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2,1,3-Benzothiadiazole (BTD)-moiety-containing red emitter conjugated amphiphilic poly(ethylene glycol)-*block*-poly(*ε*-caprolactone) copolymers for bioimaging

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Summary

2,1,3-Benzothiadiazole (BTD)-containing red emitter was chemically conjugated onto amphiphilic poly(ethylene glycol)-*block*-poly(*ε*-caprolactone) (PEG-*b*-PCL) copolymers to form two new fluorophore-conjugated block copolymers (**P5** and **P7**). **P5** is a cationic amino group-containing polymer, whereas, **P7** is a neutral polymer. The polymers formed micelles in aqueous solution with average diameters of 45 nm (**P7**) and 78 nm (**P5**), which were characterized using dynamic light scattering (DLS) and atomic force microscopy (AFM). Cell internalization of the micelles using mouse macrophage RAW 264.7 was investigated. The micelles formed from **P5** were endocytosed into the cell's cytoplasm through a non-specific endocytosis process, which was affected by temperature and calcium ions. Micelles formed from **P7** could not be endocytosed. The dramatic difference of cell uptake between **P5** and **P7** indicated the cationic amino groups had a strong influence on the cell internalization to enhance the endocytosis pathway. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was used to evaluate the cytotoxicity of the **P5** micelle and no significant toxicity was observed. This study is the first report regarding the synthesis of BTD-conjugated block copolymers and the application of the biomacromolecules for bioimaging.

1. Introduction

Micelles, formed from amphiphilic block copolymers (ABCs), with hydrophobic cores and hydrophilic coronas have been demonstrated as a powerful tool for cell imaging, disease diagnosis, and delivery of various water insoluble materials (including quantum dots, magnetic nanoparticles, and drugs) into cancer cells.[1a,1b,1c,1d,1e] In order to understand the internalization of block copolymers with cells and clarify the payload delivery mechanism, it is extremely important to directly monitor the subcellular distribution of micelles. Thus, fluorophore-conjugated block copolymers were synthesized and investigated, which include, to name a few, rhodamine derivative-conjugated poly(*ε*-caprolactone)-*block*-poly(ethylene oxide) (TMRCA-PCL-*b*-PEO),[2a] fluorescein-conjugated poly(ethylene oxide)-*block*-poly(*β*-

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Supplementary Data: Data regarding the CMC determination of the two block copolymers and concentration and time dependent cell internalization of the micelles of **P5** are shown in the supplementary data.

benzyl L-aspartate) (PEO-*b*-PBLA-FITC),[2b] rhodamine-conjugated poly(ethylene glycol)-*block*-phosphatidylethanolamine (Rhodamine-PEG-*b*-PE),[2c] and fluorescein-conjugated poly (ethylene glycol)-*block*-poly(lactic acid) (FITC-PEG-*b*-PLA).[2d] Cellular internalization of these block copolymers were found either through a non-specific endocytosis[2a,2b,2c] or a receptor mediated endocytosis[2d] process depending on the cell line and polymer structure with or without targeting groups on the micelles. As can be seen above, most of the biomarkers used in the fluorophore-conjugated block copolymers are ionic fluorescein derivatives emitting in the green spectral window and rhodamine derivatives that are red emitters.

2,1,3-Benzothiadiazole (BTD)-containing materials have been widely studied as emitters in organic/polymeric light emitting diodes,3a,b,c two-photon absorbing materials,3d and chromophores for solar cells3e because these materials tend to have a strong charge transfer ability and emit in the red spectral window. Some amphiphilic BTD moiety-containing conjugated polymers have also been synthesized and investigated as DNA sensors.3f However, little attention has been paid to the application of the BTD-containing fluorophores as intracellular biomarkers. Previously, we physically incorporated a BTD-containing two-photon absorbing fluorophore into micelles to achieve large two-photon cross-sections in the red spectral window and used these micelles as nanocarriers to deliver the hydrophobic two-photon absorbing fluorophore into mouse macrophage RAW 264.7 cells.[4] Herein, we report the synthesis and bioimaging of two new BTD-fluorophore-containing block copolymers, of which the BTD-containing fluorophore was chemically conjugated onto amphiphilic PEG-*block*-PCLs (Scheme 1). Block copolymer (**P5**) had cationic amino groups while the other one (**P7**) did not. The staining of RAW 264.7 cells using the two block copolymers was compared to study the influence of polymer structure on cell internalization.

2. Experimental

2.1. Materials

α,ω-Diaminopolyethylene glycol (M_n = 3400) (**P3**), methoxypolyethylene glycol amine (M_n = 2000) (**P6**), compound **1**, and compound **5** were prepared according to procedures previously described.[3a, 5, 6, 7]

ε-Caprolactone, stannous octoate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trimethylsilylethylene (compound **2**), sodium boronhydride ($NaBH_4$), potassium carbonate (K_2CO_3), dichlorobis(triphenylphosphine)palladium (II) ($PdCl_2(PPh_3)_2$), p-nitrophenyloxycarbonyl chloride (compound **8**), and trifluoroacetic acid (CF_3COOH) were purchased from Aldrich (St. Louis, MO, USA) and used without further purification.

Dialysis membranes (regenerate cellulose, M_w cut off 10000) were purchased from Pierce (Rockford, IL, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Hoechst 33342 were acquired from Invitrogen (Carlsbad, CA, USA).

2.2. General methods

^{1}H NMR spectra were measured using a Bruker 300 instrument operating at 300 MHz with tetramethylsilane (TMS) internal standard as a reference for chemical shifts. Molecular weights of polymers were determined using a Waters 1515 gel permeation chromatography (GPC) coupled with UV and RI detectors, in reference with a series of polystyrene standards with tetrahydrofuran (THF) as the eluent. UV-Vis spectra were recorded with a Hewlett Packard 8452A Diode Array UV-Vis Spectrophotometer. Fluorescence spectra were recorded with a Perkin Elmer Luminescence Spectrometer LS 50B using a Xenon lamp as a light source with the emission and excitation slit of 5 nm. Dynamic light scattering (DLS) measurements for micelle diameters were performed using a Malvern Nano-ZS instrument (Worcestershire, UK)

equipped with a 4 mW He-Ne laser (633 nm) with an output at a scattering angle of 90°. The solution was passed through a 0.45 μm Nylon micro-filter (VWR, Batavia, IL) to remove dust before the DLS measurements. Atomic force microscopy (AFM, NanoScope III, Digital Instrument) equipped with an integrated silicon tip/cantilever with a resonance frequency ~240 KHz in height and phase image models was utilized for the observation of morphologies. Polymer solutions (4 μL, 0.1 mg/mL) were dropped on a mica substrate and dried at room temperature for morphological observation. The AFM topographies showed no evidence of tip-induced modification during successive scans. Fourier transform infrared (FTIR) spectroscopy was measured using a Nicolet 6700 FTIR & IR spectrometer (Thermo Scientific, Waltham, MA).

2.3. Preparation of micelles

Stock solution of micelle 1—P5 (5.0 mg) was dissolved in THF (350 μL) and stirred for 30 minutes. The THF solutions were added slowly into 1.5 mL of a 10 mM HEPES MilliQ distilled water solution (pH : 7.2) under vigorous stirring. The micelles were transferred to a dialysis bag and dialyzed against 10 mM HEPES MilliQ water for 3 days with a water change of approximately every 8 hours. The solution was then filtered through a 200 nm micro-filter to remove possible large particles. Concentration of the conjugated fluorophore **7** (**RED**) in the micelles was determined as following: (1) micelles were freeze-dried and then dissolved in THF; (2) the absorbance spectra were measured using UV-Vis spectrometer; (3) concentration was calculated in a reference of a standard curve of the absorbance of **7** at 500 nm in THF. The concentration of **7** in micelle 1 was determined to be 110 μM. The corresponding polymer concentration was calculated to be 2.1 mg/mL.

Stock solution of micelle 2—Although the preparation procedure was similar to micelle 1, the polymer used was **P7** (5.0 mg). The concentration of **7** in micelle 2 was determined to be 80 μM corresponding to a polymer concentration of 1.3 mg/mL.

2.4. Critical micelle concentration (CMC) determination

As the block copolymers, **P5** and **P7**, contained fluorophores, their CMCs were determined using the intensity change upon micelle formation in aqueous solution.[2b] Before the micelle formation, the fluorophore emission was quenched by water and very weak fluorescence was observed. Once the micelle was formed, the fluorescence intensity increased significantly. This method was used to determine the CMC of fluorophore conjugated PEO-*b*-PBLA-FITC.[2b] Fluorescence intensity at 625 nm was plotted against the polymer concentrations in a range of 1.5×10^{-6} to 2.0×10^{-1} mg/mL in aqueous solution. No significant difference of the CMCs, 0.025 mg/mL for **P5** and 0.022 mg/mL for **P7**, was observed. All the DLS, AFM and cell uptake experiments were carried out using the polymer concentrations above their CMCs.

2.5. Determination of quantum yields

Fluorescence quantum yields (η) of samples in solutions were recorded by using rhodamine B ($\eta = 0.65$ in ethanol)[8] excited at 510 nm and were calculated according to the following equation:9

$$\eta_s = \eta_r \left(\frac{A_r}{A_s}\right)\left(\frac{I_s}{I_r}\right)\left(\frac{{n_s}^2}{{n_r}^2}\right)$$

where (η_r) and (η_s) are the fluorescence quantum yields of standards and the samples, respectively. A_r and A_s are the absorbance of the standards and the measured samples at the excitation wavelength, respectively. I_r and I_s are the integrated emission intensities of standards

and the samples, respectively. n_r and n_s are the refractive indices of the corresponding solvents of the solutions, respectively. The experimental error was ~ 10%.

2.6. Cell culture and imaging

Mouse macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum, 5% penicillin, 2 mM L-glutamine (Sigma), and incubated at 37 °C in a 5% CO_2 atmosphere. The cells were seeded onto 96-well plates at 10,000 cells per well and incubated for 1 day. The micelles made from **P5** and **P7** were dissolved and diluted in DMEM growth medium to give final **7** concentrations of 2 - 12 μM and polymer concentrations of 0.03 - 0.2 mg/mL for cell staining. The media in the wells were replaced with 100 μL of the pre-prepared medium containing micelles. The plates were then returned to the incubator and maintained in 5% CO_2 at 37 °C for 1 - 16 h. Upon removal of the micelles solutions, the cells were washed once with 100 μL of PBS solution. 10 μL of Hoechst 33342 with a concentration of 1 mM in 100 μL of fresh DMEM medium was then added into the wells and incubated for 30 minutes for nuclei staining. Live cells were imaged with a Zeiss LSM 510 confocal microscope (Thornwood, NY, USA). Blue emission of Hoechst 33342 was generated by a 405 nm laser and the emission was collected from 420 to 480 nm. Red emission of **7** was collected from 600 nm to 700 nm using an excitation wavelength of 488 nm. Negligible background fluorescence of cells was detected under the settings used.

2.7. Cytotoxicity study

The assay was performed by an *in vitro* MTT based toxicology assay kit (Sigma), which is based on the intracellular reduction of a tetrazolium (MTT) dye to a formazan product measured spectrophotometrically and is used for high-throughput screening.[10] Cells incubated with micelles for 16 hours in the 96-well plate were washed with PBS buffer and then incubated in fresh DMEM medium (100 μL) and 10 μL of MTT solution (5 mg/mL) in 5% CO_2 at 37°C for another 3 h. 60 μL of the culture medium was taken out and 50 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the internalized purple formazan crystals by gentle pipetting up and down. The absorbance was measured at a wavelength of 490 nm using SpectraMax 190 from Molecular Devices (Downingtown, PA, USA). Each experiment was conducted twice in triplicate. The result was expressed as a percentage of the absorbance of the blank control.

2.8. Factors affecting internalization: temperature and Ca^{2+} ion

Cells were grown in Petri dishes in 900 μL DMEM medium. For the influence of calcium ions on the cellular internalization, calcium chloride was added to the medium with a final concentration of 3 mM. The effects of temperatures were assessed by incubating the cells at 4 °C and 37 °C. Concentration of **7** was 6 μM in the growth medium. After a 16 h incubation period, the media was removed and cells were washed with cold PBS. The cells were lysed in 300 μL of DMSO. The DMSO solutions were then diluted with 3.7 mL of water. The resulting solutions were used for the measurement of their emissions at 650 nm (excited at 488 nm) corresponding to the amount of fluorophores endocytosized into cells.

2.9. Synthesis

2.9.1. Preparation of 4—A mixture of compound **1** (1.5 g, 3.4 mmol), compound **2** (1.5 g, 15.3 mmol), CuI (30 mg), $PdCl_2(PPh_3)_2$ (100 mg) and triethylamine (10 mL) in 10 mL of dry THF was heated under nitrogen at 70°C for 24 hours. After cooling down, the mixture was passed through a silica gel column eluted using hexane/ethyl acetate (20:1 by volume). The intermediate **3** was suspended with a stirring in 30 mL of methanol in the presence of 300 mg of potassium carbonate to remove the trimethylsilyl group to get 800 mg of **4**. Yield: 61%. ^1H

NMR (300 MHz, $CDCl_3$, 25°C, TMS) δ =10.08 (s, 1H), 7.89 (m, 3H), 7.73 (d, 1H), 7.54 (m, 2H), 3.20 (s, 1H), 2.05 (m, 4H), 1.10 (m, 12H), 0.99 (t, 6H), 0.50 (m, 4H).

2.9.2. Preparation of 6—A mixture of compound **4** (550 mg, 1.42 mmol), compound **5** (250 mg, 0.55 mmol), CuI(I) (20 mg), $PdCl_2(PPh_3)_2$ (35 mg) and triethylamine (5 mL) in 10 mL of dry THF was heated under nitrogen at 70°C for 36 hours. After the solvent was removed, the residue was purified with column chromatography using hexane/ethyl acetate (5:1 by volume) as an eluent to get 260 mg of compound **6** with a yield of 44%. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS) δ = 10.09 (s, 1H), 8.08 (d, 2H), 7.95-7.79 (m, 10H), 7.50 (m, 4H), 7.43 (d, 2H), 2.07 (m, 8H), 1.22 (m, 24 H), 0.80 (t, 12H), 0.64 (m, 8H). FTIR (ν, cm^{-1}): 2930 and 2855 (strong, CH_2, CH_3), 1690 (strong, aldehyde), 1604 (strong, aromatic).

2.9.3. Preparation of 7—40 mg of $NaBH_4$ was added slowly into a solution of compound **6** (180 mg, 0.168 mmol) in 20 mL of THF and the mixture was stirred at room temperature overnight. The mixture was poured into water and then extracted using ethyl acetate. After the solvent was removed under vacuum, the residue was purified through a column chromatography (methylene chloride/ethyl acetate 20:1 by volume) to get compound **7** for a yield of 80%. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS) δ = 8.08 (dd, 2H), 7.92 (d, 2H), 7.69 (m, 3H), 7.56 (m, 3H), 7.36 (m, 6H), 7.23 (dd, 2H), 4.81 (s, 4H), 2.03 (m, 8H), 1.22 (m, 24 H), 0.80 (t, 12H), 0.64 (m, 8H). FTIR (ν, cm^{-1}): 3400 (broad, weak, -OH), 2919 and 2849 (strong, CH_2, CH_3), 1604 (weak, aromatic).

2.9.4. Syntheis of P1—Compound **7** (160 mg, 0.15 mmol) was weighed into a dry flask and ε-caprolactone (1.03 g, 8.76 mmol) was subsequently added. The reaction mixture was stirred for 5 min at 110 °C in a preheated oil bath before the catalyst (stannous octoate, 1 drop) was added and the polymerization was performed for 12 h. The resulting viscous solution was rapidly cooled, upon which it solidified. The crude polymer was dissolved in dichloromethane and precipitated into cold methanol. The isolated yield was 60%. M_n (GPC) = 12000, M_w/M_n = 1.40. M_n (NMR) = 5200. Degree of polymerization (DP) of PCL = 18. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS) δ = 8.08 (dd, 2H), 7.92 (d, 2H), 7.69 (m, 3H), 7.56 (m, 3H), 7.36 (m, 6H), 7.23 (dd, 2H), 5.20 (s, 4H), 4.07 (t, 72H), 3.66 (t, 4H), 2.34 (t, 72H), 2.03 (m, 8H), 1.8-1.3 (m, 216H), 1.22 (m, 24 H), 0.80 (t, 12H), 0.64 (m, 8H). FTIR (ν, cm^{-1}): 2940 and 2864 (strong, CH_2, CH_3), 1721 (strong, -COO- of ester).

2.9.5. Synthesis of P4—P1 (0.23 g, 0.038 mmol of the corresponding hydroxyl group), 50 mg of compound **8** (0.25 mmol), and 30 mg of pyridine (0.3 mmol) were dissolved in 10 mL of anhydrous methylene chloride and stirred at room temperature overnight to generate the intermediate **P2** (0.22 g), which was precipitated from the reaction mixture into ether, filtrated and dried. The **P2** was then dissolved into 10 mL methylene chloride again. 450 mg of **P3** (2.6 mmol of the free amino group) in 5 mL of dimethylformamide (DMF) was added and the mixture was stirred at room temperature for 1 day. 1 mL of additional pyridine was added into the mixture and the reaction was stirred for one more day. The solvent was removed under reduced pressure and the polymer was precipitated into ether. The solid was washed with water overnight, filtrated and dried to yield 305 mg of polymer. Further purification was carried using dialysis. The powder (75 mg) was dissolved in 5 mL of THF and the solution was slowly added to 20 mL of water to form the micelles. The mixture was dialyzed against the regenerated cellulose membrane with a M_w cut off of 10,000-12,000 for 4 days and then lyophilized to get 54 mg of the product. Basing on the conversion of the dialysis process, the yield was calculated to be 49%. ^{1}H NMR ($CDCl_3$): 8.08 (dd, 2H), 7.92 (d, 2H), 7.69 (m, 3H), 7.56 (m, 3H), 7.36 (m, 6H), 7.23 (dd, 2H), 5.20 (s, 4H), 4.07 (t, 72H), 3.66 (m, 610H), 2.34 (t, 72H), 2.03 (m, 8H), 1.8-1.3 (m, 216H), 1.22 (m, 24 H), 0.80 (t, 12H), 0.64 (m, 8H). M_n (GPC) = 18800, M_w/

M_n = 1.43. M_n (NMR) = 12100. FTIR (ν, cm^{-1}): 2940 and 2883 (strong, CH_2, CH_3), 1721 (strong, -COO- of ester).

2.9.6. Synthesis of P5—P4 (50 mg) was dissolved into 5 mL of methylene chloride and then 150 mg of CF_3COOH was added. The mixture was stirred at room temperature for 30 minutes. After the solvent was removed the residue (50 mg) was used directly without further purification. FTIR (ν, cm^{-1}): 2940 and 2883 (strong, CH_2, CH_3), 1722 (strong, -COO- of ester).

2.9.7. Synthesis of P7—P1 (30 mg, 0.005 mmol of the corresponding hydroxyl groups), 5 mg of compound **8** (0.025 mmol), and 3 mg of pyridine (0.03 mmol) were dissolved in 5 mL of anhydrous methylene chloride and stirred at room temperature overnight to generate the intermediate **P2**. **P2** was precipitated from the reaction mixture into ether, filtrated, dried, and dissolved again in 5 mL methylene chloride. 100 mg of the **P6** (0.05 mmol of the free amino group) in 10 mL of CH_2Cl_2 and 100 μL of triethylamine was added and the mixture was stirred at room temperature for 2 days. The CH_2Cl_2 was removed under reduced pressure and the residue was dissolved in 5 mL of THF. The THF solution was slowly added into 10 mL of water and dialyzed against water using regenerated cellulose membrane with a M_w cut off of 10,000-12,000 for 5 days and then lyophilized to get 23 mg of the product with a yield of 58%. ^{1}H NMR ($CDCl_3$): 8.08 (dd, 2H), 7.92 (d, 2H), 7.69 (m, 3H), 7.56 (m, 3H), 7.36 (m, 6H), 7.23 (dd, 2H), 5.20 (s, 4H), 4.07 (t, 72H), 3.66 (m, 370H), 2.34 (t, 72H), 2.03 (m, 8H), 1.8-1.3 (m, 216H), 1.22 (m, 24 H), 0.80 (t, 12H), 0.64 (m, 8H). M_n (GPC) = 16200, M_w/M_n = 1.39. M_n (NMR) = 9200.

3. Results and discussion

3.1. Synthesis of the new block copolymers P5 and P7

A new red emitter-conjugated PEG-*b*-PCL block copolymer (**P4**), having two active amino-groups at the end of the polymer for further chemical modifications, was prepared as shown in Scheme 1. The final polymer and its intermediates were characterized using NMR and GPC. It has been shown that the PEG-*b*-PCL type block copolymers have low CMC and can be biocompatible and biodegradable.[2a] Neutral BDT-containing fluorophore **7** was chemically conjugated onto the block copolymer and the rigid fluorophore was constructed in the center of the block copolymer. The fluorophore **7**, which has two hydroxyl groups, was used as the initiator for the polymerization of ε-caprolactone through the typical ring-opening polymerization[11] to prepare **P1**. **P1** was activated using compound **8** to afford polymer **P2**, which has two active nitrophenyl esters. **P2** was then reacted with a polyethylene oxide that has two amino end groups. In order to get the amino-substituted polymer **P4**, a large excess of the diamino-PEG (**P3**) was used. The purified **P4** shows a single-modal GPC curve (Figure 1), indicating its desired structure. In order to enhance the delivery efficiency, **P4** was mixed with trifluoroacetic acid and dried to yield **P5** with two cationic amino groups. The use of polymers that contain cationic amino groups has been reported to effectively enhance the intracellular delivery of dyes and nanoparticles.[12] For an demonstration of whether the cationic amino groups can enhance non-specific endocytosis, a neutral block copolymer **P7** was prepared as a counterpart. The relative molecular weights measured by GPC calibrated using linear polystyrenes as standards are different from those measured by ^{1}H NMR. This discrepancy may be attributed to the difference between the hydrodynamic volumes of PCL, PCL-*block*-PEG and the polystyrene standards used for molecular weights determination using GPC.[13] Thus, ^{1}H NMR data were used for the degree of polymerization calculation.

3.2. The micelle formation and characterization

The powders of **P7** and **P5** were insoluble in water after 12 hours. However, using the micelle preparation methods described in the experimental section, the polymers could be well

dispersed in aqueous solutions due to micelle formation (Scheme 2). The PCL segment and fluorophore **7** acts as the hydrophobic core and the hydrophilic PEG segment functionalizes as the shell - a brush-like protective corona to ensure a complete dispersion of the micelles in water. Fluorophore **7** is extremely hydrophobic, however, after the conjugation of the water-insoluble material onto the amphiphilic block copolymer and the formation of micelles, the hydrophobic material in the micellar core can be well dispersed in aqueous solutions. Therefore this method was a feasible way to enable the application of hydrophobic materials in biocompatible environment by taking the advantages of the micelles. It was also possible to change the fluorophore, hydrophobic segment, and hydrophilic polymer using the synthetic route outlined in Scheme 2.

CMCs of the two polymers were determined using the fluorescence dependence upon the micelle formation[2b] and were found to be 0.025 mg/mL for **P5** and 0.022 mg/mL for **P7**, corresponding to 1.31 μM and 1.35 μM, respectively. These CMCs were higher than those of other PEG-*b*-PCL copolymers,[2a, 4] most likely the rigid hydrophobic fluorophore in the center of block copolymer causes the micelle formation to be relatively difficult compared with flexible PEG-*block*-PCLs. Depending on the polymers (**P5** and **P7**) used, the micelles have average diameters of 45 and 78 nm (Figure 2A) determined using DLS. Dried micelles of **P5** in the form of particles were visualized under AFM (Figure 2B), showing the sizes of dried micelles are 68 ± 23 nm, which is in accordance with the sizes measured using DLS (78 nm) in aqueous solution.

3.3. Photophysical properties

The photophysical properties of **P5** and **P7** were studied in THF, a typical organic solvent, and the micellar aqueous solution (Figure 3; Table 1). Absorption spectra of each polymer containing fluorophore **7** had two bands around 330-420 and 420-650 nm arising from π-π* and charge-transfer transitions, respectively, and are the typical characteristics of BTD-containing fluorophores.[3, 4] The fluorophore emits in the red spectral widow at the wavelength region of 550-800 nm. Quantum yields of fluorophore **7** decreased from 0.25 in THF to 0.17 in micelle 1 and to 0.12 in micelle 2, showing an influence of polymer structures on the photophysical properties of the red emitters. The low quantum yields within micelles were probably due to (1) aggregates of the fluorophores in the micellar cores which may quench the fluorescence and (2) interactions of the fluorophores with water. Although the fluorophores were encapsulated by the hydrophobic PCL segments of the micellar cores, they may still have had some interactions with water at the boundaries between the micellar cores and shells. As the micelle 1 and 2 have the same hydrophobic cores, the interactions of the fluorophores with water may result in the difference of their quantum yields. When the absorbance and emission of the fluorophores in THF and micellar solution were compared, it was observed that (1) the absorbance maxima in THF and micelle did not differ; (2) the emission maximum in micelle showed 6 nm of a bathochromic shift. These are the typical characteristics of the BTD-containing fluorophores, as their emissions are usually sensitive to the polar environment, whereas the absorptions are not.[3d, 4] This observation also indicated that the PCL segment was slightly more polar than THF.

3.4. Bioimaging

Cellular uptake of micelles by mouse macrophage RAW 264.7 was studied using confocal laser scanning microscopy. Cells were incubated with micelle 1 and micelle 2-containing DMEM medium to study the effect of peripheral groups of the micelles (e.g. the shells of the micelles) on internalization. It was found that micelle 2 with the neutral PEG peripheries did not stain the cells (data not shown) even after 24 hours incubation at a high dye concentration of 10 μM. Thus, the micelles were cell impermeable. However, the cells could be successfully stained by micelle 1 with the cationic corona. The fluorescence intensities inside the cells

became strong with the increase of uptake times and concentrations (see supplementary materials). In order to confirm whether the micelles were located intracellularly or on the membrane, sixty z-slices (Figure 4) were acquired at a sampling distance of 30 μm along z-axis using a 63x oil objective (Zeiss LSM 510 confocal microscope). Clear distance dependent fluorescence intensity was observed, indicating the micelles were located inside the cells. For a more detailed understanding of the subcellular locations of the micelles, a nuclei staining dye of Hoechst 33342 was used following the cellular uptake of micelle 1 (Figure 5). We observed only very minor co-localization of the red emission with the blue emission of the nuclei-selective emitter Hoechst 33342, which indicated cytoplasmic localization of the micelles. As indicated by the arrows in Figures 5A and 5C, two nuclei in one cell were observed, showing under our experimental conditions, the cells were most likely healthy and in the process of dividing and proliferation.

The different staining results between the two micelles were most likely due to the difference in the micelle surfaces. Micelle 2 had neutral PEG segments at its corona, whereas, micelle 1 had the cationic amino groups on the shells. Most likely, the neutral PEG was prohibitive of endocytosis. However, the cationic amino groups were observed to enhance the non-specific endocytosis process.[12] It should be noted that micelles with neutral PEG as the corona have been observed to be taken up by cells. For example, Savic et al.[2a] studied the cellular internalization of TMRAC-PCL-*b*-PEG by rat pheochromocytoma cells (P12) and mouse embryonic fibroblast cells (NIH 3T3) and concluded that the micelles were internalized through a non-specific endocytosis. However, it was later argued that the micelles were not endocytosed but were taken up by a strong interaction of the cationic fluorescent markers (TMRACs) with the cell's membrane.[14] Nevertheless other researchers, namely Tang et. al.,2c reported that a cationic rhodamine-conjugated PEG-*b*-PE was not able to stain human lung cancer cells (A549). These discrepancies indicate that cellular uptake of micelles is affected by the materials and cell lines utilized.

Within this study, cellular internalization of micelle 1 was observed under varying conditions in order to determine if the modality of cellular uptake was through endocytosis. It has been reported that several basic criteria must be fulfilled if a given material is internalized by endocytosis.[15-21] The process is time and energy dependent, thus the extent of internalization is reduced at low temperature[20, 21] but enhanced by calcium ion.[15-19] In the present studies, RAW 264.7 cells treated with micelle 1 and incubated at 4 °C internalized a significantly smaller amount of micelles than the cells incubated at 37 °C (Figure 6). In addition, the internalization of micelle 1 in the presence of calcium ions (3 mM) was higher than that without the addition of calcium ions. These data, in addition to confocal z-stack imaging, strongly indicated that the cellular internalization of micelle 1 was through endocytosis.

3.5. Cytotoxicity

Cytotoxicity of the micelle 1 to RAW 264.7 was investigated using an MTT assay [10] (Figure 7). Concentration dependent cell viability was observed. Greater than 88% of the cells were viable after the cells were stained for 16 hours using the dye concentrations of 3 – 6 μM. These observations demonstrated the biocompatibility of the dye-conjugated block copolymers using the newly synthesized **P5**.

4. Conclusion

BTD-containing fluorophore exhibiting red emission was chemically conjugated onto amphiphilic poly(ε-carpolactone)-*block*-poly(ethylene glycol) copolymers to enable the BTD-containing emitters as a new type of bioimaging agent. Using the block copolymer approach, neutral and hydrophobic emitters were made to be biocompatible by integrating them into micelles. Using micelles as delivery vehicles may also enable a wide array of other organic

and polymeric fluorophores to be used as new biomarkers. The polymers form micelles in aqueous solutions with average diameters of 45 nm and 78 nm depending on polymer structures. The micelles formed from polymer **P5** with the cationic amino groups were observed to be endocytosed by mouse macrophage RAW 264.7 cells by means of confocal microscopy. Whereas, the micelles formed from polymer **P7** with neutral PEGs as the shells were not taken up by the mouse macrophage. The difference in results highlights the influence of the micelle's corona on cellular internalization. The effects of temperature and calcium ion concentration when combined with imaging data suggests that micelles formed from **P5** were internalized by means of an endocytosis pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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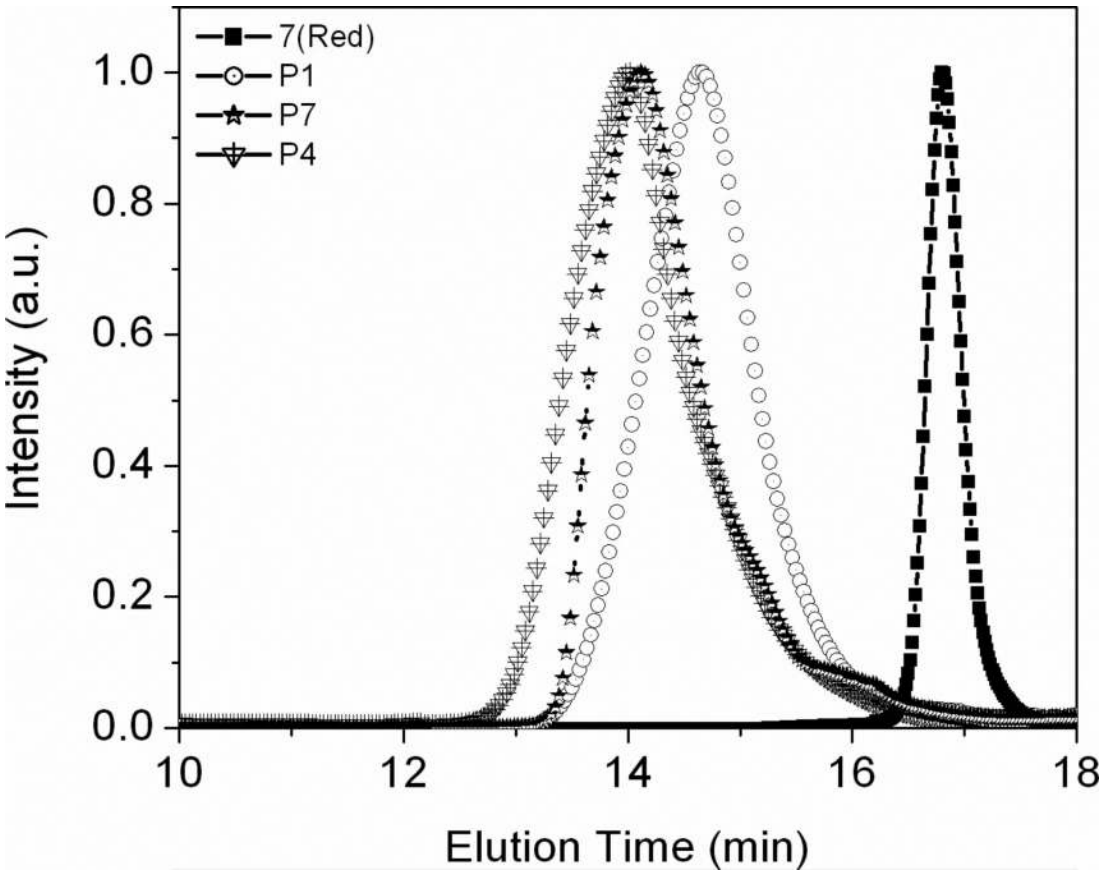


Figure 1.
GPC curves of **7**, **P1**, **P7** and **P4**.

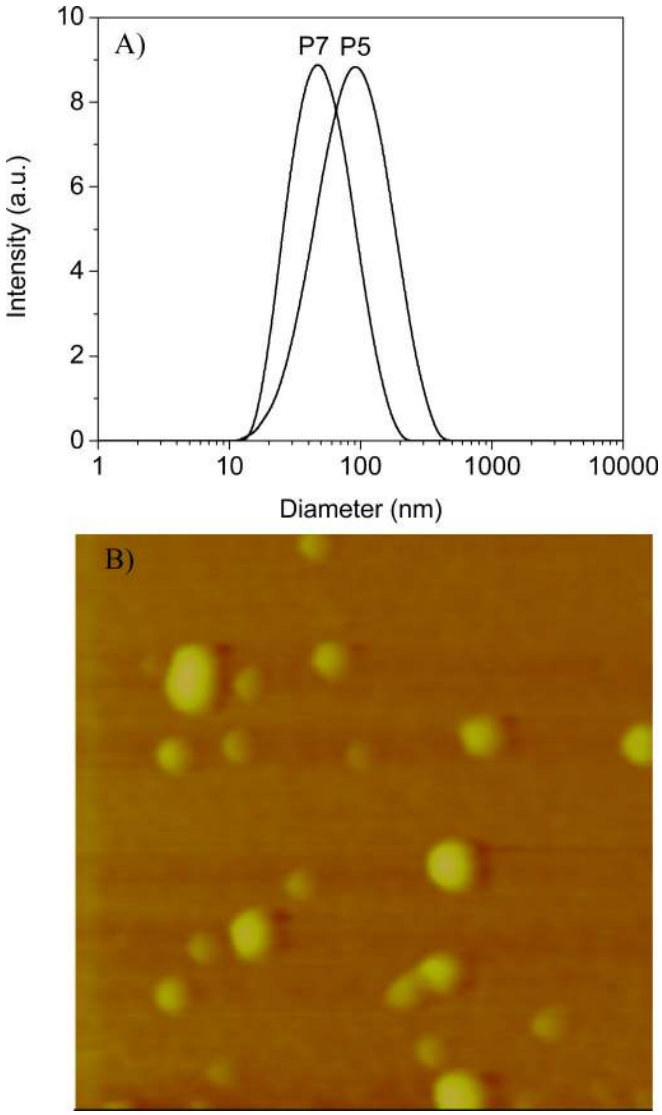


Figure 2.
A) DLS of the micelle 1 formed from **P5** and micelle 2 formed from **P7**. Average diameter of micelle 1 is 78 nm with a polydispersity of 0.27. Average diameter of micelle 2 is 45 nm with a polydispersity of 0.19. B) AFM height image (1 μm × 1 μm) of the micelle 1 formed from **P5**.

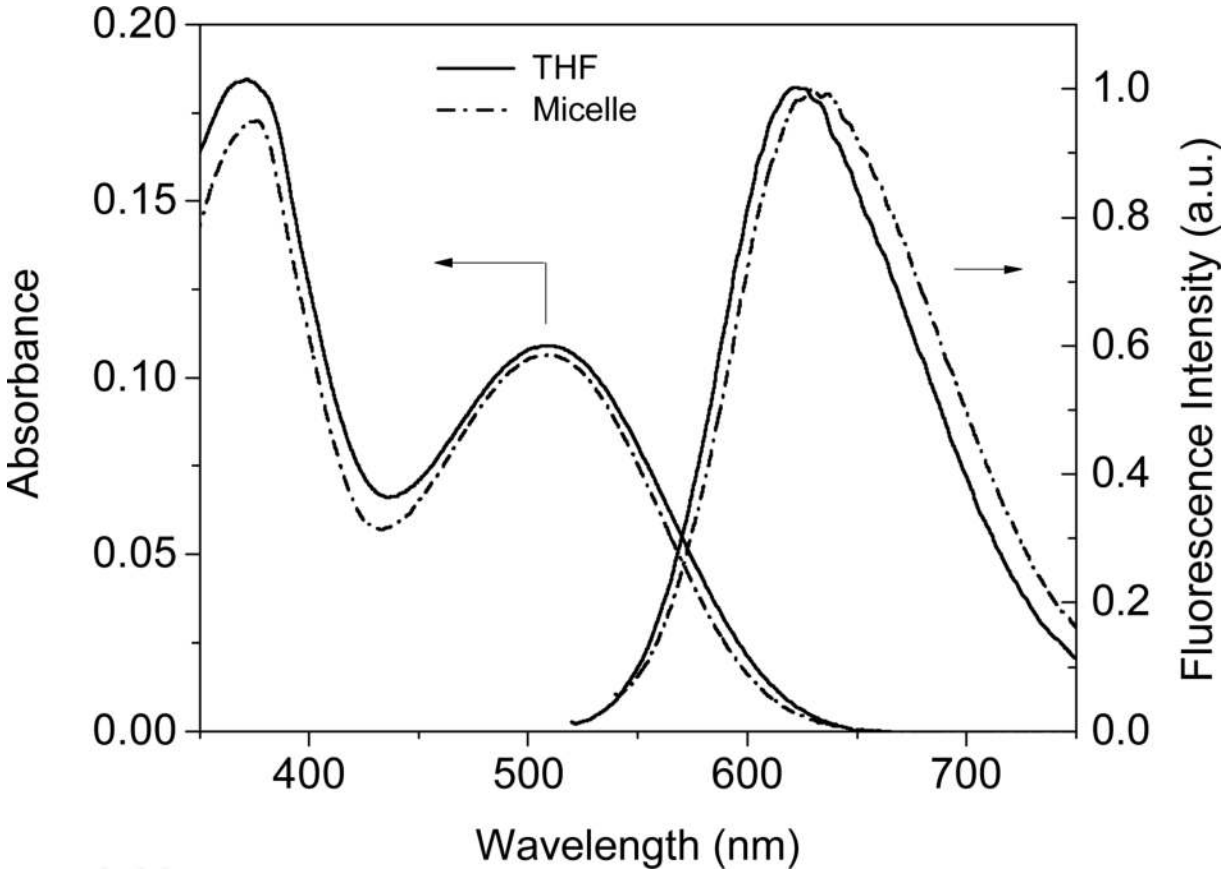


Figure 3.
UV-vis spectra and the fluorescence spectra of polymer **P5** in THF and micellular aqueous HEPES buffer solution.

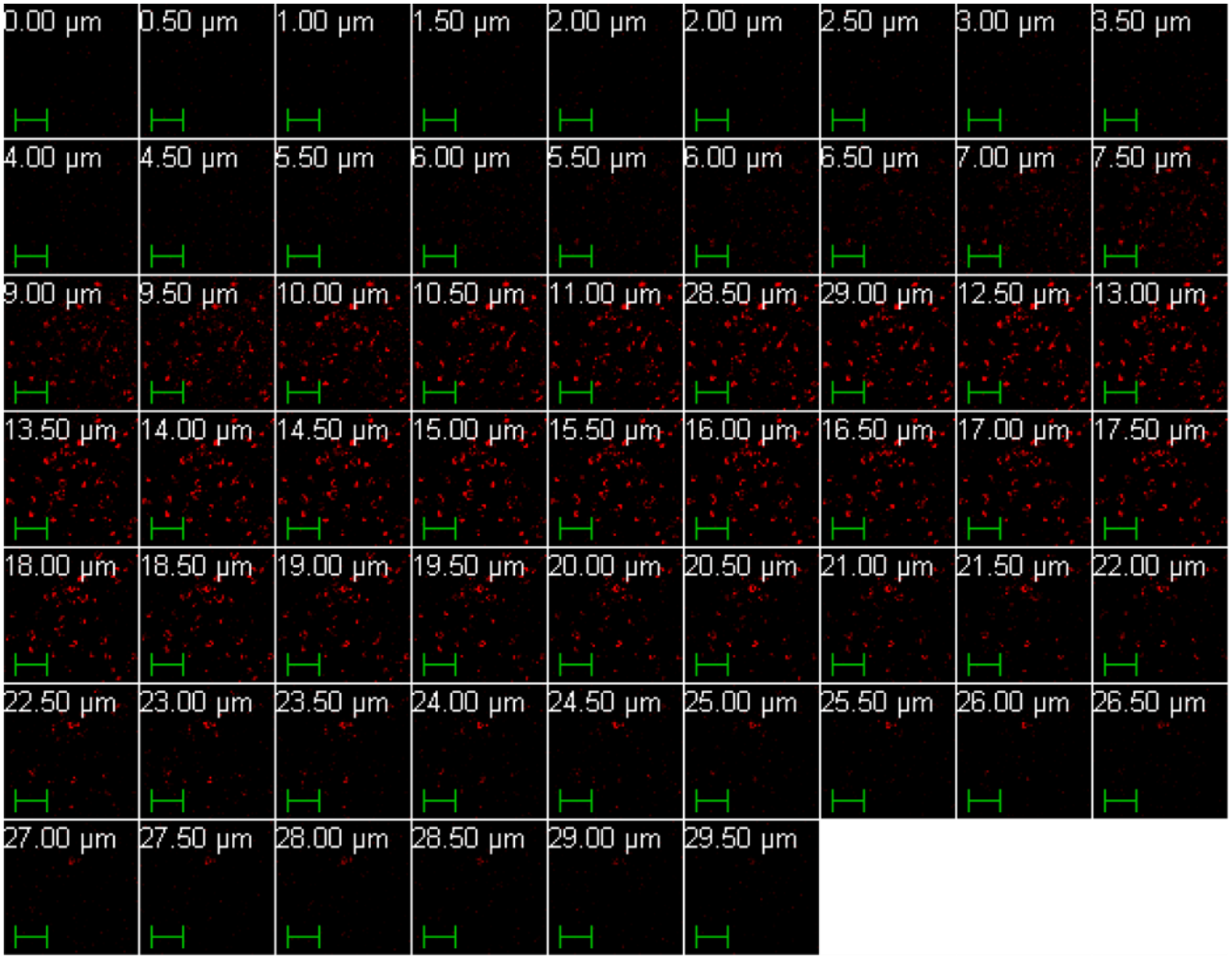


Figure 4.
Z-stack of micelle 1 in RAW 264.7 cells. Fluorophore concentration is 4 µM and internalization time is 16 hours. Scale bar is 50 µm.

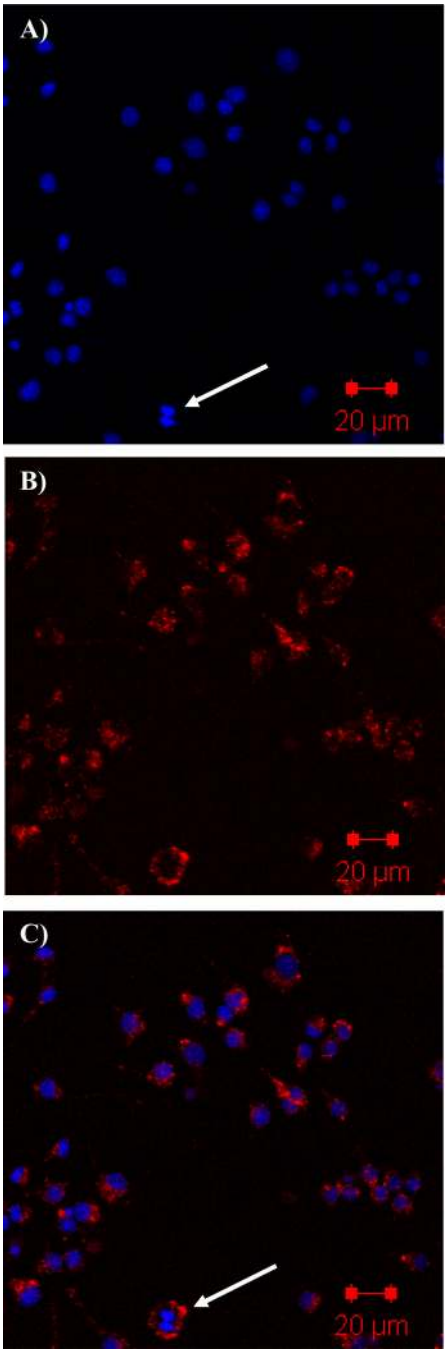


Figure 5.
Confocal imaging of RAW 264.7 cells with endocytosed micelle 1. A) Blue emission of Hoechst 33342. B) Red emission of micelles 1. C) Overlay of A and B. The white arrows in A and C indicate a dividing cell. Cells were incubated with micelles for 16 hours. Fluorophore concentration is 4 μM in medium corresponding to a polymer concentration of 0.08 mg/mL. Scale bar is 20 μm.

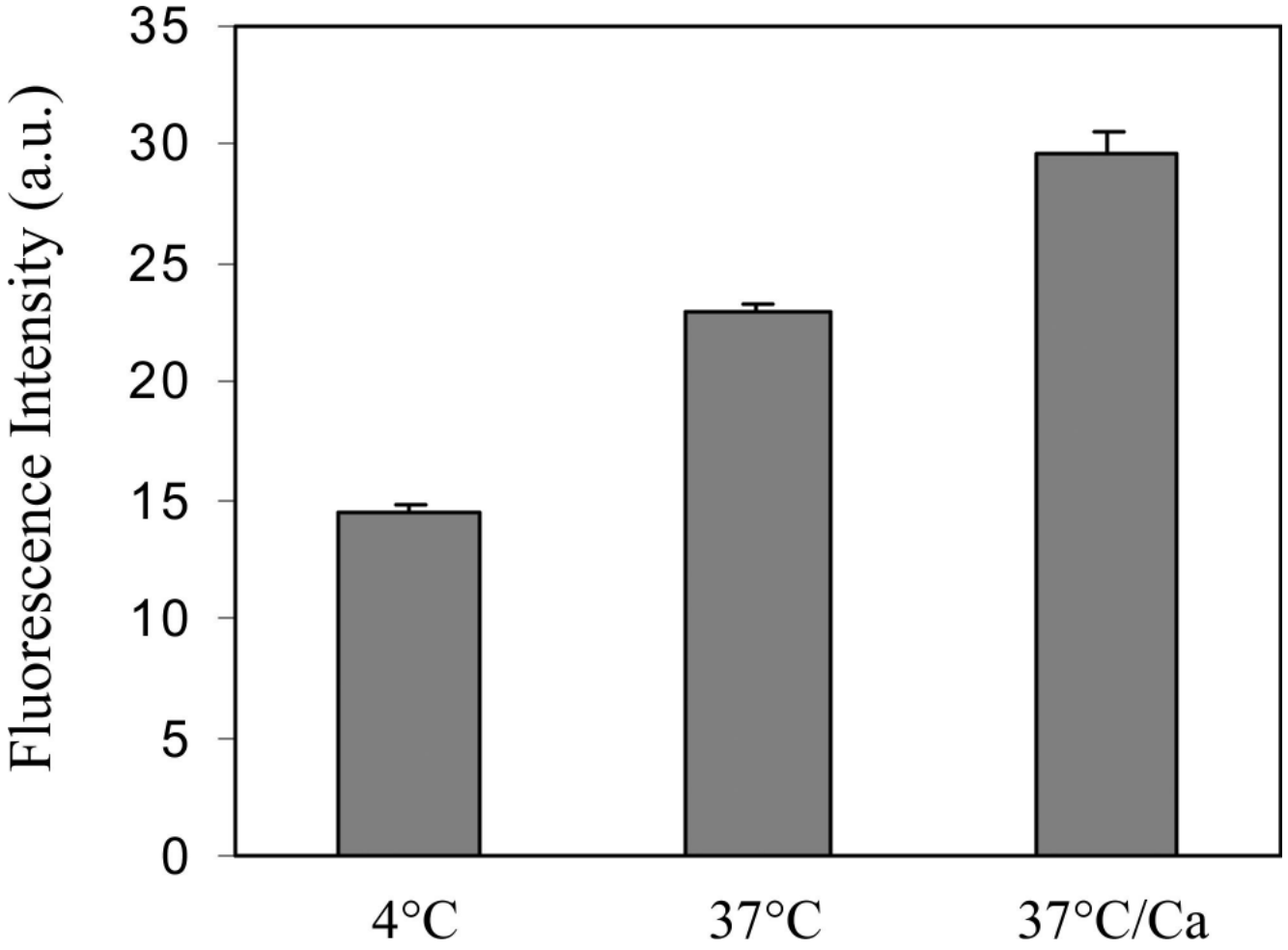


Figure 6.

The effect of temperature and calcium ion concentration on the internalization of micelle 1 by RAW 264.7 cells. Polymer concentration of **P5** is 0.08 mg/mL. Concentration of fluorophore **7** in **P5** is 4 μM. The x-axis labeled with 4°C and 37°C indicate that the cells were cultured at the two different temperatures in DMEM. The x-axis labeled with 37°/Ca means the cells were cultured at 37°C in DMEM medium with an addition of 3 mM of calcium chloride.

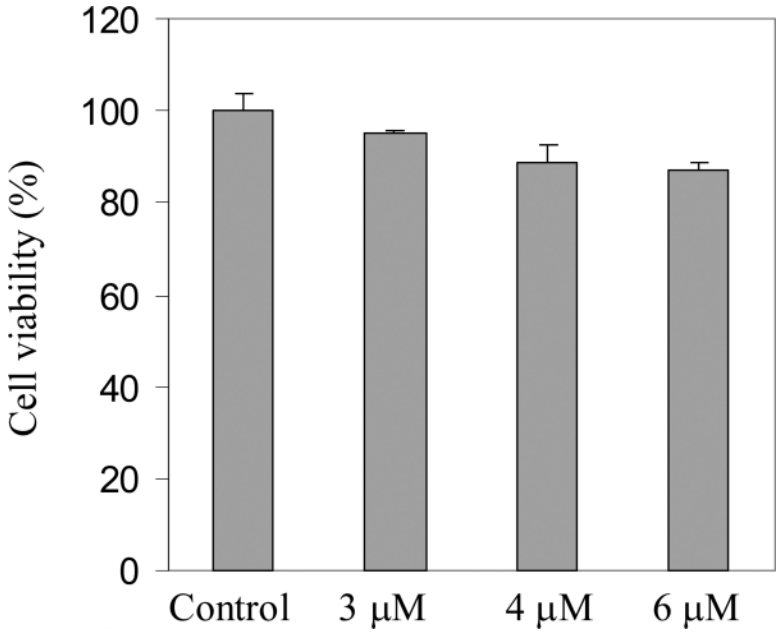
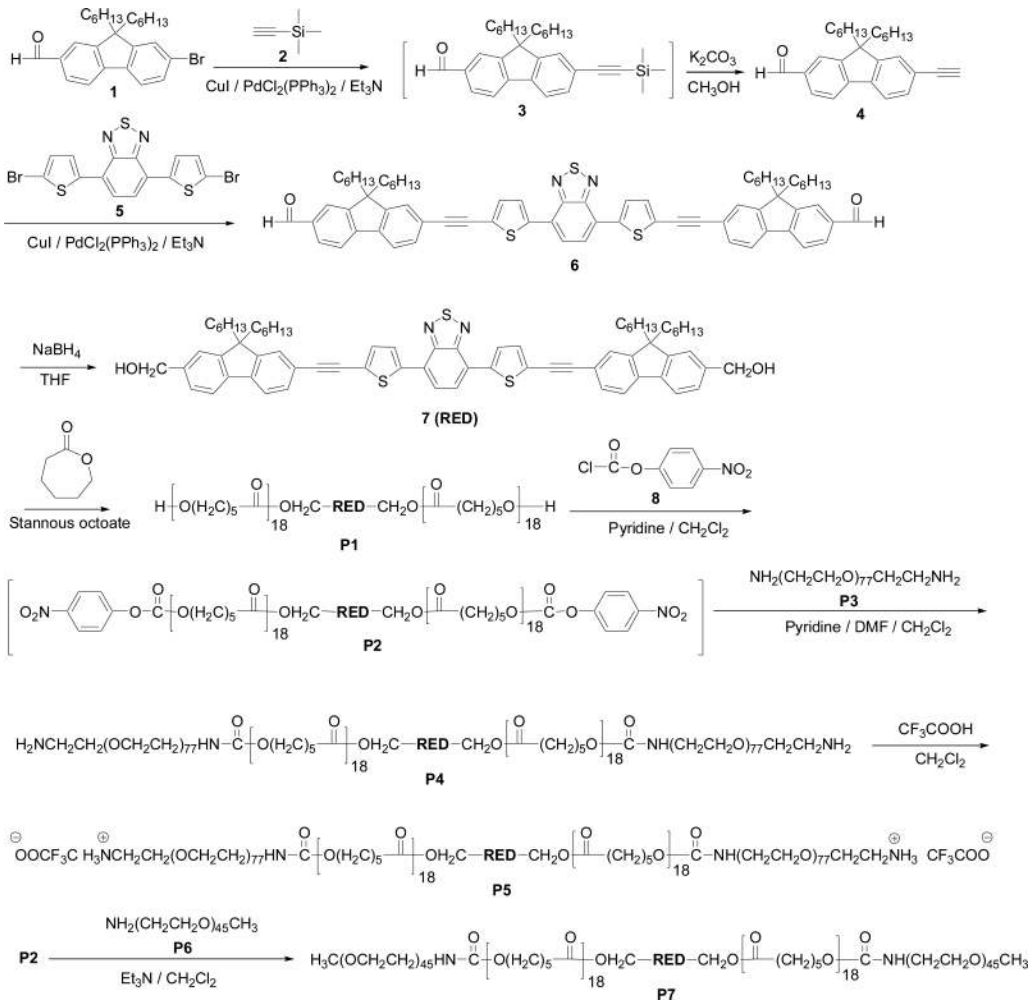
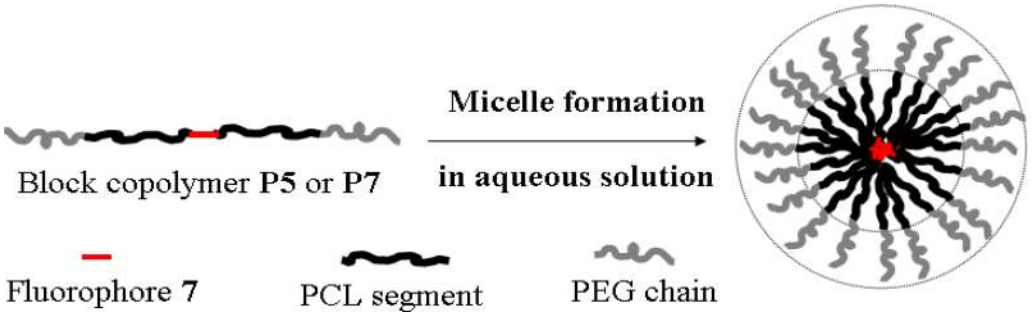


Figure 7.
Cell viability (MTT assay) of RAW 264.7 incubated in the presence of micelle 1 in DMEM medium for 16 hours. The 3, 4 and 6 μM in the x-axis were the fluorophore **7**'s concentrations corresponding to **P5**'s concentrations of 0.06, 0.08, and 0.12 mg/mL.



Scheme 1.
Synthesis of block copolymers of **P5** and **P7**.



Scheme 2.
Schematic drawing of the preparation of micelles. Micelle 1 was prepared from **P5**. Micelle 2 was prepared from **P7**.

Table 1

Photophysical properties of **P5** and **P7** in their micellar aqueous and THF solutions.

	λ_{max} [a]	λ_{max} [b]	η [c]	Average diameter	
				DLS	AFM
P5 in Micelle 1	509 nm	630 nm	0.17	78 nm	68 ± 23 nm
P5 in THF	509 nm	624 nm	0.25	-----	-----
P7 in micelle 2	509 nm	630 nm	0.12	45 nm	----- [d]
P7 in THF	509 nm	624 nm	0.25	-----	-----

[a] absorbance maximum

[b] emission maximum

[c] quantum yield.

[d] not measured.