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Adsorption of Monovalent Cations on Negatively Charged Liposomes Evaluated from the Spectral Change of Methylene Blue

JUZIRO NISHIJO,*,[a] KIYOKO KAWABATA,[a] and MASAYUKI NAKAGAKI[b]

Kobe Women's College of Pharmacy,[a] Motoyamakita-machi, Higashinada-ku, Kobe 658, Japan and Faculty of Pharmaceutical Science, Kyoto University,[b] Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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The adsorption of monovalent cations on negatively charged liposomes (membranes) was studied by the following the spectral changes of methylene blue. The strength of adsorption of inorganic monovalent cations (as chlorides) was in the order lithium > sodium > ammonium > potassium > rubidium > cesium ions, and that of organic monovalent cations (alkylammonium ions as hydrochlorides) was in the order *n*-octyl > *n*-heptyl > methyl > *n*-hexyl > ethyl > *n*-propyl > *n*-butyl > *n*-amylammonium ions.

Keywords——adsorption; methylene blue; negatively charged liposome; inorganic monovalent cation; alkylammonium ion

When a positively charged probe, methylene blue (MB), is introduced into a negatively charged liposome suspension, the dye binds electrostatically to the membrane from the aqueous phase and forms dimers having a smaller molar extinction coefficient in the neighborhood of the negative charge on the membrane surface.[1-3] The result is a decrease in the absorbance of MB. When a cation is added to this system, MB is released from the membrane phase and the absorbance decrease due to partitioning of the dye into the membrane phase is reversed.[3] It can therefore be presumed that the stronger the adsorption of a cation on the negatively charged membranes the greater the recovery of absorbance of MB. On the basis of this principle, we examined the adsorption of inorganic monovalent cations (metallic and ammonium ions) on negatively charged membranes. As biological fluids contain monovalent cations, it is important to know whether ions such as sodium and potassium are adsorbed specifically on negatively charged membranes.[4-6] It has already been reported that inorganic monovalent cations at high concentration (0.1 M or more) are specifically adsorbed on negatively charged liposomes (membranes) based on measurements of ζ potential[5] or electron paramagnetic resonance.[6]

On the other hand, it has been reported that no specific interaction occurs between liposomes (membranes) made of phosphatidylcholine or phosphatidylethanolamine and inorganic monovalent cations such as potassium and sodium based on studies with a fluorescent probe.[7]

Experimental

Materials——L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-dipalmitoylphosphatidic acid (DPPA), L-α-dioleoylphosphatidic acid (DOPA), L-α-distearoylphosphatidic acid (DSPA), and L-α-dimyristoylphosphatidic acid (DMPA) were obtained from Sigma Chemical Co. Reagent-grade MB was supplied by Nakarai Chemical Co., Ltd. and further recrystallized according to the method of Pal and Schubert.[8] Alkylammonium chlorides prepared by the reaction of the corresponding amines with hydrogen chloride gas in dry ether were recrystallized twice or more from a mixed solvent of methanol and acetone. Alkali metal chlorides and ammonium chloride were reagent-grade products and were further recrystallized from water. Water that had been deionized and twice distilled in an all-glass apparatus was used.

Preparation of Liposomes (Membranes)——DPPC and phosphatidic acid in a given mole ratio were dissolved in chloroform. The solvent was distilled off, and the lipid mixture obtained in this way was suspended in Hepes buffer of pH 6.0 (5 mM Hepes, 0.2 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA) and 0.2 mM Tris). The lipids were dispersed by ultrasonification under a nitrogen atmosphere. The solvent was cooled in ice during this process. The ultrasonifier was a UR-200P apparatus from Tomy Seiko Co., Ltd. The ultrasonically dispersed lipids were then centrifuged for 40 min at 30000 *g* at 4 °C to remove titanium dust from the ultrasonic tip and a small amount of undispersed lipids. The supernatant fluid obtained was used.

Measurements of Absorption Spectra——The MB stock solution was added to an aqueous solution containing liposomes to give a final concentration of 2×10^{-5} M. The solution thus obtained was incubated at 25 °C for 15 min and then the absorption spectrum was measured at the same temperature. MB stock solution was prepared with Hepes buffer of pH 6.0. Absorption spectra to examine the effect of adding cations were measured in the same way as mentioned above except that the cation was added to the aqueous solution containing liposomes and MB. Preliminary experiments revealed that the absorption spectrum of MB was not changed by adding any of the cations used without liposomes. Therefore, it was concluded that there is no interaction between MB and the cations used.

Results and Discussion

As shown in Fig. 1, MB in buffer solution (2×10^{-5} M) shows a monomer absorbance maximum at 664 nm (1.62 in absorbance unit). This absorbance decreases with increase in the amount of the negatively charged liposomes added. The liposomes were composed of DPPC and DOPA with a mole fraction of DOPA, $X_{pA}=0.14$. When the amount of liposomes added is 24 mg in 100 ml, the absorbance at 664 nm decreases to 1.05 in absorbance unit (Fig. 1). The absorbance decrease, the relative increase of the maximum at the shorter wavelength, and the presence of an isosbestic point, at 593 nm for MB are typical phenomena of the interaction of the dye with polyanions.[1] Addition of lithium chloride to this system (1.05 in absorbance unit) restored the absorbance of MB solution which had been decreased by the addition of liposomes. As shown in Fig. 1, the return of absorbance increased with an increase in the concentration of lithium chloride added. The correlation between the percentage (%) of absorbance increase and lithium chloride concentration in solution is shown in Fig. 2. MB spectral changes upon addition of other inorganic monovalent cations were investigated in the same manner and the results are shown in Fig. 2. The level of the absorbance increase (%) at the same concentration of the inorganic monovalent cation was in the order; $Li > Na > NH_4 > K > Rb > Cs$. When other types of phosphatidic acid were used in place of DOPA, the level of the decrease in MB absorbance on adding the liposomes and the level of absorbance increase (%) on adding inorganic monovalent cation were almost identical with those in the case of DOPA. Some of the results are shown in Table I. As mentioned above, the order of the level of absorbance increase (%) represents that of the strength of adsorption of cations on the negatively charged liposomes. In the absence of specific adsorption, the double layer theory predicts that all monovalent cations should exert identical effects on the surface potential of a membrane containing negatively charged lipids and that the level of absorbance increase (%) caused by all monovalent cations should be identical at the same concentration of the ions.[5] Therefore, these results show that there is specific adsorption of inorganic monovalent cations on negatively charged liposomes. Also, it was found that the order of specific adsorption is the same as that obtained by the measurements of ζ potential or electron

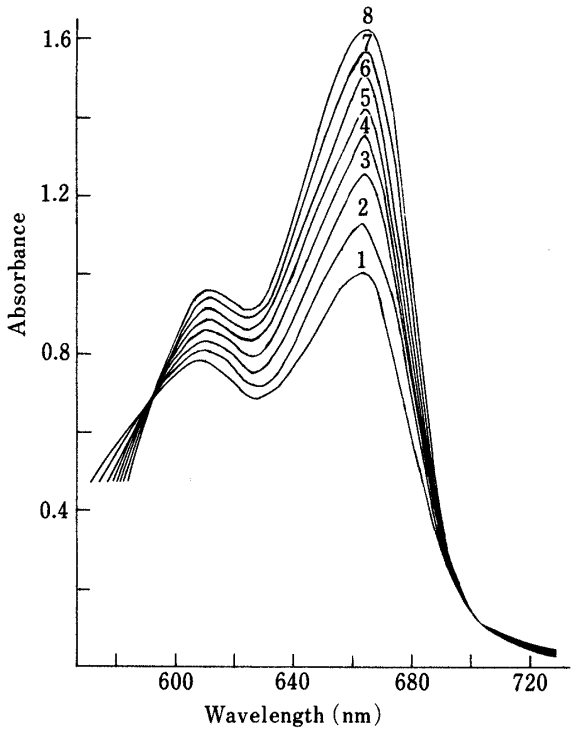

Fig. 1. MB Spectral Change upon Addition of LiCl

1, 0; 2, 0.56; 3, 0.84; 4, 2.10; 5, 3.00; 6, 4.54; 7, 7.00; 8, 10.56 (mM).
The liposomes (membranes) were formed from DPPC and DOPA in the molar ratio of 0.86:0.14. The amount of the liposomes was 24 mg in 100 ml. The absorbance of MB without the liposomes coincides with that of 8 above.

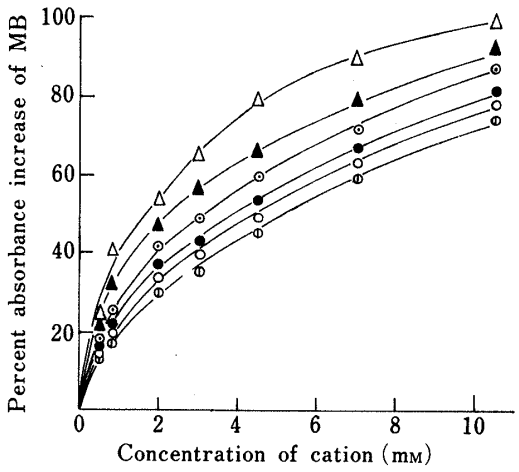

Fig. 2. Percent Absorbance Increase of MB on Addition of Inorganic Monovalent Cations

△, LiCl; ▲, NaCl; ⊙, NH_4Cl; ●, KCl; ○, RbCl; ⓘ, CsCl.
The liposomes and amounts are the same as in Fig. 1.

TABLE I. Percent Absorbance Increase of MB upon Addition of Inorganic Monovalent Cations to Negatively Charged Liposomes[a)]

Cation (3 mM) as chloride	% of absorbance increase of MB			
	DPPC–DOPA	DPPC–DSPA	DPPC–DPPA	DPPC–DMPA
Li	65	60	58	61
Na	56	53	52	55
NH_4	48	46	46	47
K	43	40	39	42
Rb	39	37	36	38
Cs	35	34	33	33

a) The amount of the liposomes was 24 mg in 100 ml and the mole fraction of phosphatidic acid, X_{pA}, was 0.14.

paramagnetic resonance. Consequently, the method using MB was found to be useful to estimate the adsorption of inorganic cations on negatively charged liposomes.

Next, as a simple drug model, the adsorption of aliphatic monoamines (normal chain type) on the negatively charged liposomes was examined. The amines (employed as the hydrochlorides) used are shown in Fig. 3. These amines are present as monovalent cations (ammonium ions) at pH 6.0. The levels of absorbance increase (%) of MB on addition of monoamine hydrochlorides are shown in Fig. 3. The liposomes (membranes) are composed of DPPC and DPPA (mole fraction of DPPA, $X_{pA}=0.20$). The effectiveness of ammonium ions

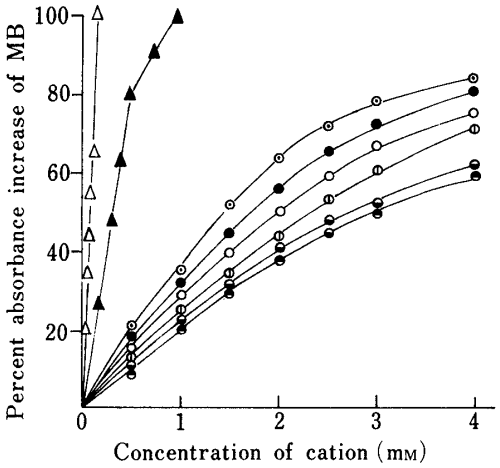

Fig. 3. Percent Absorbance Increase of MB on Addition of Alkylammonium Ions

△, *n*-octylamine·HCl; ▲, *n*-heptylamine·HCl; ⊙, methylamine·HCl; ●, *n*-hexylamine·HCl; ○, ethylamine·HCl; ⓘ, *n*-propylamine·HCl; ◒, *n*-butylamine·HCl; ◓, *n*-amylamine·HCl.
The liposomes were formed from DPPC and DPPA in the molar ratio of 0.80:0.20. The amount of the liposomes was 20 mg in 100 ml.

on the absorbance increase (%) was in the order; *n*-octyl > *n*-heptyl > methyl > *n*-hexyl > ethyl > *n*-propyl > *n*-butyl > *n*-amylammonium ions. This order of the strength of adsorption can be explained as follows: when the bulk of a cation is smaller and its electron density is larger, it can approach more easily to the surface of liposomes and be adsorbed more strongly on the liposomes. Similar considerations can explain why the adsorption of ammonium ions decreases gradually up to *n*-amylammonium ion having a methylene number of five. However, the adsorption of hexylammonium ion having a methylene number of six was stronger than that of *n*-amylammonium ion and was between those of methyl- and ethylammonium ions. Moreover, *n*-heptylammonium ion is adsorbed more strongly than methylammonium ion and the adsorption of *n*-octylammonium ion increases even more than that of *n*-heptylammonium ion. Though *n*-octyl and *n*-heptylammonium ions are adsorbed strongly on the liposomes, it can be presumed that lysis of the liposomes does not occur under the present experimental conditions because the absorbance increase (%) is continuous in the range of low to high concentration of each ammonium ion. If lysis of the liposomes occurs, MB molecules should be exposed to the internal negative charge of the liposomes and should form new dimers; as a result the absorbance increase (%) may not be continuous.

On the basis of the results presented here, the following conclusions may be reached. (1) Ammonium ions having a methylene number of not more than five (amyl) are adsorbed on negatively charged liposomes by electrostatic interaction between the negatively charged phosphate group of phosphatidic acid in membranes and the positively charged ammonium group of the alkylammonium ion. (2) The methylene moiety of alkylammonium ions having a methylene number of more than six (hexyl) can penetrate into membranes and form hydrophobic bonds with methylene moieties of phospholipid. Therefore, alkylammonium ions having a methylene number more than six are adsorbed on the negatively charged liposomes both by the same electrostatic interaction as (1) and by hydrophobic bond formation, which increases with the methylene number of the alkylammonium ion.

It is concluded that the weak adsorption of monovalent cations with negatively charged liposomes can be examined in terms of the MB spectral change.

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