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Synthesis and physicochemical properties of new tripodal amphiphiles bearing fatty acids as a hydrophobic group

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ABSTRACT

Saturated fatty acids (FA) were grafted using tyrosine as a spacer group to the cyclotriphosphazene ring along with equimolar hydrophilic methoxy poly(ethylene glycol) (MPEG) in *cis*-nongeminal way. Seven new cyclotriphosphazene amphiphiles were prepared from combinations of hydrophilic MPEGs with different molecular weights of 350, 550, 750 and 1000 and four different fatty acids of different hydrophobicity including lauric, myristic, palmitic and stearic acids. These steric amphiphiles bearing fatty acids as a hydrophobic group were found to form more stable micelles with very low critical micelle concentrations (CMC) (2.95–7.80 mg/L) compared with oligopeptide analogues, and their highly hydrophobic core environment is unique and potentially useful for various biomedical applications.

Polymeric micelles have been extensively studied for last decades aiming at clinical applications as drug delivery systems for polymer therapeutics.[1,2] Polymeric micelles exhibit various advantageous properties associated with solubilization of hydrophobic drugs, potential tumor targeting by enhanced permeability and retention (EPR) effect, controlled drug release, and possible long blood circulation.[3–6] However, success of clinical applications of polymeric micelles has been limited, because it is not easy to design a polymeric micelle that can satisfy simultaneously all the clinical requirements including stability, selectivity and releasing kinetics of the drug-loaded micelle as well as biodegradability and nontoxicity of the polymeric micelle itself.

Polymeric micelles are one of the soft nanostructures self-assembled from amphiphilic block copolymers or graft copolymers, but most of the spherical micelles are composed of linear di- or tri-block copolymers constituted of hydrophilic and hydrophobic segments. Intermolecular hydrophobic interaction among the hydrophobic segments of the copolymer molecules (unimer) in aqueous solution lead to formation of the hydrophobic core with the hydrophilic shell of spherical micelles. Typical size of polymeric micelles is between 20 and 100 nm in diameter and their critical micelle concentration (CMC) is in the range of 10–100 mg/L depending on the kind and molecular weight of the block copolymers.[3,6] The hydrophobic core of polymeric micelles can accommodate hydrophobic drugs, which therefore can be solubilized in aqueous solution.

However, one of the most essential properties of polymeric micelles required for clinical application is the stability of drug-loaded polymeric micelles rather than that of the polymeric micelles themselves. For example, most of the conventional di-block copolymers including PEG-b-PLA,[7] PEG-b-CL,[8] and PEO-b-PBO[9] are known to form stable micelles in aqueous solution, but when loaded with docetaxel, these polymeric micelles cannot hold the drug, which precipitates within 24 h after drug loading. In fact, it is known that important physicochemical properties such as stability, loading capacity and releasing kinetics of drug-loaded polymeric micelles are greatly dependent upon the properties both of the micelle core and the drug molecules loaded.[4,10]

Therefore, in order to overcome such limited properties attributed from the conventional linear block copolymers and to expand the properties of the micelle core environment, it is urgent to develop new types of polymeric micelles. In this context, we have recently developed a new class of tripodal amphiphiles of a general formula $[N=P(PEG)(OPE)]_3$ bearing poly(ethylene glycol) (PEG) as a hydrophilic group and oligopeptide (OPE) as a hydrophobic group.[11] These novel steric amphiphiles were found to self-assemble in aqueous solution into very stable spherical micelles with a mean diameter of 7.4 to 13.9 nm,[12] and the resultant hydrophobic core composed of multifunctional oligopeptide groups provides a unique polar hydrophobic core environment, which can stably

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encapsulate docetaxel.[10] In this study we have found that extremely hydrophobic lipid core environment can be created to accommodate nonpolar hydrophobic drugs in the micelle core by introducing saturated fatty acids instead of oligopeptides into the cyclic phosphazene template as a hydrophobic group. Herein we report synthesis and properties of steric amphiphiles bearing equimolar methoxy poly(ethylene glycol) and saturated fatty acid.

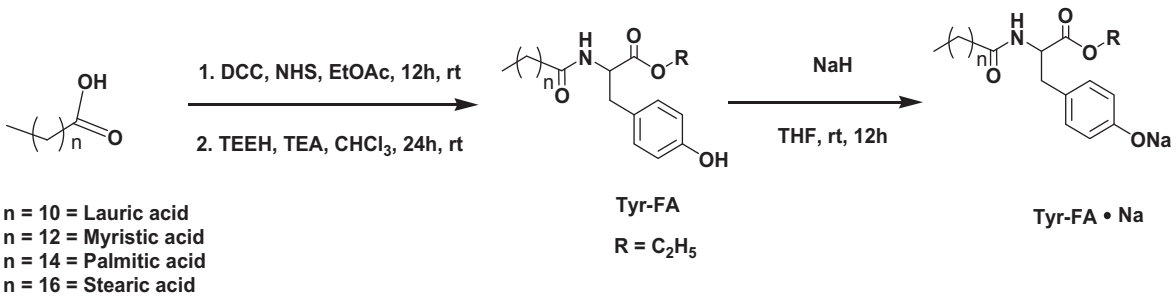
Since saturated fatty acids have only one functional group, carboxylic acid, which is known to lead to phosphazene ring cleavage reaction,[13] it was necessary to employ a spacer group with two functional groups such as tyrosine to link fatty acid to the phosphazene ring. Among the saturated fatty acid ethyl esters, $CH_3(CH_2)_nCOOEt$, lauric ($n = 10$), myristic ($n = 12$), palmitic ($n = 14$), and stearic ($n = 16$) acids were selected considering their hydrophobicity defined as $\log \boldsymbol{P}$ where $\mathbf{P} = [\text{solute}]_{n\text{-octanol}}/[\text{solute}]_{\text{water}}$[14] and the hydrophilicity of methoxy poly(ethylene glycol) (MPEG) to be employed. Thus, in order to link fatty acid to the cyclic phosphazene ring, we have prepared in the first step tyrosine-fatty acid conjugates according to the following reaction Scheme 1 (Supplementary data). Fatty acids were easily coupled with tyrosine through amide bond using N-hydroxysuccinimide (NHS) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) as coupling agents. The resultant tyrosine-fatty acid conjugates were transformed to sodium salts for nucleophilic substitution with the chlorine atoms of the cyclic phosphazene trimer.

In order to obtain pure tripodal cyclotriphosphazene amphiphiles, it is important to perform the initial PEGylation reaction of the cyclotriphosphazene ring at low temperature (<−20 °C) so that the first three chlorine atoms of hexachlorocyclotriphosphazene can be substituted with PEG in *cis*-nongeminal way. Then the tyrosine-fatty acid conjugates (Tyr-FA) were grafted by substitution with the remaining three chlorine atoms of the cyclotriphosphazene ring according to the following reaction Scheme 2 (Supplementary data). The ^{1}H and ^{31}P NMR spectra measured using Varian 500 MHz NMR spectrometer are illustrated in Figure 1. In particular, it is clearly seen from the single peak of phosphorus resonance that both of hydrophilic and hydrophobic side groups are grafted in *cis*-nongeminal way to form a tripodal steric amphiphile as shown in the Scheme.

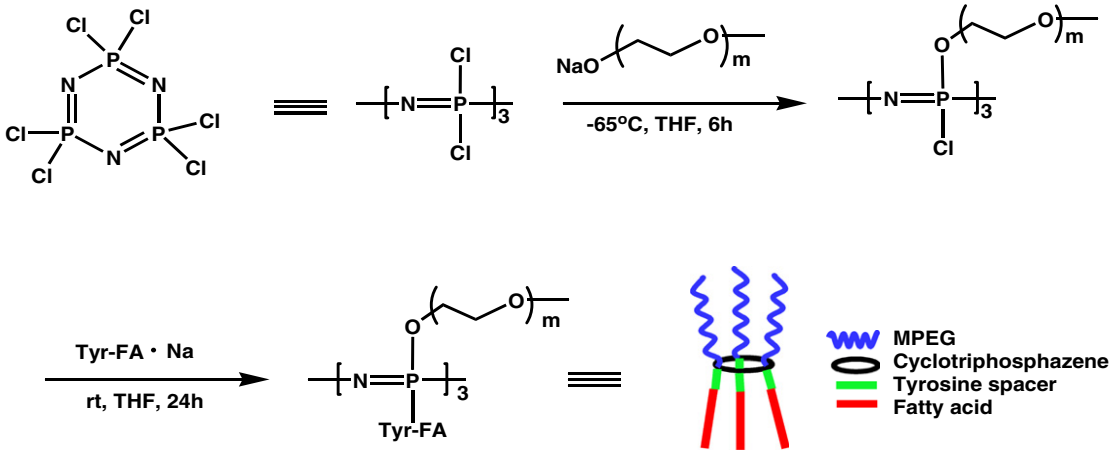
We have attempted to prepare water soluble analogues of amphiphilic cyclotriphosphazenes bearing fatty acids by different combinations from four MPEGs of different hydrophilicity and four fatty acids of different hydrophobicity shown in Table 1. We have found that water insoluble products are resulted when the overall hydrophobicity ($\log \boldsymbol{P}_{\text{sum}}$), that is, the sum of the hydrophobicity of the hydrophobic group, Tyr-FA ($\log \boldsymbol{P}_{\text{Tyr-FA}}$) and that of the hydrophilic group MPEG ($\log \boldsymbol{P}_{\text{MPEG}}$) is larger than 4.5. For example, MPEG350 ($\log \boldsymbol{P} = -1.42$) gave a water soluble cyclotriphosphazene product with Tyr-Lau conjugate ($\log \boldsymbol{P} = 5.26$) but insoluble products with Tyr-Myr conjugate ($\log \boldsymbol{P} = 6.17$) and other more hydrophobic tyrosine-fatty acid conjugates (Tyr-FA) in the table. However, MPEG550 and MPEG750 produced water soluble derivatives with both Tyr-Lau and Tyr-Myr conjugates. More hydrophobic Tyr-Pal and Tyr-Ste conjugates gave rise to water soluble products only with more hydrophilic MPEG1000. Thus we have prepared from appropriate combinations of MPEGs and Tyr-FA conjugates shown in the table seven soluble amphiphilic cyclotriphosphazenes, which were clearly characterized by means of elemental analysis, ^{1}H and ^{31}P NMR, dynamic light scattering (DLS), critical micelle concentration (CMC), etc. (Supplementary data). All these amphiphilic cyclotriphosphazene products were obtained in little sticky white solids, which are very soluble in water and polar organic solvents.

The morphology of the present trimer amphiphiles bearing fatty acids was found to be spherical micelles, as shown in the TEM image of $[NP(MPEG1000)(Tyr\text{-}Ste)]_3$ measured by JEM-2100F[15] and illustrated in Figure 2. Also the particle size distributions of the present fatty acid grafted amphiphiles were measured for 0.5% aqueous solution by dynamic light scattering (DLS) method using Malvern Zetasizer (Nano-ZS), and the representative results are displayed in Figure 3. Thus the fatty acid grafted amphiphiles exhibit the same morphology as the oligopeptide analogues but have shown quite different behavior in aqueous solution. For example, we have shown that, in case of oligopeptide analogues,[16] the morphology of the trimer amphiphiles is determined mainly by the hydrophobicity of the oligopeptide employed ($\log \boldsymbol{P}_{\text{olig}}$). Thus if $\log \boldsymbol{P}_{\text{olig}}$ is in the range of 0–1, the trimer amphiphiles initially form spherical micelles of 10–20 nm in aqueous solution but slowly reassemble into larger double layered polymersomes of 100–1000 nm in diameter. However, if $\log \boldsymbol{P}_{\text{olig}}$ is larger than 1, the initially formed micelles of 7–8 nm in diameter remain unchanged in morphology. In contrast to such a variable morphology of the trimer amphiphiles bearing oligopeptides, the trimeric analogues of tyrosine-fatty acid conjugates with very high hydrophobicity ($\log \boldsymbol{P}_{\text{Tyr-FA}} = 5.26$–$7.84$) were found to form only highly stable micelles with extremely law CMC values in aqueous solution as will be shown later. Such a different behavior in morphology seems to be attributed to the difference in the chemical nature of the two hydrophobic groups. In other words, oligopeptides have many functional groups such as amine and carbonyl groups around the peptide backbone, which can afford partially polarized micelle core environments while fatty acids are mainly composed of aliphatic hydrocarbon chains without polar groups. Such a difference in the chemical environment of the micelle cores composed of the trimer amphiphiles bearing fatty acids and oligopeptides is expected to affect the physicochemical properties such as micelle size and stability, thermal sensitivity, drug loading capacity, and drug releasing kinetics of the micelles.

In order to examine the physicochemical properties of the fatty acid grafted cyclotriphosphazene amphiphiles as new drug delivery systems, in addition to the DLS measurements abovementioned, we have measured their lower critical solution



Scheme 1. Synthetic route to conjugation of fatty acids to a spacer group tyrosine.



Scheme 2. Synthetic route to amphiphilic cyclotriphosphazenes bearing fatty acids as a hydrophobic group.

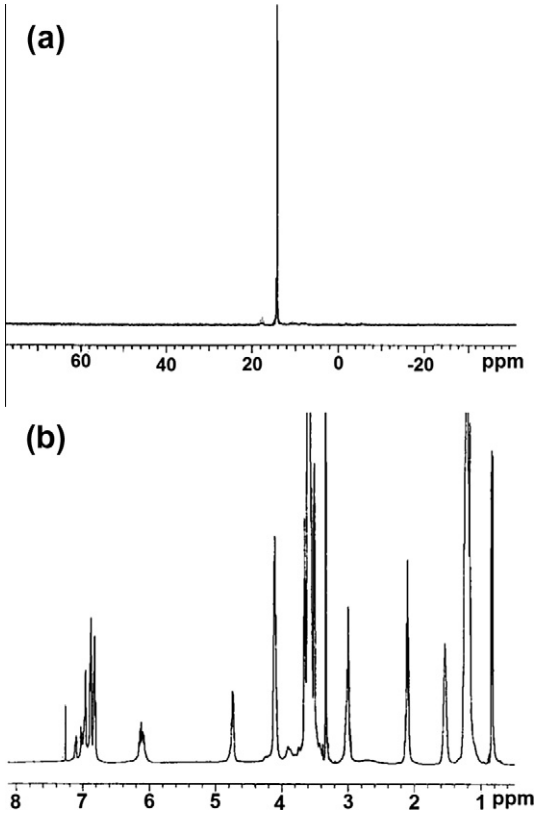


Figure 1. ^{31}P NMR (a) and ^{1}H NMR (b) spectra of amphiphilic cyclotriphosphazene **1**.

temperature (LCST) by cloud point method[17] for 5% polymer solution in distilled water and PBS contained in a glass capillary immersed in an oil bath. We have also measured their critical micelle concentration by the fluorescence probe technique using pyrene.[18] All the measured data of the present cyclotriphosphazene amphiphiles are listed in Table 2.

The micelle size of the trimer amphiphiles bearing oligopeptide was reported to be in a wide range of 7.4–27.8 nm in diameter depending on their chemical structure and hydrophobicity of the hydrophobic oligopeptide,[12] but the present fatty acid analogues exhibit quite smaller size of 5.47–10.3 nm in diameter with narrower size distribution (Fig. 3) probably due to their much higher hydrophobicity. Also the critical micelle concentration (CMC) of the fatty acid analogues measured by the fluorescence probe technique (Fig. 4) displayed much lower values of 2.95–7.80 mg/L compared with 7–20 mg/L of the oligopeptide analogues, which indicates that the micelle stability of the present fatty acid analogues is much higher compared with that of the oligopeptide analogues. To our knowledge, the CMC values of the present fatty acid grafted cyclotriphosphazenes are among the lowest of the known polymeric micelles.

The present trimer amphiphiles bearing fatty acids are thermosensitive like the oligopeptide analogues. As seen in Table 2, the LCSTs of all the fatty acid analogues are abnormally high (39.4–82.2 °C) compared with those of the oligopeptide analogues.[12] It is generally known that the LCST of an amphiphile is lowered if the hydrophobicity of the hydrophobic group is increased for the same hydrophilic group of the amphiphile due to easier aggregation of the micelle particles for precipitation by stronger intermolecular hydrophobic interactions among the unimers.[12,19] On the other hand, if the hydrophilicity of the hydrophilic group is increased for the same hydrophobic group, the LCST of the amphiphile is increased because of stronger intermolecular interaction between the hydrophilic group of the micelle corona and the solvent water molecules, which inhibit aggregation of the micelle particles. According to this rule, the LCSTs of all the fatty acid analogues in Table 2 should be lower than those of the oligopeptide analogues with the same hydrophilic group MPEG because of much higher hydrophobicity of the fatty acids compared with the oligopeptide employed. However, comparing directly two representative analogues bearing the same hydrophilic group MPEG550 but different hydrophobic groups Tyr-Lau (log***P*** = 5.26) and $(GlyPheLeu)_2Et$ (log***P*** = 1.11), the LCST of the fatty acid analogue $[NP(MPEG550)(Tyr\text{-}Lau)]_3$ appears at much higher 72.6 °C in water than that of the hexapeptide analogue $[NP(MPEG550)(GlyPheLeu)_2Et]_3$ at 48 °C.[12] Similarly, other fatty acid analogues exhibit higher LCSTs compared with the oligopeptide analogues bearing the same MPEG. Such a surprising result seems to be attributed to the different chemical nature of the two hydrophobic groups. It may be conjectured that if the hydrophobic group of the amphiphile is too hydrophobic like fatty acids, strong intermolecular hydrophobic interaction within the micelle core may hamper the dynamic interaction among micelles to precipitate. As seen in Table 2, the LCSTs of the fatty acid analogues range from body temperature to over 80 °C, and all the fatty acid analogues except for trimer **1** are potentially useful for intravenously injectable drug carrier.

Finally, the drug loading capacity and the stability of drug-loaded micelles are more critically dependent on the nature of

Table 1
Hydrophobicity of tyrosine-fatty acid conjugates and methoxy poly(ethylene glycol)s employed as side groups

Hydrophobic and hydrophilic groups[a] employed as side groups	Empirical formula	Mol. Wt.	Hydrophobicity ($\log \boldsymbol{P}$)[b]
Tyrosine-lauramide (Tyr-Lau)	$C_{23}H_{37}NO_4$	391.5	5.26
Tyrosine-myristamide (Tyr-Myr)	$C_{25}H_{41}NO_4$	419.6	6.17
Tyrosine-palmitamide (Tyr-Pal)	$C_{27}H_{45}NO_4$	447.6	7.00
Tyrosine-stearamide (Tyr-Ste)	$C_{29}H_{49}NO_4$	475.7	7.84
MPEG350	$C_{15}H_{32}O_8$	340.2	−1.42
MPEG550	$C_{25}H_{52}O_{13}$	560.5	−2.24
MPEG750	$C_{33}H_{68}O_{17}$	736.4	−2.80
MPEG1000	$C_{45}H_{92}O_{23}$	1000.5	−4.00

[a] Empirical formula of methoxy poly(ethylene glycol) was estimated from the general formula of $CH_3O(CH_2CH_2O)_nH$ assuming $n = 7$ for MPEG350; $n = 12$ for MPEG550; $n = 16$ for MPEG750; $n = 22$ for MPEG1000.
[b] Hydrophobicity is defined as $\log \boldsymbol{P}$ where $\mathbf{P} = [\text{solute}]_{n\text{-octanol}}/[\text{solute}]_{\text{water}}$.[14]

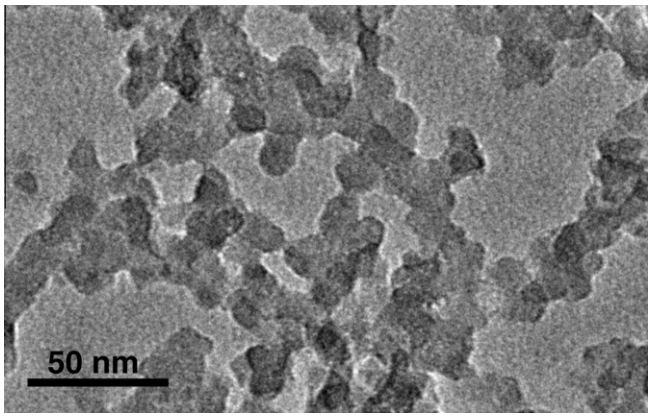


Figure 2. Transmission electron microscopic image of $[NP(MPEG1000)(Tyr\text{-}Ste)]_3$.

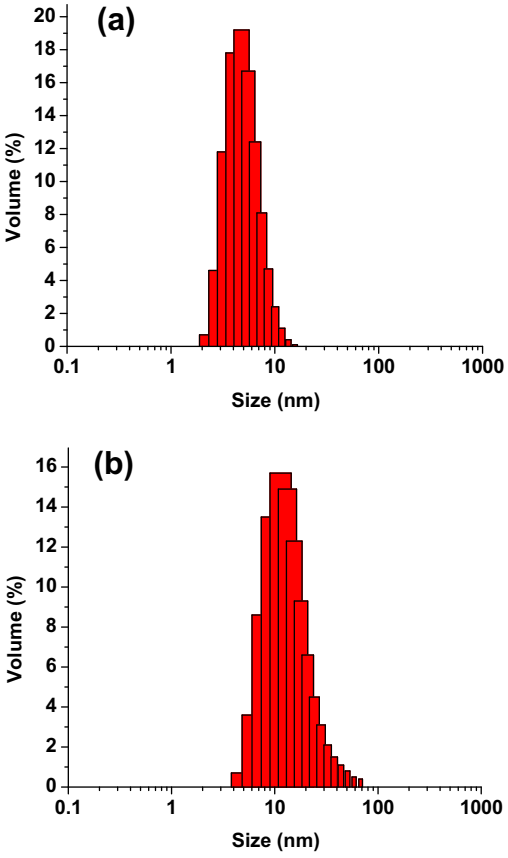


Figure 3. The particle size distributions of amphiphilic cyclotriphosphazenes **1** (a) and **7** (b).

the micelle core because of the intermolecular interactions between the drug molecules and micelle core environment as were illustrated in recent reports. For example, adriamycin (ADR) could not be properly loaded into the micelle composed of poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymers (PEG-PBLA) because of poor affinity of the hydrophobic PBLA segment with ADR.[4] As early mentioned in the introductory section in this Letter, docetaxel can be solubilized by micelle-encapsulation using the conventional block copolymers,[7,8] but their micelle cores cannot hold docetaxel probably due to weak intermolecular interaction between the docetaxel molecules and micelle core environment. However, we have shown that our cyclotriphosphazene amphiphile bearing hydrophilic MPEG750 and hydrophobic hexapeptide represented as $[NP(MPEG750)(GlyPheLeu)Et]_3$ (CP750) has not only a large loading capacity of docetaxel (>25%) but also high stability of drug-loaded micelles[10] probably because of the locally polarized core environment composed of the hexapeptide groups as above-mentioned. Interestingly, we have most recently found that the present fatty acid analogues $[NP(MPEG750)(Tyr\text{-}FA)Et]_3$ can solubilize docetaxel by micelle-encapsulation like the hexapeptide analogue CP750 but cannot hold the drug molecules in their micelle core unlike CP750, and docetaxel precipitated in a few days.

On the contrary, if the drug to be formulated by micelle-encapsulation changes from docetaxel ($\log \boldsymbol{P} = 2.59$) to a highly hydrophobic drug like propofol (2,6-diisopropyl phenol) ($\log \boldsymbol{P} = 4.11$), the situation may be reversed. Propofol is currently one of the most widely used anesthetic agent which is marketed as 1% propofol formulated in oil emulsion.[20] We have tested loading capacity and stability of propofol micelle-encapsulated by the present fatty acid grafted amphiphile $[NP(MPEG750)(Tyr\text{-}Lau)Et]_3$ and the hexapeptide analogue CP750 for comparison. Propofol could be loaded into CP750 up to 10% of the carrier by our solvent evaporation method,[10] but when the propofol formulated by micelle-encapsulation using CP750 was attempted to dissolve up to 1% propofol in distilled water, no clear solution was obtained due to precipitation of propofol. In other words, the propofol loaded into CP750 was not stable. On the other hand, the loading capacity (13%) of propofol into the present fatty acid grafted amphiphile $[NP(MPEG750)(Tyr\text{-}Lau)Et]_3$ was higher compared with CP750, and furthermore, clear and stable 1% propofol solution was obtained without precipitation of propofol at least for a few months. The highly hydrophobic core environment composed of the present hydrophobic group Tyr-Lau ($\log \boldsymbol{P} = 5.26$) seems to be more favorable to propofol compared to docetaxel ($\log \boldsymbol{P} = 2.59$), while the polar micelle core composed of the polar hexapeptide with less hydrophobicity ($\log \boldsymbol{P} = 1.11$) favors docetaxel to propofol. We believe that the present fatty acid grafted amphiphiles are useful for micelle formulation particularly of any highly hydrophobic drugs.

Table 2
Characteristic properties of amphiphilic cyclotriphosphazenes bearing equimolar hydrophilic MPEG and hydrophobic fatty acid

Trimer No.	Compounds	Mol. Wt.	$\log P_{sum}$[a]	LCST (°C)[b]		DLS[c] (nm)	CMC[d] (mg/L)
				[H_2O]	[PBS]		
1	$[NP(MPEG350)(Tyr\text{-}Lau)]_3$	2323.3	3.84	39.4	37.2	5.47	2.95
2	$[NP(MPEG550)(Tyr\text{-}Lau)]_3$	2983.7	3.02	72.6	65.1	8.02	6.77
3	$[NP(MPEG550)(Tyr\text{-}Myr)]_3$	3067.8	3.93	69.5	64.3	8.91	4.73
4	$[NP(MPEG750)(Tyr\text{-}Lau)]_3$	3512.0	2.46	81.4	76.2	8.58	7.80
5	$[NP(MPEG750)(Tyr\text{-}Myr)]_3$	3596.1	3.37	74.7	71.5	9.21	5.51
6	$[NP(MPEG1000)(Tyr\text{-}Pal)]_3$	4472.7	3.00	82.2	76.6	9.42	4.33
7	$[NP(MPEG1000)(Tyr\text{-}Ste)]_3$	4556.8	3.84	77.3	71.7	10.3	3.32

[a] $\log P_{sum} = \log P_{Tyr\text{-}FA} + \log P_{MPEG}$.
[b] The lower critical solution temperature was identified as the temperature at which the polymer solution (5.0 wt %) became turbid.
[c] The mean diameter of the particles measured for 0.5% solutions at 25 °C by dynamic light scattering method.
[d] The critical micelle concentration was measured by the pyrene method[18] at ambient temperature.

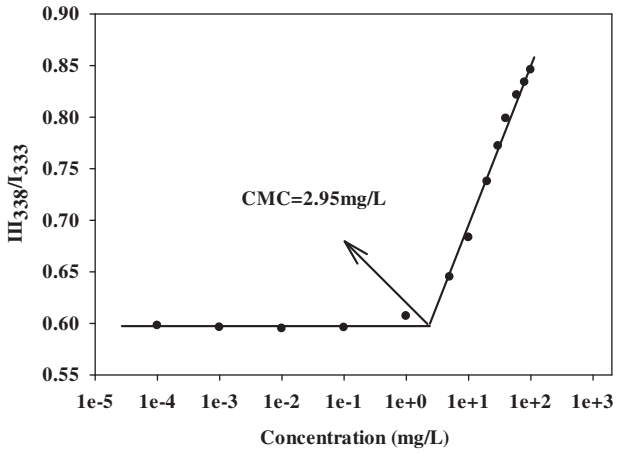


Figure 4. The critical micelle concentration (CMC) of cyclotriphosphazene **1** measured by fluorescence probe technique using pyrene.[18]

In conclusion, it is very important to design or apply polymeric micelles for clinical applications based on the intermolecular interactions between the target drug molecules and the chemical environment of the micelle core composed of the hydrophobic segments of the polymer amphiphiles.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.052.

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