

ELECTROACTIVE POLYMER- PEPTIDE CONJUGATES FOR ADHESIVE BIOINTERFACES

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CHEMICAL SYNTHESIS

The synthetic procedures used to obtain the intermediates **1-5** (Figure 1) required for the preparation of protected RG^{ED} as well as their chemical characterization can be described as follow:

Methyl 2-(N-tert-butoxycarbonyl)amino-3-(3,4-ethylenedioxy-2-thienyl) propanoate (1). The synthesis of **1** was reported in reference 1.

2-(N-tert-butoxycarbonyl)amino-3-(3,4-ethylenedioxy-2-thienyl) propanoic acid (2). A 2N solution of KOH in methanol (15 mL) was added to **1** (1.44 g, 4.20 mmol) and the reaction mixture was stirred at room temperature for 2 hours. After evaporation of the solvent, the remaining residue was re-dissolved in water (15 mL), neutralized with HCl 2N and extracted with dichloromethane (3×30 mL). The combined organic layers were dried over magnesium sulphate. Evaporation of the solvent gave **2**, a colourless oil that crystallized with a mixture of hexane/ether 2:1 v/v. The resulting white solid was collected by filtration under reduced pressure and washed several times with hexane (1.37 g, 4.16 mmol, 99% yield). Mp 132–133 °C. IR (nujol) ν : 3341, 1704, 1676 cm^{-1} . ^1H NMR (CDCl_3 , 400 MHz, 60 °C): δ 1.44 (s, 9H), 3.13 (dd, 1H, $J = 15.0$ Hz, $J = 6.7$ Hz), 3.23 (dd, 1H, $J = 15.1$ Hz, $J = 5.0$ Hz), 4.16 (m, 4H), 4.49 (m, 1H), 5.29 (m, 1H), 6.17 (s, 1H), 8.45 (bs, 1H). ^{13}C NMR (CDCl_3 , 100 MHz, 60 °C): δ 28.5, 28.9, 54.4, 64.8, 64.9, 80.6, 97.8, 110.7, 139.8, 141.7, 155.7, 175.8. HRMS (ESI) $\text{C}_{14}\text{H}_{19}\text{NO}_6\text{NaS}$ $[\text{M}+\text{Na}]^+$: calcd. 352.0825, found 352.0817.

2-Amino-3-(3,4-ethylenedioxy-2-thienyl) propanoic acid hydrochloride, [HCl·H-DL-GIE-OH] (3). A 3N solution of HCl in anhydrous ethyl acetate (10 mL) was added to **2** (750 mg, 2.28 mmol), and the reaction mixture was stirred at room temperature for 1 h. After evaporation of the solvent, the residue was taken up in water and lyophilized to afford pure **3** as a white solid (604 mg, 2.28 mmol, 100% yield). Mp 223–224 °C

(dec). IR (nujol) ν : 3300–2200, 1739 cm^{-1} . ^1H NMR ($\text{DMSO}-d_6$, 300 MHz): δ 3.07 (dd, 1H, $J = 15.5$ Hz, $J = 5.8$ Hz), 3.18 (dd, 1H, $J = 15.3$ Hz, $J = 6.2$ Hz), 3.97–4.07 (m, 1H), 4.11–4.23 (m, 4H), 6.48 (s, 1H), 8.39 (m, 2H). ^{13}C NMR ($\text{DMSO}-d_6$, 75 MHz): δ 26.7, 52.6, 64.3, 64.5, 97.9, 108.0, 140.3, 141.4, 170.0. HRMS (ESI) $\text{C}_9\text{H}_{12}\text{NO}_4\text{S}$ $[\text{M}+\text{H}-\text{HCl}]^+$: calcd. 230.0482, found 230.0472.

2-(*N*-fluorenylmethoxycarbonyl)amino-3-(3,4-ethylenedioxy-2-thienyl)

propanoic acid [Fmoc-DL-GIE-OH] (4). A solution of **3** (600 mg, 2.26 mmol) in water (15 mL) was cooled at 0 °C and potassium carbonate (626 mg, 4.53 mmol) was added. Then, a solution of *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (991 mg, 2.94 mmol) in acetonitrile (45 mL) was added and the reaction mixture was stirred at 0 °C 1 hour and overnight at room temperature. The solvent was evaporated *in vacuo* and the remaining residue re-dissolved in water (15 mL) and extracted with diethyl ether (3×15 mL). The aqueous phase was neutralized with HCl 2N and extracted with dichloromethane (3×15 mL). The combined organic layers were dried over magnesium sulphate, filtered and the solvent evaporated to dryness to give **4** as a white solid (815 mg, 1.81 mmol, 80% yield). Mp 201–202 °C. IR (nujol) ν : 3303, 1695 cm^{-1} . ^1H NMR (Acetone- d_6 , 400 MHz): δ 3.05 (dd, 1H, $J = 15.0$ Hz, $J = 8.4$ Hz), 3.34 (dd, 1H, $J = 15.0$ Hz, $J = 4.6$ Hz), 4.10–4.20 (m, 4H), 4.20–4.40 (m, 3H), 4.49 (ddd, 1H, $J = 8.4$ Hz, $J = 8.4$ Hz, $J = 4.7$ Hz), 6.25 (s, 1H), 6.64 (d, 1H, $J = 8.4$ Hz), 7.26–7.41 (m, 2H), 7.54–7.60 (m, 2H), 7.71 (d, 2H, $J = 7.5$ Hz), 7.85 (d, 2H, $J = 7.5$ Hz). ^{13}C NMR (Acetone- d_6 , 100 MHz): δ 29.0, 48.1, 55.4, 65.5, 65.6, 67.3, 97.7, 112.4, 120.9, 126.3, 128.0, 128.6, 140.4, 142.2, 142.6, 145.2, 156.8, 172.8. HRMS (ESI) $\text{C}_{24}\text{H}_{21}\text{NO}_6\text{NaS}$ $[\text{M}+\text{Na}]^+$: calcd. 474.0982, found 474.0972.

Fmoc-DL-GIE-L-Asp(O^tBu)-O^tBu (5). To a solution of **4** (451 mg, 1 mmol) in dichloromethane (60 mL) cooled to 0 °C in an ice bath, was added 1-

hydroxybenzotriazole hydrate (HOBt) (184.3 mg, 1.2 mmol) and *N*-[3-(dimethylamino)-propyl]-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) (230 mg, 1.2 mmol) followed by a solution of L-aspartic acid di-*tert*-butylester (338 mg, 1.2 mmol) and NMM (0.15 ml, 1.32 mmol) in dichloromethane (10 mL) and finally NMM (0.11 ml, 1 mmol). The reaction mixture was stirred at room temperature for 24 hours. Then, the solution was washed with 5% NaHCO₃ (3×30 mL) followed by 5% KHSO₄. The organic phase was dried over magnesium sulfate and evaporated to dryness. The crude product was purified by column chromatography (eluent: hexane/ethyl acetate 7/3) to provide **5** (mixture of diastereoisomers) as a white solid (645 mg, 0.95 mmol, 95% yield).

IR (KBr) ν : 3321, 1733, 1683 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz): δ 1.42 (sm 9H), 1.44 (s, 9H), 2.58–2.75 (m, 1H), 2.82–2.91 (m, 1H), 3.04–3.22 (m, 2H), 4.04–4.18 (m, 4H), 4.18–4.25 (m, 1H), 4.29–4.49 (m, 3H), 4.59–4.70 (m, 1H), 6.62–5.73 (m, 1H), 2.16 (2s, 1H), 7.00–7.10 (m 1H), 7.27–7.34 (m, 2H), 7.36–7.44 (m, 2H), 7.52–7.61 (m, 2H), 7.72–7.79 (m, 2H). ¹³C NMR (CDCl₃, 125 MHz): δ 28.0, 28.15, 28.18, 29.3, 29.4, 37.45, 37.54, 47.3, 49.1, 49.4, 55.9, 64.7, 64.8, 67.2, 81.6, 81.7, 82.5, 97.6, 97.7, 111.2, 111.3, 120.1, 125.22, 125.24, 125.3, 127.2, 127.8, 139.31, 139.33, 141.4, 141.44, 143.9, 143.96, 144.03, 156.1, 169.4, 169.6, 170.2, 170.3, 170.4. HRMS (ESI) C₃₆H₄₂N₂O₉NaS [M+Na]⁺: calcd. 701.2503, found 701.2516.

CHARACTERIZATION

Contact profilometry. The thickness of PEDOT and conjugate films prepared in this work was determined using a Dektak 150 stylus profilometer (Veeco, Plainview, NY). Different scratches were intentionally provoked on the films and measured to allow statistical analysis of data. Imaging of the films was conducted using the following

optimized settings: tip radius= 2.5 μm ; stylus force= 1.5 mg; scan length= 1 μm ; speed= 1.5 nm/s. The thickness was measured using the average step height (ASH), which measures the difference between the average height of the polymer and the average height of the steel substrate.

FTIR spectroscopy. FTIR spectra of PEDOT and PEDOT-I were recorded on a Nicolet 6700 spectrophotometer. For this purpose, films were scrapped off from the electrode and dried under vacuum. FTIR spectra were recorded using KBr discs at 6 cm^{-1} resolution (60 scans).

X-ray photoelectron spectroscopy (XPS). XPS analyses were performed in a SPECS system equipped with a high-intensity twin-anode X-ray source XR50 of Mg/Al (1253 eV/1487 eV) operating at 150 W, placed perpendicular to the analyzer axis, and using a Phoibos 150 MCD-9 XP detector. The X-ray spot size was 650 μm . The pass energy was set to 25 and 0.1 eV for the survey and the narrow scans, respectively. Charge compensation was achieved with a combination of electron and argon ion flood guns. The energy and emission currents of the electrons were 4 eV and 0.35 mA, respectively. For the argon gun, the energy and the emission currents were 0 eV and 0.1 mA, respectively. The spectra were recorded with a pass energy of 25 eV in 0.1 eV steps at a pressure below $6 \cdot 10^{-9}$ mbar. These standard conditions of charge compensation resulted in a negative but perfectly uniform static charge. The C1s peak was used as an internal reference with a binding energy of 284.8 eV. High-resolution XPS spectra were acquired by Gaussian/Lorentzian curve fitting after S-shape background subtraction. The surface composition was determined using the manufacturer's sensitivity factors.

Standard ion chromatography. The percentage of doping ClO_4^- in PEDOT was obtained from reduction of approximately 2 mg of sample with carbon, followed by determination of the amount of chloride ions released by standard ion chromatography.

This analysis was performed with a Kontron 600 HPLC liquid chromatograph fitted with a Waters IC-Pak anion column at 30 °C and equipped with a Wescan conductimetric detector.

Scanning electron microscopy (SEM). SEM studies were performed to examine the effect of RG^{ED} on the surface morphology of PEDOT, respectively. Dried samples were placed in a Focussed Ion Beam Zeiss Neon 40 scanning electron microscope operating at 5 kV, equipped with an EDX spectroscopy system. Samples were mounted on a double-side adhesive carbon disc and sputter-coated with a thin layer of carbon to prevent sample charging problems.

Atomic force microscopy (AFM). Topographic AFM images were obtained with an AFM Dimension 3100 microscope and an AFM MultimodeTM microscope using a NanoScope IV controller (Bruker) under ambient conditions in tapping mode. The root-mean-square roughness (R_q), which is the average height deviation taken from the mean data plane, was determined using the statistical application of the NanoScope Analysis software (1.20, Veeco). AFM measurements were performed on various parts of the films, which produced reproducible images similar to those displayed in this work. The scan window size was 25×25 μm².

The AFM scratching technique was used to measure the thickness of the films. Specifically, contact mode AFM was used to intentionally scratch the films deposited on the steel substrate. Thus, the force was set to completely remove the layer in a scratch area of 0.7× 0.7 μm². After scratching, a topographic image was then obtained with tapping mode AFM and used to determine the films thickness from the depth of the scratch. It should be noted that such a topographic image was taken of a big region to accurately measure the step between the coated and the uncoated surfaces.

Wettability. Contact angle measurements were carried out using the water sessile drop method. Images of 0.5 μL distilled water drops were recorded after stabilization with the equipment OCA 15EC (Data-Physics Instruments GmbH, Filderstadt). SCA20 software was used to analyze the images and determine the contact angle value, which was obtained as the average of at least six independent measures for each sample.

Cyclic voltammetry. The electrochemical behavior of PEDOT and PEDOT- RG^{ED} was determined by cyclic voltammetry (CV) considering two different environments: films directly obtained from electropolymerization (*i.e.* as prepared samples) and films covered with cells. For this purpose, a sterilized phosphate buffer saline solution (PBS; pH = 7.2) was used as the electrolyte in the three electrode cell. All CV assays were performed with an Autolab PGSTAT302N equipped with the ECD module (Ecochimie, The Netherlands) using a three-electrode cell under a nitrogen atmosphere (99.995% in purity) at room temperature. The working compartment was filled with 50 μL of the electrolyte solution. Steel AISI 316 sheets of 1 cm^2 were used as both the working and the counter electrodes, and an Ag|AgCl electrode was used as the reference electrode which contained a KCl saturated aqueous solution (offset potential versus the standard hydrogen electrode, $E^0 = 0.222 \text{ V}$ at 25 $^{\circ}\text{C}$). All potentials given in this report are referenced to this electrode. Cyclic voltammograms were registered at a scan rate of 50 mV/s in the potential range from -0.4 to $+0.8 \text{ V}$.

The electroactivity, which indicates the ability to exchange charge reversibly, was evaluated by examining the similarity between the anodic and cathodic areas of the control voltammogram. The electrochemical stability (*i.e.* loss of electroactivity, LEA), which decreases with the oxidation and reduction areas of consecutive control voltammograms, was determined using the following expression:

$$LEA = \frac{\Delta Q}{Q_{ii}} 100 \quad (1)$$

where ΔQ is the difference of voltammetric charge between the second cycle and the last cycle and Q_{ii} is the voltammetric charge corresponding to the second cycle. In this work all values of LEA were referred to 10 consecutive oxidation-reduction cycles.

Conformational analyses. Density Functional Theory (DFT) calculations at the B3LYP/6-31+G(d,p) level were performed in aqueous solution. The solvent was described as a dielectric medium using a well established Self-Consistent Reaction Field (SCRF), that is, the Polarizable Continuum Model (PCM) of Tomasi and co-workers.²

The conformational potential energy surface of the RG^{ED} peptide was systematically explored using a procedure inspired in the build-up method early developed by Scheraga and coworkers.³ This approach is based on the assumption that short-range interactions play a dominant role in determining the conformation of a given peptide. Accordingly, the accessible conformations of a given peptide result from the combination of N independent rotamers (*i.e.*, the rotational isomeric approximation⁴), in which each independent rotational state corresponds to the most favored conformations of each residue. In practice, accessible starting geometries of RG^{ED} were constructed by combining all the minima identified for each of the three involved residues (*i.e.* Arg, Glu and Asp). Specifically, the minima of Glu and Asp were taken from the literature^{5,6} while those of Arg were explicitly calculated in this work. For this purpose, starting geometries for calculations on Arg were constructed using the minima previously obtained for *homo*-Arginine, that is, the Arg homologue containing one more methylene unit.⁷

Starting from the five conformers computed with PCM(water)-B3LYP/6-31++G(d,p) and lying in a 5 kcal/mol energetic gap, a re-optimization has been performed in the

experimental condition (in acetonitrile) using a PCM(acetonitrile)- ω B97XD/6-311++G(d,p) level of theory that has already shown satisfying results to study conformational preferences of dipeptides⁸ and EDOT residues⁵. No negative frequencies were detected. For each conformer the forty first excited states were evaluated with the CAM-B3LYP/6-311++G(d,p) level of theory and the electronic density difference (EDD) between the excited state and the ground state have been plotted to qualitatively understand the variation of the maximum absorption wavelength (λ_{max}) depending on the conformation. All Quantum mechanics computations have been realized with Gaussian09 software.⁹

UV-vis spectroscopy. UV-vis absorption spectra were obtained using a UV-vis-NIR Shimadzu 3600 spectrophotometer equipped with a tungsten halogen visible source, a deuterium arc UV source, a photomultiplier tube UV-vis detector, and a InGaAs photodiode and cooled PbS photocell NIR detectors. Spectra were recorded in the absorbance mode using the integrating sphere accessory (model ISR-3100), the range wavelength being 200-900 nm. The interior of the integrating sphere was coated with a highly diffuse BaSO₄ reflectance standard. Single-scan spectra were recorded at a scan speed of 60 nm·min⁻¹. Measurements, data collection and data evaluation were controlled by the computer software UVProbe version 2.31.

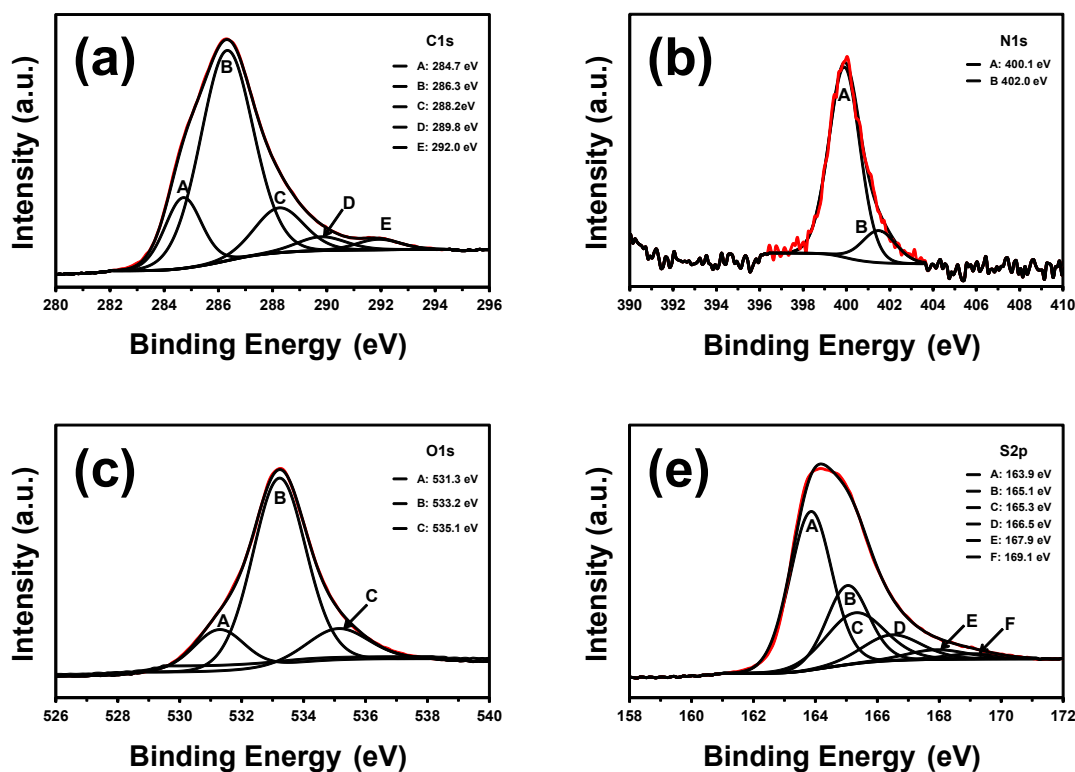


Figure S1. (a) High resolution XPS spectra and peaks from deconvolution for PEDOT-RG^{ED}/A: (a) C1s region, where the five Gaussian curves have been attributed to saturated and conjugated C–C (284.7 eV) and C=C–O (286.3 eV) bonds of PEDOT chains, the C=O of amide (288.2 eV), the C–N bonds of the Arg guanidinium group (289.8 eV) and the C–F₃ component of residual TFA molecules (292.0 eV) arising from the deprotection step in the synthesis; (b) N1s region, where the peak centered at 400.1 eV has been attributed to the guanidinium and amide groups while the peak at 402.0 eV corresponds to impurities of the substrate; (c) O1s region, where the three Gaussian curves have been attributed to the carboxylate groups in the conjugated RG^{ED} peptide (531.3 eV), the C–O–C bond in the ethylene bridge of PEDOT (533.2 eV) and C=O of contamination products produced during anodic polymerization (535.1 eV); and (d) S2p region, where the Gaussian curves have been assigned to the spin-split sulfur coupling S2p_{3/2} and S2p_{1/2} for the C–S–C bond of the thiophene ring (163.9 and 165.1 eV, respectively) in PEDOT, its homologous with positively charged sulfur (*i.e.* C–S⁺–C at 167.9 and 169.1, respectively), and the C–S–C bond of the side chain of the GIE residue in the peptide (165.3 and 166.5 eV, respectively).

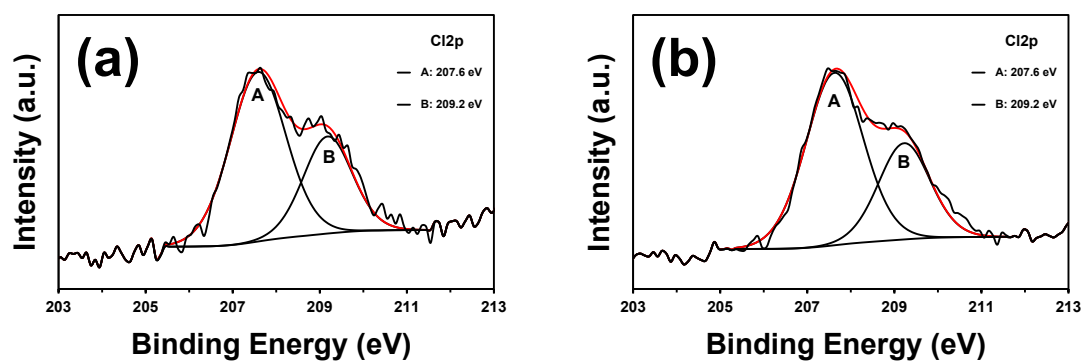


Figure S2. (a) High resolution XPS spectra in the Cl2p region and peaks from deconvolution for: (a) PEDOT-RG^{ED}/A and (b) PEDOT-RG^{ED}/B.

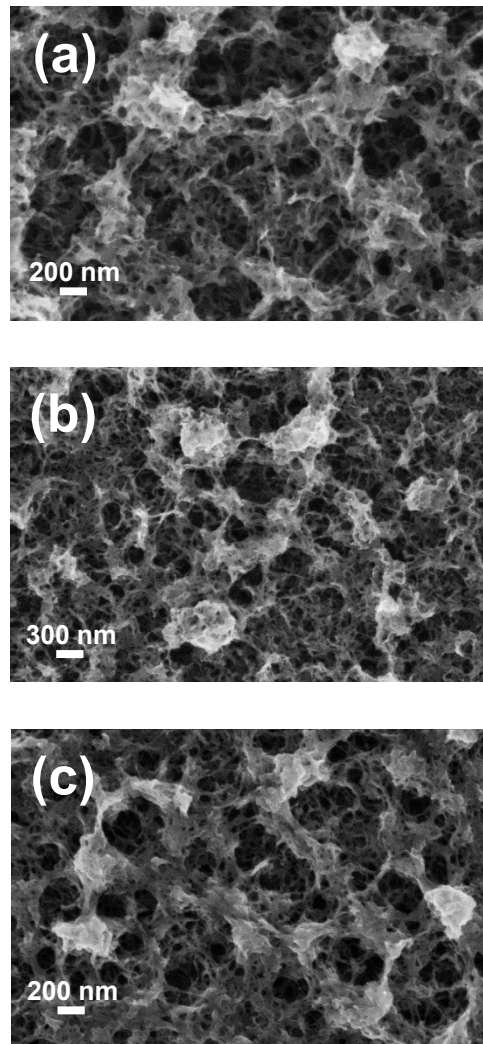


Figure S3. SEM micrographs of: (a) as prepared PEDOT; (b) PEDOT treated with 1:1 TFA:DCM by 2 hours and, subsequently, washed with DCM, emulating the step used to eliminate the protection of peptide fragments in PEDOT-(protected RG^{ED}) systems; and (c) PEDOT-RG^{ED}/A.

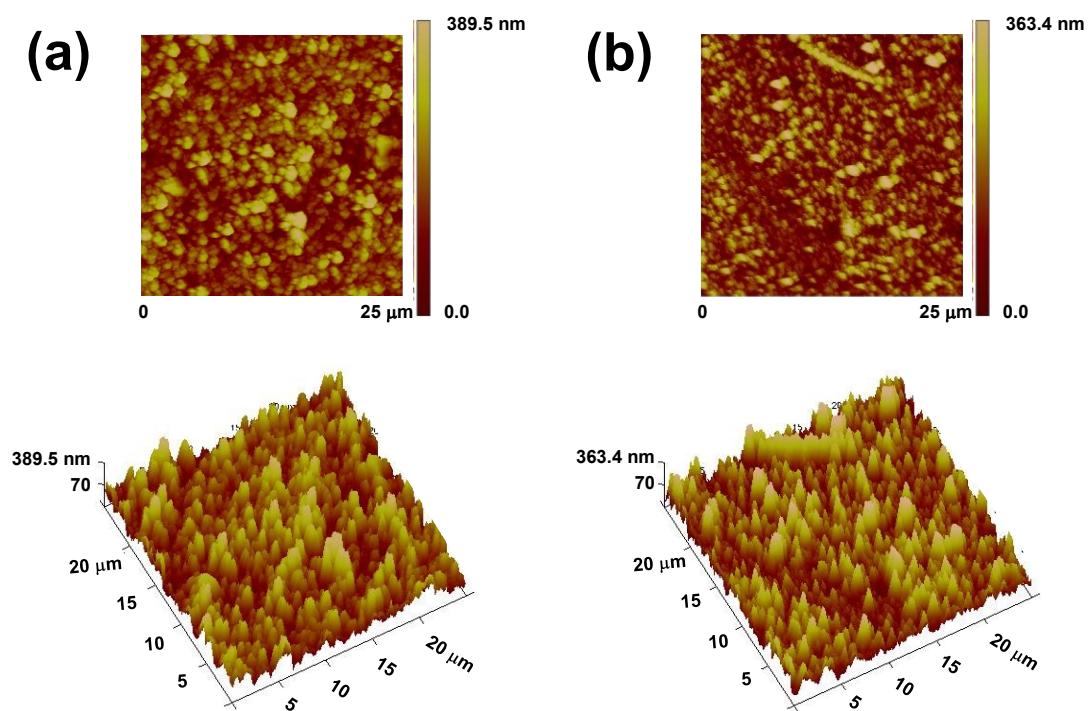


Figure S4. 2D and 3D topographical AFM images: (a) as prepared PEDOT; and (b) PEDOT-RG^{ED}/A.

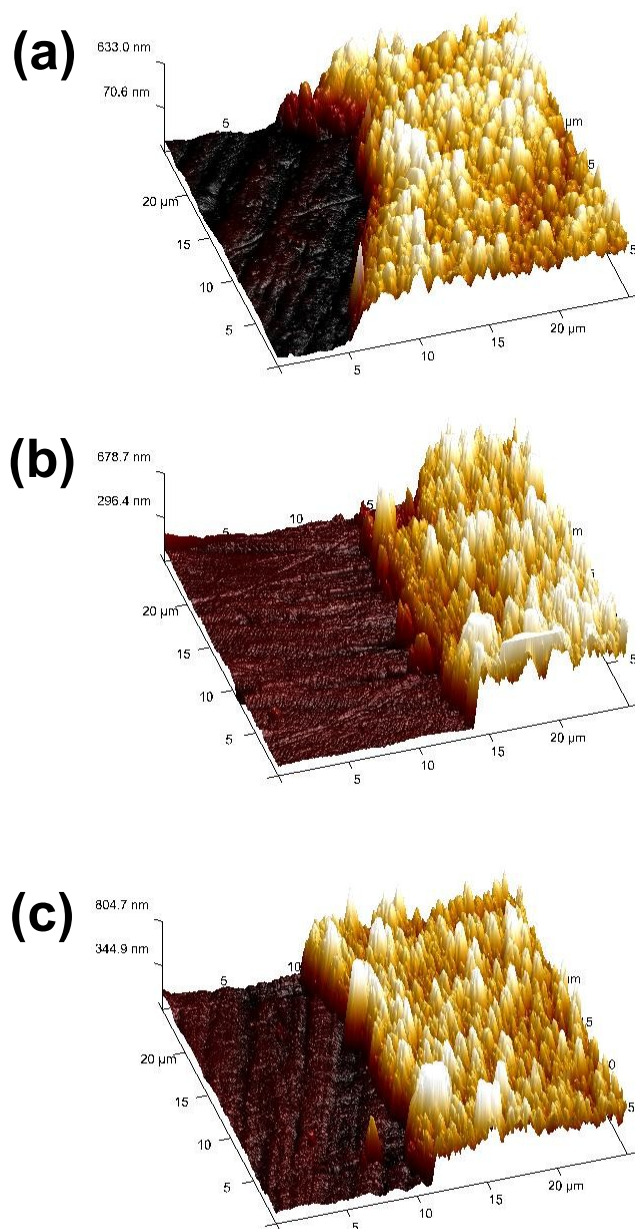


Figure S5. Determination of the thickness by scratch AFM for: (a) as prepared PEDOT; (b) PEDOT-RG^{ED}/A; and (c) PEDOT-RG^{ED}/B.

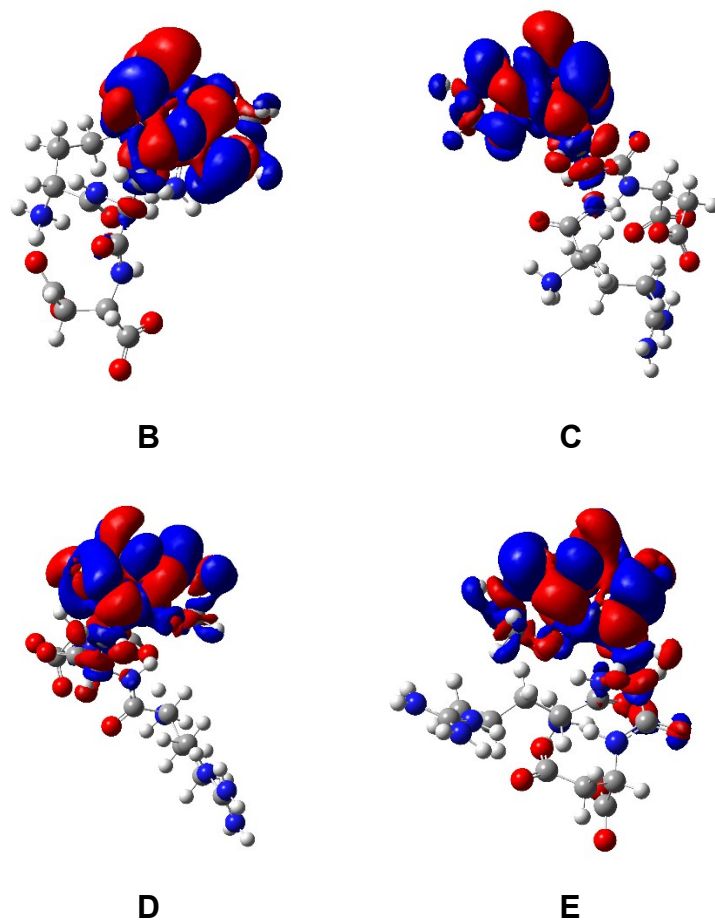


Figure S6. Electronic density difference (excited - ground) corresponding to the first and second electronic transition ($\lambda^{(1)}_{\text{max}}$) of conformation B-E. Blue (red) regions indicate an increase (decrease) of electron density upon electronic transition.

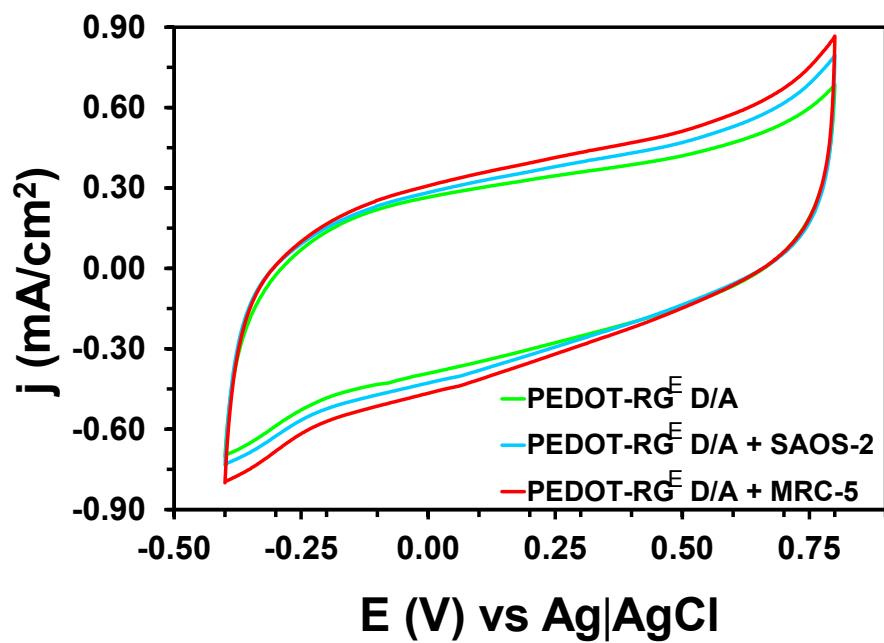


Figure S7. Control voltammograms in PBS of PEDOT-RG^ED/A uncoated and coated with Saos-2 and MRC-5 cells.

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