Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supplementary Information

An amphiphilic, heterografted polythiophene copolymer containing biocompatible/biodegradable side chains for use as (electro)active surface in biomedical applications

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2. Experimental section

2.3.1. Structural characterization

1H-NMR and 13C-NMR spectra were recorded at room temperature on a Bruker Avance DRX-400 spectrometer at 400 MHz and 100.61 MHz, respectively, in acetone-d_6 solution. The chemical shifts are reported in ppm relative to residual peak of the solvent. The FTIR spectra 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. The absorption was measured in a wavenumber range from 4000 to 600 cm^{-1}, 64 scans for each spectrum and with baseline correction. The relative molecular weight of Th-PEG macromonomer was determined by gel permeation chromatography (GPC) using a PL-EMD instrument, polystyrene standards for the calibration plot, and tetrahydrofuran (THF) as elution solvent. The relative molecular weights of the Th-PCL macromonomer and that of PTh-g-(PEG-r-PCL) copolymer, respectively, were determined by GPC instrument WGE SEC-3010 multidetection system, consisting of a pump, two PL gel columns (PLgel 5micro Mixed C Agilent and PLgel 5micro Mixed D Agilent), dual detector RI/VI (Refractometer/Viscometer) WGE SEC-3010, using chloroform ($CHCl_3$), at a flow rate of 1.0 ml/min at 30ºC. The RI/VI detector was calibrated with polystyrene standards (580-467,000 DA) having narrow molecular weight distribution. The system was also equipped with a UV detector WGE SEC-3010 and Bi-MwA Brookhaven multi-angle SLS detector.

2.3.2. Characterizations in solution

UV-vis absorption spectra were measured using a Specord 200 Analytik Jena spectrophotometer. Fluorescence measurements were carried out using a Perkin Elmer LS 55 apparatus. All the measurements were done in acetone by keeping constant the concentration of solutions as 1mg/mL.

Particles size measurements were carried out by dynamic light scattering (DLS) using Delsa Nano C Submicron Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA). This device is equipped with dual 30 mW laser diodes emitting at 658 nm. The intensity weighted mean hydrodynamic size (Z average) and the polydispersity factor were obtained from

analysis of the autocorrelation function. The solvent used for sample preparation was filtered through NY-0.45µm filter to ensure the minimization of dust and other particulates. Samples were used as prepared, without filtration at a concentration of 1 mg/ml in acetone. The reported values represent the average of three measurements made for each sample, at 25^{o} C with an equilibration time of 5 minutes before starting each measurement. Quartz cells were used for organic solvent.

2.3.3. Morphology and particle size measurements

The morphology of the micro- and nanoparticles was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM micrographs were obtained using a Focussed Ion Beam Zeiss Neon 40 scanning electron microscope operating at 5 kV, equipped with an EDX spectroscopy system. A mother solution of the copolymer in acetone (100mg/mL) was used for the following experiments. Before use, the former solution was sonicated in a bath ultrasound for 5 minutes, for complete dissolution. Afterwards, an aliquot of 5 µL from this mother solution was deposited on a silicon plate of 1 cm^2 and the solvent was evaporated by using a vacuum system. The sample was sputter-coated with a thin layer of carbon for SEM experiments, to prevent sample charging problems. For TEM analysis one aliquot of 10 µL from the mother solution was diluted in 1mL of acetone and dripped to copper grids. TEM images were taken from a Philips TECNAI 10 electron microscope operated at 100 kV. The copolymer was observed with and without uranyl acetate coating. In the former case, a solution of 0.5% v/v in distilled water was dripped onto the sample previously deposited in a copper grid and dried at room temperature. Dynamic light scattering (DLS) NanoBrook Omni Zeta Potential Analyzer (from Brookheaven Instruments) was also employed for the particle size evaluation. The particles were dispersed in acetone solutions, by using one aliquot of 10 µL from mother solution and diluting it to a total volume of 1mL. Sonication of the mixture is also necessary in this step.

2.3.4. Electrochemical studies

Electrochemical characterization was carried out by cyclic voltammetry (CV) using an Autolab PGSTAT302N and NOVA software. Experiments were conducted in a three-electrode cell using a Screen-Printed Carbon Electrodes (SPCE, model C150, supplied by DropSens Co.) with carbon, as working electrode-WE (4 mm diameter), Pt as counter electrode, and an Ag|AgCl electrode containing a KCl saturated aqueous solution (E^0 = 0.222 V at 25 °C) as reference electrode. The two latter being external to the inner connections available in the SPCE substrate. A phosphate buffered saline (PBS) solution 0.1 M (pH = 7.4) at room temperature was used as electrolyte solution in all electrochemical assays. The initial and final potentials were −0.2 V, and the reversal potential was +0.6 V. Different scan rates were studied from 20 to 200 $mV \cdot s^{-1}$, considering the 50 $mV \cdot s^{-1}$ the best option for the successive assays. The small WE was covered with one aliquot of 5 µL of a 100 mg/mL, from the copolymer in acetone, and after careful sonication.

The ability to exchange charge reversibly (i.e. electroactivity) and the electrochemical stability (*i.e.* electrostability) were determined through direct measurement of the anodic and cathodic areas in the control voltammograms recorded at 50 mV s^{-1}. The loss of electroactivity (LEA, in %) was expressed as:

$$LEA\ (\%)\frac{\Delta Q}{Q_i} * 100$$

where ΔQ is the difference in voltammetric charges (in Coulombs) between the second and the n cycle, and Q_i is the voltammetric charge corresponding to the second cycle.

The electrochemical detection of NADH and NAD^+ was studied by CV, differential pulse voltammetry (DPV) and chronoamperometry (CA). CV assays were conducted using the experimental conditions previously described but incorporating 10 mM to the electrolyte solution. DPV assays were carried out also in a three electrode cell. The applied potential range was from +0.5V to +0.8 V with 80 mV as modulation amplitude and a scan increment of 2 mV. The modulation and interval time were 0.05 and 40 s, respectively. The DPV signal corresponding to electrochemical oxidation of NADH was recorded in PBS (pH 7.4) solutions with concentrations ranging from 2 mM to 10 mM. Chronoamperometric measurements were performed using a steady

current of +0.65 V and increasing the concentrations of NADH from 0.2 to 2 mM each 50 s. In order to prove the sensitivity of PTh-*g*-(PEG-*r*-PCL) as sensor, CV and DPV assays were also carried out in presence of ascorbic acid (AA), which was used as interfering substance.

2.3.5. Biocompatibility studies

Cellular assays were performed using Cos-1 and Vero cells, which were selected due to their rapid growth. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cultures were maintained in an incubator chamber (Air-Jacketed Automatic CO_2 Incubator) with an atmosphere of 5% of carbon dioxide and 95% of oxygen, at 37 °C. Culture media were changed every two days. When the cells reached 80-90% confluence, they were detached using 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.

The procedure was similar to that used to the deposition of the macromonomer for the electrochemical tests. Small aliquots of 5 μL of a solution containing 100 mg of copolymer per mL of acetone, were dropped onto steel AISI 316L sheets with an area of 0.5 × 1 cm^2. After 3 days drying in vacuum, the coated steel sheets were placed in plates of 24 wells and sterilized using UV irradiation for 15 min in a laminar flux cabinet. Controls were simultaneously performed by culturing cells on the surface of steel. For adhesion and cytotoxicity assays, 2×10^4 and 5×10^4 of cells respectively, were deposited on the surface sample of each well. Then, attachment of cells to the film surface was promoted by incubating under culture conditions for 30 min. Finally, 2 mL of the culture medium were added to each well. After 24 h, cells adhered to the materials were quantified. Furthermore, after 7 days, all cells inside the wells were quantified to evaluate the cytotoxicity of the materials. The cellular proliferation was determined by quantifying exclusively the cells attached to the copolymer surface.

Cellular viability was evaluated by the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] 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 colour change: the characteristic pale yellow of MTT transforms into the dark-blue of formazan crystals. Specifically, 50 mL 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 was measured in a plate reader at 570 nm. The viability results, derived from the average of three replicates (n= 3) for each independent experiment, were normalized to the control, for relative percentages.

3. Results and discussion

3.1. Molecular design and structural characterization of PTh-*g*-(PEG-*r*-PCL) copolymer

Table S1. Data on synthesized compounds obtained by calculation and by GPC measurements.

Sample	**$M_{n,\ ^1H\text{-}NMR}$**	**$M_{n,\ GPC}$**	**IPD**
Th-PEG	2110	2486	1.04
Th-PCL	2283; PD=19		1.47
PTh-*g*-(PEG-*r*-PCL)	-	5113	1.44

IPD: Index of Polydispersity; PD: Polymerization Degree

Table S2. Physical properties of side chains and acetone

Compound	Hildebrand Solubility Parameter δ ($cal^{1/2}\ cm^{-3/2}$)	Acetone-Polymer interaction parameter(χ)
PEG	10.17	0.46
PCL	10	0.47

Acetone	9.77	-

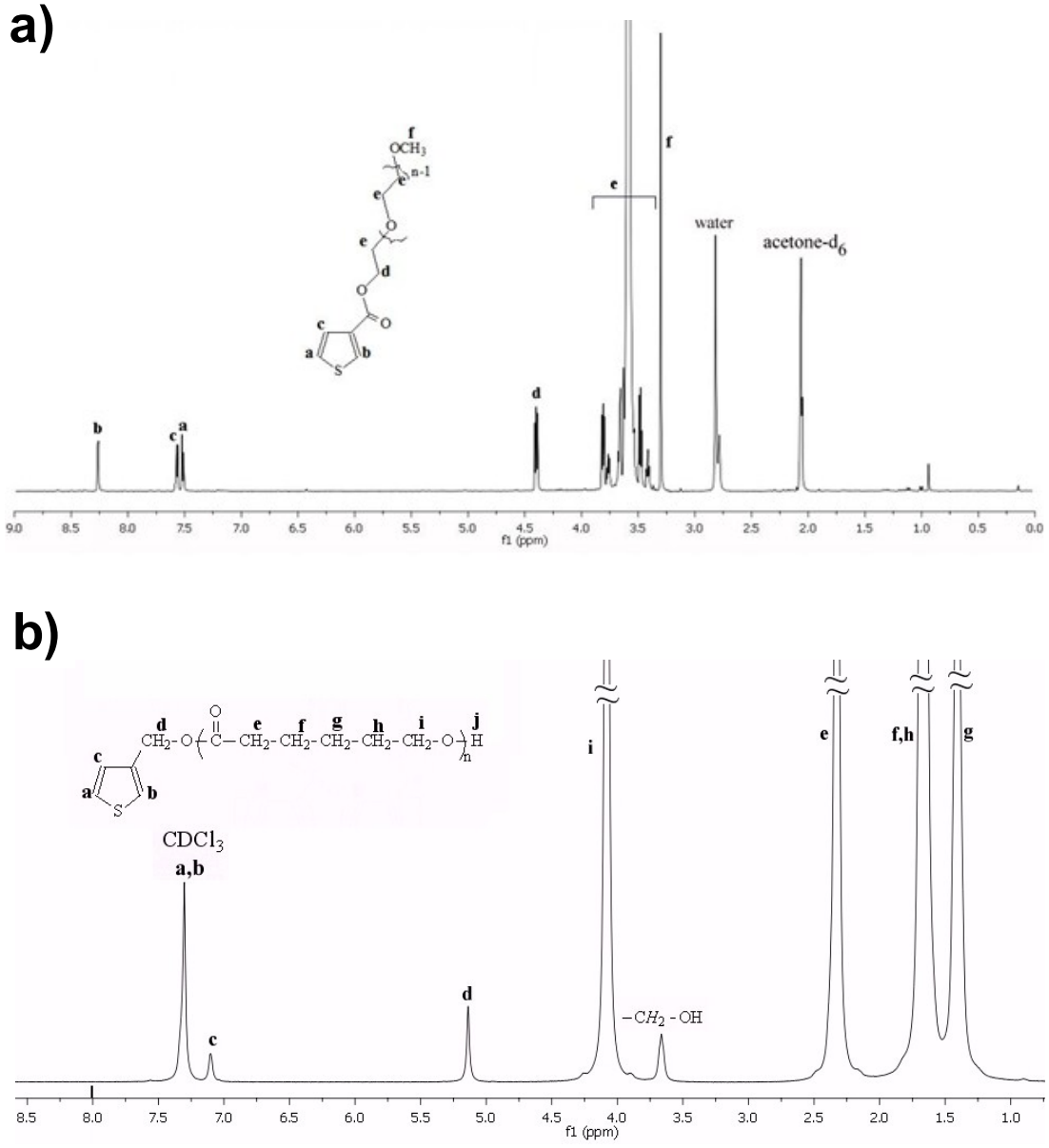

Figure S1. ^{1}H-NMR of macromonomers Th-PCL (a) and Th-PEG$_{2000}$ (b), precursors of PTh-*g*-(PEG-*r*-PCL) copolymer.

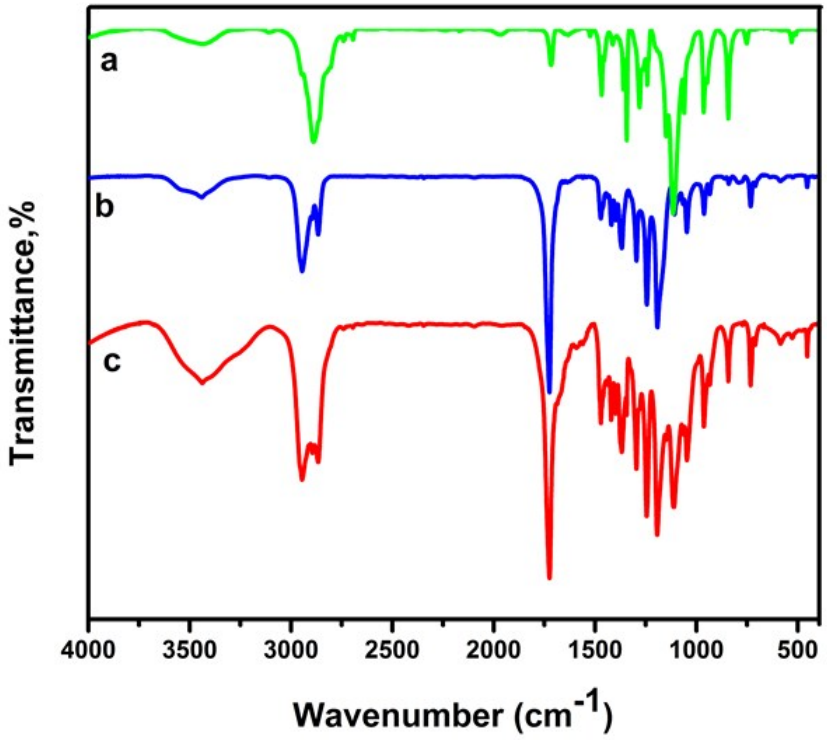

Figure S2. FTIR spectra of starting materials, macromonomers Th-PEG$_{2000}$ (a); Th-PCL (b); and of the final copolymer PTh-*g*-(PEG-*r*-PCL) (c).

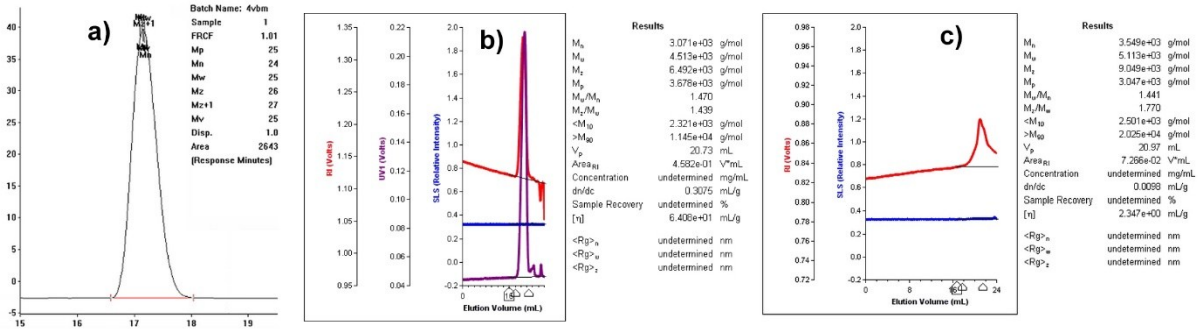

Figure S3. GPC traces of macromonomers Th-PEG$_{2000}$ (a), Th-PCL (b) and copolymer PTh-*g*-(PEG-*r*-PCL) (c).

Additionally, for clear assignment of the amphiphilic side chains presence in PTh-*g*-(PEG-*r*-PCL), a more accurate study with DEPT 135 ^{13}C-NMR registration was performed (Figure 1c, main text). Being more sensitive than normal acquisition, this method allowed for the separation of peak corresponding to atom **13** from those of acetone used as registration solvent and for definitely assignment of the atoms **2**, **5**, **6** having particular positions in PEG side chains. A similar separate placement of peaks can be seen for carbon atoms **15** and **16** in oligo-ε-caprolactone final structural unit, that appeared separately from their homologues **13** and **14**. What it is intriguing in Figure 1b is the fact that from eight carbon atoms belonging to the two types of thiophene rings in the main chain of copolymer only two of them are visible, the other ones being hardly discernible. Moreover, the information given by DEPT 135 ^{13}C-NMR registration allowed for the assignment of carbon atoms **17** and **18** from thiophene rings, as explained in the main text (Figure 1c).

Given the side chains different nature and their inherited incompatibility, from the obtained results also it can get the conclusion that, in acetone, the copolymer chains segregated lengthwise by intramolecular self-assembling, as schematically suggested in Figure 8 (main text).

3.3. Influence of the solvent on the morphology of amphiphilic PTh-*g*-(PEG-*r*-PCL) copolymer

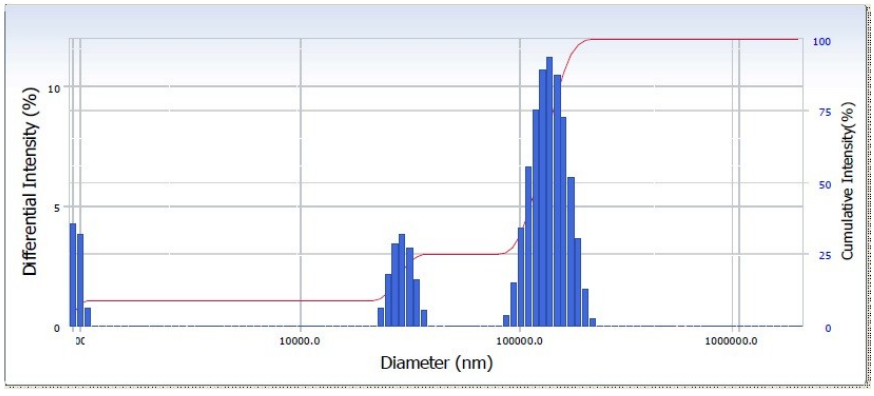

Figure S4. PTh-*g*-(PEG-*r*-PCL) copolymer particle distribution by DLS in diluted acetone solutions.

General references

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