

Covalently stabilized temperature and pH responsive four-layer nanoparticles fabricated from surface ‘clickable’ shell cross-linked micelles

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Alkynyl-terminated double hydrophilic ABC triblock copolymer, poly(oligo(ethylene glycol) monomethyl ether methacrylate)-*b*-poly(2-(dimethylamino) ethyl methacrylate)-*b*-poly(2-(diethylamino) ethyl methacrylate) (*alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA), was synthesized *via* atom transfer radical polymerization (ATRP) by sequential monomer addition using propargyl 2-bromoisobutyrate (P_gBiB) as the initiator. The obtained triblock copolymer dissolves molecularly in acidic media and self-assembles into alkynyl surface-functionalized three-layer “onion-like” micelles consisting of a PDEA core, a PDMA inner shell, and a POEGMA corona at alkaline pH. Selective cross-linking of the PDMA inner shell with 2-bis(2-iodoethoxy)ethane (BIEE) results in structurally stable and surface ‘clickable’ shell cross-linked (SCL) micelles with pH-responsive PDEA cores. This new kind of SCL micelles could be further surface functionalized or conjugated with other azido-terminated polymer chains, functional groups, or biomolecules *via* Click chemistry. Four layer nanoparticles (SCL-PNIPAM) which have pH-responsive PDEA cores and temperature responsive PNIPAM outer coronas were fabricated from surface ‘clickable’ shell cross-linked (SCL) micelles and azide-terminated poly(*N*-isopropylacrylamide) (PNIPAM-*N*₃) using Click chemistry. These novel four layer nanoparticles might act as suitable nano-sized drug delivery vehicles for the encapsulation and release of hydrophobic drugs as a function of either temperature or pH of the environment

Introduction

Block copolymers can self-assemble in selective solvents into mesophases with various morphologies such as micelles or vesicles.^{1–6} In order to enhance the structural stability of block copolymer micelles and broaden their potential applications, non-covalent and covalent stabilization of these assembled structures have been developed. The covalent stabilization strategy mainly involves core cross-linking^{7–12} and shell cross-linking.^{13–21}

Shell cross-linked (SCL) micelles were originally reported by Wooley *et al.* from the self-assembly of amphiphilic block copolymers and cross-linking.^{15,16,21} Armes *et al.* then applied this principle to the structural fixation of stimuli-responsive double hydrophilic block copolymer (DHBC) micelles.^{17,18} They also developed the triblock copolymer approach for the preparation of SCL micelles at high solids, taking advantage of steric stabilization of the soluble block located in the outer coronas of three-layer “onionlike” micelles.^{19,22–24}

Recent progress in this field includes surface functionalization of SCL micelles. Extensive work has been done by the Wooley research group, focusing on the introduction of bioactive moieties such as peptide,^{25–27} folate,²⁸ and saccharide²⁹ at the

periphery of SCL micelles. Two primary synthetic strategies have been established for the preparation of these functionalized SCL micelles and these involve either the use of a functionalized initiator species together with a mixed-micelle assembly or a secondary functionalization step after formation of the nanoparticles. The first functionalized initiator strategy has the advantage of controlling the degree of surface coverage by the functional groups. However, for each new functional group that was used, a new functionalized initiator has to be created. Moreover, during the preparation of the polymers and SCL micelles, complicated protection/deprotection chemistry has to be considered, thus limiting the practical application of this strategy. As for the latter post-preparation functionalization strategy, the one major drawback is the difficulty in controlling the efficiency of incorporating the functional groups into the hydrophilic shell of SCL micelles. Therefore, a more versatile, reliable, and robust strategy for the functionalization of SCL micelles should be developed.

In the past few years, Click chemistry has been employed to synthesize and functionalize various polymers and dendrimers based on the characteristics quantitative yields, easily isolated products and readily accessible starting materials.^{30–36} Click chemistry is also tolerant to a range of functional groups and the reaction can be conducted both in organic and aqueous media.³⁰ Therefore, Click chemistry has the potential to overcome all of the above problems for the functionalization of SCL micelles. Recently, many interesting SCL micelles with Click-reactive azide and alkynyl functional groups within the shell surface or the cores have been studied by Wooley’s group.^{37–41} However, the polymers involved were amphiphilic diblock copolymers and

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organic solvent was used during the preparation of the stabilized nanoparticles which limits their potential applications in biomedicine. The preparation of SCL micelles with surface Click-reactive functional groups based on double hydrophilic triblock copolymers in purely aqueous solutions should therefore be an interesting and fascinating approach.

Herein, we describe the preparation of novel four-layer SCL micelles *via* Click chemistry between surface functionalized SCL micelles formed from an alkynyl-terminated double hydrophilic ABC triblock copolymer (DHBC), poly(oligo(ethylene glycol) methyl ether methacrylate)-*b*-poly(2-(dimethylamino)ethyl methacrylate)-*b*-poly(2-(diethylamino)ethyl methacrylate) (*alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA) and an azido-terminated PNIPAM homopolymer (PNIPAM- N_3). The obtained PNIPAM functionalized four-layer SCL micelles (SCL-PNIPAM) with thermo-sensitive outer PNIPAM corona and pH-responsive inner PDEA core were investigated in detail. To the best of our knowledge, this is the first report concerning the preparation of four-layer SCL micelles *via* Click chemistry starting from an alkynyl-terminated pH-responsive DHBC and well-defined thermo-responsive homopolymer.

Experimental

Materials

Poly(ethylene glycol) monomethyl ether methacrylate (OEGMA, $M_n = 475$ g/mol, Aldrich) was passed through an alumina column to remove the inhibitor. 2-(Dimethylamino)ethyl methacrylate (DMA, 99%, Aldrich) and 2-(diethylamino)ethyl methacrylate (DEA, 99%, Aldrich) were dried with calcium hydride, vacuum-distilled, and stored at -20 °C prior to use. *N*-Isopropylacrylamide (NIPAM, 97%, Tokyo Kasei Kagyo Co.) was recrystallized from a mixture of benzene and *n*-hexane (1:3 (v/v)). Propargyl alcohol, sodium azide (NaN_3), 2-bromoisobutryl bromide, ethyl 2-chloropropionate (ECP), CuCl, tris(aminoethyl)amine (TREN), 2,2'-bipyridine (bpy), and 1,2-bis(2-iodoethoxy)ethane (BIEE) were purchased from Aldrich and used as received. Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) was prepared from TREN according to literature procedures.⁴² All the other chemicals were purchased from Shanghai Chemical Reagent Co. and used as received unless otherwise specified. Anhydrous dichloromethane (CH_2Cl_2) and triethylamine (TEA) were dried by calcium hydride (CaH_2) and distilled prior to use.

Synthesis of propargyl 2-bromoisobutyrate (PgBiB)

Propargyl alcohol (5.61 g, 0.1 mol) and TEA (16.7 mL, 0.12 mol) were dissolved in 100 mL anhydrous CH_2Cl_2 . The reaction mixture was cooled in an ice-water bath, and 40 mL of a CH_2Cl_2 solution of 2-bromoisobutryl bromide (14.8 mL, 0.12 mol) was added dropwise within 1 h. The reaction was quite exothermic and a white precipitate was observed during addition. After stirring overnight at room temperature, the reaction mixture was filtered to remove the precipitated ammonium salt. The filtrate was concentrated under reduced pressure, and then purified by silica gel column chromatography using CH_2Cl_2 as the eluent. The solvent was removed on a rotary evaporator, and the product was distilled under vacuum. A colorless liquid was obtained (14.35 g, yield: 70%). $^1\text{H NMR}$ analysis of the obtained

product was consistent with that reported by Matyjaszewski *et al.* which proved the successful synthesis of PgBiB initiator.⁴³

Preparation of alkynyl-terminated POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer

Alkynyl-terminated POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer was synthesized *via* ATRP by monomer addition method using PgBiB as the ATRP initiator. A typical procedure was as follows: OEGMA monomer (3.04 g, 6.4 mmol), PgBiB initiator (66 mg, 0.32 mmol), and bpy ligand (100 mg, 0.64 mmol) were added into a round-bottomed flask containing IPA (4 mL) sealed with a rubber septum, and the flask was degassed *via* two freeze-pump-thaw cycles, and then placed in an oil bath preheated at 45 °C. After ~ 5 min, CuCl (32 mg, 0.32 mmol) was added to start the polymerization under nitrogen atmosphere. The reaction solution turned dark brown and became progressively more viscous.

Aliquots were withdrawn at regular time intervals and analyzed by $^1\text{H NMR}$ and THF GPC for monitoring the degree of polymerization of OEGMA (and also DMA, DEA, see below). After 7 h, the OEGMA conversion reaches $\geq 99\%$. Degassed DMA monomer (2.52 g, 16 mmol) in 3 mL IPA was then added through a double-tipped needle under nitrogen flow. The reaction mixture turned deep brown within a few minutes after the addition. After 4 h, the DMA monomer conversion had reached about 96%. Degassed DEA monomer (3.56 g, 19.2 mmol) in 5 mL IPA solution was then transferred into the flask *via* a double-tipped needle. The third polymerization of DEA monomer was allowed to proceed for 12 h.

The polymerization was quenched by immersing the reaction flask into liquid nitrogen and exposing to air thus leading to the oxidation of the brown Cu(I) catalyst. The blue reaction mixture was then diluted with IPA and passed through a neutral alumina column to remove the copper catalysts. The solvent in the mixture was removed by rotary evaporation, and the residual monomers were eliminated by dissolving the mixture in THF and then precipitated into cold *n*-hexane (-70 °C). After discarding the supernatant solution and evaporating the solvents, the colorless and viscous solid product was obtained and dried under vacuum overnight at room temperature. The overall yield was 80%.

Synthesis of azide-terminated PNIPAM homopolymer (PNIPAM- N_3)

The synthesis of azide-terminated PNIPAM homopolymer (PNIPAM- N_3) with target degree of polymerization of 60 was as follows: NIPAM (1.36 g, 12 mmol), Me_6TREN (46 mg, 0.2 mmol), ethyl 2-chloro-propionate (27 mg, 0.2 mmol), and IPA (3 mL) were charged into a 25 mL reaction flask sealed with a rubber septum. The reaction mixture was degassed by two freeze-pump-thaw cycles and back-filled with N_2 . After heating to room temperature, CuCl (20 mg, 0.2 mmol) was introduced into the reaction flask under nitrogen atmosphere. After polymerization at room temperature for 4 h, the flask was quenched into liquid nitrogen, exposed to air, and then diluted with 10 mL IPA. After passing through a neutral alumina column to remove the copper catalysts and evaporating the solvent under reduced

pressure, the obtained solid was thoroughly washed with an excess of *n*-hexane and ethyl ether, successively, to remove the residual NIPAM monomer. The obtained product was dried under vacuum overnight at room temperature. The yield was 70%. GPC analysis of the obtained PNIPAM-Cl homopolymer revealed an M_n of 6120 g/mol and an M_w/M_n of 1.23. The actual DP of the PNIPAM block was determined to be 54 by ^1H NMR analysis in CDCl_3 . Thus the homopolymer was denoted as PNIPAM₅₄-Cl.

In order to synthesize the azide-terminated PNIPAM homopolymer, the resulting PNIPAM₅₄-Cl homopolymer (0.61 g, 0.1 mmol) and NaN_3 (26 mg, 0.4 mmol) were dissolved into 5 mL DMF in a sealed 10 mL round-bottomed flask. The solution was stirred at 45 °C for 48 h. After removing the DMF solvent under vacuum, the residue was dissolved in THF and precipitated into an excess of ethyl ether. After filtration, the obtained precipitate was further dissolved in CH_2Cl_2 and passed through a neutral alumina column to remove sodium salt and excess sodium azide. The solvent was then removed by rotary evaporation, and the final product was dried under vacuum overnight at room temperature.

Preparation of surface alkyne-functionalized micelles and shell cross-linked (SCL) micelles

The *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer was dissolved molecularly in deionized water at pH 3 and a concentration of 2.0 g/L. Micellization was induced by adjusting the solution pH to 9 with 0.1 M NaOH solution. Shell cross-linking was achieved by adding BIEE and stirring the solution for at least 3 days at room temperature to ensure effective covalent stabilization of the PDEA-core micelles. The used amount of BIEE was 50 mol% with respect to that of DMA, corresponding to a target degree of cross-linking of 100%.

Preparation of four-layer shell cross-linked (SCL) micelles with outer thermo-sensitive PNIPAM coronas via Click chemistry

PNIPAM₅₄-N₃ (0.12 g, 0.02 mmol), CuSO_4 (2 mg, 0.01 mmol), H_2O (140 mL) and sodium ascorbic acid (4 mg, 0.02 mmol) were added into a 250 mL round-bottomed flask equipped with a magnetic stir bar. The mixture was stirred at room temperature for 30 min and then transferred into a 500 mL round-bottom flask containing 140 mL aqueous solution (2.0 g/L) of alkyne-functionalized SCL micelles (0.01 mmol alkyne group). The reaction mixture stirred for 2 days at room temperature, and then dialyzed against deionized water for 3 days using presoaked dialysis membrane tubes (MW cutoff, 14 000 Da) to remove excess PNIPAM-N₃ homopolymer and copper catalyst. Lyophilization gave the PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM) as a white solid. The concentrations of all the solutions used in this study were 1.0 g/L unless otherwise specified. The fabrication of SCL-PNIPAM was as shown in Fig. 1.

Instrumentation

All ^1H NMR spectra were recorded in D_2O or CDCl_3 using a Bruker 300 MHz spectrometer. Molecular weight and molecular weight distributions were determined by THF GPC equipped with a series of three linear Styragel columns HT2, HT4, and

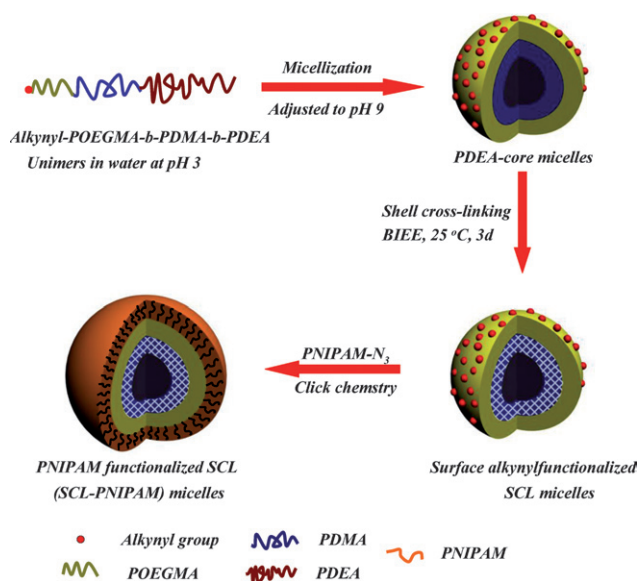


Fig. 1 Schematic illustration of the preparation of surface alkyne-functionalized SCL micelles from *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer and the subsequent fabrication of PNIPAM-functionalized SCL micelles (SCL-PNIPAM) via Click chemistry.

HT5 and an oven temperature of 45 °C. The eluent was THF at a flow rate of 1.0 mL/min. Waters 1515 pump and Waters 2414 differential refractive index detector (set at 30 °C) were used. A series of low polydispersity polystyrene standards were employed for the GPC calibration. The FTIR spectra were collected on a Bruker VECTOR-22 IR spectrometer, the interferogram was averaged 64 times and the IR spectra were obtained from 400–4000 cm^{-1} with a resolution of 4 cm^{-1} . The transmittance of the solution was measured by a Unico UV-vis 2802PCS spectrophotometer at a wavelength of 600 nm using a thermostatically controlled cuvette.

A commercial spectrometer (ALV/DLS/SLS-5022F) equipped with a multi-tau digital time correlator (ALV5000) and a cylindrical UNIPHASE He-Ne laser (22 mW, $\lambda_0 = 632$ nm) as the light source were employed for dynamic laser light scattering (LLS) measurements. Scattered light was collected at a fixed angle of 90° for a duration of ~10 min. The intensity-average hydrodynamic diameter, $\langle D_h \rangle$, and polydispersities (μ_2/Γ^2) of the micelles were calculated by cumulants analysis of the experimental correlation function. All data were averaged over three measurements.

TEM images of SCL-PNIPAM were recorded using a Philips CM120 electron microscope at an acceleration voltage of 200 kV, the specimen was prepared by spreading 10 μL aqueous solution (1.0 g/L, pH = 9) on copper grids that had been coated with thin films of Formvar and carbon successively. No staining was required.

Results and discussion

Synthesis of *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer and PNIPAM-N₃ homopolymer

Atom transfer radical polymerization (ATRP) has been shown to be a versatile technique for the controlled polymerization of

many hydrophilic monomers.^{44–46} The controlled ATRP of OEGMA, DMA, NIPAM, and DEA monomers and the facile preparation of POEGMA-*b*-PDMA-*b*-PDEA triblock copolymers by sequential monomer addition in methanol or IPA using CuX/bpy catalysts have been well documented.^{22,24,47–52} Thus, in order to synthesize the alkynyl-terminated double hydrophilic triblock copolymer of *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA, this triblock copolymer was prepared in IPA at 45 °C by sequential monomer addition using CuCl/bpy catalysts and propargyl 2-bromoisobutyrate as the ATRP initiator as shown in Scheme 1(a).

In the first stage, *alkynyl*-POEGMA precursor was polymerized using CuCl/bpy as catalysts and propargyl 2-bromoisobutyrate as the ATRP initiator, then DMA and DEA were added successively to synthesize *alkynyl*-POEGMA-*b*-PDMA diblock copolymer precursor and the final triblock copolymer, *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA. The GPC chromatograms of each step during the synthesis are shown in Fig. 2, in which the chromatogram (a) of *alkynyl*-POEGMA sampled at almost 100% conversion reveals a mono-modal and symmetric peak with an M_n of ~9550 g/mol and M_w/M_n of 1.16. Chromatogram (b) shows the GPC traces of the *alkynyl*-POEGMA-*b*-PDMA diblock copolymer just before the addition of DEA, the polymerization of DMA was almost complete at that point (96% conversion). No shoulder peak corresponding to homopolymer can be observed, indicating the high initiator efficiency of *alkynyl*-POEGMA precursor. The M_n and M_w/M_n of the diblock copolymer were determined to be 18 550 g/mol and 1.25, respectively. The GPC chromatogram of the *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer is given as Fig. 1(c). As we can see, there is no evidence of tailing in the GPC traces due to

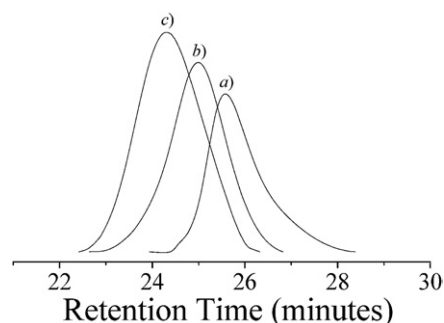
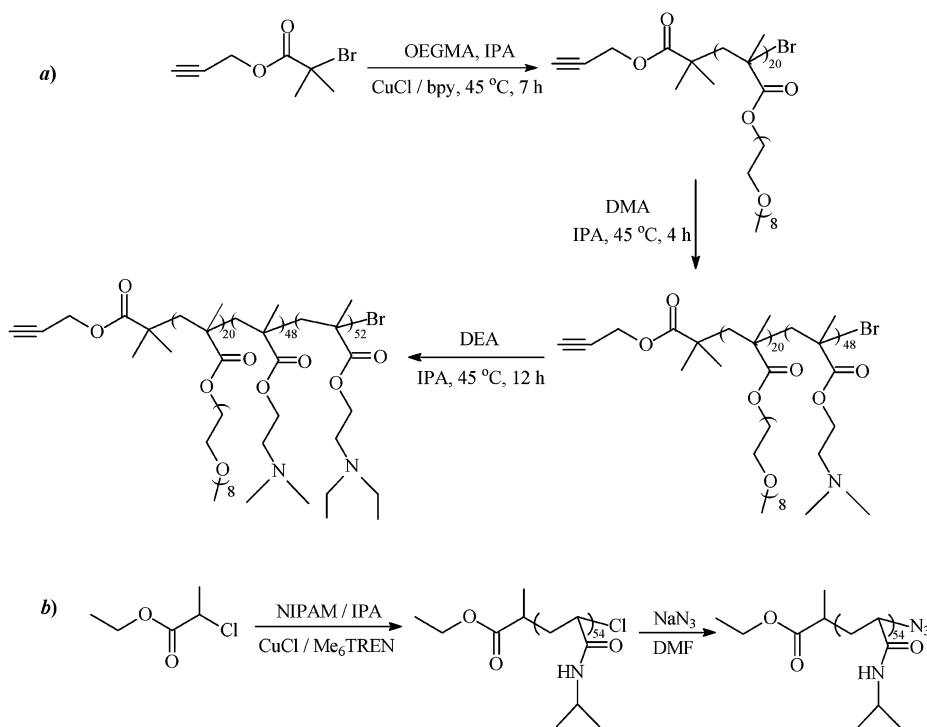


Fig. 2 GPC traces of (a) *alkynyl*-POEGMA precursor ($M_n = 9,500$, $M_w/M_n = 1.16$), (b) *alkynyl*-POEGMA-*b*-PDMA diblock copolymer precursor ($M_n = 18,600$, $M_w/M_n = 1.25$), and (c) *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer ($M_n = 29,300$, $M_w/M_n = 1.29$).

homopolymer and diblock copolymer contaminants. The GPC peak was also mono-modal and quite symmetric, revealing an M_n of 29 250 g/mol and M_w/M_n of 1.29.

The ¹H NMR spectrum of *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer recorded in CDCl₃ is displayed in Fig. 3 with the relevant signals labeled. It should be noted that besides those signals characteristic of POEGMA, PDMA, and PDEA blocks, a slight resonance signal at about 4.6 ppm could be seen which is typically assigned to the –OCH₂ (*j*) of alkynyl initiator. By comparing the well-defined peak integrals of the POEGMA, PDMA, and PDEA blocks with that of the alkynyl initiator, the degrees of polymerization, DP, of the POEGMA, PDMA, and PDEA blocks were calculated to be 20, 48, and 52, respectively, which are quite close to the target DP. Thus, the



Scheme 1 Reaction schemes for the preparation of (a) alkynyl terminally functionalized POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer (*alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA), and (b) azide-terminated PNIPAM (PNIPAM-N₃).

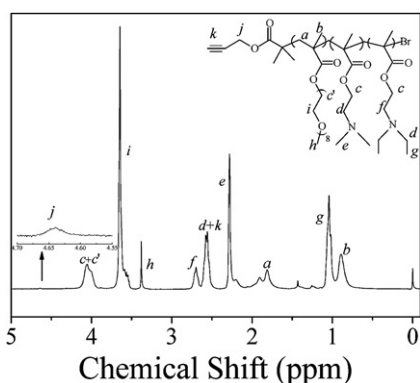


Fig. 3 ^1H NMR spectrum of *alkynyl*-POEGMA₂₀-*b*-PDMA₄₈-*b*-PDEA₅₂ triblock copolymer in CDCl_3 .

obtained alkynyl-functionalized triblock copolymer can be described as *alkynyl*-POEGMA₂₀-*b*-PDMA₄₈-*b*-PDEA₅₂.

pH-Induced formation of surface-functionalized “clickable” three-layer “onion-like” micelles

The DMA and DEA homopolymers are both weak polybases with $\text{p}K_a$'s of about 7.0 and 7.3, respectively.^{53–58} DMA homopolymer is water-soluble over a wide pH range, while DEA homopolymer is water-insoluble at neutral or alkaline pH. Below pH 6.0, it is soluble as a weak cationic polyelectrolyte due to protonation of the tertiary amine groups. As reported in the literature, PDMA-*b*-PDEA diblock copolymers synthesized *via* ATRP can dissolve molecularly in acidic media, but at pH 7–8 they form micelles with hydrophobic DEA cores and neutral (or only weakly cationic) DMA coronas due to deprotonation of both blocks. At higher pH, the micelles aggregate and finally precipitate. However, using ABC triblock copolymers, such as PEO-*b*-PDMA-*b*-PDEA triblock copolymer instead of DMA-*b*-PDEA diblock copolymers can increase the micelles stability in alkaline media due to the steric stabilization imparted by the hydrophilic PEO block.²⁴ The micelles formed have a three-layer “onion” structure with the DEA blocks consisting of the micelle cores and the DMA and PEO blocks constructing the inner shell and coronas, respectively. Having similar block structures, the *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymers will also form three-layer “onion-like” micelles consisting of PDEA cores, PDMA inner shell, and permanently hydrophilic POEGMA outer coronas. NMR and DLS studies confirmed this structural arrangement. Furthermore, the surface of the micelles was modified by functional alkynyl groups that can be reactive with azide groups, as illustrated in Fig. 3.

The ^1H NMR spectra of the *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer recorded in acidic and alkaline media are shown in Fig. 4(a) and (b), respectively. As shown, the *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymer is fully solvated at pH 3 and all the signals expected for each block are visible. On the other hand, at pH 9, the signal due to the PDEA blocks at δ 1.4 ppm completely disappeared, indicating the formation of relatively compact, hydrophobic PDEA-core micelles. In addition, the signal corresponding to PDMA blocks at about δ 3.4 ppm is attenuated as a result of the partial

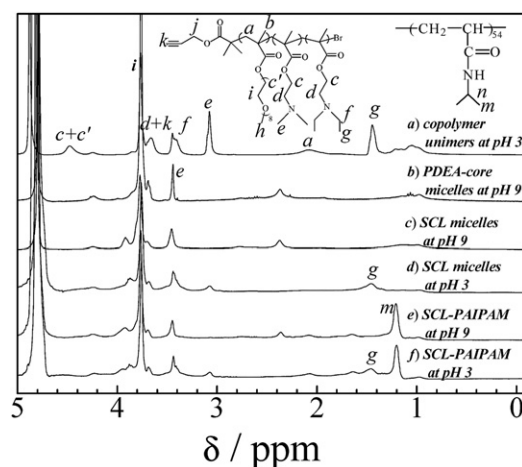


Fig. 4 ^1H NMR spectra of *alkynyl*-POEGMA₂₀-*b*-PDMA₄₈-*b*-PDEA₅₂ triblock copolymer obtained in D_2O (a) at pH 3; (b) at pH 9; (c) after shell cross-linking at pH 9; (d) after shell cross-linking at pH 3, and that of PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM) at (e) pH 9 and (f) pH 3, the target degree of cross-linking was 100%.

desolvation of the hydrophilic PDMA chains in the inner-shell. However, the signals due to POEGMA blocks are still prominent confirming the existence of the POEGMA outer coronas.

Surface-functionalized “clickable” SCL micelles with pH-responsive cores

As we know, these micellar structures formed by double hydrophilic block copolymers are unstable, and therefore, a variety of methods were employed to covalently stabilize their intriguing assemblies, involving either the core or the corona.^{12,17,18} In particular, recent efforts have focused on the synthesis of shell cross-linked micelles from ABC triblock copolymers since shell cross-linking can be carried out at high solids with little or no intermicellar cross-linking with increased interests.^{19,22–24,52,59–66}

In the current case, after preparation of the three-layer “onion-like” PDEA-core micelles from *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymers, 1,2-bis(2-iodoethoxy)ethane (BIEE) was added to the micellar solution as a cross-linking agent. The reaction solution was stirred for 3 days at room temperature, leading to the quaternization of DMA residues and subsequently cross-linking of the inner shell of the “onion-like” micelles (see Fig. 1). The resulting SCL micelles were studied in detail by means of DLS and ^1H NMR.

As shown in Fig. 5, the D_h of SCL micelles ranges from 5 to 90 nm with a $\langle D_h \rangle$ of 27 nm, which is very similar to the micelle diameter prior to cross-linking, thus confirming the expected intramicellar cross-linking mechanism. In order to identify the successful covalent stabilization of the SCL micelles, the solution pH was adjusted to pH 3 using concentrated HCl solution. If no shell cross-linking had occurred, micellar dissociation into individual triblock copolymer chains would be expected, since the DEA core blocks become soluble at acidic pH. However, both DLS studies and visual inspection confirmed the presence of typical light scattering characteristic of micellar solutions and hence indicating successful covalent stabilization. DLS studies of the SCL solutions at pH 3 revealed an increase of $\langle D_h \rangle$ to 37 nm

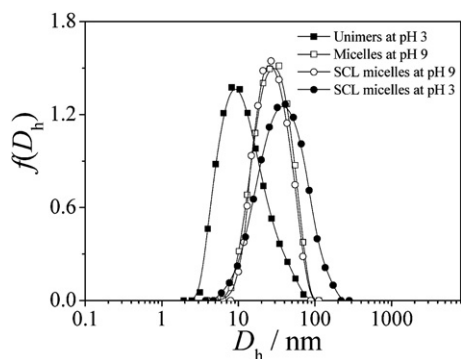


Fig. 5 Typical hydrodynamic diameter distributions, $f(D_h)$, obtained at 25 °C for 1.0 g/L aqueous solutions of unimers, non-cross-linked micelles, and SCL micelles of *alkynyl*-POEGMA₂₀-*b*-PDMA₄₈-*b*-PDEA₅₂ triblock copolymer at different pH.

compared to that of 27 nm at pH 9, the micellar swelling should be due to the electrostatic repulsion between cationic protonated tertiary amine residues of the hydrophilic PDEA blocks in the cores. The polydispersity indexes (μ_2/I^2) calculated by cumulants analysis were typically less than 0.15 for the micelles formed at different pH, indicating that the SCL micelles are relatively monodisperse and also confirming the expected intramicellar cross-linking reaction mechanism, *i.e.*, essentially no intermicellar cross-linking occurred.

Fig. 4(c) and (d) show the ¹H NMR spectra of SCL micelles obtained in D₂O solutions at pH 9 and pH 3, respectively. As we can see, for the SCL micelles at pH 9 the signal at δ 3.4 due to DMA residues are still evident, indicating the incomplete cross-linking of the PDMA blocks. Comparing the integral of the unquaternized DMA signal at δ 3.4 ppm to that of POEGMA block at δ 3.8 ppm enables the actual degree of quaternization, as well as the actual degree of cross-linking, to be estimated, which is about 70%. When the solution pH was adjusted to pH 3 by addition of DCl, as shown in Fig. 4(d), the signal at δ 1.4 ppm assigned to PDEA blocks reappeared, indicating that the PDEA-core became hydrophilic and swollen under these conditions.

Preparation of novel multi-responsive SCL micelles (SCL-PNIPAM) *via* Click chemistry

To further extend the potential application of SCL micelles as targeted drug delivery systems, the synthesis of SCL micelles with specific functional groups in the shell or corona^{25–29} have been reported. Synthetic strategies for surface functionalization of SCL micelles have been established based upon mixed micelle formation or post-functionalization reactions. Although these methods have been proven successful and valuable, a unique strategy should be developed that would eliminate the creation of a new functionalized initiator and polymer for each functionality desired and that would be tolerant to both the functional groups present in the SCL micelles and for any further chemistry performed. Due to all of these requirements, Click chemistry was employed for the functionalization of SCL micelles.

In the present study, based on the micelles formed by the *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA triblock copolymers, surface alkynyl-functionalized SCL micelles were obtained by intramicellar cross-linking of approximately 70% of the DMA

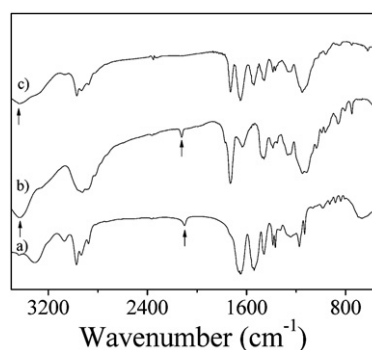


Fig. 6 FT-IR spectra of (a) PNIPAM₅₄-N₃ homopolymer, (b) surface alkynyl-functionalized SCL micelles, and (c) PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM) prepared from *alkynyl*-POEGMA₂₀-*b*-PDMA₄₈-*b*-PDEA₅₂.

residues in the shell. The alkynyl functionality was confirmed by FTIR spectrum shown in Fig. 6(b), in which well-defined absorption peaks at about 2200 cm⁻¹ and 3400 cm⁻¹ (as indicated by arrows) characteristic of the alkynyl groups can be observed. With the aim to prepare novel SCL micelles with valuable thermo-responsive PNIPAM outer coronas, azido-terminated PNIPAM homopolymer (PNIPAM-N₃) synthesized *via* ATRP was then chosen as a Click-ready polymer.

The synthesis of PNIPAM-N₃ homopolymer can be divided into two steps according to Scheme 1(b). The first step involves the polymerization of NIPAM in IPA using ethyl 2-chloropropionate as an initiator and CuCl/Me₆TREN as a catalyst to give the chloride-terminated precursor, PNIPAM-Cl homopolymer with an M_n of 6120 g/mol and an M_w/M_n of 1.23. The second step is the conversion of the terminal Cl groups to azide groups by reacting the PNIPAM-Cl homopolymer with excess NaN₃ in DMF. The successful introduction of azide groups was confirmed by the appearance of a characteristic absorbance band at about 2100 cm⁻¹ corresponding to a N=N=N stretch in the FTIR spectrum of PNIPAM₅₄-N₃ homopolymer shown in Fig. 6(a). The actual DP of the PNIPAM block in the polymer was determined to be 54 by ¹H NMR analysis in CDCl₃, so the obtained azido-terminated PNIPAM homopolymer was denoted as PNIPAM₅₄-N₃.

The Click chemistry reaction between the alkynyl-functionalized SCL micelles and azido-terminated PNIPAM-N₃ homopolymer was carried out for 2 days at ambient temperature in the presence of a Cu(I) catalyst (generated from the reduction of Cu(II) with ascorbic acid). The product was purified by dialysis against deionized water for 3 days and then lyophilized. Although the chemistry is straightforward, confirmation of the covalent coupling requires the combination of many characterization techniques.

Fig. 6(c) shows the FTIR spectrum of the PNIPAM surface-functionalized SCL micelles, SCL-PNIPAM. By comparison with the spectra of PNIPAM₅₄-N₃ homopolymer and surface alkynyl-functionalized SCL micelles, it can be seen that the intensities of the absorption bands at both 2100 cm⁻¹ assigned to azide groups and 2200 cm⁻¹ assigned to alkynyl groups almost disappeared, and the absorption peak at 3400 cm⁻¹ corresponding to alkynyl groups considerably decreased. It can therefore be concluded that during the Click chemistry reaction,

the azide groups are almost completely consumed, while a small proportion of alkynyl groups remained unreacted, which affords the SCL micelles the possibility of further corona functionalization. The attachment of the PNIPAM chains onto the surface of the SCL micelles also can be demonstrated from the ^1H NMR spectra shown as Fig. 4(e) and 4(f), in which the signals at about δ 1.2 ppm attributed to PNIPAM can be discerned.

The successful azido-alkynyl coupling between the alkynyl-functionalized SCL micelles and azido-terminated PNIPAM- N_3 homopolymer was clearly confirmed by optical transmittance characterization, the temperature-dependent transmittance of the aqueous solution of SCL-PNIPAM micelles and the mixture of SCL micelles and PNIPAM homopolymer with the same molar ratio are compared in Fig. 7. As expected, the polymers based on NIPAM show a thermo-sensitive behavior in aqueous solution, since the NIPAM block is hydrophilic at temperatures below the lower critical solution temperature (LCST) and hydrophobic above LCST. The solubility of the NIPAM-containing polymers below LCST is due to the formation of hydrogen bonds between water and *N*-isopropyl groups on the polymer chain. The hydrogen bonds are broken when the solution temperature increased above LCST. In the case of the PNIPAM homopolymer, the LCST commonly reported is about 31–35 °C depending on its molecular weight; the higher the molecular weight, the lower the LCST.^{67,68} The copolymers of NIPAM with hydrophilic or hydrophobic polymer segments may increase or decrease the LCST,^{69,70} respectively, as compared to pure PNIPAM homopolymer. Here in our study, the LCST of the PNIPAM₅₄- N_3 homopolymer with a molecular weight of $M_n = 6100$ g/mol is about 39 °C. As shown in Fig. 7, for the aqueous solution mixture of SCL micelles and PNIPAM homopolymer, the temperature at which the optical transmittance decreased sharply is almost the same as the LCST of PNIPAM₅₄- N_3 homopolymer. The corresponding LCST for SCL-PNIPAM increases to about 44 °C, which clearly indicates that all the PNIPAM chains have covalently linked with the hydrophilic POEGMA corona of the SCL micelles. As a result, the aqueous solution of prepared SCL-PNIPAM can be stable for several months at room temperature. Moreover, even at a higher temperature (for example, 50 °C) and higher concentration (5.0 g/L), the micelles still remain aggregated for about two weeks and do not precipitate.

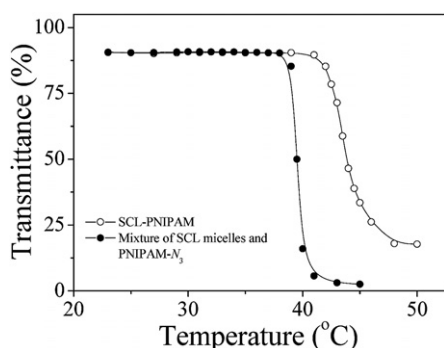


Fig. 7 Temperature-dependence of optical transmittance at 600 nm obtained for 1.0 g/L aqueous solutions (pH 9) of PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM), and mixtures of SCL micelles with PNIPAM₅₄- N_3 .

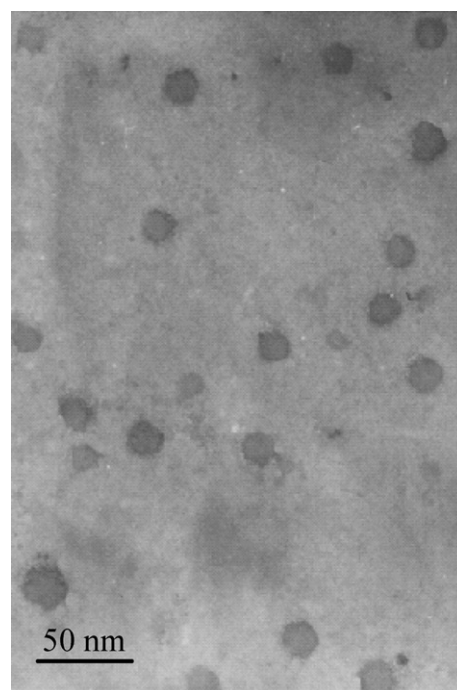


Fig. 8 Typical TEM image obtained by drying an aqueous solution of PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM) at pH 9.

TEM was used to study the morphology of the SCL-PNIPAM micelles, a typical TEM micrograph of the dried SCL-PNIPAM is shown in Fig. 8, in which the approximately spherical micelles can be observed and the mean diameter of the micelles was around 20–30 nm. The D_h of SCL micelles measured by DLS ranges from 13 to 92 nm with a $\langle D_h \rangle$ of 30 nm at pH 9 (Fig. 9). Taking into account the hydration and polydispersity effects, this TEM value is in good agreement with that obtained from DLS.

As for the PDEA cores of the SCL micelles, it can be seen from the ^1H NMR spectrum shown in Fig. 4(e) and (f), when the solution pH of SCL-PNIPAM changed from pH 9 to pH 3 on addition of DCl, the signals at δ 1.4 ppm ascribed to PDEA blocks reappeared, which indicates that the pH-responsive nature of the PDEA-core does not change upon covalent attachment of the PNIPAM chains. The pH-responsive property of SCL-PNIPAM can also be identified by DLS measurements,

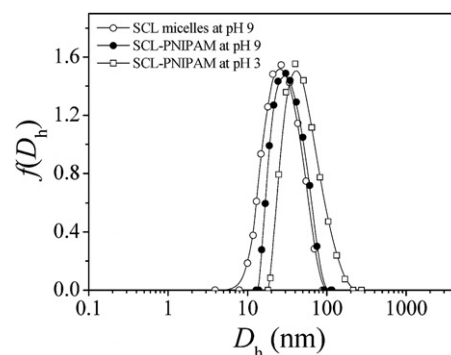


Fig. 9 Typical hydrodynamic diameter distributions, $f(D_h)$, obtained for 1.0 g/L aqueous solutions (25 °C) of SCL micelles and PNIPAM surface-functionalized SCL micelles (SCL-PNIPAM) at different pH.

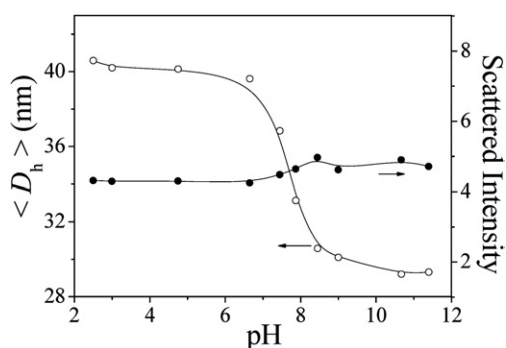


Fig. 10 Variation of intensity-average hydrodynamic diameter, $\langle D_h \rangle$, and scattered light intensity as a function of pH obtained for aqueous solution of 1.0 g/L SCL-PNIPAM.

the calculated intensity-average diameter, $\langle D_h \rangle$, and scattering intensity for SCL-PNIPAM are illustrated in Fig. 10. There are several noteworthy features. First of all, the scattering intensity of the SCL solution remains almost unchanged upon addition of NaOH, which further confirms the successful shell cross-linking; the SCL micelles exhibit excellent colloid stability within the pH range of 2–12. Second, compared to three-layered SCL micelles, the $\langle D_h \rangle$ of SCL-PNIPAM at pH 9, which is calculated to be 30 nm, increased slightly, indicating the successful formation of four-layered SCL-PNIPAM by the azido-alkynyl coupling. Finally, based on the known pK_a value (~ 7.3) of PDEA homopolymers, the dramatic change in particle size caused by swelling/deswelling of the PDEA cores takes place in the pH range of 6–8, which is just around the physiological pH. Such SCL-PNIPAM nanoparticles can find application in drug delivery since an abrupt environmental change (such as temperature or pH) could allow the release of encapsulated hydrophobic drugs.

Conclusions

In summary, an alkynyl-terminated functional triblock copolymer, *alkynyl*-POEGMA-*b*-PDMA-*b*-PDEA was synthesized by sequential polymerization of OEGMA, DMA, and DEA *via* ATRP using propargyl 2-bromoisobutyrate as the initiator. The triblock copolymers dissolved molecularly in aqueous solution at low pH; they self-assembled into three-layer “onion-like” micelles with PDEA cores, a PDMA inner shell and POEGMA coronas bearing clickable alkynyl functional groups at the surface at basic conditions. Efficient shell cross-linking of the micelles was achieved in aqueous solution at room temperature using 1,2-bis(2-iodoethoxy)ethane (BIEE) as a cross-linking agent to selectively quaternize the DMA residues. The introduction of the clickable alkynyl groups on the surface of the SCL micelles with pH-responsive cores provides a convenient route for functionalization of these well-defined nanostructures. Multi-responsive four-layer SCL micelles (SCL-PNIPAM) with pH-responsive PDEA cores and thermosensitive PNIPAM outer coronas can be prepared *via* a Click reaction between the alkynyl-functionalized SCL micelles and azido-terminated PNIPAM homopolymer by alkynyl-azide coupling. The enhanced colloid stabilities at elevated temperatures, as well as the pH-responsive behavior, makes these novel SCL-PNIPAM potential candidates for controlled encapsulation and drug delivery.

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