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Thermally Regulated Reversible Formation of Vesicle-Like Assemblies by Hexaproline Amphiphiles

Carles Felip-León, Francisco Galindo, and Juan F. Miravet*

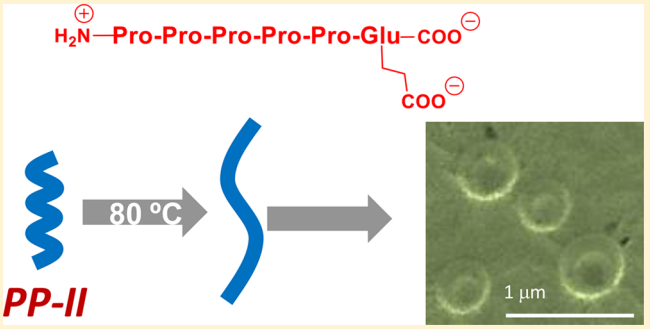
Departament de Química Inorgànica i Orgànica, Universitat Jaume I, Avda Sos Baynat s/n, 12071 Castelló, Spain

Valeria Castelletto and Ian W. Hamley*

Department of Chemistry, University of Reading, Whiteknights, Reading RG6 6AD, U.K.

Supporting Information

ABSTRACT: Peptides composed of hexaproline and glutamic acid (P_6E) or lysine (P_6K) as C-terminal units show thermally promoted aggregation, affording vesicle-like assemblies upon heating to 80 °C. The aggregation is analyzed by dynamic light scattering (DLS), with number-averaged diameters of ca. 600 and 300 nm, respectively, for P_6E and P_6K. NMR studies reveal that upon heating the amount of NMR-visible species is reduced to ca. 50% and that an important conformational change is experienced by the molecules in solution. Circular dichroism (CD) shows that at 20 °C the peptides present a polyproline II (PP-II) conformation which is disorganized upon heating. Scanning electron microscopy for samples which were fast frozen at 80 °C reveals vesicle-like assemblies. Using pyrene as a fluorescence probe, a critical aggregation concentration of ca. 30 μM was estimated for P_6E, while that of P_6K was above 0.6 mM. The aggregation process is found to be fully reversible and could serve as a basis for development of stimuli responsive carriers.

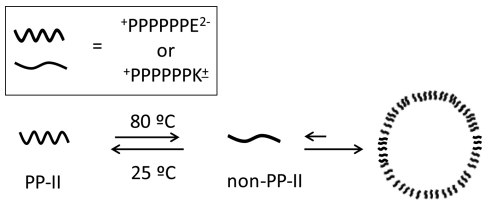


INTRODUCTION

Peptide self-assembly has received increasing interest in the last years due to its relevance in difference biological processes. In particular, aggregation of peptides to give fibers is common as a result of 1-D growth of fibrils promoted by intermolecular H-bonding interactions. Fundamental studies of this process have implications in the diseases such as amyloid-type fibrillization and in the formation of peptide-derived supramolecular gels.[1,2] Vesicle formation by peptides is much less common than fibrillization. Several cases have been reported using, for example, glycine-rich surfactant-like peptides,[3,4] sequenced peptides,[5] and diphenylglycine in aqueous solution[6] or Boc-diphenylalanine [Boc: *tert*-butoxycarbonyl] in organic solvents.[7,8]

Here we report on unprecedented formation of vesicle-like structures by oligoproline amphiphiles (Scheme 1). Polyproline (PP) domains are present in proteins prone to fibrillation such as collagen and play an important role in protein−protein and protein−nucleic acid interactions.[9] PP peptides are considered to be rather rigid domains in protein structures and have received attention due to their use as molecular rulers for fluorescence resonance energy transfer (FRET) calibration studies.[10,11] PP in water presents predominantly the so-called PP-II arrangement, a left-handed helix with an all-trans amide configuration and a repeat distance of 3.1 Å.[12] The PP-I structure, with an all-cis amide configuration, is a right-handed helix observed in organic solvents.[13] The aggregation of PP motifs in water has been scarcely studied. Tertiary amides lack amide protons for intermolecular H-bonding, and one would expect that PP cannot aggregate in a similar way to other peptides. However, it has been described recently that PP precipitates in aqueous solution to give a thin film above a certain concentration threshold (ca. 1 mg/mL) at high temperatures.[14] In earlier reports, the precipitation upon heating above ca. 55 °C of PP in water was studied by IR.[15,16] The interaction of the carbonyl groups of the PP with the solvent seems to be a key issue in the observed behavior. Upon heating, the weakening of these interactions leads to

Scheme 1. Schematic of the Process of Formation of Vesicle-Like Assemblies for Peptide P_6E



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precipitation which is probably associated also with a slight conformational change. Circular dichroism spectroscopy and vibrational circular dichroism (VCD) spectroscopy along with Raman and FTIR methods have been used to investigate the conformation of a model triproline peptide in water.[17] VCD has been used to investigate in detail the conformation of oligoproline peptides (P_2–P_{12}) in aqueous solutions.[18,19] A reversible conformational transition from PP-I to PP-II upon heating has been observed for a P_{12} peptide in *n*-propanol by CD, and it was associated with the cis–trans isomerization of amide bonds.[20] The transition kinetics and thermodynamics were also analyzed. Peptide P_6 also shows a concentration-dependent transition between PP-I and PP-II in aqueous *n*-propanol.[21] However, no aggregation process has been reported for unmodified oligoproline peptides, to our knowledge.

RESULTS AND DISCUSSION

Hexaproline peptides containing as a C-terminal unit the ionizable amino acids glutamic acid (P_6E) or lysine (P_6K) were studied in phosphate-buffered water at pH 7 (Scheme 1). Assuming that the pK_a values do not differ to a great degree from those reported for proline and glutamic acid in peptides, at neutral pH, P_6E should exist as a singly charged anion with partial positive charge in the N-terminal proline unit and the C-terminal glutamic acid unit as a dianion. On the other side, P_6K should predominantly form a cationic species at pH 7 as a result of the protonation of N-terminal proline and C-terminal lysine units together with the anionic character of the C-terminal carboxylate (Figure 1).

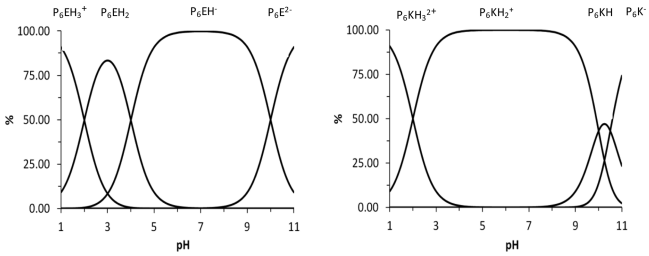

Figure 1. Species distribution diagrams calculated for P_6E and P_6K in aqueous media (estimated pK_a values = 2.0, 4.0, 10.0, and 10.5 for C terminus, E residue, N terminus, and K residue, respectively).

Dynamic light scattering (DLS) studies carried out at 25 °C indicate the presence of free, nonaggregated species both for P_6E and P_6K with an average size of ca. 1 nm (Figure 2). Remarkably, upon heating up to 80 °C, aggregation into objects with low polydispersity objects ascribable to vesicle-like assemblies is observed. In the case of P_6E, a number-averaged diameter (D_n) of ca. 600 nm is detected, while the assemblies formed by P_6K were smaller with a diameter of ca. 300 nm (Figure 2). The aggregation process is found to be fully reversible, and upon cooling down back to 25 °C, free P_6E and P_6K is observed again by DLS.

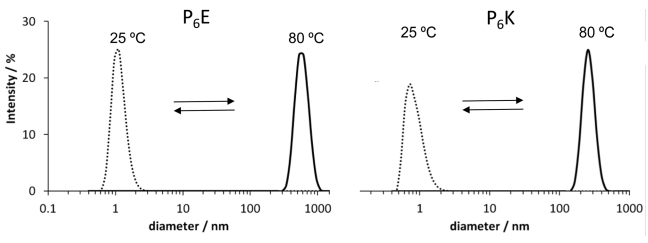

Figure 2. DLS analysis (number-averaged diameters) of P_6E and P_6K (1% w/w, pH 7) at 25 and 80 °C.

Circular dichroism experiments were carried out at 0.1% w/w to avoid signal saturation. At 25 °C, the characteristic pattern of a PP-II conformation[18,22,23] with positive ellipticity at 228 nm was observed for both peptides (Figure 3). Upon heating up to 80 °C, the PP-II pattern disappears, indicating an important conformational change taking place. The change was found to occur gradually with the increasing temperature with no critical point observed (Figure S2). In accordance with DLS measurements, the process is fully reversible, and upon cooling back to 20 °C, the PP-II conformation is recovered. Difference spectra were plotted, showing an increasing deviation from PP-II conformation on heating with a development of a spectrum with a maximum near 200 nm and a minimum near 220 nm, suggesting the appearance of turn or β-sheet structures[24−27] (Figure S3).

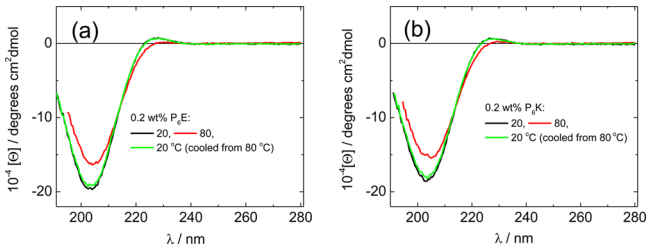

Figure 3. CD spectra of P_6E and P_6K (0.1% w/w, pH 7) at 20 and 80 °C.

These results are fully consistent with NMR measurements. Upon heating to 80 °C, the ^{1}H NMR-visible species were reduced to ca. 50% using an internal standard for integration. This fact proves the formation of large, NMR-silent aggregates. Interestingly, ^{1}H NMR spectra revealed that the multiplicity pattern of the signals corresponding to the diasterotopic protons at position 3 in the proline ring is heavily modified, indicating important conformational changes (Figure 4). Additionally, the traces of ^{1}H–^{15}N long-range correlations, carried out for the ^{15}N-enriched sample at proline unit number 5, also reveal important changes in the complex multiplicity pattern of the signals corresponding to the proline ring (Figure S4).

Imaging the vesicle-like assemblies by cryo-TEM and AFM was found to be challenging because of their instability at room temperature. Nevertheless, scanning electron microscopy (SEM) images were obtained from samples at 80 °C that were fast frozen and lyophilized. As can be seen in Figure 5, round shaped objects are observed which are ascribable to vesicle-like assemblies with variable diameters. These objects do not correspond to artifacts arising from salts in the buffer solution, which emerged as brilliant not-round spots that can be easily distinguished from the vesicles (Figure S9). The diameter of the vesicle-like assemblies is only in rough agreement with DLS, since the SEM images show considerable polydispersity in size. It has to be taken into account that the manipulation process of the sample (fast freezing and lyophilization) could affect the structures present in solution, leading to aggregation and increased polydispersity. Some of the observed vesicle-like assemblies revealed their hollow structure as a result of the

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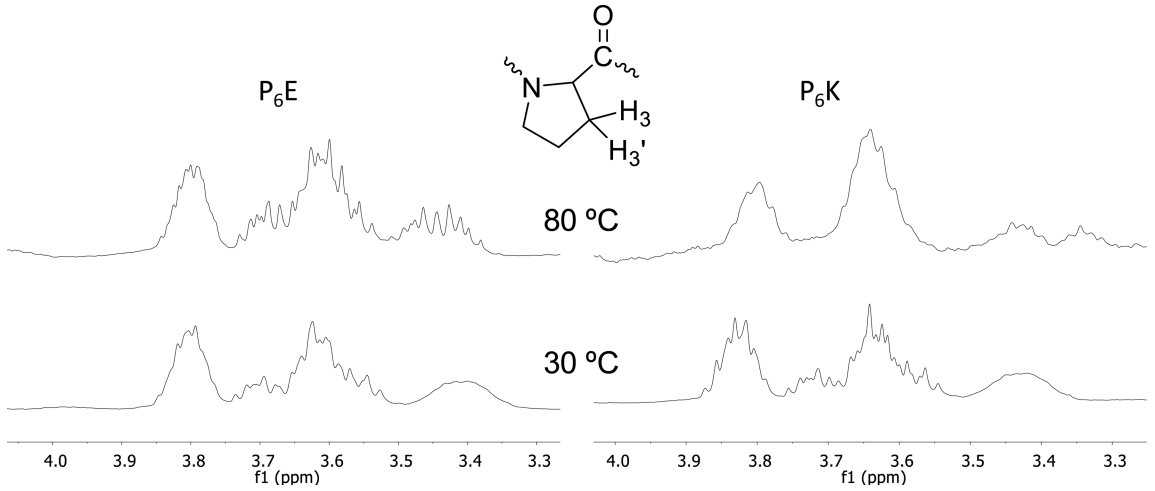

Figure 4. Partial ^{1}H NMR spectra of P_6E and P_6K (0.1% w/w, pH 7) at 25 and 80 °C.

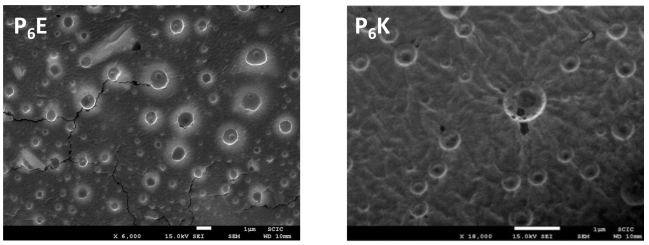

Figure 5. Scanning electron microscopy images of vesicle-like assemblies of P_6E and P_6K (1% w/w) formed at 80 °C in phosphate buffer at pH 7. The sample was fast frozen and lyophilized prior to observation. Scale bars are 1 μm long.

observation under the electron beam, resulting in donut-like objects.

The self-assembly process was followed using pyrene as a fluorescent probe (Figure 6). Upon heating, and subsequent

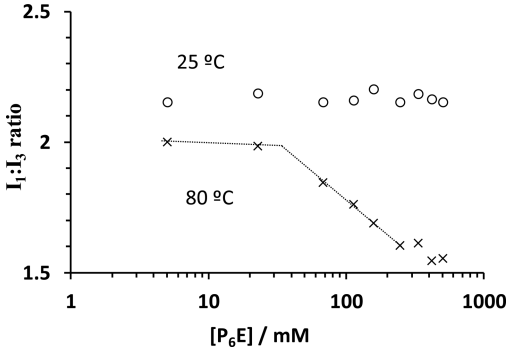

Figure 6. Variation of the relative intensity of fluorescence signals I_1 and I_3 of pyrene (1 μM) in the presence of P_6E in phosphate buffer, pH 7. λ_{exc} = 350 nm, λ_1 = 370.5 nm, λ_3 = 381 nm.

P_6E aggregation, incorporation of pyrene into a hydrophobic environment[28,29] was clearly detected by measuring the relative intensity of its vibronic fluorescence bands 1 and 3. The trend in I_1/I_3 as a function of concentration is similar to that noted for conventional amphiphiles for which I_1/I_3 decreases at the critical micelle concentration, from a typical value of around 1.7−1.8 to a lower value.[30,31] The critical concentration for aggregation at 80 °C of P_6E was found to be as low as ca. 30 μM, revealing a strongly thermodynamically favorable aggregation process. In constrast to P_6E, no critical point was observed in the fluorescence of pyrene in the presence of P_6K at 80 °C in the range of concentrations used (upper limit was 0.6 mM). This result indicates that the aggregation of P_6K is thermodynamically less favorable than that of P_6E.

CONCLUSIONS

Peptide amphiphiles P_6E and P_6K at pH 7 and 25 °C present a PP-II conformation as revealed by CD, showing no aggregation. Upon heating, an important conformational change takes place, with the peptides losing their secondary structure according to CD. NMR studies confirm an important conformational reorganization which strongly affects the multiplicity pattern of the proline ring protons. The conformation adopted at 80 °C is accompanied by aggregation into vesicle-like assemblies which are detected as rather monodisperse objects by DLS with diameters of ca. 600 and 300 nm for P_6E and P_6K, respectively. The aggregation is found to be fully reversible, with the system recovering the PP-II conformation upon cooling back to 25 °C. Scanning electron microscopy images from samples at 80 °C which were fast frozen confirm the formation of vesicle-like assemblies. The aggregation onset at 80 °C was studied monitoring the fluorescence of pyrene. It was found that P_6E shows a quite low critical aggregation concentration of ca. 30 μM in contrast to that for P_6K, which is above 0.6 mM. In summary, we have observed the remarkable thermoreversible formation of vesicle-like assemblies by the surfactant-like peptides P_6E and P_6K. This transition is accompanied by, or driven by, a change in peptide conformation from PPII. To our knowledge, this is the first observation of such a phenomenon for peptide materials. The discovery of polyproline-rich peptide vesicles has great potential in the development of responsive delivery systems (e.g., drug delivery) among other future applications. In this regard, design of related polyproline amphiphiles with a lower transition temperature or responsive to other physicochemical stimuli could be evaluated in future work.

ASSOCIATED CONTENT

Supporting Information

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Details on DLS, CD, and fluorescence studies as well as on electron microscopy (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: miravet@uji.es.

*E-mail: i.w.hamley@reading.ac.uk.

ORCID

Francisco Galindo: 0000-0003-0826-6084

Juan F. Miravet: 0000-0003-0946-3784

Valeria Castelletto: 0000-0002-3705-0162

Ian W. Hamley: 0000-0002-4549-0926

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Hamley, I. W. The Amyloid Beta Peptide: A Chemist's Perspective. Role in Alzheimer's and Fibrillization. *Chem. Rev.* **2012**, *112*, 5147−5192.

(2) Tomasini, C.; Castellucci, N. Peptides and Peptidomimetics that Behave as Low Molecular Weight Gelators. *Chem. Soc. Rev.* **2013**, *42*, 156−172.

(3) Santoso, S.; Hwang, W.; Hartman, H.; Zhang, S. Self-Assembly of Surfactant-Like Peptides with Variable Glycine Tails to Form Nanotubes and Nanovesicles. *Nano Lett.* **2002**, *2*, 687−691.

(4) Vauthey, S.; Santoso, S.; Gong, H.; Watson, N.; Zhang, S. Molecular Self-Assembly of Surfactant-Like Peptides to Form Nanotubes and Nanovesicles. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 5355−5360.

(5) van Hell, A. J.; Costa, C. I. C. A.; Flesch, F. M.; Sutter, M.; Jiskoot, W.; Crommelin, D. J. A.; Hennink, W. E.; Mastrobattista, E. Self-Assembly of Recombinant Amphiphilic Oligopeptides into Vesicles. *Biomacromolecules* **2007**, *8*, 2753−2761.

(6) Reches, M.; Gazit, E. Formation of Closed-Cage Nanostructures by Self-Assembly of Aromatic Dipeptides. *Nano Lett.* **2004**, *4*, 581−585.

(7) Adler-Abramovich, L.; Gazit, E. Controlled Patterning of Peptide Nanotubes and Nanospheres Using Inkjet Printing Technology. *J. Pept. Sci.* **2008**, *14*, 217−223.

(8) Adler-Abramovich, L.; Kol, N.; Yanai, I.; Barlam, D.; Shneck, R. Z.; Gazit, E.; Rousso, I. Self-Assembled Organic Nanostructures with Metallic-Like Stiffness. *Angew. Chem., Int. Ed.* **2010**, *49*, 9939−9942.

(9) Adzhubei, A. A.; Sternberg, M. J. E.; Makarov, A. A. Polyproline-II Helix in Proteins: Structure and Function. *J. Mol. Biol.* **2013**, *425*, 2100−2132.

(10) Stryer, L.; Haugland, R. P. Energy Transfer: A Spectroscopic Ruler. *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *58*, 719−726.

(11) Dolghih, E.; Ortiz, W.; Kim, S.; Krueger, B. P.; Krause, J. L.; Roitberg, A. E. Theoretical Studies of Short Polyproline Systems: Recalibration of a Molecular Ruler. *J. Phys. Chem. A* **2009**, *113*, 4639−4646.

(12) Bochicchio, B.; Tamburro, A. M. Polyproline II Structure in Proteins: Identification by Chiroptical Spectroscopies, Stability, and Functions. *Chirality* **2002**, *14*, 782−792.

(13) Kakinoki, S.; Hirano, Y.; Oka, M. On the Stability of Polyproline-I and II Structures of Proline Oligopeptides. *Polym. Bull.* **2005**, *53*, 109−115.

(14) Tooke, L.; Duitch, L.; Measey, T. J.; Schweitzer-Stenner, R. Kinetics of the Self-Aggregation and Film Formation of Poly-L-Proline at High Temperatures Explored by Circular Dichroism Spectroscopy. *Biopolymers* **2010**, *93*, 451−457.

(15) Grishkovskiĭ, B. A.; Khromova, T. B.; Lazarev, I. A. Aggregation of Poly-L-Proline in Aqueous Solution. *Molekulyarnaya Biologiya* **1981**, *15*, 310−315.

(16) Swenson, C. A.; Formanek, R. Infrared Study of Poly-L-Proline in Aqueous Solution. *J. Phys. Chem.* **1967**, *71*, 4073−4077.

(17) Schweitzer-Stenner, R.; Eker, F.; Perez, A.; Griebenow, K.; Cao, X. L.; Nafie, L. A. The Structure of Tri-Proline in Water Probed by Polarized Raman, Fourier Transform Infrared, Vibrational Circular Dichroism, and Electric Ultraviolet Circular Dichroism Spectroscopy. *Biopolymers* **2003**, *71*, 558−568.

(18) Dukor, R. K.; Keiderling, T. A. Reassessment of the Random Coil Conformation - Vibrational CD Study of Proline Oligopeptides and Related Polypeptides. *Biopolymers* **1991**, *31*, 1747−1761.

(19) Dukor, R. K.; Keiderling, T. A.; Gut, V. Vibrational Circular-Dichroism Spectra of Unblocked Proline Oligomers. *Int. J. Pept. Protein Res.* **1991**, *38*, 198−203.

(20) Kuemin, M.; Engel, J.; Wennemers, H. Temperature-Induced Transition between Polyproline I and Ii Helices: Quantitative Fitting of Hysteresis Effects. *J. Pept. Sci.* **2010**, *16*, 596−600.

(21) Zanna, N.; Milli, L.; Del Secco, B.; Tomasini, C. Factors Affecting the Stabilization of Polyproline II Helices in a Hydrophobic Environment. *Org. Lett.* **2016**, *18*, 1662−1665.

(22) Chen, K.; Liu, Z. G.; Kallenbach, N. R. The Polyproline II Conformation in Short Alanine Peptides Is Noncooperative. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15352−15357.

(23) Woody, R. W. Circular Dichroism Spectrum of Peptides in the Poly(Pro)II Conformation. *J. Am. Chem. Soc.* **2009**, *131*, 8234−8245.

(24) Kelly, S. M.; Jess, T. J.; Price, N. C. How to Study Proteins by Circular Dichroism. *Biochim. Biophys. Acta, Proteins Proteomics* **2005**, *1751*, 119−139.

(25) Bulheller, B. M.; Rodger, A.; Hirst, J. D. Circular and Linear Dichroism of Proteins. *Phys. Chem. Chem. Phys.* **2007**, *9*, 2020−2035.

(26) Nordén, B.; Rodger, A.; Dafforn, T. R. *Linear Dichroism and Circular Dichroism: A Textbook on Polarized-Light Spectroscopy*; RSC: Cambridge, U.K., 2010.

(27) Rodger, A.; Nordén, B. *Circular Dichroism and Linear Dichroism*; Oxford University Press: Oxford, U.K., 1997.

(28) Kalyanasundaram, K.; Thomas, J. K. Environmental Effects on Vibronic Band Intensities in Pyrene Monomer Fluorescence and Their Application in Studies of Micellar Systems. *J. Am. Chem. Soc.* **1977**, *99*, 2039−2044.

(29) Winnik, F. M. Photophysics of Preassociated Pyrenes in Aqueous Polymer-Solutions and in Other Organized Media. *Chem. Rev.* **1993**, *93*, 587−614.

(30) Wilhelm, M.; Zhao, C.-L.; Wang, Y.; Xu, R.; Winnik, M. A.; Mura, J.-L.; Riess, G.; Croucher, M. D. Poly(Styrene-Ethylene Oxide) Block Copolymer Micelle Formation in Water: A Fluorescence Probe Study. *Macromolecules* **1991**, *24*, 1033−1040.

(31) Johnsson, M.; Hansson, P.; Edwards, K. Spherical Micelles and Other Self-Assembled Structures in Dilute Aqeuous Mixtures of Poly(Ethylene Glycol) Lipids. *J. Phys. Chem. B* **2001**, *105*, 8420−8430.

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