Electronic Supplementary Material (ESI) for Polymer Chemistry

Dynamically Functionalized Polymersomes via Hydrazone Exchange

*René P. Brinkhuis, Frank de Graaf, Morten Borre Hansen, Taco R. Visser, Floris P. J. T. Rutjes and Jan C. M. van Hest**

Table of contents:

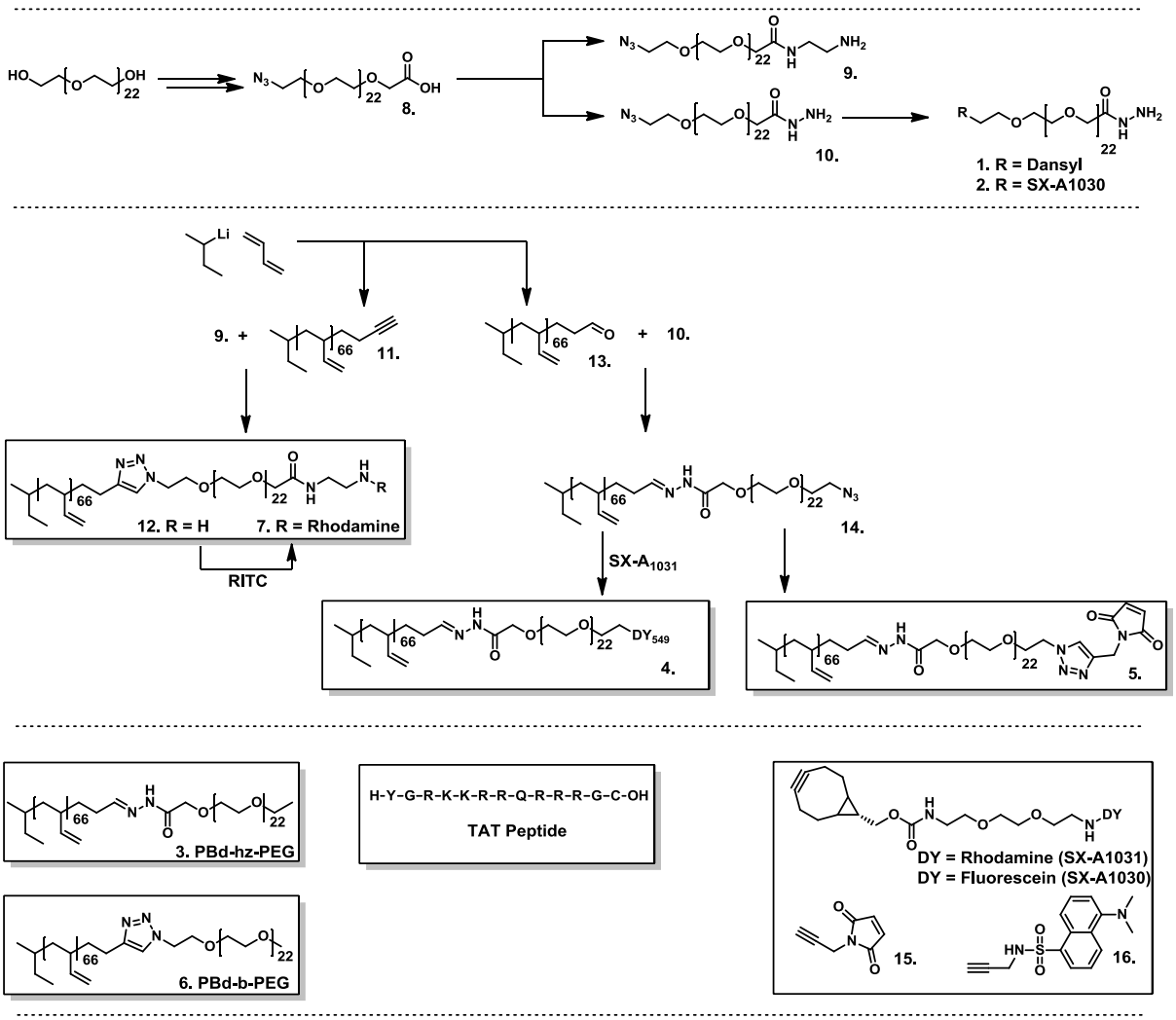
Figures

Materials. BCN dyes SX-1030 and SX-1031 were purchased from Synaffix and copper bromide was washed in ethyl acetate and dried under vacuum prior to use. All other reagents were at least 98% pure and used without further purification. Tetrahydrofuran (THF) (ACROS ORGANICS, 99+% extra pure, stabilized with BHT) was distilled under argon from sodium/benzophenone and triethyl amine (TEA) (BAKER) was distilled from calcium hydride under an argon atmosphere prior to use. Polymersome extrusions were performed using 200 nm filters (Acrodisc 13 mm Syringe Filter, 0.2 µm Nylon membrane).

Instrumentation. MilliQ water was obtained from a Labconco water pro PS system. Thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F-254 plates (layer thickness 0.25 mm). Compounds were visualized by UV, ninhydrin and/or permanganate reagents. Column chromatography (CC) was carried out using silica gel, Acros (0.035-0.070 mm, pore diameter ca. 6 nm). Infrared (IR) spectra were obtained using a Thermo Matson IR 300 FTIR spectrometer. Data are presented as the frequency of absorption (cm^{-1}). Proton nuclear magnetic resonance (1HNMR) spectra were recorded on a Varian Unity Inova 400 FTNMR spectrometer. Chemical shifts are expressed in parts per million (δ scale) relative to the internal standard tetramethylsilane (δ=0.00 ppm). Molecular weight distributions were measured using size exclusion chromatography (SEC) on a Shimadzu (CTO-20A) system equipped with a guard column and a PL gel 5 µm mixed D column (Polymer Laboratories) with differential refractive index and UV (λ=254 nm and λ=340nm) detection, using tetrahydrofuran (SIGMA ALDRICH chromasolv 99.9%) as an eluent at 1 mL/min and T = 30 °C. Particle size distributions were measured on a Malvern instruments Zetasizer Nano-S. A TECAN Infinite 200 PRO plate reader was used to perform fluorescence intensity measurements. Confocal Laser Scanning Microscopy (CLSM) images were recorded on a Leica DM IRE2 TCS SP2 AOBS inverted microscope. An Argon laser and a Vioflame diode laser were used to excite the different fluorophores.

General note. Details of the synthesis of compounds **8, 9** and **12** can be found in "Brinkhuis and Stojanov et al. Bioconjugate Chemistry **2012**, 23, 958". Details of the synthesis of compounds **3**, **6**, **11** and **13** can be found in " Brinkhuis et al. Polymer Chemistry **2011**, 2, 550-552" Details of the synthesis of compound **15** can be found in "Karlèn et al. J. Med. Chem. **1970**, 13, 651" Details of the synthesis of compound **16** can be found in "Deiters et al. J. Am. Chem. Soc. **2003**, 125, 11782-11783"

1. Synthesis and Characterization



Scheme S1. Overview of the synthetic route to all structures. Details on the synthetic procedures are described below.

α-dansyl ω-hydrazine poly(ethylene glycol) (1) Polymer **10** (100 mg, 0.1 mmol) was dissolved in THF (10 mL) under an argon atmosphere. Next, CuBr (5 mg, 34 μmol), 1 drop of N,N,N',N',N"-pentadimethyldiethylenetriamine (PMDETA, ca. 50 mg, 0.28 mmol) and dansyl probe **16** (26 mg, 0.95 equiv.) were added and the mixture was heated to reflux overnight. All of **16** was consumed as determined by TLC. Next, all solvents were removed and the products were dissolved in dichloromethane (1 mL). The product was purified on a silica column, eluting with dichloromethane to remove any traces of **16**. Compound **1** was obtained by eluting with 8% methanol in dichloromethane (70 mg, 65%, Rf = 0.55 as a bright -single- fluorescent spot). ^{1}HNMR (400 mHz, $CDCl_3$) δ (ppm) 9.33 (s, 1H, CON*H*NH_2), 8.55 (1H), 8.27 (2H), 7.54 (2H), 7.19 (1H), 4.42 (1H, SN*H*CH_2), 3.79 (2H, C*H*$_2$N), 2.89 (6H, N(CH_3)$_2$), 4.06 (2H, OCH_2CO), 3.64 (90H,

CH_2CH_2O), 1.70 (2H, NHNH_2) SEC (THF): Mn = 1.1 kg/mol, Mw/Mn = 1.26 (detection at 340 nm, whereas the starting material, **10**, does not absorb at 340 nm).

Partial fluorescein labeling of α-azido ω-hydrazine poly(ethylene glycol) (2) Polymer **10** (100 mg, 0.1 mmol) was dissolved in methanol (10 mL) and fluorescein probe SX-A1030 (5 mg, 0.06 equiv.) was added. The reaction was allowed to proceed overnight, after which all solvents were removed. TLC, eluting with acetonitrile:MilliQ (9:1) showed full consumption of dye SX-A1030 (Rf = 0.75) and a new bright fluorescent product (Rf = 0.05) at the same height as **10** was formed (note that mixing of PEG and SX-A1030 results in a clear separation under these conditions). The product was dissolved in ice-cold dichloromethane (1 mL) and filtered over a 0.1 µm syringe filter. The product, was obtained by removing all dichloromethane (85 mg, ca. 60%). SEC (THF): Mn = 1.4 kg/mol, Mw/Mn = 1.31 (detection at 340 nm, whereas the starting material, **10**, does not absorb at 340 nm). ^{1}HNMR (400 mHz, $CDCl_3$): no significant deviation from **10**, due to labeling with only 0.06 equiv. of dye.

Partial Rhodamine labeled Polybutadiene-*hydrazone*-poly(ethylene glycol) (4) Polymer **14** (100 mg, 0.021 mmol) was dissolved in THF (10 mL) and Rhodamine probe SX-A1031 (5 mg, 0.2 equiv.) was added. The reaction was allowed to proceed overnight. Solvents were removed and product **4** was purified by preparative GPC (THF) to yield 70 mg product. The product was highly fluorescent and in contrast to **14** showed absorption in the GPC detector at 340 nm (see figure S2). Mw/Mn = 1.22, Mn: 4.7 kg/mol. ^{1}HNMR (400 mHz, $CDCl_3$) no significant deviation from **14**, due to the low concentration of dye.

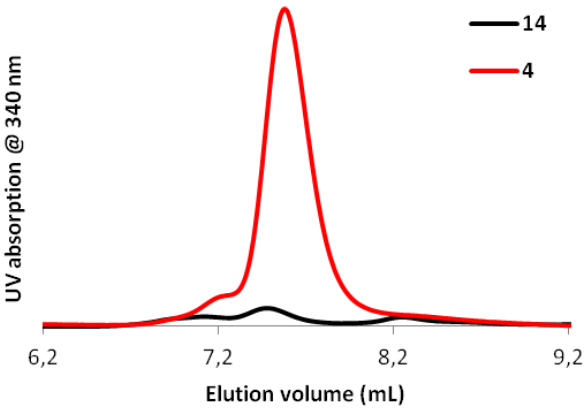


Figure S2: Using equal polymer concentrations for SEC (THF) analysis. UV absorption at 340 nm can be detected for polymer **4**, due to the presence of SX-1031, whereas only limited absorption is observed before the coupling (**14**).

Maleimide end-functional Polybutadiene-*hydrazone*-poly(ethylene glycol) (5) Polymer **14** (100 mg, 0.021 mmol) was dissolved in THF (10 mL) under an argon atmosphere. Next, CuBr (5 mg, 34 µmol), PMDETA (1 drop, ca. 50 mg, 0.28 mmol) and maleimide **15** (5 mg, 1.6 equiv.) were added and heated to reflux overnight. All solvents were removed and the products were dissolved in dichloromethane (1mL). The product was purified on a silica column, eluting with a gradient of 0-8 v% methanol in dichloromethane to obtain the product (55 mg, 50 %, Rf in 8%

methanol/DCM = 0.65). The product showed, in contrast to **14,** good UV (254 nm) absorption in SEC analysis. ^{1}HNMR (400 mHz, $CDCl_3$) δ (ppm) 6.96 (s, 2H, maleimide), 5.45 (m, 67H, C*H*CH_2), 4.94 (m, 134H, CHC*H*$_2$), 3.65 (m, 90H, C*H*$_2$C*H*$_2$O), 2.11 (m, 67H, CH_2C*H*), 1.16 (m, 134H, C*H*$_2$CH). SEC (THF): Mw/Mn = 1.26, Mn: 4.7 kg/mol (see Figure S3).

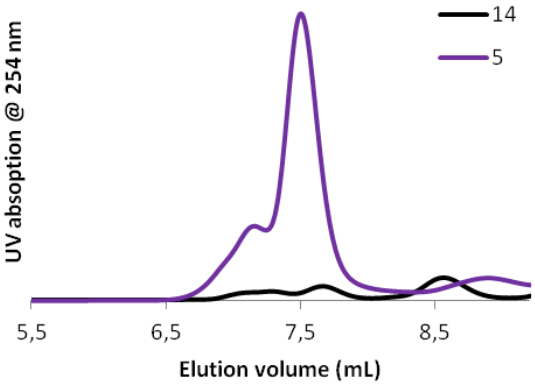


Figure S3: Using equal polymer concentrations for SEC (THF) analysis. UV absorption at 254 nm can be detected for polymer **5**, due to the presence of triazoles and a maleimide, whereas only limited absorption is observed before the introduction of a maleimide (**14**).

Rhodamine labeled Polybutadiene-*triazole*-poly(ethylene glycol) (7) Polymer **12** (50 mg, 10 µmol) was dissolved in THF (10 mL) and Et_3N (1 mL) was added. Next, rhodamine B isothiocyanate (7.5 mg, 1.5 equiv.) was added and allowed to react for 48 hours. Solvents were removed and product **7** was purified by preparative SEC (THF) to yield the product (25 mg, 45%). The product was a single spot on TLC (8 percent methanol in DCM; UV and permanganate stain) which appeared brightly fluorescent. Note that the isothiocyanate coupling of rhodamine B proceeded slowly and the reaction did not go to completion as can be seen from the SEC traces (absorption @ 340 nm) in figure S4. The elution volume that was collected in preparative SEC (THF) is depicted in the box (6.7 to 8.1 mL)

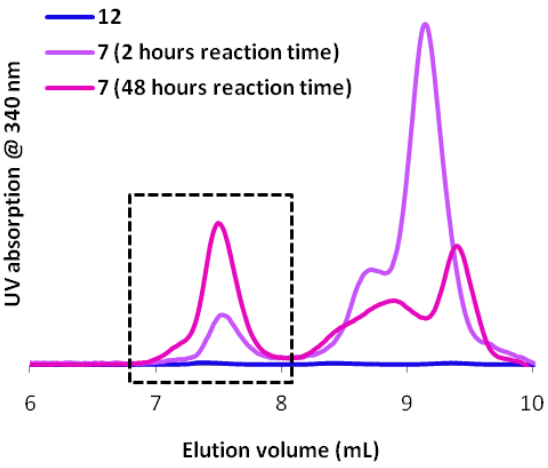


Figure S4: SEC (THF) analysis of block copolymer **12**, which does not absorb at 340 nm before the addition of rhodamine B isothiocyanate. After 2 hours the labeled polymer (top~7.5 mL) does absorb at 340 nm, which increases over the next 46 hours. The dashed box indicates the volume, containing product **7**, that was collected by preparative SEC in THF.

α-azido ω-hydrazine poly(ethylene glycol) (10) Compound **8** (1g, 1 mmol) was dissolved in methanol (50 mL) and concentrated sulphuric acid (5 drops) were added. The mixture was heated under reflux overnight, after which methanol was removed. The product was dissolved in water (50 mL) and extracted with dichloromethane (5x 50 mL). The organic layer was dried over $MgSO_4$ and all solvents were removed. TLC: Rf = 0.65, permanganate staining, running in DCM:MeOH (92:8). Indicative ^{1}HNMR shifts: (400 mHz, $CDCl_3$) δ (ppm) 4.17 (s, 2H, OCH_2COOMe), 3.75 (s, 3H, COOCH_3) and IR: 1748 cm^{-1} (carbonyl/ester)

The product was dissolved in methanol (50 mL) and hydrazine (15 mL, 1M in THF) was added. The mixture was heated under reflux for 48 hours, after which ***almost*** all solvents were removed. The product was dissolved in dichloromethane (50 mL) and washed with hydrochloric acid (1M, 50 mL). The aqueous layer was extracted with dichloromethane (4x 50 mL). The combined organic layers were dried over $MgSO_4$ and all solvents were removed to yield the product (800 mg, 80%). TLC: Rf = 0.55, permanganate and ninhydrin staining, eluent DCM:MeOH (92:8). ^{1}HNMR (400 MHz, $CDCL_3$) δ (ppm) 3.39 (t, 2H, CH_2N$_3$), 3.64 (m, 90H, CH_2CH_2O), 4.01 (s, 2H, OCH_2CONH). FTIR: 2098 cm^{-1} (azide), 1696 cm^{-1} (C=O). SEC (THF): Mn = 1 kg/mol, Mw/Mn = 1.22.

ω-azido-Polybutadiene-*hydrazone*-poly(ethylene glycol) (14) Polymer **10** (80 mg, 0.8 mmol) and **13** (800 mg , 2.5 equiv. 3.7 kg/mol) were dissolved in dry dichloromethane (2 mL). The mixture was allowed to react for 24 hours while gently stirring. The mixture was poured on a short silica column, which was eluted with dichloromethane. After all non-reacted **13** was flushed off the product was eluted with 8 percent methanol in dichloromethane. After removal of all solvents the product (240 mg, 65%) was obtained. ^{1}HNMR (400 mHz, $CDCl_3$) δ (ppm) 5.45 (m, 67H, C*H*CH$_2$), 4.94 (m, 134H, CHCH_2), 3.65 (m, 90H, CH_2CH_2O), 2.11 (m, 67H, CH_2C*H*), 1.16 (m, 134H, CH_2CH). SEC (THF): Mw/Mn = 1.18, Mn: 4.7 kg/mol (see figure S5).

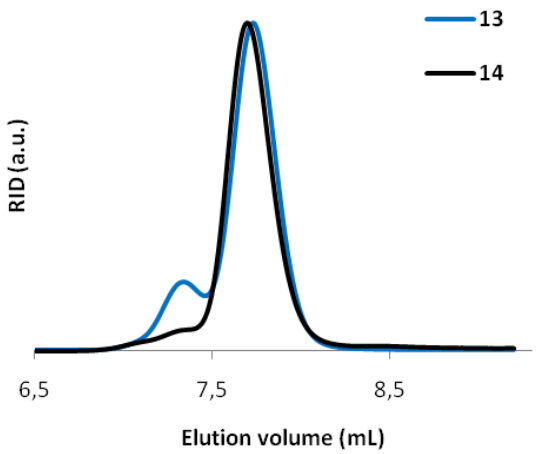


Figure S5: Synthesis of ω-azido-polybutadiene-*hydrazone*-poly(ethylene glycol) (**14**) from polymers **10** and **13**. Due to an increase in molecular weight the GPC trace shifts to shorter elution time.

Tat-peptide TAT peptide was synthesized by means of standard Fmoc chemistry. The purity was more than 95 percent as analyzed by means of HPLC eluting in water/acetonitrile both containing 0.1% v/v trifluoroacetic acid. The volume fraction of acetonitrile was increased from zero to hundred percent over 30 minutes. Maldi-TOF (cyano-4-hydroxycinnamic acid): [M+H] calc: 1719.0 g/mol and [M+H] found: 1718.7 g/mol.

2. Polymersome formation

General procedure 20 mg of the desired (ratio) of block copolymer(s) was dissolved in THF (2 mL) and slowly diluted with MilliQ water (6 mL) to obtain an opaque solution. The solution was extruded three times through a 200 nm syringe filter. THF was removed by purification over a Sephadex G200 column, eluting with demineralized water, after which the opaque polymersome fractions were combined. If needed the pH was adjusted to neutral (0.05 M NaOH) and the total volume was adjusted to 10 mL (2 mg/mL, 0.4 mM). The average size was determined by dynamic light scattering and was in all cases around 200 nm with a PDI below 0.2.

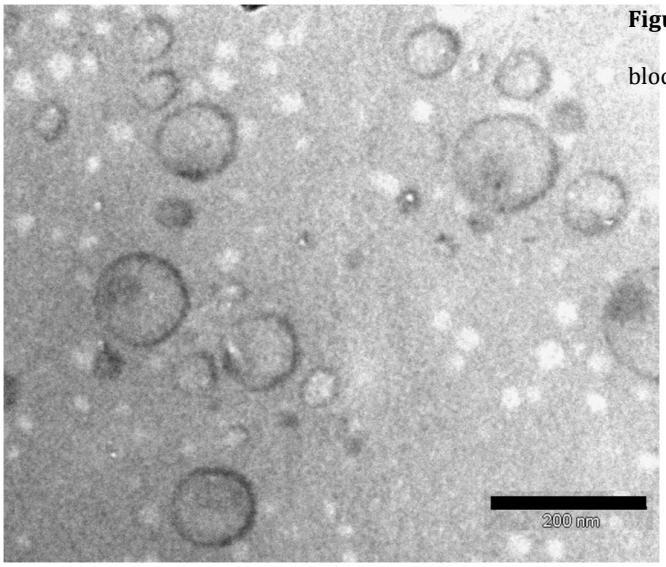


Figure S6: TEM image of polymersomes formed from block copolymer **6**. The black bar represents 200 nm.

3. Exchange experiments

Dansyl-PEG solution exchange Polymersomes from **3** and polymersomes from **6** were prepared as described in the general procedure for polymersome formation. Next, three sample were prepared containing: A) 3 mL polymersomes formed from **3** (0.4 mM), 0.8 mg of **1** (0.26 mM) and 1.7 mg aniline (6 mM) B) 3 mL polymersomes formed from **3** (0.4 mM) and 0.8 mg of **1** (0.26 mM) C) 3 mL polymersomes formed from **6** (0.4 mM), 0.8 mg of **1** (0.26 mM) and 1.7 mg aniline (6 mM)

At different time points a 600 µL sample of A, B and C was withdrawn and purified over a Sephadex G200 column to remove free PEG and aniline (elution with milliQ). The average size at a fixed attenuator and measurement position was determined by DLS and if necessary the samples were diluted to equally derived count rates. No significant change in size and polydispersity in time was observed. Samples were prepared in a 96 well plate (200 µL) and measured in a plate reader for dansyl emission (340 nm (excitation) and 560 nm (emission)).

Dansyl-PEG/Rhodamine-PEG solution exchange Polymersomes were formed from **3** *and* **4** in a ratio of 95:5 as described above. The experiment was started by mixing 3 mL of polymersomes (0.4 mM), 3 mg of **1** (1.0 mM) and 1.7 mg aniline (6.0 mM).

At different time points a 600 µL sample was withdrawn and purified over a Sephadex G200 column to remove free PEG and aniline (eluting in MilliQ). The average size at a fixed attenuator and measurement position was determined by DLS and if necessary the samples were diluted. No significant changes in size and polydispersity in time were observed. Samples were prepared in a 96 well plate (200 µL) and measured in a plate reader. Both the dansyl and rhodamine probe were selectively visualized *subsequently* (dansyl excitation 340 nm, emission 540 nm; rhodamine excitation 540 nm, emission 592 nm).

Fluorescein-PEG/Rhodamine-PEG solution exchange This experiment was performed to visualize the co localization of two fluorophores at polymersomes. As dansyl was not a suitable probe for CLSM due to fast quenching, it was replaced by fluorescein (compound **2**).

Polymersomes were formed of **3** and **4** in a ratio of 95:5 as described above. The experiment was started by mixing 600 µL of polymersomes (0.4 mM), 0.5 mg **2** (0.56 mM) and 3 mg aniline (50 mM). After 24 hours a sample was purified over a Sephadex G200 column eluting with MilliQ water. The average size at a fixed attenuator and measurement position was determined by DLS and no significant change in size and polydispersity was observed. The emission spectrum of both fluorophores was determined *simultaneously* in the plate reader (480 nm excitation). For the CLSM experiment both fluorophores were excited with 476 nm laser. Simultaneously, Rhodamine was selectively visualized by recording emission between 580-610 nm and fluorescein was recorded between 500-530 nm (Fig S7).

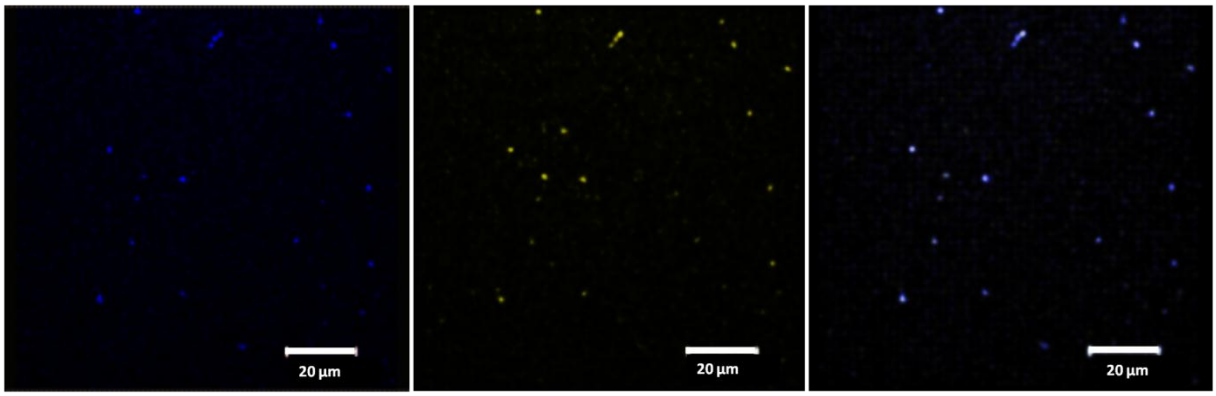


Figure S7: Left, selective detection of rhodamine emission after 24 hours of catalyzed exchange. Middle, selective detection of fluorescein emission and right, the overlay of both images showing the presence of both fluorophores at the same polymeric vesicles after 24 hours of equilibration.

4. Cell studies

TAT-PEG/Rhodamine-PEG surface exchange Three types of polymersomes (P1, P2 and P3) were formed as described in the general section on polymersome formation. P1 was formed from **3** and **4** in a ratio of 9:1 (rhodamine-labeled) (10 mL, 0.4 mM); P2 was formed from **6** and **7** in a ratio of 9:1 (same as P1 but no hydrazone bonds) (10 mL, 0.4 mM); P3 was formed from **3** and **5** in a ratio of 8:2 (Maleimide, but no fluorescent label) (10 mL, 0.4 mM)

P3 polymersomes were functionalized with tat-peptide; 1.1 mg tat (~0.5 equiv towards maleimides) was dissolved in 1 mL MilliQ and 5 mg TCEP gel (Piercenet) was added. The mixture was incubated for 30 minutes after which the solution was filtered and added to the full 10 mL of maleimide-functional polymersomes P3. Residual tat-peptide was removed by spin column (4000 rpm, 0.1 nm pores) until the eluate showed negative on Kaiser test (ninhydrin for free amines). The total volume was adjusted to 10 mL to have equal concentrations compared to P1 and P2 (0.4 mM).

Next, three samples to test polymersome-polymersome surface exchange were prepared as follows:

A) 600 µL P1 + 600 µL P3 + 1 mg aniline (1.2 mL sample; 0.4 mM in polymer and 9 mM in aniline)

B) 600 µL P2 + 600 µL P3 + 1 mg aniline (negative control) (1.2 mL sample; 0.4 mM in polymer and 9 mM in aniline)

C) 600 µL P1 and 0.5 mg aniline (negative control) (0.6 mL sample; 0.4 mM in polymer and 9 mM in aniline)

After 16 hours all samples were purified over a Sephadex G200 column to remove any free PEG and aniline (eluting with milliQ). The opaque fractions were combined and used in the Hela Cell Studies as discussed next.

Hela Cell Studies The cellular adhesion experiments were performed with HeLa cells, which were seeded one day before the experiment in 8-well microscopy chambers (Nunc, Wiesbaden, Germany) at a density of 40,000 cells/well. At the time of the experiment, cells had grown to approximately 50% confluence. The polymersome samples (20 μL) were diluted with Dulbecco's Modified Eagle Medium DMEM (380 μL) and added to the cells, which were incubated for 1.5 h at 37 °C. The cells were washed with DMEM (3 × 400 μL) and imaged immediately. Confocal laser scanning microscopy was performed using a Leica Microsystems TCS SP2 AOBS system (Mannheim, Germany). Excitation of rhodamine was achieved with an argon laser [488 nm (47%), 514 nm (39%) and 561 nm (36%)] and the resulting emission was acquired between 575 and 725 nm as an average of four scans.

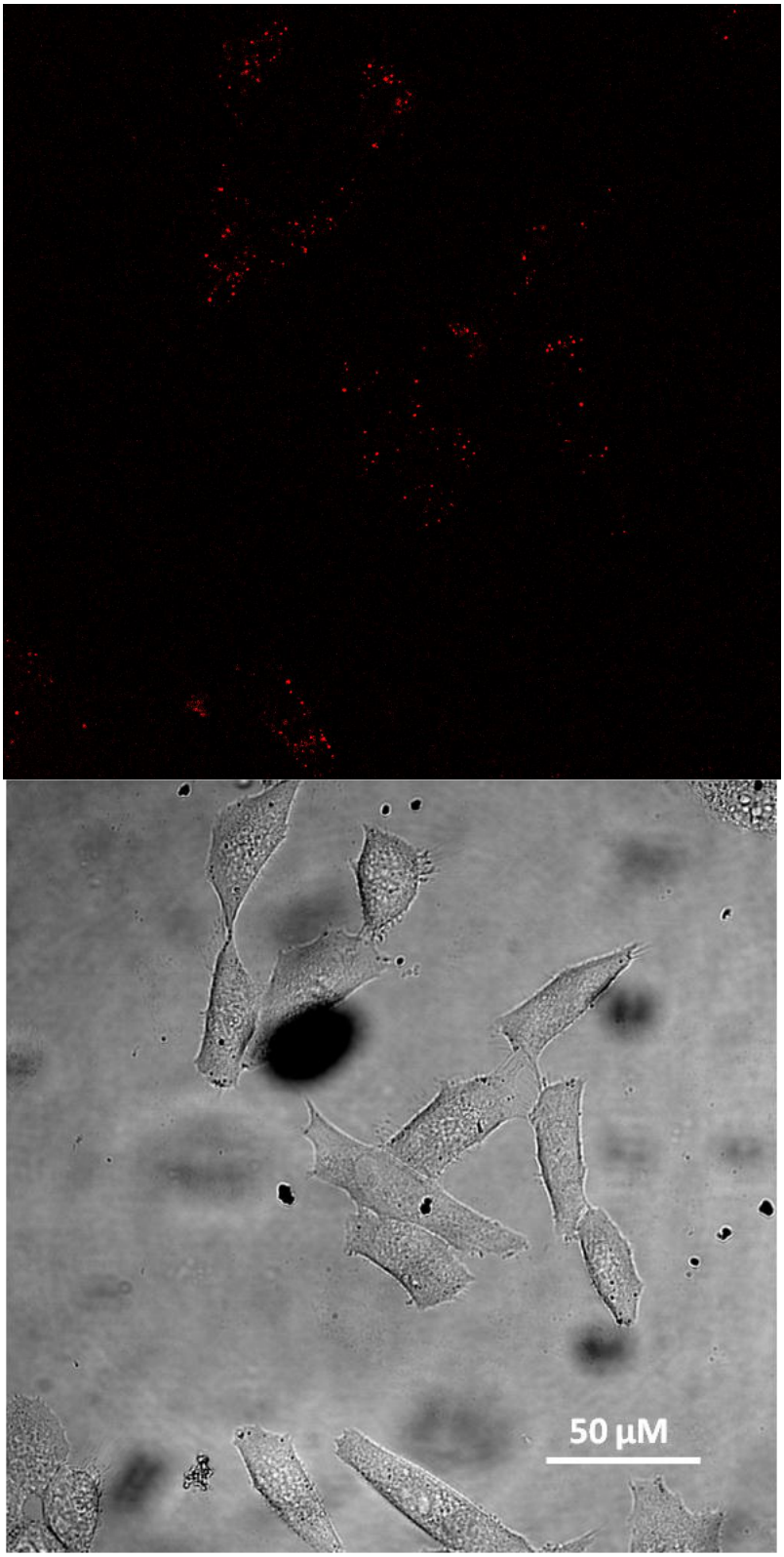


Figure S8: Aniline-catalyzed dynamic exchange between a set of Tat-functional polymersomes and a set of rhodamine-functional polymersomes results in a new set of polymersomes that are both cell penetrating (Tat, Hela cells) and visible under CLSM. Top) confocal fluorescent image showing fluorescent cell adhering polymersomes as red dots. Bottom) Corresponding bright field image of Hela cells after incubation with the polymersome sample.

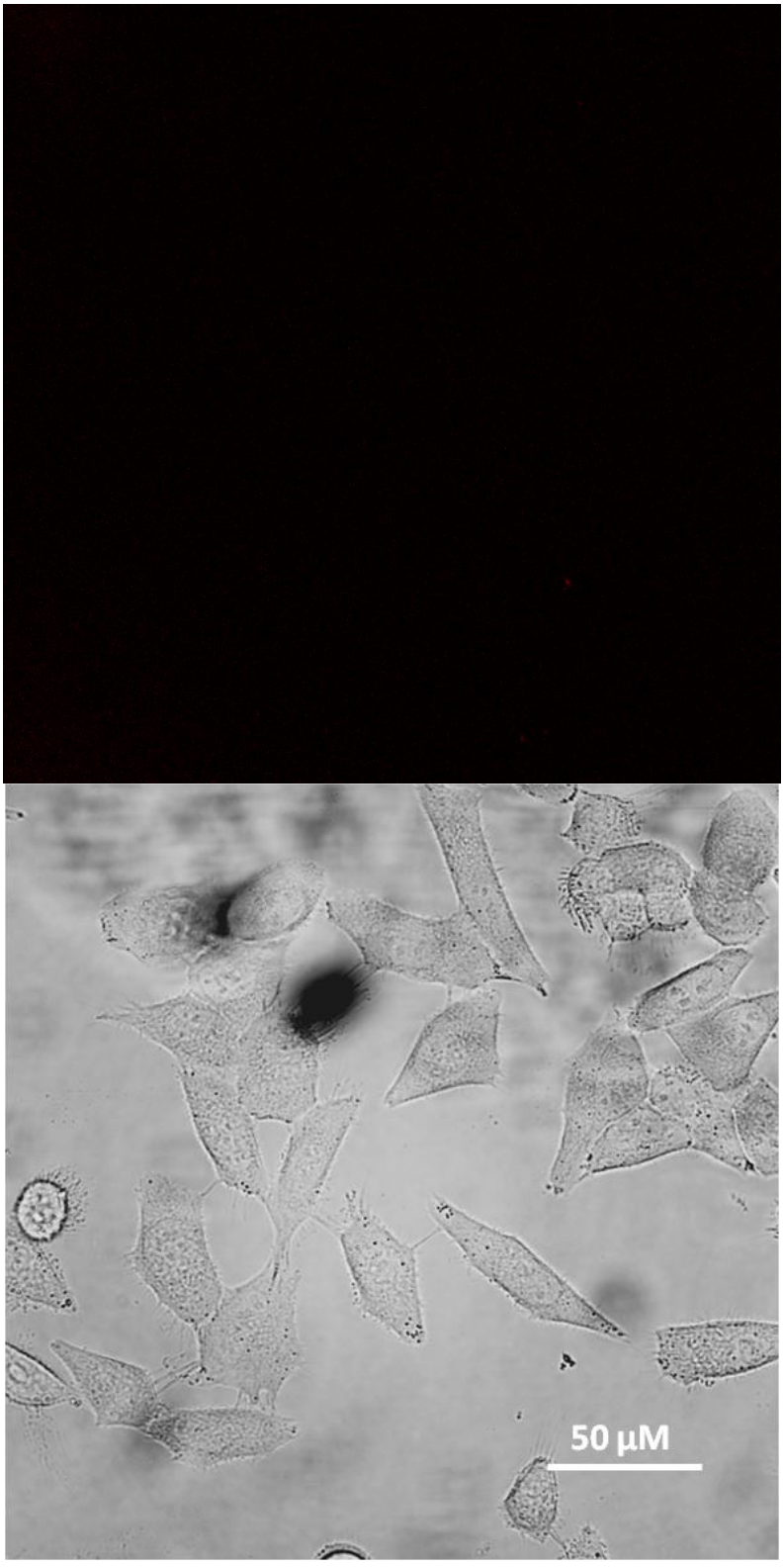


Figure S9: Negative control: Aniline-catalyzed dynamic exchange between a set of Tat-functional polymersomes and a set of irreversibly functionalized (no hydrazone bond) rhodamine polymersomes does not result in polymersomes that are both cell penetrating (Tat, Hela cells) and visible under CLSM. Top, confocal fluorescent image showing the absence of fluorescence of polymersomes which would appear as red dots. Bottom, corresponding bright field image of Hela cells after incubation with the polymersome sample.

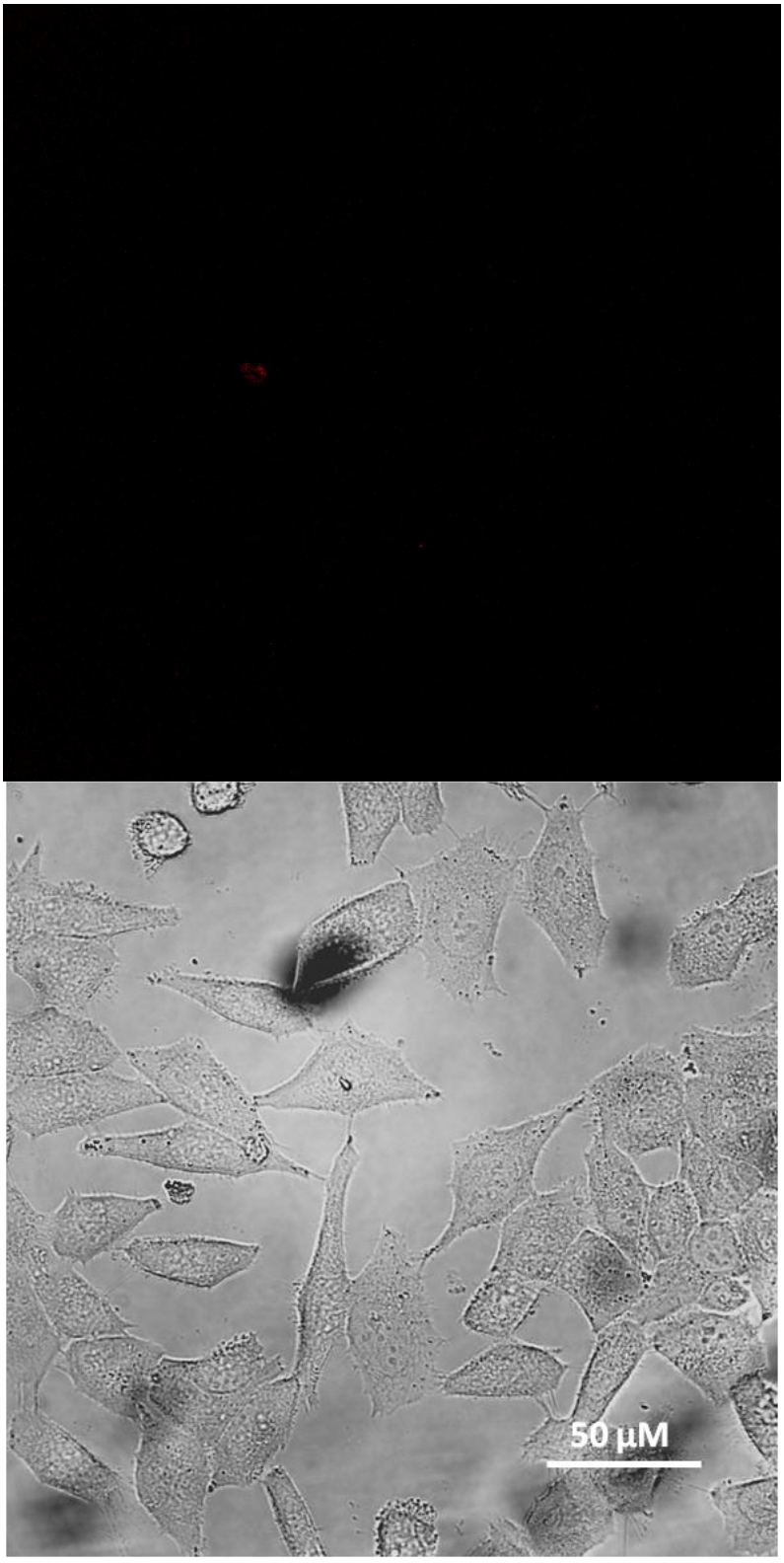


Figure S10: Negative control: Aniline-catalyzed dynamic exchange involving rhodamine-functionalized polymersomes in ***absence*** of Tat polymersomes does not result in a new set of polymersomes that are both cell penetrating (Hela cells) and visible under CLSM. Top, confocal fluorescent image showing the fluorescence of polymersomes. If polymersomes would be fluorescent and cell adhering they would color as red dots.; Bottom, corresponding bright field image of Hela cells after incubation with the polymersome sample.