Polymer-Stabilized Perfluorobutane Nanodroplets for Ultrasound Imaging Agents

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All reagents were purchased from commercial sources and used without further purification unless otherwise noted. Grubbs third-generation catalyst (IMesH\(_2\))(C\(_5\)H\(_3\)N)\(_2\)(Cl)\(_2\)Ru=CHPh (‘Ru’) and norbornene OEG compound 4 were prepared according to literature procedures.\(^1\) Polymer polydispersity and molecular weight were measured by size-exclusion chromatography (SEC) (Phenomenex Phenogel 5u 10, 1K-75K, 300 x 7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (0.05 M LiBr in DMF)) using a Chrom Tech® Series 1500 pump equipped with a multi-angle light scattering detector (DAWN-HELIOS: Wyatt Technology), a refractive index detector (Wyatt OptiLab T-rEX) and a UV-Vis detector (Shimadzu SPD-10AVP) normalized to a 30,000 MW polystyrene standard. The dn/dc value used (0.179 in DMF) was calculated by averaging several runs of norbornene-phenyl homopolymers assuming 100% mass elution from the columns.\(^3\) \(^1\)H and \(^19\)F NMR spectra were recorded on a Varian Mercury Plus spectrometer (400 MHz) or 300 Bruker AVA spectrometer (300MHz). Chemical shifts (\(^1\)H) and (\(^19\)F) are reported in (ppm) relative to the residual solvent peak. TEM images were acquired on carbon grids (Ted Pella, INC.) on a FEI Tecnai G2 Sphera at 200 KV. Mass spectra were obtained at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. DLS data was obtained on a Wyatt DynaPro Nanostar. Emulsions were made using a Probe Sonicator (S-4000, Misonix Sonicators, 30% amplitude, 30s). UV-induced thiol-ene crosslinking was performed with UV lamp (8W) from UVP, LLC. All ultrasound images were obtained using an Acuson Sequoia 512 ultrasound instrument with a 15L8 probe.
Synthesis of Nor-PFUA (Compound 1)

BOC-protected norbornene amine (0.60 g, 1.98 mmol) \(^3\) was dissolved in a 1:1 TFA: CH\(_2\)Cl\(_2\) solution (20 mL) and stirred at room temperature for 6 hours. The reaction was concentrated to dryness to give a brown residue that was precipitated by the addition of ether. Removal of the solvent gave the free norbornene amine as a white solid. The amine (352.30 mg, 1.10 mmol) and 2H,2H,3H,3H-Perfluoroundecanoic acid (PFUA, 492.13 mg, 1.00 mmol) were dissolved in DMF (5 mL) to give a pale yellow transparent solution. HBTU (1327.34 mg, 3.50 mmol) and triethylamine (487.84 \(\mu\)L, 3.50 mmol) were subsequently added to the solution which was stirred at room temperature for 3 days and turned dark brown in color. The solution was concentrated under reduced pressure to yield a dark brown solid, that was purified by silica-gel chromatography (CH\(_2\)Cl\(_2\): Ethyl Acetate, 10:3) to give compound 1 (Nor-PFUA) as a white powder in 67% yield. \(^1\)H NMR in CDCl\(_3\): 6.29 (a, 2H), 6.05 (h, 1H), 3.68 (f, 2H), 3.48 (g, 2H), 3.27 (e, 2H), 2.71 (b, 2H), 2.43 (i, j, 4H), 1.53 (c, 1H), 1.19 (d, 1H) [Figure S1]. \(^19\)F NMR: -81.14 (a, 3F), -115.14 (b, 2F), -122.34- -122.13 (c, 6F), -123.15 (f, 2F), -123.93 (e, 2F), -126.54 (d, 2F) [Figure S2]. ES-MS: found m/z 702.99, expected 703.10 [M+Na]+; found m/z 681.02, expected 681.10 [M+H]+; found m/z 718.96, expected 718.10 [M+K]+ [Figure S3].

Figure S1. \(^1\)H-NMR spectrum of compound 1.
Figure S2. $^{19}$F-NMR spectrum of compound 1.

Figure S3. Mass Spectrum of compound 1.
Synthesis of Norbornene Alcohol (Compound 2)
A round-bottom flask was charged with norbornene anhydride (2.07 g, 10.00 mmol), 15 mL toluene, followed by 2-aminoethanol (800 µL, 10.50 mmol) and triethylamine (200 µL, 1.1 mmol.). A Dean-Stark trap was attached to the flask, and the reaction mixture was heated at reflux for 8 h. The reaction mixture was concentrated under reduced pressure to yield a pale yellow solid. The residue was dissolved in 40 mL CH₂Cl₂ and washed with 0.1N HCl (10 mL) and brine (10 mL). The organic layer was dried over MgSO₄ and concentrated under vacuum to yield compound 2 as a white solid in 93% yield. ¹H NMR in CDCl₃: 1.35 (d, 1H), 1.53 (c, 1H), 2.08 (h, 1H), 2.73 (b, 2H), 3.30 (e, 2H), 3.71 (g, 2H), 3.78 (f, 2H), 6.30 (a, 2H).

![Diagram of compound 2]

Figure S4. ¹H-NMR spectrum of compound 2.

Synthesis of Norbornene methacrylate (NMA) (Compound 3)
Norbornene alcohol 2 (100.00 mg, 0.48 mmol) and 4-dimethylaminopyridine (DMAP) (1.62 mg, 0.10 mmol) were dissolved in anhydrous CH₂Cl₂ under a nitrogen atmosphere, followed by addition of N,N'-Diisopropylcarbodiimide (DIPC) (84.05 mg, 0.67 mmol) and methacrylic acid (45.03 mg, 0.52 mmol). The mixture was stirred at room temperature overnight, concentrated under reduced pressure, and purified by silica-gel chromatography (CH₂Cl₂: Ethyl Acetate, 1:1) to give 3 as a pale yellow solid in yield 85%. ¹H NMR in CDCl₃: 6.26 (a, 2H), 6.02 (h, 1H), 5.54 (i, 1H), 4.27 (f, 2H), 3.79 (g, 2H), 3.25 (c, 2H), 2.68 (b, 2H), 1.87 (j, 3H), 1.43 (c, 1H), 1.17 (d, 1H).
**Polymerization of PFUA_{5}-NMA_{10}-OEG_{32}**

To a stirred solution of compound 1 (10.00 mg, 14.70 µmol) in dry CH\textsubscript{2}Cl\textsubscript{2} (500 µL) was added a solution of Grubbs third-generation catalyst (2.13 mg, 2.94 µmol) in dry CH\textsubscript{2}Cl\textsubscript{2} (50 µL). The reaction was left to stir under nitrogen at room temperature for 2 hrs after which a solution of compound 3 (8.09 mg, 29.40 µmol) was added and left to stir for an additional 30 min. A solution of compound 4 (33.25 mg, 94.10 µmol) was added to the reaction and stirred for 40 min. Finally, the reaction was quenched by addition of an excess of ethyl vinyl ether (EVE). After 20 min the solution was concentrated to 1/3 of its volume then precipitated by addition of cold diethyl ether to give the copolymer as an off white solid.
Figure S6. The schematic diagram for UV-induced thiol-ene crosslinking.

Figure S7. General method utilized in polymerization reactions for copolymer PFUA_{m-NMA_{n-OEG_{p}}}

Figure S8. SEC-MALS trace of copolymer PFUA_{5-NMA_{10-OEG_{32}}}, light scattering (LS, red) and refractive index (dRI, black).
Figure S9. $^1$H-NMR of copolymer PFUA$_5$-NMA$_{10}$-OEG$_{32}$.

UV-induced crosslinking of PFUA$_5$-NMA$_{10}$-OEG$_{32}$

The copolymer PFUA$_5$-NMA$_{10}$-OEG$_{32}$, PETMP and DMPA, with a ratio of alkene: thiol: photoiniator of 1:1:0.2, were dissolved in 1 mL THF cooled in an ice bath and irradiated at 365 nm for 30 min. The polymer was precipitated by addition of cold diethyl ether, dried under vacuum, and characterized by SEC and $^1$H-NMR.

Figure S10. SEC-MALS dRI trace showing the change in elution time of polymer PFUA$_5$-NMA$_{10}$-OEG$_{32}$ after UV-induced crosslinking.
Preparation of PFC-containing emulsions

The copolymer PFUA$_5$-NMA$_{10}$-OEG$_{32}$, PETMP and DMPA, with a ratio of alkene:thiol: photoiniator of 1:1:0.2, were dissolved in 100 µL of pre-cooled THF, then 60 µL PFH or PFP was added to the solution. The mixture was subjected to probe sonication at 70% amplitude for 30 s immediately after addition of 1000 µL PBS buffer. Half of the resulting emulsion was irradiated at 365 nm UV for 30 min in an ice bath. For PFB-loaded emulsions, all procedures were conducted at approximately -20 °C (mass ratio of ice to sodium chloride =3:1). In order to prevent freezing the buffer was changed to a mixture of PBS: Propylene glycol: Glycerol (16:3:1).
Figure S12. TEM images (left) and DLS measurements (right) of non-crosslinked and crosslinked PFH-emulsions made from polymer PFUA5-NMA10-OEG32.

**Thermal Stability of PFH-containing emulsions observed by optical microscopy**

PFH-containing emulsions with and without crosslinking were spotted on clear glass slides and were gently covered by cover slips to avoid the generation of bubbles. The glass slides were heated for 3 min at room temperature (~20 °C), the boiling point of PFH (56 °C) and at 70 °C. Images were taken by optical microscopy to observe the evaporation of the PFH.
Figure S13. Optical microscopy images of PFH emulsions made by polymer PFUA₅-NMA₁₀-OEG₃₂ with and without crosslinking heated to different temperatures.

**Ultrasound imaging**

30 µL PFCs-encapsulated emulsions with and without crosslinking were diluted in 3 ml PBS buffer, and evaluated by ultrasound imaging. The ultrasound power was increased by 1 dB/s to trigger the PFCs’ phase transition from liquid to gas; upon reaching maximum power (0dB, MI = 1.1) it was then decreased at 1 dB/s to stop the PFCs phase transition. The sample chambers were put in water bath with different temperatures including room temperature (RT) and body temperature (37 ºC). The ultrasound images were analyzed using OsiriX and matlab to calculate the intensity of ultrasound signal.

Figure S14. Ultrasound images of PFH-containing emulsion made from copolymer PFUA₅-NMA₁₀-OEG₃₂ at 37 ºC.
Figure S15. DLS data of PFP-emulsion with and without crosslinking made from polymer PFUA$_5$-NMA$_{10}$-OEG$_{32}$.

**Polymerization of PFUA$_5$-NMA$_{20}$-OEG$_{45}$**

To a stirred solution of 1 (10.00 mg, 14.70 µmol) in dry CH$_2$Cl$_2$ (500 µL) was added a solution of Grubbs third-generation catalyst (2.13 mg, 2.94 µmol) in dry CH$_2$Cl$_2$ (50 µL). The reaction was stirred under nitrogen at room temperature for 2 hrs. A solution of 3 (16.18 mg, 58.80 µmol) was added to the reaction mixture and left to stir for an additional 30 min. A solution of compound 4 (46.76 mg, 132.30 µmol) was added to the mixture, which was stirred for another 40 min. Finally, the reaction was quenched with ethyl vinyl ether (EVE). After 20 min the solution was concentrated to 1/3 of its volume then precipitated by addition of cold diethyl ether to give the copolymer as an off white solid.

Figure S16. SEC-MALS trace of copolymer PFUA$_5$-NMA$_{20}$-OEG$_{45}$, light scattering (LS, red) and refractive index (dRI, black).
Figure S17. Ultrasound images of non-crosslinked PFB-containing emulsions made with copolymer PFUA$_{5}$-NMA$_{20}$-OEG$_{45}$ at 37 °C.

Figure S18. a) DLS measurements for size changes of noncrosslinked and crosslinked PFP emulsions after 7 days; b) DLS measurements for size changes of noncrosslinked and crosslinked PFB emulsions after 7 days.
Figure S19. a) Size changes of crosslinked PFP-emulsions before and after US burst; b) Size changes of crosslinked PFB-emulsions before and after US burst.
Figure S20. US images of PFP emulsions fabricated with PFUA$_{5}$-NMA$_{10}$-OEG$_{32}$ in 55% FBS in PBS buffer along with changes of ultrasound MI at physiological temperature (37 ºC) respectively, and region of interest (ROI) analysis of US signal intensity at the electronic focus of the transducer in one US test cycle. The blue line in each plot represents MI over time. The red line in each plot represents mean ROI signal intensity over time.

Figure S21. US images of PFB emulsions fabricated by PFUA$_{5}$-NMA$_{20}$-OEG$_{45}$ in 55% FBS in PBS buffer along with changes of ultrasound MI in physiological temperature (37 ºC) respectively, and region of interest (ROI) analysis of US signal intensity at the electronic focus of the transducer in one US test cycle. The blue line in each plot represents MI over time. The red line in each plot represents mean ROI signal intensity over time.

**Cell Viability Assay**
HeLa cells ($10^4$ cells per well) were incubated in 96-well plates (Corning, New York, USA) in an air atmosphere with 5% CO$_2$ at 37 ºC using DMEM with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin for 24 hours. 0.1 mL crosslinked and non-crosslinked PFP-emulsions and PFB-emulsions with different concentrations ranging from 0.1 µg/ml to 1 mg/ml were added and left
to incubate with cells for 24 hours and 48 hours respectively. Cell viabilities were determined by CCK-8 assay.

Figure S2. Cell viabilities of PFP-emulsions and PFB-emulsions with and without crosslinking after incubating with HeLa cells for 24 hours.

References