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BIPHASIC SUBSTRATE-INCORPORATION BY SYNTHETIC MEMBRANE VESICLES

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The substrate-binding property of single-walled vesicles formed with N,N-ditetradecyl-N^{α}-(6-trimethylammoniohexanoyl)-L-alaninamide bromide was investigated from both static and dynamic viewpoints, hexyl p-nitrophenyl ether being used as a hydrophobic pseudo-substrate. The substrate-binding equilibrium was achieved through a two-step mechanism.

Currently, much attention has been focused on the development of biomembrane-mimic agents[1] which spontaneously form bilayer membranes in aqueous media in a manner as observed with phospholipids.[2] Single-compartment vesicles formed with synthetic amphiphiles involving amino acid residue or residues are structurally stable and useful for understanding intrinsic functions of biological membranes as reported previously.[3] Such work prompted us to characterize the dynamic aspects of synthetic bilayer assemblies for incorporating substrate speceis. We report in this communication a novel substrate-binding property of single-walled vesicles formed with synthetic amphiphile $N^{+}C_5Ala\text{-}2C_{14}$[4] in aqueous media. Hexyl p-nitrophenyl ether (HNPE)[5] was employed as a pseudo-substrate which is structurally related to p-nitrophenyl esters, i.e., hydrolytic substrates of frequent use.

$$N^{+}C_5Ala2C_{14}: \quad (C_{14}H_{29})_2NCOCH(CH_3)NHCO(CH_2)_5N^{+}(CH_3)_3 \quad Br^{-}$$

Multilayered vesicles, having diameters distributed from 1000 to 8000 Å, were observed for an aqueous dispersion of $N^{+}C_5Ala2C_{14}$ (Fig. 1a), like its synthetic analogues.[3] The present amphiphile forms single-compartment vesicles with relatively uniform size (130-500 Å) in aqueous media upon sonication (Fig. 1b) in a manner as described previously.[3] The single-walled vesicles were used for investigation of the substrate-binding property. HNPE showed a characteristic absorption band in the UV region; namely, λ_{max} at 324 nm in water and 310 nm in the vesicle. The dynamic behavior in connection with the substrate-binding equilibria was examined by the stopped-flow technique,[6] a monitoring wavelength being set at 310 nm.[7] The biphasic feature of substrate-binding dynamics became apparent in the light of absorbance change shown in Fig. 2.

In order to characterize the substrate-binding behavior exercised by the single-walled vesicles, the binding constant for formation of the vesicle-substrate complex was evaluated. An amount of the substrate entrapped in the vesicular domain was determined upon separating the free substrate from the vesicle-bound one by means of gel-filtration chromatography. As a typical exercise, a 1.0-ml sample of a buffer solution (0.05 mol dm^{-3} phosphate - 0.04 mol dm^{-3} borate, pH 6.72) containing $N^{+}C_5Ala2C_{14}$ (1.0×10^{-3} mol dm^{-3}) and a 1.0-ml sample of the same buffer solution but containing HNPE ($1.0\text{-}8.0 \times 10^{-4}$ mol dm^{-3}) were mixed and incubated for one or two hours at 25.0 ± 0.2 °C. The substrate entrapped in the vesicular phase was separated by gel-filtration chromatography on a column of Sephadex G-50 thermostated at 25.0 ± 0.2 °C with the same phosphate-borate buffer as an eluant.

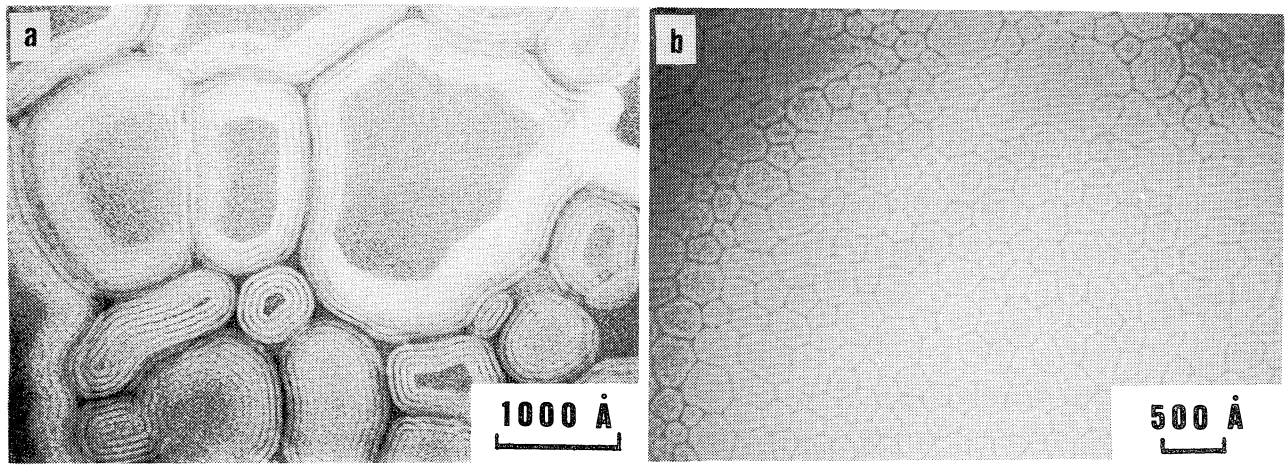

Fig. 1. Electron micrographs negatively stained with uranyl acetate: (a) 5 mmol dm^{-3} aqueous dispersion of $N^{+}C_5Ala2C_{14}$; (b) 5 mmol dm^{-3} aqueous solution of $N^{+}C_5Ala2C_{14}$ sonicated for 2 min with a probe-type sonicator at 30 W and allowed to stand for 30 min at 5 °C; taken on a JEOL JEM-200B electron microscope installed at the Research Laboratory for High Voltage Electron Microscopy of Kyushu University.

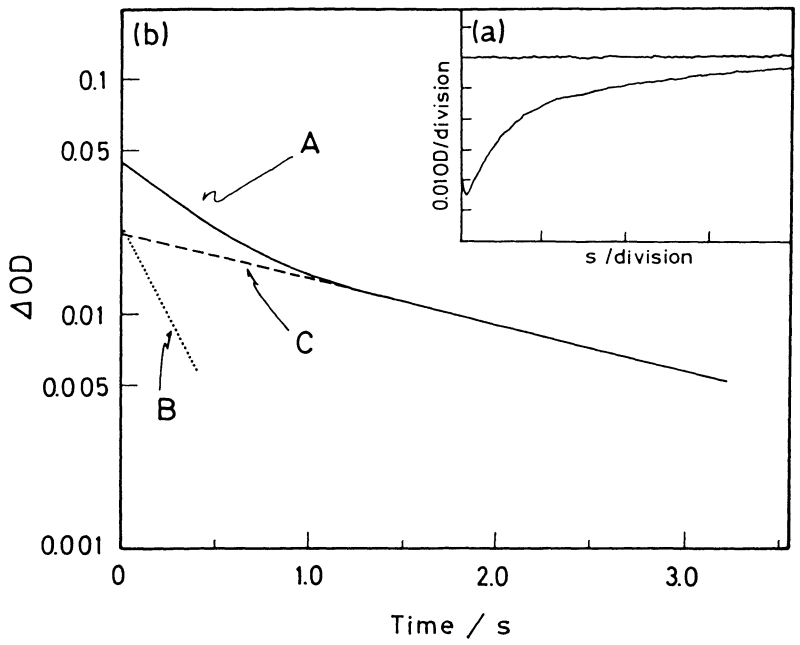

Fig. 2. Absorbance change at 310 nm caused by incorporation of HNPE into the vesicular assembly formed with $N^{+}C_5Ala2C_{14}$ at pH 6.72 (0.05 mol dm^{-3} phosphate - 0.04 mol dm^{-3} borate) and 25.0 ± 0.1 °C with initial concentrations of $N^{+}C_5Ala2C_{14}$ and HNPE at 1.96 x 10^{-3} and 1.80 x 10^{-5} mol dm^{-3}, respectively: (a) an absorbance decay recorded on a X-Y recorder, the upper trace being the final base-line recorded at 200 s after mixing; (b) a semilogarithmic plot of the data given in (a) (curve A), being resolved into two exponential components with relaxation times of 0.71 and 2.17 s (B and C, respectively).

The vesicular fraction was collected in the column void volume, evaporated to dryness, and sonicated for 2 min with a probe-type sonicator at 30 W upon addition of 3 ml of 1%(w/w) aqueous Triton X-100 (ca. 6 x 10^{-2} mol dm^{-3}). An absorbance at 320 nm, λ_{max} of the substrate observed in the sonicated aqueous medium, was measured and a concentration of the entrapped substrate was determined in reference to the calibration curve established independently. The substrate-binding equilibrium in the vesicular system is represented by Eq. 1, under the assumption that each binding site in the vesicular domain can incorporate only one substrate molecule; a binding equilibrium constant being denoted by K_b^S, where S, V_b, and $S \cdot V_b$ stand for HNPE, a binding site in the vesicular system, and a complex

formed with HNPE and a binding site, respectively.

$$S + V_b \underset{}{\overset{K_b^S}{\rightleftharpoons}} S \cdot V_b \qquad (1)$$

The static binding constant, K_b^S, is represented by Eq. 2. A new parameter defined as $\alpha = S_b/n$ is

$$K_b^S = [S \cdot V_b]/([S]_f[V_b]_f) \qquad (2)$$

introduced, where S_b is a number of the entrapped substrate molecules per vesicle as given by $[S]_b/[\text{vesicle}]$[8] and n denotes a number of the binding site per vesicle on average; $[S]_b$ and $[S]_f$ are concentrations of the bound and free substrates, respectively. The following relations are then derived, where $[V_b]_T$ stands for a total concentration of the binding site.

$$[S \cdot V_b] = \alpha[V_b]_T \; ; \quad [V_b]_f = (1 - \alpha)[V_b]_T$$

Eq. 2 is rearranged by the aid of the above relations to give Eqs. 3 and 4.

$$K_b^S = \alpha/\{(1 - \alpha)[S]_f\} \qquad (3) \; ; \qquad 1/\alpha = n/S_b = 1/(K_b^S[S]_f) + 1 \qquad (4)$$

These equations lead to formulation of Eq. 5.

$$[\text{vesicle}]/[S]_b = 1/(nK_b^S[S]_f) + 1/n \qquad (5)$$

Amounts of the substrate entrapped in the vesicular domain were determined after incubation for either one or two hours at 25.0 ± 0.2 °C. Good linear correlations of $[\text{vesicle}]/[S]_b$ with $1/[S]_f$ were obtained for all the measurements. The linear correlations based on the data of one-hour incubation were in good agreement with those of two-hour incubation. This result indicates that the binding equilibrium is completely attained within one hour for incorporation of HNPE. Thus, the value of K_b^S and n were determined to be (5.0 ± 1.3) x 10^4 mol^{-1} dm^3 and 140 ± 10, respectively.

The substrate-binding processes were treated kinetically as follows. The relaxation times for the HNPE-binding equilibria were calculated from the logarithmic plots of absorbance change at 310 nm vs. time as typically shown in Fig. 2. The obtained τ_1 and τ_2 values for the first (fast) and second (slow) steps were plotted against the amphiphile concentration (Fig. 3); being confirmed to be bimolecular

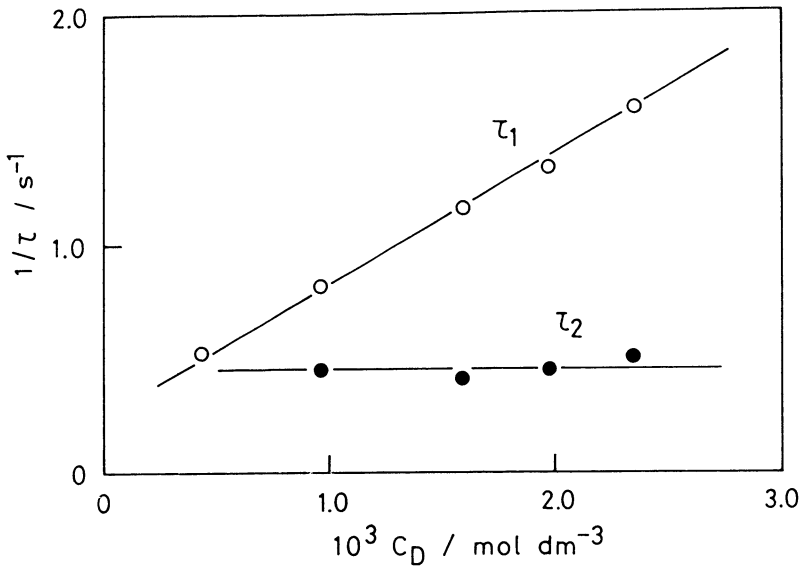

Fig. 3. Correlations of relaxation times with amphiphile concentration (C_D) for incorporation of HNPE into the vesicular assembly formed with $N^+C_5Ala2C_{14}$ at pH 6.72 and 25.0 ± 0.1 °C with initial concentration of HNPE at 1.80 x 10^{-5} mol dm^{-3}.

and unimolecular, respectively. This seems to indicate that the substrate molecules located in the bulk phase are incorporated readily into the surface area as the first step and such incorporated guest molecules penetrate rather slowly from the surface area into the hydrophobic interior of vesicular assembly. This specific feature is consistent with the presence of a potential barrier between the surface and interior hydrophobic regions of the vesicular system for translocation of such a relatively hydrophobic substrate. Such a barrier must be identical with the so-called hydrogen-belt region located in the intra-membrane region suggested previously.[3c]

In conclusion, the single-walled vesicles formed with synthetic amphiphiles involving an amino acid residue as a molecular component provide two different binding sites separated by the hydrogen-belt barrier, and the substrate-binding equilibrium is achieved through the two-step mechanism.

References

1) For example: T. Kunitake, N. Nakashima, M. Shimomura, Y. Okahata, K. Kano, and T. Ogawa, J. Am. Chem. Soc., 102, 6642 (1980), and references therein; J. H. Fendler, Acc. Chem. Res., 13, 7 (1980), and references therein.

2) A. D. Bangham, M. M. Standish, and J. C. Watkins, J. Mol. Biol., 13, 238 (1965); C. Huang, Biochemistry, 8, 344 (1969).

3) a) Y. Murakami, A. Nakano, and K. Fukuya, J. Am. Chem. Soc., 102, 4253 (1980); b) Y. Murakami, A. Nakano, A. Yoshimatsu, and K. Fukuya, ibid., 103, 728 (1981); c) Y. Murakami, Y. Aoyama, A. Nakano, T. Tada, and K. Fukuya, ibid., 103, 3951 (1981); d) Y. Murakami, A. Nakano, and H. Ikeda, J. Org. Chem., 47, 2137 (1982).

4) N,N-Ditetradecyl-N^{α}-(6-trimethylammoniohexanoyl)-L-alaninamide bromide: liquid crystal with final mp 225 °C, $[\alpha]_D^{25}$ -18.6 ° (c 1.08, EtOH). Found: C, 66.24; H, 11.45; N, 5.77%. Calcd for $C_{40}H_{82}BrN_3O_2 + 1/2\,H_2O$: C, 66.17; H, 11.52; N, 5.78%. ^{1}H-NMR signals observed in $CDCl_3$ with Me_4Si as an internal reference were consistent with the assigned structure. Differential scanning calorimetry for 0.022 mol dm^{-3} aqueous dispersion: T_m, 2.0 °C; ΔH, 4.9 kcal/mol for the gel-liquid crystal phase transition.

5) Prepared from the reaction of p-nitrophenol with the corresponding alkyl bromide in the presence of potassium carbonate and recrystallized from methanol: mp <30 °C. Found: C, 64.50; H, 7.76; N, 6.26%. Calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27%.

6) Rapid reaction rates were measured on a Union Giken RA-401 stopped-flow spectrophotometer. An absorbance change at 310 nm vs. time was displayed on an oscilloscope and recorded on a X-Y recorder. The sonicated amphiphile solution was clear and transparent and remained so for a reasonably prolonged period of time during the kinetic runs. Any morphological change was not observed during the kinetic runs as confirmed by electron microscopy and good kinetic reproducibility was secured.

7) The molar extinction coefficient of HNPE increases as the solvent polarity decreases: 7960 (H_2O), 13500 (MeOH), and 14300 (hexane).

8) A vesicle concentration is defined as [vesicle] = C_D/N, where C_D stands for an amphiphile concentration and N is an aggregation number per vesicle on average. An aggregation number for the single-walled vesicles formed with $N^+C_5Ala2C_{14}$ was calculated by the equation, $N = 2\pi \times (D - h)^2/A$, where D and h refer to average diameter and bilayer thickness of a single-compartment vesicle in Å, respectively, and A is a cross section area of the double chain portion of an amphiphile molecule. These values used in this work are: D, 250 Å; h, 47 Å (both from the electron micrograph, Fig. 1b); A, 65 $Å^2$. The N-value was then evaluated to be approximately 4000.

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