Preparation of Liquid-Filled Micelles Based on an Amphiphilic Triblock Copolymer

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Received 4 December 2008; accepted 7 May 2009
DOI 10.1002/app.30745
Published online 12 August 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A series of novel amphiphilic triblock poly(ethylene glycol)-*b*-poly(2-aminoethyl methacrylate hydrochloride)-*b*-poly(heptadeca-fluorodecyl acrylate) (PEG-*b*-PAEMA-*b*-PHFDA) comprised of two hydrophilic PEG and PAEMA segments and one hydrophobic PHFDA segment was designed and synthesized. The structure of the triblock copolymer was characterized by ^{1}H-NMR and GPC analysis. The amphiphilic triblock copolymer was capable of self-assembling into liquid-filled micelles that consisted of PHFDA and liquid perfluorocarbons (PFCs) as the core and PEG as outer shell. PAEMA can be used as cross-linking sites to increase the stability of the liquid-filled micelles. The shape, size, and Acoustic properties of the obtained liquid-filled micelles were investigated. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 3472–3478, 2009

Key words: amphiphilic block copolymer; copolymer micelle; self-assembly; cross-link

INTRODUCTION

Well-defined nanostructured materials containing various species (e.g., catalysts, DNA, drugs, enzymes) have attracted considerable interest over the past decade, due to the unique behaviors of nanoscale objects that can be exploited in a wide range of applications such as in diagnostics, catalysis, therapy, and bioengineering in both aqueous and nonaqueous media.[1] Colloidal hollow microparticles that traps for gases or liquids is one of the most successful examples of such special nanostructured materials.[2] The advantages of these structures are their small (and usually variable) size and low toxicity, and the controlled permeability of the microcontainer wall. A remarkable property of these materials is their efficiency as ultrasound contrast agents (UCAs) in ultrasound imaging, a nowadays established diagnostic method, provided three-dimensional information, and permitted evaluation of dynamic physiologic processes such as blood flow or tissue motion.[3]

These UCAs are generally composed of internal gas or liquid surrounded by a thin shell, which maintains the shape of the microcapsule and prevents diffusion of the internal gas or liquid. For example, liposomes are one of the membrane-enclosed vesicles that composed of a lipid bilayer shell (which can trap hydrophobic and amphipathic drugs) surrounding an aqueous core (which can encapsulate hydrophilic drugs).[4] From microcapsule having a hard plastic shell the internal gas cannot be ejected completely, and a portion of the internal gas remains inside the broken shell.[5] These agents can improve the accuracy of assessing left ventricular function during echocardiography and allow imaging of tissue perfusion.[6] Unfortunately, the fluid structure of liposomes makes them weakly echogenic and not readily manipulated with ultrasound radiation force.

To increase the stability of UCAs, many authors have shown interest in polymers since polymeric shells are more resistant to ultrasonic waves than monomolecular layers of lipids or surfactants usually stabilizing commercial UCAs such as Levovist

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor: Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT); contract grant number: 0654.
Contract grant sponsor: Scientific Research Foundation of Zhejiang Sci-Tech University; contract grant number: 0901808-Y.
Contract grant sponsors: Training Foundation for the Excellent Young Talents by the Key Laboratory of Advanced Textile Materials and Manufacturing Technology (ATMT), Ministry of Education; contract grant number: 2008QN10.

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or Optison.[7] On the other hand, perfluorocarbons (PFCs), because of their difference of density with air and their poor solubility in water, have been shown to increase both the stability and the echogenicity of UCAs. We therefore chose to encapsulate PFCs in the liquid state within polymeric particles because of its easier feasibility. The challenge to overcome for PFCs encapsulation is their very low miscibility with hydrogenated organic solvents. We developed innovative UCAs encapsulating liquid PFCs and surrounded by a polymeric shell. Water-soluble copolymers bearing small hydrophobic segments have attracted much attention due to their strong hydrophobic self-associating properties in aqueous solutions. This kind of amphiphilic copolymers can form micelles structure in the aqueous environment. This method can afford well-defined phase-separated spherical micelles which contain a hydrophobic central domain which is surrounded and stabilized in aqueous media by the hydrophilic shell layer. Fluorocarbon functional groups are interesting moieties and are of industrial importance because of their low surface energy, biocompatibility, and extreme hydrophobic properties.[8] Fluorocarbon-functionalized hydrophobic segments can be dissolved in PFCs. This afforded a hydrophilic shell layer surrounding a liquid-filled core which was capable of hydrophobic guest sequestration and further functionalization by the reintroduction of hydrophobicity in the amphiphilic copolymer.[9] There has been significant recent interest in the covalent stabilization of the outer corona to afford robust shell cross-linked nanoparticles which are stable to changes in concentration and temperature.[10] With limitation of cross-links to the copolymer chain segments that compose the peripheral micelle shell, shell cross-linked knedel-like nanoparticles (SCKs) can be formed, which consist of a hydrophobic core domain and a cross-linke hydrophilic shell layer.[11]

Our interest is primarily focused on these amphiphilic nanostructures, due to their analogy to biological constructs. In this study, we aim to develop a liquid-filled micelles that consisting of PHFDC and liquid perfluorocarbons (PFCs) as the core and PEG as outer shell. PAEMA can be used as cross-linking sites to increase the stability of the liquid-filled micelles. The shape, size, and Acoustic properties of the obtained liquid-filled micelles were investigated.

EXPERIMENTAL SECTION

Materials

2-Aminoethyl methacrylate hydrochloride (AEMA, 90%), anhydrous dichloromethane (CH_2Cl_2), copper (I) bromide (Cu(I)Br, 98%), and anhydrous isopropanol [$(CH_3)_2CHOH$] were purchased from Sigma Chemical (St. Louis, MO). Perfluoropentane (PFP, 97%) triethylamine (TEA, 96%) and 1,1,4,7,7- pentamethyldiethylenetriamine (PMDETA, 98%) were purchased from Alfa Aesar Chemical (Ward Hill, MA). 2-Bromoisobutyryl bromide (BIBB, 97%) and 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl acrylate (HDFDA, 90%) were purchased from Fluka Chemical (Milwaukee, WI). Glutaraldehyde (70% solution) was obtained from Electron Microscopy Science (Hatfield, PA). Dialysis tubings (MWCO 8 000) were from Spectrum Medical Industries (Los Angeles, CA). α-Aminopropyl-ω-methoxy polyoxyetylene (MeO-PEG-NH_2, 97.9%) was supplied by Nippon Oil & Fats (NOF; Tokyo, Japan).

Synthesis of amphiphilic Triblock copolymer

MeO-PEG-NH_2 (0.15 g, MW = 5 KDa, 2.85×10^{-5} mole) was dissolved in 1.0 mL anhydrous CH_2Cl_2 with triethylamine (0.1 mL). After cooling to 0°C, bromoisobutyryl bromide (0.65 g, 2.85×10^{-3} mole) was then added dropwise to the solution. Triethylamine was used to trap the hydrobromic acid produced as an insoluble ammonium salt and thus completely displace the equilibrium. After the completion of the addition, the reaction mixture warmed up to room temperature and continuous stirring was applied for 24 h. Afterward, the macromolecular initiator in CH_2Cl_2 solution was filtered to remove the ammonium salt, and the product (MeO-PEG-Br) was precipitated in diethyl ether and then dried under vacuum for 12 h at 30°C (0.12 g, Yield 80%). MeO-PEG-Br (0.10 g, $\sim 1.90 \times 10^{-5}$ mole) was dissolved in reaction tube containing 0.5 mL of 2-propanol/H_2O (80 : 20). AEMA (0.042 g, 2.53×10^{-4} mole) was then added. The Cu(I)Br catalyst (0.0027 g, 1.88×10^{-4} mole) was added carefully into the mixture. Finally, the ligand 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 10 uL, $\sim 3.60 \times 10^{-4}$ mole) was added, and the reaction tube was connected to the Schlenk line system, and three freeze-pump-thaw cycles were used to remove the trace of oxygen. The reactive system was immersed into an oil bath at 50°C for 24 h. The crude product was dissolved in DI H_2O and dialysis against DI H_2O (MWCO = 5000) was performed to remove the catalyst. The copolymer (PEG-*b*-PAEMA-*b*-PHFDA) was obtained after freeze-drying (0.075 g, Yield 75%). PEG-*b*-PAEMA (0.07 g, $\sim 1.00 \times 10^{-5}$ mole) was dissolved in reaction tube containing 0.5 mL of 2-propanol/H_2O (80 : 20). Perfluorodecyl acrylate (0.10 g, 1.93×10^{-5} mole) was then added. The Cu(I)Br catalyst (1.43×10^{-3} g, 1.00×10^{-5} mole) was added carefully into the mixture. Finally, the ligand HMTETA (5.70 μL, 2.00×10^{-5} mole) was added, and the reaction tube was connected to the Schlenk line system, and three freeze-pump-thaw cycles

were used to remove the trace of oxygen. The reactive system was immersed into an oil bath at 70°C for 24 h. The crude product was dissolved in DI H_2O and dialysis against DI H_2O (MWCO = 8000) was performed to remove the catalyst. The copolymer (PEG-*b*-PAEMA-*b*-PHFDA) was obtained after freeze-drying (0.12 g, Yield 70.6%).

Preparation of micelles

Typically, 1×10^{-3} g of PEG-*b*-PAEMA-*b*-PHFDA sample was dissolved in 1 mL of perfluoropentane and the resultant stock copolymer solution was stored in 0°C for further use. 1 mL DI H_2O was placed in a 10 mL-glass flask containing a magnetic stir bar. The flask was submerged in an ice-water bath and stirred for 30 min. Then, 100 μL of copolymer solution was added into the flask. The mixture was stirred for 24 h at 0°C to get the copolymer micelles. For the cross-linked micelles, shell cross-linking was achieved by adding 20% glutaraldehyde aqueous solution after adding the copolymer solution and stirring the solution for at least 1 day at 0°C.

Determination of critical micellar concentration

PEG-*b*-PAEMA-*b*-PHFDA was dissolved in perfluoropentane (concentration = 1×10^{-3} g/mL). Pyrene was used as a hydrophobic agent. Aliquots of pyrene solution (5×10^{-5} mole/L, 100 uL) were added into 96-well plate, and acetone was allowed to evaporate at room temperature. Taking the different volume of copolymer solution (10–100 μL) was diluted by DI water to 1 mL mixture solution which was stirred at 0°C to prepare the micelle solutions. Taking 100 μl of the prepared micelles solution into another 96-well plate and measure the absorbance at 337 nm with a plate reader. From the pyrene excitation spectra, the intensities at 337 nm were analyzed as function of the copolymer concentrations.

Acoustic testing

Acoustic properties of copolymer micelles were characterized as described previously.[12] Briefly, a pulse-echo setup was used for *in vitro* studies, employing a single-element, broadband, 12.7-mm element diameter, 50.8 mm spherically focused transducer with a center frequency of 50 MHz (Panametrics, Waltham, MA). The transducer was inserted in water bath ($0.5 \times 26.7 \times 25.4$ cm^3 custom-built acrylic tank filled with deionized water) and focused through an acoustic window of a custom-made sample vessel. During the insonation, the transducer was positioned within the water bath at the depth of 14 cm from the top of the liquid in the sample vessel. A pulser/receiver (model 5072 PR, Panametrics, Waltham, MA) was used to pulse the transducer at a pulse repetition frequency of 100 MHz. The received signals were amplified and fed to the digital oscilloscope (Lecroy 9350A, Lecroy, Chestnu Ridge, NY). The digitized data was stored and analyzed using LabView (National Instruments, Austin, TX).

Measurments

^{1}H-NMR spectra were recorded on a 300 MHz Varian Mercury 300 at ambient temperature. Gel permeation chromatographic (GPC) analysis was carried out using a Waters 1525 pumping system at the flow rate of 0.5 mL/min with an Ultrahydrogel 500 column (Waters). The eluent was deioned water. The light transmittance was measured the absorbance on a Thermo multiskan spectrum. Micelles morphology was observed on JEOL 2010F transmission electron microscope (TEM) at an acceleration voltage of 200 kV. The size distribution and zeta potential of micelles was determined by 91 Plus particle size analyzer (Broolhaven Instruments). The determination was repeated three times/sample for three samples.

RESULTS AND DISCUSSION

Design and synthesis of copolymers

The low immunogenicity of PEGs makes them attractive copolymers for biomedical applications.[13] Hydrophobically modified PEGs, called also PEG-surfactants or PEO-lipids, consisting of a hydrophilic PEG chain covalently attached to a hydrophobic aliphatic double chain moiety have been prepared. It is well-known these amphiphilic block copolymers can self-assemble into aggregates with various morphologies in water.[14–16] However, due to the presence of dynamic exchange between assembled aggregates and individual unimers, the equilibrium between the aggregates and unimers is governed by a delicate balance of weak intermolecular forces. The disruption of such a balance can be easily triggered by temperature, dilution, and salt concentration.[13,17,18] In many applications, it is advantageous to have a covalently-stabilized nanostructure, which is robust and maintains its integrity under a variety of conditions. As a result, there has been significant effort in recent years towards the stabilization of self-assembled copolymeric nanostructures.[19] If one block bears reactive functional groups, the morphologies of the aggregates can be fixed by chemical cross-linking. In particular, shell cross-linked (SCL) micelles combine the properties of micelles, microgels, nanoparticles, and dendrimers, and various applications such as targeted

TABLE I
Summary of PEG-*b*-PAEMA-*b*-PHFDA Amphiphilic Triblock Copolymers

Run	Composition[a]	MP (Da)[b]	Solubility[c]	CMC[d] (mg/L)
1	86.7 : 5.9 : 7.4	10,590	+	12.0
2	86.1 : 5.8 : 8.1	11,787	+	13.5
3	85.5 : 5.7 : 8.7	12,144	±	13.8
4	77.6 : 5.9 : 16.5	–	–	–

[a] Block ratio calculated on the basis on the ^{1}H-NMR Data.
[b] Molar ratio calculated on the basis on the linear poly(ethylene glycol) stands with low polydispersity index.
[c] Complete solubility (+), part solubility (±), and no solubility (–) in water.
[d] CMC data were determined from the fluorescence method, pyrene was used as a hydrophobic fluorescent probe.

drug delivery, sequestration of metabolites, and entrapment of environmental pollutants have been suggested.[20] In the present study, we synthesized a series of triblock copolymer PEG-*b*-PAEMA-*b*-PHFDA involoved three steps as illustrated in Scheme S1. First, PEG-based initiator was synthesized by reaction of MeO-PEG-NH_2 and BIBB. Then, the second, PAEMA as cross-link sites, was attached to the PEG by ATRP method. The amino groups on the PAEMA can be used as reactive functional groups to react with glutaraldehyde to form the cross-linking shell. At last, by using the similar method, a hydrophobic block was introduced in the copolymer to form a triblock copolymer of PEG-*b*-PAEMA-*b*-PHFDA.

The molecular weight of copolymers was verified by GPC as shown in Table I. From the difference of molecular weight of copolymers, it was inferred that PHFDA segment in the copolymers having different chain length. The solubility of the copolymers depends on the composition of them. The longer of the hydrophobic chain, the lower solubility can be obtained. ^{1}H-NMR was further employed to characterize the structure of the copolymers. Supporting Information Figure S1 shows the ^{1} H-NMR spectrum of the copolymer (PEG-*b*-PAEMA-*b*-PHFDA-1) in $CDCl_3$. The singals at δ 4.05 and 4.42 ppm are assigned to the methylene protons linked to oxygen of ester group in PHFDA and PAEMA. Because of the singal overlap of main chain protons, we choose the methylene protons from ester groups to confirm the composition of copolymer. Compare the integration values of peaks 3, 7, and 10, wich provide building units ratio of PEG: PAEMA: PHFDA equal to 85.3 : 6.2 : 8.5. We used the copolymer, PEG-*b*-PAEMA-*b*-PHFDA-1, as represent for the further research in the below.

Micelle formation and characterization

Information about the onset of micellization of PEG-*b*-PAEMA-*b*-PHFDA is drove from steady-state fluorescent probe studies. Pyrene was chosen as the fluorescent probe because of its photophysical property. It was well known that pyrene, as a fluorescene probe, shows little fluorescence when it was in aggregation state and shows strong fluorescence when it was separated molecularly. The hydrophobic environment is also favorable to improve the fluorescent intensity of pyrene. In the critical micellar concentration (CMC) measurements, keeping a relatively high concentration of pyrene is to make sure the pyrene is in its aggregation state in pure water. So, under this condition, if the concentration of the pyrene was kept the same, the fluorescence intensity would be greatly improved when the micelle existed in the solution because the pyrene molecules can be separated due to the fact that they entered into the hydrophobic core of micelles.[21] The solutions were kept at room temperature for 24 h to reach the equilibrated solubilization of pyrene in the aqueous phase. Emission was measured at 390 nm, and excitation spectra were recorded ranging from 240 to 360 nm. Both excitation and emission bandwidths were 10 nm. From the pyrene excitation spectra, the intensities at 337 nm were analyzed as a function of the copolymer concentrations. A CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration (Supporting Information Figure S2). The longer of the hydrophobic chain, the higher CMC data can be obtained.

Micelle morphology was confirmed by means of TEM as shown in Figure 1(A,B). TEM images clearly show the existence of "core-shell" structure micelles derived from the triblock copolymer. The self-assembled micelles were dispersed as individual micelles with spherical shape with a diameter of $\sim$ 150 nm. The origin of this phenomenon is likely due to the amphiphilic property of the copolymer.

To study the effect of temperature on the size of micelles, the prepared micelle solution contained in scintillation vials were incubated at 22°C, 29°C, 37°C, and 40°C water bath. Supporting Information Figure S3 shows the size change of copolymer micelles at different temperature. Both of the

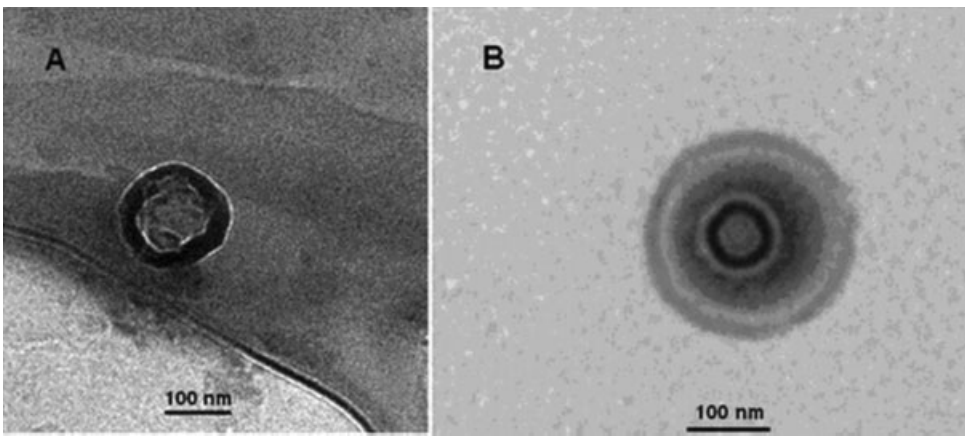

Figure 1 TEM micropicture of PEG-*b*-PAEMA-*b*-PHFDA-1 micelle without (A) and with cross-link (B).

copolymer micelles with and without cross-link have the stable size around 120 nm at the 22 and 29°C. The smaller diameter from TEM study may be due to the collapse of the free segment of the hydrophilic chain of the copolymer as well as the dehydration of the copolymer chain.[21] The size of the copolymer micelles keep relatively stable under these temperatures. However, they showed different behaviors at higher temperature. In the case of copolymer micelles without cross-link, the biggest size can be reached at 15 and 25 min for 37 and 40°C, respectively. After that, the size of copolymer micelles decreased quickly (Supporting Information Figure S3A). While, in the case of copolymer micelles after

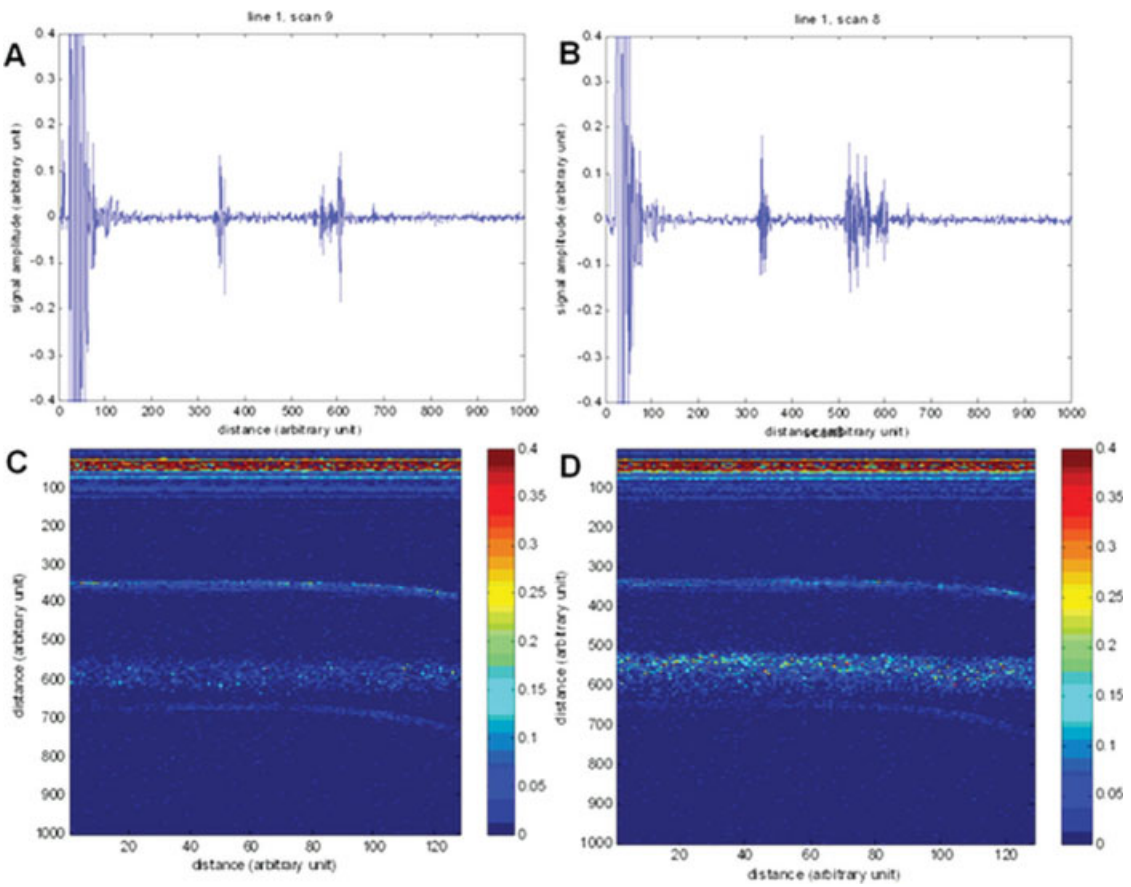

Figure 2 Ultrasound signals of copolymer micelles without cross-link (A and B) and with cross-link (C and D) performed at physiological temperature (~ 37°C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

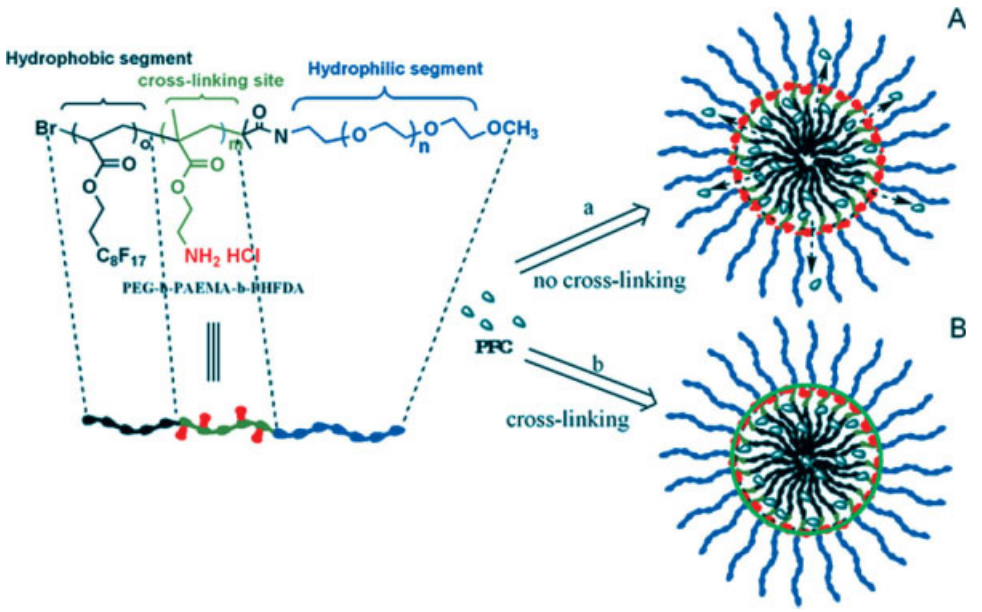

Figure 3 Self-assembly of copolymer micelles from an amphiphilic triblock copolymer PEG-*b*-PAEMA-*b*-PHFDA for encapsulating PFC (perfluoropentane in this case) to form liquid-filled micelles using cross-link (A) or not (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cross-linked, the size of copolymer micelles started to increase at 5 and 10 min for 37 and 40°C, respectively. Then, the size of the micelles can be kept stable for 15–20 min at these temperatures (Supporting Information Figure S3B). The boiling point of perfluoropentane is 28–30°C. At the 22 and 29°C, the perfluoropentane is still in liquid state. And the size of copolymer micelles keeps stable. Their sizes increase when the perfluoropentane change from liquid to gas state at the higher temperature above its boiling point. The gas can be come out from the core of micelles if the outer corona that have no robust shell, just like the copolymer micelles without cross-link. The size of micelles decreases due to the leak of the gas and collapse of the free segment of the shell of the micelles. After the shell of micelles has been chemical cross-linked, the copolymer micelles are robust and maintain its integrity under higher temperatures. And the perfluoropentane gas can not leak from the micelle quickly, so the micelles can maintain the stable size in a longer time. The zeta potential of the micelles is zero, which means no charges are on the surface of the micelles (Supporting Information Figure S4) which give it hope of passing through the negatively charged plasma membrane as medical applications.

Acoustic properties of micelles

The principle behind using PFC liquid-filled micelles as ultrasound contrast agents is based on their lower boiling point and compressibility. At the physiological temperature (~ 37°C), the PFC liquid-filled micelles transfer into the gas-filled micelles which are several orders of magnitude more compressible than water or tissue, and are smaller than the wavelength of the applied ultrasound field in the diagnostic frequency range. Therefore, they undergo volumetric oscillation, whereby compression occurs during the pressure peaks and expansion occurs during the pressure nadirs of the ultrasound wave. This vibration of the PFC liquid-filled micelles in the ultrasound field produces a strong backscattered Acoustic signal that can be detected and reproduced as an opacification on ultrasound imaging.[3]

The sample vessel containing 1 mL of copolymer micelles was held in the water bath. The strong ultrasound signals for copolymer micelles with and without cross-link can be obtained when the temperature of water is 40°C. No great difference for these two samples can be found. The perfluoropentane can be transferred into gas from liquid state at this temperature. All the perfluoropentane in the free state can be escaped from the micelles solution. Meanwhile, some of them in the core of copolymer micelles also can be leaked if no robust shell present in the copolymer micelles. If the outer shell of copolymer micelles has been cross-linked that can afford robust corona, the perfluoropentane in the core are stable to changes in temperature.

To verify our hypothesis, the ultrasound experiments of copolymer micelles were performed at physiological temperature (~ 37°C) after these samples were inserted in a water bath with temperature at 40°C for 10 min. Figure 2(A,C) shows the ultrasound signals of copolymer micelles without cross-link and

with cross-link at $\sim$ 37°C. The ultrasound wave amplitude of copolymer micelles without cross-link is lower than that of copolymer micelles with cross-link. It suggested the less gas can be produced in the former sample for the most of perfluoropentane gas has been leaked at 40°C [as shown in Fig. 3(A)]. Figure 2(B,D) show the ultrasound signal at horizontal direction. More signal point for copolymer micelles with cross-link can be observed which is corresponding to the results of ultrasound amplitude signals. From these results, we believe that the perfluoropentane in the core copolymer is more stable to changes in environment temperature after their shell has been cross-linked [as shown in Fig. 3(B)].

CONCLUSION

A series of novel amphiphilic triblock poly(ethylene glycol)-*b*-poly(2-aminioethyl ethacrylate hydrochloride)-*b*-poly(heptadecafluorodecyl acrylate) (PEG-*b*-PAEMA-*b*-PHFDA) comprised of two hydrophilic PEG and PAEMA segments and one hydrophobic PHFDA segment was designed and synthesized. The structure of the triblock copolymer was characterized by ^{1}H-NMR and GPC analysis. The amphiphilic triblock copolymer was capable of self-assembling into liquid-filled micelles that consisted of PHFDC and liquid perfluorocarbons (PFCs) as the core and PEG as outer shell. PAEMA can be used as cross-linking sites to increase the stability of the liquid-filled micelles. The perfluoropentane in the core copolymer micelles with cross-link is more stable to changes in environment temperature. These features make the attractive candidates as molecular nanocarriers for potential applications including controlled drug delivery and release, dye phase transfer, UCAs and building blocks for nanomaterials.

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