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Supporting Information to

Peptide functionalized nanomotor as efficient cell penetrating tool

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1 Materials and instruments

Materials.

Unless otherwise indicated all reagents are used as received. Copper bromide (CuBr), N,N,N',N",N"-pentamethyldi-ethyleentriamine (PMDETA), styrene, tert-butyl α-bromoisobutyrate, 1-phenyl-1-trimethylsiloxyethene, ethylenediaminetetra acetic acid (EDTA), α-ω-amino-poly(ethylene glycol), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 6-maleimidohexanoic acid N-hydroxysuccinimide ester, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris(2-carboxyethyl)phosphine (TCEP), polyvinylpyrrolidone (Mn10000), potassium tetrachloroplatinate (II) and L (+) ascorbic acid were purchased from Sigma-Aldrich. Tetrahydrofuran (THF), anisole and N,N-Diisopropylethylamine (DIPEA) were obtained from Acros. Hydrochloric acid (37%) and hydrogen peroxide were purchased from J.T. Baker. 1,4-dioxane (Biosolve BV), dichloromethane (CH_2Cl_2, Fisher Chemical) were also used. Methanol of HPLC grade was purchased from Avantor Performance Materials and used without further purification. Spectra/Por dialysis membrane with a MW cut-off of 12000-14000 was used for dialysis. All solutions were prepared with MilliQ water, which was obtained with a MilliQ QPOD purification system, with an electrical resistance > 18.2 MΩ.

Instruments.

Gel permeation chromatography (GPC) was performed on a Shodex GPC column equipped with UV SPD 20A detectors (254 nm, 215 nm). THF was used as eluent and the flow rate was kept constant at 1 mL/min. ^{1}H nuclear magnetic resonance spectra (^{1}H NMR) were measured on a Varian Inova 400 MHz spectrometer, with $CDCl_3$ as solvent. Dynamic light scattering (DLS) analysis was performed on Malvern Zetasizer Nano S with following settings: temperature 20 °C, He-Ne laser wavelength 633 nm and detector angle 170°. Infrared (IR) spectroscopy was performed with an IR-ATR instrument (Bruker TENSOR 27). Transmission electron microscopy was performed with a JEOL 1010 Transmission Electron Microscope at an acceleration voltage of 60 kV.

2 Supramolecular nanomotors preparation and structure characterization

2.1 Synthesis of poly(ethylene glycol)$_{44}$-*b*-polystyrene$_{190}$ (PEG$_{44}$-*b*-PS$_{190}$)

Based on a modified literature method poly(ethylene glycol)$_{44}$-*b*-polystyrene$_{190}$ was synthesized.[1] For monitoring the polymerization process, ^{1}H NMR and GPC were used to determine the molecular weight of the block copolymer. The reaction was terminated

once the desired MW of 24000 was attained. According to the GPC, PEG_{44}-b-PS_{190} block copolymer was obtained with a PDI of 1.07.

2.2 Synthesis of NH_2-poly(ethylene glycol)$_{66}$-*b*-polystyrene$_{190}$ (NH_2-PEG_{66}-*b*-PS_{190})

Poly(ethylene glycol)$_{66}$-*b*-polystyrene$_{190}$ with amine as ending group was synthesized as following. The schlenk tube with CuBr (45 mg, 0.32 mmol) was evacuated for 15 min and refilled with argon for three times. PMDETA (66 µL, 0.32 mmol) in anisole (0.5 mL) was added, followed by 15 min of vigorously stirring. Styrene (5.74 mL, 50 mmol) in anisole (0.5 mL) was added via a syringe and degassed for 15 min. After cooling the mixture to 0 °C, tert-butyl α-bromoisobutyrate (27 µL, 0.14 mmol) in anisole was injected and the solution was degassed for another 15 min. The schlenk tube was transferred into an oil bath at 90 °C. ^{1}H-NMR was used for monitoring the reaction process. Upon attainment of the required molecular weight, the reaction was terminated by adding 1-phenyl-1-trimethylsiloxyethene (1.91 mL, 9.28 mmol). The mixture was left to stir for 2h. The solution was diluted with CH_2Cl_2 and extracted with an aqueous solution of EDTA (65 mM). The organic layer was collected and dried with $MgSO_4$ before concentration with vacuum pump. The polymer obtained was precipitated in MeOH for three times and dried under vacuum for an overnight, giving polymer α-tert-Butyloxycarbonyl-polystyrene. Then α-tert-Butyloxycarbonyl-polystyrene (3 g) was dissolved in 1, 4-dioxane (30 mL) and concentrated HCl (1.5 mL, 37%) was added. The reaction was refluxed at 110 °C overnight. The mixture was dried using a rotary evaporator and then dissolved in CH_2Cl_2. The polymer (α-carboxylic acid-polystyrene) was obtained after precipitation in MeOH (3x) and then dried under vacuum for an overnight. The dried polymer α-carboxylic acid-polystyrene (1 g, 43.5 µmol), α-ω-amino-poly(ethylene glycol) (521.7 mg, 260 µmol) and DIPEA (17.4 µL, 100 µmol) were dissolved in DMF (12 mL). The solution was cooled to 0 °C and PyBOP (42 mg, 80 µmol) was added. The reaction was stirred overnight, while slowly warming to room temperature. The progress of the coupling was monitored by GPC. After that, the mixture was diluted with CH_2Cl_2 and extracted with $NaHCO_3$ solution (4 wt%) and saturated NaCl solution. The organic layer was collected and dried with $MgSO_4$ and concentrated. The pure polymer was obtained after precipitation in MeOH (3x) and dried under vacuum overnight. The final polymer was characterized by ^{1}H-NMR in $CDCl_3$.

2.3 Preparation of PtNP with PVP coating

To 2 mL K_2PtCl_4 (20 mM), 20 mg poly(vinyl pyrrolidone) (PVP, MW≈ 10,000) was added. The mixture was allowed to stir at room temperature for 48 hours. Then 1 mL ascorbic acid aqueous solution (35 mg/mL) was added to the above solution. After stirring for 1 min, the mixture was incubated in a sonication bath (VWR Ultrasonic Cleaner Model 75D) at room temperature for 1 h. The appearance of a black color indicated the formation of PtNP. The average size of particles was determined to be 74 nm with DLS.

2.4 Preparation of the platinum nanoparticles (PtNP)- loaded stomatocytes

The block copolymer poly(ethylene glycol)$_{44}$-*b*-polystyrene$_{190}$ and poly(ethylene glycol)$_{66}$-*b*-polystyrene$_{190}$ with amine ending (for 5% functionalization, 9.5 mg:0.5 mg; for 1% functionalization, 9.9 mg: 0.1 mg) was fully dissolved in 1 mL organic solvent (tetrahydrofuran:dioxane = 4 : 1). Deionized water (0.35 mL) was subsequently slowly added to the solution, followed by addition of 0.65 mL preformed PtNP aqueous solution. After dialysis for 48 hours, PtNP entrapped stomatocytes were obtained.

2.5 Functionalization of amine ended poly(ethylene glycol)-*b*-polystyrene stomatocytes with fluorescein isothiocyanate labelled tat peptide (FITC-tat)

The amine exposed poly(ethylene glycol)-*b*-polystyrene (2.5 mg polymer/mL) stomatocytes solution (800 µL) was diluted with 333 µL of phosphate buffer (pH 7.4, 80 mM), followed by addition of 6-maleimidohexanoic acid N-hydroxysuccinimide ester (2 mg/mL, for 5% functionalization 15 µL, 1% functionalization 3 µL). The mixture was allowed to stir vigorously for 2 hours before ultrafiltration with (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer for three times to remove unreacted 6-maleimidohexanoic acid N-hydroxysuccinimide ester and concentrate the solution to 4 mg polymer/mL. To 400 µL above obtained solution, FITC-tat (1 mg/mL) aqueous solution (for 5% 16 µL, 1% 3.2 µL), Tris(2-carboxyethyl)phosphine (TCEP, 1 mg/mL) aqueous solution for (5% 8.2 µL, 1% 1.7 µL and 100 µL HEPES buffer was added. The reaction mixture was agitated in dark for 16 hours. Then three cycles of wash and centrifugation (6000 rpm, 10 min) was performed to remove unreacted FITC-tat and the stomatocytes pellet was redispersed with MilliQ water (4 mg polymer/mL).

2.6 Functionalization of amine ended poly(ethylene glycol) -*b*-polystyrene stomatocytes with fluorescein isothiocyanate (FITC)

The amine exposed poly(ethylene glycol)-*b*-polystyrene (2.5 mg polymer/mL) stomatocytes solution (800 µL) was diluted with 200 µL of borate buffer (pH 9, 80 mM), followed by addition of fluorescein isothiocyanate (FITC) in DMSO (2 mg/mL, for 5% 20 µL, 1% 4 µL). The reaction mixture was left to stir in dark before ultrafiltration for three times to remove unreacted FITC.

2.7 Transmission electron microscopy (TEM)

Samples were prepared with the following protocol: 6 µL of diluted sample aliquots were dropped onto carbon-coated copper grids. Excessive liquid was removed with filter paper and the grid was dried overnight at room temperature. Image acquisition was performed with iTEM software (Olympus).

2.8 Dynamic light scattering (DLS)

Dynamic light scattering analysis of PtNP in aqueous solution and zeta potential measurement of functionalized stomatocytes were performed using a Malvern Zetasizer Nano S instrument. Samples were typically loaded in Malvern disposable capillary cells.

3 Procedure to measure motion of nanomotors by nanosight

To investigate the motion of stomatocytes nanomotors, nanoparticle tracking analysis was carried out with a nanosight LM10 at a magnification of 20×. The nanoparticle tracking analysis provides visualization of the particles. We used this technique to study the effect of fuel concentration on the movement of the stomatocytes. In this experiment we analysed the movement of Dox and PtNP loaded stomatocytes at four concentrations of hydrogen peroxide (final concentration 0 v/v %, 0.015 v/v %, 0.06 v/v %, 0.15 v/v %). 30 µL of Milli Q water or freshly prepared hydrogen peroxide (5 v/v %, 2 v/v %, 0.5 v/v %) was added to 1 mL stomatocytes nanomotors solution with the appropriate concentration (6×10^8 particles/mL) before loading into the sample cell. A typical video of 90 seconds was recorded. By analyzing the video, x, y coordinates of each particle were determined as a function of time intervals. Mean square displacements obtained by averaging over 25 particles from the major size distribution observed with Nanosight were plotted versus the time intervals.

4 Cell culture and cellular uptake evaluation by confocal microscopy and flow cytometry

Hela cells were cultured in a T flask at 37 degrees with Dulbecco's modified Eagle's medium (DMEM) buffer which was supplemented with 100 U/mL penicillin, streptomycin and 10% fetal bovine serum. When reaching 90% confluency, cells were harvested by trypsinization and centrifugation at 1000 rpm for 5 minutes and then resuspended in fresh culture medium (2×10^5 cells/mL) before being seeded into 8 well micro dish (ibidi GmbH) and incubated for an overnight. After removing the cell culture medium, the cells were rinsed with PBS for 3 times and incubated with 200 µL FITC PEGPS stomatocytes or FITC-tat PEGPS stomatocytes (particle concentrations were kept constant at 7×10^9 particles/mL, as determined with nanoparticle tracking analysis) with hydrogen peroxide free or hydrogen peroxide added (final hydrogen peroxide concentration is 0.015%) in DMEM buffer at 37 degrees for 6 hours. After 6 hours, the culture medium was removed followed by two washing steps with PBS. Then the cells were stained with live cell nucleus dye Hoechst 33342 and cell cellmask deep red cell membrane dye before another washing step with PBS and addition of fresh DMEM buffer. The cellular uptake was examined with a confocal microscope (Leica TCS SP2 AOBS). FITC was excited with an Ar laser at 488 nm and the emission was collected at 510 – 530 nm. The laser beam was focused on the sample through a 63× oil immersion objective. A pinhole of 400 was selected. For cellular uptake analysis with flow cytometry, culture medium was removed from the well after 6 hours of incubation followed by one washing step with PBS. Then cells were detached by trypsinization (trypsin-EDTA) for 5 minutes. The cells were collected by centrifugation (1000 rpm, 5 min) and resuspended in PBS supplemented with 1% BSA before measurement with a flow cytometer (laser line 488 nm).

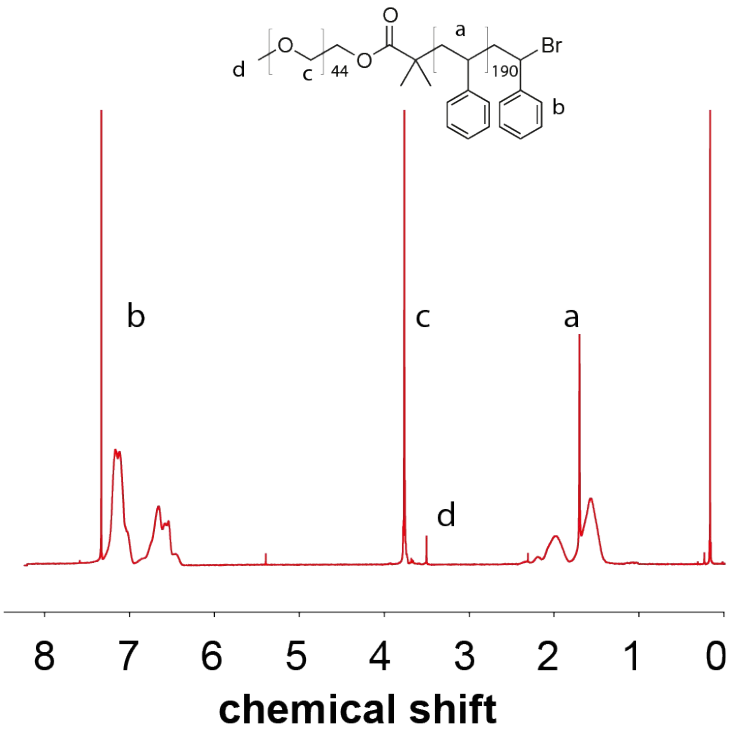

Figure SI. ^{1}H NMR spectrum of poly(ethylene glycol)$_{44}$-*b*-polystyrene$_{190}$.

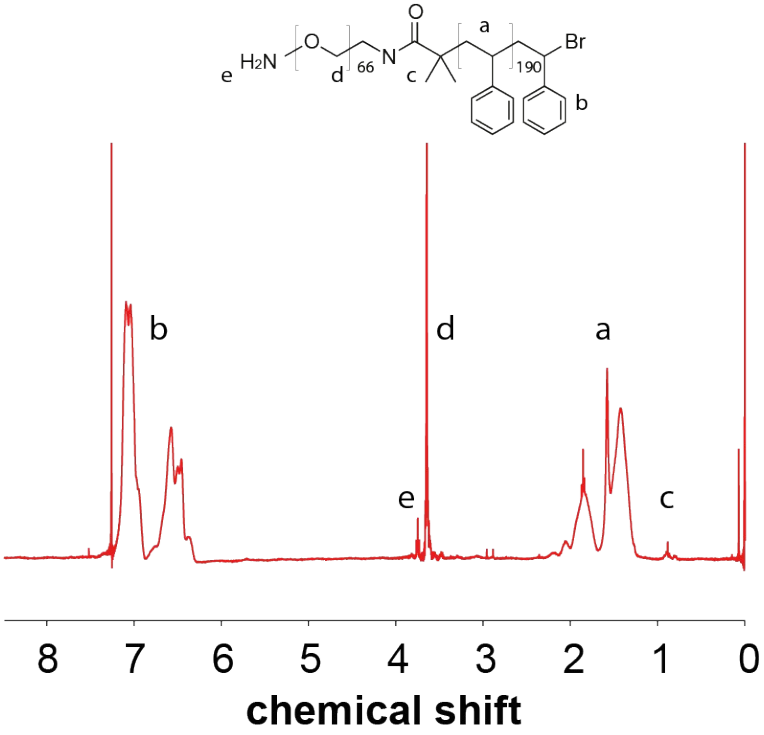

Figure S2. ^{1}H NMR spectrum of amine functionalized poly(ethylene glycol)$_{66}$-*b*-polystyrene$_{190}$.

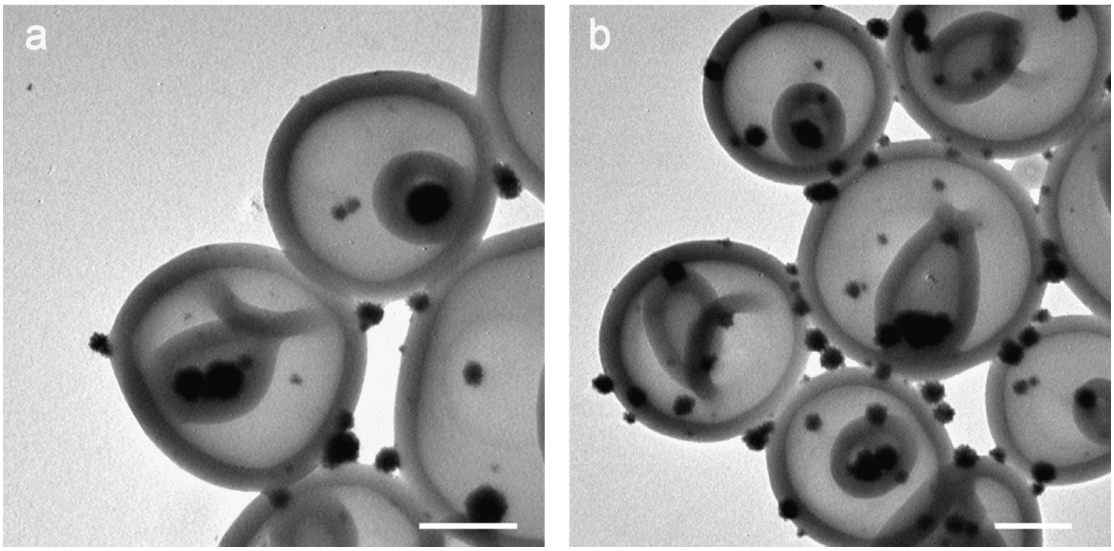

Figure S3. TEM images of a) 1% amine functionalized poly(ethylene glycol) -*b*-polystyrene stomatocytes; b) 5% amine functionalized poly(ethylene glycol) -*b*-polystyrene stomatocytes.

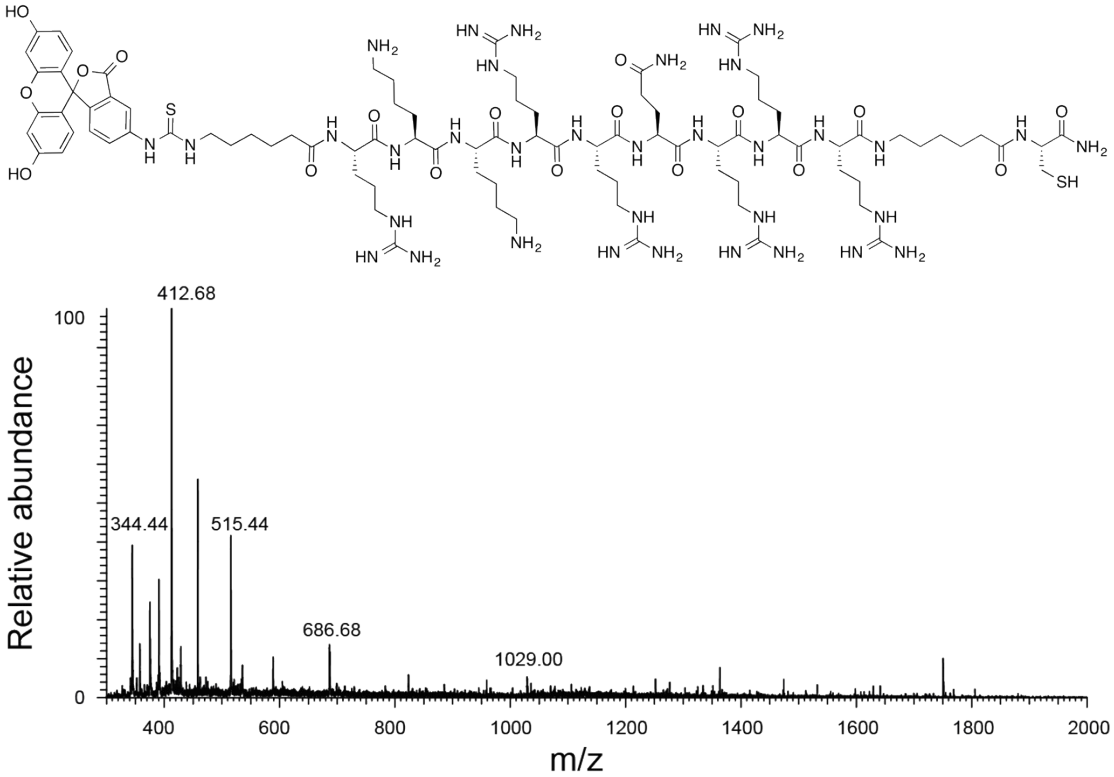

Figure S4. Molecular structure and electrospray ionisation mass spectrometry (ESI-MS) spectrum of FITC labelled tat peptide (expected molecular mass 2056.09), found m/z 344.44 at +6 charge, m/z 412.68 at +5 charge, m/z 515.44 at +4 charge, m/z 686.68 at +3 charge, m/z 1029.00 at +2 charge.

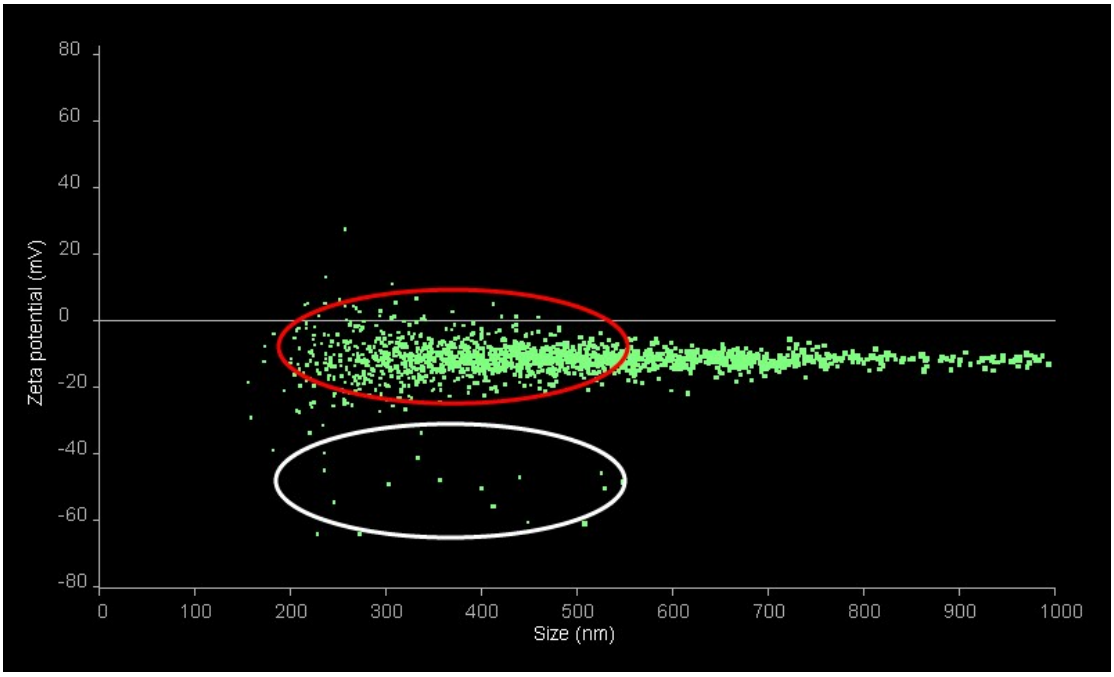

Figure S5. Zeta potential and size distribution of tat functionalized stomatocytes nanomotors determined with nanoparticle tracking analysis.

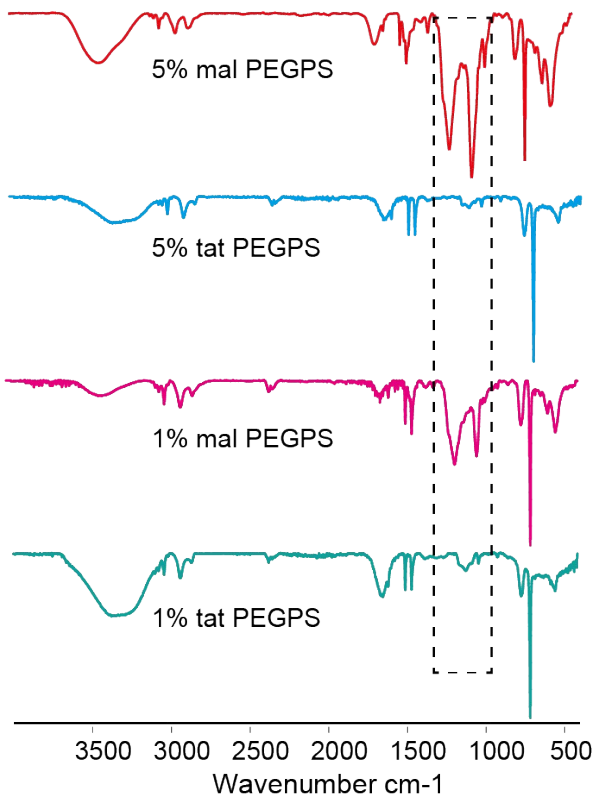

Figure S6. Infrared spectra of (from top to bottom) 5% mal functionalized poly(ethylene glycol)-*b*-polystyrene stomatocytes; 5% tat functionalized poly(ethylene glycol) -*b*-polystyrene stomatocytes; 1% mal functionalized poly(ethylene glycol)-*b*-polystyrene

stomatocytes; 1% tat functionalized poly(ethylene glycol)-*b*-polystyrene stomatocytes.

List of abbreviations in the manuscript:

tat: trans-activator of transcription peptide

FITC: fluorescein isothiocyanate

PEGPS: poly(ethylene glycol)-*b*-polystyrene

NHS-Mal: 6-maleimidohexanoic acid N-hydroxysuccinimide ester

FITC PEGPS Pt stomatocytes: FITC incorporated and platinum loaded PEGPS stomatocytes

FITC-tat PEGPS Pt stomatocytes: FITC incorporated/tat functionalized and platinum loaded PEGPS stomatocytes

References

(1) K. T. Kim, J. J. L. M. Cornelissen, R. J. M. Nolte, J. C. M. van Hest, Adv. Mater. 2009, 21, 2787-2791.