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Research paper

Modification of ceftibuten transport by the addition of non-ionic surfactants

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Abstract

The effects of non-ionic surfactants on the carrier-mediated transport of ceftibuten by rat intestinal brush-border membrane vesicles (BBMVs) were investigated. Ceftibuten uptake by BBMVs was measured by a rapid filtration technique. The concentration of surfactants for the uptake experiments was determined by a decrease in the turbidity of BBMV suspension and by the release of an impermeable probe, 2′,7′-bis(carboxyethyl)-4(5)-carboxyfluorescein, from the vesicle inside. In fact, the surfactant concentration of 0.03% (w/v) was selected to maintain the stability of BBMVs. The extent of ceftibuten uptake by BBMVs with various surfactants was correlated with their physicochemical properties, i.e. hydrophile–lipophile balance (HLB), critical micelle concentration (c.m.c.), average diameter of micelle colloid, and polydispersity determined by particle size distribution. The surfactants used were divided into two groups on the basis of polydispersity index (d_w/d_n), i.e. low polydispersity ($d_w/d_n \cong 1$) and high polydispersity $d_w/d_n > 2$). The ceftibuten uptake due to the addition of surfactants with low polydispersity increased with a decrease in the HLB number. These results indicate that the ceftibuten transport is modulated by the size distribution and hydrophobicity of surfactants. In addition, the effects of surfactants on the membrane lipid fluidity monitored by diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) were investigated. There was significant correlation between ceftibuten uptake and the fluorescence anisotropy of TMA-DPH-labeled membranes due to the addition of surfactants with low polydispersity ($r = -0.81$, $P < 0.0001$). These results suggest that surfactants with low polydispersity, in part, increase or decrease the outer membrane leaflet, thereby enhancing or suppressing the ceftibuten transport by BBMVs, and that ceftibuten transport caused by surfactants with low polydispersity may be strongly dependent on the hydrophobic interaction.

Keywords: Non-ionic surfactant; Carrier-mediated transport; Ceftibuten; Brush-border membrane vesicle; HLB number; Polydispersity; Particle size distribution; Hydrophobicity; Membrane lipid fluidity

1. Introduction

As oral pharmaceutical adjuvants, non-ionic surfactants are much safer for the biological membranes than ionic surfactants [1]. Therefore, many kinds of non-ionic surfactants are used as emulsifiers to improve the aqueous solubility and oral bioavailability of drugs. There are many reports describing the function of non-ionic surfactants in the drug intestinal absorption from the viewpoint of biopharmaceutics [2,3]. In recent years, it has been reported that the transport of Na^+-dependent D-glucose by rat intestinal brush-border membrane vesicles (BBMVs) was inhibited by the addition of unsaturated fatty acid and octyl-β-D-glucopyranoside [4,5]. Thus there are a number of reports about the action of non-ionic surfactants as pharmaceutical adjuvants, but the effects of surfactants on carrier-mediated drug transport in BBMVs have not been fully elucidated.

The aim of this study was to elucidate the effects of physicochemical properties of surfactants on the modulated transport of ceftibuten, i.e. hydrophile–lipophile balance (HLB), critical micelle concentration (c.m.c.), average particle size (diameter of micelle colloid), and the weight average diameter to number average diameter ratio (d_w/d_n) as a measurement of polydispersity. For this study, we selected 25 kinds of non-ionic surfactants with various hydrophobicities that are used as oral pharmaceutical adjuvants.

It is known that ceftibuten is transported via a proton-coupled peptide transporter in rat intestinal BBMVs [6,7].

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Table 1
List of non-ionic surfactants and their physicochemical parameters

Chemical name	Product name	HLB number[a]	c.m.c.[b] (mg/l)	Average diameter[c]	d_w	d_n	d_w/d_n[d]
Sorbitan monolaurate	Span 20	8.6	12.6	553	1879	540	3.5
Sorbitan sesquioleate	Span 30	3.7	11.9	596	1369	235	5.8
Sorbitan monooleate	Span 80	4.3	11.3	903	1635	267	6.1
Sorbitan trioleate	Span 85	1.8	10.9	741	1915	289	6.6
Polyoxyethylene(20)sorbitan monolaurate	Tween 20	16.7	27.0	96	6	5	1.2
Polyoxyethylene(20)sorbitan monopalmitate	Tween 40	15.6	17.0	278	8	6	1.2
Polyoxyethylene(20)sorbitan monostearate	Tween 60	14.9	15.9	491	1488	301	4.9
Polyoxyethylene(20)sorbitan monooleate	Tween 80	15.0	15.6	184	7	6	1.1
Polyoxyethylene(20)sorbitan trioleate	Tween 85	11.0	11.5	151	50	42	1.2
Polyoxyethylene(6)sorbitan monolaurate	Tween 21	13.3	15.8	117	30	27	1.1
Polyoxyethylene(6)sorbitan monooleate	Tween 81	10.0	13.6	228	300	98	3.1
Sucrose stearate palmitate(7:3)monoester	SS	19.0	13.0	61	7	6	1.1
Sucrose stearate palmitate(7:3)polyester[e]	F-140	13.0	47.5	408	505	111	4.6
Sucrose stearate palmitate(7:3)polyester[f]	F-160	15.0	30.9	396	490	115	4.3
Tetraglycerol monooleate	MO-310	10.2	19.0	371	6	6	1.0
Hexaglycerol monooleate	MO-500	12.2	19.9	143	50	48	1.0
Decaglycerol monooleate	MO-750	14.5	21.6	155	166	161	1.0
Tetraglycerol monolaurate	ML-310	11.9	19.7	140	63	61	1.0
Hexaglycerol monolaurate	ML-500	13.8	23.1	98	18	17	1.0
Decaglycerol monolaurate	ML-750	15.7	26.6	98	29	29	1.0
Tetraglycerol monostearate	MS-310	10.2	13.0	438	820	798	1.0
Hexaglycerol monostearate	MS-500	12.2	20.4	502	163	152	1.1
Hexaglycerol sesquistearate	SS-500	10.1	28.3	461	491	434	1.1
Hexaglycerol tristearate	TS-500	6.5	14.8	706	668	631	1.1
Decaglycerol tristearate	TS-750	9.1	15.9	525	371	352	1.1

[a] The HLB numbers of surfactants were calculated from Griffin's equation.
[b] The c.m.c. values of surfactants were determined by measurement of pyrene fluorescence intensity at 25°C using a Hitachi Spectrofluorometer 850 by the method of Ollmann [34].
[c] Average diameters were determined by a cumulant analysis at the concentration of 0.03% (w/v).
[d] The d_w/d_n values were the ratio of weight average diameter d_w to number average diameter d_n.
[e] Monoester compound polyester compound (62:38).
[f] Monoester compound polyester compound (74:26).

Some studies have reported that the transport of drugs or nutrients in rat intestinal BBMVs is affected by membrane lipid fluidity [8–10]. In particular, it is important to consider that ceftibuten transport may be modified by a change in the lipid fluidity induced by the addition of a surfactant. Therefore, we also investigated the effects of surfactants on the membrane lipid fluidity using fluorescent probes.

2. Materials and methods

2.1. Materials

The physicochemical parameters of non-ionic surfactants used in the present study are summarized in Table 1. Sorbitan (Spans) and polyoxyethylene (Tweens) surfactants were purchased from Sigma (St. Louis, MO, USA). Fatty acid sucrose esters and fatty acid glycerol esters were kindly donated by Daiichi Kogyo Seiyaku Co. (Kyoto, Japan) and Sakamoto Chemicals Co. (Tokyo, Japan), respectively. Ceftibuten was a gift from Shionogi Co. (Osaka, Japan). 2′,7′-Bis(carboxyethyl)-4(5)-carboxyfluorescein (BCECF) was purchased from Dojindo Lab. (Kumamoto, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene iodide (TMA-DPH) and pyrene were purchased from Wako Pure Chemical Ind. (Osaka, Japan). BCA protein assay kita was obtained from Pierce Chemical Co. (Chicago, IL, USA). All other chemicals were analytical reagent grade.

2.2. Dynamic light scattering (DLS) studies

Measurements of the DLS of surfactants in buffered solution [20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/Tris buffer, pH 5.5, containing 100 mM D-mannitol and 100 mM KCl; buffer A] were carried out using a laser particle analyzer (DLS-7000 and LPA-3000/3100, Otsuka Electronics Co., Osaka, Japan) at 25°C. A DLS-7000 with a 488.0 nm Ar laser at a power of 75 mW was used to measure Tween 20, Tween 40, Tween 80, and sucrose stearate palmitate(7:3)monoester (SS). An LPA-3000/3100 using a 632.8 nm He–Ne laser at a power of 5 mW was used to measure other surfactants. The average diameters of the surfactants were evaluated as a *Z*-average using a monomo-

dal method (a cumulant analysis) and particle size distribution as a ratio of d_w to d_n using a multimodal method (exponential sampling algorithm) [11,12]. The range for the analysis in the multimodal method was set from 1 nm to 5 μm in diameter at the first run of each measurement. All surfactants were assayed at the concentration of 0.03% (w/v).

2.3. Membrane vesicle preparations

BBMVs were prepared from the small intestine of male Wistar rats (220–250 g) by $MgCl_2$ precipitation [13]. Final BBMVs were suspended in 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/Tris buffer (pH 7.5) containing 100 mM D-mannitol and 100 mM KCl (buffer B) at a concentration of 10 mg protein/ml.

The quality of the preparation was determined by assay of the activities of alkaline phosphatase and sucrase which are known as marker enzymes in BBMVs [14]. The activities of these two enzymes in BBMVs were enriched about 15 and 10 times that of the original mucosa.

2.4. Stability of BBMVs

The effects of surfactants on the solubility and integrity of BBMVs were evaluated at 25°C by the change in the turbidity of the membrane suspension (0.7 mg protein/ml) with a surfactant [15] and the release of an impermeable fluorescent probe, BCECF, loaded into BBMVs [16], respectively. The turbidity assay was carried out after 5 min incubation (at final surfactant concentrations of 0.005 to 0.1%, w/v). The BCECF-loaded BBMVs were prepared as follows: after incubation of the isolated rat intestinal mucosa with 5 μM BCECF for 30 min at 37°C, the intestinal mucosa was homogenized using an Oster blender (Osaka, Japan) at 16 000 rpm. The prepared BBMVs were centrifuged at 70 000*g* for 15 min to remove the untrapped BCECF, and then the precipitate was resuspended in buffer B at a final concentration of 2 mg protein/ml. To 1.5 ml of the BCECF-loaded BBMVs, 1.5 ml of a surfactant (range 0–0.1%, w/v) or 0.5% Triton X-100 was added. The mixture was then incubated for 10 min at 25°C and centrifuged at 27 000*g* for 20 min. The fluorescence intensity of the supernatant was measured at $E_x = 490$ nm and $E_m = 526$ nm using a Hitachi spectrofluorometer 850 (Tokyo, Japan) at 25°C. The permeability of BCECF from the vesicle inside, f (%), is defined as

$$f = 100(f_s - f_b)/(f_a - f_b)$$

where f_s is the fluorescence intensity of BCECF in BBMVs with a surfactant, f_b the fluorescence intensity in BBMVs without a surfactant, and f_a the fluorescence intensity in BBMVs thoroughly dissolved by 0.5% Triton X-100, respectively.

2.5. Uptake studies

Ceftibuten uptake was studied out by rapid filtration using a Millipore filter (HAWP, 0.45 μm, 25 mm diameter, Japan Millipore Ltd., Tokyo, Japan) in the presence and absence of an inward H^+-gradient ($pH_{in} = 7.5$, $pH_{out} = 5.5$ or 7.5) at 25°C. The reaction was initiated by the addition of 110 ml of buffer A or buffer B containing 0.06% surfactant and 1.5 mM ceftibuten to 110 ml of BBMV suspension. The reaction medium containing BBMVs was stopped by the addition of 3 ml ice-cold buffer B at 0.5, 1, 2, 5 and 20 min after incubation, and then filtered immediately. The millipore filter trapping the membranes was dried at room temperature. Extraction and measurement of ceftibuten trapped in the millipore filter were carried out according to the method of Sugawara et al. [17].

2.6. Fluorescence studies

It is known that DPH and TMA-DPH reflect the fluidity of inner (hydrophobic core domain) and outer (superficial domain) layers of the membrane lipids, respectively [18]. DPH- or TMA-DPH-labeled membrane vesicles were prepared by the method described previously [19]. It is believed that the fluorescence probes distribute from BBMVs to surfactant micelles. Therefore, the changes (P) in the fluorescence intensity of two probes were measured using the following equation,

$$P = (I_b - I_c)/I_a$$

where I_a is the fluorescence intensity of DPH or TMA-DPH in BBMVs (as a final concentration of 0.2 mg protein/ml) dissolved in 0.5% Triton X-100, I_b the fluorescence intensity in BBMVs with a surfactant, and I_c the fluorescence intensity in BBMVs without a surfactant, respectively. Fluorescence intensity was measured in the presence and absence of the surfactants (0.03%, w/v) at 25°C. Steady state polarization studies were routinely performed with a Hitachi spectrofluorometer 850 equipped with a rhodamine B quantum counter. The excitation and emission wavelengths used were 363 and 428 nm for DPH, and 365 and 428 nm for TMA-DPH. The fluorescence anisotropy (γ) was calculated using the following equation [20],

$$\gamma = (I_V - I_H)/(I_V + 2I_H)$$

where I_V and I_H represent the fluorescence intensities of the vertically and horizontally polarized excitation, respectively.

3. Results and discussion

3.1. Effects of surfactants on the stability of BBMVs

Our first purpose was to determine the concentration range within which surfactants have no effect on the solubility and integrity of BBMVs. The turbidity of the membrane suspensions decreased with increasing concentration of the surfactants (range 0–0.1%, w/v) at 25°C. Decreases in the turbidity due to the addition of surfactants

Table 2
Effects of surfactants on turbidity of membrane vesicle suspension at 25°C at various surfactant concentrations (%, w/v)

Surfactant	HLB number	Turbidity (% of control)				
		0.005	0.01	0.03	0.05	0.1
MO-310	10.2	99.4	96.4	90.2	84.9	79.7
MO-500	12.2	99.3	96.3	90.0	85.6	80.8
F-140	13.0	99.0	96.4	90.2	84.9	79.7
MO-750	14.5	98.6	95.1	88.1	83.3	75.1
F-160	15.0	98.1	95.0	86.6	82.2	74.2
Tween 20	16.7	98.1	94.3	82.6	78.2	71.2
SS	19.0	97.9	94.4	82.1	78.0	68.2

with low HLB number (HLB < 10) were hardly observed (data not shown). On the other hand, the addition of a surfactant with a high HLB (≥ 10) decreased turbidity with increasing HLB number (Table 2). In particular, the turbidity of BBMV suspensions with F-160, Tween 20, and SS at the concentration of 0.1% (w/v) were 74.2, 71.2 and 68.2 of that without surfactants, respectively.

The BCECF released from BBMVs increased in proportion to the HLB number (Table 3). The permeabilities (f) of BCECF in the presence of MO-750, F-160, Tween 20, and SS at the concentration of 0.1% (w/v) were 22.4, 23.8, 26.1 and 26.8%, respectively, compared to those in the absence of surfactants. Based on these results, a surfactant concentration of 0.03% (w/v) was selected to maintain the turbidity of BBMVs at more than 80% and the permeability of BCECF at less than 12%, indicating that the membrane function as a vesicle was being maintained in the present experiments.

3.2. Physicochemical properties of surfactants

The physicochemical properties of the surfactants in the absence of ceftibuten are shown in Table 1. The HLB numbers and d_w/d_n values represent hydrophobicity and polydispersity, respectively [21,22]. Further, the HLB number and c.m.c. value are known to be key factors in the effects of surfactants on biological membranes [23]. In particular, some studies have reported that differences in the hydrophobicity of surfactants affect membrane lipid fluidity [24] and the alkaline phosphatase of intestinal BBMVs [25]. When the particle size distribution of pharmaceutical adjuvants in aqueous solution is broad, the d_w/d_n value is a more suitable parameter to estimate the pharmaceutical characteristics of surfactant micelle colloids [22,26], fat emulsion [27], and liposomes [28]. The d_w/d_n values of several surfactants (Spans, Tween 60, Tween 81, F-140, and F-160) were clearly above 2, which meant a high polydispersity. Fig. 1A shows typical size distributions as d_w and d_n for Span 20, Tween 60, and F-160. Their size distributions showed 2 peaks which indicated that there were a small fraction of small size micelle colloids and a large fraction of large size micelle colloids. On the other hand, the d_w/d_n values of the other surfactants were approximately 1, indicating low polydispersity. Typical size distributions of surfactants with low polydispersity are shown in Fig. 1B,C. Therefore, the surfactants used could be divided into two groups ($d_w/d_n \cong 1$ and $d_w/d_n > 2$) on the basis of particle size.

Table 3
Effects of surfactants on the release of an impermeable probe, BCECF, from membrane vesicles at 25°C at various surfactant concentrations (%,w/v)

Surfactant	HLB number	Permeability (%)				
		0.005	0.01	0.03	0.05	0.1
MO-310	10.2	0.4	3.1	4.3	12.4	13.6
MO-500	12.2	0.5	3.2	5.1	13.2	17.4
F-140	13.0	0.9	3.2	5.2	13.7	18.4
MO-750	14.5	1.2	4.2	6.8	15.1	22.4
F-160	15.0	1.9	4.2	7.7	16.8	23.8
Tween 20	16.7	1.7	5.0	11.4	19.9	26.1
SS	19.0	1.9	5.4	11.3	19.7	26.8

Table 4
Effects of surfactants on ceftibuten uptake by rat intestinal brush-border membrane vesicles in the absence and presence of an inward H^+-gradient condition at 25°C[a]

Surfactant	Ceftibuten uptake (nmol/mg protein at 1 min)			
	$pH_{in} = 7.5$, $pH_{out} = 7.5$		$pH_{in} = 7.5$, $pH_{out} = 5.5$	
	0.03%	% of control	0.03%	% of control
Control	0.23 ± 0.04	100	1.24 ± 0.14	100
Span 20	0.21 ± 0.05	91	1.11 ± 0.15	89
Span 30	0.22 ± 0.03	96	1.16 ± 0.09	93
Span 80	0.22 ± 0.03	96	1.18 ± 0.11	95
Span 85	0.22 ± 0.03	96	1.21 ± 0.16	98
Tween 20	0.17 ± 0.03*	74	0.82 ± 0.09*	66
Tween 40	0.15 ± 0.02*	65	0.88 ± 0.07*	71
Tween 60	0.22 ± 0.04	96	1.08 ± 0.13	87
Tween 80	0.20 ± 0.03	87	0.98 ± 0.06	79
Tween 85	0.26 ± 0.03	113	1.29 ± 0.12	103
Tween 21	0.24 ± 0.04	104	1.12 ± 0.13	90
Tween 81	0.21 ± 0.03	91	1.04 ± 0.09	84
SS	0.17 ± 0.03*	78	0.88 ± 0.06*	71
F-140	0.23 ± 0.02	100	1.08 ± 0.11	87
F-160	0.23 ± 0.02	100	1.11 ± 0.08	89
MO-310	0.26 ± 0.04	113	1.70 ± 0.19*	137
MO-500	0.25 ± 0.03	109	1.33 ± 0.13	106
MO-750	0.21 ± 0.03	91	0.94 ± 0.14	76
ML-310	0.24 ± 0.04	104	1.44 ± 0.17	116
ML-500	0.27 ± 0.03	117	1.31 ± 0.12	105
ML-750	0.24 ± 0.04	104	1.16 ± 0.12	93
MS-310	0.26 ± 0.04	113	1.78 ± 0.22*	143
MS-500	0.29 ± 0.04	126	1.93 ± 0.21**	155
SS-500	0.30 ± 0.05	130	2.09 ± 0.24**	168
TS-500	0.32 ± 0.04*	139	2.45 ± 0.22**	197
TS-750	0.30 ± 0.05	130	1.94 ± 0.19*	156

[a] The procedures and conditions of the ceftibuten uptake experiments are described in Section 2. Data represent the mean ± S.D. of three to five experiments. Statistical significance between the systems without and with surfactants is indicated by $*P < 0.05$, $**P < 0.01$ (vs. control by Dunnett's multiple range test).

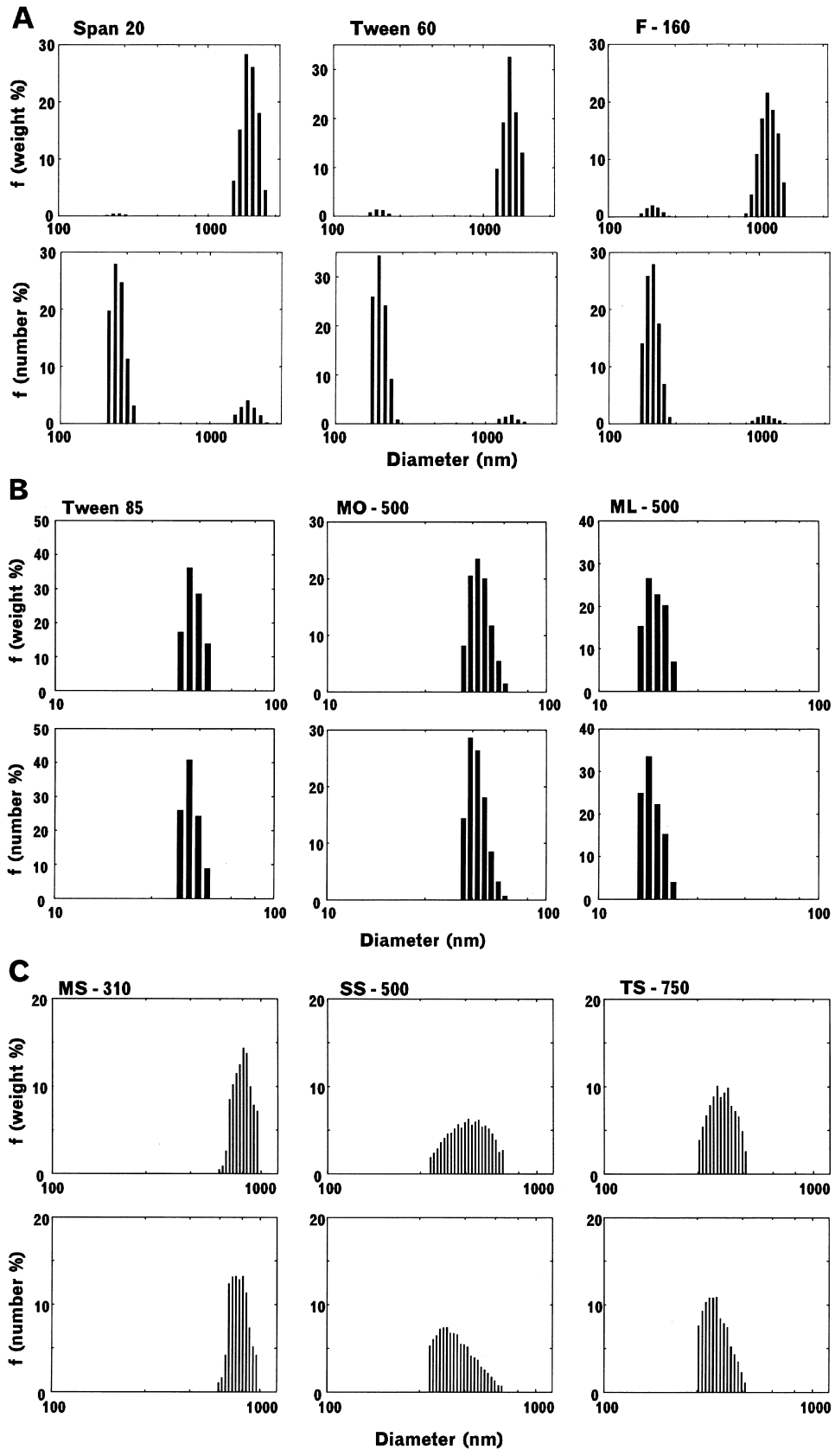

Fig. 1. Weight- and number-particle size distributions of Span 20, Tween 60 and F-160 (A), Tween 85, MO-500 and ML-500 (B), and MS-310, SS-500 and TS-750 (C) at 25°C. Each surfactant concentration was 0.03% (w/v). Accumulation times for scattering analysis were 50.

3.3. Effects of surfactants on ceftibuten uptake

The overshoot phenomenon of ceftibuten transport by BBMVs with or without surfactants was observed at 1 min after incubation under an inward H^+-gradient at 25°C (data not shown). Therefore, the effects of surfactants on ceftibuten uptake in BBMVs were compared at 1 min after incubation in the presence of surfactants.

Table 4 shows the effects of surfactants on the ceftibuten uptake in the absence and presence of an inward H^+-gradient. Tween 20, Tween 40, and SS significantly suppressed ceftibuten uptake in both the presence and absence of the H^+-gradient. TS-500 significantly enhanced the drug uptake under the same conditions. MO-310, MS-310, MS-500, SS-500, and TS-750 significantly enhanced the drug uptake only under the H^+-gradient. Other surfactants did not affect the uptake. These results indicated that ceftibuten uptake in BBMVs was modulated by several surfactants, which were classified into groups that had no effect, enhanced, or suppressed ceftibuten uptake. In particular, the enhancement was remarkable when glycerol esters with stearate were used.

3.4. Contribution of physicochemical properties of surfactants to ceftibuten uptake

The effects of surfactants on ceftibuten uptake in BBMVs were evaluated from the physicochemical properties of surfactants. Fig. 2 shows the relationship between the ceftibuten uptake and HLB, c.m.c., average diameters, or d_w/d_n ratios of surfactants.

The extent of the ceftibuten uptake due to the addition of, a surfactant, except for the Spans, was related to the HLB number ($r = -0.79$, $P < 0.001$), suggesting that the hydrophobicity of surfactants contributes to the modification of ceftibuten transport. It has been reported that surfactants interact with membranes in close proportion to their hydrophobicity in interactions such as binding to [29] and perturbation of membrane lipid [30]. However, the Spans, with a low HLB number, did not affect the uptake which suggests that the Spans, whose hydrophobic group is sorbitan, may have difficulty affecting the hydrophobic interaction between membranes because of their low molecular weights. Because surfactants with low HLB numbers such as the Spans are not stable in water, it is thought that the

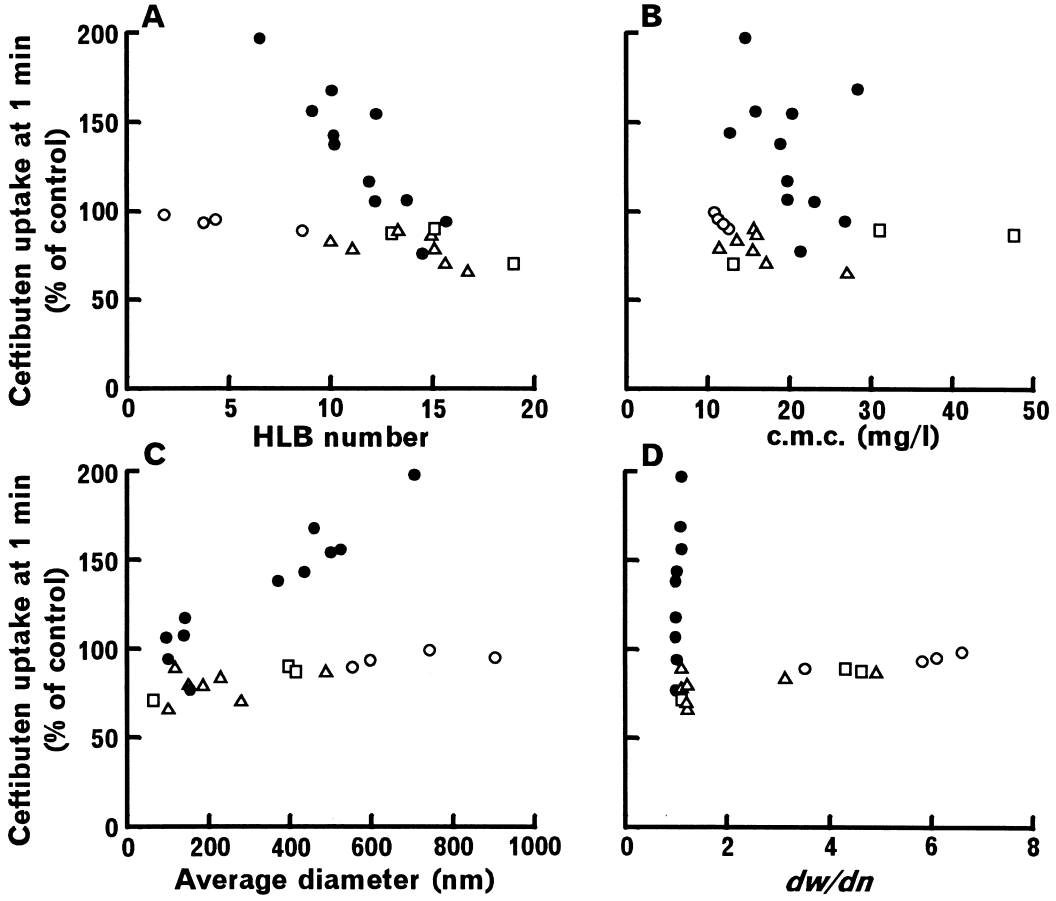

Fig. 2. Relationship between the uptake of ceftibuten in BBMVs and the HLB (A), c.m.c. (B), average diameters (C), or d_w/d_n values (D) at 25°C. Symbols: (○), Spans; (△), Tweens; (□), fatty acid sucrose esters; (●), fatty acid glycerol esters. Each surfactant concentration was 0.03% (w/v), and the physicochemical parameters of surfactants are shown in Table 1. The maximum uptake of ceftibuten in the absence of surfactants was 1.24 nmol/mg protein.

surfactant molecule may easily flocculate in the experimental conditions. Further, two peaks were observed in particle size distribution for the surfactants with high polydispersity (Fig. 1A). On the assumption that the uptake characteristics of the Spans are not solely dependent on the HLB, we investigated the contribution of other parameters to ceftibuten transport.

The c.m.c. of the surfactants used was less than 0.03% (w/v) (Table 1), indicating that all of these surfactants formed micelles under the present conditions. Fig. 2B shows that no correlation was found between the ceftibuten uptake and the c.m.c. of the surfactants, suggesting that the ceftibuten uptake is not affected by differences in c.m.c. values at the surfactant concentration of 0.03% (w/v).

A strong correlation was observed between the average diameter of fatty acid glycerol esters and the ceftibuten uptake, whereas the different average diameters of other surfactants had almost no effect on the ceftibuten uptake (Fig. 2C). We had predicted that the average diameters of fatty acid glycerol esters would be dependent on measures of hydrophobicity, such as HLB. Further, the average particle sizes of the surfactants used ranged from 61 to 903 nm so some micelle colloids were larger than the BBMVs (240 nm average diameter) and thus had difficulty interacting with them. In general, non-ionic surfactants do not produce electrostatic forces of attraction and repulsion. Therefore, the modulation of ceftibuten transport may be caused by interaction between lipid and/or protein of BBMVs and the acyl chain of surfactants on the basis of hydrophobic effect. This hypothesis is supported by the perturbation mechanism of membrane lipids described by Muranishi [31].

An interesting relationship was obtained between the ceftibuten uptake and the d_w/d_n values of surfactants (Fig. 2D). Namely, the surfactants used were clearly divided into two groups by their d_w/d_n values. The ceftibuten uptake was modulated in the presence of several surfactants with low polydispersity ($d_w/d_n \cong 1$), but the drug uptake was similar to that of the control in the presence of surfactants with high

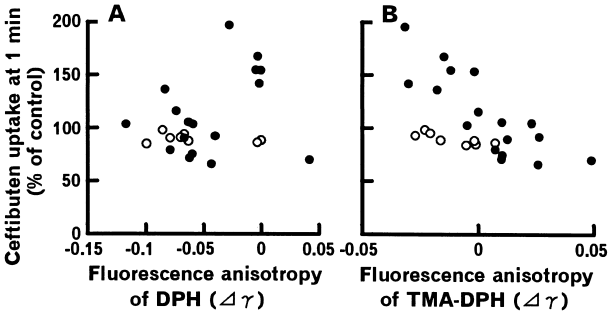

Fig. 3. Relationship between the uptake of ceftibuten in BBMVs and the fluorescence anisotropy of DPH- (A) or TMA-DPH-labeled membranes (B) at 25°C. Symbols: (○), surfactants with high polydispersity ($d_w/d_n > 2$); (●), surfactants with low polydispersity ($d_w/d_n \cong 1$). Each surfactant concentration was 0.03% (w/v). The fluorescence anisotropies of DPH- and TMA-DPH-labeled membranes in the absence of surfactants were 0.250 and 0.285, respectively.

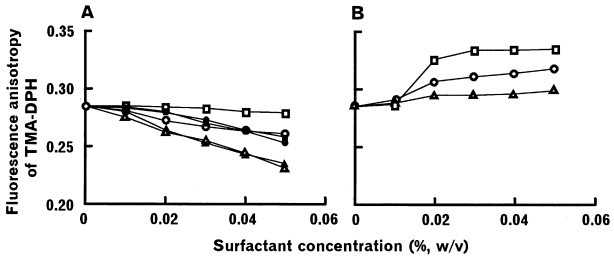

Fig. 4. Effects of surfactants on fluorescence anisotropy of TMA-DPH-labeled membranes at 25°C. Symbols: (A) (○), MO-310; (△), MS-310; (□), MS-500; (●), SS-500; (▲), TS-500; (■), TS-750; (B) (○), Tween 20; (△), Tween 40; (□), SS. Data represent the mean of three experiments. Standard deviation bars from the means were less than 0.005.

polydispersity ($d_w/d_n > 2$). Low polydispersity may explain the modulation effects of surfactants because of the differences in the Gibbs free energy of a monomer molecule surfactant. Stecker and Benedek [32] described the theoretical relationship between the Gibbs free energy of the micellar system and micelle colloid distribution, i.e. the state of polydispersity in size distribution of micelles which have sufficiently finite width as one of balance between two factors: the entropy of mixing micelles and the formation two- or three-component micelles. Therefore, surfactants with high polydispersity may not interact with the transporter of ceftibuten. The results of these experiments lead to our presumption that the modification of ceftibuten uptake by the addition of surfactants depends on the number of monomer molecules which have high Gibbs free energy because it has been reported that alterations in the permeability of drug by a surfactant are determined by the surfactant monomer [33]. In other words, it is reasonable to suppose that a monomer molecule with high energy that is supplemented by a surfactant with low polydispersity has higher capacity to modulate the ceftibuten transport.

More interestingly, surfactants with high polydispersity appeared to function independently of the HLB number from the results in Fig. 2A. In general, the type of surfactant that has high polydispersity is uncertain. Therefore, further studies will be required to elucidate the dispersity of surfactant in aqueous solution.

3.5. Effects of surfactants on membrane lipid fluidity

To examine the changes in the fluorescence anisotropy of DPH and TMA-DPH, the changes (P) in the fluorescence intensity of these probes were measured in the presence and absence of surfactants. The P values with surfactants were less than 0.18, indicating that the distribution of the probes from BBMVs to surfactant micelles was small. This confirmed that the changes in the fluorescence anisotropy of the probes reflected the membrane lipid fluidity.

In the present study, no strong correlation between the fluorescence anisotropy of DPH- or TMA-DPH-labeled membranes and ceftibuten uptake in BBMVs was found

(Fig. 3). Surfactants with low polydispersity, however, which enhanced ceftibuten uptake (MO-310, MS-310, MS-500, SS-500, TS-500, and TS-750) decreased the anisotropy of TMA-DPH, and the surfactants that inhibited the drug uptake (Tween 20, Tween 40, and SS) increased the anisotropy of TMA-DPH. Furthermore, we investigated the relationship between the anisotropy of TMA-DPH and the concentration of the surfactants that significantly modulated the ceftibuten uptake (Fig. 4). The anisotropy of TMA-DPH-labeled membrane with surfactants that enhanced the ceftibuten uptake was decreased with an increase in the surfactant concentration (Fig. 4A), whereas uptake with surfactants that inhibited the drug uptake was increased depending on the concentration (Fig. 4B). These results indicate that each surfactant modulates the fluidity of the membrane outer lipid layer depending on the surfactant concentration, and agrees with reports using rat intestinal BBMVs and epithelial cells: (i) the addition of 2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl)octanate increased Na^+-dependent D-glucose transport with an increase in membrane lipid fluidity [9], and (ii) progressive starvation decreased the D-glucose transport with a decrease in membrane lipid fluidity [10]. Therefore, it is suggested that ceftibuten transport is sensitive to the low polydispersed surfactant-induced increase in membrane fluidity. However, we could not clarify the mechanism of action of the surfactants for the efficiency of transport in detail. As far as the mechanism is revealed from present studies, we propose, at least in part, the possibility that ceftibuten uptake is independent of changes in membrane lipid fluidity due to the addition of surfactants with high polydispersity, and the enhancing or inhibitory effects on ceftibuten uptake in BBMVs may require the phenomenon in the presence of surfactants with low polydispersity, that is, an increase or a decrease in the fluidity of outer lipid layers monitored by TMA-DPH.

3.6. Conclusion

In this study, we found that the surfactants used were divided into two groups by a measurement of size distribution, polydispersity. Ceftibuten transport was modulated by only the surfactants with low polydispersity. The extent of the transport alterations of ceftibuten by BBMVs was dependent on indicators of hydrophobicity such as the HLB number of a surfactant. For this reason, we assumed the possibility of the hydrophobic interaction between surfactant molecules and membrane components such as lipids and proteins including the peptide transporter. A strong correlation was observed between the ceftibuten transport and membrane lipid fluidity monitored by TMA-DPH. Therefore, it is suggested that surfactants with low polydispersity, in part, increase or decrease the outer leaflet fluidity of the membrane, thereby enhancing or suppressing ceftibuten transport by BBMVs.

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