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Preparation and Characterization of Bovine Serum Albumin-loaded Cationic Liposomes: Effect of Hydration Phase

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ABSTRACT − Although liposomes have been applied as drug delivery systems in various fields, the usage was limited due to the low encapsulation efficiency compared to other carrier systems. Here, cationic liposomes were prepared by mixing 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP) as a cationic lipid, 1,2-dioleoyl-sn-glycerol-phosphoethanolamine (DOPE) and cholesterol (CH), and the liposomes were hydrated by varying the aqueous phases such as phosphate-buffered saline (PBS), 5% dextrose, and 10% sucrose in order to improve the encapsulation efficiency of bovine serum albumin (BSA). The particle size and zeta potential were determined by dynamic light scattering method and *in vitro* release patterns were investigated by spectrophotometry. Particle size and zeta potential of liposomes were varied depending on the ratio of DOTAP/DOPE/CH in range of 270-350 nm and 0.8-9.7 mV, respectively. Moreover, the addition of polyethylene glycol (PEG) improved the encapsulation efficiency from 37% to 43% as well as reduced particle sizes of liposomes while the liposomes were hydrated in PBS. When the liposomes were hydrated with 10% sucrose, the encapsulation efficiency of BSA was higher than any other groups. Whereas PBS was used as hydration solution, lower encapsulation efficiency was obtained compared with other groups. More than 60% of BSA was released from the liposomes hydrated with 10% sucrose; thereafter another 20% of BSA was released. Therefore, release pattern of BSA from cationic liposomes was extended release in this study. From the results, cationic liposomes dispersed in 10% sucrose would be potential carrier with high encapsulation efficiency.

Key words − Liposomes, Bovine serum albumin, Encapsulation, *In vitro* release

Recent advance of biotechnology has increased a number of peptides and proteins as therapeutic drugs. However, these bioactive materials are usually administered by parenteral route because of low bioavailability (Debs et al., 1990). Moreover, it is well known that peptide or protein drugs usually possess very short biological half-life and extreme instability because of proteolytic inactivation and degradation (Meyenburg et al., 2000; Katayama et al., 2003). In order to obtain a valid therapeutic range of protein drug, frequent injection is unavoidable in spite of pain, local tissue necrosis, microbial contamination, and nerve damage. Therefore, safe and sustained injectable delivery systems for protein should be developed to reduce the injection frequency and toxicity.

There are a variety of strategies to improve the half-life of proteins (Werle and Bernkop-Schnürch, 2006). For example, chemical modification of the protein drugs, such as modification of *N*- and *C*-terminals of protein, replacement of labile amino acids, cyclization and increment of molecular mass by PEGylation or oligomerization, enzyme inhibition and introduction of drug delivery systems, such as liposomes, microspheres consisted of biodegradable polymers, emulsions and cyclodextrins. Among these delivery systems, liposomes have been investigated as a sustained drug delivery system (Blume and Cevc, 1990; Lian and Ho, 2001; Lee and Yuk, 2007). Liposomes have advantages over other delivery systems since these are biodegradable, non-toxic and non-immunogenic. Moreover, it is able to encapsulate both hydrophilic and hydrophobic drugs into liposomes.

However, the encapsulation efficiency of various drugs in liposomal drug delivery is of pharmaceutical importance. In order to achieve optimum efficacy and cost effectiveness for a drug delivery system, it is necessary to encapsulate the maximum possible quantity of drug in the carriers. Therefore, it has been tried by altering the various approaches including reverse liposomes, freeze-drying, prodrug, aqueous phase, etc. in order to improve the encapsulation efficiency of liposomes. Comparison of the encapsulation efficiency of the drug in liposomes with aqueous phase indicates whether liposomes can be used as a delivery system for the drug. Suitable hydration media include distilled water, buffer solutions, saline, and nonelectrolytes such as sugar solutions. Physiological osmolality

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(290 mOsm/kg) is recommended for *in vivo* applications. Generally accepted solutions with meet these conditions are 0.9% saline, 5% dextrose, and 10% sucrose.

In this study, cationic liposomes were investigated as delivery system of BSA as a model protein drug, since positive charged carriers showed smaller size and higher or comparable encapsulation efficiency of peptide drug than anionic or neutral ones (Vandana and Sahoo, 2009; Manosroi et al., 2010). For comparison of hydration, the liposomes were hydrated with phosphate buffered saline (PBS), 5% dextrose, and 10% sucrose. Then, encapsulation efficiency of liposomes was compared upon hydration solution and *in vitro* release of BSA from liposomes was investigated.

Materials and Methods

Materials

1,2-dioleoyl-9-trimethylammonium propane (DOTAP), 1,2-dioleoyl-sn-glycerol-phosphoethanolamine (DOPE), cholesterol (CH), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG(2000) Amine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Bovine serum albumin (BSA), dextrose, and sucrose were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of reagent grade and used without further purification.

Preparation of cationic liposomes

Cationic liposomes were prepared by lipid film method (Jeong et al., 2009). Stock solutions of each lipid were mixed at the various molar ratio of DOTAP/DOPE/CH in chloroform. The amounts of total lipids were fixed as 5 μmol. The organic phase was removed at 50°C on a rotary evaporator (KU-NLW, Sunil Instrument Co., Korea). The dried lipid film was flushed with nitrogen gas to remove traces of organic solvent and hydrated with hydration solution such as PBS, 5% dextrose and 10% sucrose. After sonication at about 30°C for 30 min, the solution was extruded 10 times through a 100-nm polycarbonate membrane using an extruder (Northern Lipids Inc., Canada). BSA was incorporated by adding 100 μL of stock solution (BSA 4 mg/mL). Moreover, DSPE-PEG 2000 as 5 mol% of total lipids was added to the liposomes formula to determine the effect of DSPE-PEG on the encapsulation efficiency.

Measurement of particle size and zeta potential

Particle size distribution of cationic liposomes with or without BSA was determined by light scattering spectrophotometer (ELS-8000, Otsuka Electronics Co., Japan). The samples were diluted with deionized water, and then transferred into a quartz cuvette in an ELS-8000 dynamic light scattering instrument. Zeta potential of liposomes was measured by electrophoretic light scattering spectrophotometer. Data were analyzed using a software package (ELS-8000 software) supplied by the manufacturer.

Encapsulation efficiency

The encapsulation efficiency of BSA was expressed as the ratio between the concentration of BSA in the liposomes and the concentration of BSA added to the system. In order to determine the concentration of BSA, Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used. Before analysis, the samples were centrifuged at 10,000 rpm for 30 min to remove briefly unencapsulated BSA (Mokhtar et al., 2008). One hundred microliters of each standard and sample were added into a labeled test tube with replication. The working reagent 2 mL was added to each tube and mixed well. Then, the tubes were covered and incubated at 37°C for 30 min and cooled to room temperature. Subsequently, the absorbance of all the samples was measured within 10 min. The absorbance of BSA was measured spectrophotometrically at 562 nm after destruction of the liposomes in triton X-100 by UV-VIS spectrophotometer (Mini 1240, Shimadzu, Japan).

In vitro release test

In vitro release of BSA from the liposomes was carried out in PBS (pH 7.4) at 37°C with stirring. Briefly, 1 mL of BSA liposomes hydrated with 10% sucrose was suspended in a temperature-controlled and jacketed 4-mL vial containing 3 mL of PBS. After various time intervals, aliquots of the medium were withdrawn and centrifuged. To maintain sink condition, an equal volume of fresh medium was compensated. Then, the amount of released BSA was determined by measuring the concentration of BSA in supernatant with the BCA protein assay kit.

Results and Discussion

Particle size and zeta potential

In order to determine the liposomes formulation, cationic liposomes were prepared three molar ratios of DOTAP/DOPE/CH such as 4/3/4, 2/1/1, and 2/2/1. Particle size of each formulated liposomes was about 274.4 nm, 345.0 nm, and 314.8 nm, respectively (Table I). Zeta potentials of liposomes were varied on the molar ratio of liposomes from 9.73 mV to 0.83

Table I. *Particle Sizes and Zeta Potentials of Cationic Liposomes (n=3)*

Composition	Particle size (nm)	Zeta potential (mV)
DOTAP/DOPE/CH=4:3:4	274.4 ± 33.1	9.73 ± 3.42
DOTAP/DOPE/CH=2:1:1	345.0 ± 53.8	2.50 ± 0.64
DOTAP/DOPE/CH=2:2:1	314.8 ± 12.8	0.83 ± 0.32

mV (Table I). From the results, the molar ratio of DOTAP/DOPE/CH=4/3/4 was selected by considering particle size and zeta potential.

Effect of PEGylation on physical properties of liposomes

PEGylated liposomes presented smaller particle size than conventional liposomes as shown in Figure 1. Moreover, particle size reducing effect of PEG was clear even in BSA-loaded liposomes (Figure 2). It was reported that particle size of liposomes could be decreased by PEG (Kim et al., 2009). It was supposed that reduced electrostatic repulsion by adding PEG to liposomes might result in the smaller particle size via preventing the feasible aggregation of liposomes (Kim et al., 2003). Meanwhile, the addition of DSPE-PEG was evaluated for PBS-based liposomes. The encapsulation efficiency of BSA was increased from 37% to 43% by the addition of DSPE-PEG (Table II). Here, the brief centrifugation method was selected for removal of unencapsulated BSA even though incomplete separation as expected. However, it is also suggested that brief centrifugation is more convenient than exhaustive dialysis technique (Mokhtar et al., 2008). Furthermore, liposomes with PEG-derivatized lipids have been reported to be stable *in vitro* (Blume and Cevc, 1990) and in the circulation (Gabizon and Martin, 1997), which might contribute to the sustained release of encapsulated drugs.

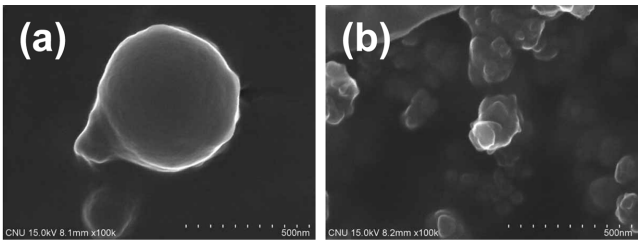

Figure 1. SEM images of (a) conventional blank liposomes and (b) PEGylated blank liposomes.

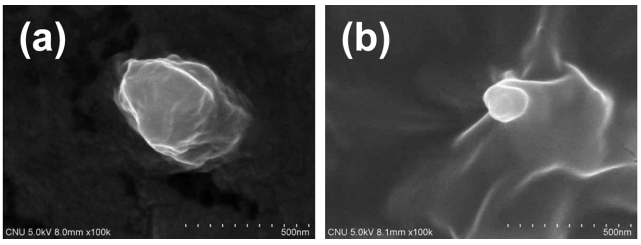

Figure 2. SEM images of (a) BSA-loaded PEGylated liposomes in PBS and (b) BSA-loaded PEGylated liposomes in 10% sucrose.

Effect of aqueous phase on encapsulation efficiency

Encapsulation efficiency of BSA was varied depending on the hydration media as shown in Table III. Briefly, the encapsulation efficiency of BSA in liposomes was decreased in order of 10% sucrose, 5% dextrose and PBS. Highest encapsulation efficiency (78%) was obtained by hydration with 10% sucrose, whereas PBS, generally used as aqueous phase, showed lowest encapsulation efficiency (43%). It is assumed that free BSA hydrated in 10% sucrose or 5% dextrose is able to be encapsulated in liposomes. In practice, carbohydrate excipients such as sucrose and dextrose have been commonly used in protein formulation to inhibit aggregation of many proteins including bovine insulin (Wang, 2005). It is supposed that the encapsulation enhancement by sucrose is caused by membrane-protecting effect from damage during process (Crowe et al., 2007; van Winden, 2003). Also, the sugar molecules may prevent disruption of the membrane bilayer by maintaining the phospholipid (Crowe et al., 2007; van Winden, 2003). Moreover, it is reported that sucrose proved to be very effective in reducing the size of nanoparticles (Vandana and Sahoo, 2009). Thus, suitable aqueous phase for hydration as well as PEG addition should be selected to increase the encapsulation efficiency of liposomes.

***In vitro* release profiles of BSA from liposomes**

In vitro release of BSA from liposomes was performed for 72 hr at 37ºC as shown in Figure 3. An initial burst release of

Table II. *Effect of PEGylation on the Encapsulation Efficiency and Particle Size of Bovine Serum Albumin in Cationic Liposomes*

Liposomes	Encapsulation efficiency (%)	Particle size (nm)
Conventional	37.06±3.65	287.8±44.4
PEGylated	43.11±1.08	176.7±8.0

Table III. *Effect of Aqueous Phase on the Encapsulation Efficiency of Bovine Serum Albumin in Cationic Liposomes*

Aqueous phase	Encapsulation efficiency (%)
PBS	43.11 ± 11.08
5% Dextrose	58.64 ± 3.23
10% Sucrose	78.57 ± 3.45

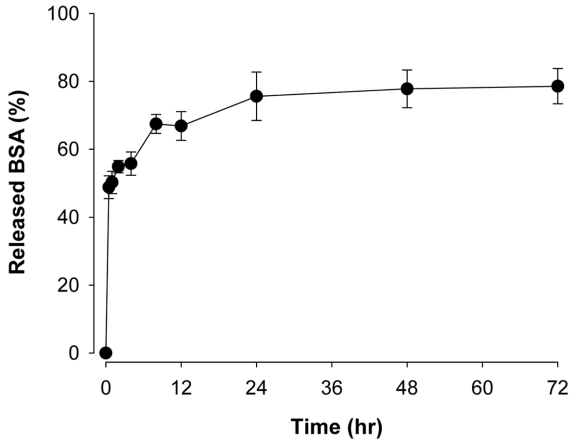

Figure 3. *In vitro* release of BSA from PEGylated liposomes. Each value represents the mean±s.d. (n=4).

49% of BSA from the liposomes was observed at 0.5 hr, followed by a continuous release of 50-79% BSA for up to 72 hr. It is thought that the burst release in 0.5 hr would result from the remaining BSA on the surface of liposomes even though centrifugation process. The other experiments are needed to prevent burst release in initial state. However, it should be noted that this initial 50% release of BSA would be sufficient to deliver model protein into target site. This result corresponds with a previous study that release rates of tPA from PEGylated liposomes were slower than of conventional liposomes (Kim et al., 2009).

Conclusion

The BSA-loaded cationic liposomes were prepared by optimizing the lipid ratio of DOPE/DOPE/CH. The addition of PEG reduced particle size of liposomes as well as improved the encapsulation efficiency. Moreover, aqueous phase did enhance the encapsulation efficiency. Thus, the aqueous phase could be an important parameter to prepare liposomes and to increase the encapsulation efficiency.

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