Supplementary Information

Water-soluble poly(2,7-dibenzosilole) as an ultra-bright fluorescent label for antibody-based flow cytometry

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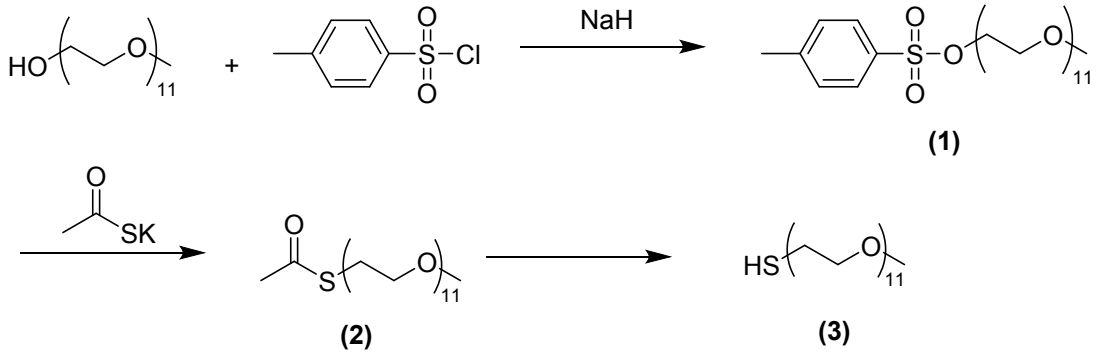
General Methods

^{1}H NMR spectra were recorded on Bruker 400 MHz instruments using $CDCl_3$. UV-Vis spectra and molar extinction coefficients were measured using a PerkinElmer LAMBDA 35 Spectrophotometer. Fluorescence spectra were recorded on a LS 45 Fluorescence Spectrometer. Fluorescent quantum yields were measured using a Hamamatsu PL quantum yield measurement system. The absorbance of polymer solution in THF was adjusted to between 0.003 and 0.005 at 400 nm, and quantum yield was obtained with 400 nm excitation. Gel permeation chromatography (GPC) was carried out in THF at 50 ^oC using a 5 μm Waters Styragel® HR3 and a HR4 GPC column system on a Waters 2960 Alliance HPLC Separations Module with a Waters 2996 PDA Detector (Waters Corporation, Milford, MA) using a flow rate of 0.5 mL/min. The system was calibrated with polystyrene standards in the range of 3500 to 200,000 g/mol (Sigma-Aldrich). Dynamic light scattering (DLS) was performed on a Malvern Zetasizer Nano ZS system equipped with red 633 nm laser. Reagents and solvents were used as obtained from commercial suppliers except where indicated otherwise.

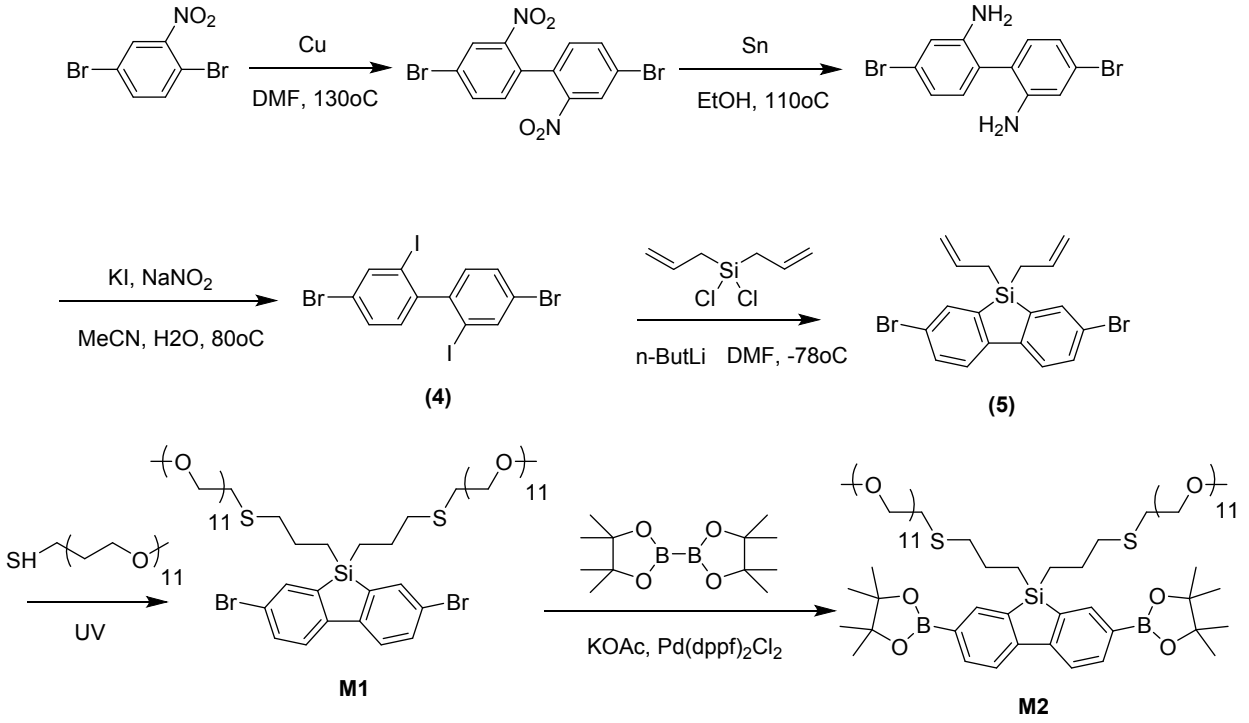
1. Syntheses of PEG11-Dibenzosilole Monomers



2,5,8,11,14,17,20,23,26,29,32-Undecaoxatetratriacontan-34-yl-4-methylbenzenesulfonate (1). A 60% dispersion of sodium hydride (1.80 g, 45.5 mmol) was added to a solution of 2,5,8,11,14,17, 20,23,26,29,32-undecaoxate-tratriacontan-34-ol, (18.0 g, 34.9 mmol, ChemPep Inc.) in dry THF (60 mL) over 10 min while the reaction mixture was cooled in an ice-water bath and stirred for 2 h. 4-methylbenzene-1-sulfonyl chloride (10.0 g, 52.5 mmol, Fisher Scientific) was added, and the reaction mixture was stirred at room temperature overnight. The resulting solid was filtered off, and the filtrate was concentrated under vacuum. The crude product was purified by column chromatography over silica gel eluting with 2 % methanol in chloroform to yield (**1**) (19.3 g, 82 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, 2H), 7.37 (d, 2H), 4.18 (t, 2H), 3.72-3.57 (m, 42H), 3.40 (s, 3H), 2.47 (s, 3H).

S-2,5,8,11,14,17,20,23,26,29,32-Undecaoxatetratriacontan-34-yl ethanethioate (2). A mixture of 2,5,8,11,14,17,20,23,26,29,32-undecaoxatetratriacontan-34-yl 4-methyl-benzenesulfonate (**1**) (10.0 g, 14.9 mmol) and potassium thioacetate (10.2 g, 89.6 mmol) in dry acetonitrile (120 mL) was heated at 90 ^{o}C for 2 h. The reaction mixture was cooled to room temperature and the solvent removed under vacuum. Chloroform (150 mL) was added to the resulting residue, and the mixture stirred at room temperature for 10 min. The resulting solid was removed by filtration, and the filtrate was concentrated under vacuum. The crude product was purified by column chromatography over silica gel eluting with 4 % water in acetonitrile to yield product (**2**) (7.2 g, 83 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67-3.61 (m, 42H), 3.40 (s, 3H), 3.11 (t, 2H), 2.36 (s, 3H).

2,5,8,11,14,17,20,23,26,29,32-Undecaoxatetratriacontane-34-thiol (3). Hydrochloric acid (36 %, 10 mL) was added to a solution of S-2,5,8,11,14,17,20,23,26,29,32-undecaoxatetratriacontan-34-yl ethanethioate (**2**) (7.0 g, 2.0 mmol) in methanol (100 mL). The solution was stirred at room temperature for 48 h and then concentrated under vacuum. The resulting crude product was purified by column chromatography eluting with 1 % methanol in chloroform to yield product (**3**) as a colorless oil (6.0 g, 93 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.69-3.62 (m, 40H), 3.58-3.55 (m, 2H), 3.40 (s, 3H), 2.72 (q, 2H)



4,4'-dibromo-2,2'-diiodobiphenyl (4) was obtained using a previously reported protocol. ^{1}H NMR data and yields were consistent with those reported in the literature.[1]

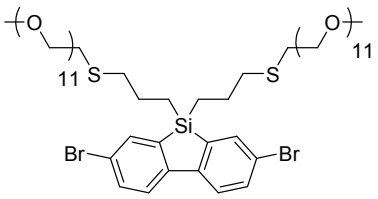
5,5-diallyl-3,7-dibromo-5H-dibenzo[b,d]silole (5) n-Butylithium (1.6 M in hexane, 11.1 mL, 17.8 mmol, Aldrich) was added dropwise over 15 min to a solution of 4,4'-dibromo-2,2'-diiodo-1,1'-biphenyl (4) (2.0 g, 3.6 mmol) in anhydrous THF (20 mL) while the reaction mixture was stirred in a dry ice-acetone bath under argon. After stirring the reaction mixture for an additional 1.5 h, diallyldichlorosilane (1.4 mL, 8.2 mmol, Gelest Inc.) was added and stirred for additional 1.5 h at room temperature. The reaction mixture was quenched with water (2 mL), and the solvent was removed under vacuum. The crude product was then dissolved in

chloroform (100 mL) and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated. The crude material was purified column chromatography over silica gel eluting with hexanes to yield product (**4**) as a white solid (0.81g, 54% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 2H), 7.64 (d, 2H), 7.59 (d, 2H), 5.83 (d, 2H), 5.0 (d, 4H), 1.85 (d, 4H).

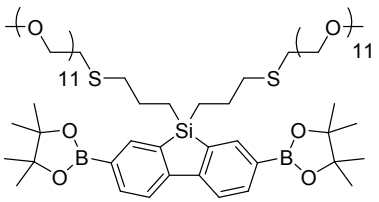
3,7-Dibromo-5,5-di-(2,5,8,11,14,17,20,23,26,29,32-undecaoxa-35-thiaoctatriacontan-38-yl)-5H-dibenzo[b,d]silole (M1). A solution of 5,5-diallyl-3,7-dibromo-5H-dibenzo[b,d]silole (**5**) (100 mg, 0.24 mmol), 2,5,8,11,14,17,20,23,26,29,32-undecaoxatetratriacontane-34-thiol (**3**) (380 mg, 0.71 mmol) and 2,2-dimethoxy-2-phenyl-acetophenone (26 mg, 0.10 mmol) in THF (BHT free, 4.5 mL) was prepared in a quartz flask equipped with a condenser and illuminated (photolysis) with BLAK-RAY long wavelength UV light under argon for 2 h. The photolysis reaction mixture was concentrated under vacuum, and the resulting crude product was purified by column chromatography over silica gel eluting with 5 % methanol in chloroform to yield product (**M1**) (270 mg, 76 % yield). ^{1}H NMR (400 MHz, CDCl3) δ 7.69 (s, 2H), 7.65 (d, 2H), 7.57 (d, 2H), 3.67-3.53 (m, 84H), 3.39 (s, 6H), 2.59 (t, 4H), 2.46 (t, 4H), 1.57-1.55 (m, 4H), 1.11-1.07 (m, 4H).

5,5-Di(2,5,8,11,14,17,20,23,26,29,32-undecaoxa-35-thiaoctatriacontan-38-yl)-3,7-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5H-dibenzo[b,d]silole (M2). A mixture of 3,7-dibromo-5,5-di(2,5,8,11,14,17,20,23,26,29,32-undecaoxa-35-thiaoctatriacontan-38-yl)-5H-dibenzo[b,d]silole (**M1**) (500 mg, 0.34 mmol), bis(pinacolato)diboron (190 mg,0.75 mmol, Oakwood Chemical), [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (97 mg, 0.12 mmol, Fisher Scientific) and potassium acetate (200 mg, 2.0 mmol) in anhydrous dioxane (12 mL) was heated under reflux for 3 h under argon. After the reaction mixture was cooled to room temperature, the solid was removed by filtration, and the filtrate was concentrated under vacuum. The resulting crude product was purified by column chromatography eluting with 10 % methanol in dichloromethane to give product (**M2**) as an oil (220 mg, 41 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (s,2H), 7.91 (d, 2H), 7.86 (d, 2H), 3.72-3.50 (m, 84H), 3.40 (s, 6H), 2.57 (t, 4H), 2.43 (t, 4H), 1.58-1.50 (m, 4H), 1.35 (s, 24H), 1.09-1.06 (m, 4H).

2. Polymerization and Functionalization of PEG11-Dibenzosilole Polymers



M1



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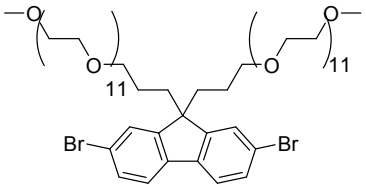

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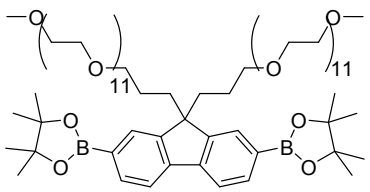


M4

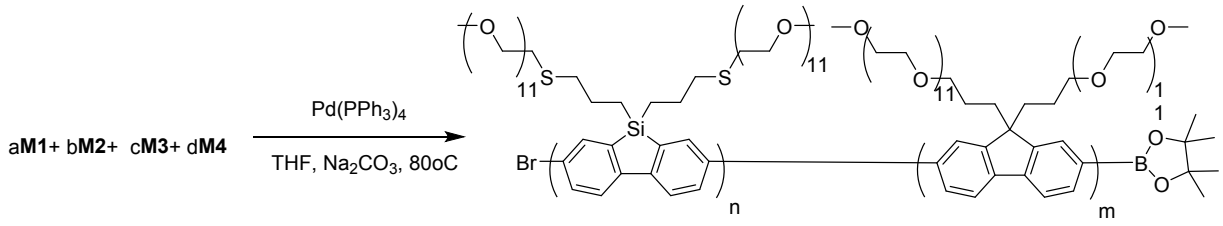


Table S1. Compositon of PEG11-dibenzosilole homopolymer and copolymers

	a	b	c	d	m/n	PF	PSi
PF	0	0	1	1	N/A (n=0)	100%	0%
P5SiF	0.1	0	0.9	1	19	95%	5%
P20SiF	0.4	0	0.6	1	4	80%	20%
P50SiF	1	0	0	1	1	50%	50%
PSi	1	1	0	0	N/A (m=0)	0%	100%

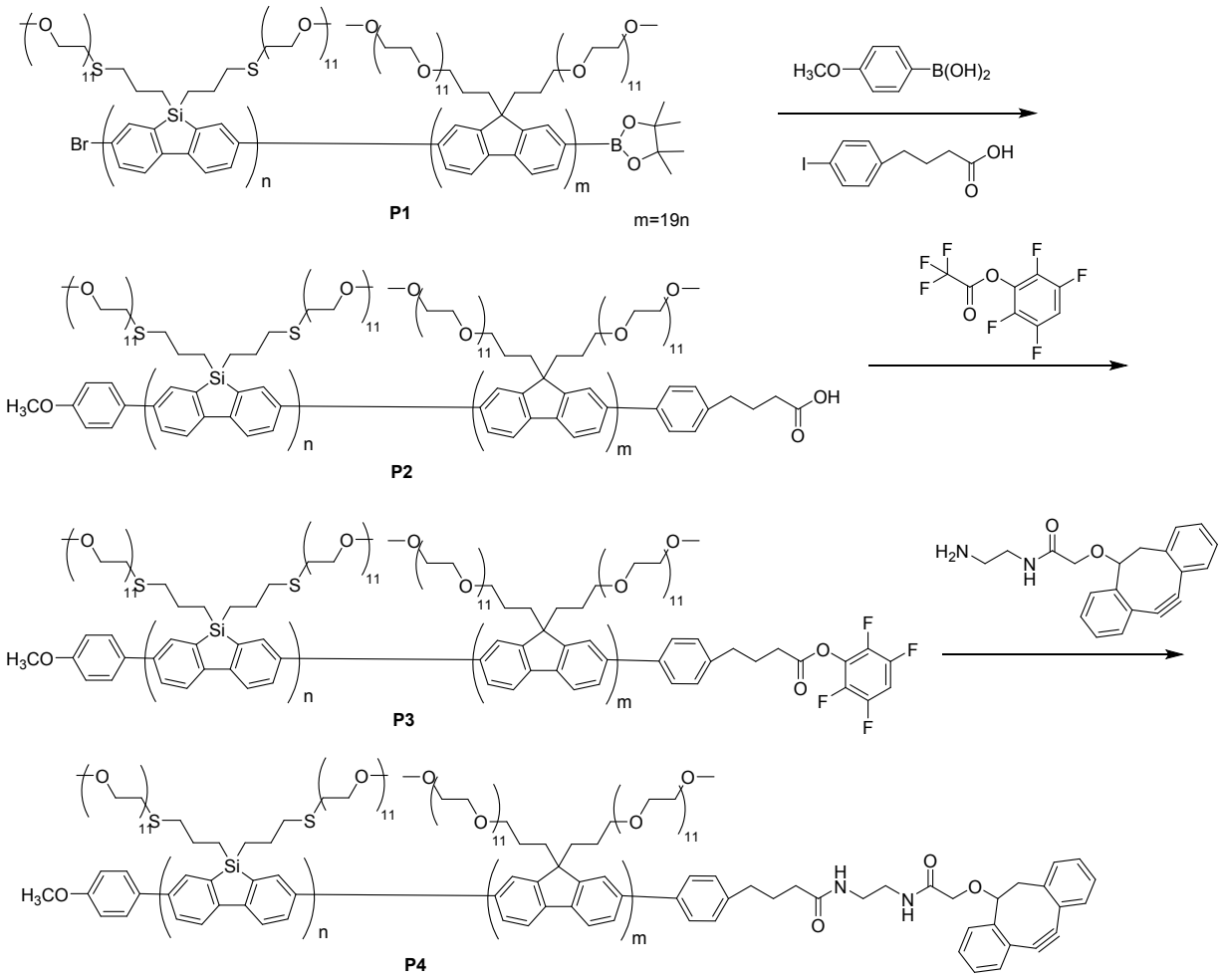
Monomers **M3** and **M4** were obtained using a previously reported protocol. NMR data and yields were well consistent with what were reported in the literature.[2]

Poly(PEG11- dibenzosilole) (PSi). 3,7-dibromo-5,5-di(2,5,8,11,14,17,20,23,26,29,32-undecaoxa-35-thiaoctatriacontan-38-yl)-5H-dibenzo[b,d]silole (**M1**) (155 mg, 0.105 mmol), 5,5-di(2,5,8,11,14,17,20,23,26,29,32-undecaoxa-35-thiaoctatriacontan-38-yl)-3,7-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5H-dibenzo[b,d]silole (**M2**) (165 mg, 0.105 mmol) and tetrakis(triphenylphosphine)palladium(0) (36 mg, 0.03 mmol) were dissolved in 0.5 mL tetrahydrofuran in a 15 mL round-bottom flask equipped with a condenser and vacuum adaptor. 0.3 mL of 2M sodium carbonate solution was added and the flask was connected to a Schlenk line. The reagent mixture was carefully degassed through four freeze-pump-thaw cycles, and after the last cycle the flask was refilled with argon. The reaction mixture was heated at 80^{o}C with vigorous stirring under argon. After 24 h, the reaction was stopped and cooled to room temperature. The organic layer was carefully collected, evaporated to dryness and redissolved in 4 mL chloroform. The solution was filtered through a 0.45 μm glass fiber filter and then poured into 60 mL hexane to precipitate the polymer. The polymer was collected by centrifugation and redissolved in chloroform. The precipitation-centrifugation-redissolution cycle was repeated twice more. The crude polymer product was then dissolved in 10 mL DI water and filtered 3 times through Amicon® Ultra-4 Centrifugal Filter Units (MWCO, 30K) (EMD Millipore Corporation, Billerica, MA) to remove low molecular weight polymers. The purified polymer product was dried under reduced pressure and collected as an amber wax (190 mg; 60 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97-7.78 (brm), 3.67-3.55 (brm), 3.39 (s), 2.65-2.56 (brm), 2.13 (br), 1.72 (br), 1.45 (s), 1.23 (br). GPC (M_w= 42,000 g/mol, PDI= 1.2).

Other polymers were synthesized similarly, except for the ratio of monomers used in the reactions (see Table S1). The molecular weights are shown in Table S2.

Table S2. Molecular weight of PEG11-dibenzosilole homopolymer and copolymers

	M_w	M_n	PDI
PF	49,000	39,000	1.3
P5SiF	70,000	51,000	1.4
P20SiF	43,000	33,000	1.3
P50SiF	55,000	43,000	1.3
PSi	42,000	36,000	1.2



P5SiF with C4-COOH end-capping (P2). 4-(4-iodophenyl)butanoic acid (42 mg, 0.10 mmol) was mixed with polymer **P5SiF** (**P1**) (130 mg, 0.10 mmol) and tetrakis(triphenylphosphine) palladium(0) (5 mg, 0.0043 mmol), and the mixture was dissolved in 0.9 mL THF. 0.6 mL of 2M sodium carbonate solution was added. The reaction mixture was degassed through 3 cycles of freeze-pump-thaw and then heated overnight at 80 °C under nitrogen. The organic layer was carefully collected, evaporated to dryness and redissolved in 4 mL chloroform. The solution was filtered through a 0.45 μm glass fiber filter and poured into 60 mL hexane to precipitate the polymers. Polymers were collected by centrifugation and redissolved in chloroform. The precipitation-centrifugation-redissolution cycle was repeated twice more. The crude polymer was then dissolved in 10 mL DI water and filtered through a 0.45 μm glass fiber filter. The solution was washed 3 times with ethyl acetate and then evaporated under high pressure. The crude product was dissolved in 20% EtOH/80% H_2O and filtered 3 times through Amicon®

Ultra-4 Centrifugal Filter Units (MWCO, 30K) to remove excessive reagents. The polymer product was dried under vacuum and collected as an amber wax (125 mg; yield: 96 %). The polymer was further end-capped similarly using 4-methoxybenzenboronic acid (13 mg, 0.086 mmol) to yield terminal carboxylic acid capped polymer (**P2**) (52 mg, yield: 47%).

P5SiF C4-TFP (P3). 2,3,5,6-tetrafluorophenyl 2,2,2-trifluoroacetate (50 mL) and polymer (**P2**) (25 mg, 0.019 mmol) were dissolved in a dry 5 mL reaction vessel in 0.5 mL anhydrous pyridine. The reaction was stirred vigorously at room temperature for 30 min. The reaction mixture was filtered through 0.45 μm glass fiber filter and then poured into 10 mL hexane to precipitate the polymer. The polymer was collected by centrifugation and redissolved in chloroform. The precipitation-centrifugation-redissolution cycle was repeated once more. The polymer was dissolved in 5 mL $CHCl_3$ and washed once with 1M HCl, then once with dilute $NaHCO_3$ solution and then once with saturated NaCl solution. The polymer product was dried over anhydrous $MgSO_4$, and solvent removed under vacuum to provide (**P3**) as an amber wax (20 mg; yield 80 %).

P5SiF DIBO (P4). N-(2-aminoethyl)-2-(5,6-dihydro-11,12-didehydro-dibenzo[a,e] cyclooctyn-5-oxy)acetamide (DIBO-amine, 3 mg, 0.0075 mmol, Life Technologies) and polymer-capped with TFP ester (**P3**) (10 mg, 0.0075 mmol) were dissolved in a dry 3 mL vial in 0.3 mL anhydrous DMF. 0.1 mL of N,N-diisopropylethylamine was added to the mixture, and the reaction was stirred vigorously overnight at room temperature. The solution was evaporated to dryness and then dissolved in 2 mL DI water. The aqueous solution was filtered through a 0.45 μm glass fiber filter and then centrifuged at 12,000x for 10 min. The supernatant was collected and washed 6 times with ethyl acetate. The solution then was filtered 3 times through an Amicon® Ultra-4 Centrifugal Filter Units (MWCO, 10K) to remove impurities. The polymer product (**P4**) was dried in vacuum and collected as an amber wax (6.5 mg; yield: 65 %).

PSi was functionalized using a similar procedure.

3. Stability and aggregation test of PEG11-Dibenzosilole Polymers

PF and PSi were dissolved in PBS buffer (pH 7.4), and stored at room temperature for 24 hours. Fluorescent spectra of the solution were obtained before and after 24 hour storage (Figure 1S). The results indicate that PF almost lose its fluorescence completely, while PSi retains about 50%.

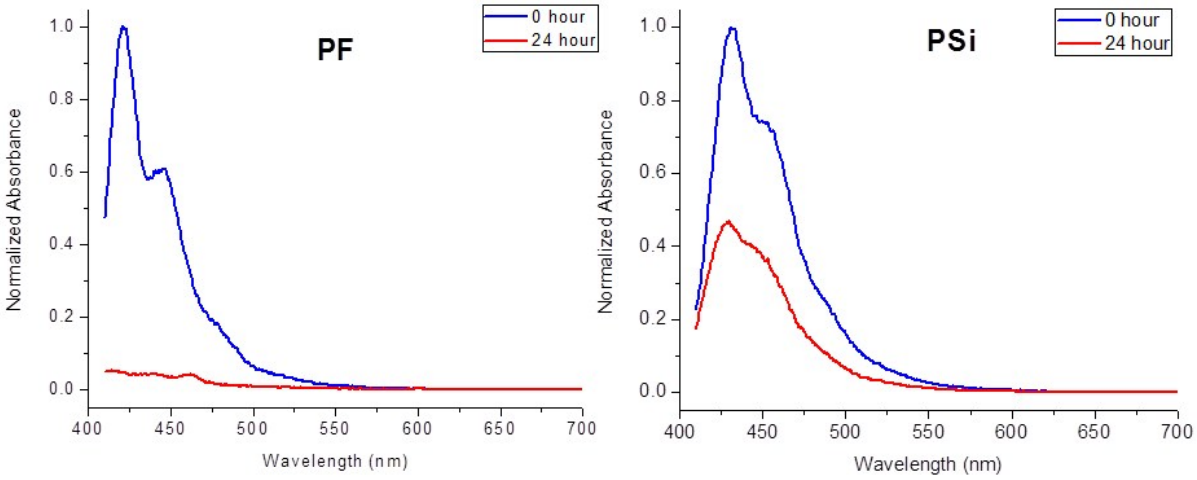


Figure S1. Fluorescent spectra of PF and PSi solution in PBS buffer (blue) and after 24 hours storage (red).

Polymer PF, P5SiF, P20SiF, P50SiF and PSi were dissolved in PBS buffer (pH 7.4), and solutions were filtered with a 0.45 μM filter before DLS measurement. DLS spectra and diameter data are shown in Figure S2 and Table S3. The results suggested no significant aggregation and the sizes of polymers are consistent with what was estimated by molecular structure.

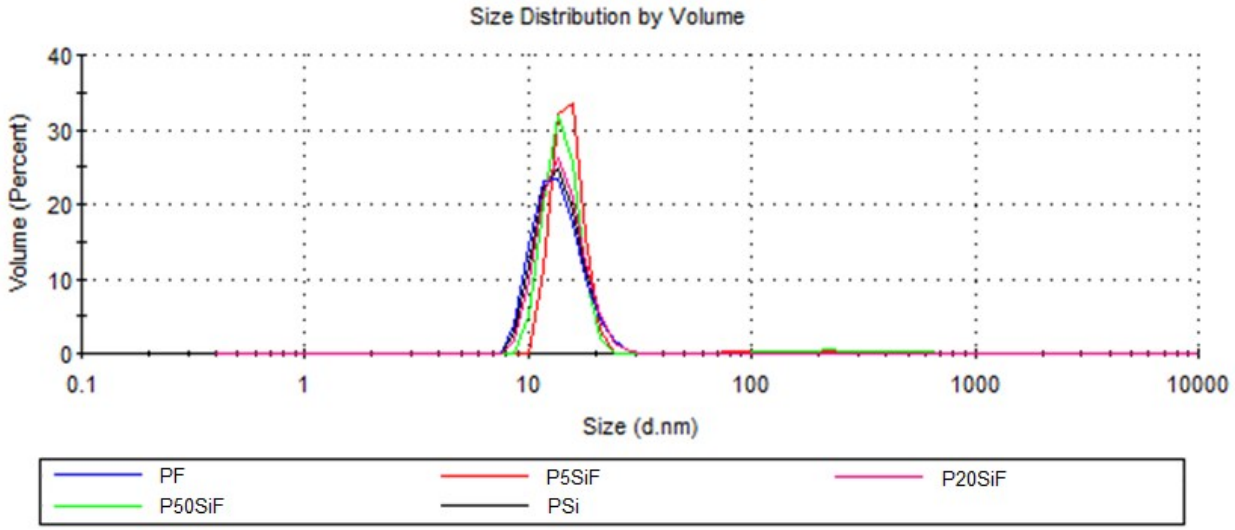


Figure S2. DLS spectra of size distribution by volume of polymer solution in PBS.

Table S3. Mean diameter of polymers in PBS measured by DLS

Polymer	PF	P5SiF	P20SiF	P50SiF	PSi
Mean Diameter (nm)	26.5	18.5	18.8	25.6	17.7

4. Conjugation of Polymers to Antibodies

A method for conjugation of antibodies to **P5SiF DIBO (P4)** is described. A mouse monoclonal antibody against human CD4 was modified with an amine-reactive azido reagent using a molar ratio of 20:1 azido reagent:antibody. The antibody was brought to 6.25 mg/mL in sodium azide free PBS, pH 7.4. Sodium bicarbonate (1M, pH 9.0) was added to a final concentration of 100 mM. Lysines on the antibody were modified by adding 10 mM azido $(PEO)_4$ propionic acid, succinimidyl ester in DMSO to a final concentration of 990 μM. After incubation for 2 h at 25°C excess reagent was removed with centrifugal filters with 50K MW cut off, washed 4 x with TBS, pH 7.4. Azide-modified antibody from the retentate was purified with 2 mL disposable spin columns using P-30 Gel (medium) in TBS. The product with a degree of substitution of ~ 22 azide molecules per antibody was used at 0.5 mg/mL in TBS for modification with **P4** at 100 μM final concentration for 72 h at 25 °C. These antibody-polymer conjugates were evaluated by flow cytometry of human lymphocytes.

PSi was conjugated to anti-CD4 using a similar procedure.

The antibody-polymer conjugates were analyzed using the Novex® NuPAGE® SDS-PAGE Gel System (Life Technologies) at 4-12% in MOPS running buffer. For gel analysis, antibodies were applied on NuPAGE® 4-12% Bis-Tris gels and run in MOPS buffer. 1 μg antibody was applied per lane. Fluorophore-containing proteins were detected on the gels by imaging with a UV-scanner BioSpectrum® 300 Imaging System, using a Benchtop 2UV Transilluminator at 365 nm (Fig. S1B). Total protein staining was achieved with SYPRO®Ruby Protein Stain (Life Technologies), and the gels were imaged with an FLA-9000 image scanner with an excitation of 473 nm and a 575LP filter (Fujifilm Life Science) (Figure S1A).

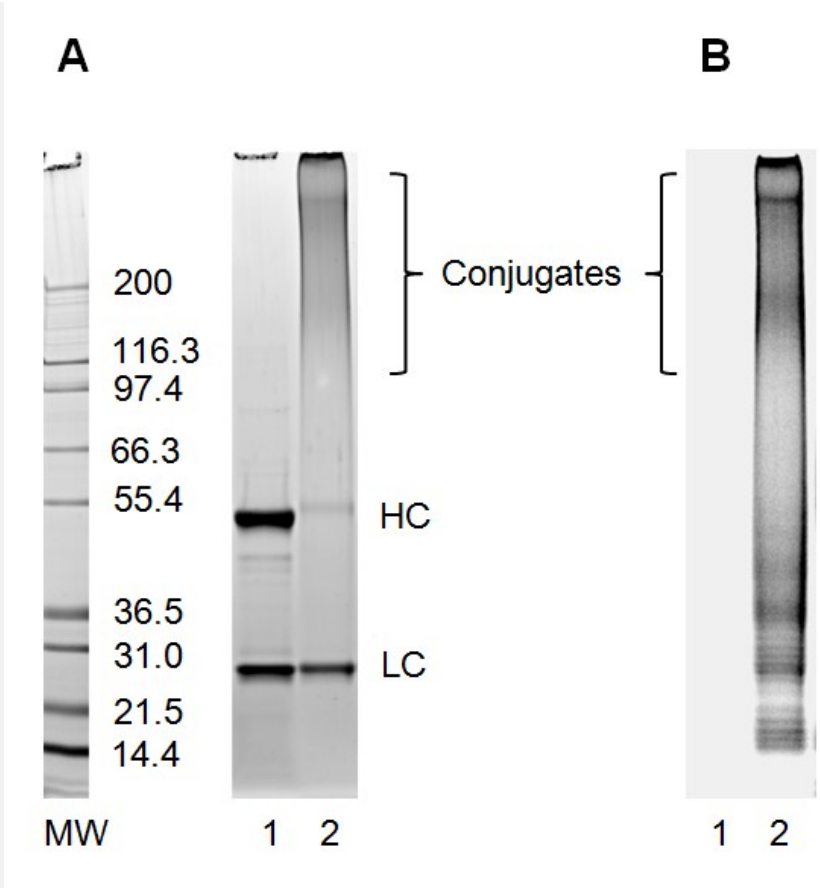


Figure S1. Gel image of antibody against human CD4 (control, lane 1) and of azide modified antibody against human CD4 and that was conjugated to **P5SiF** (conjugate, lane 2). Mark12™ Unstained Standard (Life Technologies; Carlsbad, CA) was used as molecular weight standard ladder (MW). The decrease of free heavy chain (HC) by conjugation to fluorescent polymer was >90%, as determined by densitometric quantitation.

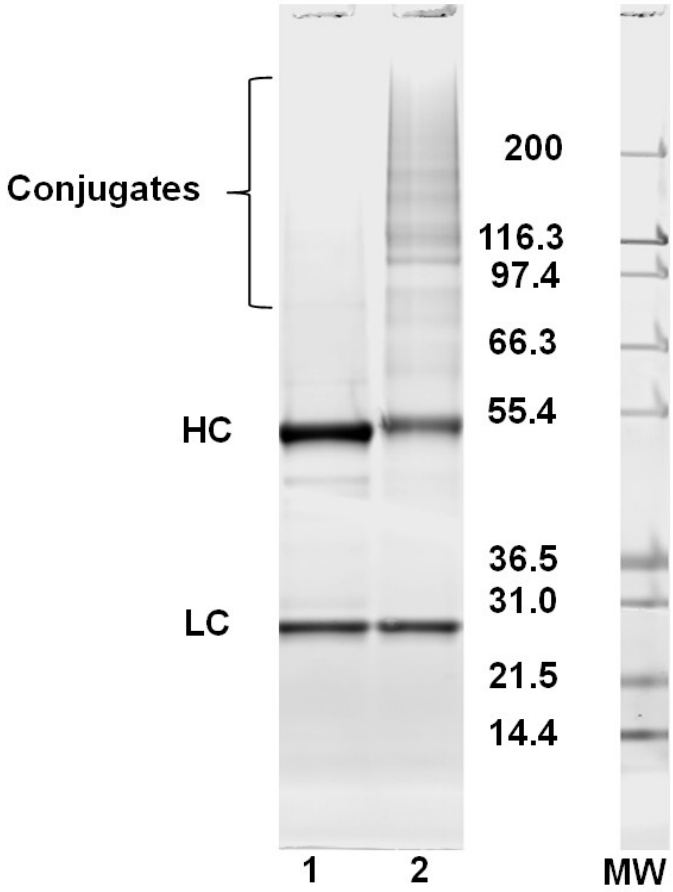


Figure S2. Gel image of antibody against human CD4 (control, lane 1) and of modified antibody against human CD4 and that was conjugated to **PSi** (conjugate, lane 2). Mark12™ Unstained Standard (Life Technologies; Carlsbad, CA) was used as molecular weight standard (MW). The decrease of free HC by conjugation to polymer-dye was 60%, as determined by densitometric quantitation.

5. Cellular Analysis by Flow Cytometry

Polymers **P5SiF** and **PSi** were separately conjugated to a mouse monoclonal antibody against human CD4. White blood cells were prepared from lysed whole blood. Pacific Blue™ anti-human CD4 antibody was purchased from Life Technologies. 125 ng of antibody conjugates were diluted in 1% BSA in PBS, pH 7.4, and 10 μL of the resulting conjugate solutions were added to 90 μL of 1 X 10^6 cells. Conjugates and cells were incubated for 20 minutes at room temperature. The cells then were washed 2 times in 1% BSA in PBS, pH 7.4. After the final wash, the cells were resuspended in 500 μL of 1% BSA in PBS, pH 7.4.[3] After 30 min of staining, cells were analyzed using the Attune® Acoustic Cytometer (Life Technologies Corporation) equipped with a 405-nm violet laser using the standard 440/50 VL1 emission filter setting. For CD4 specific staining, 10,000 lymphocyte events were collected and data were represented as histograms. Unstained cells were used for reference.

Reference

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