Synthesis of Reversible Shell Cross-Linked Micelles for Controlled Release of Bioactive Agents†

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The synthesis and subsequent self-assembly of amphiphilic diblock and triblock copolymers into micelles have been the focus of considerable research. The recent advent of controlled/"living" polymerization techniques now allows the precise construction of nanoscale carriers with potential for controlled delivery of pharmaceutics, proteins, and genes.[1,2] An important consideration in delivery applications is that such copolymer micelles invariably dissociate into unimers when the copolymer concentration falls below the critical micelle concentration (cmc) on dilution under physiological conditions, resulting in the premature release of the active compound.[3] In principle, this problem can be overcome by using shell cross-linked (SCL) micelles, which were first reported by Wooley and co-workers in 1996.[4] To date, several methods have been employed for the formation of SCL micelles[5] including our recently reported cross-linking of reactive *N*,*N*-acryloxysuccinimide (NAS) units incorporated within an ABC triblock.[6] One drawback of using SCL micelles in drug delivery applications is that the large size precludes renal excretion.[7] In this paper we report SCL micelles formed with cystamine, a reversible cross-linking agent. An important advantage of such micelles is that, in principle, the block copolymer chain should be readily eliminated from the body after in-situ micelle degradation. In this work, we also demonstrate that the individual copolymer chains produced after cleavage of the disulfide bonds in the cystamine linkages can be reassembled into micelles and subsequently re-cross-linked. The key features of micelle assembly, reversible cross-linking, and thermoresponsive behavior are illustrated in Scheme 1.

The triblock copolymer, poly(ethylene oxide)-*block*-[(*N*,*N*-dimethylacrylamide)-*stat*-(*N*-acryloxysuccinimide)]-*block*-(*N*-isopropylacrylamide), [PEO-*b*-P(DMA-*stat*-NAS)-*b*-NIPAM], was synthesized in dioxane at 70 °C using a poly(ethylene oxide)-based chain transfer agent (macroCTA) using a reversible addition−fragmentation chain transfer polymerization protocol.[6] Size exclusion chromatography (Figure S1, Supporting Information) and ^{1}H NMR analysis confirm the triblock copolymer structure to be PEO_{45}-*b*-[DMA_{98}-*s*-NAS_{30}]-*b*-$NIPAM_{87}$. At room temperature this triblock copolymer is molecularly dissolved and has a hydrodynamic diameter of approximately 7−8 nm (Figure 1a). Increasing the solution temperature to 37 °C leads to the formation of micelles with PNIPAM cores. (Further details of the micellization vs temperature behavior are given in the Supporting Information.) Spherical micelles with hydrodynamic diameters of 38 nm are formed at 45 °C. The NAS unit in the center block of the triblock copolymer was then cross-linked using 1 mol equiv of cystamine, a disulfide-based bifunctional primary amine. Dynamic light scattering (DLS) studies indicated that the micelle size did not change during cross-linking (Figure 1b). After cross-linking, the solution temperature was lowered to room temperature. DLS results showed an increase in the micelle diameter to 57 nm (Figure 1c). This larger micelle size is due to swelling of the micelle cores as the core-forming PNIPAM chains become hydrophilic under these conditions.

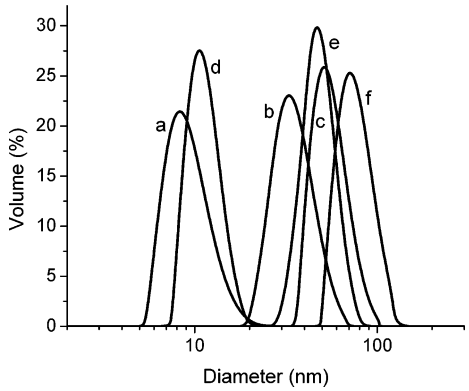

Figure 1. Dynamic light scattering size distribution of the PEO_{45}-*b*-[DMA_{98}-*s*-NAS_{30}]-*b*-$NIPAM_{87}$ triblock copolymer under specific conditions: (a) 0.5% aqueous solution at 25 °C; (b) 0.5% aqueous solution at 45 °C; (c) 0.5% shell cross-linked (SCL) micelle solution at 25 °C; (d) at 25 °C, after cleavage of the SCL micelles using dithiothreitol (DTT); (e) at 45 °C, after cleavage of the SCL micelles using DTT; (f) at 25 °C, after re-cross-linking the cleaved SCL micelles using cystamine.

Disulfide bonds can be easily cleaved by tris(2-carboxyethyl)-phosphine (TCEP).[8] In the presence of 0.05 mol/L TCEP, the SCL micelles are cleaved to produce individual copolymer chains within 30 min at room temperature. The disulfide bonds can also be cleaved by a well-known thiol−disulfide exchange reaction using compounds such as dithiothreitol (DTT).[9] DLS studies confirmed that all SCL micelles can be completely de-cross-linked after 10 h at 45 °C using excess DTT (Figure 1d). The mean hydrodynamic diameter decreased from 60 to 9−10 nm, which is comparable to that of the triblock copolymer precursor. Disulfide cleavage was also confirmed by SEC analysis (Figure S3, Supporting Information); the retention volume of the DTT-treated SCL micelles is significantly higher than that of the SCL micelles, but it is in the same range as that of the triblock copolymer precursor. In both cases the excess TCEP or DTT and their corresponding byproducts can be removed by dialysis.

After cleavage with either TCEP or DTT, the disulfide-containing cystamine groups of the triblock copolymer are converted to thiol units, and the triblock copolymer chains retain their thermoresponsive character. Micelles are re-formed when the solution temperature is raised to 45 °C (Figure 1e, 50 nm). These micelles are slightly larger than those formed by the original PEO-*b*-[DMA-*stat*-NAS]-*b*-NIPAM triblock copolymer (Figure 1a). A possible explanation is that, after cleavage, the middle block becomes slightly more hydrophilic than the original DMA-*stat*-NAS block, thus increasing the micelle size. These micelles can be re-cross-linked using a further charge of cystamine. In contrast to the original cross-linking of the NAS units by the cystamine residues, this second charge of cystamine

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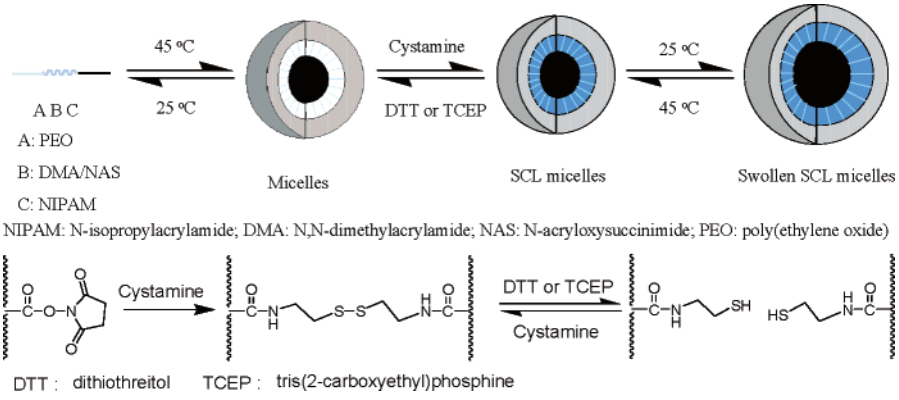
Scheme 1. Schematic Illustration of the Formation of Reversible Shell Cross-Linked (SCL) Micelles from PEO-*b*-(DMA-*s*-NAS)-*b*-NIPAM Triblock Copolymers



acts as a thiol/disulfide exchange reagent. DLS studies confirm that there was no significant size change during the re-formation of the SCL micelles. After re-cross-linking, the solution temperature was lowered to room temperature. DLS indicated a mean particle diameter of about 75 nm (Figure 1f), which confirmed that cross-linking had been successful (otherwise, micelle dissociation would have occurred below the LCST of the PNIPAM chains). This is also confirmed by TEM analysis (Figure S4, Supporting Information). The final degree of cross-linking of the re-formed SCL micelles depends on the precise conditions (e.g., micelle concentration, cystamine concentration, reaction temperature and time) selected for the thiol/disulfide exchange reaction.[10] After thiol/disulfide exchange, the chemical structure of the re-formed SCL micelles is the same as that before cleavage (Scheme 1). Thus, the SCL micelles can be un-cross-linked and re-cross-linked repeatedly, in a fully reversible process.

Dipyridamole (DIP) was used as a model compound for controlled release studies using these novel SCL micelles. By mixing DIP with the thermoresponsive micelles at 45 °C, the DIP can be loaded into the core of the micelles. Subsequently, lowering of the solution temperature to 25 °C elicits micelle dissociation, which leads to triggered release of the DIP. The rate of release can be directly monitored by visible absorption spectroscopy at 415 nm, which is the characteristic absorption for DIP. Shell cross-linking the DIP-loaded micelles using cystamine can significantly retard the rate of release (Figure 2). The release of DIP could not be detected after 7 days, meaning that the remaining DIP was stabilized within the core of the SCL micelles. Drug release from DIP-loaded SCL micelles was also monitored by visible absorption spectroscopy using a conventional dialysis method, as shown in Figure 3. The release rate is much faster in the presence of DTT which causes cleavage of the SCL micelles; therefore, the diffusion of the DIP from the core of the micelles to the environmental PBS buffer will be facile. Since the rate of cleavage of the SCL micelles can be controlled by using different types of chemical agents (such as TCEP or DTT) or by adjusting their concentration or reaction temperature, the release rate of the DIP can be easily controlled. The SCL micelles can also be cleaved by using an oxidizing agent such as H_2O_2,[9d] which leads to complete cleavage of the SCL micelles within 10 h at 60 °C. Since H_2O_2 is produced in the mammalian immune system,[11] in-situ cleavage of the SCL micelles should be feasible, possibly facilitating the elimination of the individual copolymer chains after drug delivery via renal excretion.

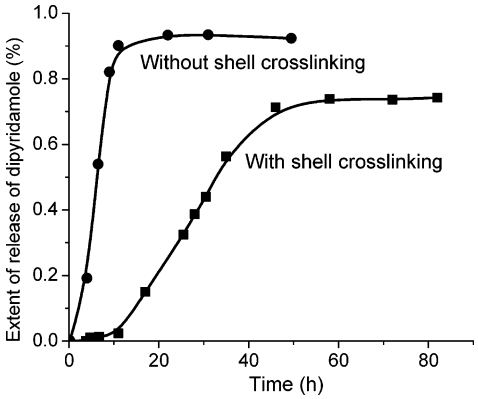


Figure 2. Cumulative dipyridamole release to phosphate buffered saline solution from shell cross-linked and non-cross-linked micelles at 25 °C.

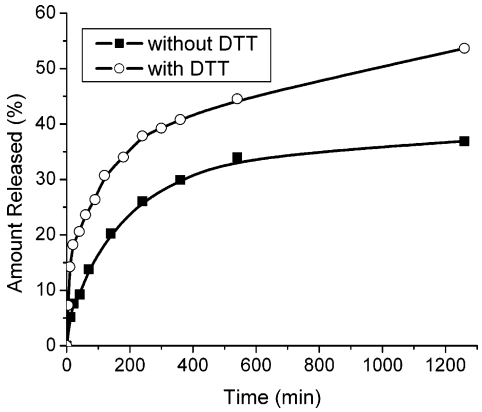


Figure 3. Cumulative dipyridamole release to phosphate buffered saline solution from shell cross-linked micelles with or without dithiothreitol (DTT) at 37 °C.

In summary, triblock copolymer micelles were prepared from a novel thermoresponsive ABC triblock copolymer that self-assembles in aqueous solution. SCL micelles were readily obtained using cystamine as a diamine cross-linker. These SCL micelles can be reversibly cleaved using either DTT or TCEP; the degraded micelles can be re-cross-linked using cystamine as a thiol-exchange compound. The rate of drug release can be easily controlled from these SCL micelles, demonstrating their potential as nanoscale drug delivery vehicles.

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Supporting Information Available: Experimental details, GPC, DLS, TEM, and DIP release rate measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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