ELECTRONIC SUPPLEMENTARY MATERIAL

Conducting polymer nanoparticles for voltage-controlled release of pharmacological chaperones

Hamidreza Enshaei,[1] Anna Puiggalí-Jou,[1,2,*] Núria Saperas[1,*] and Carlos Alemán[1,2,*]

[1] *Departament d'Enginyeria Química, EEBE, Universitat Politècnica de Catalunya, C/ Eduard Maristany 10-14, Ed. I2, 08019 Barcelona, Spain*

[2] *Barcelona Research Center for Multiscale Science and Engineering, EEBE, Universitat Politècnica de Catalunya, C/ Eduard Maristany 10-14, Ed. C, 08019 Barcelona, Spain*

METHODS

Synthesis of unloaded poly(3,4-ethylenedioxythiohene) nanoparticles (PEDOT NPs). A 30 mL Corex tube was filled with 15.8 mL of milli-Q water. After this, 96 µL of dodecyl benzenesulfonic acid (DBSA) was added and the solution was stirred for 1 h using a magnetic stirrer set at 750 rpm at 40 °C and protected from light with aluminum foil. Next, 72 µL of 3,4-ethylenedioxythiophene (EDOT) monomer and 2 mL of methanol were slowly added. The mixture was allowed to stir for 1 h at 750 rpm at 40 °C. Finally, 0.73 mg of ammonium persulfate (APS) dissolved in 2 mL of milli-Q water were added drop by drop while stirring. The reaction was maintained in agitation at 40 ºC overnight. In this process, the color of the reaction mixture changed from light grey to dark blue. No sedimentation was observed after the reaction occurred, reflecting a good colloidal stability. The side products and unreacted chemicals were removed by a sequence of three centrifugations at 11000 rpm for 40 min at 4 ºC. After each centrifugation, the resulting supernatants were decanted and the pellet was dispersed in 15 mL of deionized water by using a vortex and a sonication bath (15 min at room temperature). The last pellet was left under vacuum in the same tube for two days, then weighted (33 mg) and dispersed in the corresponding medium at the desired concentration.

Synthesis of pyrimethamine (PYR)-loaded PEDOT NPs (PEDOT/PYR NPs). 96 µL of DBSA were added to a 30 mL tube filled with 15.8 mL of milli-Q water and the solution was stirred for 1 h at 750 rpm at 40 °C. After this, 72 µL of EDOT and 2 mL of drug solution (8 mg/mL PYR in methanol) were added drop by drop while stirring and the resulting solution was stirred at 750 rpm at 40 °C during 1 h. Finally, 0.73 mg of APS dissolved in 2 mL of milli-Q water was added to the mixture. The reaction was

protected from light (aluminum foil) and maintained in agitation at 40 ºC overnight. The color of the reaction mixture changed from light grey to dark blue. No sedimentation was observed after the reaction occurred, indicating good colloidal stability. The side products, extra drug and unreacted chemicals were removed by a sequence of 3 centrifugations at 11000 rpm for 40 min at 4 ºC. The resulting supernatants were decanted and the pellet was re-dispersed in deionized water by using a vortex and a sonic bath (15 min at room temperature). The last pellet was left under vacuum for two days, then weighted and re-dispersed in the corresponding medium at the desired concentration.

Characterization. FTIR transmittance spectra were recorded on a FTIR Jasco 4100 spectrophotometer. Samples were deposited on an attenuated total reflection accessory (Top-plate) with a diamond crystal (Specac model MKII Golden Gate Heated Single Reflection Diamond ATR). For each sample, 64 scans were performed between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}.

Micro-Raman spectroscopy assays were performed using a commercial Renishaw inVia Qontor confocal Raman microscope. The Raman setup consisted of a laser (785 nm with a nominal 300 mW output power) directed through a microscope (specially adapted Leica DM2700 M microscope) to the sample, after which the scattered light is collected and directed to a spectrometer with a 1200 lines·mm^{-1} grating. The exposure time was 10 s, the laser power was adjusted to 0.001-0.05% of its nominal output power, depending on the sample, and each spectrum was collected with 30 accumulations.

X-ray photoelectron spectroscopy (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 current of the electrons were 4 eV and 0.35 mA, respectively. For the argon gun, the energy and the emission current were 0 eV and 0.1 mA, respectively. The spectra were recorded with pass energy of 25 eV in 0.1 eV steps at a pressure below 6×10^{-9} mbar. These standard conditions of charge compensation resulted in a negative but perfectly uniform static charge. The C 1s peak was used as an internal reference with a binding energy of 284.8 eV. The surface composition was determined using the manufacturer's sensitivity factors.

UV analyses were performed using a Cary100 UV-Vis spectrophotometer controlled by the UVProbe 2.31 software.

Dynamic Light Scattering (DLS) studies were performed using NanoBrook Omni Zeta Potential Analyzer from Brookheaven Instruments. Measurement consisted of 3 runs of 120 s duration each one, which were averaged to obtain the effective diameter. Samples were analyzed at 25 ºC using a scattering angle of 90º. In order to know the zeta (ζ)-potential, particles were re-suspended in 1 mM KCl solution and 30 consecutive measurements were taken of each sample.

The morphology of the PEDOT/PYR and PEDOT NPs was studied by scanning electron microscopy (SEM). Micrographs were obtained using a Focused Ion Beam Zeiss Neon 40 scanning electron microscope operating at 5 kV. 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) studies were conducted to obtain topographic images of the NPs surface using 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 Molecular Imaging PicoSPM microscope using a NanoScope IV controller under ambient conditions in tapping mode. AFM measurements were performed on various parts of the samples, which produced reproducible images similar to those displayed in this work.

Electrochemical characterization was performed by cyclic voltammetry (CV) using an Autolab PGSTAT302N Galvano stat equipped with the ECD module (Ecochimie, The Netherlands). Measurements were performed on 15 µL of 10 mg/mL NPs solution dried on a screen printed carbon electrode (SPCE; 4 mm diameter) coated with chitosan. For this purpose, three rounds of 5 µl of the 10 mg/ml NPs solution were placed on the SPCE and dried under the hood after each round. Then, dried NPs were covered with 5 µL of chitosan solution (20 mg/mL chitosan in 0.1 M HCl) and dried again. All electrochemical assays were performed using a three-electrode one compartment cell at room temperature. The cell was filled with 1.5 mL of phosphate buffered saline (PBS) solution 1× as a supporting electrolyte. A covered or bare SPCE was used as the working electrode, platinum as the counter electrode, while an Ag|AgCl electrode containing a KCl saturated aqueous solution was the reference electrode (offset potential versus the standard hydrogen electrode, E° = 0.222 V at 25 °C). Oxidation-reduction cycles were registered within the potential range of −0.5 to +1.4 V at a scan rate of 100 mV/s.

Determination of the PYR loading ratio. The drug content was determined by taking 10 µL of PEDOT/PYR NPs suspension (10 mg/mL NPs in milli-Q water) into 990 µL

of methanol (PYR solvent). The suspension was sonicated and vortexed for 10 min, leading to a complete drug release in the alcoholic medium. Then, the NPs dispersion was centrifuged with a micro-centrifuge for 15 min at 2500 rpm. Finally, the supernatant was evaluated using UV-Vis spectroscopy. The calibration curve was prepared with the drug dissolved in methanol and read at 280 nm (Figure S2). The same procedure was applied to determine the drug released during the dialysis or after the electrical stimuli assays (see below).

The loading capacity (LC, in %) was calculated using the following equation:

$$LC = \frac{(W_i - W_f)}{W_{NPs}} \times 100 \qquad \text{(S1)}$$

where W_i, W_f and W_{NPs} refer to the PYR initial mass, PYR final mass and total NPs mass, respectively. Thus, the weight of PYR entrapped was determined by subtracting the weight of the total PYR fed (the drug introduced in the solution for the synthesis of PEDOT/PYR NPs) from the weight of the non-encapsulated drug or free drug (drug remaining in the supernantant after the synthesis of PEDOT/PYR NPs). The amount of the free PYR in the supernatant was determined by measuring the absorbance at a maximum wavelength of 280 nm.

PYR release. 25 µL of PEDOT/PYR NPs (10 mg/mL) were deposited into a 30 µL dialysis button, covered by a 3.5 kDa MWCO (Molecular Weight Cut-Off) dialysis membrane, immersed in 1.5 mL of PBS (pH 7.4) and kept in a shaker at 37 ºC at 80 rpm. Each day all the immersion solution was taken out to quantify the released drug and the solution was replaced by 1.5 mL of new media. For the first experiment, the release process was evaluated for 80 days in PBS solution. For the second experiment, instead, the releasing media was weekly changed from hydrophilic to hydrophobic

during three weeks by adding ethanol (EtOH) to PBS. More specifically, for the first, second and third week the release was evaluated in PBS alone, 90:10 PBS:EtOH and 30:70 PBS:EtOH, respectively. In order to compare the kinetics of the release process, results were normalized by the total amount of PYR encapsulated within the NPs or used as a free drug. The amount of released drug was evaluated by UV spectroscopy. Calibration curves were obtained by plotting the absorbance measured at 280 nm against the PYR concentration. All drug release tests were carried out using at least three replicas and the average was plotted.

Electrical stimulation for PYR release. Washed PEDOT/PYT NPs were re-suspended in milli-Q water to have a final concentration of 10 mg/mL. Then, NPs were placed on SPCEs and covered with chitosan, as described above. A three electrode configuration was used: the SPCE coated with the corresponding NPs as a working electrode, platinum as counter electrode, and Ag|AgCl as reference electrode. 1.5 mL of PBS 1× was used as electrolytic medium. The appropriate voltage was applied for a particular period of time. After the electrical stimulation, the medium was removed to determine the concentration of released PYR and substituted by fresh medium. The absorbance was measured at 280 nm. The influence of the time was evaluated by applying a constant voltage of 1.00 V and CV from -0.5 V to 0.5 V during 5, 15 and 30 min. A control experiment was performed in the absence of the stimulus to compare the results. All the measures were repeated at least three times and the average with the corresponding standard deviation were represented in the graphs.

Cytotoxicity evaluation: *In vitro* cytotoxicity evaluation of free PYR, PEDOT NPs, and PEDOT/PYR NPs for MG-63 cell line was determined by the MTT assay. Free

PYR was dissolved in methanol and then diluted in ethanol (the final concentration of ethanol in cell media was smaller than 10 %). All the other substances were prepared in milli-Q water. Cells were seeded at a density of 20×10^4 cells per well (100 μL each) in 96-well plates and incubated overnight. Subsequently, cells were exposed to a series of increasing free PYR, PEDOT NPs, and PEDOT/PYR NPs concentrations. Free PYR concentrations were 0.1, 1, 10, 50, 100, 500 and 1000 μg/mL, whereas PEDOT and PEDOT/PYM NPs concentrations were 0.0655, 0.125, 0.25, 0.5 and 1 mg/mL. Cells were incubated with the treatment for 24 h. Next day, 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. 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 2-way ANOVA using Tukey's test for pair-wise comparison with $p < 0.05$.

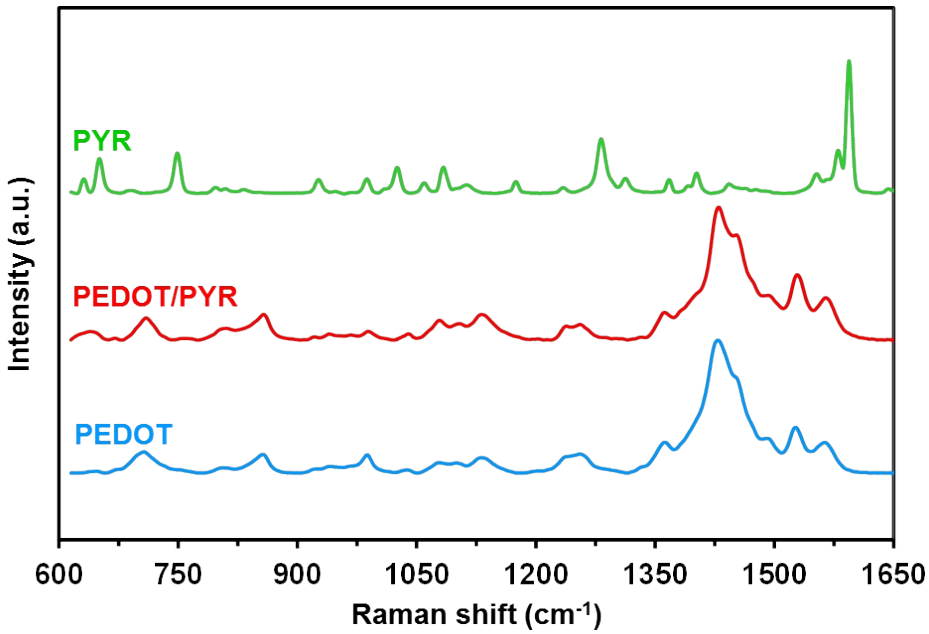

Figure S1. Raman spectra of free PYR, PEDOT NPs and PEDOT/PYR NPs.

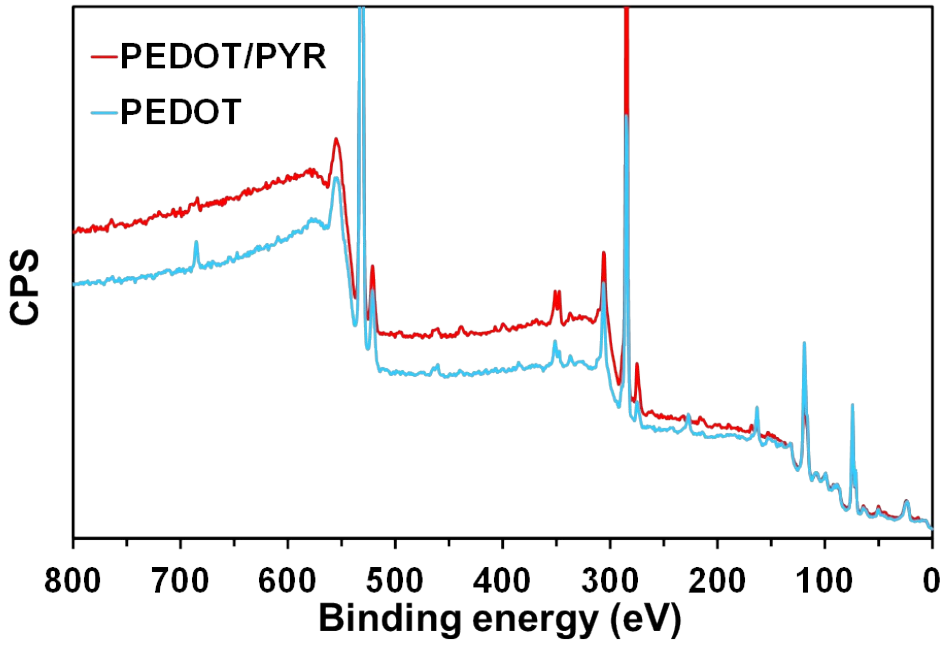

Figure S2. XPS spectra of PEDOT NPs and PEDOT/PYR NPs.

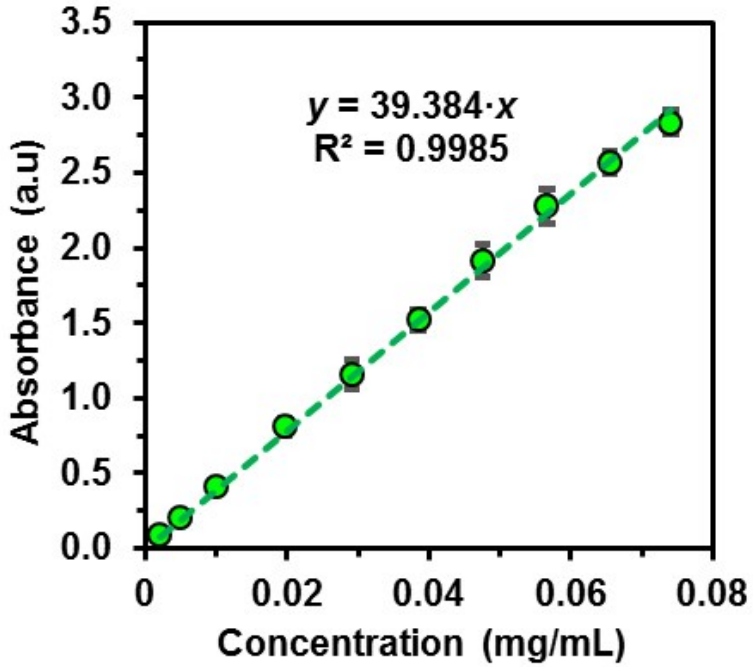

Figure S3. Calibration curve for PYR in methanol.

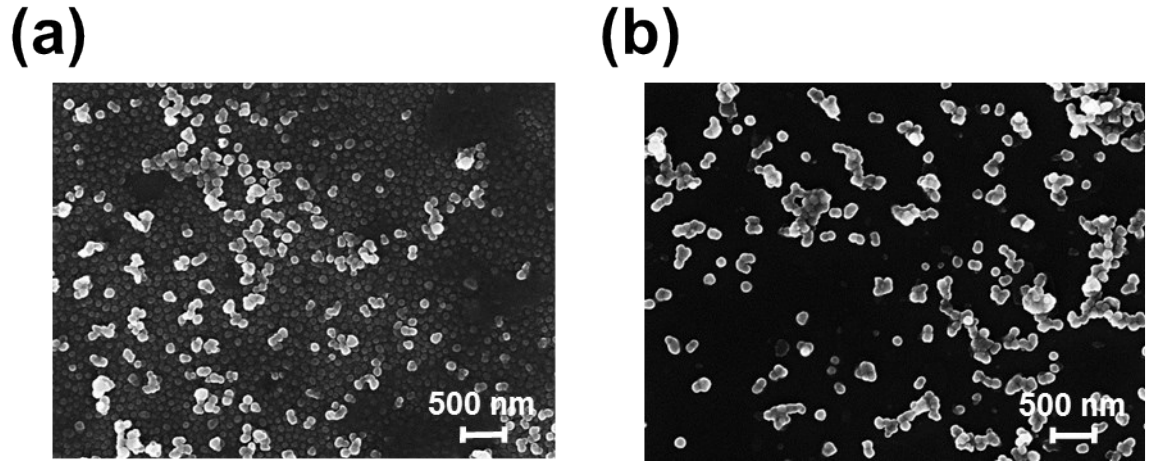

Figure S4. SEM micrographs at 50kX magnification for (a) PEDOT/PYR NPs and (b) PEDOT NPs.

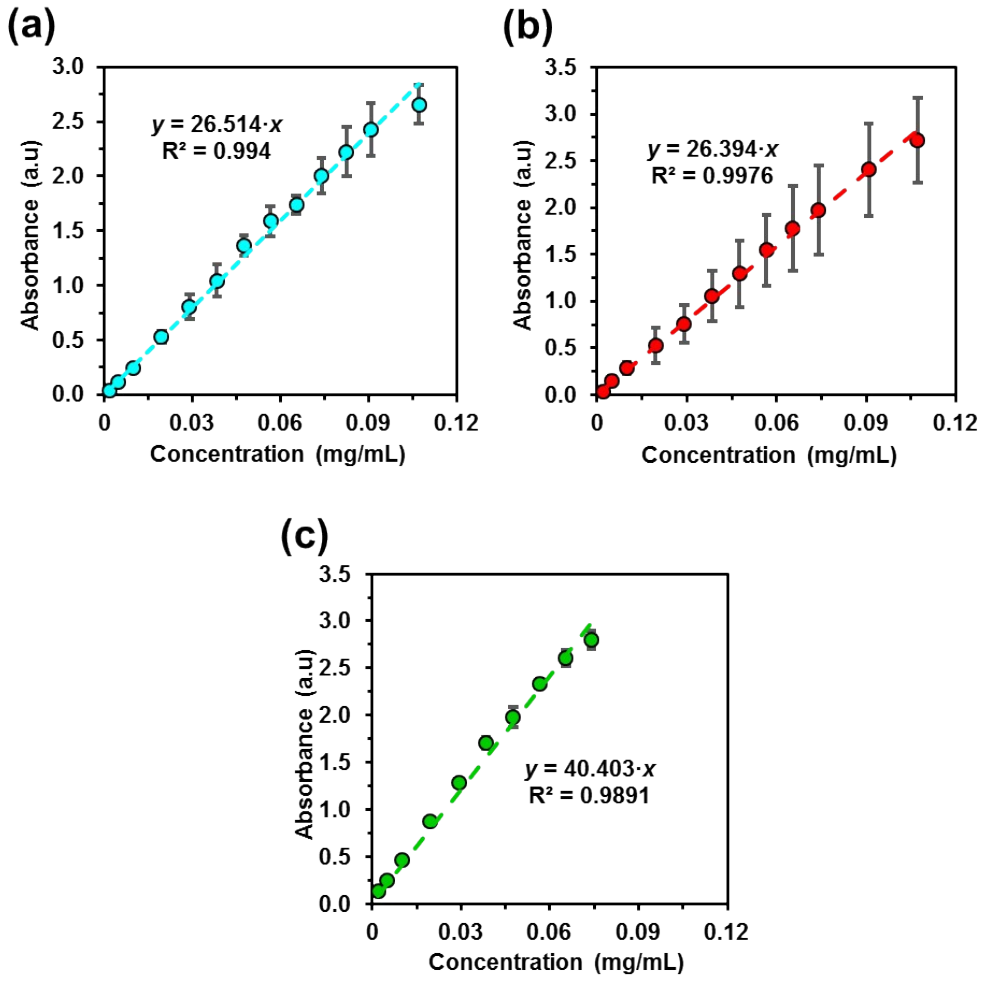

Figure S5. Calibration curve for PYR in (a) PBS, (b) 90:10 PBS:EtOH and 30:70 PBS:EtOH.

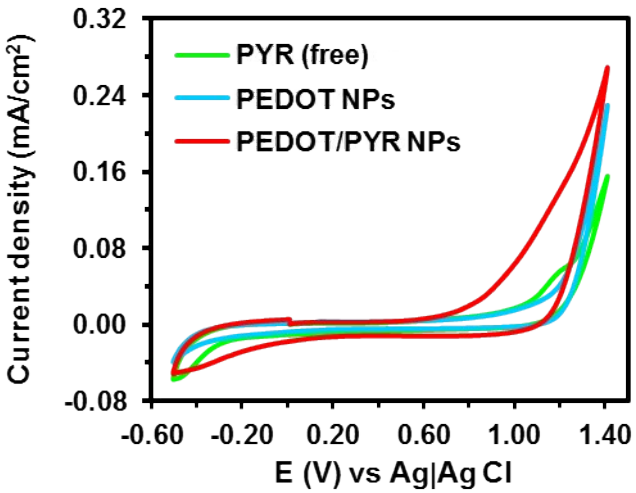

Figure S6. Cyclic voltammograms recorded from -0.5 to 1.4 V (scan rate: 100 mV/s) SPCEs coated with free PYR, PEDOT NPs or PEDOT/PYR NPs.