Peptide-directed assembly of functional supramolecular polymers for biomedical applications: electroactive molecular tongue-twisters (oligoalanine-oligoaniline-oligoalanine) for electrochemically enhanced drug delivery

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Experimental

Materials and Synthetic/Analytical Methods

Unless otherwise stated, all chemicals were of ACS grade, purchased from Sigma-Aldrich and used as received without further purification. ^{1}H and ^{13}C NMR spectra were recorded on an Agilent 600 MHz NMR spectrometer, using residual solvent ^{1}H peaks as internal references for the ^{1}H NMR spectra and the solvent ^{13}C peaks as references for the ^{13}C NMR spectra. The following notation is used for the ^{1}H NMR spectral splitting patterns: multiplet (m), broad (br). Infrared spectroscopy was carried out on a Thermo Scientific Nicolet 380 FT-IR Spectrometer (Thermo Fisher Scientific Inc., USA). Spectra were recorded in ATR mode at 21 ^{o}C, with a 1 cm^{-1} resolution and 64 scans (corrected for background and atmosphere using OMNIC software provided with the spectrometer).

Synthesis of Nitro-Terminated Aniline Tetramers (O_2N-An_4-NO_2)

The synthesis of the nitro-terminated aniline tetramers was carried out in accordance with the literature and analytical data were in agreement with the literature (Scheme S1).[23,24]

Synthesis of Amine-Terminated Aniline Tetramers (H_2N-An_4-NH_2)

The synthesis of the amine-terminated aniline tetramers was carried out in accordance with the literature and analytical data were in agreement with the literature (Scheme S1).[23,24]

Synthesis of Nitro-Terminated Aniline Hexamers (O_2N-An_6-NO_2)

The synthesis of the nitro-terminated aniline hexamers was carried out in accordance with the literature and analytical data were in agreement with the literature (Scheme S1).[23,24]

Synthesis of Amine-Terminated Aniline Hexamers (H_2N-An_6-NH_2)

The synthesis of the amine-terminated aniline hexamers was carried out in accordance with the literature and analytical data were in agreement with the literature (Scheme S1).[23,24]

Synthesis of L-Alanine N-Carboxyanhydride (Ala-NCA)

The synthesis of the L-alanine N-carboxyanhydride was carried out in accordance with the literature and analytical data were in agreement with the literature (Scheme S2).[25]

Synthesis of Tetraaniline-Based Molecular Tongue Twister 1 (MTT1)

MTT1 was synthesised by adaption of the literature methodology.[22,25] 1 molar eq. of amine-terminated aniline tetramer (**H_2N-An_4-NH_2**) and 10 molar eq. L-alanine N-carboxyanhydride (**Ala-NCA**) were stirred in anhydrous DMF (solids at 100 mg mL^{-1}) at room temperature under an inert atmosphere of argon for 4 days. The product was precipitated in diethyl ether, the solids isolated by filtration, washed with diethyl ether and dried under high vacuum for 48 hours. The solids were suspended in hexafluoroisopropanol (HFIP) and the HFIP-soluble fraction was

extracted by shaking the suspension in 15 mL of HFIP at 1000 rpm for 24 hours, after which the solids were removed by filtration and the product isolated by evaporation of the HFIP with a rotary evaporator, followed by drying under high vacuum for 48 hours (Scheme 1). MTT1 was isolated in a yield of 10 mass %, relative to the total mass of starting materials (**ALA-NCA** and **H_2N-AN_4-NH_2**). ^{1}H NMR (600 MHz, HFIP-d_2): δ_H = 7.57-6.58 (*br m*, 16H, Ar-*H*), 4.14 (*br m*, 10H, C*H*CH_3), 1.37 (*br m*, 30H, C*H*$_3$). IR (ATR) ν_{max} cm^{-1} 1648 (C=O, amide 1), 1560 (C=N and C=C stretch, oligoaniline), 1530 (C=O, amide 2), 1496 (C-C stretch, oligoaniline).

SYNTHESIS OF HEXAANILINE-BASED MOLECULAR TONGUE TWISTER 2

MTT2 was synthesised by adaption of the literature methodology.[22,25] 1 molar eq. of amine-terminated aniline hexamer (**H_2N-AN_6-NH_2**) and 10 molar eq. L-alanine N-carboxyanhydride (**ALA-NCA**) were stirred in anhydrous DMF (solids at 100 mg mL^{-1}) at room temperature under an inert atmosphere of argon for 4 days. The product was precipitated in diethyl ether, the solids isolated by filtration, washed with diethyl ether and dried under high vacuum for 48 hours. The solids were suspended in hexafluoroisopropanol (HFIP) and the HFIP-soluble fraction was extracted by shaking the suspension in 15 mL of HFIP at 1000 rpm for 24 hours, after which the solids were removed by filtration and the product isolated by evaporation of the HFIP with a rotary evaporator, followed by drying under high vacuum for 48 hours (Scheme 1). MTT2 was isolated in a yield of 9 mass %, relative to the total mass of solids (**ALA-NCA** and **H_2N-AN_6-NH_2**). ^{1}H NMR (600 MHz, HFIP-d_2): δ_H = 7.57-6.58 (*br m*, 24H, Ar-*H*), 4.14 (*br m*, 26H, C*H*CH_3), 1.31 (*br m*, 78H, C*H*$_3$). IR (ATR) ν_{max} cm^{-1} 1644 (C=O, amide 1), 1560 (C=N and C=C stretch, oligoaniline), 1532 (C=O, amide 2), 1496 (C-C stretch, oligoaniline).

Film Preparation and Characterization

FILM PREPARATION

Films were prepared by casting solutions of the copolymers in hexafluoroisopropanol (HFIP, typically 0.05 g/mL) onto HFIP insoluble substrates, (e.g., microscope slides with dimensions of 2.5 cm x 5 cm or glassy carbon electrodes with surface areas of 0.0314 cm^2). The solvent was allowed to evaporate in a fume hood, and the films were subsequently dried under vacuum for 48 hours at room temperature. The polymers were doped with camphorsulfonic acid (CSA) by the addition of CSA to the HFIP solution prior to casting on microscope slides. Unless otherwise stated CSA doping was at a mass ratio of 1:10 CSA:MTT.

PROFILOMETRY

Profilometry was carried out using a Veeco Dektak 150 Stylus Profilometer (Veeco Instruments Inc., NY) fitted with a diamond stylus tip. The profilometer was isolated on an air table to reduce ambient vibrations. The profilometer was operated at 10 mg of stylus force, and used to record profiles of distances of ca. 1 cm, recording data points every 555 nm. Data analysis was carried out with the software provided by the manufacturer, which allowed the determination of the thickness and roughness of the films. The surface roughness parameters are analyzed and reported in accordance with the ISO 25178 series. The average roughness (R_a) is the arithmetic average of the deviation from the mean line, and is the most used international parameter of roughness, and the root-mean-square roughness (R_q) is based upon this.

CONDUCTIVITY DETERMINATION

The conductance of films of the polymers were measured in accordance with protocol IPC-TM-650, number 2.5.17.2 described by the Institute for Interconnecting and Packaging Electronic Circuits. Films supported on glass slides were examined by chronoamperometry using a CHI900C electrochemical workstation (CHI instruments, Austin, TX). Chronoamperometric measurements were made with a two-point probe system (copper alligator clips), by connecting counter and reference electrodes together. Briefly, two thin strips of adhesive-backed copper tape (Ted Pella, Inc., Redding, CA) were attached to the films, parallel to one another, separated by a distance of 0.5 cm. The working and counter electrodes were clipped on the strips of copper tape, and the current measured for 50 seconds during a potential step experiment at 10 V. The electrodes were moved to different positions after each measurement, and the

current passed was recorded in at least five different positions. The resistance (R, Ω) of the films was determined in accordance with equation 1:

$$R = V/I \quad (1)$$

The resistivity (Ω/cm) of the films was determined in accordance with equation 2:

$$\rho = Rwt/L \quad (2)$$

In which: w corresponds to the width of the film in cm (2.5 cm); t corresponds to the thickness of the film in cm (as determined via profilometry); and L corresponds to the length of the film in cm (0.5 cm). The conductivity (S/cm) of the films was determined in accordance with equation 3:

$$\sigma = 1/\rho \quad (3)$$

X-RAY DIFFRACTION (XRD)

XRD data was collected on a Rigaku R-Axis Spider diffractometer with an image plate detector using a graphite monochromator with CuKα radiation (λ = 1.5418 Å) at room temperature. The instrument was controlled using Rapid/XRD diffractometer control software (Rapid/XRD Version 2.3.8., Rigaku Americas Corporation, The Woodlands, TX). The integration of the two dimensional data into a one dimensional pattern was accomplished using 2DP (2DP Version 1.0., Rigaku Americas Corporation, The Woodlands, TX). Percentage crystallinities were determined by taking the ratio of the crystalline area to the amorphous area in the XRD spectrum.

In Vitro Drug Delivery and Cell Culture Studies

PREPARATION OF DRUG DOPED FILMS

Films of ca. 3-4 mg were prepared by casting solutions of the polymers and DMP (at a mass ratio of 10:1 polymer:DMP) in hexafluoroisopropanol (typically 0.05 g/mL) onto a glassy carbon electrode (0.0314 cm^2, CH Instruments, Inc.). The solvent was allowed to evaporate in a fume hood, and the films were subsequently dried under vacuum for 48 hours at room temperature.

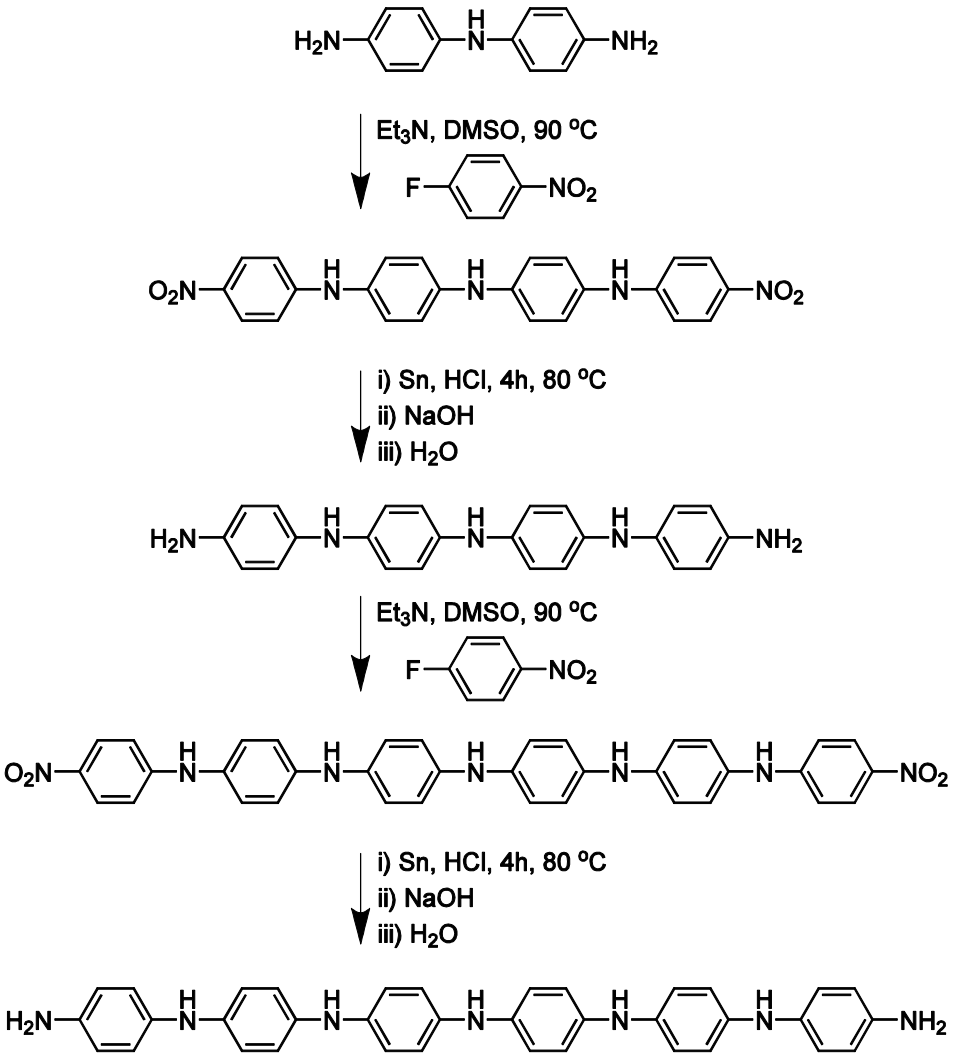
DRUG DELIVERY STUDIES

Voltammetry experiments were carried out using a CHI6273C electrochemical analyzer (CH Instruments, Inc.). Phosphate buffered saline (PBS, pH 7.0) was deoxygenated for 10 minutes with argon before the electrochemical measurements were made. Electrochemically-triggered release (i.e., de-doping) of DMP from the films deposited on glassy carbon substrates by potential cycling, was achieved using a three-electrode system consisting of one polymer film-coated glassy carbon working electrode, a Pt mesh counter electrode, and an Ag/AgCl reference electrode in 4 mL PBS. Prior to each experiment there was a 10 s "quiet time", the initial potential was 0 V, the high potential was 0.7 V, the low potential was -0.5 V, the initial scan was positive, the current was measured at intervals of 0.001 V, the scan rate used in all experiments was 50 mV s^{-1}, and this stimulation lasted 62 seconds. The films were allowed to rest for 14 minutes after which the quantity of DMP in solution was quantified by UV spectroscopy. The medium was unchanged between cycles, and the data are reported as cumulative release as a percentage of the total mass of drug in the film over the period of the experiment. These data are compared to passive DMP release from unstimulated films measured every 15 minutes. DMP release was quantified by UV spectroscopy using a BioTek Epoch ® plate reader (BioTek US, Winooski, VT) equipped with a Take3 Micro-volume Plate and Gen5 v2.04 Software supplied with the plate reader. Two samples of 2 μL were removed from the release medium at specific time points and absorbance readings were carried out at 242 nm (the characteristic absorbance band of DMP). Absorptions were corrected by subtracting the reading of PBS alone from each sample. A standard calibration curve for DMP was plotted to define the quantitative relationship between the observed absorbance and the concentration of DMP. Prior to the experiment, the mass of the drug-doped polymer film on the substrate was determined by subtracting the mass of the substrate from the mass of the polymer-coated substrate. Data are plotted as % DMP release relative to the quantity of DMP theoretically in the film at the beginning of the experiment, and all data are the average of at least three samples.

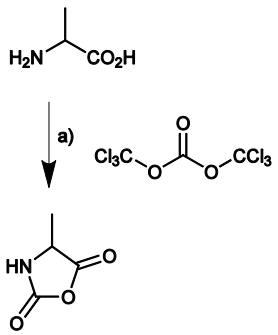
CELL CULTURE

All reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Human dermal fibroblasts (HDFs) were purchased from Lonza (Gaithersburg, MD). Commercially available tissue-culture treated Corning® Costar® tissue culture plates were used for control experiments and sections of glass slides coated with polymer films of ca. 1 cm^2 (optionally doped with CSA) were inserted in tissue culture plates and sterilized by incubation in 70 % ethanol solution, followed by exposure to UV for 30 minutes. After sterilization, films were incubated for 30 minutes under 3 mm of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} amphotericin, 0.1 mM non-essential amino acids, and 1 ng ml^{-1} basic fibroblast growth factor. Medium was aspirated and replaced prior to HDF seeding. Cell viability before starting the experiment was determined by the Trypan Blue (Sigma, USA) exclusion method, and the measured viability exceeded 95 % in all cases. Cells were seeded on tissue-culture treated Corning® Costar® tissue culture plates at 10,000 cells per cm^2, and all other substrates (i.e. MTT-based films) at 2,500 cells per cm^2, under 3 mm of medium, and incubated at 37 °C, 95 % humidity, and a CO_2 content of 5 %. The cell adhesion and quantification studies were carried out using the AlamarBlue® assay in accordance with the manufacturers instructions. After 2 days the medium was aspirated and the films were washed gently with PBS, followed by the addition of fresh medium containing 10% v/v AlamarBlue® reagent. After 2.5 hours of culture, the medium was aspirated and replaced with fresh medium, and 100 µL of the aspirated medium containing the AlamarBlue® reagent was placed in a 96 well plate, and the fluorescence was measured with a fluorimeter (Synergy HT Multi-Mode Microplate Reader, Biotek US, Winooski, VT). Two controls were considered during the measurement of the fluorescence: the first was wells containing the medium alone (i.e. no cells of AlamarBlue® reagent), which was not fluorescent; and the second was wells that contained the AlamarBlue® reagent but no cells (used for background correction). Numbers of cells adhered to the various surfaces studied herein are reported relative to their initial seeding density, which was assigned an arbitrary value of 100%. After another 2 days (i.e. at 4 days after initial seeding) the viability of the cells was evaluated using a LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). The medium was removed and the cells were incubated with 4 µM ethidium and 2 µM calcein AM in PBS for 15 min at 37 °C in the dark. Live cells were stained green because of the cytoplasmic esterase activity, which results in reduction of calcein AM into fluorescent calcein, and dead cells were stained red by ethidium, which enters into cells via damaged cell membranes and becomes integrated into the DNA strands. Fluorescence images of cells were captured using an Olympus DP80 camera (Olympus America Inc., Miami, FL, USA) attached to a fluorescence microscope (IX-70; Olympus America Inc.) running CellSens 1.12 software (Olympus America Inc.). Cells were counted with ImageJ (http://imagej.nih.gov/ij/).

Figures and Schemes



Scheme S1 Synthesis of oligoanilines.



Scheme S2 Synthesis of alanine-N-carboxyanhydride. A) α-pinene, EtOAc, 90 °C, 4h.

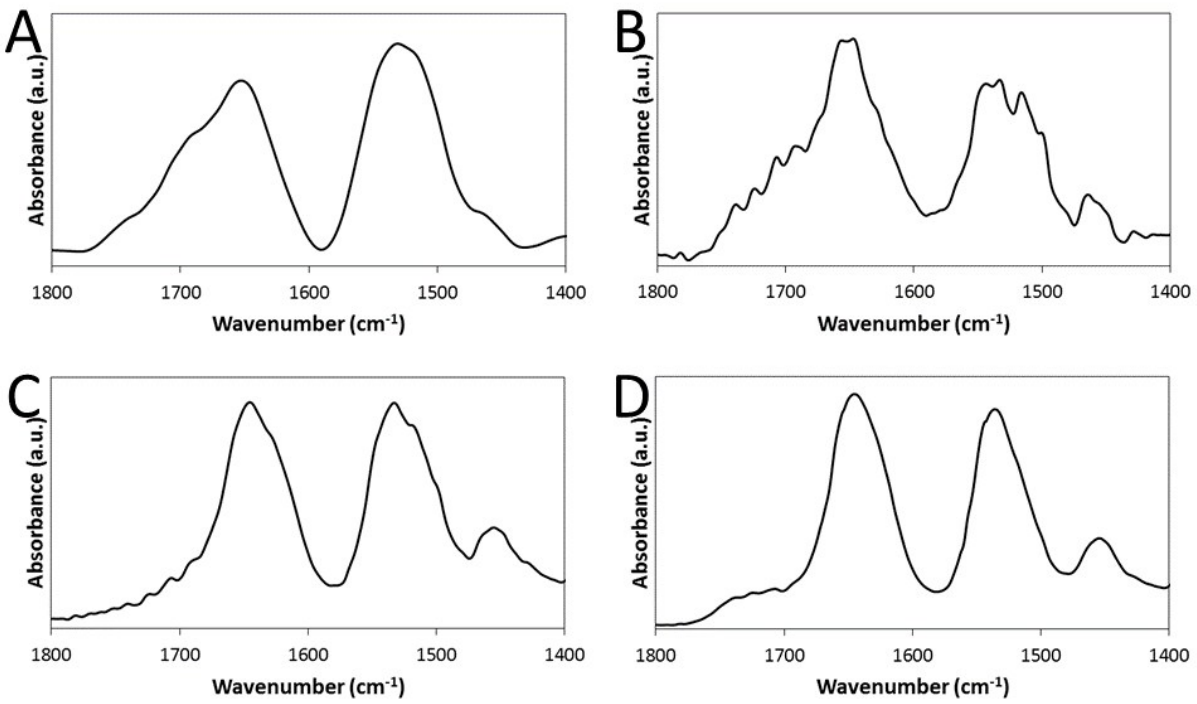


Fig. S1 FTIR spectra of the electroactive molecular tongue twisters. A) MTT1 undoped. B) MTT1 doped with CSA. C) MTT2 undoped. D) MTT2 doped with CSA.

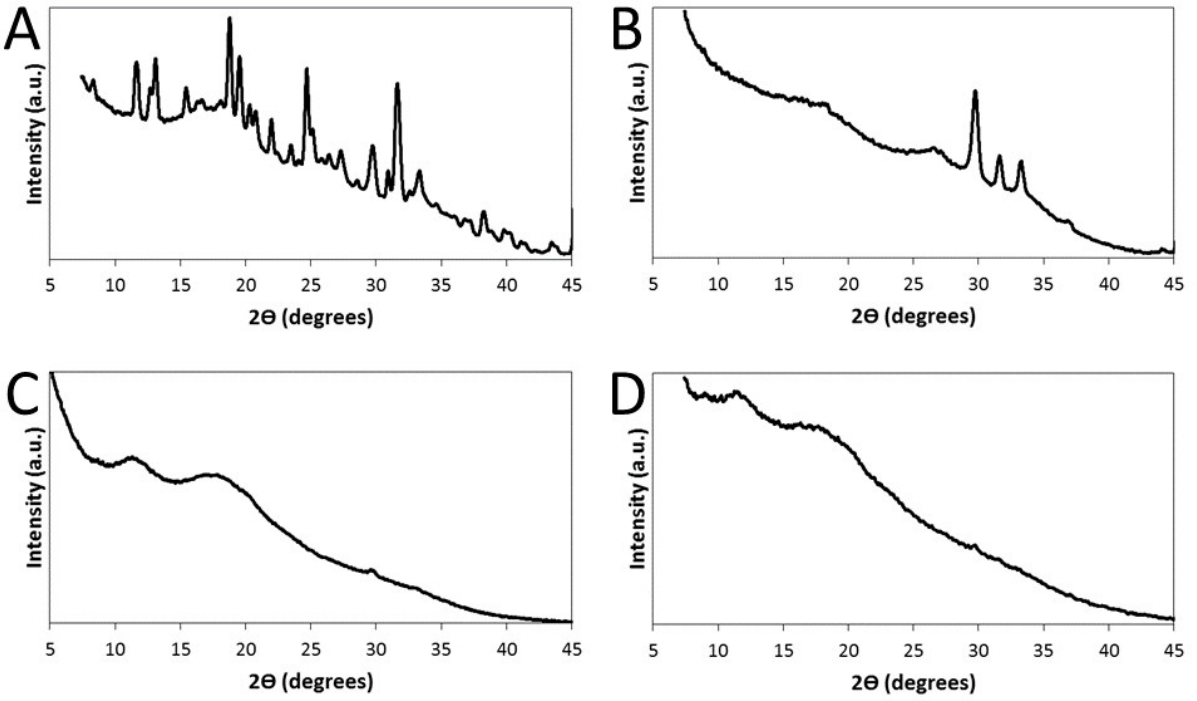


Fig. S2 XRD spectra of the electroactive molecular tongue twisters. A) MTT1 undoped. B) MTT1 doped with CSA. C) MTT2 undoped. D) MTT2 doped with CSA.

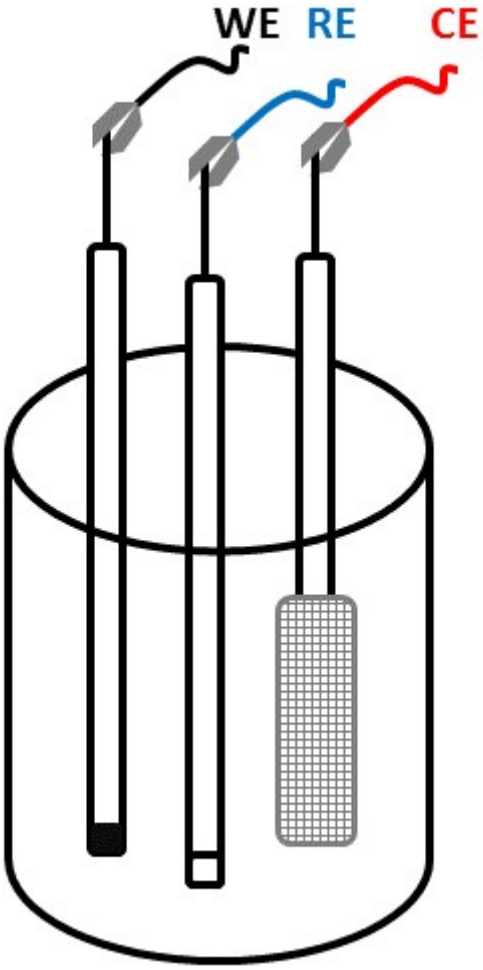


Fig. S3 Experimental setup for electrochemically-triggered drug delivery. Pt mesh counter electrode (CE), Ag/AgCl reference electrode (RE), DMP-doped polymer film coated on a glass carbon working electrode (WE).

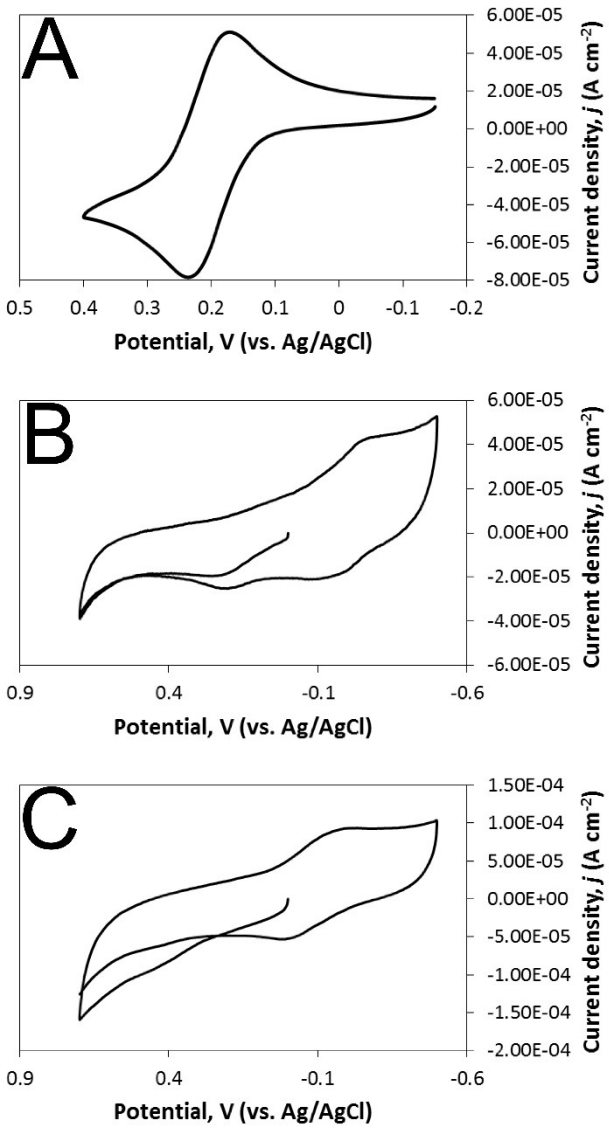


Fig. S4 A) Voltammogram of 0.6 mM ferrocenemethanol in 0.1 M KCl. Scan rate = 10 mV s^{-1}. See the literature or a comparison: A. Heras, A. Colina, J. Lopez-Palacios, A. Kaskela, A. Nasibulin, V. Ruiz, E. Kauppinen, Electrochem. Commun. 2009, 11, 442-445. B) Voltammogram of DMP-doped MTT1. Scan rate = 50 mV s^{-1}. C) Voltammogram of DMP-doped MTT2. Scan rate = 50 mV s^{-1}.

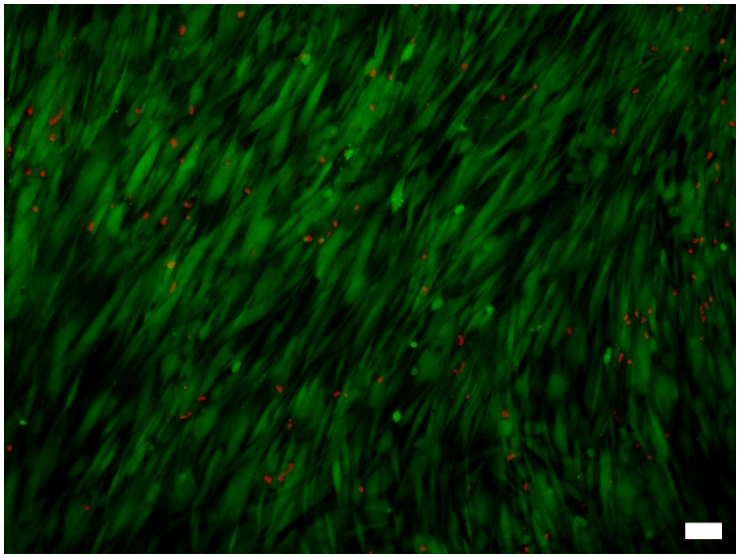


Fig. S5 HDF adhesion on the surface of Corning Costar® Tissue Culture Plates (TCP control). Live cells were stained green by calcein and dead cells were stained red by ethidium using a LIVE/DEAD® Viability/Cytotoxicity Kit. The scale bar represents 100 μm.