is presented in Figure 3 and Figure 4.

The preservation of PPy's intrinsic properties in the PPy-α-COOH composite (**2**), particularly the electrical conductivity, is the key advantage of carboxy-endcapped **2** over alternative modification approaches. Dopant-based[6b–d] and monomer-based[6e] modification approaches have the potential to adversely affect bulk properties such as conductivity and mechanical properties. Previously explored poly(*N*-(2-carboxyethyl)pyrrole) (P(Py-*N*-COOH)), for

example, has a conductivity that is 4 orders of magnitude lower than that of PPy,[6e] whereas the presently described monolayer modification does not alter the matrix doping level, mechanical integrity, or conductivity. The doping levels, calculated from Cl/N ratios (atomic concentration, %) of both **1** and **2** at a 45° takeoff angle, were found to be ca. 31.0%. The electrical conductivity of **2** was measured to be 2.78 ± 0.12 $S \cdot cm^{-1}$, which is statistically indistinguishable from that of unmodified **1** that was measured to be 2.81 ± 0.05 $S \cdot cm^{-1}$. These measurements indicate that the bulk properties of **1** are conserved in the composite (**2**) as summarized in Table 1.

To demonstrate the potential for this material to influence cellular activity, carboxy-endcapped PPy (PPy-α-COOH) (**2**) was chemically grafted with a cell-adhesive model peptide, GRGDSP, resulting in RGD-*grafted* PPy-α-COOH (**3**). Grafting occurred through an amide (−NHCO−) bond between the primary amine ($-NH_2$) of the *N* terminus of the peptide and the carboxylic acid (−COOH) at the top-surface layer of **2**.[6e] HUVECs were subsequently seeded and cultured on the polymer surfaces to investigate cell adhesion and viability qualitatively and quantitatively, respectively.Two negative controls, ungrafted **2** and unmodified **1**, underwent identical cell-seeding procedures. Unmodified **1** was also exposed to an identical grafting process to test for the effects of nonspecific binding of RGD on the control surface.

Figure 3 illustrates that cells attach and spread on the **3** surface to a greater extent than on the negative (−) control **1** in which the cells remain rounded. Endothelial cells excrete RGD-containing extracellular matrix proteins such as fibronectin, to which membrane integrin proteins anchor and spread.[12] The preferential spreading on the grafted surface indicates that the endothelial cells are able to exploit the exposed RGD motif presented on the polymer surface to further facilitate their own adhesion.

Cell quantification was conducted to determine the extent of HUVEC adhesion on **3** surfaces using the MTS cell assay.[10] The viability of HUVECs was assessed on the basis of the reduction of MTS (tetrazolium salt) to a colored formazan compound by viable cells in culture. Metabolism in the viable cells produces a reducing equivalent, NADH, that passes its electron to an intermediate electron-transfer reagent that can reduce MTS to the aqueous formazan product. The concentration of the product is proportional to the number of viable cells. The absorbance of the colored formazan product at multiple time points provides a more complete understanding of how cells respond to the surface. The data in Figure 4 are the average absorbance values at 490 nm of the formazan product derived from the reduction of MTS, and the error bars reflect the standard deviation (SD) at each incubation time point. The results indicate that HUVECs had improved viability at all time points on **3** compared with that of ungrafted **2** and unmodified **1**: the absorbance at the 3 h time point for **3** (0.305 ± 0.021; $n = 3$) was 41% higher than for ungrafted **2** (0.216 ± 0.007; $n = 3$) and 88% higher than for unmodified **1** (0.162 ± 0.012; $n = 3$), which was statistically significant for both comparisons ($p < 0.05$). The 33% higher viability ($p < 0.05$) of the HUVECs on **2** compared to that on unmodified **1** can be attributed to the surface charges on the carboxylic acid-functionalized surface, which can also facilitate cell adhesion.[13] The results suggest that the conjugated RGD is the critical factor in promoting cell adhesion. The superior ability of the **3** surface to promote cell proliferation validates the utility of the carboxy-endcapped **2** as a bioactive platform. The simple functionalization approach demonstrated provides an avenue for tethering growth factors and other biologically important moieties to the conductive polymer surface.

Conclusions

Carboxy-endcapped PPy (PPy-α-COOH) was electrochemically synthesized by chemically conjugating Py-α-COOH at the surface of PPy. The introduction of the carboxylic acid functionality was verified by angle-resolved XPS and further confirmed by grafting the surface

with an RGD containing the model GRGDSP peptide, which was found to promote HUVEC adhesion and spreading. This bioactive conductive platform provides a functional surface capable of tethering biomolecules that direct cell behavior without the drawback of reduced conductivity. Numerous applications requiring immobilization techniques capable of tight and functional connections exist in the areas of tissue engineering, bioelectronics, electrode coatings, and biosensors.[14] Research is currently underway to integrate additional functional groups that would increase the versatility of the bioactive conductive platform and also to investigate the synergistic effects of modified surfaces and electrical conductivity on various cell types.

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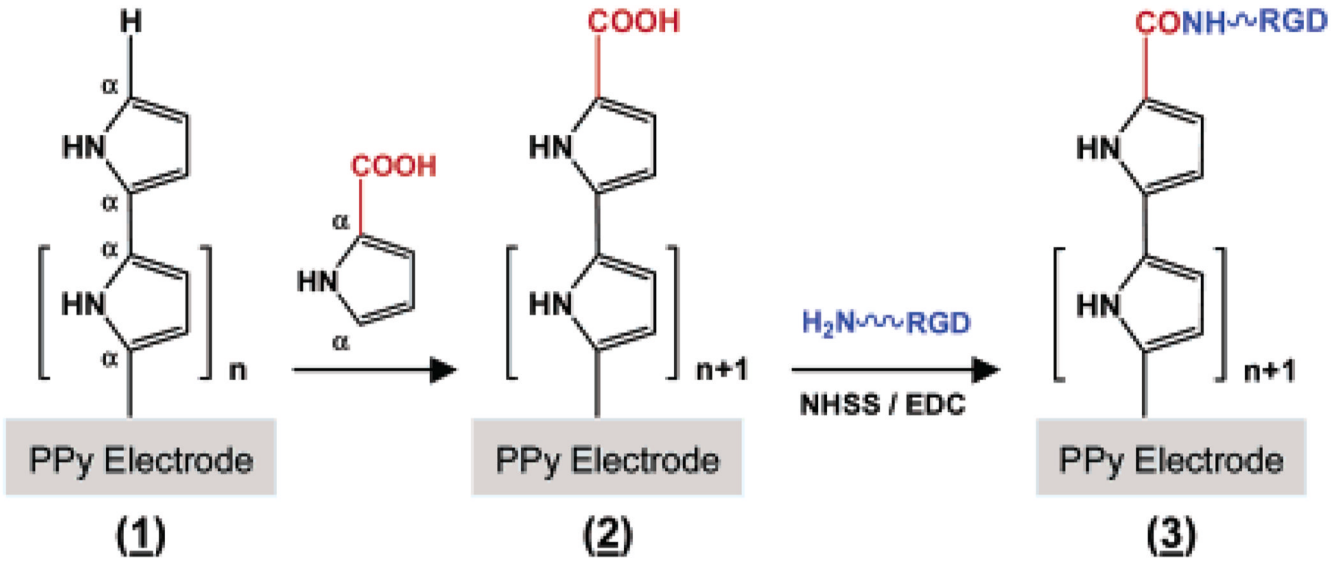

Figure 1.
Synthetic scheme of carboxy-endcapped conductive polypyrrole (PPy-α-COOH) (**2**) from polypyrrole (PPy) (**1**) and the subsequent surface modification with a cell-adhesive RGD motif to RGD-*grafted*-PPy-α-COOH (**3**).

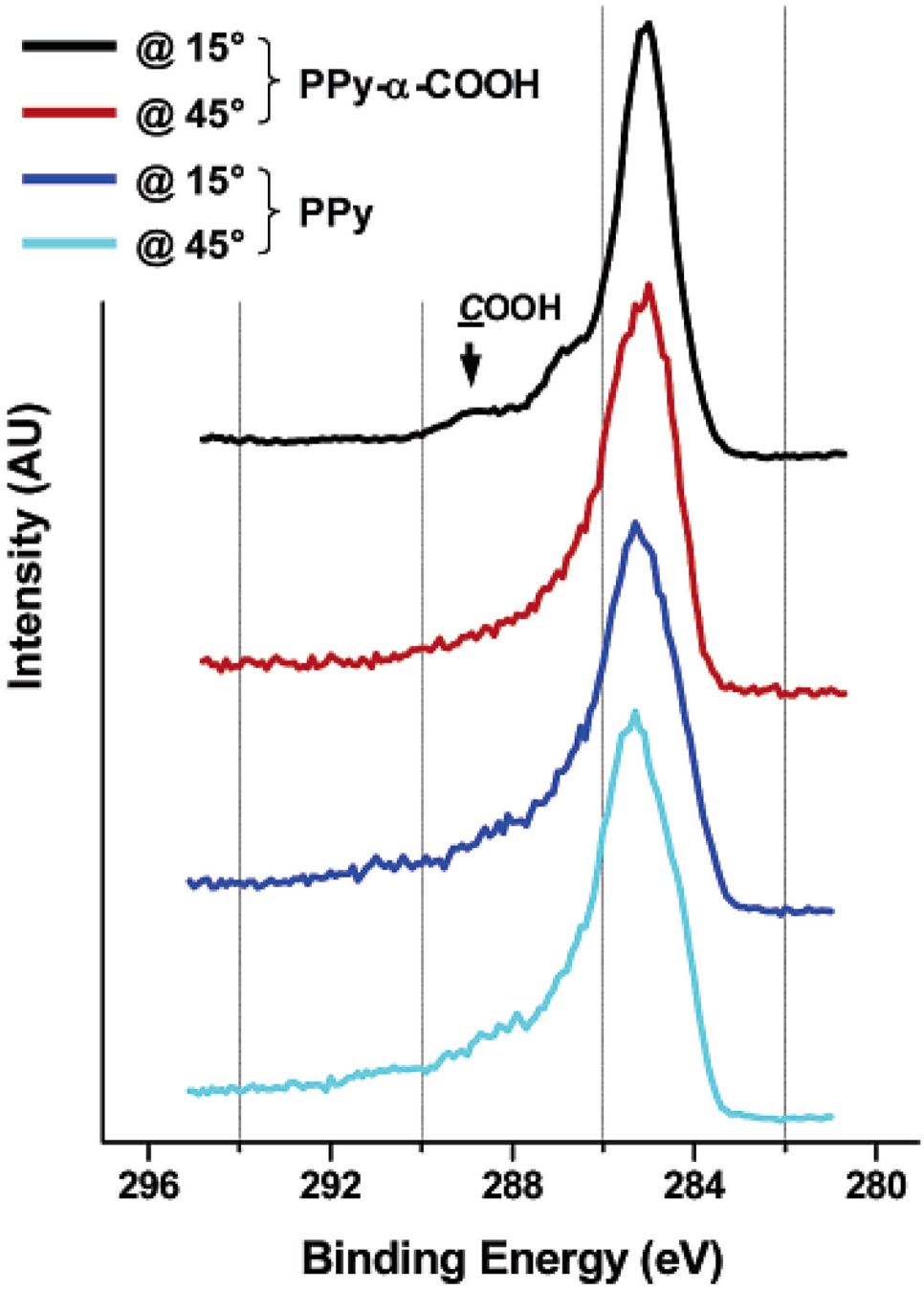

Figure 2.

High-resolution X-ray photoelectron spectroscopy (XPS) spectra relevant to C 1s envelopes of carboxy-endcapped conductive polypyrrole (PPy-α-COOH) and polypyrrole (PPy) at 15 and 45° photoelectron takeoff angles, respectively. A characteristic peak of PPy-α-COOH (black arrow) observed at 288.8 eV is assigned to the carboxylic acid (−*C*OOH) functionality.

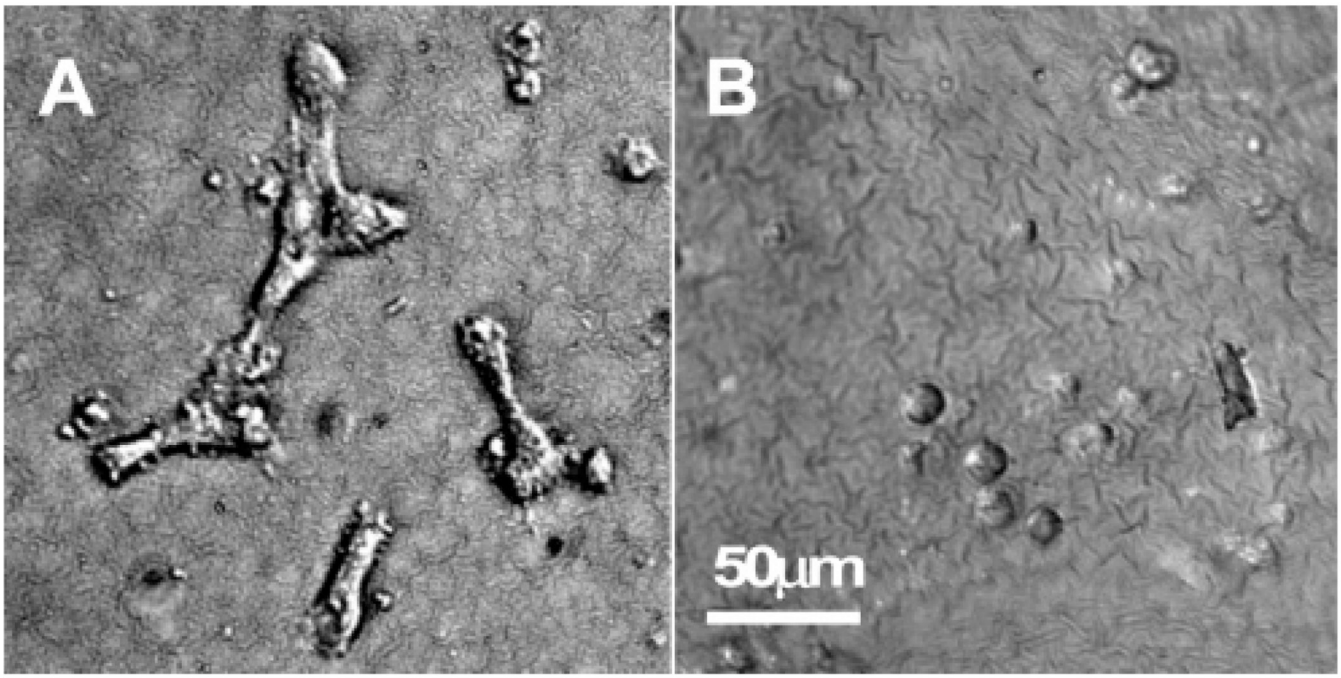

Figure 3.
Typical phase-contrast images of human umbilical vein endothelial cells (HUVECs) on theRGD-*grafted*-PPy-α-COOHfilms (A) and on the negative (−) control unmodified PPy films (B) cultured for 3 h at an initial density of 30 000 cells/cm^2.

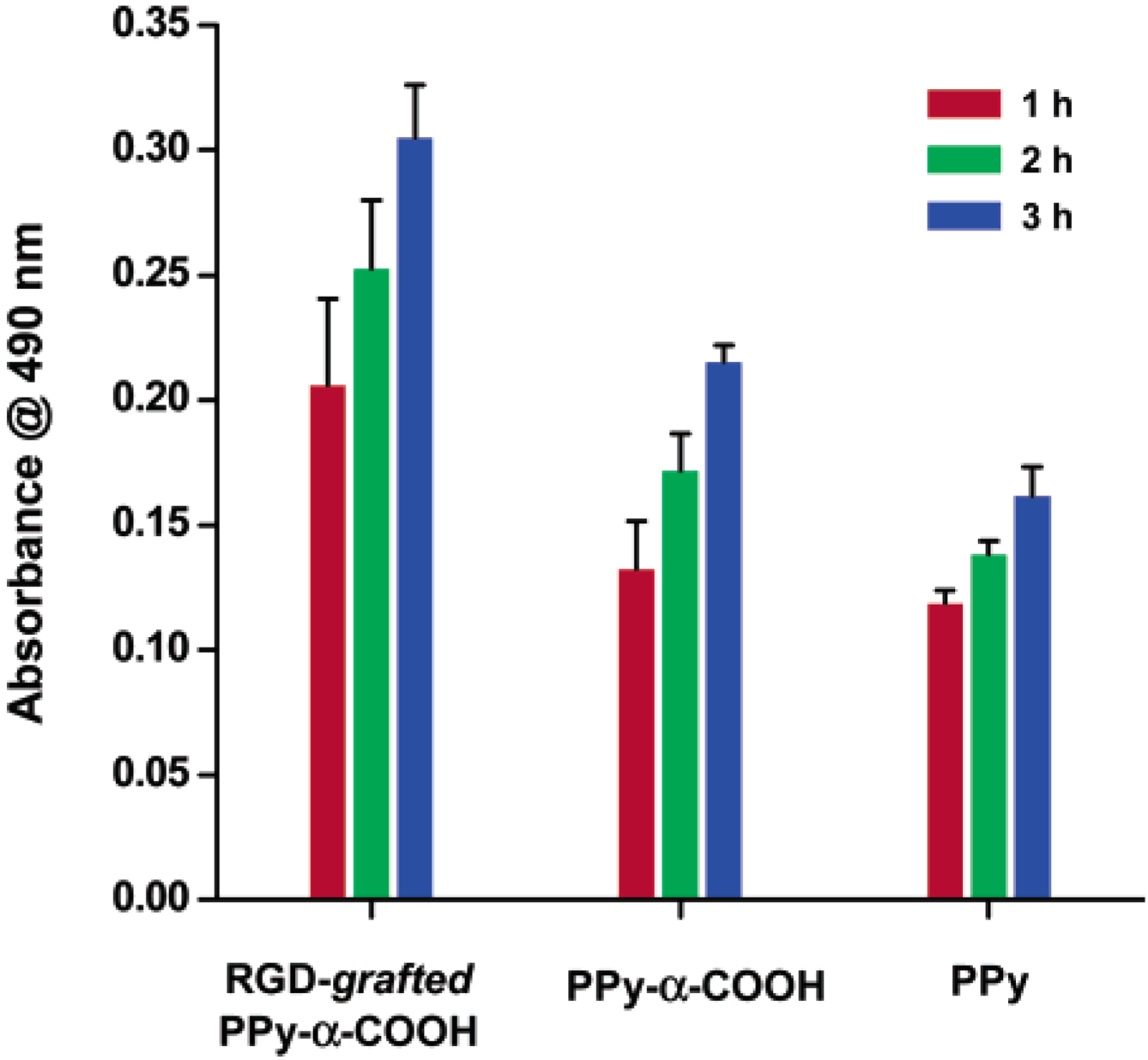

Figure 4.
HUVEC viability measured on the RGD-*grafted*-PPy-α-COOH and the negative controls, ungrafted PPy-α-COOH, and unmodified PPy as a function of incubation time using an MTS cell proliferation assay. For all samples, n = 3–5, and typically n = 3. Error bars represent the standard deviation (SD).

Table 1

Physical Properties of PPy-α-COOH and PPy Films

	sample	
property	**PPy-α-COOH**	**PPy**
doping level[a] (%, Cl/N Ratio)	30.76 ± 0.23[b] (*n* = 3)	29.86 ± 0.94 (*n* = 3)
conductivity, σ ($S \cdot cm^{-1}$)	2.78 ± 0.12[b] (*n* = 3)	2.81 ± 0.05 (*n* = 3)
thickness (nm)	243 ± 5[b] (*n* = 3)	239 ± 8 (*n* = 3)

[a] From high-resolution XPS spectra at a 45° takeoff angle.

[b] The doping level, conductivity, and thickness of PPy-α-COOH are not statistically distinguishable from those of PPy.