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ORIGINAL CONTRIBUTION

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Formation and properties of HCO-10 vesicles

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Abstract The characteristics of poly(oxyethylene) hydrogenated caster oil ether (HCO-10) vesicles were studied for the standpoints of encapsulation efficiency, stability, solubilization and permeability or barrier efficiency. The vesicles of 5% HCO-10 had 6.24% of calcein-entrapment efficiency and 240 nm of mean diameter. The stability of HCO-10 vesicle suspensions was dependent on their concentrations. In the vesicle suspensions of 10% HCO-10 or more, both the size of the vesicles and the fluidity of the suspensions obviously varied with incubation time, indicating that a flocculation occurred; whereas, the vesicle suspension of 5% HCO-10 was relatively stable. The solubilization process of HCO-10 vesicles by SDS was similar to that of EggPC liposomes.

The rate constants for permeation of Cl ion and calcein were $2.46 \times 10^{-3}\ s^{-1}$ and $5.79 \times 10^{-5}\ s^{-1}$, respectively, suggesting that HCO-10 vesicles possessed some barrier potential for Cl ion and calcein although they were smaller than those of liposomes. Furthermore, the efflux of the solute such as calcein from HCO-10 vesicles was maximum at 37 °C, where the vesicle membrane was presumably destabilized by dehydration of EOs in HCO-10 molecules.

Key words HCO-10 – vesicles – encapsulation – stability – solubilization

Introduction

Since Handjani-Vila et al. [1] and Kunitake et al. [2] introduced some new types of vesicles prepared from surfactants, various surfactants have been used for the preparation of non-ionic surfactant vesicles (NSVs), such as polyoxyethylene alkyl ethers [3], polyglycerol alkyl ethers [4] and glucosyl dialkyl ethers [5]. Early work as well as most current studies on NSVs have dealt with a drug delivery carrier made from non-ionic surfactants. Many reports concerning NSVs, involving animal experiments, have been published, for example, the distribution of drugs incorporated in NSVs has been proved to be similar to that obtained after administration of drugs encapsulated in liposomes [6]. The developments of NSVs in pharmaceutics have demonstrated that NSVs have a potential as a drug delivery carrier. Furthermore, the chemical structures and cost of these surfactants might be taken into consideration when NSVs are adopted as a vehicle for drug delivery. Using NSVs is not only of interest from the technical viewpoint, but also allows a wide study of the influence of chemical composition on the biological fate of vesicles.

Recently, vesicle formation by poly(oxyethylene) hydrogenated caster oil ether (HCO-10, Fig. 1) was reported [7–9]. Tajima et al. reported that in the suspensions below

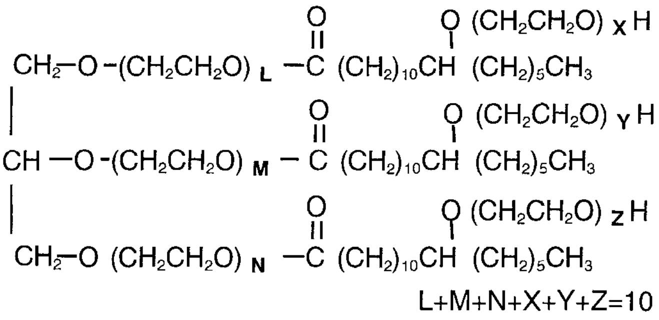


Fig. 1 Chemical structure of polyoxyethylene hydrogenated caster oil ether (HCO-10)

20% HCO-10 the cross nicols were observed by polarized microscopy, indicating a concentric lamellar phase [9]. Tanaka et al. used a freeze-fracture electron microscope to display the vesicular system of HCO-10 [8]. Horiuchi et al. investigated the properties of HCO-10 vesicle suspensions by ESR, ^{1}H-NMR spectra, etc. and found that HCO-10 vesicles prepared by mechanical stirring can entrap solutes, and that the membrane fluidity is temperature-dependent [10]. The preliminary findings indicated that HCO-10 vesicles behave like liposomes.

We started to study the features of HCO-10 vesicles as a drug delivery carrier. This paper is confined to the investigation of encapsulation, stability and retention of solutes, as a prelude to further pharmaceutical studies.

Materials

Technical grade of poly(oxyethylene) hydrogenated Caster Oil ether (HCO-10, Fig. 1), a non-ionic surfactant, was purchased from Nikko Chemicals (Japan) and used without further purification. L-α-phosphatidylcholine from egg yolk (EggPC) was from Nichiyu Liposome Co., Ltd. All other reagents were of analytical grade.

Methods

Preparation of HCO-10 suspension

The vesicle suspension of HCO-10 was prepared by dispersing HCO-10 in an aqueous buffer (20 mM Tris-HCl, pH = 7.4), in some cases, containing calcein; HCO-10 was placed in a tube and an aqueous solution was added. This dispersion was stirred for 10 min by a vortex mixer. The resulting vesicle suspension, in the case of controlling the size of vesicles, was further extruded through two stacked Nuclepore filters (0.6 or 0.2 μm) 5 times after being frozen in liquid nitrogen and thawed in hot water 5 times. Mean diameter of vesicles was determined by quasielastic light scattering (QLS) using Otsuka Electronics LPA 3000/3100.

Calcein-entrapment efficiency

The calcein-entrapment efficiency of vesicles was determined according to the method described by Oku et al. [11]. The vesicles were prepared in 10 μM of a calcein solution; the remaining fluorescence intensities were measured after the fluorescence of untrapped calcein was quenched by the addition of an appropriate amount of 100 mM $CoCl_2$. The entrapment efficiency was calculated by the following equation:

$$\text{entrapment efficiency (\%)} = 100 \times (f_{in} \cdot r_1 - f_{total \cdot q} \cdot r_2)/(f_{total} - f_{total \cdot q} \cdot r_2) \qquad (1)$$

where f_{in} is the fluorescence intensity of the internal calcein; f_{total} and $f_{total \cdot q}$ are the total fluorescence intensity of calcein before and after vesicle destruction, respectively; r_1 and r_2 are correction factors for the volume increase upon adding a $CoCl_2$ solution and the Triton X-100 solution, respectively. They were 2620/2600 and 2625/2600 in our experiments.

Viscosity measurement

The viscosity of HCO-10 suspensions was measured by a cone and plate viscometer (visconic ED, Tokyo Keiki, Japan). The vesicle sizes were measured before and after the viscosity measurements. Both of the experiments were carried out at 25 ± 1 °C.

Cl ion permeability

Cl ion permeability was determined by electrometric measurement of Cl ion efflux into $NaNO_3$ solution using an Ionalyer (Orion Research, Model 701A) with a Cl ion selective solid membrane electrode. The buffer solution used was usually composed of 250 mM NaCl/1 mM EDTA/20 mM Tris buffer (pH = 7.4).

Calcein-permeation kinetics

The vesicle suspensions of HCO-10 were prepared in 100 mM calcein/20 mM Tris buffer (pH = 7.4, 388 mOsm), in which the fluorescence intensity of calcein is self-quenched. The vesicles were separated from untrapped calcein by gel permeation chromatograph using a Sephadex G-75 gel (0.5 × 10 cm column) equilibrated with an isotonic buffer, glucose/20 mM Tris buffer (pH = 7.4). The vesicles eluted with the void volume; free calcein was retarded. The osmolarity of buffers was monitored with an Osmometer (Semi-micro Osmometer, Knauer).

The separated vesicles, suspended in a cold isotonic buffer, were rapidly diluted (1:200) into the well-stirred isotonic buffer equilibrated to the experimental temperature. The fluorescence emission intensity of a sample was recorded continuously subsequent to this dilution (Zero time) on a RF-5000 fluorescence spectrophotometer, Shimadzu (excitation $\lambda = 490$ nm, emission $\lambda = 520$ nm) equipped with temperature control accessories and a magnetic stirrer.

Solubilization of the vesicles by SDS

The addition of SDS (sodium dodecyl sulfate) into the vesicle suspension was performed gently through a dialysis membrane, as follows: the vesicle suspension and an appropriate concentration of SDS, which were separated by a dialysis membrane (size 8, Viskase Sales Corporation), were incubated together 1 day to make SDS enter the HCO-10 suspension slowly. The mean diameter of vesicles and OD_{500} (optical density at 500 nm) of the suspensions were measured as an index of solubilization of the vesicles.

The method of Arand [12] was adopted to determine SDS concentration. EggPC concentration was determined according to Ames [13].

Results

Characterization of HCO-10 vesicle suspensions

Vesicles of 10% HCO-10 prepared by vortex had 11.6% of calcein-entrapment efficiency, which was approximately two-fold higher than 5% HCO-10 vesicles (6.2%), and 368 nm of the mean diameter. The mean diameter of 5% HCO-10 was 240 nm. Mean diameter and size distribution of HCO-10 vesicles also depended on vortex time. In general, the mean diameter of HCO-10 vesicles prepared by vortex was smaller and the size distribution was narrower than those of EggPC.

The mean diameter and entrapment efficiency of HCO-10 vesicles were plotted against time periods on standing, as shown in Fig. 2. In the vesicle suspension of 10% HCO-10 the mean diameter of the vesicles increased greatly after the incubation for 40 h at room temperature and simultaneously the reduction of fluidity of the suspension was observed; however, the entrapment efficiency remained constant after rapidly decreasing in the initial period, suggesting that the vesicles aggregated. On the other hand, in the case of 5% HCO-10 the time-dependence of the mean diameter and entrapment efficiency were rather small. These observations suggested that 10% HCO-10 suspension was a relatively unstable colloid system. In order to confirm the above observation, we further investigated the fluid characteristics of HCO-10 suspensions.

Rheology of HCO-10 vesicle suspensions

Only a few rheological studies on vesicle suspensions have been carried out for all their potential for determining hydrodynamic features of the suspensions [14]. We measured the viscosity of HCO-10 suspensions in an attempt to provide an indication of fluid characteristics of HCO-10 suspensions under certain conditions. Some results are shown in Fig. 3. The arrows indicate upward and downward shear rates, respectively. The viscosities of 5% and 10% HCO-10 suspensions, when prepared by vortex, were 1.26 cp and 1.69 cp, respectively. As shown in Fig. 3, the shear rate versus shearing stress plot for both 5% and 10% HCO-10 suspensions immediately after preparation

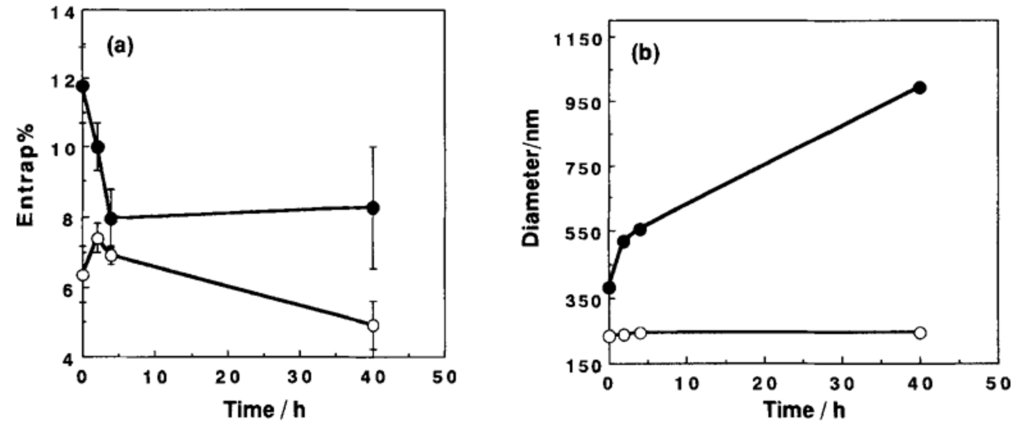


Fig. 2 Time dependences of the calcein entrap% (a) and average particle sizes (b) of HCO-10 vesicles. The vesicle suspensions of 5% HCO-10 (○) and 10% HCO-10 (●) were incubated at room temperature

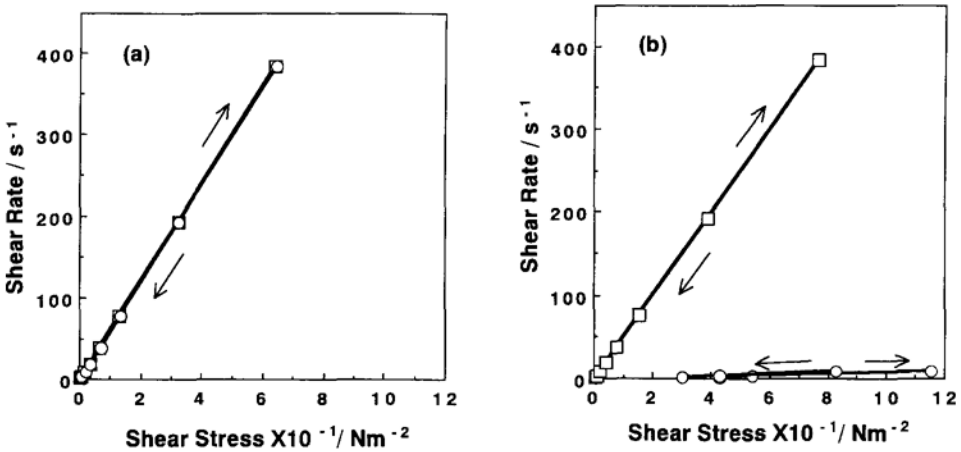


Fig. 3 Rheogram of HCO-10 suspension. Viscosities of 5% HCO-10 (a) and 10% HCO-10 (b) suspensions were measured immediately (□) and 24 h (○) after preparation. The arrows indicated upward and downward shear rates

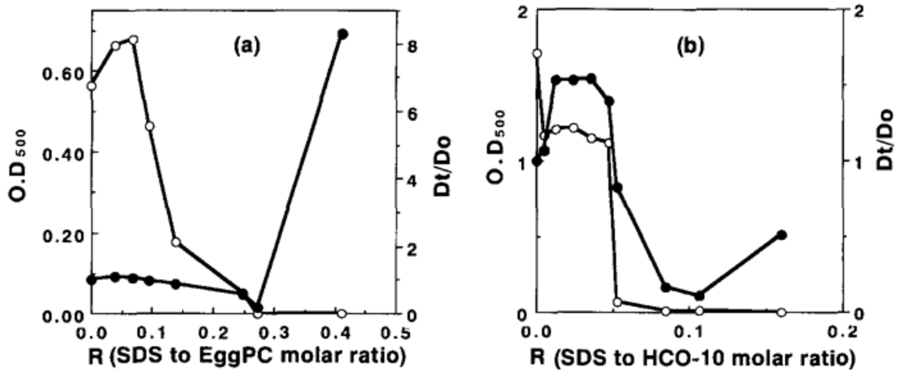


Fig. 4 Variation of turbidity and particle size of EggPC (a) and HCO-10 (b) vesicles containing SDS. Optical density at 500 nm (○) and size (●) was observed 1 day after SDS-addition. Size was represented as a relative value against SDS-free vesicles

showed a straight line and passed through the starting point, demonstrating a Newtonian fluid. After standing for 24 h, the suspension of 10% HCO-10 turned to be a high-viscosity fluid, which was so viscous that the viscosity experiment was restricted to 19.2 s^{-1} or more of shear rate, and a hysteresis in shear stress vs. shear rate curve between upward and downward shear rates was observed, indicating a deviation apparently from the Newtonian fluid, whereas 5% HCO-10 suspension showed almost no change in viscosity after 24 h of standing. Therefore, 5% HCO-10 suspension has a relatively high stability compared with 10% HCO-10 suspension.

Solubilization of HCO-10 vesicles by SDS

To avoid the possible breakdown of a part of vesicles due to a locally high concentration of the surfactant, SDS was added into the suspension through a dialysis membrane with stirring of the suspension. The turbidity of the resultant suspensions and the apparent diameters of vesicles were plotted against a wide range of the molar ratio (R) of bound SDS to HCO-10 or EggPC comprising the region in which vesicles were completely transformed into another aggregate structure, as shown in Fig. 4. In general, there appeared to be a similar solubilization process between HCO-10 and EggPC. However, if Fig. 4a is compared with Fig. 4b in detail, some differences in the process can be found; in the initial stage of SDS addition to EggPC vesicles (up to $R = 0.075$) an increase of turbidity was observed. The turbidity increase is accompanied by an increase of the mean diameter of vesicles due to incorporation of the detergent. In the case of HCO-10 vesicles (up to $R = 0.05$), the mean diameter of the vesicles increased while the turbidity of the suspension decreased rapidly followed by slight increase (Fig. 4b).

Membrane barrier efficiency

The efflux rates of either Cl ion or calcein was measured to evaluate the barrier efficiency of the vesicle membranes.

If Cl ion or calcein permeation obey first-order kinetics:

$$\ln(C_\infty - C) = \ln(C_\infty - C_0) - kt \qquad (2)$$

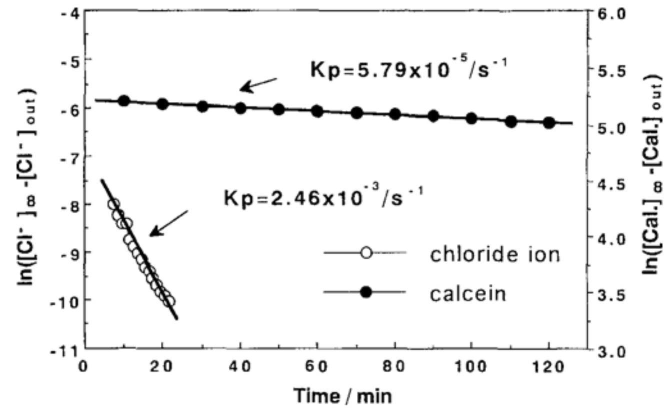


Fig. 5 Cl^- and calcein permeation in HCO-10 vesicles

where k is the rate constant for permeation of Cl ion or calcein, C is the measured concentration at time t, C_0 is the initial concentration, and C_∞ is the concentration after complete liberation of trapped Cl ion or calcein attained by adding triton X-100. k was calculated as the slop of the line obtained by plotting $\ln(C_\infty - C)$ against time t.

Figure 5 shows a typical efflux data of Cl ion and calcein, plotted according to Eq. (2). In the experiment of Cl ion efflux, the total ion-trapped volume was determined from the difference between C_∞ and C_0, the external concentration at zero time. Zero time was defined as the mid-point of the time required to exchange the external medium (replacing Cl^- by NO_3^-), which was usually about 7 min before the first potentiometric measurement for the mean diameter of about 200 nm. In the case of HCO-10 vesicles, the efflux rate constant (k) was 2.46×10^{-3} s^{-1}, indicating a rapid release of Cl ion from HCO-10 vesicles.

The rate constant of calcein efflux (k) was 5.79×10^{-5} s^{-1}, about 100 times smaller than that of Cl ion. Furthermore, HCO-10 vesicle suspensions, in which the calcein outside the vesicles was removed by gel chromatograph, were stored for 5–6 days and when they were destructed by addition of Triton X-100 the fluorescence intensity of those systems increased obviously. All this means that HCO-10 vesicles possess the relative high barrier efficiency for calcein efflux.

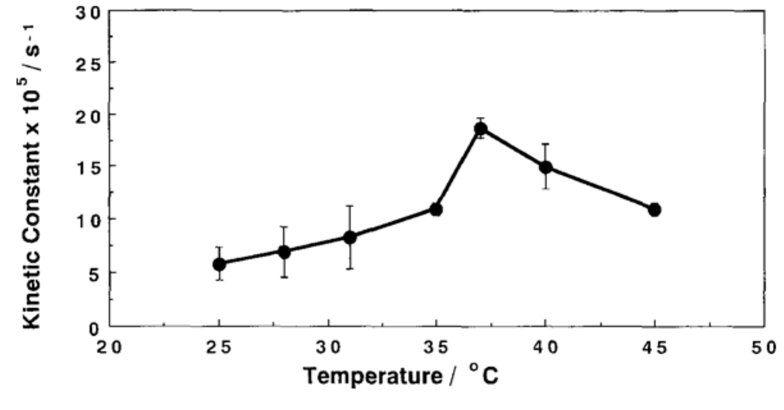
The efflux rate of calcein, measured over a broad range of temperatures, was shown in Fig. 6. There existed a direct dependence of calcein efflux rate on temperature with a superimposed discontinuity. This discontinuity exerted an influence over a relatively broad temperature range (31–40 °C) which corresponds to the phase transition temperature range of the HCO-10 membrane caused by dehydration of HCO-10 molecules [10]. The temperature dependence of calcein efflux kinetics of HCO-10 vesicles seems to resemble that of phosphatidylcholine liposomes [15], which revealed a maximal release at T_c, the melting point of a phospholipid membrane.

Discussion

The sample preparative methods described here produced a closed vesicular system of HCO-10 as judged from the latency of calcein fluorescence, which is analogous to that described for the liposome. Although HCO-10 vesicles were large, multilamellar and polydisperse, the reproducibility of the vesicles in average size, size distribution and other physical properties appeared to be good, indicating that the sample preparative methods adopted here are suitable for the research and development of HCO-10 vesicles.

The stability of the vesicles was evaluated by the changes in the particle size, entrapment efficiency and fluidity. In this study, the suspensions were kept under a natural condition and not disturbed. Therefore, the changes in the mean diameter, entrapment efficiency and fluid flow could be regarded as a spontaneous process. We investigated the stability of 5% and 10% HCO-10 vesicle suspensions and found that in 10% HCO-10 vesicle suspension the apparant size of vesicles increased greatly with incubation time, while the entrapment efficiency became constant after decreasing within several hours. Simultaneously the fluid characteristics of the suspension changed from the

Fig. 6 Temperature dependence of kinetic constants of calcein leakage from HCO-10 vesicles. Error bars represent standard deviations ($n \geq 3$)



Newtonian flow to a non-Newtonian one, as described in the report of Horiuchi and Tajima [16]. When this non-Newtonian fluid was restirred, it was restored to the Newtonian fluid, which suggests the formation of a scaffold structure with a weak interaction. Those results showed that the vesicles easily flocculated in 10% HCO-10 suspension. On the other hand, the vesicle suspension of 5% HCO-10 showed the Newtonian flow, even after 24 h incubation, demonstrating its stable dispersion. Besides, we pursued a vesicle size change before and after viscosity measurements to allow for a possible destruction and fusion of vesicles by rotation of cone plate of the viscometer. There was no change in sizes, showing that the destruction and fusion in the process can be practically neglected in our experimental conditions. The stability of colloidal suspensions is usually governed by three effects: a) the electrostatic repulsion between charges on the surface, b) the hydration force, and c) the steric hindrance. HCO-10 used in our experiments is a non-ionic surfactant, polyoxyethylated hydrogen caster oil ether, which do not introduce any charge into the system. Arnold et al. demonstrated by a small-angle x-ray experiment [17] that phospholipid membrane-fixed poly(oxyethylene) chains increase the membrane repulsion. The instability of HCO-10, resulting from the increase of the concentrations, may be due to the reason that the repulsive hydration force on the surface of HCO-10 vesicles is not strong enough to protect HCO-10 vesicles from flocculation in such a high concentration as 10% HCO-10.

The solubilization of the vesicles by SDS was studied as an indicator to evaluate the stability of the vesicles in the presence of an amphipathic compound. Recently, the solubilization mechanisms of EggPC liposomes by some detergents have been proposed, that is, a transformation from large vesicles to mixed micelles occurs via small vesicle formation, followed by intermediate transition structures [18]. It was also demonstrated that the transformation was not specific for liposomes but was found for NSVs [19]. HCO-10 vesicles in this study showed a similar behavior to EggPC vesicles in the solubilization process by SDS. A difference, that is an abrupt decrease in turbidity in the initial stage, was observed (Fig. 4b). This might result from a polydispersion of HCO-10 aggregates due to its heterogeneous compositions with varying EOs. Some "weak" vesicles or aggregates of HCO-10 might be solubilized rapidly. This difference mentioned above was not displayed in the mean diameter curve of Fig. 4b. This is due to the fact that photo correlation spectroscopy emphasizes the contribution of large structures rather than the mean diameter.

The efflux rate constant of Cl ion from HCO-10 vesicles was $2.46 \times 10^{-3}\ s^{-1}$, much less than that of EggPC liposomes. In our experiment the efflux rate of Cl ion from the EggPC liposome was $3.27 \times 10^{-5}\ s^{-1}$, comparable to $2.3 \times 10^{-5}\ s^{-1}$ of Ueno et al. and $2.1 \times 10^{-5}\ s^{-1}$ of Mimms et al. [20, 21]. This low barrier efficiency of HCO-10 vesicles may be ascribed to the characteristics of HCO-10 molecules: e.g., some of ethylene oxides (EOs) of HCO-10 are located in the trialkyl chains (Fig. 1). ESR experiment results suggested the association of water to EOs located in the membrane core [10]. It likely increases the hydrophilicity of HCO-10 membrane.

A relationship between the efflux rate constants of calcein and temperatures was demonstrated in Fig. 6. The efflux rate constants of calcein from HCO-10 vesicles reached a maximum at the transition temperature. The reduction of the barrier efficiency at the temperature might be due to the unstabilization of hydrophilic region caused by dehydration of EOs and/or the intermolecular packing change in hydrophobic moiety brought about by dehydration of EOs located in membrane matrix. The proposed mechanisms of the abrupt reduction of phospholipid membrane barrier efficiency at T_c (gel–liquid phase transition temperature) were summarized as 1) the appearance of a boundary between the gel phase and the liquid crystalline phase accompanying lateral phase separation, 2) a change in the lateral compressibility between adjacent phospholipid molecules with an increase in membrane fluidity, and 3) the formation of statistical pores by the rotation of phospholipid molecules [23]. Apparently, the mechanism of increase of the calcein release at the transition temperature is different from the above mechanisms, dominated by the unstabilization of the membrane caused by the dehydration of EOs of HCO-10 molecules.

References

1. Handjiani-Vila RM, Ribier A, Rondot BA, Vanlerberghie G (1979) Int J Cosm Sci 1:303–314
2. Kunitake T, Okahata Y (1977) J Am Chem Soc 99:3860
3. a) Hofland HEJ, Bouwstra JA, Talsma H, Junginger HE (1988) Proceed Intern Symp Control Rel Bioact Mater 15:406–407 b) Hofland HEJ et al (1989) Ibid 16:136–137 c) Hofland HEJ et al (1990) Ibid 17:39–40 d) Hofland HEJ et al (1991) J Control Rel 16:155–168
4. Baillie AJ, Florence AT, Hume LR, Muirhead GT, Rogerson A (1985) J Pharm Pharmacol 37:863
5. Kiwada H, Niimura H, Kato Y (1985) Chem Pharm Bull 33:753–759
6. Ozer AY, Hincal AA, Bouwstra JA (1991) Eur J Pharm Biopharm 37:75–79
7. Tajima K, Horiuchi T, Tanaka M (1988) Proc ISF-JOCS World Congn 2:741
8. Tanaka M, Fukuda H, Horiuchi T (1990) J Am Oil Chem Soc 67:55–60

9. Horiuchi T, Tajima K (1992) J Jpn Oil Chem Soc 41:1191–1196
10. Horiuchi T, Tajima K (1992) J Jpn Oil Chem Soc 41:1197–1202
11. Oku N, Knedall DA, MacDonald RC (1982) Biochim Biophys Acta 691:332
12. Arand M, Friedberg T, Oesch F (1992) Analytical Biochemistry 207:73–75
13. Ames S (1966) Methods Enzymol 8:115–118
14. Florence AT (1993) In: Gregoriadis G (eds) Liposome Technology. CRC Press Inc, Vol 1, pp 157–175
15. John Bramhall, Jan Hofmann, Ron DeGuzman, Silvia Montestruque, Richard Schell (1987) Biochemistry 26:6330–6340
16. Horiuchi T, Tajima K, Kanai J (1995) J Jpn Oil Chem Soc 44:1060–1066
17. Anold K, Lvov YM, Szogyi M, Gyorgri S (1986) Studia Biophysica 113–117
18. Ueno M (1993) Membrane (Japan) 18:96–106
19. Lesieur S, Grabielle-Madelmont C, Paternostre MT, Moreau JM, Handjani-Vila RM, Ollivon M (1990) Chem Phys Lipids 56:109–121
20. Ueno M, Tanford C, Reynolds JA (1984) Biochemistry 23:3070–3076
21. Mimms LT, Zampighi G, Nozaki Y, Tanford C, Reynolds JA (1981) Biochemistry 20:833–840
22. Ueno M, Yoshida S, Horikoshi I (1991) Bull Chem Soc Jpn 64:1588–1593