Synthesis, characterization, micellization and application of novel multiblock copolymers with the same compositions but different linkages

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Several novel multiblock copolymers, \((\text{PEO}-b-\text{PS}-b-\text{PEO-Dyne})_n\), \((\text{PEO}-b-\text{PS}-b-\text{PEO}-(\text{OH})_n)_n\), and \((\text{PEO}-b-\text{PS}-b-\text{PEO-Acetal})_n\), with the same compositions but different linkages were constructed, and their micellization and application were studied. First, the precursor \(\text{HO-PEO}-b-\text{PS}-b-\text{PEO}-\text{OH}\) was prepared by sequential LAP and ROP mechanisms, and the precursor Propargyl-PEO-\(b-\text{PS}-b-\text{PEO}-\text{Propargyl}\) was achieved by a subsequent modification procedure. Subsequently, using the efficient Glaser coupling reaction, the multiblock copolymer \((\text{PEO}-b-\text{PS}-b-\text{PEO-Dyne})_n\) was synthesized and the dyne groups embedded in the main chain were modified by a thiol-yne reaction to give multiblock copolymer \((\text{PEO}-b-\text{PS}-b-\text{PEO-(OH)})_n\). Also, using the efficient Williamson reaction, the multiblock copolymer \((\text{PEO}-b-\text{PS}-b-\text{PEO-Acetal})_n\) was obtained. Finally, the micellar morphologies formed from the synthesized copolymers were investigated and compared by DLS and TEM measurements, and the in vivo distribution of the micelles was also studied by loading them with a fluorescent probe. The results revealed that, under the same conditions, the multiblock copolymers can form micelles of different sizes. Due to the hydrophilicity of the introduced dyine groups and PS segments, smaller sized micelles can be formed, which traverse the BBB and therefore might result in a therapeutic application in the treatment of brain disease. However, the hydrophilicity of the acetal and hydroxyl groups gave a similar effect to that of the PEO segment, and larger sized micelles were formed.

Introduction

Over the past twenty years, with the innovative presentation and rapid development of various “living”/controlled polymerization mechanisms and efficient coupling reactions, plenty of polymers with complicated topologies and multiple compositions have been constructed using certain synthetic routes.\(^1\sim\)\(^3\) Correspondingly, the successful synthesis of these polymers further helped related research in polymer theory and the polymer materials field due to their unique physical properties and potential applications.\(^4\sim\)\(^6\) Among the various synthesized polymers, the focus has been on amphiphilic polymers because their unique self-assembled aggregates, polymeric micelles, provide some promising to alter the pharmacokinetic profile of drugs, reduce the off-target toxicity and side effects, and enhance the therapeutic efficiency.\(^7\sim\)\(^12\)

Polymeric micelles of a small size are distinctively poised to efficiently deliver chemotherapeutics because the small size allows them to utilize the enhanced permeation and retention (EPR) effect, which describes the preferential accumulation of micelle particles in tumor tissue via leaky blood vessels.\(^13\)

Up to now, the self-assembly behaviour of amphiphilic copolymers has received great attention and progress from an enormous number of researchers.\(^14\sim\)\(^19\) The morphologies of formed copolymeric micelles strictly depend on both the compositions and topologies of the copolymers. For example, the morphologies of the micelles formed from multiblock copolymer \([\text{poly(caprolactone)-b-poly(ethylene oxide)}]_n\) \([\text{PCL-b-PEO}]_n\) could be globules, fibers, or worms in certain selective solvents,\(^20\) and those from diblock copolymer (PEO-b-PCL) are typical spherical particles.\(^21\) Actually, the self-assembly behaviours of amphiphilic di- or triblock copolymers have been widely investigated,\(^22\sim\)\(^24\) however, the related study on multiblock copolymers is still rather limited. The largest obstacle is the difficulty in finding versatile routes to the required multiblock copolymers.

Typically, there are two approaches used to synthesize multiblock copolymers: (a) by the sequential addition of monomers to a precursor containing multiple initiator units,\(^25\) and (b) by a coupling reaction between reactive building blocks.\(^26\)
Comparatively, the latter approach (b) is widely used because the parameters of the building blocks can be easily controlled. The most adopted coupling reactions are copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) “click” chemistry, the atom transfer radical coupling (ATRC) reaction, and so on. For example, the amphiphilic multiblock copolymer poly(acrylic acid)-b-polystyrene was constructed by CuAAC “click” chemistry of α-azide, ω-alkyne heterofunctional diblock copolymers. Similarly, the multiblock copolymer poly(isoprene)-b-polystyrene, [π-b-PS]n, was also successfully synthesized using “click” chemistry. However, in approach (b), a precursor with defined azide and alkyne groups at each end must first be selectively synthesized when CuAAC “click” chemistry is used, and the possible disproportionation termination and side reactions on the formed carbon radicals also must be avoided in the ATRC reaction. Thus, finding an efficient and convenient coupling reaction is indeed the key point for multiblock copolymers.

Recently, the classic Glaser coupling reaction between alkyne groups has been evoked and its application in polymer science has been discovered since it provides access to functional materials with 1,3-conjugated structures. This reaction has the advantages of the convenient introduction of alkyne groups onto a polymeric precursor, mild operation under an oxygen atmosphere and yields with a high efficiency. As another classic and efficient coupling reaction, the Wilkinson reaction between active hydroxyl groups has also been applied in polymer science to construct polymers with acid-sensitive linkages. This reaction also has the advantage of convenient characteristics because dichloromethane (CH$_2$Cl$_2$) is simultaneously used as a coupling agent and solvent in the presence of KOH and/or NaH. In our previous work, these two coupling reactions have been successfully used as an efficient cyclization method to cyclic polymers, which have been confirmed to have versatility and potential applications in polymer science.

Herein, considering the above limitations in the synthesis, self-assembly behaviour and applications of multiblock copolymers, a series of novel multiblock copolymers, (PEO-b-PS-b-PEO-Dyne)$_n$, (PEO-b-PS-b-PEO-(OH)$_n$, and (PEO-b-PS-b-PEO-Acetal)$_n$, with the same compositions but different linkages were first realized by controlled living anionic polymerization (LAP) and ring-opening polymerization (ROP) mechanisms, and the efficient Glaser reaction, thiol-yne reaction and Williamson reaction were also adopted (Scheme 1). Subsequently, the micelles formed from these multiblock copolymers were also investigated and used for the targeted delivery of a fluorescent probe. The effects of the linkages on the self-assembly and the delivery behaviour were investigated and compared.

### Experimental

#### Materials

Styrene [St, 99%, Sinopharm Chemical Reagent Co. (SCR)] was washed with 10% NaOH aqueous solution, followed by water three times successively. It was then dried over anhydrous MgSO$_4$ for 24 h and further dried over CaH$_2$, and then distilled under reduced pressure before use. Ethylene oxide (EO, 99%, SCR) was dried over CaH$_2$ and then distilled before use. Naphthalene (AR, SCR) was purified by sublimation. Tetrahydrofuran (THF, 99%, SCR) was refluxed and distilled from potassium naphthalenide solution. Propargyl bromide (99%, Aldrich), dichloromethane (CH$_2$Cl$_2$), and cyclohexane were purified by direct distillation from CaH$_2$. n-Butyllithium (n-Bu”Li”, 1.6 M in hexane, J&K), 2-mercaptoethanol (ME, 98%, Aldrich), N,N-dimethylformamide (DMF), N,N,N′,N′-tetramethylethlenediamine (TMEDA, 98%, Aldrich), sodium hydride (NaH, 60% dispersion in mineral oil, SCR), 2,2-dimethoxy-2-phenylacetophenone (DMPA), 1,1′-diodo-3,3,3′,3′-tetramethyldiindocarbocyanine iodide (DiR iodide, Aladdin) and cuprous chloride (CuCl, 99%, SCR) were used as received. N-Phenyl-1-naphthylamine (PNA, Alfa Aesar, 97%) was purified by recrystallization in ethanol three times. Diphenylmethyliotassium (DPMK) with a concentration of 0.75 mol L$^{-1}$ was prepared as described elsewhere. The initiator of lithium naphthalenide was prepared from naphthalene and lithium according to ref. 42, and the concentration of 0.75 mol L$^{-1}$ was analyzed by titration using hydrochloric acid (0.10 mol L$^{-1}$). All other reagents and solvents were purchased from SCR and were used as received except for when declared.

#### Measurements

Gel permeation chromatographic (GPC) analysis of the polymers was performed in THF at 35 °C with an elution rate of 1.0 mL min$^{-1}$ on an Agilent 1260 equipped with a G1310A pump, a G1362A refractive index detector, and a G1314A variable wavelength detector. One 5 μm LP gel column (500 Å, molecular range 500-2 × 10$^4$ Da) and two 5 μm LP gel mixed
bed columns (molecular range 200–3 × 10^6 Da) were calibrated using PS standards. The absolute molecular weights of the polymers were determined by GPC measurements through three Waters Styragel columns (pore size 10^2, 10^3 and 10^4 Å) calibrated using PS standards, and equipped with three detectors: a DAWN H ELEOS (14–154°) (Wyatt multi-angle laser light scattering detector, He–Ne 632.8 nm), a ViscoStar (Wyatt), and an Optilab rEX (Wyatt). ¹H NMR spectra were recorded on a Bruker (500 MHz) spectrometer in CDCl₃ with tetramethylsilane (TMS) as the internal standard. The MALDI-TOF MS measurements were performed using a Perspective Biosystem Voyager-DE STR MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometer (PE Applied Biosystems, Framingham, MA). The instrument was equipped with a nitrogen laser emitting at 337 nm with a 3 ns pulse width, working in the positive mode. A matrix solution containing dithranol (20 mg mL⁻¹), polymer (10 mg mL⁻¹) and a cationizing salt of silver trifluoroacetate (10 mg mL⁻¹) in THF mixed in the ratio of matrix:cationizing salt:polymer = 10 : 1 : 2 was prepared, and 0.8 µL of the mixed solution was deposited on the sample holder (well-plate). The sizes of the polymeric micelles in water were measured on a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS90, Malvern) at 25 °C. A He–Ne laser (633 nm, 4 mW) was used to detect the scattering, and the detection angle was 90°. Transmission electron microscopy (TEM) images of the micelles were obtained by using a Tecnai G2 F20 S-Twin electron microscope. Steady-state fluorescence (FLS) spectra of the samples were recorded on an Edinburgh Instruments 920 spectrometer. The emission intensity at 418 nm was recorded to determine the critical micelle concentration (cmc), where λ_ex was 340 nm. Fluorescence images were collected using a Clairvivo OPT (SHIMADZU Corporation, Kyoto, Japan) with a 735 nm single laser, and the exposure time was 5 s for each image.

### Synthesis of triblock copolymer HO-PEO-b-PS-b-PEO-OH

In order to achieve the triblock copolymer HO-PEO-b-PS-b-PEO-OH, the difunctional α,ω-hydroxyl polystyrene (HO-PS-OH) precursor was first prepared (Scheme 2). According to our previous work,²² the precursor HO-PS-OH was synthesized by LRP of St monomers initiated by lithium naphthalenide and a subsequent end-capping reaction with excess ethylene oxide agent. In a typical procedure, cyclohexane (700 mL), styrene (50.0 mL, 0.43 mol) and THF (6.0 mL) were sequentially introduced into a 1.0 L ampoule. After the trace impurities in the ampoule were consumed by n-BuLi, the lithium naphthalenide solution (35.0 mL, 24.9 mmol) was added rapidly. Subsequently, the system was stirred at 25 °C for 1.0 h, and the EO agent (6.30 mL, 125 mmol) was injected to cap the living –Li⁺ species. The formed –O’Li⁻ species was finally terminated with acidic methanol (0.1 mol L⁻¹ HCl in CH₂OH). After all the solvents were evaporated, the product was recovered by precipitation into methanol three times and then it was dried under vacuum at 45 °C until a constant weight was achieved. Yield: 44.8 g (98%). ¹H NMR (CDCl₃) δ (ppm): 1.07–2.15 (C₆H₅CH(CH₃)), 3.23–3.46 (–CH₂OH), 6.30–7.23 (–C₆H₅). M₁,GPC = 3000 g mol⁻¹, PDI = 1.09, M₁(MALDI-TOF MS) = 2500 g mol⁻¹.

Using the above HO-PS-OH as a macroinitiator, the triblock copolymer HO-PEO-b-PS-b-PEO-OH was prepared by ROP of EO monomers (Scheme 2). Typically, the dry HO-PS-OH (10.0 g, 3.0 mmol) was dissolved in 200 mL of THF, which was added into a 500 mL dry ampoule. The calculated DPMK solution (4.0 mL, 3.0 mmol) was added dropwise using a syringe under magnetic stirring. Then, the ampoule was placed into an ice bath and the cold EO (25.0 mL, 0.49 mol) monomer was added quickly. The solution was heated to 60 °C and stirred for 96 h. Finally, the reaction was terminated by acidic methanol (0.1 mol L⁻¹ HCl in CH₂OH). After the solvents were evaporated, the copolymers were precipitated into cold petroleum ether (30–60 °C) slowly three times and then they were dried under vacuum at 45 °C until a constant weight was achieved. Yield: 29.8 g (95%). ¹H NMR (CDCl₃) δ (ppm): 3.46–3.83 (–C₆H₅CH(CH₃O)₂), 6.33–7.23 (–C₆H₅). M₁,GPC = 11 200 g mol⁻¹, PDI = 1.10, M₁,MMR = 9300 g mol⁻¹.

### Synthesis of multiblock copolymer (PEO-b-PS-b-PEO-Diyne)ₙ by the Glaser coupling reaction

The multiblock copolymer (PEO-b-PS-b-PEO-Diyne)ₙ was prepared using the functional Propargyl-PEO-b-PS-b-PEO-Propargyl with terminal propargyl groups at both ends as the precursor (Scheme 3). Typically, the precursor Propargyl-PEO-b-PS-b-PEO-Propargyl was synthesized by modifying the hydroxyl groups on HO-PEO-b-PS-b-PEO-OH with propargyl bromide. First, HO-PEO-b-PS-b-PEO-OH (3000 g mol⁻¹, 30.0 g, 10.0 mmol) was added into a 500 mL round bottom flask and dried by azotropic distillation with toluene. After HO-PEO-b-PS-b-PEO-OH was again dissolved in dry THF (300 mL), NaH (2.4 g, 100.0 mmol) was added in three batches. Then, the system was placed into an ice bath, propargyl bromide (3.0 mL, 38.3 mmol) was added dropwise over 2.0 h and the reaction was allowed to continue for another 48 h. Finally, the THF solvent was removed by evaporation, and the product was extracted with CH₂Cl₂. The organic layer was then dried over...
MgSO₄ before purification by precipitation into cold petroleum ether (30 °C–60 °C) three times. The obtained Propargyl-PEO-b-PS-b-PEO-Propargyl was dried under vacuum at 45 °C until a constant weight was achieved. ¹H NMR (CDCl₃) δ (ppm): 2.45 (–C≡CH), 3.47–3.80 (–OCH₂CH₂O–), 4.20 (–OCH₂C≡CH), 6.33–7.23 (–C₆H₅). Mₙ,NMR = 9400 g mol⁻¹.

For the multiblock copolymer (PEO-b-PS-b-PEO-Diyne)ₙ with 1,3-diyn groups as linkages (Scheme 3), Propargyl-PEO-b-PS-b-PEO-Propargyl (6.0 g, 2.0 mmol), pyridine (75 mL), CuCl (0.32 g, 3.18 mmol), and TMEDA (1.60 mL, 10.49 mmol) were sequentially added into a 250 mL round bottom flask. Then, the flask was filled with oxygen and maintained at 25 °C for 120 h. Finally, the solution was concentrated and the crude product was purified by passing it through a neutral alumina column using CH₂Cl₂ as the eluent to remove the copper catalyst. The product was precipitated into cold petroleum ether (30 °C) three times, the obtained (PEO-b-PS-b-PEO-Diyne)ₙ was dried under vacuum at 45 °C until a constant weight was achieved. ¹H NMR (CDCl₃) δ (ppm): 3.06 (–SCH₂CH₂OH), 3.92 (–SCH₂CH₂OH), 3.34–3.43 (–CH₂CH(S)CH–), 3.45–3.83 (–OCH₂CH₂O–), 6.33–7.27 (–C₆H₅).

Synthesis of multiblock copolymer (PEO-b-PS-b-PEO-Acetal)ₙ by a Williamson reaction

The multiblock copolymer (PEO-b-PS-b-PEO-Acetal)ₙ with acetal groups as linkages was prepared by a Williamson reaction using the above HO-PEO-b-PS-b-PEO-OH as a precursor (Scheme 4). Typically, the precursor HO-PEO-b-PS-b-PEO-OH (0.50 g, 0.04 mmol), dried by azetropic distillation with toluene, was dissolved in 11.0 mL of dry CH₂Cl₂ in a 50 mL round bottom flask. Then, NaH (0.10 g, 4.17 mmol) and potassium hydroxide (KOH, 1.00 g, 17.86 mmol) were added, and the reaction was carried out at reflux temperature for 72 h. After the reaction system was evaporated and extracted with CH₂Cl₂/H₂O three times, the CH₂Cl₂ phase was concentrated and precipitated into cold petroleum ether (30–60 °C) three times. The obtained (PEO-b-PS-b-PEO-Acetal)ₙ was dried under vacuum at 45 °C until a constant weight was achieved. ¹H NMR (CDCl₃) δ (ppm): 3.47–3.80 (–OCH₂CH₂O–), 4.65–4.80 (–OCH₂O–), 6.35–7.23 (–C₆H₅). Mₙ,GPC = 64 000 g mol⁻¹, PDI = 1.40.

Determination of the critical micelle concentration (cmc)

According to the literature,⁴⁸ PNA was used as a fluorescent probe to measure the cmc values of the copolymers. First, 50 µL of acetone solution of PNA (0.001 mol L⁻¹) was added into 25 mL of water to give a PNA concentration of 2 × 10⁻³ mmol L⁻¹. Then, different amounts of the copolymer solutions in THF were added into the above water containing PNA ([PNA] = 2× 10⁻³ mmol L⁻¹), and the concentrations of the block copolymer were finally modulated from 5.0 × 10⁻⁵ to 7.0 × 10⁻² mg mL⁻¹ for FLS measurements. All fluorescence spectra were recorded at 25 °C.

Observation of the micellar morphologies of the copolymers with different linkages

The micelles formed from copolymers HO-PEO-b-PS-b-PEO-OH, (PEO-b-PS-b-PEO-Diyne)ₙ, [PEO-b-PS-b-PEO-(OH)]ₙ, and [PEO-b-PS-b-PEO-Acetal]ₙ were prepared according to the following procedure. The copolymer (40 mg) was first dissolved in 5.0 mL of acetone, and then this solution was added dropwise into 6.0 mL of deionized water under stirring. Subsequently, the mixture was evaporated under reduced pressure.
to remove the acetonitrile solvent. Finally, an ultrathin carbon network was dipped into the obtained aqueous solution of the copolymer, and the samples were subjected to a freeze drying procedure. The TEM images were obtained at 200 kV on a Tecnai G2 F20 S-Twin electron microscope.

**Study on the in vivo distribution of the micelles from the copolymers with different linkages**

The micelles containing the DiR agent were prepared using a similar procedure as that described above, except that the DiR agent was first added and dispersed in the acetonitrile phase of the copolymers before their addition into the deionized water. Typically, 40 mg of copolymer was first dissolved in 5.0 mL of acetonitrile, and then 20 µL of the DiR agent (10 mg mL⁻¹ in ethanol) was added into the organic phase and vortexed. Subsequently, the organic phase was added dropwise into 6.0 mL of deionized water under stirring. After the acetonitrile solvent was removed by evaporation, only water remained and the DiR loaded micelles (33.3 µg mL⁻¹) were obtained.

The obtained micelles were then used to study the in vivo distribution of the copolymers. Typically, the nude mice were intravenously injected with 200 µL of the DiR loaded micelles. After 4.0 h, the mice were anesthetized using isoflurane in oxygen and put into a chamber. For the in vivo imaging, the mice were sacrificed by cervical dislocation, and the major organs including the hearts, livers, spleens, lungs, and kidneys were excised. The fluorescence images of the tissues were obtained and the near-infrared fluorescence signal intensities in different tissues were measured.

All animal experiments were conducted in accordance with the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985) and approved by the ethics committee of Fudan University.

**Results and discussion**

**Synthesis and characterization of copolymer HO-PEO-b-PS-b-PEO-OH**

In order to synthesize multiblock copolymers with as many PS and PEO segments as possible, the triblock copolymer precursor HO-PEO-b-PS-b-PEO-OH with defined terminal functionality and controlled compositions should first be prepared, and the formation of possible diblock copolymer PEO-b-PS and homopolymer PS should be largely avoided. Thus, the sequential LAP and ROP mechanisms with excellent controllability were adopted to synthesize the triblock copolymer HO-PEO-b-PS-b-PEO-OH.

First, the difunctional living “Li–PS–Li” species, grown from lithium naphthalenide, was carefully end-capped with the EO agent. Once the EO agent (eight-fold to the living species) was added into the red “Li–PS–Li” solution, one could observe that the color of the system changed to light yellow immediately, which meant that the alkoxides (−O–Li⁻) were formed. Furthermore, the high degree of aggregation of lithium alkoxides compared to other alkali metal alkoxides in the hydrocarbon media rendered them unreactive towards further oligomerization of the EO agent at the chain end. Additionally, different from the previous work on the functionalization of poly(styryl) lithium (PS‘Li⁻) with propylene oxide,⁴⁹ styrene oxide,⁵⁰ 1-buteneoxide,⁵¹ 3,4-epoxy-1-butene⁵² and ethoxylated glycidyl ether⁵³ agents, the EO agent without any substituted groups tends to result in fewer side reactions and provides a high functionalization efficiency on the living species, and HO–PS–OH with a high purity was obtained.

The successful LAP of St monomers and the subsequent end-capping reaction was evidenced by the monomodal peak and the symmetrical GPC curve with a narrow molecular weight distribution (PDI = 1.09) (Fig. 1A). The ¹H NMR spectrum of the synthesized HO-PS-OH was measured and it is shown in Fig. 2. Except for the characteristic resonance signals of the aromatic protons (−C₆H₅) on the PS segment at 6.29–7.25 ppm, the resonance signal appearing at 3.23–3.46 ppm, corresponding to protons (−CH₂OH⁻), confirmed the successful introduction of the EO agents onto the PS end. As a direct and efficient method, HO-PS-OH was also investigated by MALDI-TOF MS and the accurate information was collected (Fig. 3). The peak at m/z = 2487.7 Da is attributed to HO-PS-OH [HOCH₂CH₂(C₆H₅)₂−CH₂CH₂OH·Ag⁺], and the spacing between adjacent peaks is 104.0 Da, which is the mass of a St unit. In the whole and expanded MALDI-TOF mass spectrum, the absence of any sub-peaks confirmed that only the functionalized HO-PS-OH was actually present.

Then, the triblock copolymer HO-PEO-b-PS-b-PEO-OH was prepared by ROP of EO monomers using HO-PS-OH as the macroinitiator. From Fig. 1B, it can be observed that the GPC trace of the obtained HO-PEO-b-PS-b-PEO-OH has a monomodal peak and a narrow PDI. The composition of the triblock copolymer was further verified by ¹H NMR spectroscopy. As shown in Fig. 4A, the characteristic resonance signals of the aromatic protons (−C₆H₅) on the PS segment at 6.33–7.23 ppm and the methylene protons (−CH₂CH₂O⁻) on the PEO segment at 3.45–3.83 ppm were all well discriminated. Thus, HO-PEO-b-PS-b-PEO-OH has been successfully synthesized and its structure is confirmed.

![GPC traces of polymers HO-PS-OH (A, Mₙ = 3000 g mol⁻¹, PDI = 1.09), HO-PEO-b-PS-b-PEO-OH (B, Mₙ = 11200 g mol⁻¹, PDI = 1.10), (PEO-b-PS-b-PEO-Diyne)₃ (C, Mₙ = 62 500 g mol⁻¹, PDI = 1.55), and (PEO-b-PS-b-PEO-Acetal)₃ (D, Mₙ = 64 000 g mol⁻¹, PDI = 1.40).](image-url)
Synthesis and characterization of multiblock copolymers (PEO-b-PS-b-PEO-Diyne)_n, [PEO-b-PS-b-PEO-(OH)]_n and [PEO-b-PS-b-PEO-Acetal]_n

The functional Propargyl-PEO-b-PS-b-PEO-Propargyl was prepared by end group transformation of HO-PEO-b-PS-b-PEO-OH with propargyl bromide in the presence of NaH. From the 1H NMR spectrum of Propargyl-PEO-b-PS-b-PEO-Propargyl (Fig. 4B), the characteristic resonance signals attributed to the alkynyl proton (–C≡CH) and methylene protons (–OCH₂C≡CH) on the propargyl group were observed at 2.43 and 4.21 ppm, respectively.

As mentioned in the previous section, the Glaser coupling reaction between alkyne groups has been widely used in polymer science, and it results in good yields at room temperature and under an air atmosphere.41,42 Herein, the efficient Glaser coupling reaction was again used for our multiblock copolymer (PEO-b-PS-b-PEO-Diyne)_n. The GPC trace of the coupled product with a broad PDI is shown in Fig. 1C. It is in accordance with the characteristics of a step-growth polymerization mechanism. Also, the absolute molecular weight (Mₘ,MALLS = 110 000 g mol⁻¹) of (PEO-b-PS-b-PEO-Diyne)_n could be measured by the GPC instrument equipped with a multi-angle laser light scattering detector. From the above Mₘ,MALLS and the molecular weight (Mₙ,NMR = 9400 g mol⁻¹) of Propargyl-PEO-b-PS-b-PEO-Propargyl, the degree of the Glaser coupling (DG) reaction could be calculated as 11.7. On the other hand, different from the 1H NMR spectrum of Propargyl-PEO-b-PS-b-PEO-Propargyl, there was almost no obvious change in the 1H NMR spectrum except that the signal of the alkynyl protons (–C≡CH) at 2.43 ppm disappeared from the 1H NMR spectrum of (PEO-b-PS-b-PEO-Diyne)_n (Fig. 5A). Thus, the GPC trace and the 1H NMR spectrum comprehensively confirmed that the Glaser coupling reaction was successful.
resistant probe in terms of reproducibility because it has a high fluorescence activity in nonpolar environments, and it can be very easily quenched by polar solvents, such as water. The relationship of the fluorescence intensity ratio ($I_{\text{PS}}/I_{\text{OH}}$) of PNA as a function of the concentration of the copolymer aqueous solution is plotted in Fig. 7. It was found that $I_{\text{PS}}/I_{\text{OH}}$ increased sharply when the concentration exceeded a certain value, which proved that the PNA probe was incorporated into the hydrophobic core of the micelles. Therefore, the intersection of the two straight lines with a value of $3.26 \times 10^{-3} \text{mg mL}^{-1}$ was determined to be the cmc of the triblock copolymer HO-PEO-$b$-PS-$b$-PEO-OH. Similarly, the cmc values of the multiblock copolymers with the same compositions but different linkages were measured and were found to have increased cmc values, i.e., $6.48 \times 10^{-3} \text{mg mL}^{-1}$ for [PEO-$b$-PS-$b$-PEO-Diyne]$_n$, $5.56 \times 10^{-3} \text{mg mL}^{-1}$ for [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, and $5.77 \times 10^{-3} \text{mg mL}^{-1}$ for [PEO-$b$-PS-$b$-PEO-Acetal]$_n$.

Subsequently, [PEO-$b$-PS-$b$-PEO-(OH)]$_n$ was obtained by an efficient thiol–yne addition reaction between the 1,3-dyne structures and excess 2-mercaptoethanol, with DMF used as the solvent and DMPA used as the photoinitiator under 254 nm UV irradiation. From the $^1$H NMR spectrum of [PEO-$b$-PS-$b$-PEO-(OH)]$_n$ (Fig. 5B), the characteristic resonance signal attributed to the methylene protons (–OCH$_2$C≡C–) connected to the triple bond disappeared completely, which confirmed that all the 1,3-dyne groups were successfully transformed into hydroxyl groups.

Alternatively, based on the high efficiency and advantages of the Williamson reaction, a high molecular weight multiblock copolymer (PEO-$b$-PS-$b$-PEO-Acetal)$_n$ was also synthesized by a coupling reaction between the active hydroxyl groups at the end of HO-PEO-$b$-PS-$b$-PEO-OH. From the GPC result for (PEO-$b$-PS-$b$-PEO-Acetal)$_n$, a monomodal peak was also achieved. From the $^1$H NMR spectrum for (PEO-$b$-PS-$b$-PEO-Acetal)$_n$ (Fig. 6), except for the characteristic resonance signals for the methylene protons at 3.47–3.80 ppm (–CH$_2$CH$_2$O–) on the PEO segment and at 6.35–7.23 ppm (–C$_6$H$_5$) on the PS segment, the signals of the newly formed acetal protons (–OCH$_2$O–) after coupling were also observed (4.65–4.80 ppm), which showed that the Williamson reaction was successful.

Investigation of the micellar morphologies of the multiblock copolymers with different linkages

The amphiphilic polymers consisting of hydrophilic and hydrophobic segments can self-assemble into core–shell structures in selective solvents, such as water. Herein, the micelles formed from the multiblock copolymers (PEO-$b$-PS-$b$-PEO-Diyne)$_n$, [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, (PEO-$b$-PS-$b$-PEO-Acetal)$_n$, and the precursor HO-PEO-$b$-PS-$b$-PEO-OH were all examined by FLS, DLS and TEM measurements, and the results were compared.

The cmc values of the multi- or triblock copolymers in the aqueous phase were first determined by a fluorescence technique using PNA as a probe. Typically, PNA is a suitable fluorescent probe in terms of reproducibility because it has a high fluorescence activity in nonpolar environments, and it can be very easily quenched by polar solvents, such as water. The relationship of the fluorescence intensity ratio ($I_{\text{PS}}/I_{\text{OH}}$) of PNA as a function of the concentration of the copolymer aqueous solution is plotted in Fig. 7. It was found that $I_{\text{PS}}/I_{\text{OH}}$ increased sharply when the concentration exceeded a certain value, which proved that the PNA probe was incorporated into the hydrophobic core of the micelles. Therefore, the intersection of the two straight lines with a value of $3.26 \times 10^{-3} \text{mg mL}^{-1}$ was determined to be the cmc of the triblock copolymer HO-PEO-$b$-PS-$b$-PEO-OH. Similarly, the cmc values of the multiblock copolymers with the same compositions but different linkages were measured and were found to have increased cmc values, i.e., $6.48 \times 10^{-3} \text{mg mL}^{-1}$ for (PEO-$b$-PS-$b$-PEO-Diyne)$_n$, $5.56 \times 10^{-3} \text{mg mL}^{-1}$ for [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, and $5.77 \times 10^{-3} \text{mg mL}^{-1}$ for (PEO-$b$-PS-$b$-PEO-Acetal)$_n$. Obviously, the triblock copolymer HO-PEO-$b$-PS-$b$-PEO-OH gave the lowest cmc value, and the multiblock copolymers [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, and (PEO-$b$-PS-$b$-PEO-Acetal)$_n$, showed modest and almost the same cmc values. However, (PEO-$b$-PS-$b$-PEO-Diyne)$_n$, was endowed with the highest cmc value. These cmc values preliminarily reflect the fact that the topologies and compositions actually had some effect on the self-assembly behaviour.

Also, the micellar morphology was monitored by TEM measurements. As the TEM images in Fig. 8 show, all the samples formed spherical core–shell micelles. The precursor of the triblock copolymer HO-PEO-$b$-PS-$b$-PEO-OH formed the largest size of micelle, while the multiblock copolymer (PEO-$b$-PS-$b$-PEO-Diyne)$_n$ gave the smallest size of micelle. The multiblock copolymers [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, and (PEO-$b$-PS-$b$-PEO-Acetal)$_n$, tend to aggregate into medium sized micelles. The DLS measurements also gave the same derivation of micelle sizes (Fig. 9). In detail, the average sizes of the micelles formed from the samples HO-PEO-$b$-PS-$b$-PEO-OH, (PEO-$b$-PS-$b$-PEO-Diyne)$_n$, [PEO-$b$-PS-$b$-PEO-(OH)]$_n$, and (PEO-$b$-PS-$b$-PEO-Acetal)$_n$, were 273 nm, 24 nm, 40 nm and 34 nm, respectively.

The three multiblock copolymer samples have the same compositions (molar ratio of EO to St units), but they are embedded with different linkages. The acetal and –OH$_4$ linkages tend to give the copolymers a hydrophilic characteristic (similar to the contribution of the hydrophilic EO unit), while the 1,3-dyne linkages tend to bring some hydrophobic characteristic to the copolymers. During the formation of the micelles from multiblock copolymer (PEO-$b$-PS-$b$-PEO-Diyne)$_n$, except for the hydrophobicity brought by the PS segment, the 1,3-dyne linkage would also contribute a certain amount of hydrophobicity. Thus, as with the proposed mechanism for spherical micelles, due to the high solubility of the PEO segment in water, the hydrophobic dyne linkage embedded into the polymer chain can not be completely buried in the hydrophobic core, but appears to be oriented towards the hydrophilic corona. Also, because of the hydrophobicity of the 1,3-dyne groups, the geometric tension of PEO around the core was reduced by the 1,3-dyne groups in the middle of the
PEO segment (Scheme 5). Thus, the overall effect of the hydrophilicity and hydrophobicity of (PEO-\(b\)-PS-\(b\)-PEO-Diyne)\(_n\) gave a small size of micelles. As for the multiblock copolymers [PEO-\(b\)-PS-\(b\)-PEO-(OH)\(_4\)]\(_n\) and (PEO-\(b\)-PS-\(b\)-PEO-Acetal)\(_n\), the embedded hydrophilic groups –OH\(_4\) and acetal groups have a similar hydrophilicity to that of the EO units, which would lead to a
negligible effect on the micellization (Scheme 5). Additionally, compared with the above multiblock copolymers, the precursor HO-PEO-b-PS-b-PEO-OH gave the largest size of micelles, which again confirmed that the topology actually exerts an important influence on the self-assembly behaviour.

Investigation of the in vivo distribution of the micelles formed by the multiblock copolymers with different linkages

When polymeric micelles are used in the biomedical field, the micellar carrier is hoped to contribute to the effective treatment of brain disease or diagnosis in some cases.\(^{58}\) However, brain-targeted delivery of drugs or agents is usually hard to achieve due to the infiltrative nature of the blood–brain barrier (BBB).\(^{59-61}\) The BBB protects foreign organisms and noxious chemicals by the highly strengthened endothelial wall of the vasculature system and controls the passage of drugs from the blood into the brain. Thus, one of the major obstacles for brain targeted delivery is to overcome the BBB effect.\(^{62-65}\) Correspondingly, the screening of polymeric micelles with an applicable size and morphology might be the key task. Thus, the in vivo distributions of the micelles formed from the synthesized multiblock copolymers were also investigated and compared to screen for an optimized system.

Generally, the administration in vivo was carried out by intravenous injection, and the maximum distribution in the target nude mice was traced 4 h after the injection (Fig. 10). The distribution of the materials was detected by measuring the fluorescent probe loaded in the micelles.\(^{66,67}\) The in vivo results of the mice revealed that the multiblock copolymer (PEO-b-PS-b-PEO-Diyne)\(_n\) was instantaneously transported through the BBB and was highly accumulated at the brain within 4 h, which demonstrated that the copolymer (PEO-b-PS-b-PEO-Diyne)\(_n\) might have a therapeutic application in the treatment of brain disease. On the contrary, the accumulation of the micelles formed from the copolymers [PEO-b-PS-b-PEO-(OH)\(_4\)]\(_n\), (PEO-b-PS-b-PEO-Acetal)\(_n\), and HO-PEO-b-PS-b-PEO-OH was lower at the brain. According to the above difference in the self-assembly behaviour between several multi and triblock copolymers, one can speculate that the excellent permeability of (PEO-b-PS-b-PEO-Diyne)\(_n\) is mainly due to the presence of the hydrophobic linkages of the 1,3-diyne group and the multiblock structure, which collaboratively gives an appropriate size of micelle and can overcome the BBB effect, and therefore it may be an ideal drug delivery system to the brain. Thus, the simple modification of certain linkages could be used to modulate the size of the micelles and further control their in vivo distribution, which might bring some important
reference to the application of multiblock copolymers in the biomedical field.

Conclusions

The multiblock copolymers \([\text{PEO-b-PS-b-PEO-Diyne}_n]\), \([\text{PEO-b-PS-b-PEO-(OH)}_n]\), and \([\text{PEO-b-PS-b-PEO-Acetal}_n]\) with the same compositions but different linkages were realized by controlled LAP and ROP mechanisms, and the efficient Glaser reaction, thiol-yne reaction and Williamson reaction were also adopted. The micellar morphologies of the micelles formed from the synthesized copolymers were also investigated by DLS and TEM measurements and compared. The formed micelles were further used to load a fluorescent probe to study their in vivo distribution. Under the same conditions, the precursor triblock copolymer HO-PEO-b-PS-b-PEO-OH formed the largest size of micelle, while the multiblock copolymer \([\text{PEO-b-PS-b-PEO-Diyne}_n]\) gave the smallest size of micelle, and the multiblock copolymers \([\text{PEO-b-PS-b-PEO-(OH)}_n]\) and \([\text{PEO-b-PS-b-PEO-Acetal}_n]\) tend to aggregate into medium sized micelles. The hydrophobic 1,3-diyne groups exert some important effects on the self-assembly behaviour of multiblock copolymer \(\text{PEO-b-PS-b-PEO-Diyne}_n\), resulting in the smallest size of micelles, which can traverse the BBB and might have a therapeutic application in the treatment of brain disease. Thus, we can conclude that the topologies of multiblock copolymers, even the simple modification of certain linkages, actually exert some influences on the self-assembly behavior and the possible targeted delivery, which might bring some important reference to the application of multiblock copolymers in the biomedical field.

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Notes and references
