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Thermosensitive micelles from PEGylated oligopeptides

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Abstract

New thermosensitive and micelle-forming diblock copolymers were synthesized by amide coupling of carboxylated methoxy-poly(ethylene glycols) with hydrophobic oligopeptides and characterized by means of ^{1}H NMR spectroscopy, elemental analysis, and dynamic light scattering (DLS) measurements. Stable micelles were formed from copolymers composed of MPEG550 or MPEG750 as a hydrophilic segment and the hexapeptide, GlyPheLeuGlyPheLeuEt, as a hydrophobic segment. All the present diblock copolymers exhibited thermosensitive properties by showing a lower critical solution temperature (LCST) in water. The LCST of the copolymers composed of relatively shorter MPEG350 and GlyPheLeuAspEt$_2$ was observed at much higher temperature (48 °C) compared with the LCST (29 °C) of the cyclotriphosphazene analogues bearing the same hydrophilic and hydrophobic segments as side groups. The remarkably lower LCST of the cyclotriphosphazene analogue is presumed to be due to the structural effect of the *cis*-nongeminal conformation of its three hydrophobic oligopeptides, but such a structural effect was found to diminish as the chain lengths of the hydrophilic and hydrophobic blocks of the copolymers increased.

Keywords: Poly(ethylene glycol); Oligopeptide; Thermosensitive micelle

1. Introduction

Increasing efforts have been made in the last decade to search new amphiphilic block copolymers that can afford to form micelles by self-assembly in aqueous solution, because of their potential utility as drug delivery systems [1–7]. As a hydrophilic block to form the micelle corona, poly(ethylene glycols) are most popularly used, since they are known to have high water-solubility [8] and to protect protein adsorption and cellular adhesion in the blood circulation system, which results in longer blood circulation time [9]. As a hydrophobic block to form the micelle core [1,7] are employed many low-molecular-weight hydrophobic polymers such as poly(amino acids), poly(propylene oxide) and poly(lactic acid). Most of the amphiphilic copolymers reported so far are grafted or block copolymers with a linear backbone, which limits the scope of the properties to design drug delivery systems.

Most recently, we have reported as a communication on novel thermosensitive micelles formed from amphiphilic cyclotriphosphazenes grafted with equimolar amounts of a poly(ethylene glycol) (PEG) as a hydrophilic group and an oligopeptide as a hydrophobic group, in which the two side groups are in *cis*-nongeminal conformation [10]. In our previous work, we have shown many different aspects of micelles formed by cyclotriphosphazenes from those of the micelles formed by conventional linear block copolymers. However, we could not compare our cyclotriphosphazene micelles with those of directly coupled PEG–oligopeptide diblock copolymers, which we could not find in the literature.

Therefore, in the present study, we have prepared diblock copolymers without phosphazene backbone by direct amide coupling of carboxylated PEGs [11] with hydrophobic oligopeptides [12]. Here we report synthesis and properties of these new diblock copolymers to compare with our cyclotriphosphazene micelles.

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2. Experimental section

2.1. Materials

The oligopeptide ethyl esters, glycyl-L-phenylalanyl-L-leucylaspartic diethyl ester (GlyPheLeuAspEt$_2$), glycyl-L-phenylalanyl-L-leucylglycyl-L-phenylalanyl-L-leucyl ethyl ester (GlyPheLeuGlyPheLeuEt), glycyl-L-phenylalanyl-L-leucyl-glycyl-L-phenylalanyl-L-leucyl benzyl ester (GlyPheLeuGly-PheLeu-$CH_2C_6H_5$) were prepared by following the literature methods [12]. Methoxy-poly(ethylene glycols) (MPEG) from Fluka were used without further purification but thoroughly were vacuum dried and then stored over molecular sieve 4 Å before use. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) from Fluka were used without further purification. Carboxylated MPEGs (MPEG-CH_2COOH) were prepared by following the literature method [11]. Tetrahydrofuran (THF) was dried by boiling at reflux over sodium metal and benzophenone and then distilled under a nitrogen atmosphere. Chloroform and triethylamine were dried by boiling at reflux over sodium hydride and barium oxide, respectively, and then distilled under the same condition.

2.2. Instruments and measurements

^{1}H NMR spectra were recorded using Bruker DPX-250 NMR spectrophotometer operating at 250 MHz in the Fourier transform mode. Elemental analysis was carried out with a Carlo Ebra-EA1108. The phase transition of the polymer aqueous solutions (5%) was detected visually in a closed glass tube and the temperature was controlled by immersion of the glass tube in an oil bath. The LCST was identified as the temperature at which the solution became turbid. Dynamic light scattering (DLS) measurements were performed using Malvern Zetasizer (Nano-ZS). The critical micelle concentration (CMC) of copolymers was measured by pyrene fluorescence technique [13] using Shimadzu RF-5301 fluorescence spectrometer.

2.3. Synthesis

2.3.1. MPEG350-CH_2CO–GlyPheLeuAspEt$_2$ (**1**)

A solution of DCC (0.8 g, 3.9 mmol) in dry THF (50 ml) was added drop-wise to a solution of MPEG350-CH_2COOH (1.50 g, 3.7 mmol) and NHS (0.45 g, 3.9 mmol) in the same solvent (50 ml) at 0–5 °C. After the mixed solution was stirred for 5 h at room temperature, a solution containing the tetrapeptide, GlyPheLeuAspEt$_2$ (1.8 g, 3.3 mmol) and 2 equivalent of triethylamine in dry chloroform (100 ml) was added to the mixed solution, which was stirred overnight at room temperature. The reaction mixture was filtered to remove the precipitated triethylammonium chloride, NHS and *N*,*N*′-dicyclohexylurea. The filtrate was evaporated under reduced pressure and then the residue was dissolved in chloroform, which was washed successively with diluted hydrochloric acid, distilled water, sodium bicarbonate solution, and finally distilled water. The chloroform layer was dried over anhydrous sodium sulfate and then evaporated to dryness in vacuum. The residual material was purified by column chromatography with methylene dichloride and methanol as eluent (MC/MA = 13/1, $R_f = 0.37$) to obtain copolymer **1**. Other copolymers were prepared analogously using different MPEG-CH_2COOH, oligopeptides and their molar ratios. Yield: 63.3%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.87 (d, 6H, Leu-$(CH_3)_2$), 1.26 (t, 6H, Asp-OCH_2CH_3), 1.5–1.7 (m, 3H, Leu-$CHCH_2$), 2.9 (m, 2H, Asp-CH_2), 3.15 (m, 2H, Phe-CH_2), 3.37 (s, 3H, MPEG350-OCH_3), 3.55–3.65 (m, 30H, MPEG350-OCH_2CH_2), 3.92 (d, 2H, Gly-CH_2), 4.2 (m, 4H, Asp-OCH_2CH_3), 4.4 (dd, 1H, Leu-CH), 4.62 (dd, 1H, Phe-CH), 4.77 (dd, 1H, Asp-CH), 7.22 (m, 10H, Phe-arom). Elem. Anal. (%) Calcd for $C_{42}H_{70}N_4O_{16}$: C, 56.87; H, 7.95; N, 6.32. Found: C, 56.55; H, 8.02; N, 6.06. LCST: 49 °C.

2.3.2. MPEG350-CH_2CO–GlyPheLeuGlyPheLeuEt (**2**)

MPEG350-CH_2COOH (1.42 g, 3.5 mmol) and GlyPheLeu-GlyPheLeuEt (2.76 g, 3.85 mmol) were used. Yield: 55.6%. ^{1}H NMR($CDCl_3$), δ (ppm): 0.87–0.94(m, 12H, Leu-$(CH_3)_2$), 1.23 (t, 3H, Leu-OCH_2CH_3), 1.4–1.67 (m, 6H, Leu-$CHCH_2$), 3.0 (m, 2H, Phe-CH_2), 3.2 (m, 2H, Phe-CH_2), 3.37 (s, 3H, MPEG350-OCH_3), 3.52–3.65 (m, 30H, MPEG350-OCH_2CH_2), 3.85 (br, 4H, Gly-CH_2), 4.15 (q, 2H, Leu-OCH_2CH_3), 4.5 (br, 2H, Leu-CH), 4.7 (br, 2H, Phe-CH), 7.22 (m, 10H, Phe-arom). Elem. Anal. (%) Calcd for $C_{53}H_{84}N_6O_{16}$: C, 59.59; H, 7.98; N, 7.92. Found: C, 60.96; H, 8.56; N, 7.77. LCST: 34 °C.

2.3.3. MPEG550-CH_2CO–GlyPheLeuGlyPheLeuEt (**3**)

MPEG550-CH_2COOH (2.15 g, 3.55 mmol) and GlyPheLeuGlyPheLeuEt (2.8 g, 3.9 mmol) were used. Yield: 48.0%. ^{1}H NMR($CDCl_3$), δ (ppm): 0.85–0.96 (m, 12H, Leu-$(CH_3)_2$), 1.26 (t, 3H, Leu-OCH_2CH_3), 1.4–1.67 (m, 6H, Leu-$CHCH_2$), 3.0 (m, 2H, Phe-CH_2), 3.2 (m, 2H, Phe-CH_2), 3.37 (s, 3H, MPEG550-OCH_3), 3.54–3.65 (m, 48H, MPEG550-OCH_2CH_2), 3.86 (br, 4H, Gly-CH_2), 4.15 (q, 2H, Leu-OCH_2CH_3), 4.5 (br, 2H, Leu-CH), 4.7 (br, 2H, Phe-CH), 7.2 (m, 10H, Phe-arom). Elem. Anal. (%) Calcd for $C_{63}H_{104}N_6O_{21}$: C, 59.04; H, 8.18; N, 6.56. Found: C, 58.76; H, 8.05; N, 6.52. LCST: 48 °C.

2.3.4. MPEG550-CH_2CO–GlyPheLeuGlyPheLeu-$CH_2C_6H_5$ (**4**)

MPEG550-CH_2COOH (2.5 g, 4.13 mmol) and GlyPheLeu-GlyPheLeu-$CH_2C_6H_5$ (3 g, 3.85 mmol) were used. Yield: 68.2%. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–0.88 (m, 12H, Leu-$(CH_3)_2$), 1.2–1.39 (m, 6H, Leu-$CHCH_2$), 3–3.2 (br, 4H, Phe-CH_2), 3.38 (s, 3H, MPEG550-OCH_3), 3.52–3.65 (m, 48H, MPEG550-OCH_2CH_2), 3.85 (br, 4H, Gly-CH_2), 4.52 (br, 2H, Leu-CH), 4.7 (br, 2H, Phe-CH), 5.13 (s, 2H, Leu-OCH_2-C_6H_5), 7.21 (m, 10H, Phe-arom), 7.47 (m, 5H, Leu-OCH_2-C_6H_5). Elem. Anal. (%) Calcd for $C_{68}H_{106}N_6O_{21}$: C, 60.79; H, 7.95; N, 6.25. Found: C, 60.51; H, 7.82; N, 6.21. LCST: 45 °C.

*2.3.5. MPEG750-CH_2CO–GlyPheLeuGlyPheLeuEt (**5**)*

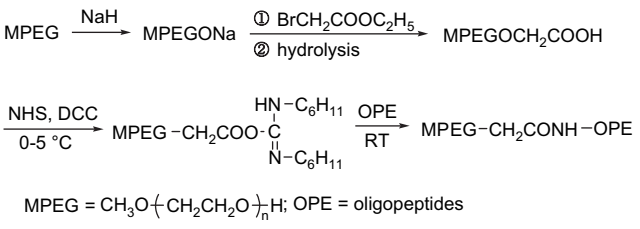
MPEG750-CH_2COOH (3.7 g, 4.58 mmol) and GlyPheLeuGlyPheLeuEt (3.6 g, 5.02 mmol) were used. Yield: 58%. ^1H NMR ($CDCl_3$), δ (ppm): 0.88–0.96 (m, 12H, Leu-$(CH_3)_2$), 1.26 (t, 3H, Leu-OCH_2CH_3), 1.4–1.67 (m, 6H, Leu-$CHCH_2$), 3–3.2 (br, 4H, Phe-CH_2), 3.38 (s, 3H, MPEG750-OCH_3), 3.53–3.66 (m, 67H, MPEG750-OCH_2CH_2), 3.85 (br, 4H, Gly-CH_2), 4.15 (q, 2H, Leu-OCH_2CH_3), 4.52 (br, 2H, Leu-CH), 4.7 (br, 2H, Phe-CH), 7.22 (m, 10H, Phe-arom). Elem. Anal. (%) Calcd for $C_{77}H_{124}N_6O_{25}$: C, 60.29; H, 8.15; N, 5.48. Found: C, 59.96; H, 8.09; N, 5.46. LCST: 58 °C.

3. Results and discussion

3.1. Synthesis and characterization

There are several methods reported for PEGylation of peptides and proteins [11,14,15], and we have attempted a few different methods for PEGylation of oligopeptides. The simplest route attempted was transesterification of oligopeptide ethyl esters with MPEG, but the resultant products were found to be not chemically stable in aqueous solution as expected. Therefore, we have prepared carboxylated PEG using bromoacetic acid [11], which was then linked to oligopeptides through amide coupling [12], as shown in Scheme 1.

We have prepared several diblock copolymers by different combinations of the hydrophilic MPEGs with different molecular weights and the hydrophobic oligopeptides selected from tetra- and hexapeptides, and listed them along with their characteristic properties in Table 1. All the products were obtained as yellow visco-elastic fluids in high yields (48.0–68.2%) and very soluble in water and polar organic solvents. The diblock copolymers were characterized by means of ^1H NMR spectroscopy and elemental analysis. A typical ^1H NMR spectrum



Scheme 1. Synthetic route to PEGylated oligopeptides.

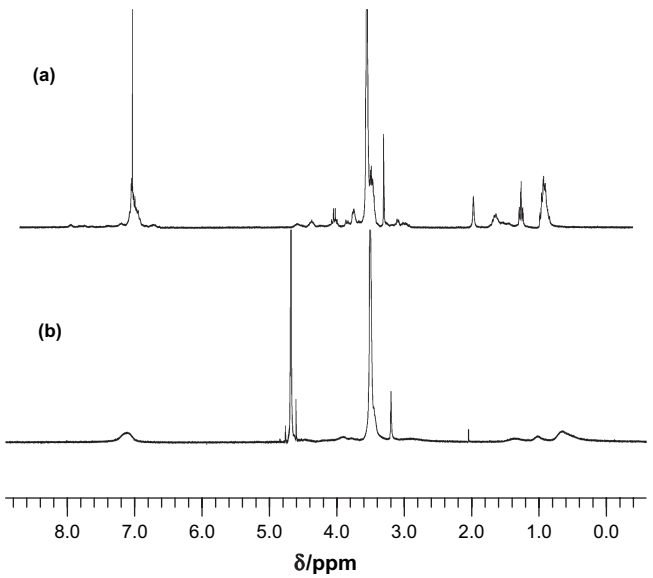


Fig. 1. ^1H NMR spectra of diblock copolymer **3** in $CDCl_3$ (a) and D_2O (b).

of copolymer **3** measured in $CDCl_3$ presented in Fig. 1(a) shows well resolved proton resonances clearly assignable, and their integration ratios were consistent with its theoretical chemical composition and elemental analysis data. However, the ^1H NMR spectrum of copolymer **3** measured in water shown in Fig. 1(b) exhibits partly disappearance or broadening of the oligopeptide proton resonances while the proton resonances of methoxy-poly(ethylene glycol) remain nearly unchanged both in intensity and chemical shift. Such results strongly indicate that the block copolymers form micelles in aqueous solution. Therefore, we have performed dynamic light scattering (DLS) measurements for all the copolymers and the results are also listed in Table 1.

3.2. Micellar properties

As shown in Table 1, copolymers **1** and **2** exhibited quite larger particle sizes compared to copolymers **3**–**5** according to their dynamic light scattering (DLS) measurements. Typical size distributions measured at 20 °C for representative copolymers **1** and **4** are illustrated in Fig. 2.

In order to further examine the nature of the particles formed from the present copolymers in aqueous solution, we

Table 1
Characteristic properties of copolymers with different MPEGs and oligopeptides

Copolymer	Molecular formula	LCST (°C)[a]		DLS[b] (nm)
		Water	PBS	
1	MPEG350-CH_2CO–GlyPheLeuAsp$(Et)_2$	48	43	64.0
2	MPEG350-CH_2CO–GlyPheLeuGlyPheLeuEt	34	31	47.5
3	MPEG550-CH_2CO–GlyPheLeuGlyPheLeuEt	48	42	16.4
4	MPEG550-CH_2CO–GlyPheLeuGlyPheLeu-$CH_2C_6H_5$	45	40	10.3
5	MPEG750-CH_2CO–GlyPheLeuGlyPheLeuEt	58	54	14.4

[a] The lower critical solution temperatures in water and PBS solution (5%).
[b] Mean diameters by volume measured using the dynamic light scattering method at 20 °C for 0.5% aqueous solutions filtered by a 0.45 μm syringe filter.

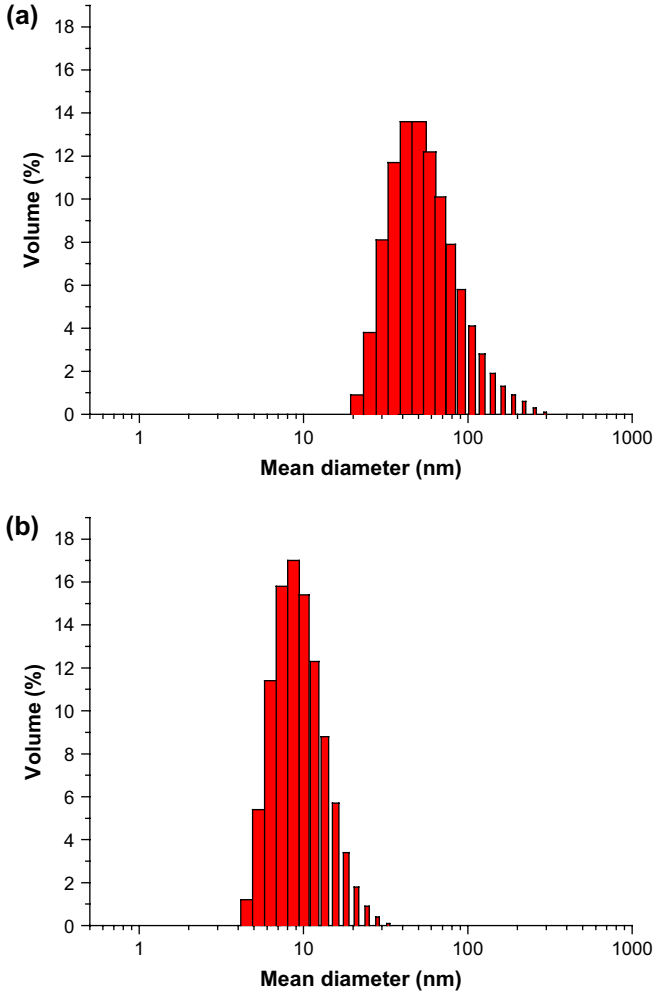


Fig. 2. Size distribution by volume of diblock copolymers **1** (a) and **4** (b).

have performed DLS measurements in the whole temperature range from 20 °C to their LCST. The temperature-dependent study has shown that the mean diameter of copolymer **1** increased gradually from 64 nm at 20 °C to about 100 nm at 40 °C followed by rapid aggregation to micro-particles at around its LCST (48 °C). Furthermore, such a temperature-dependent profile was not reproducible. Copolymer **2** also showed the similar behavior. On the other hand, the mean diameters of copolymers **3**–**5** were nearly not changed until their solutions were warmed up to their LCST as shown in Fig. 3. Therefore, we believe that copolymers **3**–**5** form stable micelles due to good hydrophilic to hydrophobic balance in aqueous solution, but copolymers **1** and **2** form unstable aggregates probably due to the lower hydrophilic (MPEG350) to hydrophobic (tetra- or hexapeptide) balance employed. In other words, MPEG350 seems not hydrophilic enough to make stable micelles with hydrophobic tetra- or hexapeptide, which means that the hydrophilic to hydrophobic balance of diblock copolymers is a critical factor to make stable micelles.

Now, it seems worthwhile to compare the present linear diblock copolymers composed of a hydrophilic PEG and a hydrophobic oligopeptide with the cyclotriphosphazene analogues bearing the same PEG and oligopeptide. Copolymer

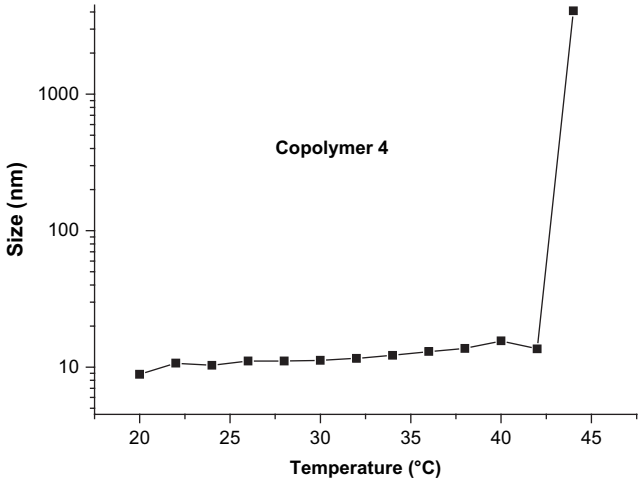


Fig. 3. Temperature-dependent micelle size of copolymer **4**.

1 composed of MPEG350 and the tetrapeptide, GlyPheLeuAsp-Et_2, did not form stable micelles but unstable aggregates as mentioned above. In contrast, we have shown in our previous works [10,16] that the cyclotriphosphazene analogue bearing the same MPEG350 and the tetrapeptide [NP(MPEG350)(GlyPheLeuAspEt_2)]$_3$, was found to form stable micelles with a mean diameter of 13.9 nm in aqueous solution. Such a difference seems to be attributed to the molecular structural difference as shown in Fig. 4. It is clearly seen from the figure that the three hydrophobic oligopeptide groups in *cis*-nongeminal conformation of the cyclotriphosphazene analogue can afford spatially more favorable intra- and intermolecular hydrophobic interactions for self-assembly to make stable micelles in aqueous solution. In case of copolymer **2**, its cyclotriphosphazene analogue bearing the same MPEG350 and the hexapeptide, GlyPheLeuGlyPheLeuEt, was found to form unstable aggregates probably due to the lowered solubility by the hydrophobic hexapeptide.

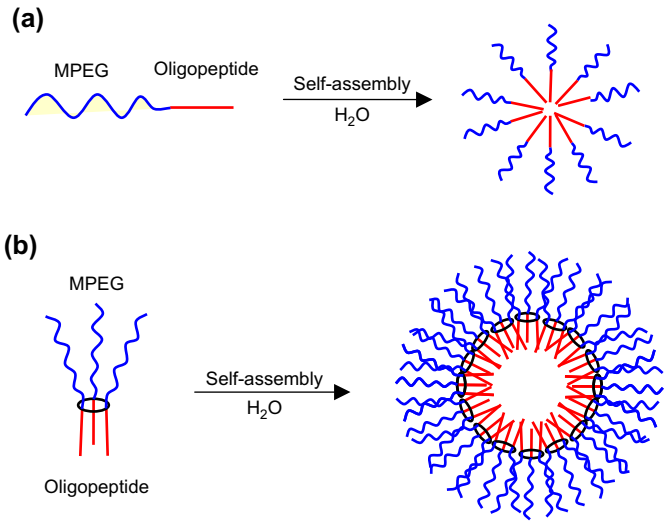


Fig. 4. Conceptual diagram for micelle formation of a linear diblock copolymer and its cyclotriphosphazene analogue.

However, copolymer **3** and its cyclotriphosphazene analogue bearing the same MPEG550 more hydrophilic than MPEG350 and the same hexapeptide not only formed stable micelles but also exhibited the same LSCT at 48 °C, which will be further discussed in the next section. Therefore, it may be presumed that the structural effect of the cyclotriphosphazene analogues bearing the same hydrophilic and hydrophobic groups in *cis*-nongeminal conformation diminishes as the lengths of the hydrophilic and hydrophobic blocks of copolymers increase.

The critical micelle concentration (CMC) is very important for biomedical applications. Therefore, we have measured the CMCs of representative copolymers **3** and **4** that form stable micelles in aqueous solution using the pyrene fluorescence technique [13]. The CMC value was taken as the concentration at which a sharp increase in the intensity ratio of I_{339}/I_{335} was observed, as shown in Fig. 5. The CMC value of copolymer **4** with benzyl ester group was found to be 86.3 mg/L remarkably lower than that of copolymer **3** with ethyl ester group (195 mg/L), which is still much lower compared with those of low molecular weight surfactants (>1 g/L). Unfortunately, we could not measure the CMC for the cyclotriphosphazene analogue bearing the same MPEG550 and the hexapeptide by the florescence method because the analogue itself emits fluorescence in the UV region.

3.3. Thermosensitive properties

As seen from Table 1, all the copolymers exhibited thermosensitivity by showing a lower critical solution temperature (LCST) in water with a significant salting-out effect [17] in PBS solution in the range of 31–58 °C including body temperature. The LCST of the diblock copolymers was found to be largely dependent upon their hydrophobic and hydrophilic segments. For instance, comparing copolymers **1** and **2** with the same hydrophilic MPEG350 but different oligopeptides, copolymer **2** with the highly hydrophobic hexapeptide, GlyPheLeuGlyPheLeuEt (log $P = 1.84$; $P = [\text{solute}]_{n\text{-octanol}}/[\text{solute}]_{\text{water}}$)

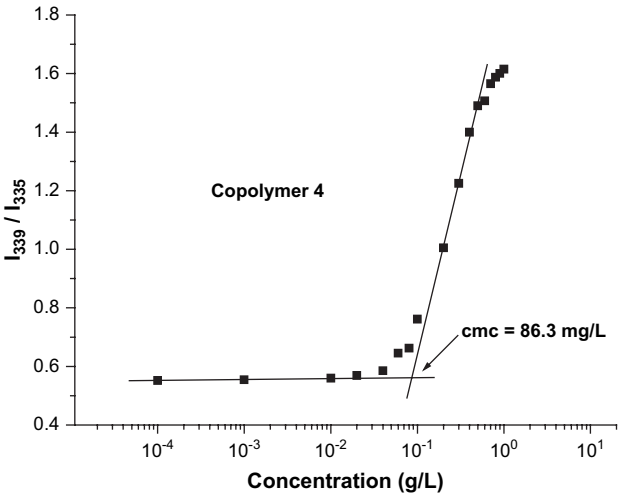


Fig. 5. Determination of the critical micelle concentration (CMC) of copolymer **4**.

Table 2
The lower critical solution temperatures of the present diblock copolymers and cyclotriphosphazene analogues (°C)

Hydrophilic–hydrophobic segments	Diblock copolymers	Cyclotriphosphazenes	ΔT[a]
MPEG350–$GlyPheLeuAspEt_2$	48	29	19
MPEG350–GlyPheLeuGlyPheLeuEt	34	20	14
MPEG550–GlyPheLeuGlyPheLeuEt	48	48	0

[a] The difference in LCST of the linear diblock copolymer and the cyclotriphosphazene analogue bearing the same MPEG and oligopeptide.

[18] showed remarkably lower LCST at 34 °C than the LCST (48 °C) of copolymer **1** with much less hydrophobic tetrapeptide, $GlyPheLeuAsp(Et)_2$ (log $P = 0.32$). Similarly, for the same hydrophobic block, GlyPheLeuGlyPheLeuEt, more hydrophilic MPEG550 of copolymer **3** gave rise to much higher LCST (48 °C) than the LCST (34 °C) of copolymer **2** having MPEG350. However, comparing copolymers **3** and **4**, the hydrophobicity of the ester groups seems not to affect very much the LCST.

Now, it may be interesting to compare the present diblock copolymers with the cyclotriphosphazene analogues bearing the same hydrophilic and hydrophobic blocks as side groups [10,16]. The present linear copolymer **1** composed of MPEG350 and the tetrapeptide, $GlyPheLeuAspEt_2$, exhibited its LCST at 48 °C in pure water, which is far higher than the LCST (29 °C) of the cyclotriphosphazene analogue $[NP(MPEG350)(GlyPheLeuAspEt_2)]_3$ [16]. However, the lowering of the LCST of the cyclotriphosphazene analogue was reduced as the hydrophobic chain length increased: copolymer **2** bearing the same MPEG350 and the more hydrophobic hexapeptide exhibited its LCST at 34 °C moderately higher than its cyclotriphosphazene analogue at 20 °C. Further interesting is that copolymer **3** bearing the more hydrophilic MPEG550 and the same hexapeptide shows the same LCST at 48 °C as the cyclotriphosphazene analogue bearing the same MPEG550 and the hexapeptide. Thus the differences in LCST between the linear copolymers and their cyclotriphosphazene analogues decrease as both hydrophobic and hydrophilic chain lengths increase as shown in Table 2. Such a reason is not clearly explainable at the moment, but it seems that the structural effect of the *cis*-nongeminal conformation of cyclotriphosphazene analogues becomes insignificant for the intermolecular hydrophobic interactions when the side groups are long enough. Such a trend is in accordance with the micelle-forming properties observed for the copolymers and their cyclotriphosphazene analogues as mentioned above.

4. Conclusions

New linear diblock copolymers were prepared by PEGylation of hydrophobic oligopeptides through amide coupling of carboxylated PEGs with oligopeptides and compared with the cyclotriphosphazene analogues bearing the same PEG and oligopeptide as side groups in *cis*-nongeminal conformation. Stable micelles were formed only from copolymers composed of MPEG550 or MPEG750 as a hydrophilic segment and

the hexapeptide, GlyPheLeuGlyPheLeuEt, as a hydrophobic segment, but MPEG350 seems not hydrophilic enough to make stable micelles with the hydrophobic oligopeptides employed. All the present diblock copolymers exhibited thermosensitive properties by showing a lower critical solution temperature (LCST) in water. The LCST of the copolymers composed of relatively shorter MPEG350 and GlyPheLeuAsp-Et_2 was observed at much higher temperature at 48 °C compared with the LCST (29 °C) of the cyclotriphosphazene analogues bearing the same hydrophilic and hydrophobic segments as side groups. The remarkably lower LCST of the cyclotriphosphazene analogue is presumed to be due to the structural effect of the *cis*-nongeminal conformation of its three hydrophobic oligopeptides, but such a structural effect was found to diminish as the chain lengths of the hydrophilic and hydrophobic blocks of the copolymers increased.

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