Micelles

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Thermoresponsive Micelles from Oligopeptide-Grafted Cyclotriphosphazenes**

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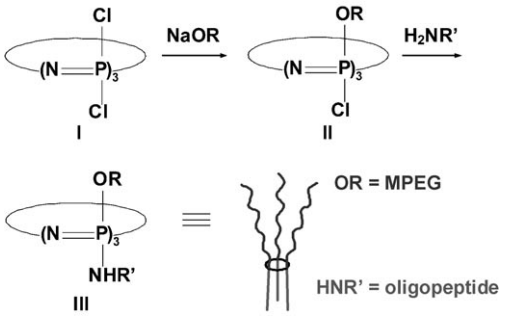
Polymeric micelles have attracted much attention because of scientific interest and their high potential utility in the biomedical field as drug carriers, surfactants, and surface modifiers.[1] Amphiphilic di- or triblock copolymers consisting of both hydrophilic and hydrophobic segments in an appropriate ratio are known to form micelles by self-assembly in aqueous solution.[2] The hydrophobic blocks of the copolymer form the core of the micelle, while the hydrophilic blocks form the corona or outer shell. A variety of drugs including genes, proteins, and hydrophobic drugs can be incorporated into the hydrophobic core of the micelle. Polyethylene glycol (PEG) is most widely used as the hydrophilic block, but many low-molecular-weight hydrophobic polymers, such as poly (propylene oxide) and poly(lactic acid), are employed as a hydrophobic block.[3] Nearly all the micelle-forming polymers reported so far are amphiphilic grafted or block copolymers with a linear backbone, but to our knowledge, no micellar polymer with a cyclic backbone has been reported.

We have synthesized amphiphilic cyclotriphosphazenes grafted with equimolar amounts of oligopeptide and methoxypolyethylene glycol (MPEG), which form strong micelles by self-assembly in aqueous solution. Herein, we report the synthesis and properties of these micellar cyclic trimers.

The amphiphilic trimers were prepared by two-step nucleophilic substitution reactions according to Scheme 1. We have shown in our previous work that nucleophilic substitution of hexachlorocyclotriphosphazene (**I**) with the sodium salt of MPEG at low temperature ($T < -60\,^{\circ}C$) gives rise to the half-substituted intermediate (**II**) with *cis* nongeminal conformation as a major product.[4] Further substitution of the intermediate with an oligopeptide ethyl ester (H_2NR') produced a final amphiphilic product (**III**), which was purified twice by precipitation at the lower critical solution temperature (LCST) in aqueous solution. The LCST is the temperature at which the polymer solution undergoes phase transition from a soluble to an insoluble state. All final products showed a clean and sharp singlet peak of ^{31}P NMR resonance at $\delta = 22$ ppm, thus confirming the *cis* nongeminal conformation of the side groups of the trimers. Three hydrophobic oligopeptides are oriented in one direction opposite to that of three hydrophilic MPEG groups with respect to the phosphazene ring (see Scheme 1). The trimeric products were obtained as a yellow viscoelastic liquid in 30–60% yields. Various trimeric derivatives with a different hydrophobic-to-hydrophilic balance were prepared using different combinations of the hydrophilic and hydrophobic groups; the products are listed in Table 1.



Scheme 1. Synthetic route to micellar cyclotriphosphazenes.

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Table 1: Characteristic properties of cyclic trimers.

Trimer	Compound	LCST [°C][a]	DLS [nm][b]	PDI[c]
1	$[NP(MTEG)GlyPheGlyEt)]_3$	34.0	2.2	1.0
2	$[NP(MPEG350)(GlyPheLeuEt)]_3$	37.0	12.3[d]	0.31
3	$[NP(MPEG350)(GlyPheLeuAspEt_2)]_3$	27.0	13.9	0.25
4	$[NP(MPEG350)(GlyPheLeuGluEt_2)]_3$	24.0	13.3	0.44
5	$[NP(MPEG550)(GlyPheLeuAspEt_2)]_3$	60.0	3.7 (94%)[e] 13.8 (6%)	0.21

[a] The lower critical solution temperature in pure water. [b] Dynamic light scattering measurements at 25 °C of 0.5 % aqueous solutions filtered with a 0.45-μm syringe filter. [c] The polydispersity index represents S/d_h, where S is the standard error in hydrodynamic diameter d_h. [d] Measured at 30 °C. [e] 94 % monomer (see text for details).

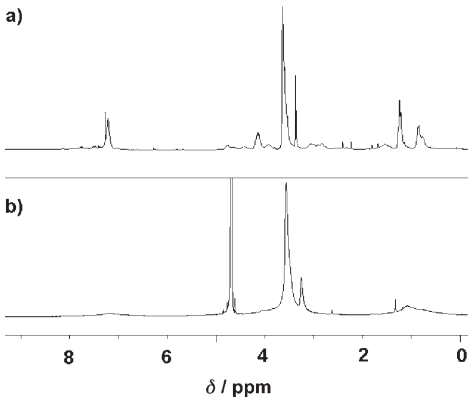

Figure 1. ^1H NMR spectra of trimer **3** in a) $CDCl_3$ and b) D_2O.

The trimers were fully characterized by multinuclear (^1H,^{31}P) NMR spectroscopy, elemental analysis, and MALDI mass spectrometry.[5a] In particular, the MALDI mass spectrum of monodisperse trimer **1** clearly shows the major parent peak at m/z 1697.7 (**1**·Na^+), which confirms the exact trimer composition. All the trimers are immediately soluble and show thermoresponsive properties with an LCST in water. Especially, except for trimer **5**, all trimers exhibit an LCST below body temperature, which is very useful for biomedical applications. The local tolerance tests for trimer **3** showed excellent biocompatibility, the results of which will be published separately. It is known that the LCST of amphiphilic copolymers is determined by their hydrophobic-to-hydrophilic balance.[6] Comparison of the LCSTs of trimers **2**–**4** that bear the same hydrophilic PEG350 indicates that their LCSTs decrease as the chain length of the oligopeptides increases.

Among the trimers listed in Table 1, trimer **1** bearing the shortest tetraethylene glycol and tripeptide side groups demonstrated remarkably different behaviors from those of the other trimers in aqueous solution. Trimer **1** showed exactly the same proton NMR spectral pattern with good resolution in both $CDCl_3$ and D_2O at 20 °C, which indicates that there is not considerable molecular aggregation in aqueous solution. However, trimer **3** showed broadened resonances of all the oligopeptide protons in aqueous solution while there was no significant change in the MPEG proton peaks at $\delta = 3.5$–3.7 ppm (see Figure 1), which is strong evidence for micelle formation in aqueous solution.

Therefore, dynamic light scattering (DLS) measurements were performed for all the trimers with a Melvern Zetasizer Nano ZS instrument, and their mean particle diameters are listed in Table 1 along with their polydispersity index (PDI) values. Interestingly, trimer **1** bearing the tetraethylene glycol with the least hydrophilicity ($\log P = -0.92$; $P = [\text{solute}]_{n\text{-octanol}}/[\text{solute}]_{\text{water}}$), calculated by a reported method,[7] and the least hydrophobic tripeptide GlyPheGlyEt ($\log P = -0.63$) does not form a micelle, but exists as a monomer in solution, as its mean diameter of 2.2 nm is almost equivalent to its unimolecular size. On the other hand, trimer **2** bearing the more hydrophilic PEG350 ($\log P = -1.42$) and a more hydrophobic tripeptide GlyPheLeuEt ($\log P = 1.11$) clearly indicates micelle formation relative to trimer **1**, with a mean diameter of 12.3 nm like that of trimers **3** and **4**, which bear larger hydrophobic tetrapeptides. The size distributions of trimers **1** and **3** (see Figure 2) are relatively narrow. Trimer **5** bearing the most hydrophilic PEG550 ($\log P = -2.24$) and the same hydrophobic tetrapeptide as trimer **3** showed a bimodal size distribution with the monomer in the majority (94 %), probably because of its high hydrophilicity. It is known that if the hydrophilicity is too high compared with the hydrophobicity in a diblock copolymer, then the copolymer exists as a monomer in water.[3a]

To examine the temperature-dependent behavior of the present trimers, the sizes of representative trimers **1**–**3** were measured as their solution temperature was increased from 20 °C to their LCST. Trimer **1** showed a constant unimolecular size of 2.2 nm up to 28 °C, near its LCST (34 °C), but beyond this temperature it started to associate to microparticles. The micellar structure of trimer **3** remained at constant size up to its LCST (27 °C), but the micelles rapidly aggregated to larger particles to precipitate beyond its LCST. Most interesting is

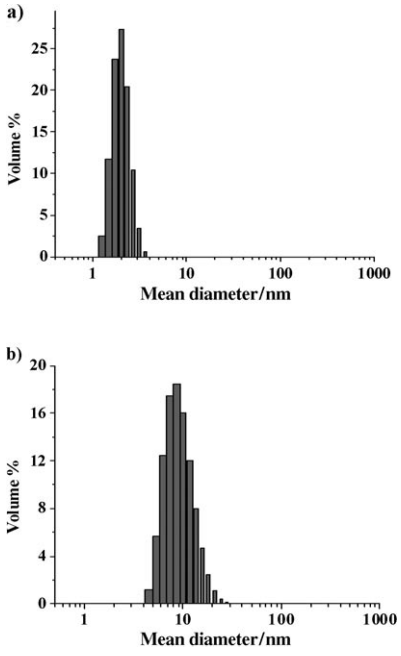

Figure 2. Size distribution by volume of trimers a) **1** and b) **3**.

the finding that trimer **2** exhibited not only an LCST at 37 °C but also an upper critical solution temperature (UCST) at 27 °C in aqueous solution. As shown in Figure 3, the micellar structure of trimer **2** is maintained between its UCST and LCST. This is the reason why the DLS measurement for trimer **2** was performed at 30 °C. The particle size of trimer **2** below its UCST was measured as approximately 150–200 nm. All other trimers showed only an LCST without a UCST.

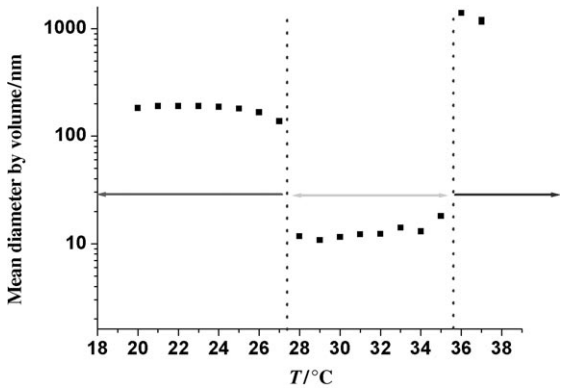

Figure 3. Temperature-dependent sizes of trimer **2**.

The LCST is frequently observed for amphiphilic copolymers, but the UCST is rarely observed. Furthermore, only a few copolymers were reported to show both an LCST and a UCST in aqueous solution.[8] The phase transition from a soluble to an insoluble state at the LCST is known to be a consequence of weakening of the hydrogen bonding between the hydrophilic part of the copolymer and the solvent water molecules.[9] The phase transition at the UCST is known to be associated with unfavorable intermolecular interactions between the polymer and solvent molecules in the low-temperature region.[10] Therefore, it seems that the UCST of trimer **2** is related to the remarkably high hydrophobicity of its grafted oligopeptide, GlyPheLeuEt ($\log P = 1.11$), relative to the other oligopeptides, GlyPheLeuAspEt$_2$ ($\log P = 0.32$) and GlyPheLeuGluEt$_2$ ($\log P = 0.57$). Such a variety of thermoresponsive properties of the trimers depending on their hydrophobic-to-hydrophilic balance can be ascribed to their unique molecular structure. The *cis* nongeminal conformation of the hydrophilic and hydrophobic side groups, in particular, may enhance effectively the intermolecular hydrophobic interaction to form micelles by self-assembly.

The morphology of the present trimeric micelles is not very clear, but from the ^{1}H NMR spectra (Figure 1), which show the near-disappearance of the oligopeptide protons of the trimer in aqueous solution, the larger hydrodynamic volume of MPEG relative to that of the oligopeptides, and the narrow size distribution of micelles we propose a spherical form of self-assembled micelles (see Figure 4). The core of the micelle seems to contain some solvent water trapped during the process of micelle formation,[2a] which is not surprising because the tetrapeptides ($\log P = 2.1$–3.7) employed are still considerably water soluble, although they are very hydrophobic compared with MPEGs.

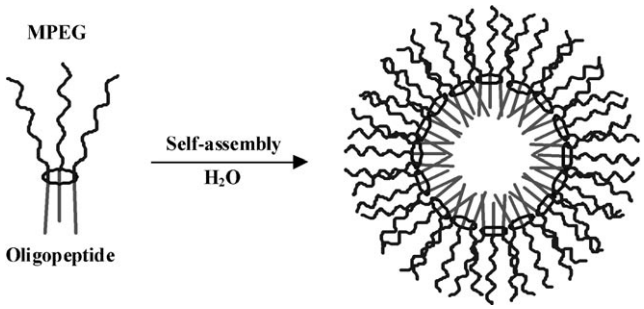

Figure 4. Micelle formation by self-assembly from trimers.

The stability of polymeric micelles is very important for biomedical applications. In particular, the critical micelle concentration (CMC) of polymers is an important measure for applications in injectable drug delivery because the micelles should remain undestroyed even after dilution by injection. Therefore, various known methods were attempted to measure the CMC of the present trimers. However, optical methods could not be used for our trimers because they showed strong absorption in the UV/Vis region and also fluorescence in the UV region. We employed the surface tension method to measure the CMC of trimer **3**,[11] which gave rise to a value of 0.11 mg L^{-1}.[5b] This value, which was further confirmed by the DLS method, is very low relative to those of known linear block copolymers, for example, 1.2 mg L^{-1} for PCL$_{21}$-*b*-PEO$_{44}$[2a] (PCL = polycaprolactone, PEO = poly(ethylene oxide)). Such a high thermodynamic stability of the trimeric micelles seems to be a result of the unique molecular structure of our trimers, as mentioned above.

In summary, new oligopeptide-grafted cyclotriphosphazenes were synthesized that show unique thermoresponsive properties depending on the hydrophobic-to-hydrophilic balance of the side groups. Strong micellar trimers could be prepared by designing appropriate hydrophobic oligopeptides that match the hydrophilicity of PEG350.

Experimental Section

All the synthetic reactions were performed under an argon atmosphere with thoroughly dried chemicals and solvents. ^{1}H and ^{31}P NMR spectra were measured with a Varian Unity INOVA-500 spectrometer operating at 500 MHz and using tetramethylsilane (TMS) and phosphoric acid, respectively, as external standards. The LCST at which trimers in aqueous solution (0.5 wt %) undergo phase transition from sol to precipitate was measured by UV/Vis spectroscopy. The MALDI-TOF mass spectrum of trimer **1** was measured with a Voyager-DE STR spectrometer. DLS measurements for the trimers in aqueous solution (0.1 %) were performed with a Melvern Zetasizer Nano ZS instrument. The CMC was measured by the Wilhelmy plate method using a Kruss K-12 surface tension apparatus equipped with a processor to acquire the data automatically.

1: Methoxytetraethylene glycol (MTEG) with a molecular weight of 208.25 (2.16 g, 10.3 mmol) was treated with sodium metal (0.24 g, 10.4 mmol) in dried THF for 24 h under an argon atmosphere to obtain the sodium salt of MTEG. The unreacted sodium was removed by filtration, the filtrate was slowly added over 30 min to a solution of hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) in dried THF in a separate reaction vessel cooled in a dry ice–acetone bath (−78 °C),

and the reaction solution was stirred for 30 min. After the dry ice–acetone bath was removed, the reaction was allowed to continue for a further 8 h at room temperature. A solution (100 mL) of triethylamine (2.61 g, 25.80 mmol) and a tripeptide (GlyPheGly(Et); 3.98 g, 12.9 mmol) in THF was added, and the reaction mixture was stirred at 50 °C for 12 h. The mixture was filtered to remove the solid by-products (NEt_3·HCl and NaCl) and the filtrate was concentrated under reduced pressure. The concentrate was dissolved in THF and an excess amount of diethyl ether or hexane was added to induce precipitation. This process was repeated twice. The resultant product was dissolved in a small amount of distilled water (20 mL) for dialysis for 24 h using the dialysis membrane (MWCO: 1000) in distilled water and then freeze dried to obtain the trimer **1**, $[NP(MTEG)(GlyPheGlyEt)]_3$. The product was further purified by reprecipitation from aqueous solution (10%) at its LCST. Other trimeric derivatives were prepared by the same procedure using different PEGs and oligopeptides. Yield: 31.5%; elemental analysis (%) calcd for $C_{72}H_{117}N_{12}O_{27}P_3\cdot 3H_2O$: C 50.00, H 7.17, N 9.72; found: C 50.33, H 7.10, N 9.86; ^{1}H NMR (250 MHz, D_2O, 25 °C): $\delta = 1.18$ (t, 3H; Gly-OCH$_2$CH_3), 3.05 (m, 2H; Phe-CH_2), 3.27 (s; MTEG-OCH_3), 3.4–3.6 (b, 16H; TetEG-OCH_2CH_2), 3.7–3.8 (d, 2H; Gly-CH_2), 4.05 (q, 2H; Gly-CH_2CH$_3$), 4.69 (m, 1H; Phe-CH), 7.14 ppm (m, 5H; Phe-arom); ^{31}P NMR (500 MHz, D_2O, 25 °C): $\delta = 22.4$ ppm; LCST: 34 °C. Characterization data for trimers **2**–**5** are also available.[5c]

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