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ELECTRONIC SUPPORTING INFORMATION

Electroactive and bioactive films of random copolymers containing terthiophene, carboxyl and Schiff base functionalities in the main chain

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METHODS

Materials. 2-Thiophenecarboxaldehyde (Aldrich, 98%) was distilled under reduced pressure before use. Benzene was dried over sodium wire and distilled before use. *p*-Toluenesulfonic acid (APTS9) (99%), and 3,5-diaminobenzoic acid (98%), Th_3 (99%), tetrabutylammonium tetrafluoroborate (TBATFB) (99.0%,) were purchased from Aldrich and used as received. The rest of the solvents were purchased from Panreac Quimica S.A.U. (Spain) and used as received.

For cell culture experiments, MG-63 cell line human and monkey kidney epithelial (Vero) cell line were purchased from ATCC (USA). Dulbecco's phosphate buffered saline solution (PBS) without calcium chloride and magnesium chloride, Dulbecco's modified Eagle's medium (DMEM, with 4500 mg of glucose/L, 110 mg of sodium pyruvate/L and 2 mM L-glutamine), penicillin-streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%) and trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA) were all purchased from Sigma-Aldrich (USA). Fetal bovine serum (FBS) and trypan blue stain (0.4%) were purchased from Gibco, UK. Dimethyl sulfoxide (99.0%) was purchased from Panreac Quimica S.A.U. (Spain). Phalloidin-TRITC (for F-actin staining; 1:100 in 0.1% Bovine Serum Albumin (BSA)/PBS) and Hoechst 33342 (1:2000 in 0.1% BSA/PBS) was purchased from Sigma Chemical.

Synthesis of the azomethine-containig bis-thienyl (AzbT) monomer: 3,5-Bis{[2-thienylmethylene]amino}benzoic acid. A 250 mL three necks round bottom flask equipped with a condenser, a Dean-Stark trap, nitrogen inlet-outlet and magnetic stirrer was charged with 2-thiophene-carboxaldehyde (5.68 mL; 0.062 mol), 3,5-diaminobenzoic acid (4.56 g, 0.03 mol), *p*-toluenesulfonic acid (0.015 g) and benzene (150 mL). Nitrogen was purged through the reaction mixture for 15 minutes. The mixture was heated to reflux with stirring for 15 hours, continuously removing the

benzene-water azeotrope. After cooling the reaction mixture, the formed solid product was filtered, dried, and purified by precipitation from acetone in toluene and filtration. Further purification was achieved by using the column chromatography method (aluminum oxide - Fluka Type 507c) and 1,2-dichloroethane as eluent. The solution was concentrated by evaporation under reduced pressure, and the solid obtained after cooling was filtrated and dried. An orange-brownish solid was obtained. Yield: 38%; m.p.: 176-178 ^{o}C.

Chemical characterization of the AzbT monomer. ^{1}H-NMR spectra were recorded at room temperature on a Bruker Avance DRX-400 spectrometer (400 MHz), using DMSO-d_6 as solvent. Chemical shifts are reported in ppm and referenced to tetramethylsilane (TMS) as internal standard.

^{13}C-NMR and 2D HMQC (Heteronuclear Multiple-Quantum Correlation) spectra of AzbT were recorded in DMSO-d_6 concentrated solution, at room temperature, at 100.61MHz on a Bruker Avance DRX Spectrometer equipped with a 5mm QNP direct detection probe and z-gradients. The chemical shifts are reported as δ values (ppm) relative to the residual peak of the solvent. The assignment of all the signals in the 1D NMR spectrum was performed using the 2D H,C- HMQC spectrum that was recorded at 100.61MHz by using a sweep width of 6.41KHz and 8 scans with 1K data points.

The melting point of the synthesized monomer was determined by using Melt-Temp II (USA Laboratory Devices). FTIR spectra of the synthesized monomer and the starting chemicals were recorded on a Bruker Vertex 70 FTIR spectrometer equipped with a diamond ATR device (Golden Gate, Bruker) in transmission mode, by using KBr pellets. Measurements of UV-vis absorption and fluorescence emission of the synthesized monomer dissolved in DMSO (1×10^{-3} M) were carried out by using a

Specord 200 Analytik Jena spectrophotometer and Perkin Elmer LS 55 apparatus, respectively.

Electropolymerization. P(AzbT-*co*-Th_3) and PTh_3 films were prepared by chronoamperometry (CA) using an Autolab PGSTAT302N equipped with the ECD module (Ecochimie, The Netherlands). Polymerization was carried out in a standard three-electrode one-compartment cell at room temperature. ITO substrates of approximately 1.0 × 0.5 cm^2 were used as working electrodes, while Pt sheets of the same area were used as counter electrode. The reference electrode was an Ag|AgCl electrode containing KCl saturated aqueous solution (E^o = 0.222 V at 25 ºC).

Stock solutions of Th_3 and AzbT monomers (2 mM) were prepared in acetronitrile containing 0.1 M TBATFB as dopant agent. For the preparation of PTh_3 films, the cell was filled with 5 mL of the 2 mM Th_3 solution. Polymerization was carried out applying a constant potential of 1.0 V during a polymerization time of θ = 150 seconds. P(AzbT-*co*-Th_3) films were prepared considering 50:50, 60:40 and 80:20 AzbT:Th_3 molar ratios. For this purpose, the cell was filled with the corresponding monomer solution prepared after mixing the appropriate amount of each stock solution. The applied potential was varied with the copolymer composition (1.05 V for PTh_3, the 50:50 and 60:40 copolymers; and 1.15V was used for the 80:20 copolymer), while the polymerization time was θ = 180 seconds in all cases. It should be noted that films obtained using $\theta \geq 300$ s were extremely brittle and peeled off easily from the ITO electrodes. As the behavior of the 50:50 and 60:40 copolymers was found to be very similar, many of the results obtained for the latter have been reported in the Electronic Supporting Information (ESI).

X-Ray photoelectron spectroscopy (XPS). XPS analyses were performed in a SPECS system equipped with a non-monochromated twin anode X-ray source XR50 of Mg/Al

(1253 eV/1487 eV). Specifically, the Al anode was operated at 150 W. The pass energy was set to 25 and 0.1 eV for the survey and the narrow scans, respectively. The C 1s peak was used as an internal reference with a binding energy of 284.5 eV. High-resolution XPS spectra were acquired by Gaussian–Lorentzian curve fitting after S-shape background subtraction.

FTIR spectra of electropolymerized polymers were recorded on a FTIR Nicolet 6700 spectrophotometer equipped with the Smart SAGA accesory, which is designed for the analysis of thin films on reflective substrates. As samples were adhered onto ITO substrates, gold-coated glass was placed as reflective substrate on top. Thus, background spectrum was collected using uncoated ITO. Samples were placed on the plate using the 5 mm aperture, and 64 scans were performed between 4000 and 600 cm^{-1} with a resolution of 2 cm^{-1}.

Optical profilometry. The thickness of the electropolymerized films was determined using a surface profilometer Dektak 150 (Veeco). Several scratches (minimum 6) were intentionally made throughout the surface of the polymer samples (n=4), and the step at several positions along the scratches was measured by the computer software Dektatk (version 9.2, Veeco Instruments Inc.) to allow statistical analysis of data.

Scanning electron microscopy (SEM). The morphology of the prepared films was examined by SEM using a Focused Ion Beam Zeiss Neon40 scanning electron microscope equipped with an energy dispersive X-ray (EDX) spectroscopy system and operating at 5 kV. All samples were sputter-coated with a thin carbon layer using a K950X Turbo Evaporator to prevent electron charging problems. Prior to SEM observation, samples covered with cells were fixed in a 2.5% glutaraldehyde PBS solution (pH= 7.2) overnight at 4 ºC. Then, they were dehydrated by washing in an

alcohol battery (30º; 50º; 70º; 90º; 95° and 100º) at 4 ºC for 30 min per wash and air-dried.

Atomic Force Microscopy (AFM). AFM was conducted to obtain topographic images of the films surface using a silicon TAP 150-G probe (Budget Sensors, Bulgaria) with a frequency of 150 kHz and a force constant of 5 N/m. Images were obtained with a AFM Dimension microscope using a NanoScope IV controller under ambient conditions in tapping mode. The row scanning frequency was set between 0.6 and 0.8 Hz. The Root Mean Square roughness (RMS Rq), 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).

Similarly to the procedure adopted during the profilometry technique, a scratch was intentionally made throughout the surface of the polymer. After scratching, a topographic image was then obtained with tapping mode AFM and used to determine the film thickness from the depth of the scratch.

Electrochemical properties. The electroactivity (charge storage ability) and electrostability (loss of electroactivity with consecutive oxidation-reduction cycles) of PTh_3 and P(AzbT-*co*-Th_3) films were determined by cyclic voltammetry (CV) assays. Ten consecutive oxidation-reduction cycles were conducted in acetonitrile containing 0.1 M of TBATFB from -0.0 to 1.1 V at scan rates of 25, 50 and 100 mV/s. The electrostability (LEA, %) was evaluated as:

$$\mathrm{LEA} = \frac{\Delta Q}{Q_I} \cdot 100 \qquad (1)$$

where ΔQ is the difference between the oxidation charge (in C) of the first and the last cycle, and Q_I is the oxidation charge corresponding to the first cycle.

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 of PTh_3 and P(AzbT-*co*-Th_3) films deposited onto ITO were recorded between 300 and 800 nm in the absorbance mode using the integrating sphere accessory (model ISR-3100). The interior of the integrating sphere was coated with highly diffuse BaO reflectance standard. Measurements, data collection and data evaluation were controlled by the computer software UVProbe version 2.31.

Cytotoxicity, cellular adhesion and cellular proliferation. MG-63 and Vero cells were cultured in DMEM high glucose supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). The cultures were maintained in a humidified incubator with an atmosphere of 5% CO_2 and 95% O_2 at 37ºC. Culture media was changed every two days. When the cells reached 80-90% of confluence, they were detached using 1-2 mL of trypsin (0.25% trypsin/EDTA) for 5 min at 37 °C. Finally, cells were re-suspended in 5 mL of fresh medium, and their concentration was determined by counting with a Neubauer camera using 0.4% trypan blue as a vital dye.

PTh_3 and P(AzbT-*co*-Th_3) films deposited onto ITO were placed in plates of 24 wells and sterilized using UV-light for 15 min in a laminar flux cabinet. For adhesion assays, an aliquot of 50 μL containing 5×10^4 cells was deposited onto each polymer surface. Then, cell attachment was promoted by incubating under culture conditions for 30 min. Finally, 500 μL of the culture medium were added to each well. After 24 h, non-attached cells were washed out, while attached cells were quantified. For proliferation assays, the 50 μL aliquots deposited on each well contained 2×10^4 cells. Quantification of proliferated cells was performed after 7 days of culture. Controls were simultaneously performed by culturing cells on the surface of the tissue culture polystyrene (TCPS) plates and uncoated ITO sheets. Cytotoxicity was also determined

after 24 h and 7 days of culture. All viability measures were relative to the TCPS control (*i.e.* 100%).

Viability for cytotoxicity, cellular adhesion and cellular proliferation were evaluated by the colorimetric MTT assay. This assay measures the ability of the mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the MTT and form formazan crystals, which are impermeable to cell membranes and, therefore, are accumulated in healthy cells. This process is detected by a color change: the characteristic pale yellow of MTT transforms into the dark-blue of formazan crystals. Specifically, 50 µL of MTT solution (5 mg/mL in PBS) were added to each well. After 3 h of incubation, samples were washed twice with PBS and stored in clean wells. In order to dissolve formazan crystals, 1 mL of DMSO/methanol/water (70/20/10 % v/v) was added. Finally, the absorbance at 540 mm was measured using a UV-vis spectrophotomether (UV-3600, Shimadzu). The resulting viability results were normalized to TCP control as relative percentages. Results were derived from the average of four replicates (n= 4) for each independent experiment. ANOVA and Tukey tests were performed to determine statistical significance, which was considered at a confidence level of 95 % ($p < 0.05$).

Fluorescence microscopy. The protocol for the immunofluorescence staining study was as follows: MG-63 and Vero cells cultured onto materials for 7 days were fixed in 2.5 % paraformaldehyde/PBS during 15 min. Then, samples were washed with PBS, and a permeabilizing buffer (0.1% Triton X-100 in 1% BSA/PBS) was added for 5 min. Next, samples were incubated for 5 min in 1% BSA/PBS and washed with 0.1% BSA/PBS for other 5 min. After this, Phalloidin-TRITC was sequentially added for 1h. Then, samples were washed twice in 0.1% BSA/PBS for 5 min, and Hoechst 33342 (1:2000 in 0.1% BSA/PBS) was added for 10 min to stain the nucleus of the cells

Samples were washed one more time before air-drying for 24 h. After that period of time, samples were ready for epifluorescence microscopy observations. The entire fluorescent staining protocol was performed at room temperature.

Fluorescence microscope (BA410 Model, Motic Spain S.L.) was used to examine the fluorescently-labelled cells onto the material substrates. Hoechst and Phalloidine images were acquired sequentially. The final analysis of the images was performed using the Image-J software.

DISCUSSION

Characterization of the AzbT monomer. Figure S1 compares the FTIR spectrum of the synthesized monomer with that of 3,5-diaminobenzoic acid. The presence of the thienyl rings in the AzbT monomer is confirmed by the following characteristic peaks: 3097 cm^{-1} (aromatic α-CH stretching); 3073 cm^{-1}(aromatic β-CH stretching); 1450 cm^{-1} (symmetric C=C stretching vibration); 836 cm^{-1} (ring β(C-H) out-of-plane bending vibration); 762 cm^{-1} (out-of-plane α(C-H) ring deformation); 710 cm^{-1} (ν C–S); 572 cm^{-1} (γ ring deformation); and 473 cm^{-1} (C–S–C ring deformation). Moreover, absorption peaks attributed to the vibration of NH_2 functional groups, which are present in the IR spectrum of starting 3,5-diaminobenzoic acid, disappear in the spectrum of the AzbT monomer. Specifically, these peaks are observed at 3339 cm^{-1} (asymmetric ν N–H), 3216 cm^{-1} (symmetric ν N–H) and 1352 cm^{-1}(ν C–N). For the new-formed azomethine bond, a shoulder at 1638 cm^{-1} is discernible. A shallow peak centered at 1910 cm^{-1} appeared in the spectrum of AzbT that could be attributable to the 1, 3, 5-trisubstituted benezene ring.[1] Finally, the C=O stretching vibration of the carboxylic function characteristic of dimerized benzoic acids appears at 1689 cm^{-1} in both spectra.

The structure of the synthesized monomer was also investigated by ^{1}H-NMR spectroscopy (Figure 1). As it can be seen, a multiplet corresponding to 9 protons from the thiophene (Th) rings as well as from the trisubstituted benzene ring is present in the interval comprised between 6.8 and 7.8 ppm. Two singlets at 8.75 ppm and at 8.9 ppm can be assigned to the *syn*- and *anti*-isomers of the azomethine group.[2,3] Protons from the carboxylic function (**g** in Figure 1) appear as a broad signal (most probably due to the presence of water in the solvent used during registration) centered at 12.75 ppm.

Polymerizartion of AzbT. Even though a number of different experimental conditions were tested for the electropolymerization of AzbT alone (*i.e.* varying the solvent, electrolyte, electrode and/or potential), no polymer was obtained. For this behaviour of AzbT in the given conditions several explanations could be possible. The first one is related to its structural peculiarities (related to the presence of carboxyl as electron-withdrawing group and to the conjugated short chain-like structure) that could hinder electrochemical polymerization by a high oxidation potential. The impossibility or difficulty of electropolymerizing monomers of high oxidation potentials has been attributed to the high reactivity of the corresponding radicals, which can thus undergo rapid reactions with the solvent or anions to form soluble products rather than to electropolymerize.[4] Moreover, the anodic polymerization of thiophenes with reactive functional groups (*e.g.* -NH_2, -OH and –COOH) has been reported to be difficult due to their substantial nucleophilicity, which allow the functional groups to attack on the radical cation intermediates formed during electropolymerization, hence inhibiting the polymerization process.[5]

On the other hand, in acetonitrile–TBATFB mixture, during the electropolymerization process, a protonation of this medium could take place most probably by ionic dissociation of the carboxyl group. Even if both the dielectric constant and the acceptor number of acetonitrile have lower values as those of water, the presence of the acidic protons in the medium could be possible in a similar fashion as that observed for films of poly(3-thiophene acetic acid).[6] In our case, the ionization process could be favored and enhanced by the presence in the medium of TBATFB electrolyte. Into an organic medium of a higher acidity, the azomethine linkages can undergo a dynamical exchange that possibly could compete with the polymerization reaction.[7]

Variation of the optical properties with the polymerization time. By analysing Figure S2, which compares the copolymers film UV-vis spectra obtained by polymerization at different time intervals (θ = 50, 100, 200 and 400 s) and at their corresponding polymerization potential (*i.e.* 1.0, 1.05 or 1.15 V, depending on the composition), it can be concluded that the estimated optical π-π^* lowest transition energy (E_g) values (Table S1) for films of P(AzbT-*co*-Th_3) copolymers do not vary considerable with the polymerization time. On the contrary, E_g values for PTh_3 films fluctuate, this phenomenon being an indication that the polymerization time affects the π-conjugation length of the polymeric chains in a greater extent for this system. However, P(AzbT-*co*-Th_3) and PTh_3 films obtained using $\theta \geq$ 300 s were extremely fragile and peeled off easily from the ITO electrodes in comparison with those produced using θ comprised between 150 and 200 s.

Reduction of the doping level in copolymers derived from 50:50 and 60:40 AzbT:Th_3 compositions. These phenomena could take place based on the following two possible scenarios. First, AzbT hiders the electropolymerization of Th_3 which is consistent with the reduction of Q_{pol}, and thus promotes the formation of α-β and β-β linkages between the Th rings of already and newly incorporated monomers with respect to the formation of α-α linkages. Therefore, it can be assumed that the 50:50 and 60:40 copolymers are mostly arranged in a closed cross-linked structure and the formation of long linear Th-chains seems to be unfavorable. Consequently, the penetration of the dopant BF_4^- into such structure is hampered, thus explaining the low doping level. Second, the ionized form of the AzbT (*i.e.* AzbT-COO^-) present in the medium could work as a doping agent in competition with BF_4^-. Such behaviour could be favoured by the structural similarity existing between the growing polymeric chains

and AzbT-COO^-. In addition to that, the π-π stacking interaction between both species in solution could also enhance such conduct. Also, while BF_4^- doping is a conventional diffusion-controlled slow process, "*rapid doping*" could be achieved by the release of protons from the ionizable carboxyl function of AzbT, resulting in so-called "*self- or autodoping polymers*".[2] All of these trends could have as a result the experimentally observed decreased value of DL in relation with BF_4^-.

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Table S1. Optical properties of PTh3 and P(AzbT-co-Th3) copolymers electropolymerized during θ = 50, 100, 200 and 400 seconds obtained from UV-vis spectra displayed in Figure 6S: onset wavelength (λ_{onset}, in nm) and π-π* transition lowest transition energy (E_g, in eV).

θ (seconds)	**PTh_3**		**P(AzbT-*co*-Th_3)**					
			50:50		**60:40**		**80:20**	
	λ_{onset}	**E_g**	**λ_{onset}**	**E_g**	**λ_{onset}**	**E_g**	**λ_{onset}**	**E_g**
50	685	1.81	803	1.54	667	1.86	685	1.81
100	914	1.36	678	1.83	632	1.96	637	1.95
200	764	1.62	673	1.84	690	1.80	635	1.95
400	892	1.39	697	1.78	648	1.91	654	1.90

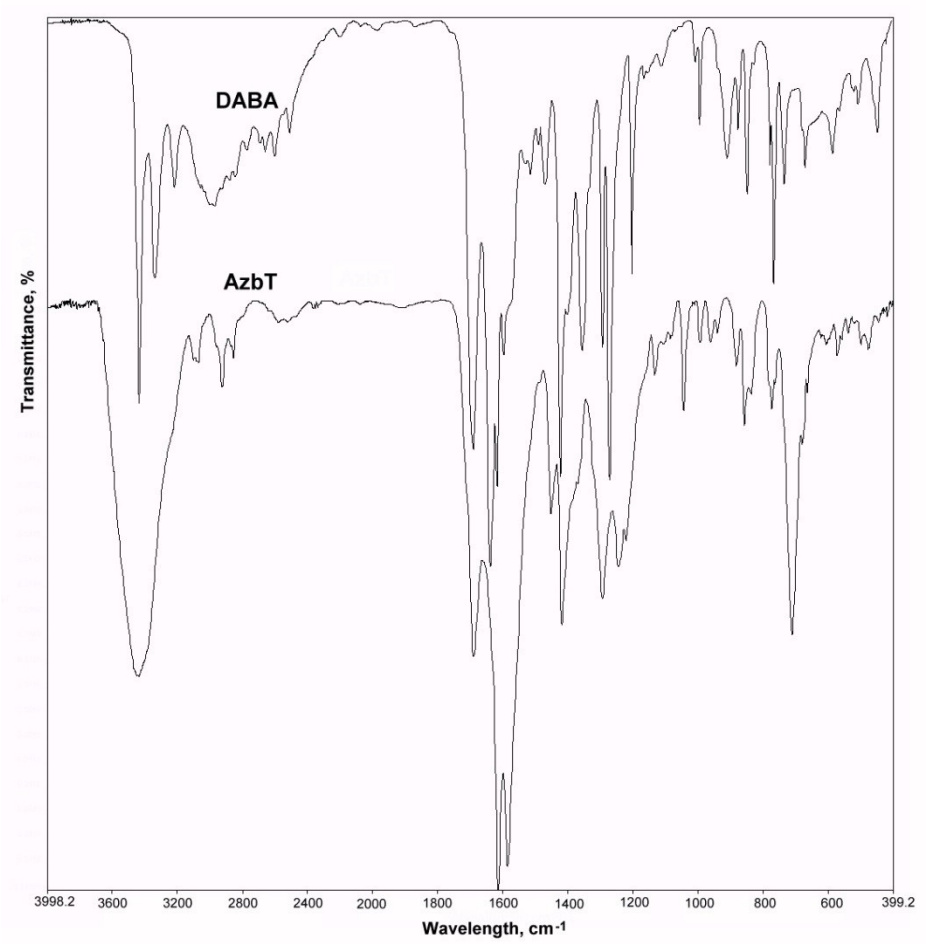

Figure S1. FTIR spectra of (1) azomethine-containing bis-thienyl (AzbT) monomer and (2) 3,5-diamonobenzoic acid.

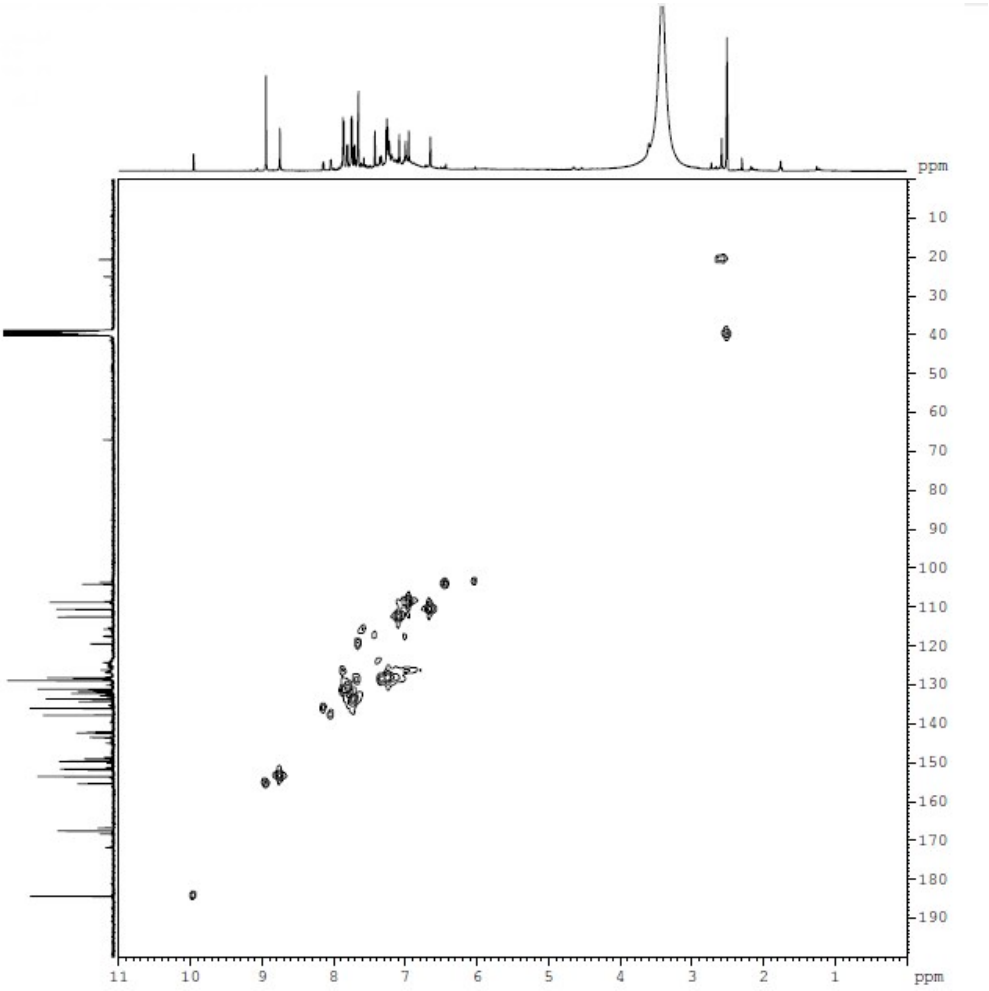

Figure S2. HMQC spectrum of AzbT in DMSO-d_6.

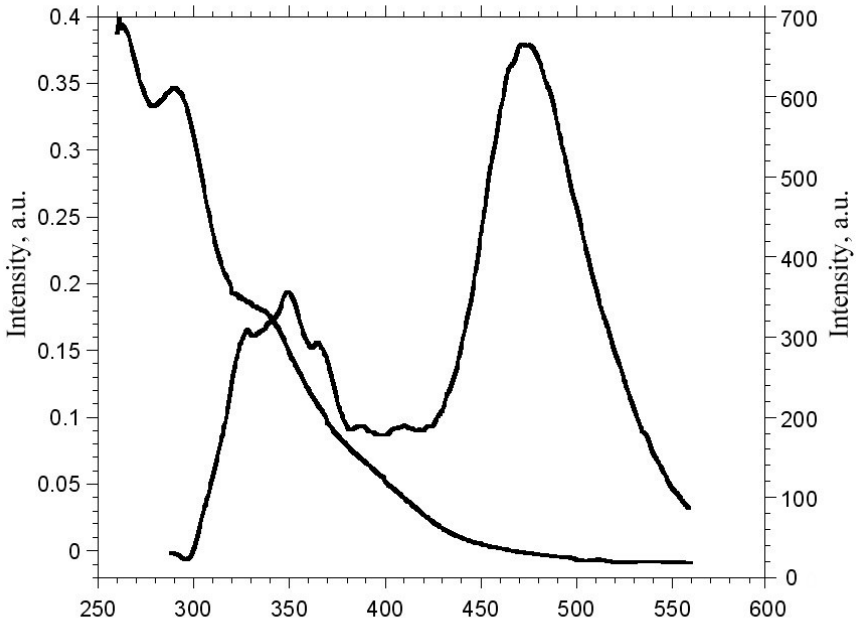

Figure S3. UV-vis absorption (left) and fluorescence (right) spectra of azomethine-containing bis-thienyl monomer.

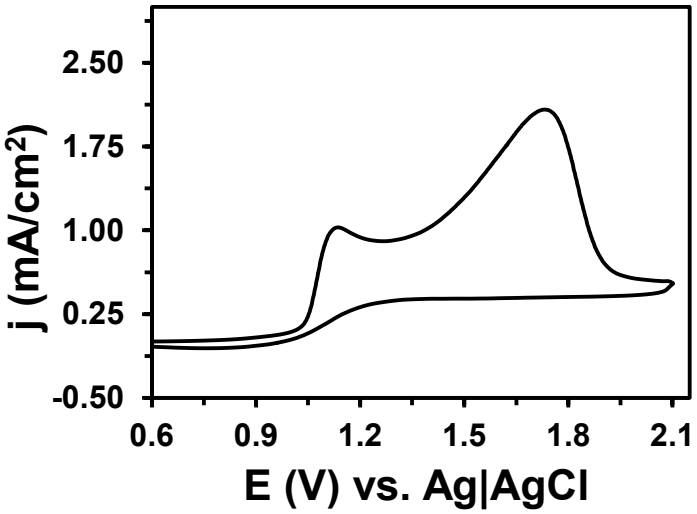

Figure S4. Control voltammogram for the oxidation of 60:40 AzbT:Th_3 in acetonitrile 0.1 M TBATFB. Voltammograms were recorded using a 1.0×0.5 cm^2 ITO substrate as working electrode. Initial and final potentials: 0.6 V; reversal potential: 2.10 V. Scan rate: 50 mV/s. Two anodic processes appear at 1.14 (O_1) and 1.74 V (O_2).

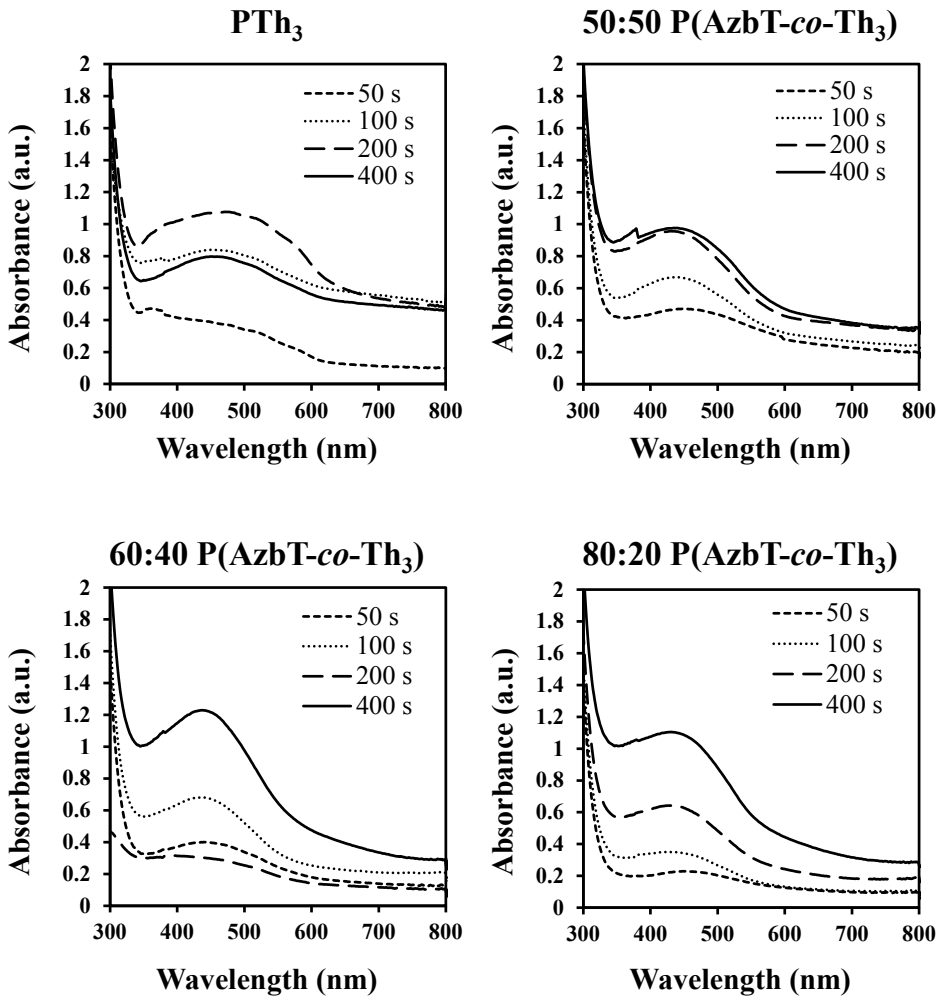

Figure S5. UV-vis spectra of PTh_3 and P(AzbT-*co*-Th_3) films prepared using polymerization times of 50, 100, 200 and 400 s at their corresponding polymerization potential.

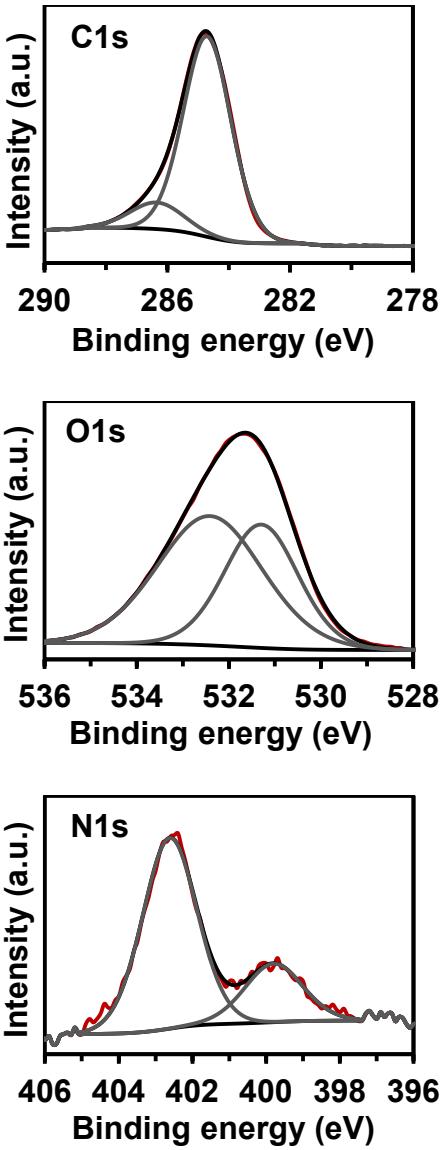

Figure S6. High-resolution XPS spectra (black line) for the 60:40 P(AzbT-*co*-Th_3) copolymer: C1s (top), O1s (middle) and N1s (bottom) regions. Peaks from deconvolution (in grey) are also displayed.

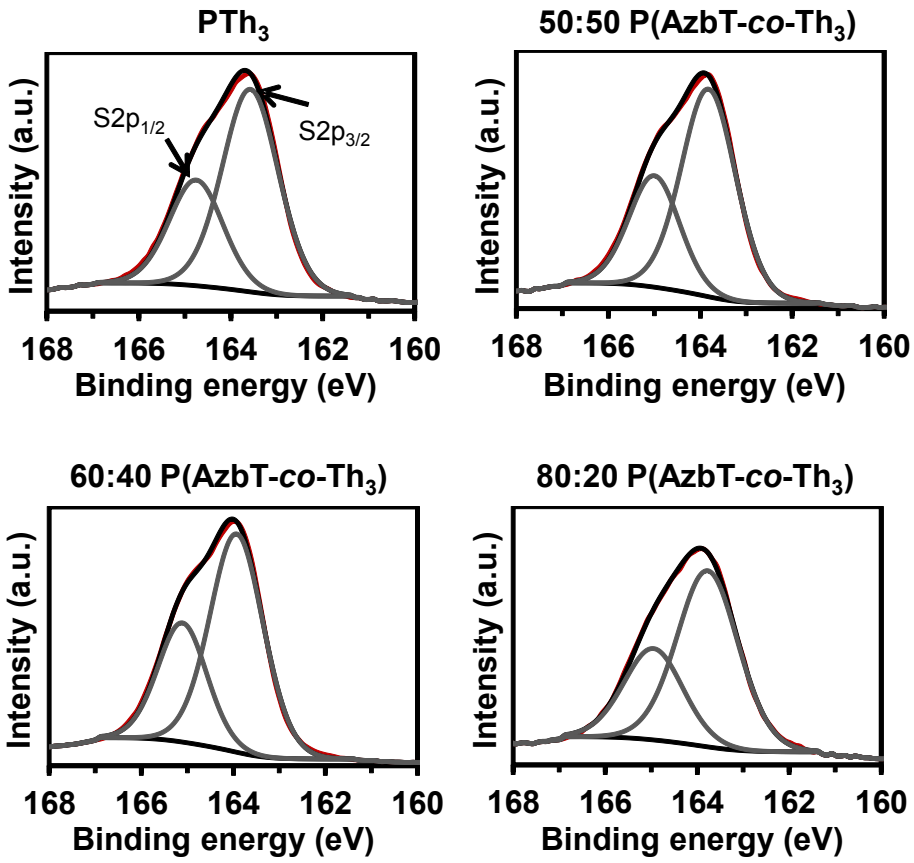

Figure S7. High-resolution XPS spectra in the S2p region for PTh_3 and the three different P(AzbT-*co*-Th_3) compositions.

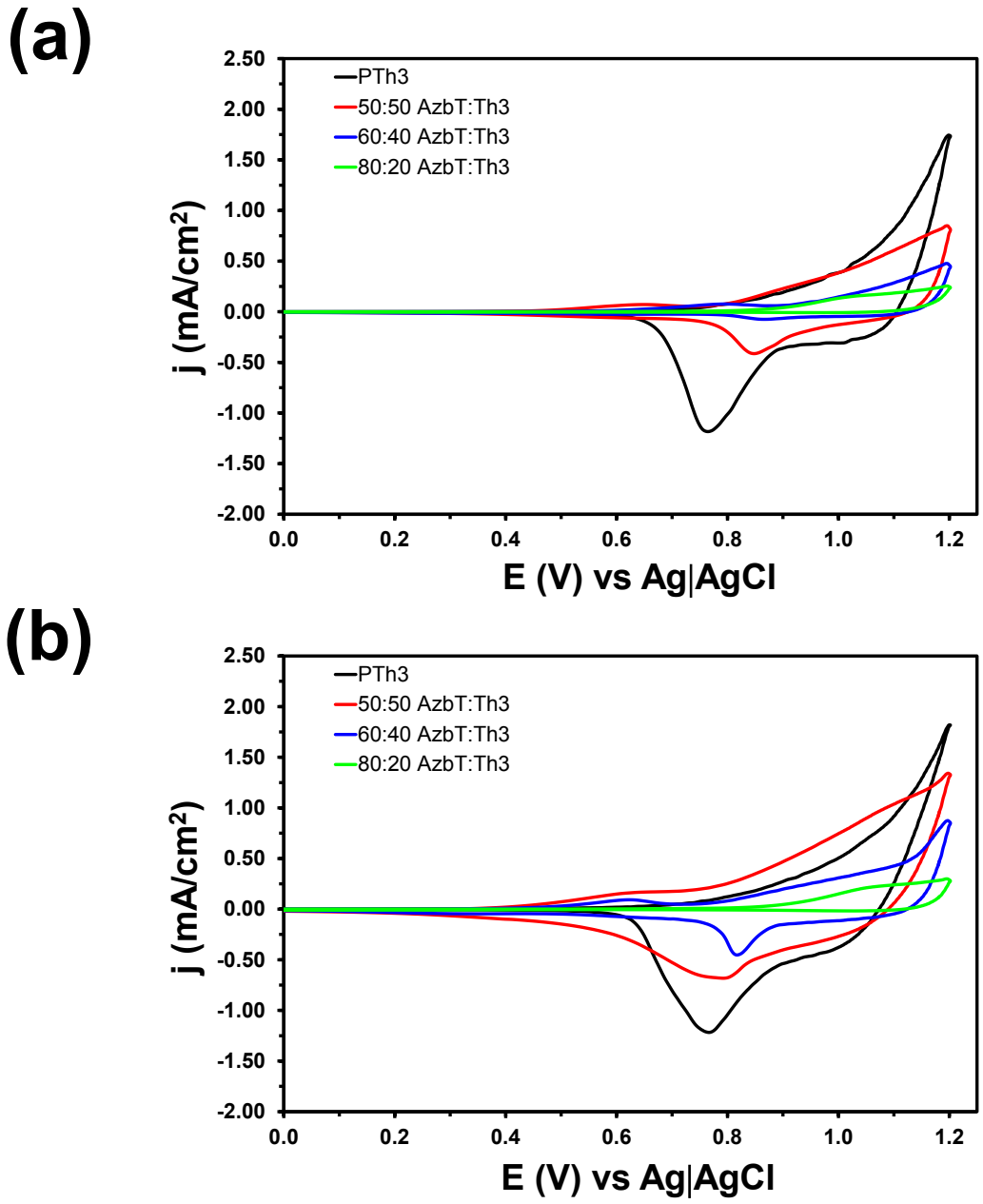

Figure S8. First control voltammograms for PTh_3 and P(AzbT-*co*-Th_3) films in acetonitrile with 0.1M TBATFB. Initial and final potential: 0.0 V; reversal potential: 1.1 V. Scan rate: (a) 25 mV/s and (b) 50 mV/s.

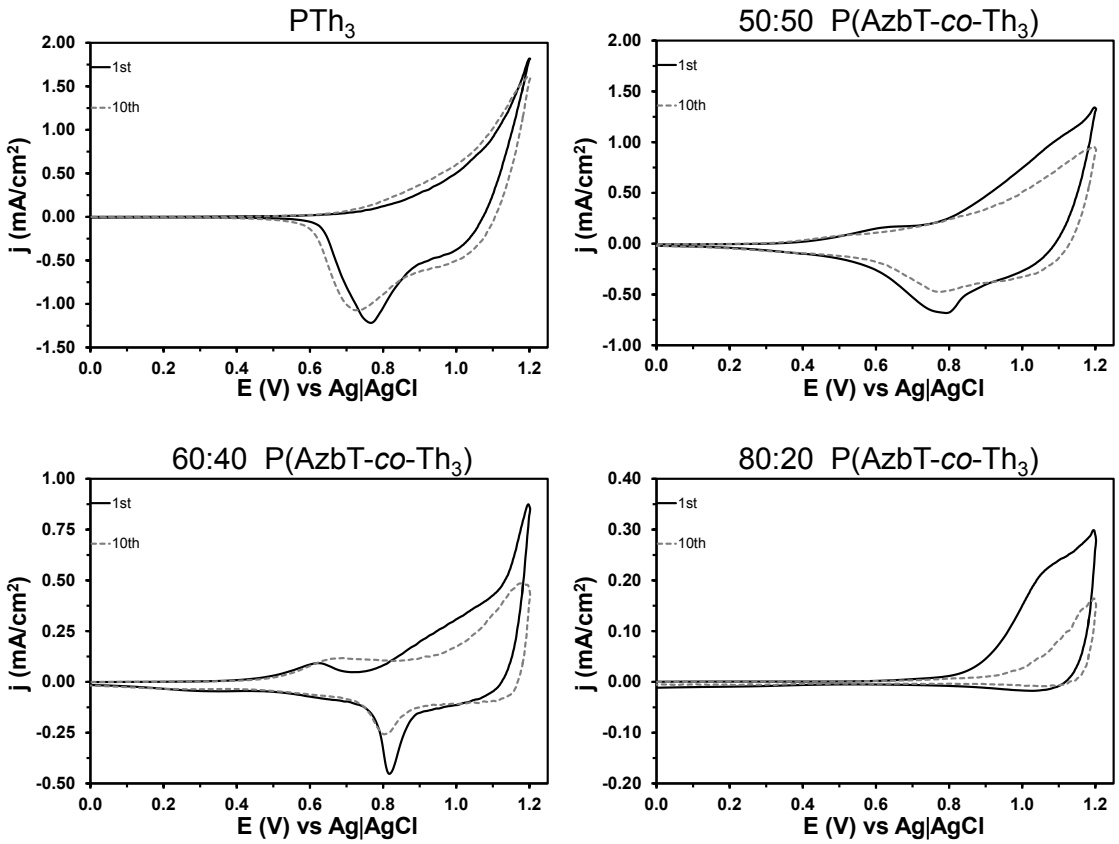

Figure S9. Voltammograms of PTh_3 and P(AzbT-*co*-Th_3) films recorded in acetonitrile with 0.1M TBATFB for the as prepared materials and after 10 consecutive oxidation-reduction cycles. Initial and final potential: 0.0 V; reversal potential: 1.1 V. Scan rate: 50 mV/s.

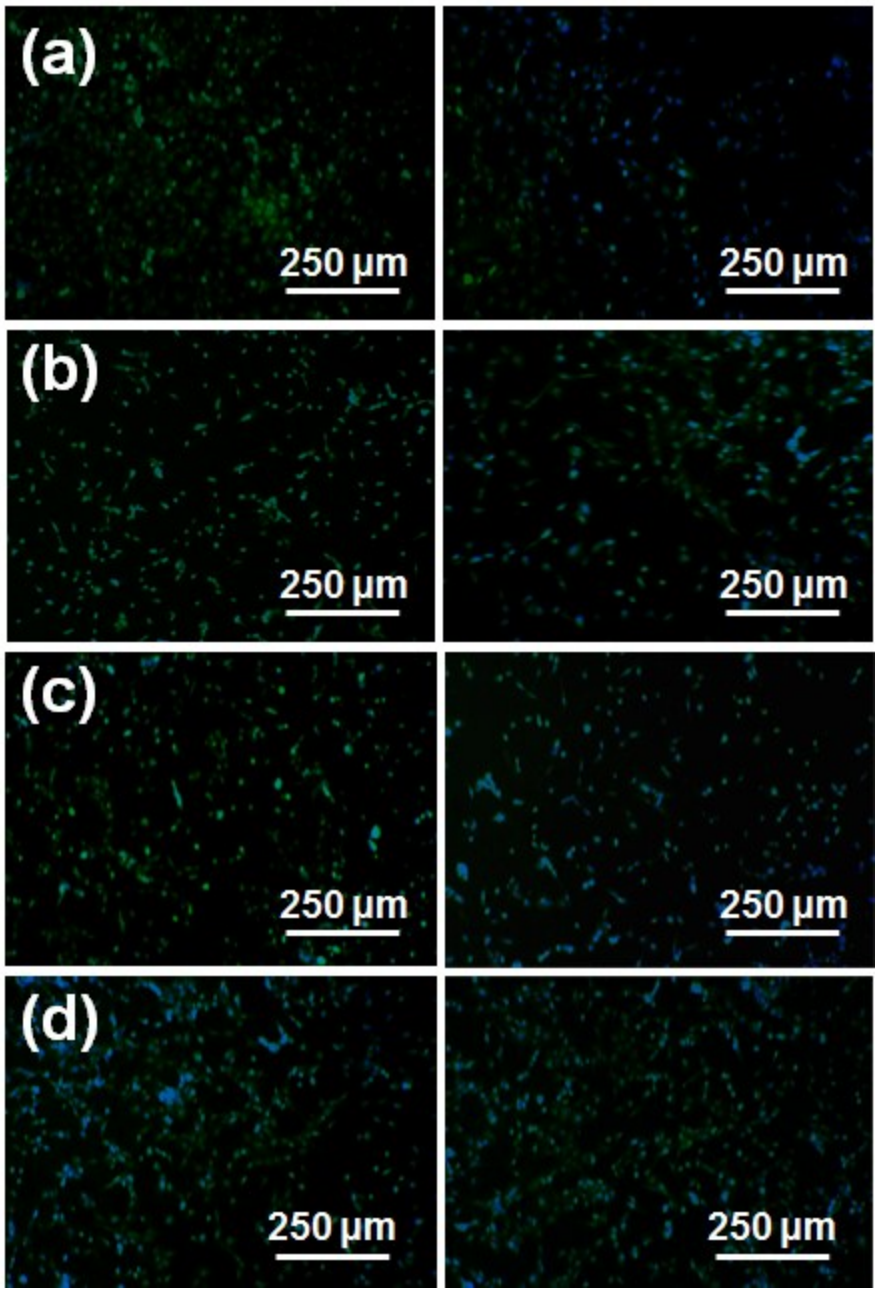

Figure S10. Fluorescence images of MG-63 and Vero cells (left and right, respectively) adhered onto: (a) ITO; (b) PTh_3; (c) 50:50 P(AzbT-*co*-Th_3); and (d) 80:20 P(AzbT-*co*-Th_3). Cell adhesion was observed with nuclei staining using Hoechst 33342.