Preparation and Mucoadhesive Test of CSA-loaded Liposomes with Different Characteristics for the Intestinal Lymphatic Delivery

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Abstract Drug delivery to the lymphatic system may be important in terms of the treatment with lymphatic involvement, such as tumor metastases and immunization. Especially, drug transport via the intestinal lymphatics after oral administration has been attracted lots of interests. The purpose of this study was to prepare cyclosporin A (CSA)-loaded liposomes, with different characteristics, and evaluate their mucoadhesivity. Three liposome preparations were formulated: cationic stearylamine liposomes (SA-Lip), anionic phosphatidylserine liposomes (PS-Lip), polymer (chitosan)-coated liposomes (CS-Lip), and characterized. The liposome preparations were found to be spherical in shape, with PS-Lip being the smallest. The liposome preparations exhibited entrapment efficiencies in the order: PS-Lip (52.5 ± 2.9%) > SA-Lip (48.8 ± 3.3%) > CS-Lip (41.7 ± 4.2%). Finally, mucoadhesive tests were carried out using rat intestine, with SA-Lip (67%) showing the best adhesive rate of the three preparations (PS-Lip: 56%, CS-Lip: 61%). These results suggest that a positive charge on the surface of drug carriers may be an important factor for the intestinal drug delivery.

Keywords: CSA, liposome, intestinal lymphatics, mucoadhesive

INTRODUCTION

Drug delivery to the lymphatic system may be important in terms of the diagnosis and treatment with lymphatic involvement, such as tumor metastases, viral and bacterial infections and immunization [1]. Therefore, many investigators have been interested in the lymphatic delivery systems entrapped immunosuppressive or anticancer agents for the prevention of autoimmune disease or cancer progression.

Cyclosporin A (CSA) among these drugs is a sparingly water-soluble cyclic peptide drug, consisting of 11 amino acids, which has been utilized clinically for immunomodulation, such as the prevention of rejection following kidney, liver, bone marrow and pancreas transplantations. The use of CSA has been limited due to its low bioavailability and serious side effects, such as nephrotoxicity and hepatotoxicity [2], which has result in the need to use a solubilizing agent, such as polyoxyethylated castor oil (Cremohpor EL), for its effective administration. Therefore, liposomal formulations have been used to overcome these side effects, and low bioavailability [3].

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Meanwhile, many drug carriers, using biodegradable polymers, lipids, such as poly lactide-co-glycolide (PLG), liposomes, and emulsions, have been used as lymphatic delivery systems [4-6]. In particular, liposomes, a nano-sized biodegradable lipid vesicles, with an aqueous space surrounded by a lipid bilayer, have received considerable interest as a lymphatic delivery system vehicle, as they can effectively entrap and deliver both hydrophilic and lipophilic drugs to the target site. The stability of liposomes and their drug delivery ability are dictated by the type of lipid and method used in their preparation [7]. Also, liposomes have been reported as a suitable vehicle for peptide drugs, with many studies carried out to investigate aspects of their use, such as drug encapsulation, and *in vivo* absorption, *etc.* [8].

Furthermore, several different routes of administration, such as oral, subcutaneous (s.c.), intramuscular (i.m.), and intraperitoneal (i.p.) [1,9], have been investigated for lymphatic delivery systems. Drug transport *via* the intestinal lymphatics, after oral administration, may confer particular delivery advantages in terms of increased bioavailability, with the possibility of directing delivery to the lymphatic system [10]. Peyer's patches (PP) in the gastrointestinal tract (GIT) is the main target site of orally administrated drug carriers. The uptake rate of PP depends on the characteristics of the drug carrier, such

as the particle surface charge [11], attachment of ligands [12,13], and surfactant coating [14]. The absorption rate and residence time of a drug orally delivered in the gastrointestinal tract are also considered important factors in controlling the bioavailability of a drug. Therefore, the mucoadhesive study is very important for evaluating usefulness of carriers in the oral lymphatic drug delivery. In the previous study, it was reported that various carriers had a good mucosal adhesion and was the effective drug carriers for the intestinal targeting [15,16]. But the extent of mucosal adhesion of liposomes, differently characterized in surface properties, was not evaluated simultaneously before study.

Consequently, the purpose of this study was to prepare CSA-loaded liposomes, with different characteristics, such as cationic stearylamine liposomes (SA-Lip), anionic phosphatidylserine liposomes (PS-Lip) and polymer (chitosan)-coated liposomes (CS-Lip) liposomes for the intestinal lymphatic delivery and characterize them according to the size distribution, zeta potential, and morphology. The entrapment efficiency and stability of liposomes were also investigated. Finally, the mucoadhesive tests were performed to evaluate the liposome preparation with the better extent of mucosal adhesion to the rat intestinal layer.

MATERIALS AND METHODS

Materials

l-α-dipalmitoylphosphatidylcholine (DPPC), phosphatidylserine (PS), stearylamine (SA), phosphatidic acid (PA), cholesterol (Chol) and 5-carboxyfluorescein (5-CF) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cyclosporin A (CSA) was a gift from Chong Kun Dang Pharm. Co. (Seoul, Korea). A water-soluble chitosan (CS, 2500 kDa) was supplied by Chitoful Co. (Yeosu, Korea). All the solvents used for the chromatographic analysis were of HPLC grade. Male Sprague-Dawley (SD) rats, weighing 100~150 g, were obtained from Orient Co., Ltd. (Sungnam, Korea).

Preparation of Liposomes

Three liposome preparations were formulated using the film method: SA-Lip, PS-Lip, and CS-Lip. The liposome preparations consisted of the following lipid composition: DPPC:PS (or SA):Chol (in the molar ratio 7:3:2) [17]. The lipid mixtures and CSA were dissolved in 3 mL of chloroform-methanol solution (2:1, v/v), the solution was evaporated and the residue was then hydrated with PBS buffer (0.01 M, pH 7.4) at the transition temperature of that of the DPPC (60°C). The prepared liposomal dispersions were sonicated for 3 min in an ice water bath using a tip-type sonicator (Sonics & Materials Inc., Danbury, CT, USA).

Polymer-coated liposomes (CS-Lip) were prepared using the different molar ratios described above: DPPC:PA (40:1). Equal volumes of the prepared liposomal dispersions and chitosan solution of various concentrations (0.1, 0.25, and 0.5%, w/v) were mixed, and then incubated in a shaking water bath at 20°C for 2 h.

Characterization of Liposomes

The size distribution and zeta potential of the liposome preparations were measured using an ELS-8000 (OTUSUKA Electronics, Osaka, Japan), and the morphology observed with a JEM-200 FXII transmission electron microscope (JEOL Ltd., Tokyo, Japan), after their negative staining using 0.2% (w/v) phosphotungstic acid buffer (pH 6.8).

Entrapment Efficiency of Liposomes

The CSA unentrapped in the liposomal dispersions was separated by ultracentrifugation (Optima™ TLX Ultracentrifuge, Beckman, USA) at 60,000 rpm for 1 h [18]. After ultracentrifugation, the supernatant was analyzed by HPLC to determine the amount of CSA that had not been entrapped. The entrapped CSA content was determined by subtracting the amount of free drug from that of the total.

The Stability of Liposomes in Storage States

The stability of the liposome preparations was determined from the relative turbidity. All the liposomal suspensions were stored at 4°C for 2 weeks and monitored at 2 day time intervals using a UV-VIS spectrophotometer (Shimadzu UV-201, Japan) at 600 nm. The relative turbidity is the ratio of the optical density at the time of monitoring compared to that at the initial stage [19].

Mucoadhesive Test

The mucoadhesive tests were carried out using rat intestine and 5-CF loaded liposomes. The intestines (about 10 cm long) were isolated from SD rats and then washed with PBS buffer. The prepared liposomes were injected into intestines tubes, which were sealed and then incubated in PBS buffer at 37°C for 1 h. The amount of liposome mucoadhesion was evaluated using a Luminometer (Turner Biosystems, USA), with the extent of adhension of the prepared liposomes to the rat intestinal mucosal layer confirmed by fluorescence microscopy.

RESULTS AND DISCUSSION

Preparation and Characterization of Liposomes

The neutral liposomes, consisting of only DPPC and Chol, were excluded from further study due to a phase separation within only a few hours.

The size distribution and zeta potential of each prepared liposome are shown in Table 1. The PS-Lip, which had the strongest surface charge, was smaller in size than the other preparations. This may be due to the stabilization effect of anionic phosphatidylserine through repulsive electrostatic interaction [20]. The size distribution of

Table 1. The characteristics of each liposome preparation before and after ultracentrifugation

Type of liposomes	Before ultracentrifugation		After ultracentrifugation	
	Mean diameter (nm)	Zeta potential (mV)	Mean diameter (nm)	Zeta potential (mV)
SA-Lip	135.3 ± 5.8	28.9 ± 1.8	135.8 ± 4.6	27.4 ± 1.7
PS-Lip	99.6 ± 7.2	-38.1 ± 3.2	100.2 ± 2.3	-36.9 ± 2.6
CS-Lip	149.3 ± 8.4	-6.9 ± 1.7	151.5 ± 7.3	-6.3 ± 2.3

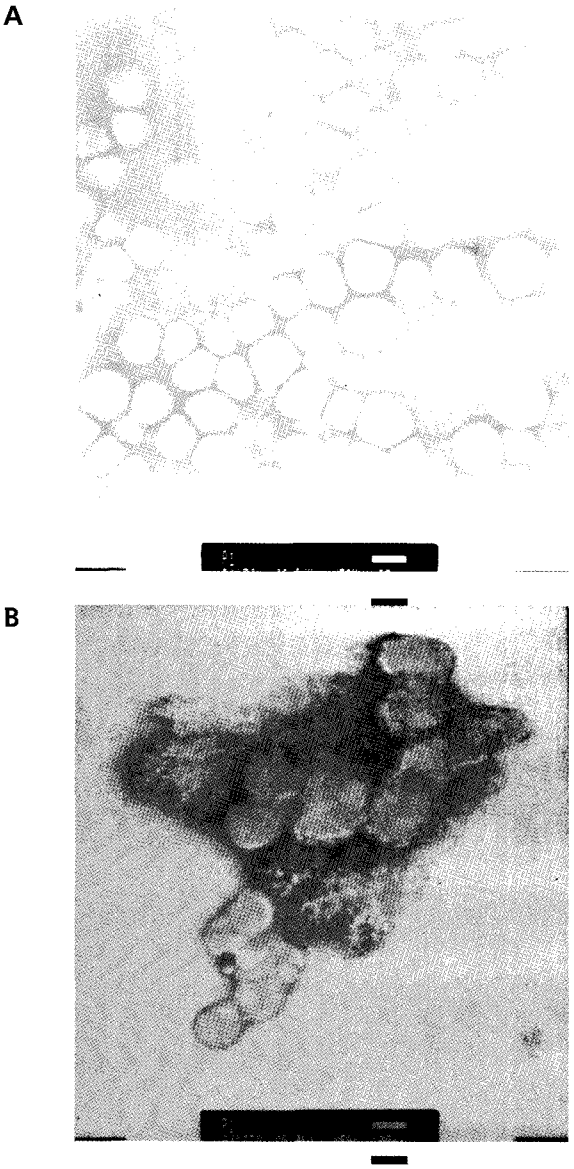


Fig. 1. TEM photographs of SA-Lip (A) and CS-Lip (B). The bar represents 100 nm.

each liposome preparations remained unchanged after ultracentrifugation. The shape of the liposomes was observed by TEM, and found to be spherical, with a chitosan coating layer observed in the CS-Lip (Fig. 1).

The CS-Lip was prepared with a different molar ratio to that used for the other liposome preparations, because of a phase separation (coagulation), as shown in Table 2(a), which was due to the high PA content [21]. Therefore, the CS-Lip was tested at various lipid ratios to seek conditions where no phase separation occurred. Finally, the CS-Lip was prepared with the lipid composition shown in Table 2(b): DPPC: PA (40:1), but with no Chol.

Table 2. The CS-Lip status based on the different lipid compositions

PA(mg) / CS(%, w/v)	DPPC:PA:CH[a]			DPPC:PA(Chol-free)[b]		
	6	3	1	3	1	0.5
0.10	○	×	× (1.0 μm)	○	×	× (132.9 nm)
0.25	○	○	× (1.4 μm)	○	×	× (149.3 nm)
0.50	○	○	× (2.6 μm)	○	○	× (172.9 nm)

○ : phase separation (causing aggregation of liposomal dispersion)
× : non-phase separation

The change in the size distribution and zeta potential of the CS-Lip is shown in Fig. 2. The size of the CS-Lip increased with increasing chitosan concentration, with similar results shown for the change in the zeta potential [22], which increased or chitosan concentrations up to 0.25%, but showed to a constant value thereafter. The formation of CS-Lip was confirmed by measuring the change in the zeta potential, with the chitosan coating layer observed in TEM image (Fig. 1B).

Entrapment Efficiency and Stability of Liposomes

The CSA that had not been entrapped was separated from the liposomal suspension using ultracentrifugation, and the supernatant then analyzed by HPLC. Generally, the supernatant was used in a quantitative process, and the pellet in further study following its redispersion [18], but the pellet was found to not redisperse well. Therefore, it could not be confirmed if the residual pellet contained the drug. In the case of the non-CSA liposomes, the residual pellet was observed to not redisperse in the solution, but the residual pellet of the CSA-loaded liposomes was confirmed to containing the drug. The concentration of the drug that had not been entrapped was determined using the supernatant and the residual pellet from the CSA-loaded liposomes. The entrapped CSA content was determined by subtracting the amount of free drug from that of the total. The entrapment efficiency was found to decrease in all the liposome preparations in relation to

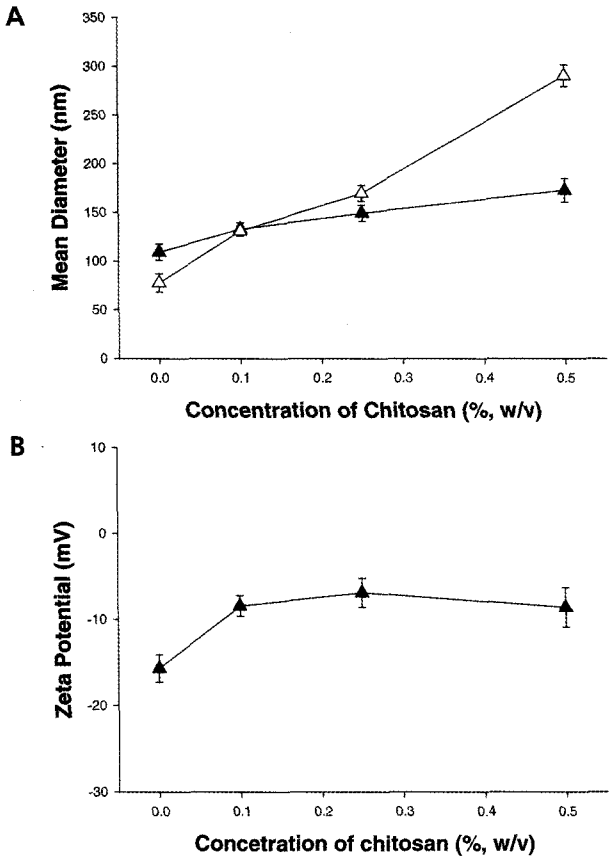


Fig. 2. Size distribution (A) and zeta potential (B) of CS-Lip preparations with respect to the different chitosan concentrations. △ : PA (0.1 mg), ▲ : PA (0.5 mg).

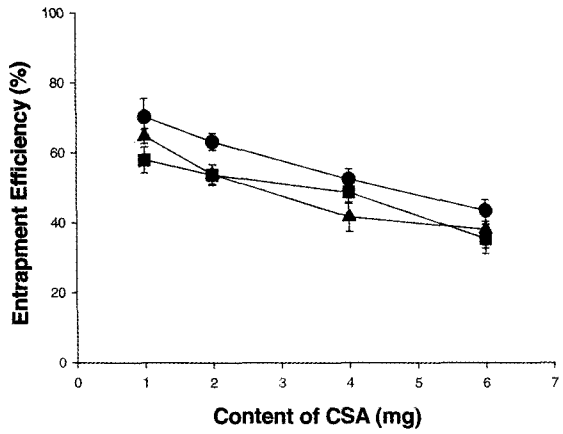


Fig. 3. The entrapment efficiencies of the different liposome preparations. ▲ : CS-Lip, ■ : SA-Lip, ● : PS-Lip.

increases in the CSA content. The liposome preparations had the following entrapment efficiencies: PS-Lip (52.5 ± 2.9%) > SA-Lip (48.8 ± 3.3%) > CS-Lip (41.7 ± 4.2%) (with a 4 mg CSA content) (Fig. 3). The reason why PS-Lip has higher entrapment efficiency than SA- and CS-Lip remains to be elucidated.

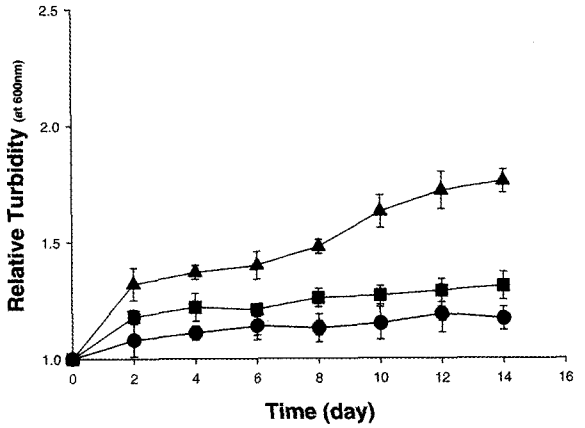
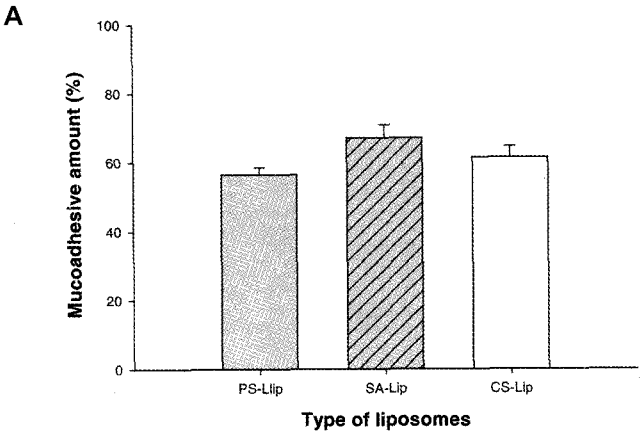


Fig. 4. Stability of the liposome preparations with respect to the storage time. ▲ : CS-Lip, ■ : SA-Lip, ● : PS-Lip.



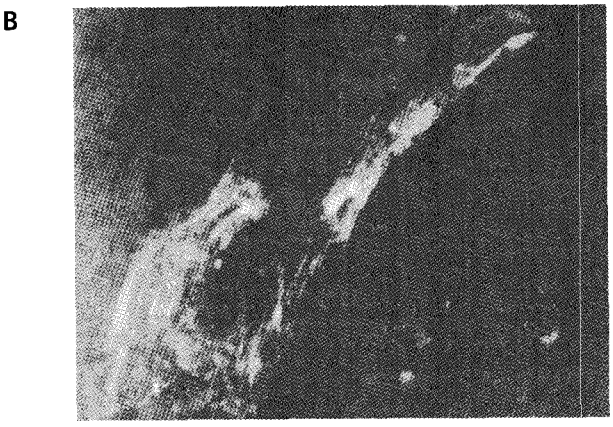


Fig. 5. The amount of mucoadhesion (A) and a fluorescence microscopy image (B) of the liposomes. (B) is a SA-Lip image. ■ : PS-Lip, ▨ : SA-Lip, □ : CS-Lip.

The stability of each liposome preparations in relation to the storage time was monitored using a UV spectrophotometer. The change in the turbidity was due to aggregation of the liposomal solution [19]. The relative turbidity of all the liposome preparations increased within 2

days. PS-Lip and SA-Lip were shown to be stable after 2 days, but the turbidity of the CS-Lip constantly increased over time (Fig. 4), and showed a phase separation (coagulation) after 12 days.

Mucoadhesive Test of Liposomes

The amount of mucoadhesive of each liposome preparation was evaluated using a Luminometer and fluorescence microscope. The SA-Lip (67%) had a higher amount of mucoadhesion than the other liposomes, CS-Lip (61%) and PS-Lip (56%) (Fig. 5). This result was due to the different zeta potential values, as shown in Table 1, SA-Lip (27.4 ± 1.7 mV) > CS-Lip (-6.3 ± 0.3 mV) > PS-Lip (-36.9 ± 2.4 mV). In the previous study, positively charged liposomes also showed higher ionic interactions with the negatively charged mucus layers on the surface of the intestines [20]. The adhesion of the prepared 5-CF loaded liposomes to the rat intestinal wall was also confirmed using fluorescence microscopy (Fig. 5).

CONCLUSION

Many drug carriers, using biodegradable polymers, lipids, have been used as lymphatic delivery systems. In particular, liposomes have received considerable interest as a lymphatic delivery system vehicle, as they can effectively entrap and deliver both hydrophilic and lipophilic drugs to the target site. Three types of CSA-loaded liposomes (anionic, cationic, polymer-coated liposomes) were prepared and characterized. All of the prepared liposomes showed a spherical, the size distributions under 150 nm and the entrapment efficiencies over 40%. Also, they show a stable state for the storage time except CS-Lip. Finally, the positive charged liposomes had a higher amount of mucoadhesion than the other liposomes in the mucoad-hesive tests. These results suggest that a positive charge on the surface of drug carriers may be an important factor for the intestinal drug delivery.

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