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Penetration enhancement across a model membrane by liposomally entrapped drugs using *N,N'*-diacylcystine as a bilayer lipid

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A liposomal system for percutaneous absorption and transdermal drug delivery was developed and evaluated using a model apparatus connecting two chambers by a membrane. When *N,N'*-long chain diacyl L-cystine was incorporated into the liposomal bilayer as well as lecithin and cholesterol, liposomally entrapped calcein as the model drug transferred well across the keratin membrane functioning as the skin model.

Liposomally entrapped drug; Penetration enhancement; *N,N'*-Diacyl L-cystine; Keratin membrane

1. INTRODUCTION

There have been many studies on percutaneous absorption and transdermal drug delivery systems [1]. Some adhesive transdermal matrices have been utilized clinically [2]. The present authors have developed new penetration enhancers [3] and studied on liposomal assemblies [4-7] for chemical sensing and drug delivery systems. In the present study, we tried to construct a liposomal system suitable for transdermal drug delivery using *N,N'*-long chain diacyl L-cystine incorporated into the liposomal bilayer with lecithin and cholesterol.

2. MATERIALS AND METHODS

2.1. *Reagents*

Egg yolk lecithin (high grade) was purchased from Asahi Chemical Industry Co. Dicetyl phosphate (DCP) and calcein were purchased from Nakarai Tesque Inc. and Wako Pure Chemical Co., respectively. *N,N'*-Diacyl L-cystines were prepared from L-cystine and octanoyl or lauroyl chloride by condensation reaction in NaOH-acetone at 0°C overnight. Isolated precipitate was purified by crystallization from ethanol and analyzed by means of IR and NMR spectroscopies. The cellophane membrane (10 μm thickness) was Visking cellophane tube. The keratin membrane (180 μm thickness) was prepared from white leghorn hen feather, which was dissolved in 0.2 M thioglycolic acid (pH 11 in NaOH) at 50°C for 24 h, and the membrane was prepared by casting after dialytic purification of the solution. Other chemicals were all reagent grade.

2.2. *Preparation of model drug entrapped liposome*

A typical procedure was as follows; egg yolk lecithin (0.028 mmol), cholesterol (0.008 mmol), *N,N'*-dilauroyl L-cystine (C_{12}-SS, 0.004 mmol) were spread on the bottom of a 100 ml round-bottomed flask from a mixed solvent of $CHCl_3/CH_3OH$ and were dried under reduced pressure. 0.4 mM calcein solution (4 ml) of PBS buffer (pH 5.2) was added to the flask, followed by vortex treatment for 15 min and a slight sonication at 40 W for 1 min using a Branson Sonifier, Cell Disruptor 185. Extraneous calcein was removed by gel permeation chromatography using Sephadex G-50 and the liposomal fractions (elution volume, 30-48 ml) in Fig. 1. The size of the liposomes averaged 178 nm. Liposomes negatively stained with phosphotungstic acid were observed using an electron microscope (Fig. 2). The amount of calcein entrapped in the loposomes was estimated at 25% from the difference between the fluorescence intensities of liposome suspensions with and without the addition of Triton X-100 [8].

2.3. *Membrane permeation procedure*

Membrane was clamped between two half cells (Fig. 3), which were placed on a platform in a water bath maintained at 37.0±0.5°C. Liposomal suspension (30 ml) was placed in the donor-half cell and 30 ml of PBS buffer (pH 7.2) in the opposite cell. The stirrer motors were started, and the stirrer propellers were controlled to 120 pm. Samples (4 ml) were removed from the recipient-half cell at suitable time intervals and assayed spectrophotometrically. Permeation experiments were continued for 300 min or more.

2.4. *Instruments*

A transmitting electron microscope (Hitachi HU-12A) was used to observe the liposomes. Liposomal sizes were measured using a Coulter N-4 Submicron Particle Analyzer. The amount of calcein was determined fluorometrically using a Shimadzu RF-540 spectrofluorometer at 520 nm at the excitation wavelength of 480 nm. The amount of *p*-aminobenzoic acid (PABA) was determined at 277 nm using a Hitachi spectrophotometer Model 101.

3. RESULTS AND DISCUSSION

3.1. *Transfer of liposomally entrapped drugs across keratin and cellophane membranes*

Keratin is known to form the stratum corneum of human skin and is a disulfide bonding-rich protein composed of amino acids with sequential networks

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Abbreviations: PBS, phosphate-buffered saline; PABA, *p*-aminobenzoic acid; C_8-SS, *N,N'*-dioctanoyl L-cystine; C_{12}-SS, *N,N'*-dilauroyl L-cystine; DCP, dicetyl phosphate

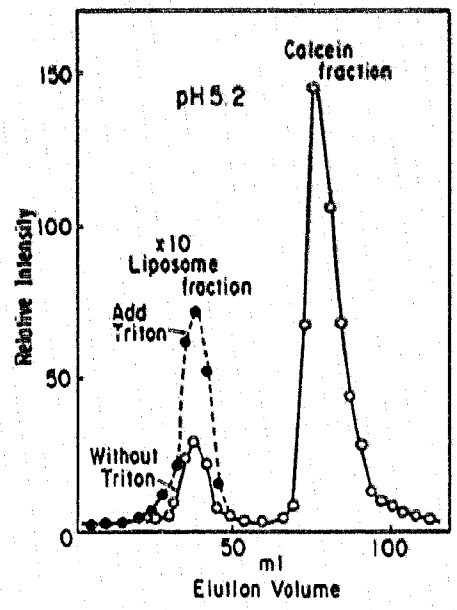


Fig. 1. Removal of extraneous calcein from the liposomal suspension using a Sephadex G-50 column and the determination of the amount of calcein entrapped inside the liposome.

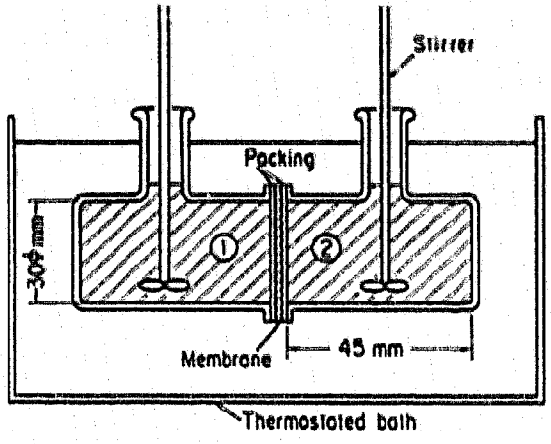


Fig. 3. Two-chamber diffusion cell.

linked by disulfide bonds between cysteine residues. We tried to utilize disulfide-bearing amphiphiles (*N*,*N*'-diacyl cystine) into the liposomal bilayer for liposomal drug entrapment [9] due to percutaneous absorption. Transferring experiments of liposomally en-

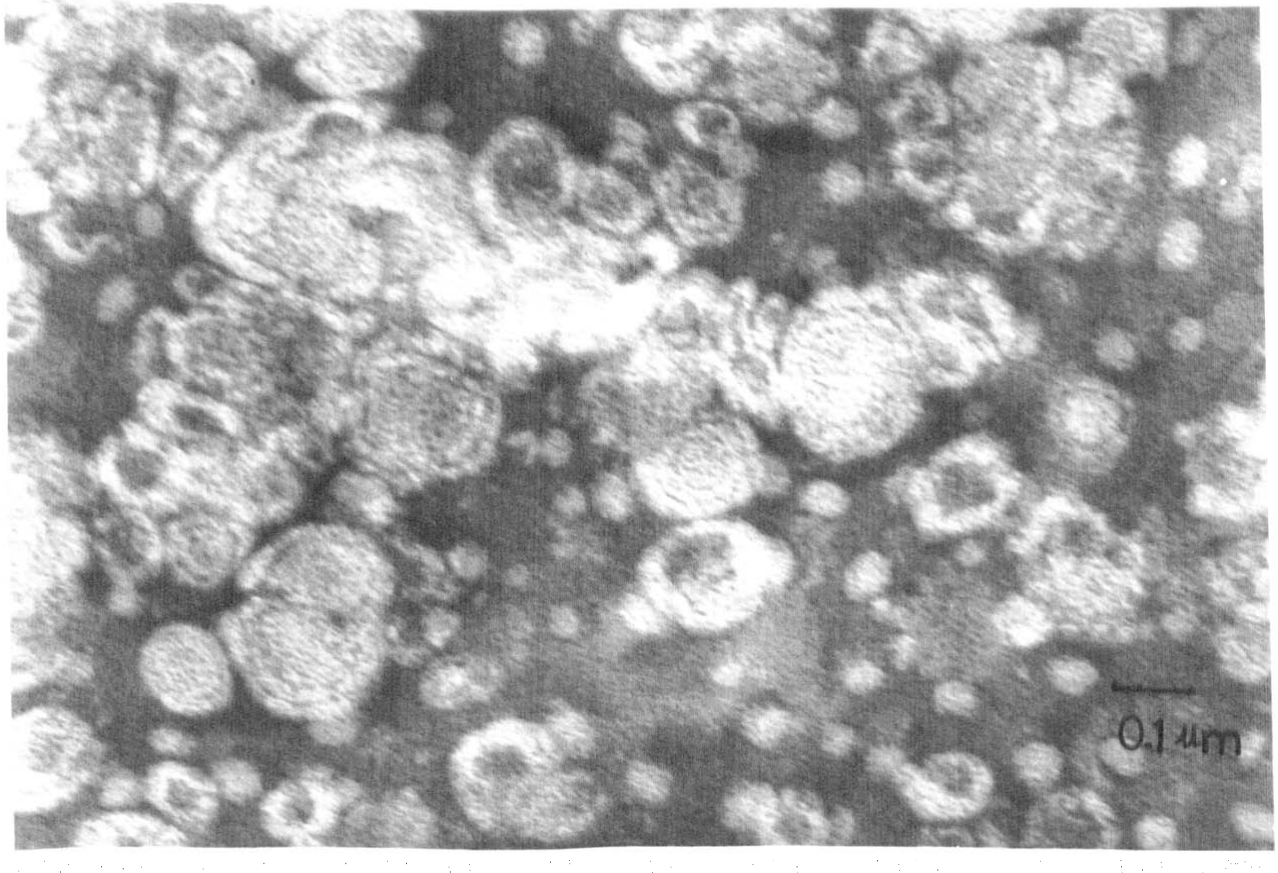
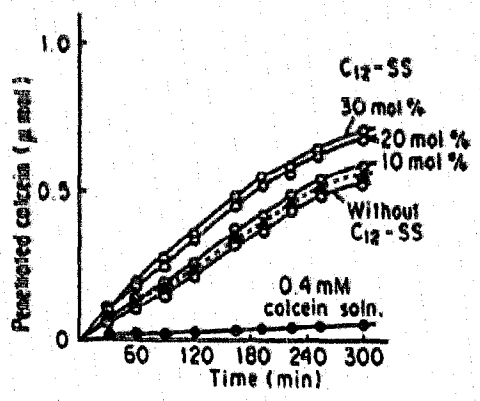


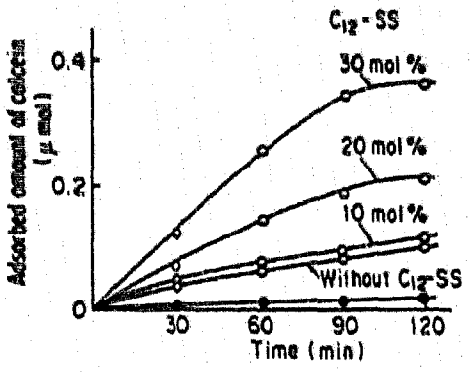
Fig. 2. Electron micrograph of negatively stained liposomes consisting of egg lecithin/cholesterol/C_{12}-SS (70:20:10).



Constitution of liposomes

	30mol%	20	10	▲	○	●
Egg lecithin	50	60	70	70	70	-
Cholesterol	20	20	20	20	20	-
C_{12}-SS	30	20	10	-	-	-
DCP	-	-	-	10	-	-

Fig. 4. Effect of C_{12}-SS content of liposomes on calcein transfer across the keratin membrane (donor cell: liposomal suspension in PBS (pH 5.2), 30 ml; recipient cell: PBS (pH 7.2), 30 ml).

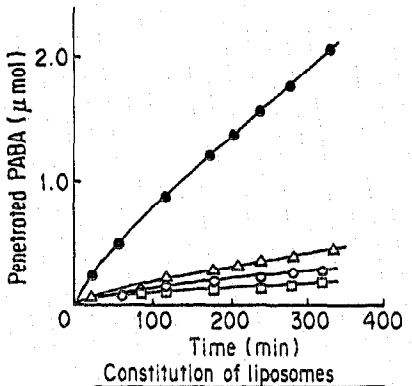


Constitution of liposomes (molar ratio)

	Keratin				Cellophane
	mol %				●
Egg lecithin	50	60	70	70	50
Cholesterol	20	20	20	20	20
C_{12}-SS	30	20	10	-	30

Fig. 6. Desorption of adsorbed calcein from the keratin and cellophane membranes immersed in PBS (pH 7.2) after finishing the experiments of Figs. 4 and 5.

trapped calcein across the keratin membrane were carried out until 300 min (Fig. 3). The effect of the constitutions of the liposomes on calcein transfer is obvious in Fig. 4. Namely, the greater the content of C_{12}-SS in the liposomal bilayers, the greater the permeation of liposomally entrapped calcein. It may be also noted that the liposomal system without C_{12}-SS (Fig. 4) also enhances calcein release in agreement with the result of Katoh [10]. In fact, C_{12}-SS (30 mol%) or DCP (10 mol%) containing liposome cause 14- or 11-fold increase in calcein permeation over the 0.4 mM calcein solution in Fig. 4. Incidentally, the amount of liposomally entrapped calcein in the donor cell was calculated to be ca. 3 μmol and reduced to 0.7 μmol at the maximum after 300 min in Fig. 4. A similar result was obtained spectrophotometrically for PABA permeation when 0.3 mM PABA solution was introduced into the donor cell instead of 0.4 mM calcein. Free



Constitution of liposomes

	○	△	□
Egg lecithin	70	70	70
Cholesterol	20	20	20
C_8-SS	10	-	-
C_{12}-SS	-	10	-

● 0.3mM PABA solution (Without liposome)

Fig. 5. Transfer of PABA across the cellophane membrane from PABA-consisting liposomes (donor cell: liposomal suspension in PBS (pH 5.2), 30 ml; recipient cell: PBS (pH 7.2), 30 ml).

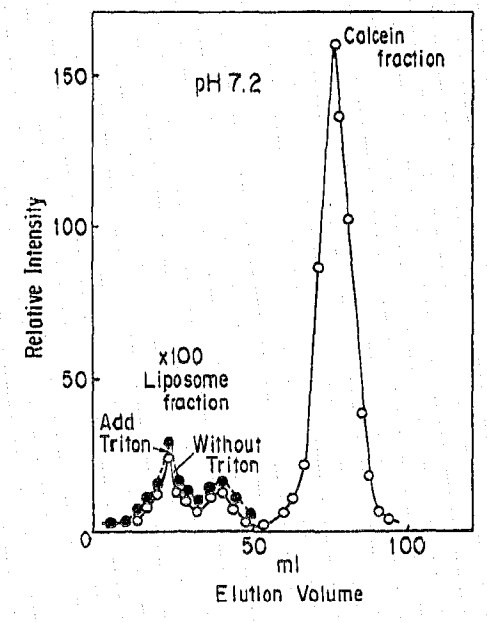


Fig. 7. Leakage of calcein in the liposomes consisting of lecithin/cholesterol/C_{12}-SS (70:20:10) prepared at pH 7.2.

PABA molecules are transferred more easily into the recipient cell across a cellophane membrane than liposomally entrapped PABA, as shown in Fig. 5. Liposomally entrapped PABA transfer is suppressed by the inhibition of liposomal bilayers and the cellophane membrane up to 1/10 at 320 min to the penetrated amount of PABA from the 0.3 mM PABA solution. C_{12}-SS and C_8-SS containing liposomes accelerate PABA transfer to more than double the amount of liposomes consisting of lecithin and cholesterol, since these liposomes may cause the occurrence of defective bilayer domains affected by the alkaline environment in the recipient cell.

3.2. *Effect of* N,N'-*long chain diacyl L-cystine incorporated into the liposomal bilayer on transfer acceleration of calcein through a keratin membrane*

A significant difference between the permeability of liposomally entrapped model drugs through keratin and cellophane was found in section 3.1. The amount of calcein adsorbed onto the keratin and cellophane was determined fluorometrically for the membranes used in the experiments of Figs. 4 and 5. The membranes were washed twice with water. The test pieces were then immersed in 4 ml PBS (pH 7.2) of fluorometric cells and their fluorescent intensities were measured until 120 min after removing the pieces outside. The results are shown in Fig. 6. The value at 120 min seems to be the equilibrium value. Keratin holds calcein and increases the amount with increases in C_{12}-SS in the liposomal bilayer. Keratin is also able to hold calcein even with liposomes without C_{12}-SS. Cellophane holds only a small amount of calcein. Therefore, it was concluded that C_{12}-SS and the basic liposomal constituents of lecithin and cholesterol have an affinity for keratin. C_{12}-SS has an especially large affinity. This seems to be due to the mediating (or accompanying) action of C_{12}-SS for calcein as an amphiphile. This is meaningful for transdermal mechanism, because stratum corneum consisting of keratin is known to be the interfering barrier for percutaneous absorption of drugs. The proper affinity of C_{12}-SS for keratin may be attributed to their interpenetration on the basis of the disulfide-rich structure of keratin. Ishii [11] discussed the interaction between liposomes and the skin in citing [9]. He took the position that liposomes enhance drug transfer inside the skin, but that liposomes do not penetrate the skin itself. It is also important to point out the pH-sensitive property of *N,N'*-diacyl L-cystine. Namely, C_{12}-SS can gain strong surface activity by changing to carboxylate ions in contact with the alkaline environment (pH 7.2) in the recipient cell. It is obvious from Fig. 7 that C_{12}-SS containing liposomes cannot hold calcein inside at pH 7.2. Thus, *N,N'*-diacyl L-cystine plays an important role in the acceleration of calcein transfer through keratin.

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