

Conducting polymer nanoparticles decorated with collagen mimetic peptides for collagen targeting

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Experimental Section

Materials

Commercial chemicals and solvents were purchased from Sigma Aldrich or Fisher Scientific and used as received unless otherwise specified. Poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,10,3)-thiadiazole)] (PFBT, M_n 10000-20000) was purchased from Sigma Aldrich. Peptide synthesis reagents and Fmoc-amino acids were acquired from Advanced ChemTech; Fmoc-Hyp(*t*Bu)-OH was purchased from EMD Millipore Chemicals. The solid phase resin for peptide synthesis was purchased from Peptides International. Deionized water was purified in a Barnstead Nanopure system to a final resistance of 18.2 m Ω ; it will be referred to as nanopure water. Azobisisobutyronitrile (AIBN, 98% Aldrich) was recrystallized from methanol before use. Styrene was purified by passage through a short alumina column. Spectrapor membranes (MWCO 10 kDa) were used for dialysis. Rat tail type I collagen solution was obtained from BD Science. Amine-terminated poly(ethylene glycol) was synthesized as previously reported.¹

Characterization

Gel permeation chromatography (GPC) was carried out on a Waters 1515 Isocratic HPLC. The equipment was equipped with two Styragel[®] columns (HR4 and HR3, 300 mm \times 7.8 mm) connected in series, a differential refractive index detector (Waters 2414), and a UV-Visible Detector (Waters 2489). HPLC grade tetrahydrofuran (THF) was used as the eluent, at a flow rate of 1 mL/min. Samples were filtered through a 0.22 μ m PVDF syringe filter (Millipore) before injection. All measurements were carried out at 30 $^{\circ}$ C. Molecular weights are reported referenced to monodisperse polystyrene standards (Shodex SL-105). NMR spectra were recorded on a Bruker AV 400 MHz spectrometer in either CDCl₃ or d₆-DMSO. Spectra were referenced to CHCl₃ (7.26 ppm) or d₆-DMSO (2.50 ppm). Mass spectrometry data of the CMP peptide were recorded on a Bruker AutoFlex MALDI-TOF mass spectrometer using standard settings and α -Cyano-4-hydroxycinnamic acid (CHCA) as matrix. Dynamic light scattering (DLS) experiments were carried out on a Malvern Instruments Nano-ZS ZetaSizer equipped with a 4 mW He-Ne laser operating at 633 nm. Measurements were performed at 25 $^{\circ}$ C at a scattering angle of 173 $^{\circ}$. Autocorrelation functions of backscattered light were analyzed using a DTS version 6.12 software. Cumulants method was used to obtain hydrodynamic radii and polydispersity. Measurements were carried out five times with a duration of 150 s each. Bright-field transmission electron microscopy (TEM) was performed on a FEI Tecnai 12 TWIN transmission electron microscope operated at an acceleration voltage of 100 kV. TEM images were recorded by a SIS Megaview III wide-angle CCD camera. Copper grids were treated under plasma to render carbon films hydrophilic. Sample grids were prepared by placing a carbon-coated copper grid (Electron Microscopy, Hatfield, PA) onto a droplet of nanoparticle suspension (50 μ L). After 5 min the grid was washed under 8 drops of Nanopure water and placed onto a drop (50 μ L) of a 2 wt% aqueous uranyl acetate solution for 20 s. Grids were then blotted with filter paper and allowed to dry at room temperature prior to imaging. Fluorescence data were acquired with a Flurolog-3 system (HORIBA Jobin Yvon Inc., NJ). Excitation and emission bandwidths were set to 2 nm.

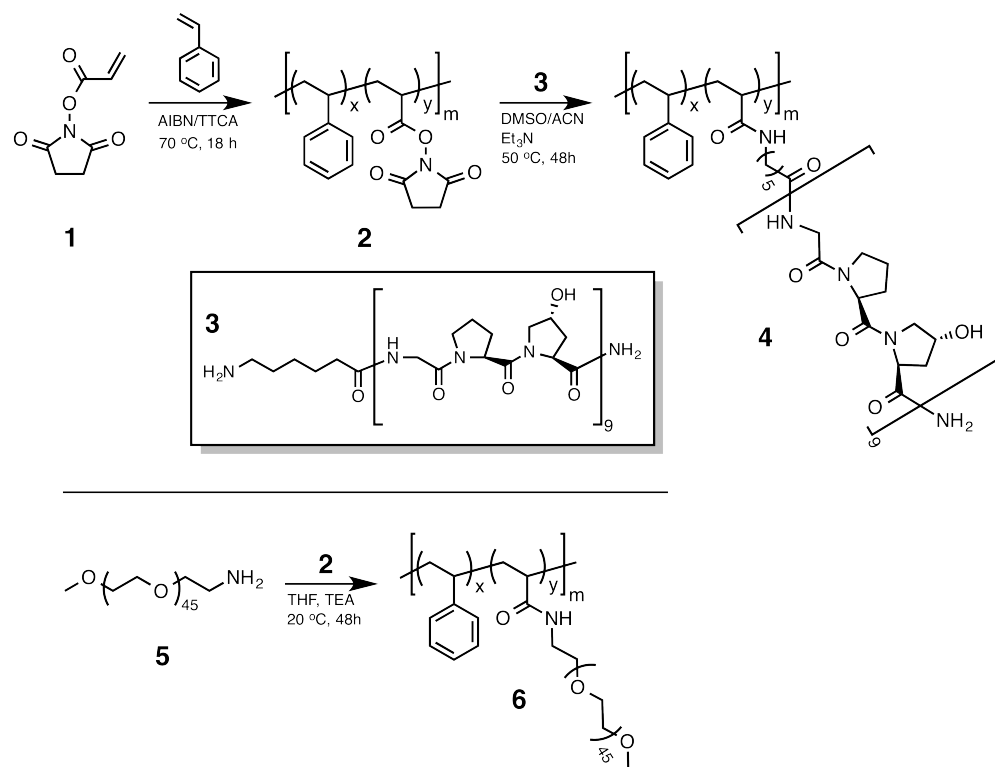
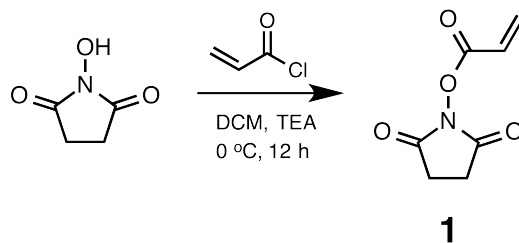


Figure S1. Summary of polymer amphiphile synthesis.

Methods

Synthesis of *N*-acryloxysuccinimide (NAS, **1**)



N-acryloxysuccinimide (NAS) was synthesized according to a previously described procedure.² Briefly, *N*-hydroxysuccinimide (3.80 g, 33 mmol) and triethylamine (4.66 mL, 33 mmol) were dissolved in anhydrous dichloromethane (DCM, 20 mL) and the mixture was cooled to 0 °C using an ice bath. Acryloyl chloride (3 g, 33 mmol) was added dropwise over 30 min under vigorous stirring. The reaction was allowed to proceed overnight at room temperature. After removing NEt_3Cl salts by filtration, the solution was washed with water (2x), then with NaHCO_3 (2x) and again with water. The organic phase was collected, dried over MgSO_4 , filtered and evaporated to afford a yellowish residue. Pure NAS monomer (**1**) was obtained by recrystallization from a mixture of ethyl acetate/hexane (20/80 v/v). Yield: 4.6 g, 82%. ^1H NMR (400 MHz, CDCl_3), δ 6.78-6.60 (d, 1H), 6.40-6.30 (q, 1H), 6.20-6.15 (d, 1H), 2.87 (s, 4H). Spectrum and peak assignment are provided in Figure S2.

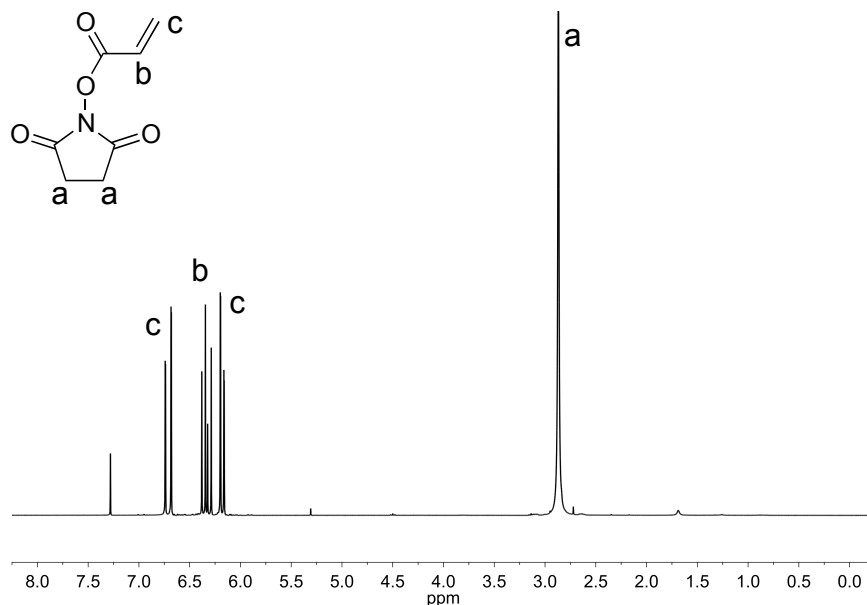
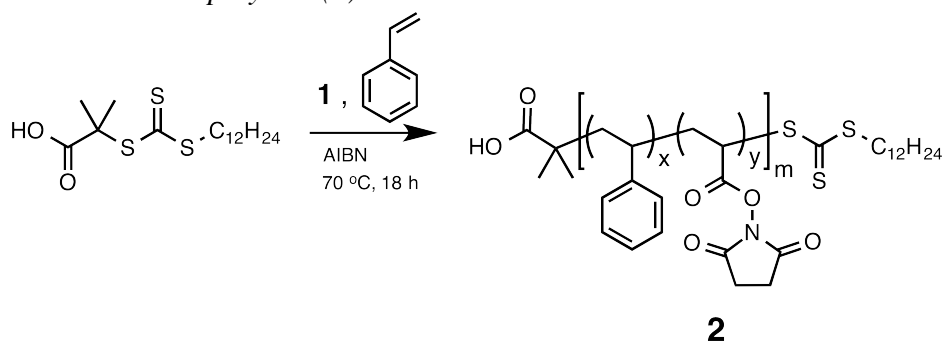


Figure S2. ^1H NMR spectrum and peak assignments of NAS (**1**).

Synthesis of PS-co-PNAS copolymer (**2**)



To a oven dried 25 mL Schlenk flask equipped with a stir bar, were added styrene (1 g, 9.6 mmol), **1** (100 mg, 0.6 mmol), AIBN (1.64 mg, 10 μ mol), the chain transfer agent TTCA (2-(dodecylthiocarbonothioylthio)-2-methylpropanoic acid) (37 mg, 0.1 mmol) and DMF (2 mL). The mixture was purged with argon for 30 min at room temperature and then placed in a 75 $^{\circ}$ C oil bath to initiate polymerization. After 16 h, the polymerization was terminated by cooling the mixture to room temperature and opening the flask to air. The mixture was concentrated and the residue taken in THF. The copolymer was isolated by precipitation from THF into methanol. This procedure was repeated 3 times to afford a light pale yellow powder. Yield (650 mg, 60 %) was calculated based on styrene conversion (80 %) as measured by ^1H NMR. ^1H NMR (400 MHz, DMSO- d_6), δ 7.45-6.25 (b, 400 H), 2.80-2.60 (b, 28 H), 2.3-1.0 (b, 304 H) $M_n = 8918$ g/mol; GPC: $M_n = 4700$, $M_w = 5374$, PDI = 1.14. Spectrum and peak assignment are provided in Figure S3. The composition of the copolymer, as determined by ^1H NMR: 92 mol% styrene and 8 mol% NAS. The degree of polymerization is approximately 87; $x=0.92$, $y=0.08$ and $m=87$. Accordingly, the number of repeat units of styrene is 80, while the number of repeat units for NAS is 6.

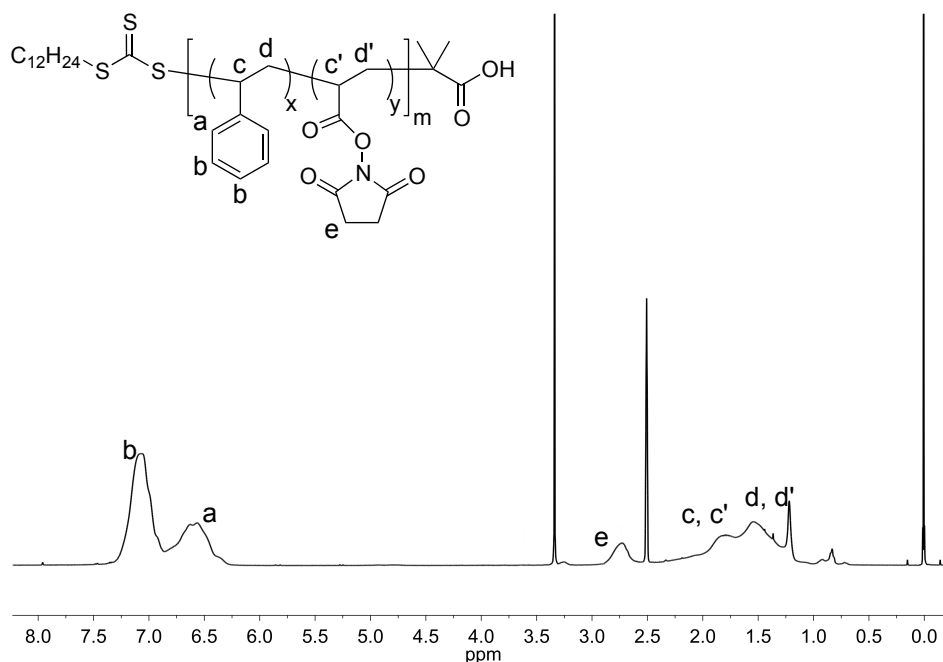
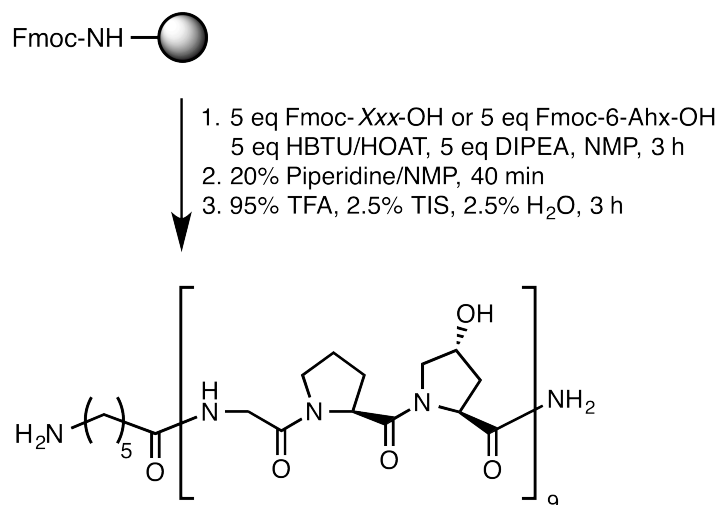


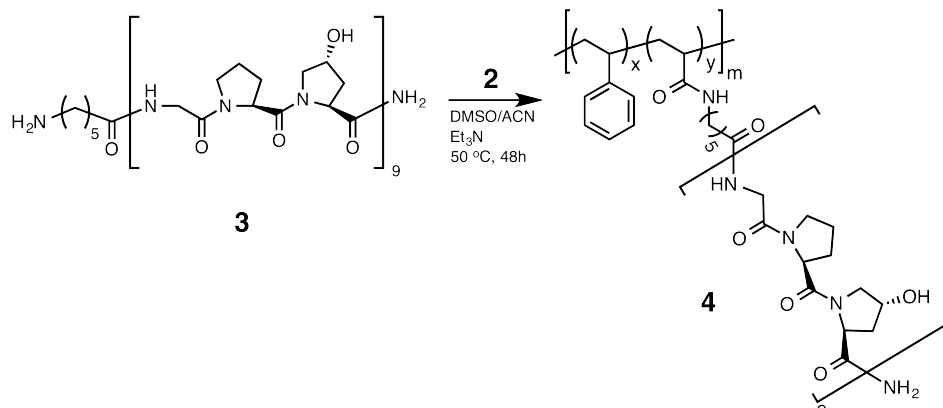
Figure S3. ^1H NMR spectrum and peak assignments of PS-co-PNAS (**2**).

Synthesis of collagen mimetic peptide (CMP, **3**)



The collagen mimetic peptide (CMP) with the linker 6-aminohexanoic acid was synthesized automatically on a peptide synthesizer (Applied BioSystems model 431) using conventional Fmoc-mediated solid-phase chemistry as described previously.^{3,4} TentaGel R RAM resin (0.19 mmol of reactive sites/g; 0.1 mmol loading level) was loaded in the reaction vessel. Coupling reactions were achieved using 5 molar equivalents of amino acids activated by 5 molar equivalents HBTU and HOAT in the presence of DIPEA, for 1 molar equivalent of reactive site on resin. Fmoc protection groups were removed by treatment with piperidine. All amino acids were double-coupled followed by acetylation using acetic anhydride to minimize the production of peptides with deleted sequence. The full length CMP was cleaved from resin by treating the resin with TFA/TIS/H₂O (95:2.5:2.5) for over 3 h and the cleaved peptide was purified by reverse phase HPLC on a Vydac C18 column using a linear gradient mixture of water (0.1% TFA) and acetonitrile (0.1% TFA) (5-30% acetonitrile gradient in 30 min). The purified peptides were analyzed by MALDI-TOF MS: *m/z* calculated 2557.7 [M + H⁺] for Ahx-(GPO)₉, found 2557.9 [M + Na⁺].

Conjugation of CMP to PS-co-PNAS (PS-g-CMP, **4**)



The CMP (**3**) was conjugated onto the PS-g-PNAS backbone through NHS pendant groups. PS-co-PNAS (1.5 mg, 1 μ mol of NHS) and CMP (2.6 mg, 1.02 μ mol) were dissolved in anhydrous DMSO/acetonitrile (2 mL, 1/1 v/v) in a 5 mL round bottom flask. The flask was sealed and purged with argon for 15 min. Then, triethylamine (10 μ L, 70 μ mol) was injected and the reaction was carried out at 50 °C for 2 days. To remove the byproducts originated by the reaction and any unreacted peptide, the mixture was dialyzed (10 kDa MWCO, Spectrapor) against nanopure water for 3 days. The final product was isolated by lyophilization. Yield: 3.3 mg, 81%. The amount of conjugated peptide was determined by ¹H NMR spectroscopy. ¹H NMR (400 MHz, DMSO-d₆), δ 7.45-6.25 (b, 400 H), 5.45-5.40 (s, 5 H) 5.12-4.95 (b, 38 H), 4.70-4.20 (b, 92 H), 2.53 (s), 2.3-0.8 (b, 580 H), M_n = 21078 g/mol. Spectrum and peak assignment are provided in Figure S4. The average number of CMP peptides per polymer backbone was determined to be 6; the degree of polymerization (m) is approximately 87; x=0.92, y=0.08.

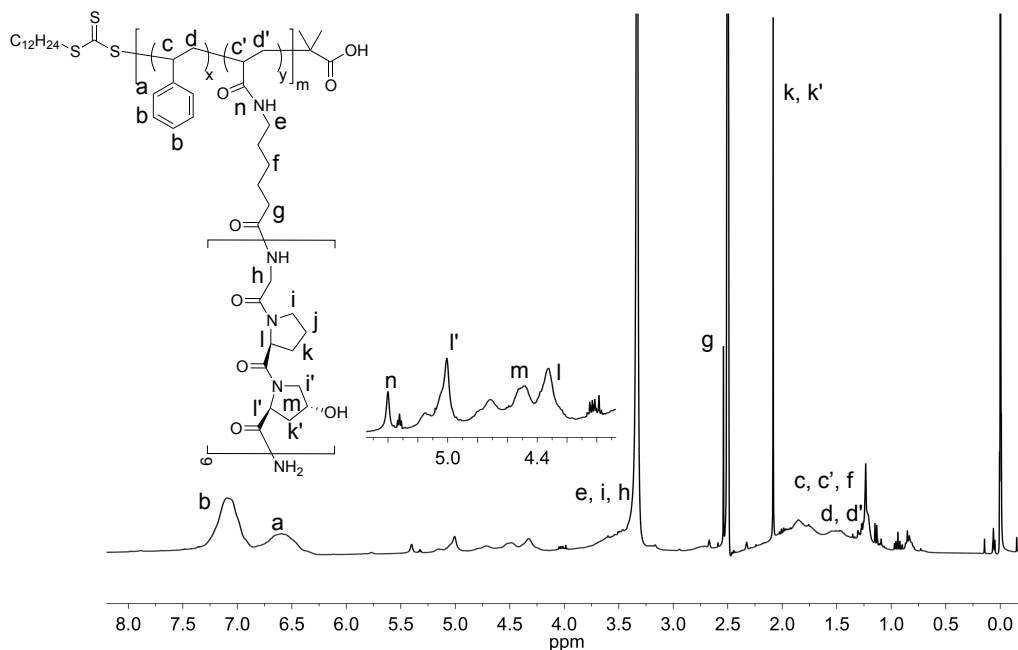
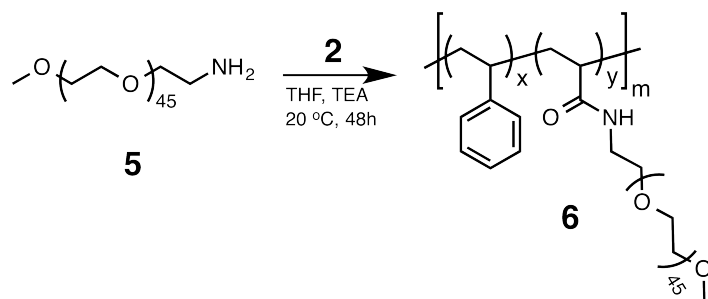


Figure S4. ¹H NMR spectrum and peak assignments of PS-g-CMP (**4**).

Conjugation of PEG to PS-co-PNAS (PS-g-PEG), **6**



A 25 mL Schlenk flask was charged with PS-co-PNAS (**2**, 100 mg, 68 μ mol of NHS), monomethoxypoly(ethylene glycol)-NH₂ (**5**, 141 mg, 69 μ mol) and 3 mL anhydrous THF. **5** was synthesized according to a previously published protocol.¹ The solution was degassed with argon for 15 min. Subsequently, anhydrous triethylamine (20 μ L, 136 μ mol) was added via syringe and the reaction was allowed to proceed at room temperature for 24 h. The crude solution was concentrated and the residue was precipitated twice in cold diethyl ether. The precipitate was collected and washed thoroughly with diethyl ether before being dried overnight at 40 °C to yield a white powder. ¹H NMR (400 MHz, DMSO-d₆), δ 7.45-6.25 (b, 400 H), 3.60-3.40 (b, 1300 H), 3.3 (s, 21), 2.3-1.0 (b, 330 H) $M_n = 22178$ g/mol; GPC: $M_n = 13004$, $M_w = 15025$, PDI = 1.15. Spectrum and peak assignment are provided in Figure S5. Chromatograms of PS-co-(PNAS-g-PEG) and the parent PS-co-PNAS are given in Figure S6.

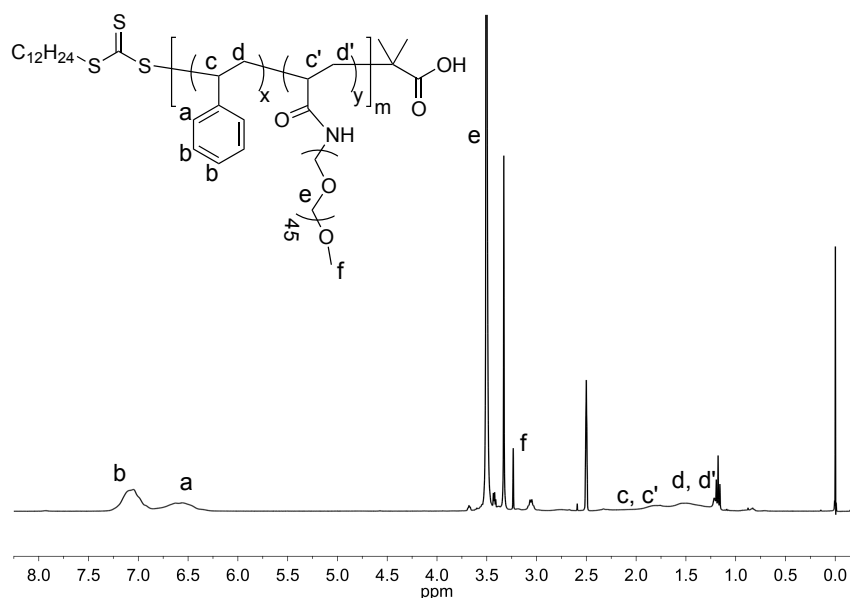


Figure S5. ¹H NMR spectrum and peak assignments of PS-g-PEG (**5**).

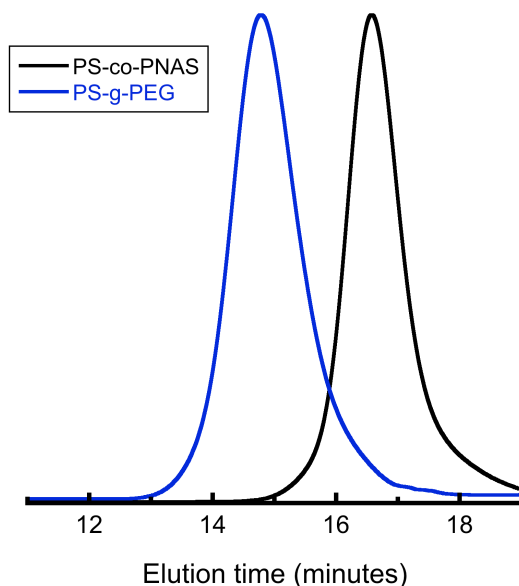


Figure S6. Gel permeation chromatograms of **6** and the parent copolymer **2**.

Preparation of CMP-semiconducting nanoparticles

CMP-semiconducting fluorescent nanoparticles were prepared by a rapid change in solvent quality inside a four stream-vortex mixer. First, the semiconducting fluorescent polymer, PFBT, was allowed to dissolve in THF at room temperature to prepare a stock solution of 0.5 mg/mL. The CMP-conjugated polymer (**4**) was dissolved in DMSO (6 mg/mL) and added to a dilute solution of PFBT to produce a final solution containing PFBT at a concentration of 50 $\mu\text{g/mL}$ and CMP-conjugate polymer concentration ranged from 25 to 150 $\mu\text{g/mL}$. The solution was stirred over a vortex mixer for 5 min before being filtered through a 0.22 μm PVDF syringe filter (Millipore). The THF:water ratio used was 1:6.75 (v/v), with mixing speeds of 16 mL/min and 108 mL/min (36 mL/min per stream) for the organic and aqueous phases, respectively. Samples were collected after 5 s of the start of mixing process to ensure steady operation of the mixer. Nanoparticle suspensions were dialyzed (10 kDa MWCO, Spectrapor) against nanopure water for 24 h. Water was replenished 4 times during the dialysis process. Nanoparticle suspensions were then concentrated using an Amicon Ultra-15 centrifugal unit (3.5 kDa MWCO, Millipore) and stored in scintillation vials. Fluorescent nanoparticles stabilized by PS-co-(PNAS-g-PEG) (**5**) were used as a negative control, and were prepared using the method described above.

The Reynolds number was calculated based on the velocities of all streams according to

$$\text{Re} = \sum_{i=1,N} \frac{V_i}{\nu_i} D$$

where D is the chamber diameter, V_i and ν_i are the velocity and kinematic

viscosity of the n -th inlet stream, and N the number of inlet streams.

To determine the concentration of PFBT in CMP-CPN and PEG-CPN solutions, a standard curve was generated using the maximum peak absorbance of the semiconducting polymer (450 nm) from known concentrations (0-200 $\mu\text{g/mL}$) of CMP- or PEG-CPN solutions. Nanoparticle solution concentration was then determined by measuring the absorbance.

Collagen/gelatin binding assays

A collagen solution (31.7 μL , 4.73 mg/mL in 0.02 N acetic acid) was diluted in 718.3 μL of 1 \times PBS buffer and heated at 70 $^{\circ}\text{C}$ for 8 min to generate a fresh solution containing 0.2 mg/mL of denatured collagen (gelatin). Wells of nunc black 96-well maxisorp plate were charged with 50 μL of this gelatin solution. After incubation at room temperature under gentle shaking overnight, the gelatin solutions were removed and the wells were washed with PBSB buffer (1 mg/mL BSA in 1 \times PBS) twice and blocked with 50 mg/mL BSA in PBS for 1 h. Two additional washings with PBSB were then carried out. The 50 mg/mL BSA blocking solutions were added to additional empty wells to create BSA coatings as a control. CMP-CPN or PEG-CPN solutions of the same PFBT concentration (20 $\mu\text{g}/\text{mL}$ in water, 50 $\mu\text{L}/\text{well}$) were directly added to the gelatin or BSA coated wells and incubated at 4 $^{\circ}\text{C}$ for 2 h. To test whether the nanoparticle-conjugated CMPs form homotrimers -which would decrease their propensity to hybridize with the collagen strands- one group of CMP-CPN solution was heated at 75 $^{\circ}\text{C}$ for 10 min before addition to the wells to ensure the CMPs are monomeric and available for binding. After the 2 h incubation, wells were washed with PBSB three times followed by fluorescence reading (ex: 460 nm, em: 535 nm) with a SpectraMax M-2 microplate reader (Molecular Devices). Each experiment group was performed in triplicate. Average background fluorescence of empty wells was subtracted from raw fluorescence data.

Ex vivo staining of mouse cornea tissue sections

Mouse cornea tissue sections were a kind gift from Huan Meng and Dr. Albert S. Jun at the Wilmer eye institute, Johns Hopkins Medicine. Cornea tissues were harvested from a 23 month-old wild-type C57BL/6 mouse, fixed with 4% paraformaldehyde in PBS solution for 1 h, and cryopreserved in Tissue-Tek O.C.T. medium. Cryosections of 8 μm thickness were obtained and mounted onto charged glass slides. Frozen fixed tissue slides were allowed to equilibrate at room temperature and dried under air flow. Tissue sections were permeabilized by cold methanol at -20 $^{\circ}\text{C}$ for 10 min, and incubated in 1 \times PBS solution. Subsequently, to each slide, 0.5 mL of blocking solution (10% v/v goat serum in 1 \times PBS) was added, and incubated for 1 h at room temperature. After blocking, the CMP-CPN and PEG-CPN solutions of the same concentration (20 $\mu\text{g}/\text{mL}$ PFBT in water) were directly applied to the tissue sections (100 μL for each slide). Tissue sections were gently covered with parafilm to prevent drying and incubated in a humidity chamber at 4 $^{\circ}\text{C}$ for over 2 h. Subsequently, DAPI solutions (1 $\mu\text{g}/\text{mL}$) were applied to the slides for 60 s before the tissue slides were washed by soaking in 1 \times PBS solutions for 5 min three times. Stained sections were imaged with a Nikon Eclipse E600 microscope (Nikon Instruments) under standard DAPI and FITC channels.

Table S1. Characteristics of CPNs used in this study.

PFBT ($\mu\text{g/mL}$)	PS- <i>g</i> -CMP ($\mu\text{g/mL}$)	PS- <i>g</i> -PEG ($\mu\text{g/mL}$)	D_h^a (nm)	PDI ^a	$\zeta^{a,b}$ (mV)
50	25		28.6 ± 1.1	0.09	-13.3 ± 1.4
50	50		23.3 ± 1.3	0.16	-12.6 ± 1.0
50	125		19.4 ± 0.4	0.12	-15.3 ± 0.7
200	125		38.4 ± 6.9	0.10	
50		125	15 ± 4.8	0.12	-11.4 ± 3.7

^a Measured by dynamic light scattering (DLS). ^b Measured in phosphate saline buffer 10 mM, pH 7.4.

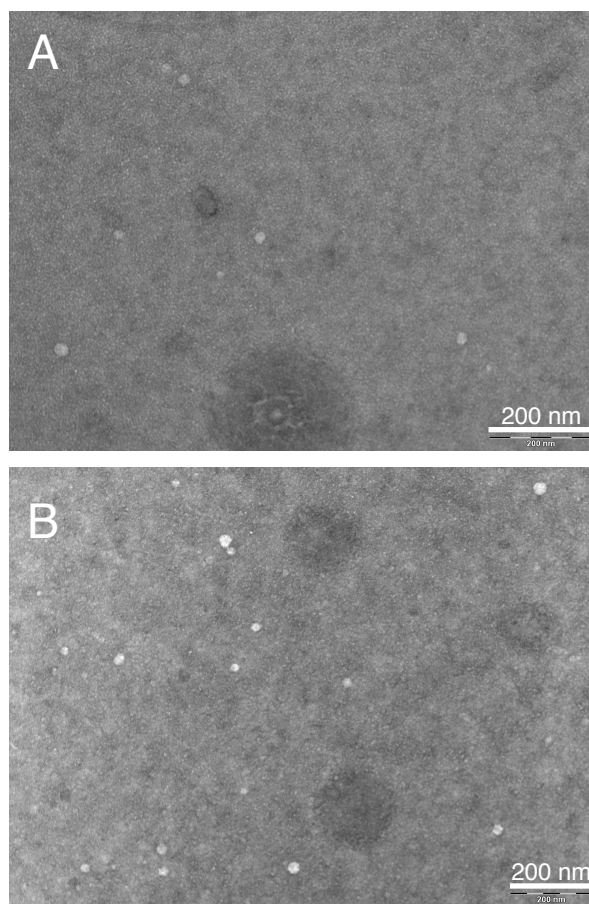


Figure S7. Representative TEM images of CMP-CPN (A) and PEG-CPN (B), prepared from a PFBT solution of 50 $\mu\text{g/mL}$.

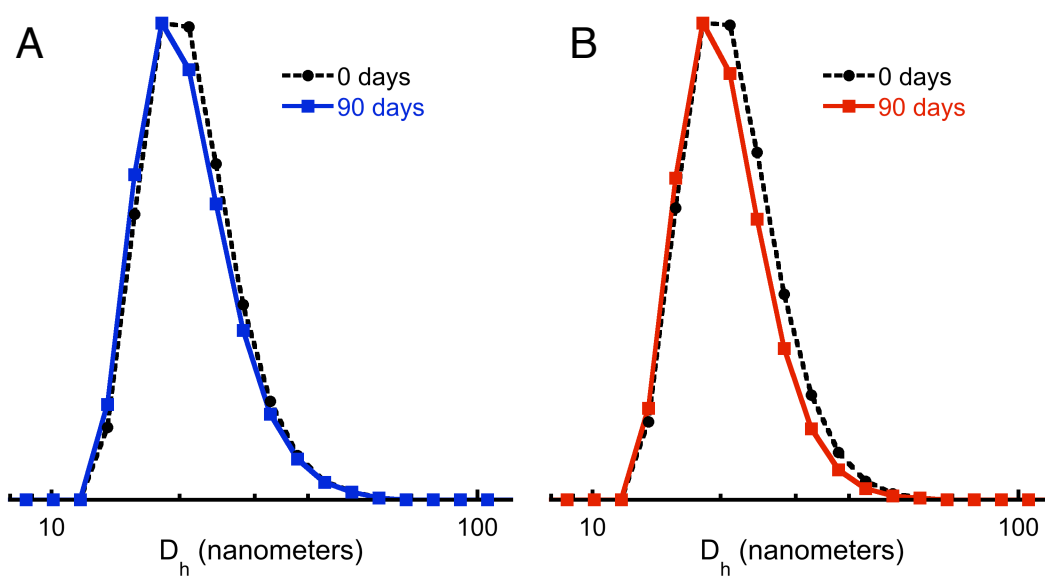


Figure S8. Dynamic light scattering data for CMP-CPNs (A) and PEG-CPNs (B) stored at 4 °C in the dark for 90 days. Preservation of particle size indicates the absence of interparticle aggregation, an effect that would be observed if peptide self-trimerization occurred.

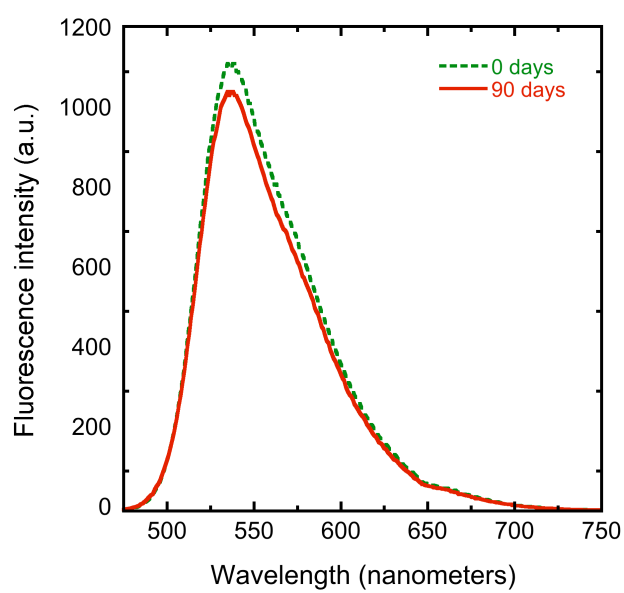


Figure S9. Stability of CMP-CPN after being stored at 4 °C in the dark for 90 days.

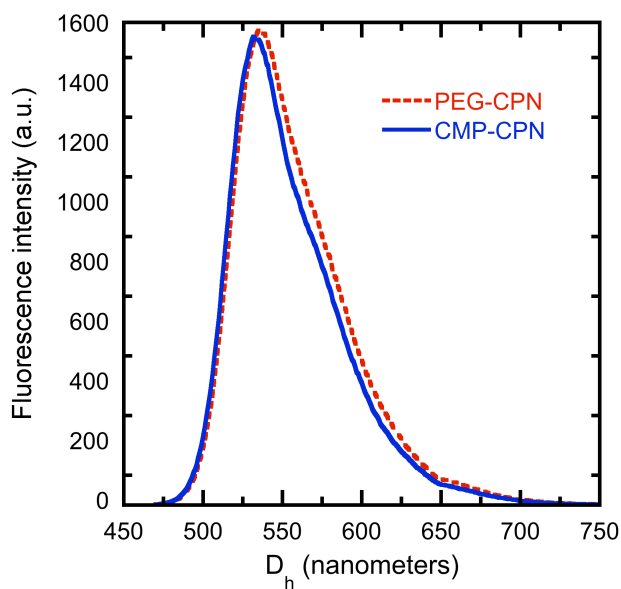


Figure S10. Comparison of fluorescence spectra from PEG-CPN and CMP-CPN, showing the negligible effect of amphiphile on nanoparticle optical properties.

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