SUPPLEMENTARY INFORMTION

Antimicrobial activity of poly(3,4-ethylenedioxythiophene) n-doped with a pyridinium-containing polyelectrolyte

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METHODS

Materials

3,4-Ethylenedioxythiophene (EDOT) monomer and acetonitrile of analytical reagent grade were purchased from Aldrich. Anhydrous $LiClO_4$, analytical reagent grade, from Aldrich, was stored in an oven at 80 ºC before using it in the electrochemical trials. Tetramethylammonium chloride (TMA; 98%) was purchased from Across. 4-aminopyridine (98%) was purchased from Sigma and 4-(chloromethyl)benzoyl chloride (>98%) was purchased from TCI Europe.

Normal rat kidney fibroblast cells (NRK) and African green monkey kidney epithelial cells (VERO) from ATCC (USA) were used in this study.

The bacterial culture was prepared with reagents and labware from Scharlab (Spain). *Escherichia coli* (*E. coli*) CECT 101 and *Staphylococcus aureus* (*S. aureus*) CECT 245 bacterial strains were obtained from Spanish Collection of Type Culture (Valencia, Spain).

Preparation of p-doped PEDOT films

Oxidized (p-doped) PEDOT films were prepared by chronoamperometry (CA) under a constant potential of +1.40 V. Electrochemical experiments were conducted on a PGSTAT204 AUTOLAB potentiostat–galvanostat connected to a PC computer controlled through the NOVA 1.6 software using a three-electrode two-compartment cell under nitrogen atmosphere at 25 ºC. The cell was filled with 10 mL of a 10 mM EDOT solution in acetonitrile with 100 mM $LiClO_4$. Steel AISI 316 sheets with an area of 2 cm^2 were employed as working and counter electrodes, respectively. Before each trial, electrodes were cleaned with acetone and dried in a nitrogen-flow. The reference electrode was an Ag|AgCl electrode containing a KCl saturated aqueous solution (E^o =

0.222 V *vs.* standard hydrogen electrode at 25 ºC), which was connected to the working compartment through a salt bridge containing the electrolyte solution. The electrodes used for synthesis of p-doped PEDOT films were also employed for de-doping and re-doping experiments.

The polymerization time was adjusted to obtain PEDOT electrodes with a polymerization charge of 0.960 C (480 mC/cm^2). The mass of PEDOT deposited onto the WE was determined as the weight difference between coated and uncoated steel sheets using a CPA26P Sartorius analytical microbalance with a precision of 10^{-6} g.

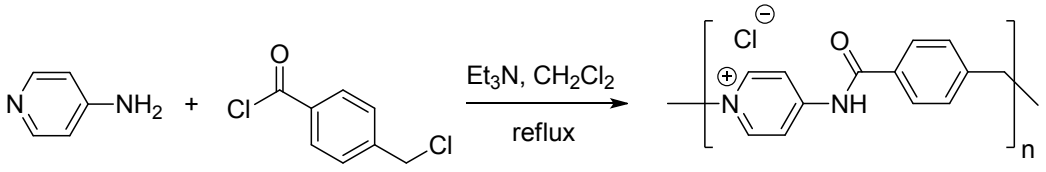
Preparation of de-doped PEDOT films

De-doping of electropolymerized p-doped PEDOT was carried out applying a fixed potential of –1.50 V during 120 s in a 10 mM $LiClO_4$ acetonitrile solution. The resulting samples were kept immersed in acetonitrile until utilization in next steps.

Synthesis of P(Py-1,4-P)

Poly[pyridinium-1,4-diyliminocarbonyl-1,4-phenylene-methylene chloride, P(Py-1,4-P), was synthesized as previously reported.[S1] A solution of 4-chloromethylbenzoylchloride (23.9 g, 130 mmol, 1.0 equiv) in dichloromethane (100 mL) was added dropwise to a suspension of 4-aminopyridine (12.2 g, 130 mmol, 1.0 equiv) and triethylamine (20 mL, 143 mmol, 1.1 equiv) in dichloromethane (200 mL). The reaction mixture was refluxed overnight. After cooling, the so formed precipitate was filtered off, washed with dichloromethane and methanol, and dried under vacuum to afford the desired polyelectrolyte as a white powder in ca. 80% yield (25 g) (Scheme S1). The structure of P(Py-1,4-P)was confirmed by ^{1}H NMR in D_2O (Figure S1), which afforded spectroscopic data in agreement with those previously reported.[S1] Moreover,

the gelation ability of P(Py-1,4-P) driven by non-covalent intermolecular interactions (*e.g.*, hydrogen-bonding, π-π and cation-π interactions) was confirmed at a minimum gelation concentration of 7.5 g/L.



Scheme S1

Anion-exchange reaction was carried out by adding lithium bis(trifluoromethanesulfonyl)imide (LiTFSI, 1.11 g) in water (10 mL) to a hot solution of P(Py-1,4-P) (190 mg) in water (25 mL). The reaction mixture was refluxed for 30 min. After cooling, precipitates were filtered off, washed with water and dried under vacuo to afford the corresponding P(Py-1,4-P)·TFSI, which was re-dispersed in DMF for SEC analysis: Mw = 2.80 x 10^3 Da; PD = 1.66 (polystyrene standards; DMF eluent containing 30 mM of LiTFSI); degree of polymerization = 13.[S1]

Preparation of n-doped PEDOT:P(Py-1,4-P) films

Re-doping experiments were conducted using aqueous solutions with 10 mM P(Py-1,4-P) or 10 mM TMA aqueous solution applying a reduction potential of –0.50, –0.70, –0.90 or –1.10 V during 300 s. Homogeneous P(Py-1,4-P) solutions were prepared according to the following procedure: 1) 5 min sonication at the 30% power was applied six times to a solution made of 0.0217 g (0.010 mol) of polyelectrolyte in 10 mL of de-ionized water; and ii) magnetic stirring at 75 ºC was applied at 75 ºC for 45 min.

Characterization measurements

FTIR spectra were recorded on a FTIR Jasco 4100 spectrophotometer. The p-doped PEDOT, de-doped PEDOT and n-doped PEDOT:P(Py-1,4-P) films were deposited on an attenuated total reflection accessory (Top-plate) with a diamond crystal (Specac model MKII Golden Gate Heated Single Reflection Diamond ATR). Samples were evaluated using the spectra manager software and, for each sample, 32 scans were performed between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}.

Scanning electron microscopy (SEM) studies were performed to examine the surface morphology of the prepared samples. Dried films were placed in a Focused Ion Beam Zeis Neon 40 scanning electron microscope operating at 5 kV, equipped with an energy dispersive X-ray (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. The size of pores was determined from the SEM images using the software SmartTIFF (v1.0.1.2.).

AFM images were obtained with a Molecular Imaging PicoSPM using a NanoScope IV controller under ambient conditions. The tapping mode AFM was operated at constant deflection. The row scanning frequency was set to 1 Hz. AFM measurements were performed on various parts of the films, which provided reproducible images similar to those displayed in this work. The statistical application of the NanoScope Analysis software was used to determine the arithmetic average of the roughness profile (R_a) and the root mean square roughness (R_q), which corresponds to the average height deviation taken from the mean data plane.

Contact angle measurements were carried out using the sessile drop method at room temperature on an OCA 15EC with SCA20 software (Data-Physics Instruments GmbH, Filderstadt, Germany). The solvent used for these experiments was deionized water, contact angle being determined for the first drop. For measurements, the sessile drop

was gently put on the surface of sample discs using a micrometric syringe with a proper metallic needle (Hamilton 500 μL). The ellipse method was used to fit a mathematical function to the measured drop contour. This procedure consists on approximate the drop contour to the line of an ellipse, deviations from the true drop shape being in the range of a few percent. The ellipse method provides accurate measure of the contact angle and holds the advantage that it is extremely fast. For each sample, no less than fifteen drops were examined.

The electrical conductivity was measured by the four probe methid method using films synthesized on steel electrodes of 4-cm^2 area.

Cytotoxicity of P(Py-1,4-P)

In vitro cytotoxicity evaluation of P(Py-1,4-P) for NRK y VERO cell lines was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is described below. P(Py-1,4-P) was dissolved in water until reach a concentration of 10 mM and then sterilized by filtration (0.2 μm). Cells were seeded at a density of 2×10^4 cells in 96-well plates and incubated overnight. Subsequently, cells were exposed to a series of increasing P(Py-1,4-P) concentration. Cells were incubated with the treatment for 24 h. Finally, the percentage of viable cells relative to untreated control was determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (MTT assay). The results were expressed as mean value ± standard deviation (SD). All the experiments were performed in triplicate. Statistical comparison of values was based on a one-way ANOVA using Tukey's test for pair-wise comparison with $p < 0.05$.

Biocompatibility

Cellular assays were performed using NRK and Vero cells. These cells were selected due to their fast growth. Cells were cultured in DMEM high glucose supplemented with 2 mM L-glutamine, 10% fetal bovine serum (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% air at 37ºC. 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.

Tested films 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 tissue culture polystyrene (TCPS) plates. For adhesion and proliferation assays an aliquot of 100 μL with 2×10^4 cells was deposited on the film 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, cellular adhesion was determined by quantifying the cells attached to the films or the control. Cellular proliferation was evaluated by quantifying the viable cells onto the evaluated materials after 7 days of culture.

Cellular viability was evaluated by the 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 phosphate buffer saline,

PBS, solution) 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 mm. The viability results, derived from the average of three replicates (n= 3) for each independent experiment, were normalized to the control, for relative percentages.

Antimicrobial test assays

E. coli and *S. aureus* were selected to evaluate the antibacterial activity of the different PEDOT films. The bacteria were previously grown aerobically to exponential phase in broth culture (LB broth, Lennox) (5 g/L beef extract, 5 g/L NaCl, 10 g/L tryptone, pH 7.2).

Growth experiments were performed placing a piece (area: 0.5 cm^2) of film in polystyrene tubes of 15 mL. After this, 5 mL of broth culture containing 1×10^3 colony forming units (CFU) was seeded in each sample-containing tube. The cultures were incubated at 37 ºC and agitated at 100 rpm. Aliquots of 100 µL were taken at pre-defined time intervals for absorbance measurement at 595 nm in a plate reader. Thus, turbidity was directly related to bacterial growth. The bacterial growth in broth culture alone (in absence of any film) was considered as the maximum growth (control) and it was used to calculate the relative growth of the bacteria in presence of the samples. All assays were conducted in triplicate and the values averaged.

Bacterial adhesion on p-doped, de-doped and n-doped PEDOT films was also determined. Upon completion of growth experiments, culture media were aspirated and the material was washed three times with distilled water. Then, 0.5 mL of sterile 0.01 M sodium thiosulfate was added to each film to detach adhered bacteria. After 1 h

agitation (100 rpm) at 37 °C, samples were removed and 1 mL of broth culture was added in each tube. After filling all tubes with fresh broth culture, the first sample (100 μL) was taken as a time 0 for adhesion assay and the absorbance was measured at 595 nm in a plate reader. Then, tubes were incubated at 37 °C and 100 rpm for 24 h. Bacterial growth was determined as above indicated. All assays were conducted in quadruplicate and values were averaged

Finally, the cytotoxicity of P(Py-1,4-P) for *E. coli* and *S. aureus* was determined using the procedure described above. 5 mL of broth culture containing 1×10^3 CFU were introduced into 15 mL tubes and incubated overnight. The bacteria were exposed to a series of increasing P(Py-1,4-P) concentration. During the incubation, aliquots of 100 μL were taken at pre-defined time intervals for absorbance measurement at 595 nm in a plate reader. Experiments were performed in triplicate and the bacterial growths were normalized to the control (bacterial growth in LB broth alone). Then, results were expressed as mean value ± standard deviation (SD).

S1. M. Yoshida, N. Koumura, Y. Misawa, N. Tamaoki, H. Matsumoto, H. Kawanami, S. Kazaoui and N. Minami, *J. Am. Chem. Soc.*, **2007**, 129, 11039-11041.

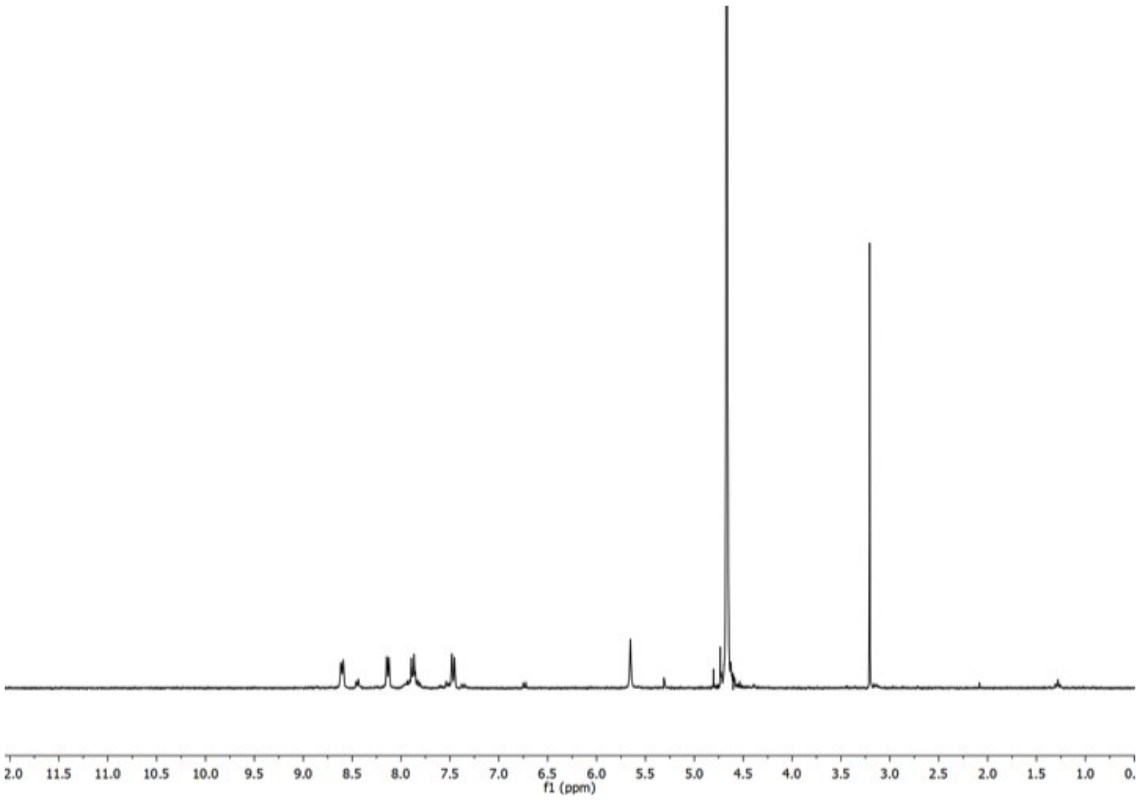


Figure S1. ^{1}H NMR of P(Py-1,4-P) in D_2O.

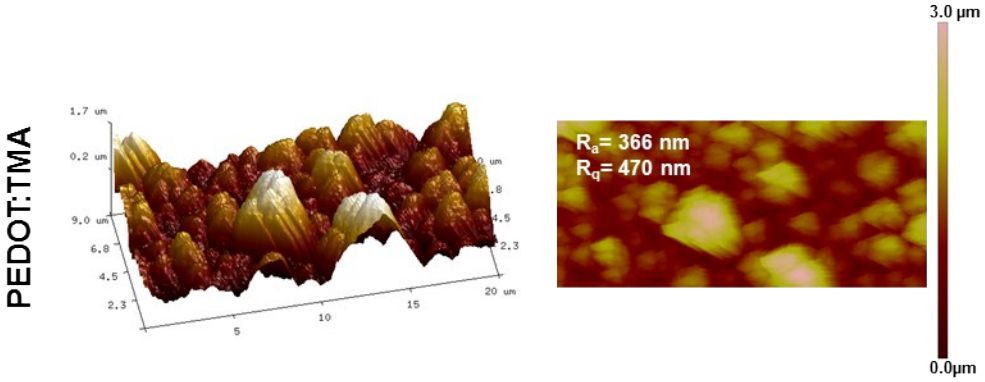


Figure S2. 3D Topographic and 2D height AFM images (9×20 µm^2) of PEDOT:TMA. Values of R_a and R_q are also displayed.