Laboratory of Molecular Pharmacokinetics[1], College of Pharmaceutical Sciences; Ritsumeikan-Global Innovation Research Organization[2]; Research Center for Drug Discovery and Development[3], Ritsumeikan University, Shiga, Japan

Effect of surface charge, particle size, and modification by polyethylene glycol of liposomes on their association with Caco-2 cells across an unstirred water layer

Y. Kono[1,2,*], A. Iwasaki[1], T. Fujita[1,2,3]

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[*]Corresponding author: Yusuke Kono, Ph.D., Laboratory of Molecular Pharmacokinetics, College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Noji-Higashi, Kusatsu, Shiga 525-8577, Japan
y-kono@fc.ritsumei.ac.jp

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For the development of orally available liposomes, understanding the interaction of liposomes with the intestinal mucosa is important. An unstirred water layer (UWL) on the intestinal epithelium surface is a considerable permeability barrier for lipophilic drugs. Therefore, the effects of an UWL on liposome transport across intestinal epithelial cells must be elucidated. We evaluated the effects of the surface charge, particle size, and polyethylene glycol (PEG) modification of liposomes on their association with Caco-2 cells across an UWL. When the association of cationic liposomes with Caco-2 cells was evaluated under a reduction in UWL thickness by shaking, the uptake and/or amount of surface-bound cationic liposomes in cells was increased significantly in a shaking rate-dependent manner. The uptake and/or amount of surface-bound neutral liposomes were increased only at the highest shaking rate. No significant differences in the cellular association of anionic liposomes and PEG-modified liposomes were observed with or without shaking. The association of large liposomes with Caco-2 cells was affected considerably by an UWL compared with that of small liposomes. These results suggest that an UWL affects the surface binding and subsequent uptake of liposomes in Caco-2 cells according to their particle size, surface charge, and PEG modification.

1. Introduction

Lipid-based drug carriers such as liposomes, emulsions, and nanoparticles are considered to be promising tools for improving the poor oral bioavailability of biologic therapeutics (Mei et al. 2013; Zhang et al. 2013; Fong et al. 2015). In particular, liposomes are among the most frequently used nanocarriers for the development of oral drug-delivery systems because they have several advantages over other carriers. For instance, liposomes can be readily prepared in a wide range of sizes and their surface characteristics can be changed. In addition, liposomes can encapsulate hydrophilic and hydrophobic drugs (Mei et al. 2013; Zhang et al. 2013; Fong et al. 2015). Therefore, they have the capability of not only increasing the membrane permeability and cellular uptake of hydrophilic drugs, but also improving the aqueous solubility of hydrophobic drugs. Moreover, liposomes have been investigated as protein/peptide delivery carriers for oral administration (Martin et al. 2007; Pawar et al. 2014). Liposomes have been reported to increase the bioavailability of insulin (Niu et al. 2014; Cui et al. 2015). In addition, several studies have demonstrated that oral administration of antigenic proteins with liposomes can elicit an intestinal mucosal immune response against infection and cancer (Minato et al. 2003; Naito et al. 2007). Thus, liposome-mediated drug-delivery systems could provide alternative formulations for oral administration of drugs to improve their absorption and therapeutic effect.

For the development of orally available liposomes, understanding how liposomes interact with the intestinal mucosa is essential. In particular, the intestinal epithelium is covered with mucus, which is a complex hydrogel composed of glycoproteins, lipids, salts, and cellular debris (Cone 2009; Sigurdsson et al. 2013). The mucus forms a viscoelastic gel layer on the surface of the epithelium, resulting in the maintenance of an unstirred water layer (UWL), which is known to be a critical barrier to drug transport across the epithelium (Cone 2009; Korjamo et al. 2009; Sigurdsson et al. 2013). Liposomes must traverse an UWL for their intestinal absorption, so elucidation of how liposomes diffuse through an UWL is required.

Previously, we prepared a method to evaluate the effect of an UWL on liposome uptake into Caco-2 cells using mechanical agitation (Kono et al. 2016). We showed that gene transfer by 500-nm cationic liposome/plasmid DNA complexes in Caco-2 cells is affected considerably by an UWL on the surface of a monolayer of Caco-2 cells (Kono et al. 2016). Thus, transport of liposomes across the epithelium seems to be restricted by an UWL. Conversely, the transport of nanoparticles across the epithelium has been reported to be influenced by their physicochemical properties, such as particle size and surface charge (Olmsted et al. 2001; Lai et al. 2009; Schleh et al. 2012; Bannunah et al. 2014). Olmsted et al. (2001) showed that the diffusion rate of particles across the mucus is reduced according to their particle diameter. Moreover, Bannunah et al. (2014) reported that the level of internalization of positively charged nanoparticles by a monolayer of Caco-2 cells is higher than that of their negatively charged counterparts. In addition to physicochemical properties, surface chemistry is an important determinant of the transport characteristics of liposomes (Lai et al. 2009; Maisel et al. 2015).

Polyethylene glycol (PEG) is one of the most commonly used materials for the coating of the surfaces of liposomes to improve their stability and blood circulation time (Harris et al. 2001; Li et al. 2003; Nogueira et al. 2013). Recent reports have shown that PEG can reduce the association of macromolecules covered with mucus (Wang et al. 2008; Xu et al. 2015; Inchaurraga et al. 2015; Andreani et al. 2015). Taking this observation into consideration, the degree of interaction of liposomes with an UWL seems to be different according to the physicochemical and surface properties of liposomes.

Table 1: Particle sizes and zeta potentials of liposomes. Each value represents the mean±SD (n=4).

	Small		Large	
	Particle size (nm)	Zeta potential (mV)	Particle size (nm)	Zeta potential (mV)
Cationic liposomes	91.3 ± 8.2	68.5 ± 2.2	581.2 ± 31.2	70.2 ± 12.3
Neutral liposomes	97.0 ± 2.0	−6.3 ± 6.3	529.8 ± 19.2	−7.8 ± 3.9
Anionic liposomes	104.8 ± 5.8	−58.4 ± 2.6	556.3 ± 19.0	−64.2 ± 15.5
PEGylated liposomes	106.4 ± 5.2	−7.8 ± 5.5	530.9 ± 14.1	−4.4 ± 10.7

In the present study, we prepared several liposomes of different particle sizes and surface charges. Then, we evaluated the association of each type of liposome with Caco-2 cells under a reduction in UWL thickness. Moreover, we investigated the effect of PEG modification of liposomes on their association with Caco-2 cells across an UWL.

2. Investigations and results

2.1. Cellular association of cationic liposomes under mechanical agitation

First, we attempted to determine the effect of the particle size of cationic liposomes on their association with Caco-2 cells across an UWL. The particle size and ζ-potential of the liposomes used in the present study are listed in the Table. A cellular association study was carried out under reduction of UWL thickness by shaking according to our method, as described previously (Kono et al. 2016).

We confirmed that shaking and liposomes did not affect the viability of Caco-2 cells (Fig. 1). The uptake and/or amount of surface-bound 100-nm cationic liposomes to Caco-2 cells was increased significantly in a shaking rate-dependent manner (Fig. 2A). In addition, an increase in cellular association by shaking was also observed in 500-nm cationic liposomes (Fig. 2B). In particular, the cellular association of 500-nm cationic liposomes was augmented by shaking at a lower rate (60 rpm) compared with that of 100-nm liposomes.

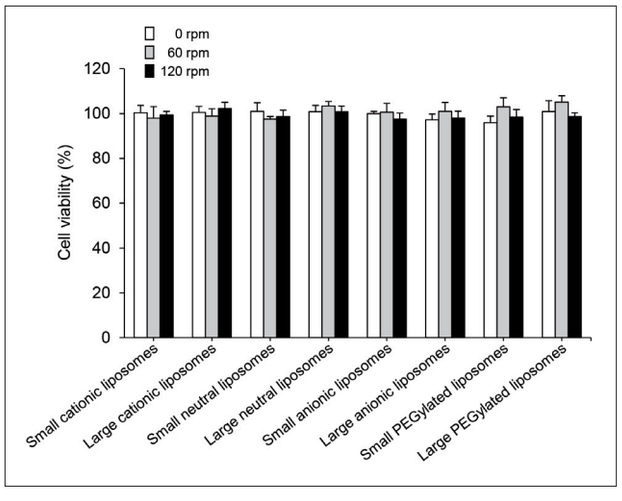

Fig. 1: Effect of shaking and liposomes addition on the viability of Caco-2 cells. Small or large cationic, neutral, anionic, or PEGylated liposomes (20 μg) were added to the well, and incubated for 120 min under shaking. Each value is the mean+SD (n = 4).

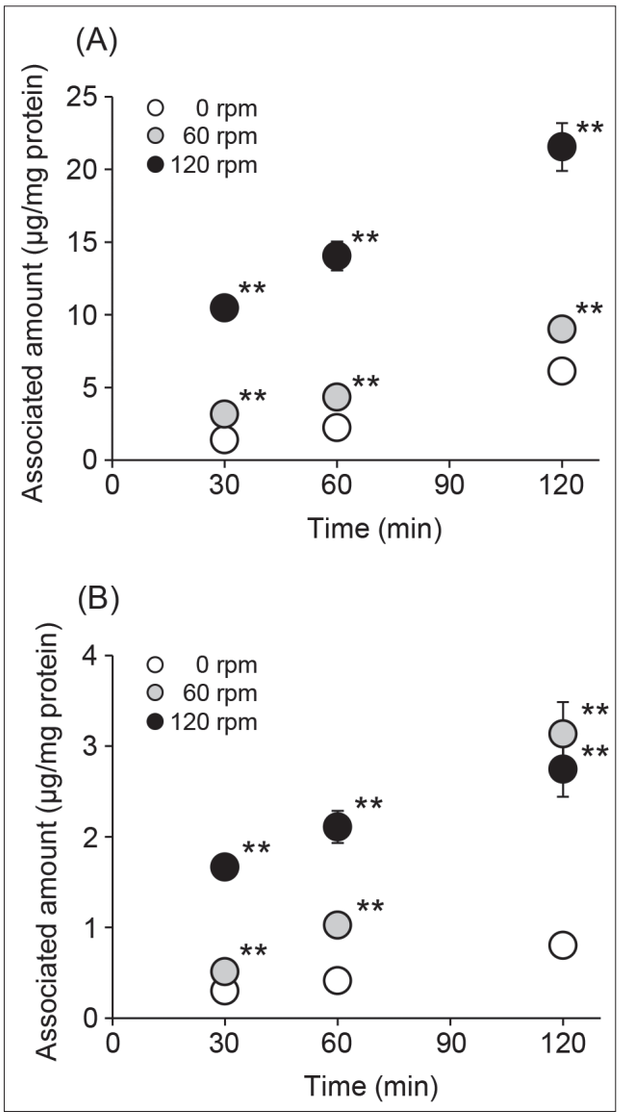

Fig. 2: Uptake and/or amount of surface-bound small (A) and large (B) cationic liposomes in Caco-2 cells under shaking. Liposomes (20 μg) were added to the well, and incubated for 30, 60, or 120 min under shaking. Each value is the mean±SD (n = 4). $^{**}P< 0.01$, compared with the corresponding group of shaking rate at 0 rpm.

2.2. Cellular association of neutral liposomes under mechanical agitation

We also investigated the effect of an UWL on the association of neutral liposomes with Caco-2 cells. No change in the association of 100-nm neutral liposomes with Caco-2 cells was observed

irrespective of whether mechanical agitation was undertaken (Fig. 3A). Conversely, the uptake and/or amount of surface-bound 500-nm neutral liposomes were increased significantly by shaking at 120 rpm (Fig. 3B).

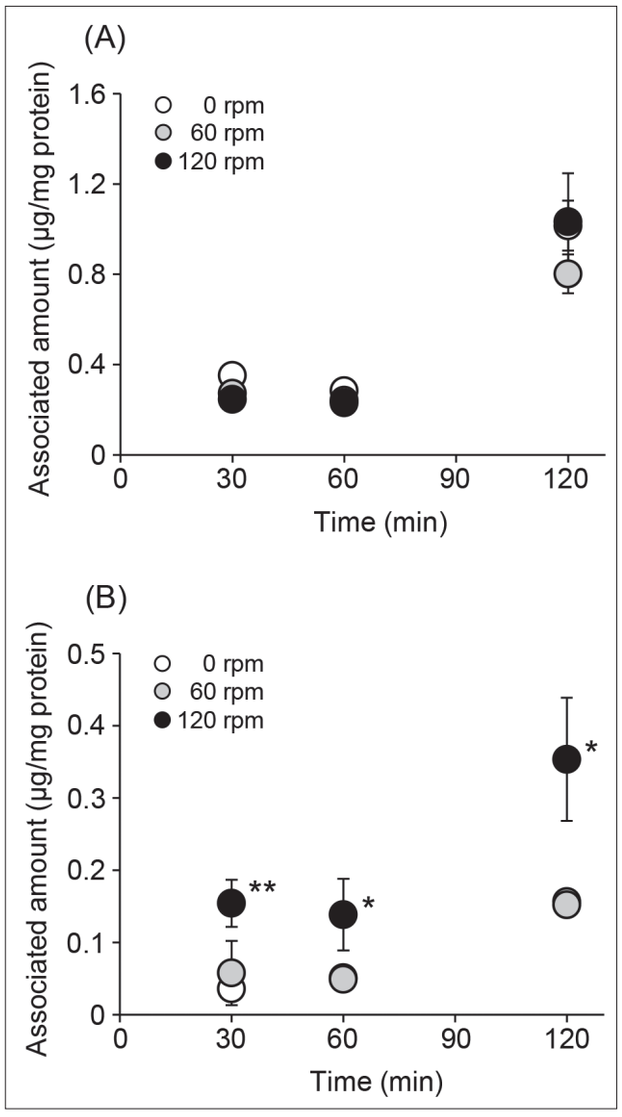

Fig. 3: Uptake and/or amount of surface-bound small (A) and large (B) neutral liposomes in Caco-2 cells under shaking. Liposomes (20 μg) were added to the well, and incubated for 30, 60, or 120 min under shaking. Each value is the mean±SD (n = 4). **P< 0.01, compared with the corresponding group of shaking rate at 0 rpm.

2.3. Cellular association of anionic liposomes under mechanical agitation

Figure 4 illustrates the cellular association of 100-nm (Fig. 4A) and 500-nm (Fig. 4B) anionic liposomes. Shaking did not affect the association of 100-nm or 500-nm anionic liposomes with Caco-2 cells.

2.4. Effect of PEG modification of liposomes on their association with Caco-2 cells under shaking

PEG modification of the surface of liposomes could be considered to be an effective method to use liposomes for oral drug delivery. Iwanaga et al. (1997, 1999) reported that liposomes coated with 5 mol% of PEG_{2000} confer resistance to digestion by bile acids. In addition, Li et al. (2003) demonstrated that surface coating of

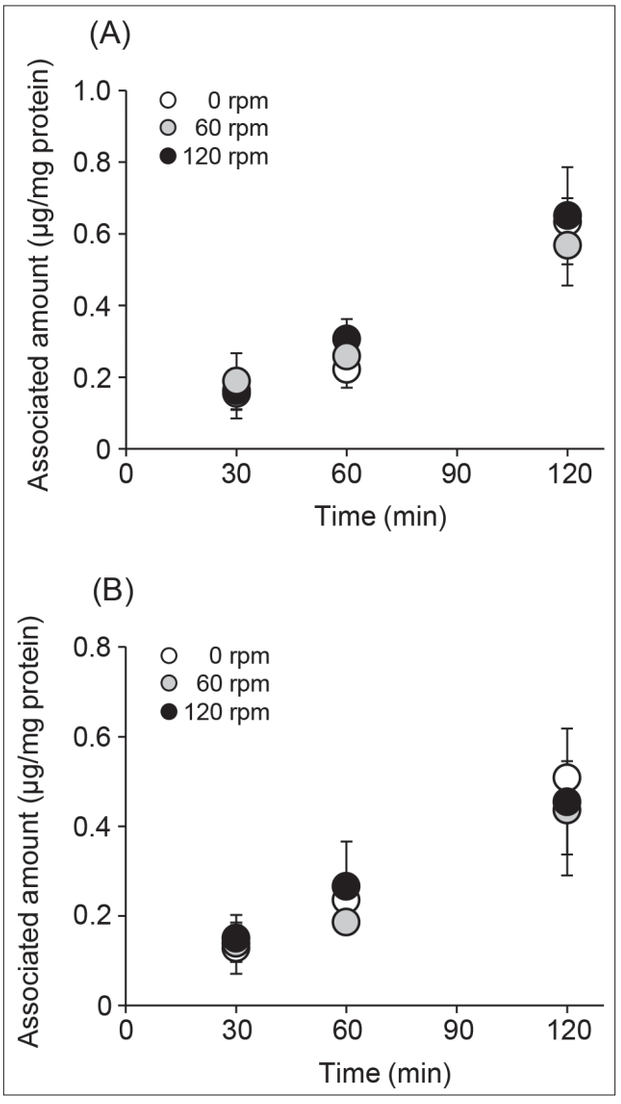

Fig. 4: Uptake and/or amount of surface-bound small (A) and large (B) anionic liposomes in Caco-2 cells under shaking. Liposomes (20 μg) were added to the well, and incubated for 30, 60, or 120 min under shaking. Each value is the mean±SD (n = 4). **P< 0.01, compared with the corresponding group of shaking rate at 0 rpm.

liposomes with 6.25 mol% of PEG_{2000} inhibits the degradation of liposomes, and they incorporated drugs into intestinal epithelial cells. Therefore, we prepared PEGylated neutral liposomes by the replacement of 5 mol% of cholesterol with PEG_{2000}-DSPE, and assessed the association of PEGylated liposomes with Caco-2 cells under a reduction in UWL thickness to determine how PEG molecules on the surface of liposomes interact with an UWL. The relative cellular uptake and/or amount of surface-bound 100-nm PEGylated liposomes was higher than that of 500-nm PEGylated liposomes (Fig. 5). This result was similar to the data obtained from the other three types of liposomes used in the experiments described above. In contrast to the "naked" neutral liposomes shown in Fig. 3, the amount of 100-nm and 500-nm PEGylated liposomes associated with Caco-2 cells was not changed by shaking.

3. Discussion

If nanoparticles are administered orally, the intestinal mucus traps them *via* steric or hydrophobic interactions according to the physicochemical characteristics of the nanoparticles employed

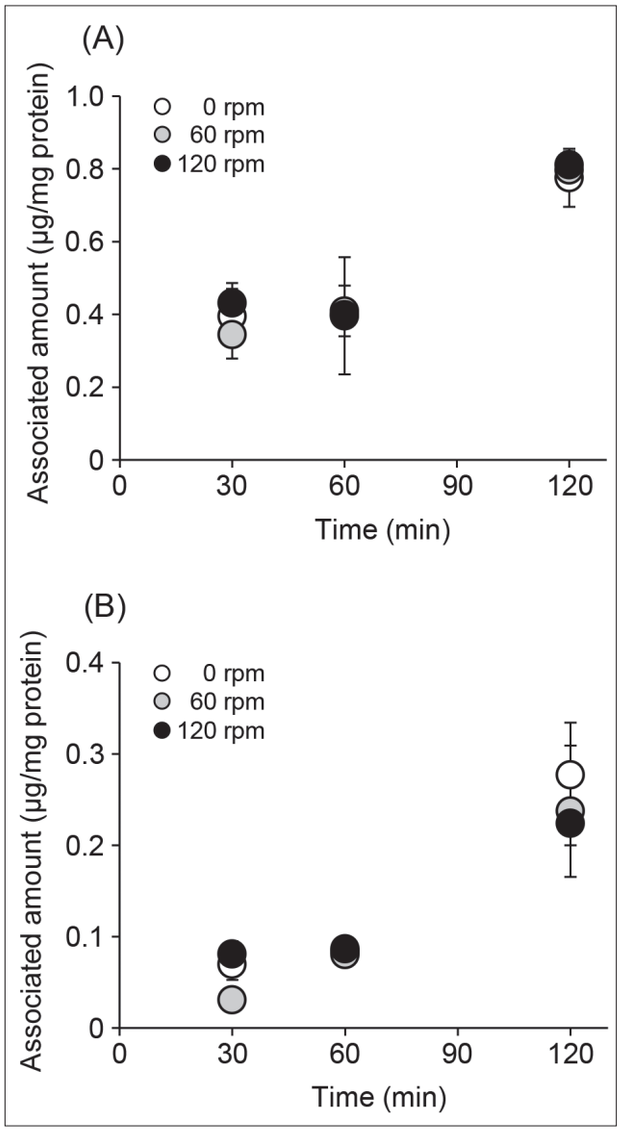

Fig. 5: Uptake and/or amount of surface-bound small (A) and large (B) PEGylated neutral liposomes in Caco-2 cells under shaking. Liposomes (20 μg) were added to the well, and incubated for 30, 60, or 120 min under shaking. Each value is the mean±SD (n = 4). $^{**}P< 0.01$, compared with the corresponding group of shaking rate at 0 rpm

(Cone 2009; Lai et al. 2009; Ensign et al. 2012). These adhered particles are eliminated by rapid mucociliary clearance. Therefore, the interaction of liposomes with an UWL composed of mucus must be understood to aid development of an oral liposome preparation that can be absorbed effectively from the small intestine. In the present study, we attempted to determine the role of surface charge, particle size, and PEG modification of liposomes on their association with a monolayer of Caco-2 cells across an UWL.

With respect to surface charge, the largest increase in the uptake and/or amount of surface binding to Caco-2 cells by shaking was observed in cationic liposomes (Fig. 2), followed by neutral liposomes (Fig. 3). The cellular association of anionic liposomes was not changed by shaking (Fig. 4). These results suggest that cationic liposomes interact strongly with an UWL. Mucin fibers, which are the primary components of mucus, contain many sulfate or carboxyl groups in their glycosylated regions (Lai et al. 2009). These regions have a negative charge at high density. Therefore, cationic liposomes are likely to interact with mucin fibers *via* electrostatic interactions, resulting in considerable limitation of their transport by an UWL (Jubeh et al. 2004; Griffiths et al. 2015). Conversely, the transport of anionic liposomes is not affected by an UWL because of electrostatic repulsion. In the case of neutral liposomes, the interaction of liposomes with an UWL seems to be based on hydrophobic interactions rather than electrostatic interactions. Olmsted et al. (2001) reported that polystyrene microspheres bind tightly to mucin *via* hydrophobic interactions. More recently, Griffiths et al. (2015) demonstrated that poly(lactic-co-glycolic acid) nanoparticles show strong hydrophobic interactions with mucin. Those reports support our suggestion that an UWL can trap neutral liposomes *via* hydrophobic forces.

In addition to surface charge, particle size is also known to have a considerable influence on the transport properties of liposomes into the epithelium (Olmsted et al. 2001; Lai et al. 2009; Schleh et al. 2012; Bannunah et al. 2014). Augmentation of the amount of 500-nm cationic liposomes associated with Caco-2 cells by shaking was observed when shaking was done at <60 rpm, whereas the cellular association of 100-nm cationic liposomes was increased only by shaking at 120 rpm (Fig. 1). In addition, the cellular association of 500-nm neutral liposomes was enhanced by shaking at 120 rpm, whereas that of 100-nm liposomes was not changed (Fig. 3). These results suggest that 500-nm liposomes are more likely to be trapped by an UWL compared with 100-nm liposomes. The mesh size of mucus has been reported to be 10–200 (mean, 100) nm (Saltzman et al. 1994; Olmsted et al. 2001), so the transport of 500-nm liposomes in Caco-2 cells could be highly restricted by the size-filtering effect of an UWL. This observation is consistent with several reports demonstrating the rapid penetration of small particles (including viruses) into mucosal tissues. Conversely, Lai et al. (2007) showed that 200-nm and 500-nm polystyrene nanoparticles moved more rapidly through human cervicovaginal mucus than 100-nm nanoparticles. This disagreement with the data of our study may be due to the difference between the materials used in each study. Lai et al. (2007) used fresh human cervicovaginal mucus, and they speculated that the mesh spacing in human cervicovaginal mucus is >500 nm. Taking this factor into consideration, an appropriate material suited to the purpose of each experiment must be chosen because the characteristics of mucus and the UWL may be different in each cell line and sample of epithelial tissue.

We also observed that the uptake and/or amount of surface-bound 100-nm liposomes were relatively higher than that of 500-nm liposomes in all liposomes (Fig. 2–4). The uptake of nanoparticles into epithelial cells is mediated by endocytosis (Fazlollahi et al. 2011; He et al. 2013; Chai et al. 2014; Bannunah et al. 2014). He et al. (2013) showed that 80-nm polymer nanoparticles are taken up into Caco-2 cells *via* multiple endocytic pathways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis. Bannunah et al. (2014) demonstrated that cationic nanoparticles internalize into Caco-2 cells *via* clathrin-mediated endocytosis and macropinocytosis, whereas anionic nanoparticles internalize *via* caveolae-mediated endocytosis. The uptake ratio of nanoparticles into cells *via* these types of endocytosis has been reported to decrease in proportion to increasing particle size (Zhang et al. 2009; Bannunah et al. 2014; Langston and Chau 2014). Hence, in the present study, the association of liposomes with Caco-2 cells was likely to have been *via* endocytosis depending on their particle size.

Next, we investigated how surface modification of liposomes by PEG_{2000}-DSPE changes the diffusion property of liposomes through an UWL. PEG modification changes the physicochemical properties of liposomes. PEG forms a hydrated shell around liposomes, resulting in "masking" of the surface charge and hydrophobic core of liposomes (Harris et al. 2001; Nogueira et al. 2013). Therefore, PEG modification could hinder the interaction of liposomes with an UWL. The cellular association of PEGylated neutral liposomes was not increased by mechanical agitation (Fig. 5). This result suggests that PEG can reduce the restriction of the transport of liposomes into Caco-2 cells by an UWL. Griffiths et al. (2015) showed that surface-grafted PEG_{2000} chains significantly reduce the interaction of hydrophobic nanoparticles with mucin. In addition, Yoncheva et al. (2005) reported that PEGylation with PEG_{2000} reduced the interaction between poly(methyl vinyl ether-

co-maleic anhydride) nanoparticles and mucin. The observations in the present study are in agreement with those reports.

In conclusion, we demonstrated that the physicochemical properties and PEG modification of liposomes strongly affect the association of liposomes with Caco-2 cells across an UWL. Cationic liposomes were trapped in large numbers by an UWL, whereas the cellular association of anionic liposomes was not affected by an UWL. In addition, the cellular uptake of large liposomes seems to be highly restricted by an UWL compared with that of small liposomes. Moreover, the surface modification of liposomes by PEG reduced the interaction of liposomes with an UWL. These findings may help in the development of orally available liposomal drugs.

4. Experimental

4.1. Cell lines

Caco-2 human epithelial colorectal adenocarcinoma cells were purchased from DS Pharm Biomedical (Osaka, Japan). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), streptomycin (100 µg/mL), and non-essential amino acids. Cells were placed in an incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air.

4.2. Preparation of liposomes

Liposomes were constructed according to methods described in our previous report (Inchaurraga et al. 2015). In brief, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids, Alabaster, AL, USA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids), and 1,2-distearoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DSPG) (Avanti Polar Lipids) were mixed in chloroform with cholesterol (Nacalai Tesque, Kyoto, Japan) at a molar ratio of 1:1 for the preparation of cationic liposomes, neutral liposomes, and anionic liposomes, respectively. For construction of PEGylated liposomes, 5 mol% of cholesterol was replaced with N-(carbonyl-methoxy PEG_{2000})-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PEG_{2000}-DSPE) (NOF, Tokyo, Japan). Cationic, neutral, and PEGylated liposomes contained 1% fluorescein (N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Thermo Fisher Scientific, Kanagawa, Japan). Anionic liposomes contained 1% cholesteryl esteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (Thermo Fisher Scientific). After the mixture had been dried on a rotary evaporator and vacuum desiccated, the resultant film was hydrated with sterile 5% glucose solution for 30 min at 70 °C under mechanical agitation for the preparation of large liposomes. Small liposomes were produced by sonication of each large liposome in a probe-type sonicator for 3 min. The particle size and ζ-potential of liposomes were measured using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK).

4.3. Preparation of a monolayer of Caco-2 cells

For a cell-viability assay and cellular association study, Caco-2 cells were seeded in 12-well culture plates at 2×10^5 cells/3.8 cm^2. For observation of a "tight-junction" structure, a culture cover glass (Matsunami Glass, Osaka, Japan) was placed on each well. Cells reached confluence 7 days later, and were cultured for an additional 7 days. The culture medium was replaced every 48 h.

4.4. Evaluation of cell viability using a WST-8 assay

After preparation of a monolayer of Caco-2 cells in 12-well culture plates, the latter were incubated in fresh Hanks' balanced salt solution (HBSS) (Sigma–Aldrich, Saint Louis, MO, USA) for 30, 60, and 120 min, with mechanical agitation at shaking rates of 60, and 120 rpm using a water-bath shaker (Taitec, Saitama, Japan). Then, the medium was removed, and Cell Count Reagent SF (Nacalai Tesque) was added to each well and incubated for 30 min. The absorbance of the supernatant was measured at 450 nm, and the results were expressed as viability (%).

4.5. Cellular association of liposomes

After preparation of a monolayer of Caco-2 cells in 12-well culture plates, cells were incubated with fresh HBSS (Sigma–Aldrich) for 30 min at 37 °C in an atmosphere of 5% CO_2 and 95% air. Then, each liposome (20 µg /well) was added to the well, and cells were incubated for 30, 60 and 120 min with or without shaking using a water-bath shaker (Taitec). After incubation, the medium was removed, and cells were washed twice with ice-cold HBSS. Then, cells were solubilized in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8), and centrifuged at 10,000 × *g* for 10 min at 4 °C. The fluorescence intensity of the supernatant was measured with an Infinite F200 Microplate Reader (Tecan Japan, Kanagawa, Japan).

4.6. Statistical analyses

Results are the mean±standard deviation (SD) of more than three experiments. Two-group comparisons were done using the Student's *t*-test.

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