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ARTICLE

Amphiphilic/fluorous random copolymers as a new class of non-cytotoxic polymeric materials for protein conjugation

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Herein, amphiphilic/fluorous random copolymers bearing poly(ethylene glycol) (PEG) chains and perfluorinated alkane pendants were developed as novel non-cytotoxic polymers for protein conjugation. Three kinds of random copolymers with different initiating terminals (carboxylic acid, pyridyl disulfide, *N*-hydroxysuccinimide ester) were prepared by reversible addition-fragmentation chain transfer (RAFT) copolymerization of a PEG methyl ether methacrylate and a perfluorinated alkane methacrylate with corresponding functional chain transfer agents. All of the polymers were soluble in water to form nanostructures with perfluorinated compartments via fluorous interaction: large aggregates from the intermolecular multi-chain association and compact unimer micelles from the intramolecular single-chain folding. Such a PEGylated and perfluorinated random copolymer was non-cytotoxic to NIH 3T3 mouse embryonic fibroblast cells and human umbilical vein endothelial cells (HUVECs). Additionally, a random copolymer with a pyridyl disulfide terminal was also successfully conjugated with a thiolated lysozyme.

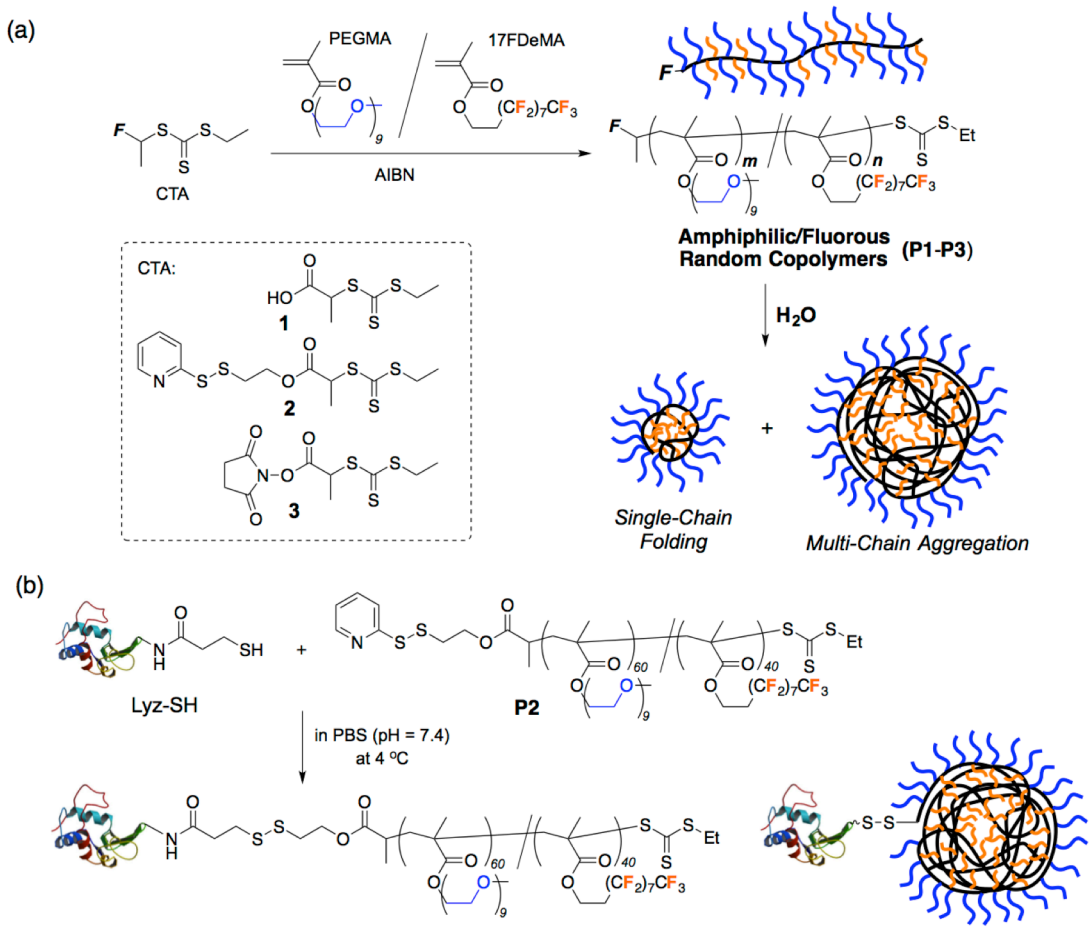
Introduction

Biocompatible synthetic polymers often play important roles in creating new technologies and function for biomedical and biochemical applications with natural materials including proteins, genes, cells, and bacteria.[1-8] Among them, proteins are one of the most widely used natural and functional biopolymers; they typically serve as therapeutic materials, as well as highly active and selective catalysts. Such functions in proteins are derived from the inherent tertiary structures carrying specific inner cavities that are formed via the self-folding of the polymer chains in water with physical (hydrophobic, hydrogen-bonding, and ionic) interactions. However, owing to the dynamic and labile conformation, proteins are often unstable and easy to denature via external stimuli (heat, desiccation, solvents, light, pH change, and lyophilization) and are typically rapidly cleaved by proteolytic enzymes and cleared from the body *in vivo*.

To enhance the stability for various applications, proteins have often been conjugated to poly(ethylene glycol) (PEG) and hydrophilic (or amphiphilic) polymers.[9-22] In particular, controlled/living radical polymerization[23-28] is a powerful tool to synthesize the latter polymers with desired properties and end-functionalization for such protein-polymer conjugates, since the polymerization system affords the direct and selective incorporation of polar functional groups and proteins into polymer segments (terminals and pendants) and the efficient control of precision primary structures and three-dimensional architectures of polymers. Recently, several hydrophilic polymers including poly[poly(ethylene glycol) methyl ether methacrylate] (poly(PEGMA))[10,13,15] and trehalose glycopolymers[11,12] have been successfully synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization[25,26] for protein conjugation. These synthetic polymers were non-cytotoxic[12,29,30] and actually effective for the improvement of protein stability.

Amphiphilic copolymers potentially include additional advantages in protein conjugation and functionalization. In contrast to hydrophilic homopolymers, amphiphilic random or block copolymers with water-insoluble segments efficiently provide globular polymeric nanomaterials (e.g., micelles, polymersomes, and nanogels) in water via the intermolecular association or intramolecular self-folding of their polymer chains.[19,21,31-36] The globular objects could further confer the properties and functions resulting from these unique environments (on the surface and/or in the interior) to proteins.[19,22]

For biomedical applications, perfluorinated alkanes (fluorocarbons)[37-42] are promising candidates as water-insoluble functional units for amphiphilic copolymers. This is because perfluorinated compounds and polymers have several attractive features: the polymers are immiscible with both water and



Scheme 1. (a) Synthesis of amphiphilic/fluorous random copolymers (**P1**-**P3**) via reversible addition-fragmentation chain transfer (RAFT) copolymerization of PEGMA and 17FDeMA with CTA (**1**-**3**). (b) Conjugation of a disulfide pyridine-bearing copolymer (**P2**) to a thiolated lysozyme (Lyz-SH) (Lyz structure PDB: 2LYZ).

common organic solvents, yet have selective interactions with fluorinated compounds resulting in stable micellization, unique association, and molecular recognition;[43-47] they also exhibit high sensitivity in ^{19}F nuclear magnetic resonance (NMR),[46-48] high oxygen affinity,[38,39] and biocompatibility (i.e. low acute toxicity, non cytotoxicity, and no hemolytic activity).[38,49-51] Based on these features, fluorinated materials have been examined in biomedical research fields, typically as oxygen transport materials (blood substitutes) and drug delivery vessels.[38,39] Thus, amphiphilic/fluorous copolymers bearing PEG chains and perfluorinated alkane units[52] would not only stably form globular nanomaterials with fluorous confined spaces in water but may be also biocompatible, with low cytotoxicity and minimal protein denaturation; furthermore, the polymers would provide unique functions resulting from the perfluorinated compartments to proteins.

Given these possibilities, we developed amphiphilic/fluorous random copolymers with PEG chains and perfluorinated pendants as a new class of biocompatible polymeric materials for protein conjugation (Scheme 1). The PEGylated and perfluorinated random copolymers (**P1**-**P3**) were synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization.[25,26] Confirmed by ^{19}F NMR spectroscopy and dynamic light scattering (DLS), their copolymers efficiently formed large aggregates with fluorous cores in water. The cytotoxicity of the copolymer (**P1**) was examined with NIH 3T3 mouse embryonic fibroblast cells and human umbilical vein endothelial cells (HUVECs). Additionally, a thiolated lysozyme was successfully conjugated to the fluorous core aggregate of the random copolymer carrying a pyridyl disulfide terminal (**P2**). To our best knowledge, the example described herein, is the first to reveal the non-cytotoxicity of amphiphilic/fluorous copolymers obtained from living radical polymerization and to conjugate protein to the large aggregates.

Experimental

Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMA: Aldrich, M_n ~ 500) and 1*H*,1*H*,2*H*,2*H*-perfluorodecyl methacrylate (17FDeMA: Aldrich, purity ~ 97%) were used as received. 2,2'-Azobisisobutyronitrile (AIBN, Aldrich, purity ~ 99%) was recrystallized twice from ethanol and dried prior to

use. Toluene (Fischer Scientific, purity > 99%) was used as received. Chain transfer agents (CTA: **1**-**3**) were synthesized according to the previous literature.[11] LIVE/DEAD® viability/cytotoxicity assay kit and CellTiter-Blue® cell viability assay were obtained from Invitrogen and Promega, respectively.

Polymer characterization

Number-average molecular weight (M_n) and Dispersity (M_w/M_n) of the polymers were measured by size exclusion chromatography (SEC) in DMF containing 10 mM LiBr at 40 °C (flow rate: 1 mL/min) on three linear-type polystyrene gel columns (Shodex KF-805L: exclusion limit = 4×10^6; particle size = 10 mm; pore size = 5000 Å; 0.8 cm i.d. × 30 cm) that were connected to a Jasco PU-2080 precision pump, a Jasco RI-2031 refractive index detector, and a Jasco UV-2075 UV/vis detector set at 270 nm. The columns were calibrated against 10 standard samples of poly(methyl methacrylate) (Polymer Laboratories: M_n = 1000–1200000; M_w/M_n = 1.06–1.22). ^{1}H and ^{19}F NMR spectra were recorded in acetone-d_6 or D_2O at 25 °C on a JEOL JNM-ECA500 spectrometer, operating at 500.16 (^{1}H), 470.62 (^{19}F) MHz. Dynamic light scattering (DLS) measurements were conduced to determine hydrodynamic radius (R_H) on Otsuka Photal ELSZ-0 equipped with a semiconductor laser (λ = 658 nm) at 30 °C ([polymer] = 2.5 mg/mL in DMF). The measuring angle was 165°, and the data were analyzed by CONTIN method. Ultraviolet-visible absorption (UV-vis) spectroscopy was measured on a BioMate 5 (Thermospectronic) instrument.

Polymer synthesis

The synthesis of amphiphilic/fluorous random polymers was carried out by standard Schlenk technique with syringe under argon. A typical procedure for **P1** is as follows: **1** (5.9 mg, 0.028 mmol), PEGMA (1.2 mL, 2.6 mmol), 17FDeMA (0.40 mL, 1.2 mmol) and 30 mM toluene stock solution of AIBN (0.47 mL, 0.014 mmol) were dissolved in toluene (3.8 mL) in a Schlenk tube. The tube was sealed and subjected to four freeze-pump-thaw cycles before immersion in a 70 °C oil bath. After 4.5 h, the tube was immersed in liquid N_2 to terminate the reaction. The conversion of PEGMA and 17FDeMA was determined as 75% and 82%, respectively, by ^{1}H NMR. The product was precipitated into hexane and purified by dialysis against MeOH to give **P1**. Mn (SEC) = 117600, M_w/M_n (SEC) = 1.13. δ_H (500 MHz; acetone-d_6; acetone) 4.5–4.2 (2H, br s, -COC$\underline{H}_2$CH$_2$CF$_2$), 4.2–4.0 (2H, br s, -COOC$\underline{H}_2$CH$_2$O), 3.8–3.4 (4H, br s, -OC$\underline{H}_2$C$\underline{H}_2$O), 3.3 (3H, br s, -OCH$_3$), 2.8–2.6 (2H, br s, -COCH$_2$C$\underline{H}_2$CF$_2$), 2.2–1.4 (2H, br s, -CH$_2$-), 1.4–0.8 (3H, br s, -CCH$_3$). δ_F (470 MHz; acetone-d_6; CF3COOH) -81.3 – -82.1 (3F, br s, -CF$_3$), -113.3 – -114.6 (2F, br s, -CH$_2$CF$_2$-), -121.7 – -124.5 (10F, br s, -CF$_2$-), -126.4 – -127.2 (2F, br s, -C$\underline{F}_2$CF$_3$). **P2** and **P3** were similarly prepared and characterized.

Terminal transformation

The trithiocarbonate end group in **P1** was transformed with AIBN before cytotoxic study. **P1** (1070 mg, 0.020 mmol) and AIBN (99.7 mg, 0.61 mmol) were dissolved by toluene (6.2 mL) and DMF (3.7 mL) in a Schlenk tube. The tube was degassed by three freeze-pump-thaw cycles and placed at 80 °C for 3 h. After purified by dialysis against MeOH, the resulting P1 was analyzed by UV-vis: the absorption derived from the trithiocarbonate in **P1** (λ = 309 nm) disappeared.

Cytotoxicity assay

The cell compatibility of **P1** to NIH 3T3 mouse embryonic fibroblast cells (NIH 3T3, ATCC) and human umbilical vein endothelial cells (HUVECs, Promocell GmbH) was evaluated with a LIVE/DEAD viability/cytotoxicity assay (Invitrogen). Controls were buffer only or media only. NIH 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin− streptomycin. HUVECs were cultured in endothelial cell growth medium (Promocell) containing 2% FCS with supplements recommended by the supplier. The cells were seeded in 48-well plates (BD Falcon) at a density of 6 × 103 cells per well. After 24 h, culture media were replaced with 200 μL of the working medium containing known polymer concentrations of 0.1, 0.5, 1 mg/ mL or the control with buffer only. After incubation for 48 h, the cells were gently washed twice with prewarmed Dulbecco's phosphate-buffered saline (D-PBS), and stained with the LIVE/DEAD reagent (2 μM calcein AM and 4 μM ethidium homodimer-1). Fluorescent images of each well were captured on a Zeiss Axiovert 200 microscope with an AxioCam MRm camera and FluoArc mercury lamp. The number of live and dead cells was counted; percent cell viability was calculated by dividing the number of live cells by the total number of cells. All experiments were conducted with four repetitions. The cell viability (%) was calculated with the following formula: 100 × (number of live cells/total number of cells). The data is provided by normalizing each set to the control without any additives (media only).

Statistical analysis for cell viability results

All the *p*-values were calculated using the independent Student's t test assuming unequal variances.

Protein conjugation

A thiolated lysozyme (Lyz-SH) was prepared according to the previous literature.[11] To a 1.5 mL Lo-Bind® centrifuge tube was added Lyz-SH (45.5 μL, 1.1 mg/mL, PBS: phosphate buffered solution, pH 7.4) and **P2** (180 μL, 51.1 mg/mL in PBS, pH 7.4, 50 eq). The total volume was thus 226 μL (PBS, pH 7.4). The solution was stored at 4 °C for 24 h before characterization by SDS-PAGE. The conjugation of **P3** and Lyz were similarly conducted.

Table 1. Synthesis of Amphiphilic/Fluorous Random Copolymers[a]

Code	CTA	***m***[a]	***n***[a]	Time (h)	Conversion (%), PEGMA/17FDeMA	M_n[b] (g/mol)	M_w/M_n[b]	***m/n*** (ratio)[c]	R_H[d] (nm) acetone	R_H[d] (nm) H_2O
P1	**1**	70	30	4.5	75/82	118000	2.10	70/35	15 (113)	(6.2) 128
P2	**2**	60	40	4	67/79	102000	1.71	60/48	13 (199)	(21) 115
P3	**3**	60	40	4	67/80	98300	1.79	60/50	13 (135)	(15) 210

[a] **P1**-**P3** were synthesized by RAFT copolymerization of PEGMA and 17FDeMA with chain transfer agents (CTA: **1** - **3**) and AIBN in toluene at 70 °C: $[PEGMA]_0/[17FDeMA]_0/[CTA]_0/[AIBN]_0$ = 500/215/5/2.5 (**P1**), 430/285/5/2.5 (**P2**, **P3**), ***m*** = $[PEGMA]_0/[CTA]_0$, ***n*** = $[17FDeMA]_0/[CTA]_0$.

[b] Determined by SEC in DMF (10 mM LiBr) with PMMA standards.

[c] Monomer composition (ratio) in copolymers: determined by ^{1}H NMR.

[d] Hydrodynamic radius (R_H) in acetone or H_2O, determined by DLS: [**P1** - **P3**] = 10 mg/mL. The values in parentheses are from minor size-distribution.

Results and discussion

Polymer synthesis

Amphiphilic and fluorous random copolymers carrying poly(ethylene glycol) chains and perfluorinated alkane pendants (**P1**-**P3**) were synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization of poly(ethylene glycol) methyl ether methacrylate (PEGMA) and 1*H*,1*H*,2*H*,2*H*-perfluorodecyl methacrylate (17FDeMA) with 2,2'-azobis(isobutyronitrile) (AIBN) and chain transfer agents (CTAs, **1**-**3**) in toluene at 70 °C. The three CTAs consist of trithiocarbonates with different functional groups: carboxylic acid (**1**); pyridyl disulfide (**2**); *N*-hydroxysuccinimide ester (**3**). Both **2** and **3**, obtained from **1**, are designed for the conjugation of the resulting random copolymers onto a protein: the polymer from **2** (**P2**) can react with a thiol group-bearing protein to give the conjugate via a cleavable disulfide linkage, while that from **3** (**P3**) may react with amino groups on a protein to provide the conjugate via an amide bond. The feed ratio of their monomers to CTAs (m = $[PEGMA]_0/[CTA]_0$, n = $[17FDeMA]_0/[CTA]_0$) was set as m/n = 70/30 (**P1**) and 60/40 (**P2**, **P3**).

In all cases, copolymerization smoothly and homogeneously proceeded up to 67% - 82% conversion in 4 or 4.5 hrs, giving amphiphilic/fluorous random copolymers (**P1**-**P3**) with high molecular weight [M_n = ~100000, M_w/M_n = 1.7-2.0, by size-exclusion chromatography (SEC) in DMF with poly(methyl methacrylate) calibration, Table 1]. The broad molecular weight distribution would be attributed to the suitability between the CTAs and methacrylate monomers.

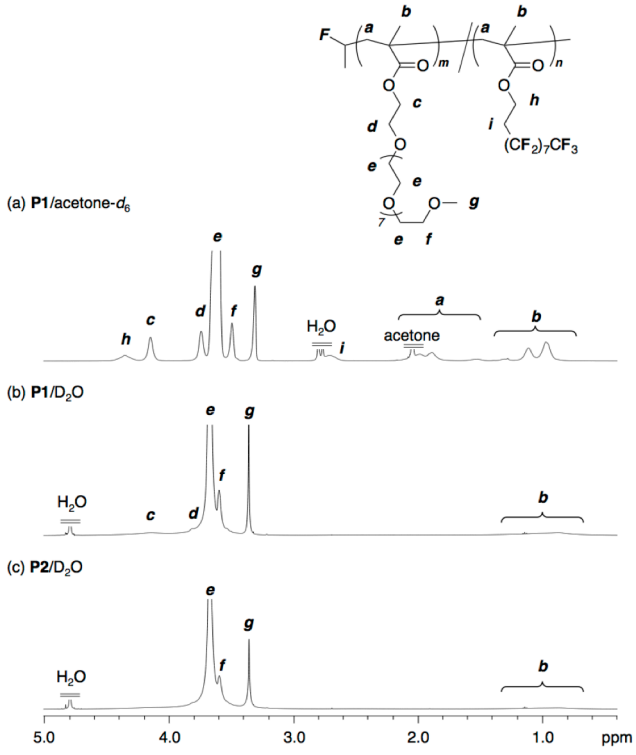


Fig 1. ^{1}H NMR spectra (500 MHz) of (a) **P1** in acetone-d_6, and (b) **P1** and (c) **P2** in D_2O at 25 °C.

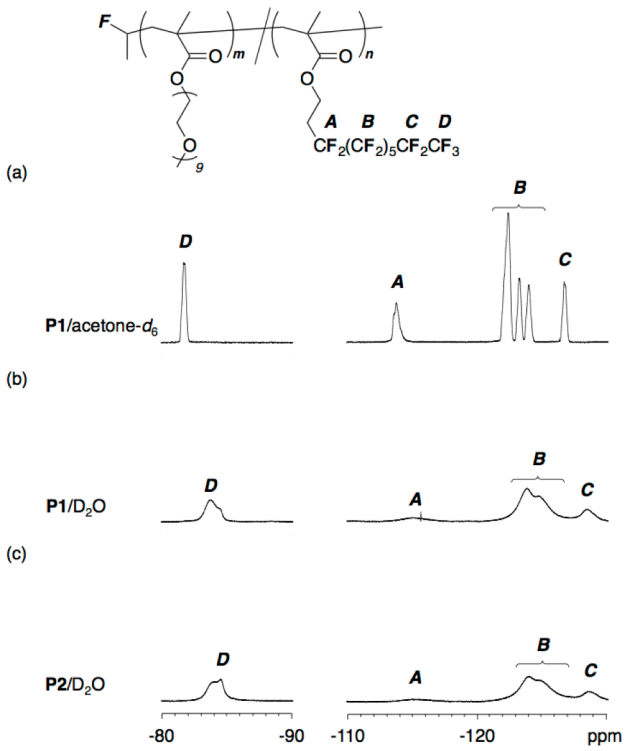


Fig 2. ^{19}F NMR spectra (470 MHz) of (a) **P1** in acetone-d_6, and (b) **P1** and (c) **P2** in D_2O at 25 °C.

Analyzed by ^{1}H NMR in acetone-d_6, all of **P1**-**P3** clearly exhibited proton signals originating from their polymer structures (Fig 1 and S1). Typically, **P1** showed methylene or methyl protons of poly(ethylene glycol) chains (*c*: 4.1 ppm, *d*: 3.8-3.7 ppm, *e*: 3.7-3.55 ppm, *f*: 3.55-3.4 ppm, *g*: 3.3 ppm) and methylene protons of perfluorinated octane pendants (*h*: 4.4 ppm, *i*: 2.7 ppm), in addition to those of a methacrylate backbone (*a*: 2.2-1.4 ppm, *b*: 1.4-0.8 ppm) (Fig 1a). The composition (molar ratio) of PEGMA and 17FDeMA was calculated from the area of their pendants (*c*/*h*): PEGMA/17FDeMA = 70/35 (**P1**), 60/48 (**P2**), 60/50 (**P3**). More importantly, despite of broad molecular weight distribution, **P2** showed small signals of the pyridyl end group at 8.5 – 6.5 ppm (Fig S1), indicating that pyridyl disulfide group was successfully introduced into **P2** with **2**. In contrast, the *N*-hydroxysuccinimide end group of **P3** was not observed owing to the overlap with the methylene groups of the polymer pendants.

P1-**P3** further clearly exhibited ^{19}F NMR signals assignable to their perfluorinated pendants in acetone-d_6 [**P1**: -CF_2- (*A*) –113, (*B*) –121- – 125, (*C*) –127 ppm; -CF_3 (*D*) – 82 ppm, Fig 2a, Fig S2]. To avoid undesirable cytotoxicity, the trithiocarbonate in the *w*-end of **P1** was removed by heating in the presence of AIBN (confirmed by UV-vis analysis, Fig S3).[13]

Folding and association in water

Owing to the hydrophilic PEG pendants, **P1**-**P3** were soluble in water but would form self-folding unimer micelles and/or large multi-chain aggregates (or nanogel) via the fluorous interaction of the perfluorinated pendants. Thus, **P1**-**P3** were analyzed by dynamic light scattering (DLS) in acetone or H_2O at 25 ^{o}C ([polymer] = 10 mg/mL). In both solvents, all of the samples showed bimodal light scattering distribution (Fig 3), whereas the major portion of the size distribution (volume fraction) was dependent on the solvent. Table 1 shows hydrodynamic radius (R_H) corresponding to both major distribution and minor counterpart (the values in parentheses).

In acetone, **P1**-**P3** mainly had small R_H of 13 - 15 nm, indicating that most of **P1**-**P3** exist as unimer in acetone.[36] In water, **P1**-**P3** in turn mainly showed DLS intensity distributions with large R_H's from 115 to 210 nm. Thus, most of their polymers intermolecularly associate with multiple chains to form large aggregates in water. In detail, the R_H (6.2 nm) for the small size fraction of **P1** (30 mol% 17FDeMA) in water was smaller than that for **P1** unimer in acetone. This suggests that a part of **P1** self-folds in water to a compact unimer micelle. Thus, **P1** dynamically form both a self-folding structure and multi-chain aggregates in water.

To evaluate aggregation properties of their perfluorinated pendants in water, **P1** and **P2** were analyzed by ^{19}F NMR in D_2O at 25 ^{o}C (Fig 2b,c). In both samples, the ^{19}F signals (*A*-*D*) broadened, compared with those in acetone. This importantly indicates that the self-folding and multi-chain association of the polymers in water are driven by the fluorous interaction of their perfluorinated pendants; both the unimer micelles and the large aggregates carry fluorinated inner cores covered by multiple short PEG chains (shell). These structures were further

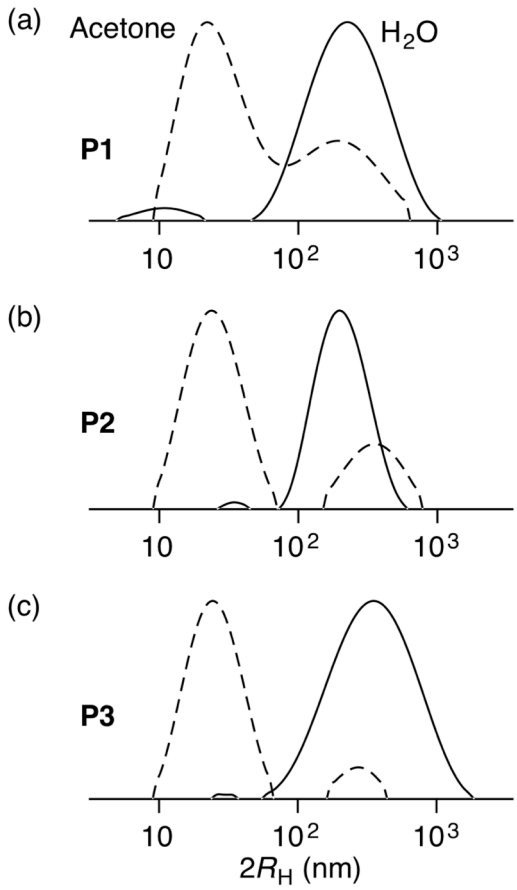


Fig 3. DLS intensity distribution of (a) **P1**, (b) **P2**, and (c) **P3** in acetone (dash) and H_2O (solid) at 25 ^{o}C: $[polymer]_0$ = 10 mg/mL.

supported by ^{1}H NMR measurements of **P1** and **P2** in D_2O (Fig 1b,c): methacrylate backbone proton signals (*a*,*b*), methylene protons adjacent to the backbones (*c*, *h*) and perfluorinated pendants (*i*) largely disappeared, while the PEG chain protons (*e*, *f*, *g*) were observed as sharp peaks.

Cytotoxicity study

To evaluate the potential biocompatibility of amphiphilic/fluorous random copolymers, cytotoxicity study of **P1** was conducted with NIH 3T3 mouse embryonic fibroblast cells and human umbilical vein endothelial cells (HUVECs) (Fig 4).[12] For this, NIH 3T3 and HUVECs were first respectively cultured in 48-well plates at density of 6000 cells per well for 24 h. The culture media was then replaced with the working medium containing **P1** at concentrations of 0.1, 0.5 and 1.0 mg/mL. After 48 h incubation, the cells were stained with the LIVE/DEAD regent, where live cells turn green and dead cells turn red. The fluorescent images of live and dead cells in their samples were counted to calculate the cell viability (%) with the following equation: 100 × (number of live cells)/(total number of cells). All experiments were conducted with four repetitions and averaged.

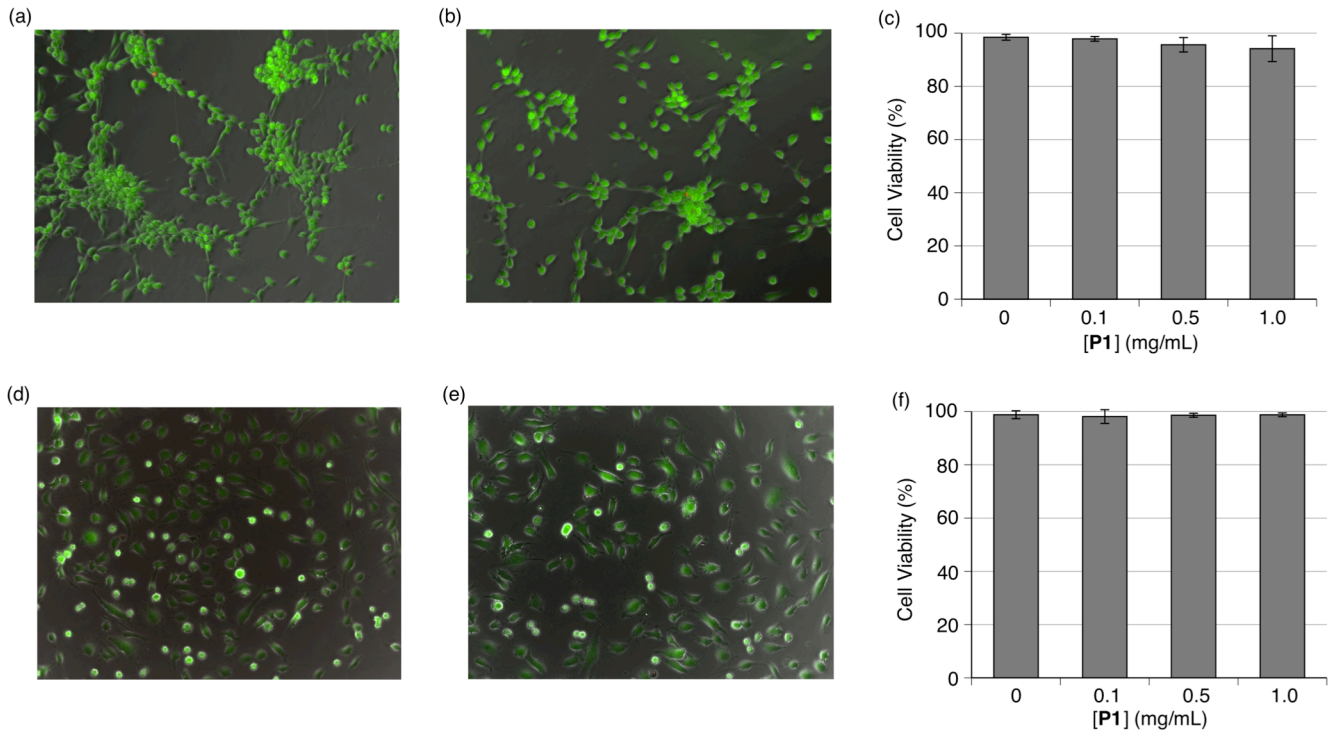


Fig 4. Live/Dead staining of (a, b) NIH 3T3 and (d, e) HUVECs in the presence of **P1** ($[\mathbf{P1}]_0$ = 0.1 (a, d), 1.0 (b, e) mg/mL). Quantification of viability of (c) NIH 3T3 and (f) HUVECs in the presence of **P1**.

As shown in Fig 4, the majority of NIH 3T3 and HUVECs were viable even in the presence of **P1** up to at least 1.0 mg/mL. This clearly demonstrates that amphiphilic/fluorous random copolymers, in spite of their perfluorinated pendants, are non-cytotoxic to NIH 3T3 and HUVECs. The high biocompatibility could be attributed to the multi-chain association and self-folding structures of **P1** in aqueous media where the perfluorinated segments are effectively confined within the inner spaces covered by PEG chains. Additionally, the potential biocompatibility of perfluorinated compounds could also contribute to the non-cytotoxicity.

Protein Conjugation

Given the non-cytotoxic properties of **P1**, protein-polymer conjugation was investigated with amphiphilic/fluorous random copolymers bearing functional α-ends (pyridyl disulfide: **P2**; *N*-hydroxysuccinimide ester: **P3**) and hen egg white lysozyme (Lyz) as a protein. For the conjugation of **P2** via disulfide linkages, thiolated lysozyme (Lyz-SH) was prepared by the treatment of Lyz with *N*-succinimidyl-*S*-acethylthiopropionate (SATP) to form a thioacetate-bearing Lyz through amide bonds and deprotection with hydroxyl amine to reveal the thiol.[11] After the removal of excess SATP, free thiol content incorporated in the resultant Lyz-SH was estimated as 4 thiol units per a protein with Ellman's assay. Lyz-SH was then treated with **P2** in PBS (pH = 7.4) at 4 °C ($[\text{Lyz-SH}]_0$ = 0.22 mg/mL, [**P2**] = 40.8 mg/mL, **P2**/Lyz-SH = 50 eq/1 eq) to induce formation of **P2**-ss-Lyz. Confirmed by DLS, **P2** still maintained a large aggregation structure in PBS solution. After 24 h, the mixture was analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 5). To visibly identify the successful conjugation, Lyz unit was stained by coomassie blue (lane 2, 3).

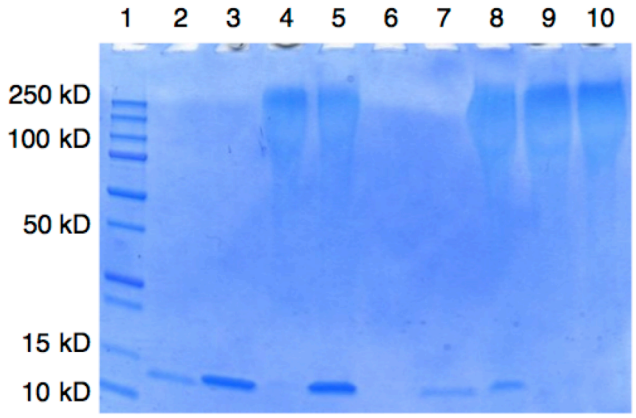


Fig 5. SDS-PAGE visualized by coomassie blue staining (lane 1: protein marker; lane 2: Lyz-SH; lane 3: Lyz; lane 4: **P2**-ss-Lyz; lane 5: **P3**-Lyz; lane 6: none; lane 7: Lyz-SH with DTT; lane 8: **P2**-ss-Lyz with DTT; lane 9: **P2**; lane 10: **P3**).

As seen in lane 4, the conjugation product only exhibited a band with high molecular weight (~250 kD) without another small molecular weight band derived from free Lyz-SH (between 10 – 15 kD), demonstrating that all Lyz-SH was successfully conjugated to **P2** to form **P2**-ss-Lyz. Under the SDS-PAGE conditions, **P2**-ss-Lyz should still maintain the

original aggregate structure because the aggregates are formed with fluorous interaction that is not reversed with water and ionic compounds.[47] Lyz would be mainly bound onto the surface of the aggregates. In the presence of D,L-dithiothreitol (DTT), the conjugate in turn showed a small molecular weight band consistent with Lyz-SH (lane 8). This is because the disulfide linkage in the conjugate was cleaved via reduction with DTT to give **P2** and free Lyz-SH therefrom. However, there was also a band still visible in high molecular weight region (~250 kD) after reduction. Owing to hydrophobicity of the perfluorinated core, **P2** large aggregate is partially stained in itself (Lane 9 for **P2** and Lane 10 for **P3**), so what is observed is cleaved polymer aggregates. Additionally, it is quite possible that the large aggregate structure of the polymer protected some of the disulfide bonds from cleavage during the short incubation time of the experiment. This has been observed previously for nanogels.[15]

Conjugation of Lyz to **P3** was similarly examined and the product was analyzed by SDS-PAGE. In contrast to **P2** via disulfide linkages, **P3** was not as effective for Lyz conjugation via amide bond formation: free Lyz was still observed in the product (lane 5). The lower efficiency for **P3**-Lyz conjugate would be attributed to the steric hindrance around the N-hydroxysuccinimide ester (NHS) that is directly connected to the polymer terminal without any spacer.[11] It is also possible that some of the NHS group was lost during the purification process due to hydrolysis of the end group.

Together these data supports the successful conjugation for **P2** to Lyz via disulfide bond formation. It also shows that RAFT polymerization was successful in forming amphiphilic/fluorous copolymers that contain a pyridyl disulfide group. The polymers with perfluorinated components and PEG side chains formed aggregates in solution that were nontoxic to a both a mouse and human cell line. Thus, the conjugates demonstrated herein could be useful for a variety of unique biomedical applications. For example, perfluorinated compound emulsions are often utilized as oxygen carriers.[38-40] Thus, these conjugates may be useful for applications where protein targeting and oxygen delivery together in one carrier would be useful, such as in hypoxic tumor therapy.

Conclusions

In conclusion, we successfully developed biocompatible and amphiphilic/fluorous random copolymers with poly(ethylene glycol) chains and perfluorinated pendants via RAFT copolymerization with functional CTAs for protein conjugation. In water, their copolymers intermolecularly associated with multi-chains to form large aggregates that effectively place the perfluorinated pendants into the inner fluorous compartment. Owing to the confined structures in water and the potential biocompatibility of the perfluorinated segments, the amphiphilic/fluorous random copolymers are non-cytotoxic against NIH 3T3 and HUVECs. Additionally, thiolated lysozyme was successfully conjugated onto the large aggregate of an amphiphilic/fluorous random copolymer bearing a pyridyl disulfide at a-end via a cleavable disulfide linkage. Such protein-polymer conjugate would serve as unique therapeutic materials by using the fluorous compartments. Thus, the PEGylated and perfluorinated copolymers reported herein open new vistas in biocompatible materials and biochemical and biomedical applications.

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Notes and references

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