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# Polymerization of amino acid under liposomal environment

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***N*-Carboxy anhydrides of amino acid derivatives with hydrophobic side chains, *N*-carboxy anhydrides of γ-dodecyl L-glutamate and γ-benzyl D-glutamate, were polymerized in bilayer membrane of large unilamellar liposomes prepared by the injection method. Infrared spectra indicated that polypeptides isolated from the liposomes existed in two different conformational forms, namely the α-helix and the β form. Studying osmotic shrinkage of liposomes, it was found that liposomal membrane was highly permeable to glucose in the presence of polypeptides in the membrane.**

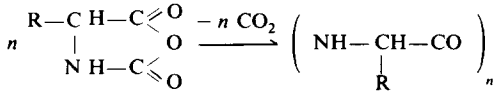
Liposomes provide compartments into which amino acids, nucleotides and nucleoside may be taken up selectively. Additionally, since liposomes are expected to catalyse reactions, amino acid, nucleotide condensation and polymerization may be enhanced in this medium. This type of study has aroused great interest because of its possible extension to the vitally important utilization of organized assemblies in such diverse areas as drug delivery, analytical chemistry, molecular self-organization, transport, and recognition [1].

In this study, the trapping of polypeptide as a protein model in large unilamellar liposome was attempted by polymerization of amino acid monomer in a liposomal environment.

Dipalmitoylphosphatidylcholine (DPPC) and dicetyl phosphate (DCP) were obtained commercially (Sigma Chem. Co.). Both γ-dodecyl L-glutamate and γ-benzyl D-glutamate were prepared by the method of Hayakawa et al. [2]. *N*-Carboxy anhydrides of γ-dodecyl L-glutamate and γ-benzyl D-glutamate were prepared by the method of Blout and Karlson [3]. The injecting solvent, dichloromethane, was distilled under nitrogen atmosphere. Water was twice distilled, the second time from alkaline potassium permanganate solution. We have employed the injection method of Deamer and Bangham [4] with some modifications for large unilamellar liposome preparations. Polymerization of *N*-carboxy amino acid anhydride was carried out in a liposomal environment. Here, two different liposomes were prepared by the following methods. (1) Large unilamellar liposomes were prepared by solubilizing DPPC, DCP and *N*-carboxy amino acid anhydride in dichloromethane at an appropriate molar ratio (1 : 0.2 : 1) in a final volume of 30 ml (2 μmol DPPC/ml). This solution was gradually injected with the aid of a mechanical drive at 0.25 ml/min through a 23 gauge needle into an aqueous phase, located within the internal chamber of the modified Liebig condensor. The aqueous phase consisted of 4.5 ml distilled water which was maintained at 60°C. (2) Large unilamellar liposomes composed of DPPC and DCP (1 : 0.2 mol/mol) were prepared. To this liposome suspension was injected a dichloromethane solution of *N*-carboxy amino acid anhydride in an amount equimolar to DPPC in the liposome. After the injection phase, nitrogen gas was bubbled immediately through the

liposome suspension for 1 min. The obtained liposomes are hereafter called a mixed lipid-polypeptide liposome for method 1 and a polypeptide-covered liposome for method 2. Gel filtration (Cellulofine GCL 2000 m) is used to provide a more homogeneous preparation and to remove residual amino acid and dichloromethane contained in the liposome suspension. The trapping volume was estimated by preparing the liposome in 0.1 M potassium chromate. The conformation of the polypeptide in liposomal membrane was studied using infrared spectroscopy (JASCO DS 701-G). For the osmotic shrinkage measurements the liposome suspension was mixed quickly with hypertonic glucose solution with mixing ratio 1 : 1. The shrinking of the liposome was followed by observing the change in turbidity at 450 nm (Hitachi 330 spectrophotometer). Rapid mixing of the liposome with glucose solution was achieved by using a mixing device (Hitachi mixing unit). In Fig. 1, a typical tracing of an experiment is given in which the shrinkage velocity is recorded when a sample of liposome is mixed with hypertonic glucose solution. The data obtained were used to calculate the relative initial shrinkage velocity, $[(dA/dt)/A_0] \times 100\%$ [5].

*N*-Carboxy amino acid anhydride represents a class of high-energy amino acid derivatives which polymerize to form polypeptides. The anion in buffered aqueous solution plays an important role in the course of the polymerization of *N*-carboxy amino acid anhydride [6].



In this study, the polymerization of *N*-carboxy amino acid anhydride in a liposomal environment was carried out using the injection method. The negative-charged portion on the liposomal surface is expected to be favorable for the spontaneous polymerization of *N*-carboxy amino acid anhydride [6]. Therefore, the procedure of liposome preparation was evolved for DPPC containing 20 mol% of DCP to provide a negative charge on the liposome. Distilled water was used as an aqueous phase for liposome preparation, since the use of ionic aqueous solution such as phosphate-buffered saline may facilitate the polymerization of *N*-carboxy

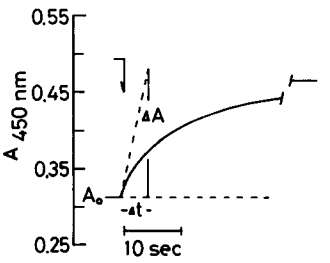



Fig. 1. Typical time-course of turbidity change at 450 nm due to osmotic shrinkage for large unilamellar liposomal membrane. At the moment indicated by the first arrow liposome suspension was mixed with hypertonic glucose solution at 39°C. $[(\Delta A/\Delta t)/A_0] \times 100$ gives the relative initial shrinkage velocity.

amino acid anhydride in the ionic aqueous solution rather than in the lipid bilayer. The trapping volume, calculated by the solute diffusion method [7], gave a large unilamellar liposome trapping volume of 9.7 l/mol lipid. Microscopic observations of the obtained liposome were made. Although each preparation was heterogeneous, no obvious difference in size (0.5–1.0 $\mu$m) of liposome in the presence and the absence of polypeptide was observed. To isolate the polypeptide from mixed lipid-polypeptide liposome and polypeptide-covered liposome (120 $\mu$mol DPPC), the liposome suspension was first lyophilized. The residue was added to 10 ml diethyl ether. After shaking, there appeared a dispersed precipitant. The precipitated polymer was filtered off and evaporation was carried out in vacuo at room temperature; yields for mixed liposomes were 17 mg (41%) for poly(γ-dodecyl L-glutamate) and 12 mg (36%) for poly(γ-benzyl D-glutamate), and yields for covered liposomes were 4 mg (9.8%) for poly(γ-dodecyl L-glutamate) and 3 mg (9.5%) for poly(γ-benzyl D-glutamate). Compared with mixed lipid-polypeptide liposomes, the yields of polypeptide-covered liposomes are considerably smaller. This seems to be due to a 'localized' adsorption of polypeptide on the membrane surface of the covered liposome.

Figs. 2 and 3 show the infrared spectra for poly(γ-dodecyl L-glutamate) and poly(γ-benzyl D-glutamate) isolated from liposomes, together with polypeptides prepared by polymerizing *N*-carboxy anhydrides of their amino acids in di-

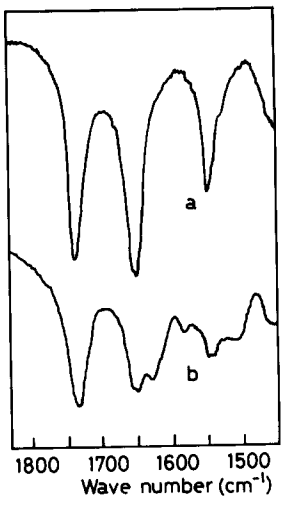



Fig. 2. Infrared spectra of poly(γ-dodecyl L-glutamate)'s polymerized in dioxane (a) and isolated from liposome (b).

oxane. The spectra which are characteristic of polypeptide having the α-helix form show major diagnostic peaks at about 1650 $cm^{-1}$ (amide I band) and 1550 $cm^{-1}$ (amide II band). The β form generally gives rise to strong bands at lower frequency (about 1635 $cm^{-1}$ for amide I band and 1520 $cm^{-1}$ for amide II band) than the α-helix form. Infrared spectra indicated that both the isolated polypeptides poly(γ-dodecyl L-glutamate) and poly(γ-benzyl D-glutamate) existed in two conformational forms, namely the α-helix and the

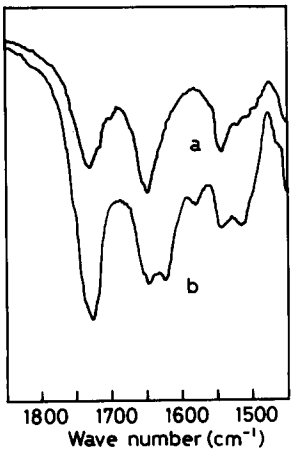



Fig. 3. Infrared spectra of poly(γ-benzyl D-glutamate)'s polymerized in dioxane (a) and isolated from liposome (b).

β form. The polypeptides polymerized in dioxane were in the α-helix form. The difference in conformation between polypeptides polymerized in membrane bilayer and those polymerized in dioxane may be attributed predominantly to the difference in polymerizing environments. From the ratio of the relative intensities of the 1650 $cm^{-1}$ (α-helix) and1635 $cm^{-1}$ (β form), the polypeptides isolated from the mixed liposome and from the covered liposome may differ in ratio of the content of α-helix and β form (data not shown). It appears that the molecular weight is also smaller for the polypeptides isolated from the covered liposome than for those from the mixed liposome.

As a test of the barrier properties of liposomal membrane at 39°C (just below the transition point) we studied the dependence of initial osmotic shrinkage velocity on the difference in glucose concentrations on both sides of the membrane. Fig. 4 shows that the initial shrinkage velocities of polypeptide-free lipid liposome, mixed lipid-polypeptide liposome and polypeptide-covered liposome were directly proportional to the glucose concentration difference. These were explicit differences in the shrinkage velocities of the liposomes prepared in the presence and the absence of polypeptide. In Fig. 4a, the rates of shrinkage are in the order of poly(γ-dodecyl L-glutamate)-covered liposome > mixed lipid-poly(γ-dodecyl L-glutamate) liposome > polypeptide-free lipid liposome and, in Fig. 4b on contrast, mixed lipid-poly(γ-benzyl D-glutamate) liposome > poly(γ-benzyl D-glutamate) covered liposome > polypeptide-free lipid liposome. Poly(γ-dodecyl L-glutamate) in mixed liposome may be mainly immersed into the membrane bilayer with direct contact and regular arrangement between dodecyl side chain and alkyl chain of the lipid in the liposomal membrane. In the case of covered liposome, adsorbed poly(γ-dodecyl L-glutamate) may exert an effect upon Coulombic hydration around the surface of the liposomal membrane. As a result of the influence on Coulombic hydration, the packing of lipid molecules in bilayer membrane are perturbed, the rate of shrinkage being larger than in the case of mixed liposome. In addition, it is noteworthy that irrespective of the low yield of poly(γ-dodecyl L-glutamate) for covered liposome,

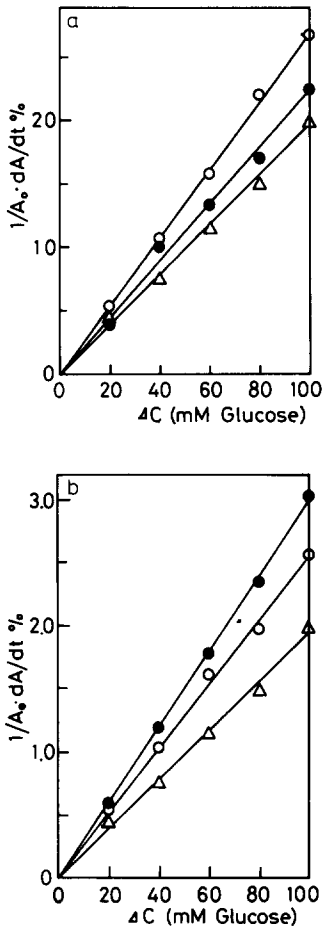



Fig. 4. Dependence of initial shrinkage velocity on the difference in glucose concentration ($\Delta c$) on both sides of the liposomal membrane at 39°C. (a) △, Polypeptide-free lipid liposome; ●, mixed lipid-poly(γ-dodecyl L-glutamate) liposome; ○, poly(γ-dodecyl L-glutamate)-covered liposome. (b) △, Polypeptide-free lipid liposome; ●, mixed lipid-poly(γ-benzyl D-glutamate) liposome; ○, poly(γ-benzyl D-glutamate)-covered liposome.

the rate of shrinkage is larger for covered liposome than for mixed liposome. As shown in Figs. 4a and 4b, the two liposomes containing either poly(γ-dodecyl L-glutamate) or poly(γ-benzyl D-glutamate) were opposite in rate of shrinkage. The barrier properties of mixed lipid-poly(γ-benzyl D-glutamate) liposomal membrane are lower than that of poly(γ-benzyl D-glutamate)-covered liposomal membrane. It can be presumed that the bulky benzyl side groups of poly(γ-benzyl D-glutamate) facilitate the modification of the orientation and packing of lipid molecules in the bilayer membrane.

It is almost certain that the conformational form of polypeptide in the bilayer membrane plays an important role in the barrier properties of the liposomal membrane. We are extending our studies on this subject at present.

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