Preparation of a Novel Aggregate Like Sugar-Ball Micelle Composed of Poly(methylglutamate) and Poly(ethyleneglycol) Modified by Lactose and Its Molecular Recognition by Lectin

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We report the preparation and characteristics of a novel micellar aggregate of an amphiphilic diblock copolymer, poly(methylglutamate) (PMG)–poly(ethyleneglycol) (PEG), whose terminus was modified by lactose lactone (LA). Due to the terminal LA moiety, this aggregate could be specifically recognized by RCA_{120} lectin. PMG-PEG-LA was synthesized by polymerizing the *N*-carboxy anhydride of L-glutamic acid γ-methyl ester with H_2N-PEG-LA as a polymerization initiator. By applying a fluorescence method using pyrene as a probe molecule, we found that PMG-PEG-LA could form the aggregate in aqueous solution. Fluorescence measurements showed that the critical aggregation concentration (C.A.C.) was 1.1×10^{-5} M. The average diameter of the aggregate was 220 nm at 25 °C, as determined by the dynamic light scattering method. Circular dichroism measurements for the aggregate solution showed that the PMG residue took an α-helical structure, and that they associated to constitute the hydrophobic core of the aggregate. By adding RCA_{120} lectin to the aggregate solution, the turbidity of the solution increased rapidly, due to association of the aggregates. This implies that the aggregate could be recognized by lectin, and also suggests that sugar residues locate at the surface of the aggregates. From these findings, we concluded that the PMG-PEG-LA molecules form an aggregate like a "sugar ball" micelle, whose surface is covered by the sugar moieties. Application of the present aggregate system as a drug carrier is briefly discussed.

Key words peptide-based amphiphile; aggregation; recognition; sugar ball; lactose; lectin

Sugar residues on biomolecules play important roles in vital phenomena in living organisms, such as differentiation, senescence and tumorigenic transformation.[1] In these processes, the sugar moieties operate as key components for molecular recognition. Thus, sugars have considerable significance for living systems, not merely as energy sources. It is important to clarify how the sugar residue performs its biofunction as a host in living systems, and in what cases it recognizes guest molecules. Substantial studies have been made to elucidate the recognition mechanism for sugars, by constructing artificial host molecules,[2,3] and furthermore, host supermolecules such as dendrimers, both of which have sugar residues.[4]

Host supermolecules also have practical significance, in terms of applications as a drug delivery system (DDS). One can solubilize hydrophobic drugs in the hydrophobic core of the dendrimer, which is available as a novel drug carrier.[4] However, because of their small sizes, the amount of the drugs included in the dendrimers is restricted. On the other hand, peptide-based amphiphilic polymers have been reported to form micelles with ordered cores consisting of peptide residues having α-helical structures. If the micelle surface is covered with sugar residues, it is expected to take a "sugar ball" like structure. These micellar systems have various advantages as drug carriers: 1) The structure is completely reversible depending on the amphiphile concentration, namely, whether it is above or below a critical micelle concentration. 2) The size of the micelle can be controlled by varying the length of the peptide and/or the hydrophilic parts and it is much larger than that obtainable by using dendrimers. Therefore, the peptide-based micellar systems can solubilize larger amounts of drugs than in the case of dendrimer systems. Thus, the "sugar ball" micellar systems might be applicable as novel drug carriers.

In this report, we examine the properties of novel peptide-based amphiphiles such as the formation of the aggregate like "sugar-ball" micelles, and their molecular recognition due to the sugar residue.[5—7] We synthesized an amphiphilic diblock copolymer composed of poly (ethyleneglycol), PEG, and poly(methylglutamate), PMG, whose ethyleneglycol terminal was modified by lactose lactone, LA, (hereafter designated as PMG-PEG-LA). Unmodified PMG-PEG block copolymer was also synthesized for comparison. The PMG-PEG-LA aggregate was recognized by RCA_{120} lectin which has a specific affinity for lactose.

Experimental

Materials The modified block copolymer PMG-PEG-LA was obtained as shown in Fig. 1 by polymerizing the *N*-carboxy anhydride of L-glutamic acid γ-methyl ester in dimethylformamide (DMF) with H_2N-PEG-[*O*-β-galactopyranosyl-(1→4)]-D-gluconamide (H_2N-PEG-LA) as a polymerization initiator.[8] H_2N-PEG-LA was synthesized from lactose lactone and H_2N-PEG-NH_2 (Degree of polymerization of PEG=75 and 113, purchased from Sigma Co., Ltd.). Lactose lactone was synthesized from lactose hydride (purchased from Sigma Co., Ltd.), and the presence of the carbonyl group was confirmed using IR spectroscopy. The IR spectrum was obtained with a 1600 series FT-IR (Perkin-Elmer Inc., CT, U.S.A.). The *N*-carboxy anhydride of L-glutamic acid γ-methylester was synthesized from L-glutamic acid γ-methylester (purchased from Sigma Co., Ltd.) using triphosgene (purchased from Nakalai Tesque Co., Ltd. Osaka, Japan) in tetrahydrofuran (THF). The molar ratio of the anhydride to the initiator was chosen to be 30. The polymerization reaction was carried out at room temperature for 72 h. The degrees of polymerization for the PMG segment (DP_{PMG}) in the copolymer were determined by ^{1}H-NMR method using a Lambda 400 spectrometer (JEOL, Co., Ltd., Tokyo) in trifluoroacetic acid (TFA) with tetramethyl silane (TMS) as an internal standard. The peak areas of β- and γ-methylene protons and methylene protons were compared to those for PMG (chemical shift θ=2.0—2.8 ppm and 3.9—4.1 ppm, respectively). The degrees of polymerization for the PEG segment (DP_{PEG}) were equal to those of H_2N-PEG-NH_2. DP_{PMG} and DP_{PEG} values were confirmed by means of TOF-Mass spec-

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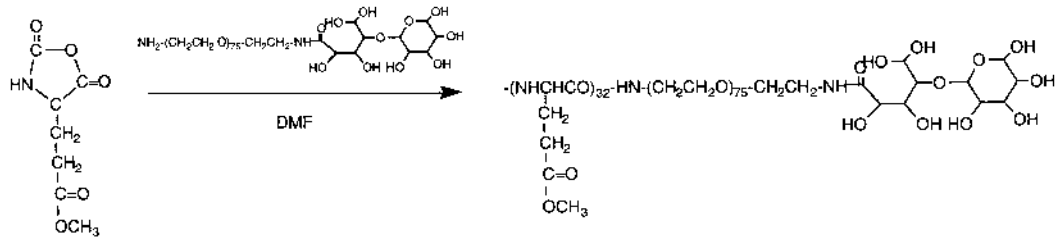

Fig. 1. Synthesis of PMG-PEG-LA

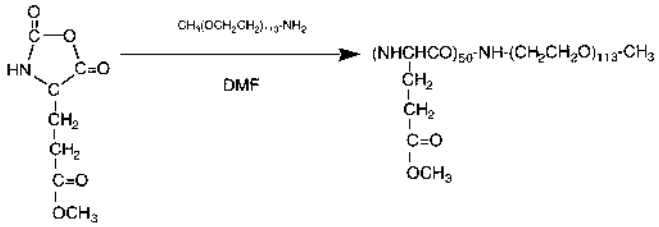

Fig. 2. Synthesis of PMG-PEG

troscopy. TOF-Mass measurements were performed using a Voyager-Elite type mass spectrometer (PerSeptive Biosystem Inc., CA, U.S.A.).

The unmodified amphiphilic polymer, PMG-PEG, was obtained as shown in Fig. 2 by polymerization of the *N*-carboxy anhydride of L-glutamic acid γ-methylester in DMF with ω-amino poly(ethyleneglycol) methylester as an initiator. Two kinds of PMG-PEG block copolymers having different segment lengths were synthesized (hereafter designated as PMG-PEG 1 and 2). The values of DP_{PEG} and DP_{PMG} were obtained in the same manner as for PMG-PEG-LA. DP_{PEG} and DP_{PMG} values for these samples are compiled in Table 1.

Determination of Critical Aggregation Concentration (C.A.C.) The critical aggregation concentrations (C.A.C.) of the amphiphilic copolymers in aqueous solutions were determined by using pyrene as a fluorescent probe.[10—13] Fluorescence intensity of the polymer solutions was measured in the presence of pyrene at various polymer concentrations, *C*, at a wavelength of 393 nm. The value of C.A.C. was estimated from the change of fluorescence intensity with *C*. The measurements were performed by using a fluorescence spectrophotometer (Type FP-77, JASCO Co., Tokyo, Japan) at room temperature.

Characterizations of the Aggregate and the Core Structures of PMG Radii of the aggregates were determined by a dynamic laser light scattering apparatus (DLS-700, Otsuka Electron Co. Ltd., Tokyo, Japan). Sample solutions were carefully filtered before every measurement to eliminate dusts. The secondary structures of the polypeptide PMG in the cores of the aggregates were studied by applying circular dichroism (CD) spectroscopy using a JASCO J-720 CD spectrometer.

Recognition of the PMG-PEG-LA Aggregate by Lectin The recognition of the aggregates was investigated by adding lectin, RCA_{120}, (Sigma Co., Ltd.) to the solutions of PMG-PEG-LA and PMG-PEG using a turbidity method. The turbidity τ was measured by UV-VIS spectrophotometer (Type U-3000, Hitachi Co. Ltd., Tokyo, Japan) at a wavelength of 500 nm. The polymer concentrations were 50 times larger than the C.A.C. values and they formed aggregates in the sample solutions. In order to confirm the binding between RCA_{120} and aggregates of PMG-PEG-LA or PMG-PEG, free lactose was added to samples containing the aggregates 34 h after preparations, and the accompanying change of τ values measured.

Results and Discussion

C.A.C. of the Peptide-Based Amphiphiles The critical aggregation concentrations (C.A.C.) of amphiphilic polymers were determined by fluorescence spectroscopy as described above. Figure 3 shows the dependence of the relative fluorescence intensity (I/I_0) on the polymer concentrations, *C*, for aqueous solutions of PMG-PEG-LA. *I* is the fluorescence intensity of the sample, and I_0 is the intensity of the solvent. I/I_0 were almost constant below $C=1.1\times10^{-5}$ M, while above this concentration it increased rapidly. Hence, the C.A.C. value for PMG-PEG-LA was 1.1×10^{-5} M. A similar trend is commonly observed for micellar systems. The increase of I/I_0 above a critical micellar concentration is attributed to an increase of micelle number (not micelle size) with *C*.[14]

Table 1. Characteristics of PMG-PEG-LA and PMG-PEG

	DP_{PEG}	DP_{PMG}	C.A.C./M
PMG-PEG-LA	75	32	1.1×10^{-5}
PMG-PEG 1	113	50	7.7×10^{-7}
PMG-PEG 2	113	20	1.4×10^{-6}

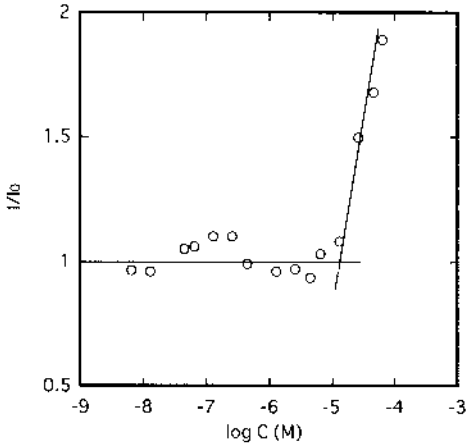

Fig. 3. Relative Fluorescent Intensity, I/I_0, *vs.* Amphiphilic Concentration, *C*, Plot for Aqueous Solutions of PMG-PEG-LA at 25 °C, Measured at Wavelength=392.6 nm

Similar measurements for PMG-PEG 1 ($DP_{PMG}=50$) and 2 ($DP_{PMG}=20$) showed that their C.A.C. values were 7.7×10^{-7} and 1.36×10^{-6} M.[15] The decrease of C.A.C. with increasing DP_{PMG} appears to be due to an increase in hydrophobicity. The higher C.A.C. value for PMG-PEG-LA compared to that for PMG-PEG 1 and 2 was attributable to an increase in hydrophilicity due to the sugar residues.

Structure of the Aggregate CD spectra of PMG-PEG-LA and PMG-PEG were measured at *C*'s above their C.A.C.'s. Figure 4 demonstrates the molar ellipticity $[\theta]_\lambda$ of PMG-PEG-LA and unmodified PMG-PEG in aqueous solutions. *C* values were 2.24×10^{-4} M and 3.88×10^{-5} M, respectively. The spectra for both amphiphiles showed a sharp maximum at $\lambda=ca.$ 190 nm followed by a shallow single minimum at $\lambda=ca.$ 230 nm. Generally, the CD spectrum of an α-helical structure for an isolated peptide shows a peak at $\lambda=190$ nm and two minima at $\lambda=208$ and 222 nm. On the

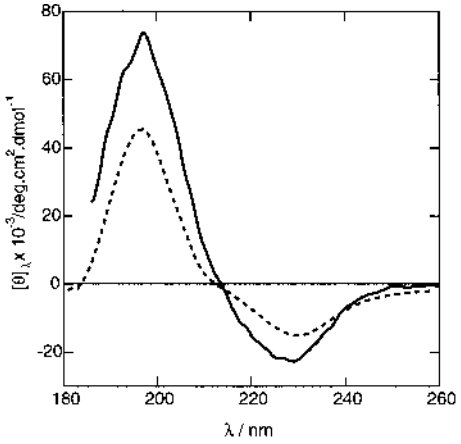

Fig. 4. Circular Dichroism Spectra of PMG-PEG-LA and PMG-PEG in Aqueous Solution

[PMG-PEG-LA]=2.24×10^{-4} M and [PMG-PEG]=3.88×10^{-5} M. (——): PMG-PEG-LA, (------): PMG-PEG.

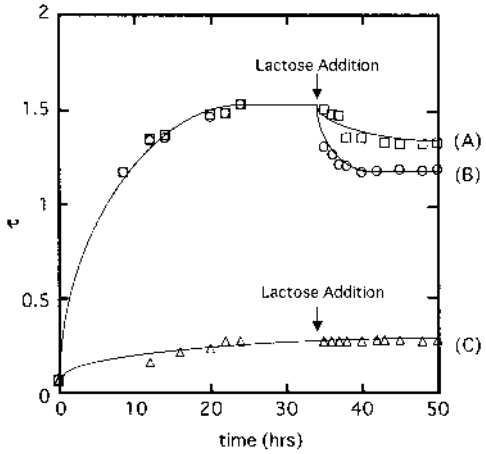

Fig. 5. Change of Turbidity, τ, with Time, t for Aqueous Solutions of PMG-PEG-LA (A, B) and PMG-PEG (C), by Addition of RCA_{120} and Lactose

RCA_{120} and lactose were added at t=0 and t=34 h, respectively. [PMG-PEG-LA]=1.12×10^{-4}, [PMG-PEG]=7.76×10^{-6}. [Lactose]=3.04 mg (A) and 30.4 mg (B, C), τ was measured at wavelength of 500 nm and 25 °C.

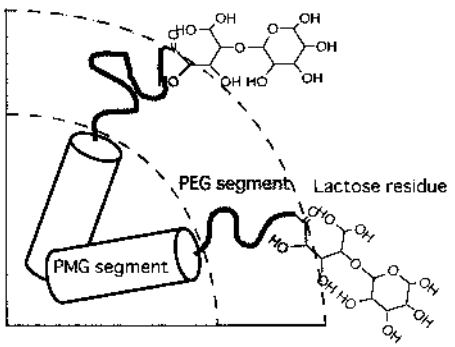

Fig. 6. Schematic Representation of Micelle of PMG-PEG-LA Formed in Aqueous Solution

other hand, when the helixes are associated, the peak wavelength shifts toward higher λ, and the first minimum is absent.[17] The present CD profiles are attributable to associated α-helixes, which presumably form the hydrophobic core of an aggregate particle.

We note that the sizes of the aggregates were comparable with the wavelengths of the incident light for the CD measurements, as will be mentioned below. In this case, diffused scattering of the incident beam by the aggregates might affect the CD spectra.[18] Although this can not readily be corrected, we believe that it does not affect the profiles quantitatively, since a similar profile for associated helical polypeptides has often successfully been measured for micellar systems.[19]

The present results indicate that PMG segments in both PMG-PEG-LA and PMG-PEG block copolymers take an α-helical structure, and that they associated to form the hydrophobic cores of the aggregates. Furthermore, the observation that the presence of a LA residue does not largely affect the helix structure strongly suggests that the LA moiety is located on the surface of the PMG-PEG-LA aggregates. This is reasonable in light of the hydrophobic nature of the PMG segments and the hydrophilic properties of the LA residue.

The average diameter d of the aggregates were determined by applying the DLS method. The d values for PMG-PEG-LA and unmodified PMG-PEG were 220 and 270 nm, respectively. Measurements were performed at C=2.24×10^{-4} M and 3.88×10^{-5} M, which were chosen to be much higher than the C.A.C. values, but sufficiently dilute to avoid interparticle hydrodynamic effects.[20] The particle size distribution was relatively narrow. For example, the standard deviation in the particle size for PMG-PEG-LA aggregate was approximately 50%, as analyzed by the cumulant method.[21] This relatively uniform particle size distribution suggests that the resulting aggregate has a micelle-like structure. This is consistent with the findings from the CD measurements. The somewhat smaller d value for the modified amphiphile can be explained in terms of augmented hydrophilicity by the LA the residue, which reduces the association number in the aggregates as seen in the micellar system.

Recognition of the PMG-PEG-LA Aggregate by Lectin

The interaction between the aggregate of PMG-PEG-LA and RCA_{120} lectin molecules in aqueous solutions was evaluated by measuring the change of turbidity, τ, at a wavelength of 500 nm. By adding RCA_{120} lectin to PMG-PEG-LA solutions at C >C.A.C., the value of τ increased after about 1 h as shown in Fig 5. This is presumably caused by association of the aggregates, since the hydrophilicity of the aggregates is largely reduced by binding of the lectin. In contrast, for the aggregate of PMG-PEG 1, the change of τ value was negligible on addition of the lectin. These results indicated that the lactose residues of the PMG-PEG-LA aggregate are located on the surface of the aggregate, and are successfully recognized by RCA_{120} lectin.

Furthermore, in order to examine the reversibility of binding between RCA_{120} and the sugar residues, free lactose was added to the solution of the complexes 34 h after preparation (Fig. 5). Two experiments were performed so that lactose concentrations in the final solutions were 19.3 and 3.2 mM. By adding lactose, τ values rapidly decreased. The drop of τ was greater with the larger amount of lactose. However, τ values decreased only by about 25—30% and did not recover the initial value. On the other hand, the τ value for PMG-PEG did not significantly change on addition of lactose as shown in Fig. 5.

From these results, we conclude that RCA_{120} lectin binds strongly to the lactose residues and that a large amount of free lactose is needed to remove lectin from the complexes, due to the competing substitution reaction with free lactose.

A possible model of the PMG-PEG-LA aggregate is shown in Fig. 6. It is reasonable to assume that PMG-PEG-LA molecules are able to form aggregate like "sugar ball" micelles with the sugar moiety on the surface and with a rigid hydrophobic core of α-helixes of PMG.

The present aggregate could be applicable as a novel drug carrier. One of the advantages of utilizing the PMG-PEG-LA system is that its size can be controlled easily by changing DP_{PMG} and DP_{PEG}. We note that the size of the present aggregate (d=220 nm) should be reduced for future use as a drug carrier. It is usually desirable to design the aggregate size to be smaller than 100 nm, *i.e.* virus size. Thus, further studies on shorter amphiphiles are required, however, aggregates with moderately large size should enable confinement of larger amounts of hydrophobic drugs than sugar-ball dendrimers.[4] Furthermore, the specific recognition ability of PMG-PEG-LA aggregate like sugar-ball micelles demonstrated above appears to be potentially useful for designed drug targeting.[22]

Conclusions

PMG-PEG-LA block copolymers form aggregate like "sugar ball" micelles, with the sugar moiety on the outside of the spherical micelle. The aggregate diameter determined by the dynamic light scattering method was approximately 200 nm. The aggregate formed a complex with RCA_{120} lectin, including the presence of recognition sites on the surface that recognized lectin specifically. These findings suggest the possibility of application of the present system as a novel drug carrier.

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