Transactions of the Materials Research Society of Japan 30[2] 337-340 (2005)

Facile Synthesis of Amphiphilic Diblock Polypeptides through Ion-Complexation and Their Self-Assembling Properties in Water

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In this study, we synthesized various polyion-complexes of poly(L-glutamic acid)-*block*-poly(oxyethylene)s (PLGA-POE) (**1**) with cationic surfactants ($\mathbf{2C_{18}N^+}$,$\mathbf{C_{18}N^+}$,$\mathbf{C_8N^+}$). These diblock polymers were dispersed in water. Secondary structures of these diblock polymers were examined by means of CD and FTIR spectroscopy. As a result, $\mathbf{1/2C_{18}N^+}$ was found to form the random coil structure based on strong interaction between alkyl chains. On the other hand, CD spectra of $\mathbf{1/C_8N^+}$ and $\mathbf{1/C_{18}N^+}$ were shown typical β-sheet and aggregated α-helical patterns, respectively. AFM images of $\mathbf{1/C_8N^+}$ and $\mathbf{1/C_{18}N^+}$ showed the formation of globular aggregates, and the globular sizes were strongly depended on the hydrophobicity of surfactants and conformation of peptide segment. It was found, however, that $\mathbf{1/2C_{18}N^+}$ formed only amorphous aggregates. In other words, these results suggest that the secondary structure and morphology of **1**-complexes could be easily controlled by selecting the appropriate surfactants.

Key words: Poly(L-glutamic acid), Amphiphilic Diblock Copolymer, Ion-complexation, Self-assembly, Conformation, Surfactants

1. INTRODUCTION.

Self-assembly is the key in the design of new functional materials. Amphiphilic macromolecules with well-defined structures on the nanometer scale are good candidates for self-assembled nano-materials and nano-deviced. A number of amphiphilic block-copolymers have been prepared and reported to self-assemble into nanostructures such as micelles, vesicles, networks, and a variety of other morphologies depending on the structure, combination of block segments, and/or environmental conditions.[1] In the view of biological applications, developing the self-assembling system by using protein-based material as building blocks is a powerful approach for the design of new functional bio-materials.[2] These self-organized protein-architectures are expected to possess potential use in applications such as bio-scaffold, nano-reactors, and nano-carrier for drug-delivery systems depending on the 3D nanostructures. From these points of view, we have developed a strategy to construct well-ordered polypeptide nano-assembly by using two-dimensional media such as water and Au surfaces, and investigated the conformational and morphological property.[3] Well-organized peptide-assemblies have provided particular properties different from those in bulk: for example, enantioselective binding of α-amino acids.[4]

In this paper, we describe the facile synthesis of water-dispersible amphiphilic diblock peptides through ion-complexation of PLGA-*block*-POE (**1**) with cationic surfactants ($\mathbf{2C_{18}N^+}$, $\mathbf{C_{18}N^+}$, **or** $\mathbf{C_8N^+}$) (Fig. 1) in order to create novel peptide-based 3D-nanoarchitectures. Effects of the surfactants on self-assembling property, conformation and morphology of the **1**-complexes in water are discussed in detail. These studies should be useful for the design of novel peptide-based biomaterials in nano-meter scale.

2. EXPERIMENTAL

2.1 Preparation of poly(L-glutamic acid)-poly(oxyethylene) diblock copolymer (1).

The poly(L-glutamic acid)-block-poly(oxyethylene) (PLGA-POE (**1**)) were prepared by ring-opening polymerization of γ-benzyl-L-glutamate *N*-carboxylic anhydride (BLG-NCA). BLG-NCA was synthesized by reacting γ-benzyl L-glutamate with triphosgen in a THF solution. α-Methoxy-ω-amino poly(oxyethylene)$_m$ (POE-NH_2, A_v.M_w: 1040, m=23) was synthesized from α-methoxy-poly(oxyethylene) (Nippon Shokubai Co.) according to the literature.[5] First, the polymerization of BLG-NCA was carried out from the primary amino groups of the POE-NH_2 in $CHCl_3$ at room temperature. Then the reaction mixture was poured into a large excess of diethyl ether, and the precipitate was separated by centrifuge and then washed with diethyl ether repeatedly and dried. The degree of polymerization of the polypeptide segment (n) was controlled by adjusting the feed-ratio of monomer (BLG-NCA) to initiator (terminal amino groups). As a result, poly(γ-benzyl-L-glutamate)-*block*-POE diblock copolymer (P(BLG)$_n$-P(OE)$_m$; (n/m) = 21/23, that was evaluated by means of ^{1}H-NMR spectroscopies (400 MHz; JEOL JNM GX-400 spectrometer)) was successfully obtained. ^{1}H-NMR ($CDCl_3$, TMS);

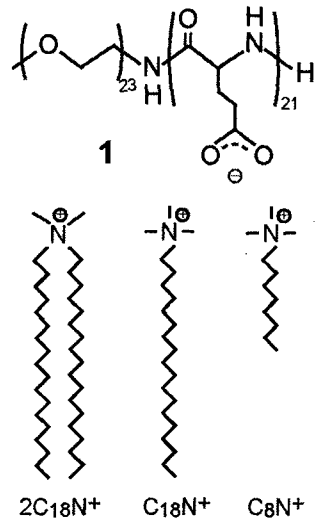

Fig. 1. Chemical structures of PLGA-*block*-POE (**1**) and surfactants used in this study.

δ(ppm) 1.4-3.0 (-CH_2CH_2-CO-(side chain of PBLG), NHC$H_2CH_2CH_2$-(main chain)), 3.6 (-CH_2CH_2O-(POE)), 3.95 (-C*H*-(main chin of PBLG)), 5.05 (-COOCH_2-Ph), 7.25 (Ph(PBLG)), 7.8-8.7 (-N*H*-). IR ($CHCl_3$); 1730 cm^{-1} ($\nu_{C=O}$ Ester), 1660 cm^{-1} ($\nu_{C=O}$ Amide), 1545 cm^{-1} (δ_{NH} Amide). Subsequently, the PBLG-POE thus obtained was dissolved in *N,N*-dimethylformamide (DMF), and the removal of benzyl groups was carried out by reduction with Pd(black)/H_2. After removal of Pd(black) by filtration, the solution was poured into a large excess of diethyl ether. As a result, the desired PLGA-POE (**1**) was obtained as a white powder. The structure was confirmed by means of ^{1}H-NMR spectroscopy on the basis of the disappearance of the proton signals of the benzyl groups at 5.05 and 7.25 ppm.

2.2 Preparation of polyion complexes.

The cationic surfactants, $\mathbf{C_{18}N^+}$ and $\mathbf{C_8N^+}$ were purchased from Nacalai tesque Co. and Tokyo Kasei Co., respectively, and used without purification. $\mathbf{2C_{18}N^+}$ was synthesized according to literature.[6] An aqueous solution (pH 8.0) of **1** (25 mM (Glu unit)) was obtained by sonication for a few minutes with a Branson Sonifier II 250. Equivalent amounts of an aqueous dispersion of surfactants (25 mM) and an aqueous solution of **1** (25 unit mM) were mixed at pH 8.0 and aged at 50°C for 20 min. The resultant solutions were freeze-dried to give white powders. The stoichiometric composition of these polyion complexes was determined by ^{1}H NMR analysis. Samples were prepared by redispersing **1**-complexes thus obtained in distilled, deionized water with appropriate concentration.

2.3 Measurements.

CD spectra were recorded on a J-720 spectropolarimeter (JASCO Ltd.) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-250 nm at ambient temperature. Final peptide concentration was 4.0-6.5 x 10^{-5} unit M in water. The pH of the sample solution was adjusted with 0.1 M HCl or 0.1 M NaOH.

Transmission FTIR spectra (Thermo Nicolet Co. Nexus 470) were obtained on mica plate using a MCT detector (resolution, 2 cm^{-1}; number of scans, 1024). The sample and the detector chamber were purged with dried nitrogen before and during measurement.

The phase-transition behavior of **1**-complex solutions was obtained from differential scanning calorimetry (DSC). The samples (300-600 unit mM) were sealed in an Ag sample pan and heated from 5 to 65 °C at a rate of 1 °C/min with an SSC-570 (Seiko Electric Co. Ltd,, Tokyo).

The AFM images were collected at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc.) operated in a tapping mode using silicon cantilevers (125 μm, tip radius 10 nm). An aliquot of **1**-complexes in water was placed on freshly cleaved mica. After adsorption for 1 min, the excess solution was removed by absorption onto filter paper and the samples were stored in a covered container to protect them from contamination until they were imaged (within 1 h). A 10 μm x 10 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz, and the original images were sampled at a resolution of 512 x 512 points.

3. RESULTS AND DISCUSSION

3.1 Conformational Studies of 1-Complexes in Water.

The secondary structures of **1**-complexes in aqueous solutions were investigated by means of circular dichroism (CD) and FTIR spectroscopies. Fig. 2 shows the CD spectra for $\mathbf{1/2C_{18}N^+}$, $\mathbf{1/C_{18}N^+}$, and $\mathbf{1/C_8N^+}$ at pH 5.8. In this figure, the data for **1** is also included for comparison. For peptide **1** (Fig. 2*a*), the spectrum give a typical pattern of right-handed α-helical polypeptides with two negative maxima, one at 222 nm and one at 208 nm. On the other hand, **1**-complexes were found to form different conformations depend on the structures of surfactants. Namely, the spectrum obtained for $\mathbf{1/C_8N^+}$ showed β-sheet structure with a

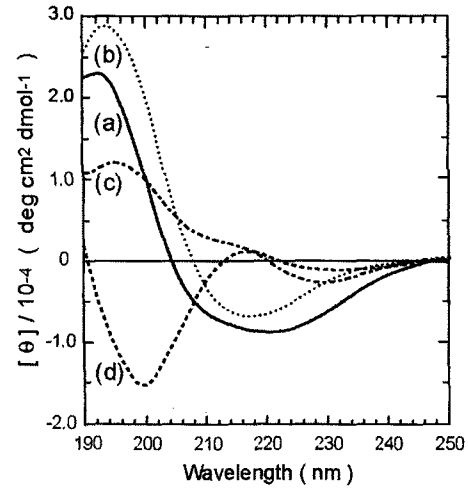

Fig. 2. CD spectra of **1** (a), $\mathbf{1/C_8N^+}$ (b), $\mathbf{1/C_{18}N^+}$ (c), and $\mathbf{1/2C_{18}N^+}$ (d) in water at pH 5.8.

single negative maximum at 216 nm (Fig. 2b). For **1/$C_{18}N^+$** (Fig. 2c), the typical aggregated α-helix pattern was observed,[7] as evidenced by the red shifting of 222 nm band toward 228 nm and the flattening of 208 nm band. In the case of **1/2$C_{18}N^+$**, CD spectrum showed random coil structure with a broad positive peak at 218 nm and negative peak at 197 nm (Fig. 2d). To obtain more quantitative information on the secondary structure, transmission FTIR spectra were measured. The **1**-complexes were adsorbed onto a CaF_2 plate at pH 5.8. In the amide I region,[8] characteristic absorptions with the random coil and α-helix structures were observed at 1660 cm^{-1} (broad peak: FWHM 60 cm^{-1}) and 1654 cm^{-1} (sharp peak: FWHM 20 cm^{-1}) in the case of **1/2$C_{18}N^+$** and **1/$C_{18}N^+$**, respectively, whereas **1/C_8N^+** took partly the β-sheet structure (peak maxima at 1654 cm^{-1} (α-helix) and 1627 cm^{-1} (β-sheet)) (data not shown). These results were well consistent with the results of CD measurements. Thus, it is clear that the structure of the surfactant was responsible for regulating the conformation of peptide chains.

In order to investigate the interactions among surfactants in **1**-complexes, the phase-transition behaviour was subsequently studied by differential scanning calorimetry (DSC) measurement (Fig. 3). The aqueous dispersion of **1/2$C_{18}N^+$** gave an endothermic peak at 48°C due to the crystal-to-liquid crystal phase transition, indicating the formation of well-developed ordered structure of surfactant. The **1/$C_{18}N^+$** also showed a single endothermic peak at 23°C, which was lower than that of **1/2$C_{18}N^+$** (Fig. 3b). On the other hand, for the **1/C_8N^+**, no thermal transitions were observed by DSC in the temperature range 5-65°C (Fig. 3c). It seems that the octyl side chains are too short to crystallize at this temperature range. From these results, the difference in conformations among **1**-complexes can be explained as follows. In **1/2$C_{18}N^+$** system in comparison with **1/$C_{18}N^+$**, the strong interactions between surfactants based on the highly hydrophobicity are supposed to prevent the α-helix formation as well as the steric bulkiness of the surfactant. On the other hand, in the case of **1/C_8N^+**, such steric bulkiness of surfactant is reduced remarkably, and this permits the tight packing of peptide chains, which induces the β-sheet formation. Appropriate hydrophobicity based on the short alkyl chains probably also contributes to such tight packing of peptide chains in water.

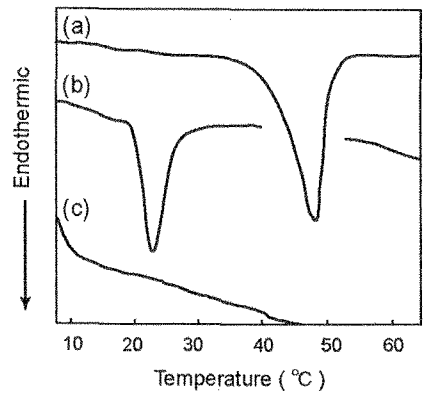

Fig. 3. DSC thermograms of **1/2$C_{18}N^+$** (a), **1/$C_{18}N^+$** (b), and **1/C_8N^+** (c) complexes on heating.

3.2 Nanostructures of 1/2$C_{18}N^+$, 1/$C_{18}N^+$, and 1/C_8N^+ aggregates.

To gain insight into the morphology of **1**-complexes in water, we performed atomic force microscopy (AFM) measurements. In the field of structural biology, AFM is a useful technique to evaluate the three-dimensional structural features of proteins and their assemblies in nano-meter scale. Fig. 4 shows the three-dimensional tapping-mode AFM images (1.5 x 1.5 μm^2) prepared from various **1**-complexes in aqueous solutions of pH 5.8. AFM image obtained for **1/2$C_{18}N^+$**(Fig. 4a), revealed the presence of poorly organized amorphous aggregates. This result is reasonable taking account of the conformation of peptide segments (random coil structure) under this condition. In contrast, an AFM image obtained for **1/C_8N^+**, in which the peptide chain took partly β-sheet form (namely, mixture of β-sheet and α-helix conformation), revealed the presence of globular aggregates (Fig. 4b). The average diameter of the globular aggregates was determined to be *ca.* 50 nm

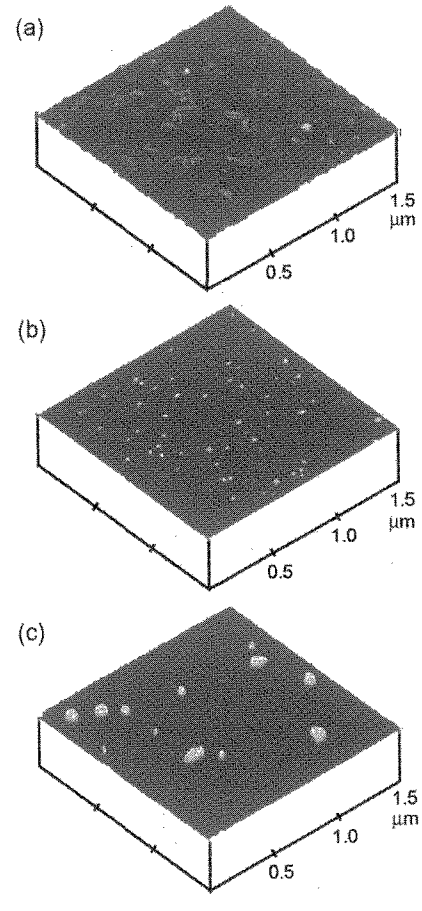

Fig. 4. Three-dimensional tapping mode AFM images (1.5 x1.5 μm^2) for **1/2$C_{18}N^+$** (a), **1/C_8N^+** (b), and **1/$C_{18}N^+$** (c).

(AFM level). It is important to note that the well-known convolution of the scanning tip leads to an overestimation of the sample width.[9] Therefore the real diameters of these globules could be somewhat smaller than the value estimated by AFM. The molecular length of the peptide segment can be estimated to be 3.2 nm in α-helical form and 7.4 nm in β-sheet form, respectively. By using these values and the completely expanded POE conformation, we can calculate the molecular length of **1** to be 13 nm in α-helix state and 17 nm in β-sheet state, respectively. Taking into account these molecular lengths of **1**, the globular aggregates of $\mathbf{1}/\mathbf{C_8N^+}$ are assumed to be micellar structures consisting of a hydrophobic β-sheet-peptide/surfactant core and the shell of a hydrophilic POE segment. Interestingly, huge globular aggregates with *ca.* 150 nm diameters were observed in the case of $\mathbf{1}/\mathbf{C_{18}N^+}$, in which the peptide chains took aggregated helix structure (Fig. 4c). These aggregates can be assumed to be vesicle-like structure rather than micelle, which will require further investigation. From these results, however, it can be concluded that the self-assembly and the resultant nanostructure of **1**/surfactant complexes were closely related to their secondary structure, which can be easily controlled by selecting the appropriate surfactant.

4. CONCLUSION

In the present study we successfully synthesized three types of amphiphilic diblock polypeptides ($\mathbf{1}/\mathbf{2C_{18}N^+}$, $\mathbf{1}/\mathbf{C_{18}N^+}$ and $\mathbf{1}/\mathbf{C_8N^+}$) through ion-complexation of PLGA-*block*-POE with appropriate cationic surfactants, and described their conformational and self-assembling properties in water. CD, FTIR, and AFM analyses suggested that the secondary structure and morphology of **1**-complexes could be easily controlled by changing the surfactants. We believe the ability to control the distribution of high-order structures (conformation and/or nanostructure) of the peptide-assembly provides important insights for developing novel peptide-based biomaterial with shape-specific nanostructure for use in biological and pharmaceutical applications.

ACKNOWLEDGEMENT

This work was supported in part by the project 'Hybrid Nanostructured Materials and Its Application' of High Technology Center at RCAST of Doshisha University.

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(Received December 24, 2004; Accepted February 24, 2005)