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Encapsulation enhancement and stabilization of insulin in cationic liposomes

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ABSTRACT

The purpose of this study was to enhance encapsulation efficiency and sustained-release delivery for parenteral administration of a protein drug. To reduce the administration frequency of protein drugs, it is necessary to develop sustained delivery systems. In this study, protein drug-loaded cationic liposomes were formulated with dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dioleoyl-3-trimethylammonium-propane (DOTAP), and cholesterol (CH) at a molar ratio of DOPE/DOTAP/CH of 2/1.5/2. Five mol% of distearoylphosphatidyl ethanolamine polyethylene glycol (DSPE-PEG) was added prior to encapsulation of the drug into liposomes. Insulin was chosen as a model protein drug and encapsulation efficiency was evaluated in various liposomes with and without DSPE-PEG. Scanning electron microscopy was used to examine the insulin-loaded cationic liposomes. Structural analysis was performed using spectropolarimetry. Additionally, the stability and cytotoxicity of insulin-loaded cationic liposomes were evaluated. Liposomes coated with DSPE-PEG showed higher insulin encapsulation efficiency than did those without DSPE-PEG, but not significantly. Moreover, among the liposomes coated with DSPE-PEG, those hydrated with 10% sucrose showed higher encapsulation efficiency than did liposomes hydrated in either phosphate-buffered saline or 5% dextrose. *In vitro* release of insulin was prolonged by cationic liposomes. Our findings suggest that cationic liposomes may be a potential sustained-release delivery system for parenteral administration of protein and peptide drugs to prolong efficacy and improve bioavailability.

1. Introduction

Increasingly, proteins and peptides are being developed for drug use, due to their biological effects and high therapeutic efficiency. However, it is well known that such drugs usually possess very short biological half-lives, and are extremely unstable, due to proteolytic inactivation and degradation (Katayama et al., 2003; Meyenburg et al., 2000). The need for frequent injections, together with the pain, tenderness, local tissue necrosis, microbial contamination, and nerve damage that can be associated with subcutaneous injections, as seen with insulin, have prompted research on alternative therapies (Hinchcliffe and Illum, 1999; Muchmore and Gates, 2006). Proper delivery systems could provide extended circulating half-lives of various pharmaceuticals (Kim et al., 2009). To reduce the injection frequency and toxicity of intravenously administered protein drugs, it would be necessary to develop safe and sustained injectable protein delivery systems. The encapsulation of peptide drugs in vesicular systems offers several advantages, decreasing toxicity while increasing stability, circulation time and absorption of the drug (Gude et al., 2002; Manosroi et al., 2005).

Liposomes have been studied as sustained drug delivery systems (Blume and Cevc, 1990). They have advantages over other delivery systems, being biodegradable, non-toxic and non-immunogenic. 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 could contribute to the sustained release of encapsulated drugs.

In this study, to evaluate liposomes as safe and sustained injectable delivery systems, insulin was chosen as a model protein drug. Insulin is an anabolic polypeptide hormone used medically to treat some forms of diabetes mellitus. Therapeutic insulin for diabetes is typically administered via subcutaneous injection. It is one of the most widely used protein drugs. It has a short half-life (30 min) after intravenous administration (Owens, 1986), but can cause hypoglycemic shock. In recent decades, a number of attempts have been made to overcome the limitations and drawbacks of conventional delivery of insulin by injection (Jeong et al., 2002; Choi and Kim, 2003; Kashyap et al., 2007). Long-term insulin delivery, over weeks or even months, can enhance patient compliance and convenience. Sustained subcutaneous insulin release systems have been reported to decrease the frequency of insulin injections, increase patient compliance, and reduce complications associated with poor glucose control (Colquitt et al., 2004; Wu et al., 2007).

The encapsulation efficiency of peptide drugs in liposomal drug delivery is of pharmaceutical importance, especially in achieving

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optimum efficacy and cost effectiveness. Various strategies have been used during preparation to improve encapsulation efficiency, including forming reverse liposomes, freeze-drying, using prodrug, and altering the aqueous phase. Of these parameters, changes in hydration media have been suggested to enhance the encapsulation efficiency of peptide drugs (Park et al., 2010).

In this study, we prepared cationic liposomes to incorporate insulin, and improved the insulin encapsulation efficiency with various hydration solutions. The physicochemical properties of insulin-loaded cationic liposomes were characterized and the stability of insulin in such liposomes was investigated in terms of particle sizes and circular dichroism spectra.

2. Materials and methods

2.1. Materials

Insulin from bovine pancreas, sucrose, and dextrose were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol (CH), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol) 2000 (ammonium salt) (DSPE-PEG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of cationic liposomes

Cationic liposomes were prepared by a lipid film method (Park et al., 2010). Stock solutions of each lipid were mixed at the various molar ratios of DOTAP/DOPE/CH in chloroform. The amount of total lipid was fixed at 5 μmol. The organic phase was removed at 50 °C using a rotary evaporator (KU-NLW, Sunil Instrument Co., Korea). The dried film was flushed with nitrogen gas to remove traces of organic solvent and hydrated with a hydration solution of PBS, 5% dextrose or 10% sucrose. After sonification at about 30 °C for 30 min in a bath-type sonicator (Branson, USA), the solution was extruded 10 times through a 100-nm polycarbonate membrane (LIPEX extruder, Northern Lipids Inc., Burnaby, Canada). For encapsulation of insulin, the crude liposome dispersion was sonicated for 30 s and 100 μl of insulin solutions (insulin 2 mg/ml; prepared in three hydration media such as PBS, 5% dextrose, and 10% sucrose) was added and vortexed for 30 s. In some preparations, DSPE-PEG 2000 as 5 mol% of total lipids was added to the liposome formula to determine its effect on the encapsulation efficiency for insulin. When the extrusion of the sample was clogged by precipitate, it was repeated slowly after dilution of the samples and was briefly centrifuged to remove unencapsulated insulin.

2.3. Measurements of particle size and zeta potential

The particle size and zeta potential of cationic liposomes were measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Co., Japan) at a fixed angle of 90° at room temperature. The system was used in the auto-measuring mode. The samples were dispersed in double-distilled water. Information about liposome size was obtained by analyzing the dynamic light scattering (DLS) data using the cumulant method.

2.4. Encapsulation efficiency

The encapsulation efficiency of insulin was expressed as the ratio between the concentration of insulin in the liposomes and the total concentration of insulin added to the system. A Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to determine the concentration of insulin. Before analysis, the samples were centrifuged (10,000 rpm, 30 min) to remove unencapsulated insulin. Each standard or sample (100 μl) was added into labeled test tubes with replication. Then, 2 ml of working reagent was added to each tube and mixed well. 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 spectrophotometrically at 562 nm within 10 min (Mini 1240 UV-VIS spectrophotometer, Shimadzu, Japan). The insulin concentration was measured after destruction of the liposomes in Triton-X100.

2.5. Scanning electron microscopy

The morphology of cationic liposomes was observed by scanning electronic microscopy (SEM, JSM-7000F, Jeol Ltd., Japan). Samples were lyophilized, coated with gold and palladium using a vacuum evaporator, and examined using SEM at 20 kV accelerating voltage.

2.6. Circular dichroism (CD)

To verify the integrity of the secondary structure of insulin when entrapped in cationic liposomes, CD studies were carried out. CD spectra were acquired at 25 °C using a spectropolarimeter (J-815 Spectropolarimeter, Jasco, Japan) in the far UV region (190–280 nm) in a 0.01 cm path length cell using a step size of 1 nm. The lamp housing was purged with nitrogen, and an average of three scans was obtained. CD spectra of the appropriate blank reference were recorded and substracted from the insulin spectra, to eliminate contributions from the aqueous phase. The mean residual molar ellipticity was calculated from the raw CD signal, using a mean residue weight of 116 Da and an insulin concentration calculated from the corrected absorbance of the insulin solution.

2.7. In vitro release study

For *in vitro* release of insulin, 400 μl of insulin-encapsulated liposomes were placed on one side of a 0.1 μm semipermeable membrane (Millipore, Bedford, MA, USA). Samples were placed in a vial containing 4 ml of PBS at 37 ± 0.5 °C. At several time points (0.5, 1, 2, 4, 8, 12, 24, 48, 72 h), 1 ml of the PBS was removed, and replaced with an equal volume of fresh buffer. The concentrations of insulin were assayed spectrophotometrically at 562 nm as described in Section 2.5.

2.8. Stability of cationic liposomes

Storage stability of insulin-loaded cationic liposomes was evaluated in terms of changes in particle size. The liposome samples were stored at 4 °C and mean particle sizes were measured at predetermined time intervals (0.5, 0, 1, 2, 3, 4, 6 weeks) with an electrophoretic light-scattering spectrophotometer. At the completion of the study, samples were transferred into clear test tubes for visual examination.

2.9. Cytotoxicity assay

The cytotoxicity of various insulin-encapsulated cationic liposomes was evaluated by MTT assay. HepG2, human hepatoma, cells were seeded at a density of 1×10^4 cells/well in 96-well plates and allowed to attach overnight. The volume of each suspension and the dose of insulin were fixed at 100 μl and 20 mg, respectively. The samples were added into each well and incubated overnight. After incubation, 100 μl of 5 mg/ml MTT in DMEM was added to each

Table 1
Particle size and zeta potential of plain cationic liposomes ($n = 3$).

Formulation ratio of DOTAP:DOPE:CH	Particle size (nm)	Zeta potential (mV)
2:1:1	334.9 ± 50.7	2.45 ± 2.6
2:1.5:2	265.4 ± 35.3	9.67 ± 4.2
2:2:1	304.8 ± 18.2	0.81 ± 3.2

well and incubated for 4 h at 37 °C. To dissolve the formazan crystal, 100 μl of MTT solubilization solution was added. After shaking the 96-well plates for 30 min, absorbance was measured at 570 nm using an ELISA plate reader (Sunrise, TECAN, Switzerland). The 100% viability standard for cytotoxicity comparison was obtained from the OD value measured in untreated cells.

3. Results and discussion

3.1. Physicochemical properties of cationic liposomes

3.1.1. Effect of lipid ratio on particle size and zeta potential of liposomes

Cationic liposomes were prepared with three molar ratios of DOTAP/DOPE/CH: 2/1/1, 2/1.5/2, and 2/2/1. Particle sizes for each empty liposome formulation were 334.9 nm, 265.4 nm, and 304.8 nm, respectively (Table 1). Normally the liposome size mainly depends on the ratio of DOTAP to DOPE. The ratio of 2/1.5/2 gave smaller particle sizes than the others. Zeta potentials of liposomes from these formulations varied from 9.67 mV to 0.81 mV (Table 1). Moreover, it is reported that the entrapment efficiency of insulin was higher in cationic niosomes than in anionic and neutral ones (Manosroi et al., 2010). From these results, the molar ratio of DOTAP/DOPE/CH of 2/1.5/2 was selected for further experimentation.

3.1.2. Effect of PEGylation on the physicochemical properties of liposomes

Because the insulin-loaded cationic liposomes were formulated for parenteral administration, size distribution was one of the crucial parameters in their preparation. PEGylated liposomes show smaller particle sizes than plain liposomes (Table 2). The addition of DSPE-PEG reduced the particle size of insulin-loaded cationic liposomes 3.7-fold compared to insulin-loaded plain liposomes. PEG could be the structural material of lipid vesicles with the object of acquisition of steric stability. This size reduction by PEG addition may be due to reduced electrostatic repulsion, preventing fusion and disruption of the liposomes (Kim et al., 2009). The zeta potential of insulin-loaded cationic liposomes was also reduced compared with that of empty liposomes (data not shown), but the zeta potential of cationic liposomes was not altered by the addition of DSPE-PEG. Further, the addition of DSPE-PEG was evaluated with PBS-based liposomes.

To deliver a sufficient amount of drug to provide a therapeutic effect, a high entrapment efficiency of drug in liposomes is required. Encapsulation efficiency of insulin in plain cationic liposomes was 45%, which was increased to 50.3% by the addition of DSPE-PEG (5 mol% of total lipids), as shown in Table 2. Theoretical loading amounts of each liposomes formulation were 18.0 g/mol of lipid for plain liposomes and 19.2 g/mol of lipid for PEGylated liposomes, respectively. The increase in encapsulation efficiency seems not to be significant, but a few studies supported that the enhancing role of PEG is critical to obtain high encapsulation efficiency (Dominak et al., 2010; Kim et al., 1999; Wang et al., 2011). However, our result disagrees with the previous report in which the encapsulation efficiency of tPA was not significantly altered by adding PEG (Kim et al., 2009) or PEG have showed marked reduction in encapsulated glucose (Nicholas et al., 2000). Moreover, the pI of insulin is 5.5. When dispersed in buffer at a pH above its pI, it has a negative charge. Thus, it is thought that insulin would be encapsulated readily into cationic liposomes at neutral pH by charge interaction. Peptide drugs that have different charges based on their pI values in a buffer system will be differentially entrapped in the liposomal system, due to the electrical double-layer effect.

Table 2
Effect of DSPE-PEG on particle size and encapsulation efficiency of insulin-loaded cationic liposomes ($n = 3$).

Cationic liposome	Particle size (nm)	Encapsulation efficiency (%)
Plain	694.6 ± 88.6	45.0 ± 3.4
PEGylated	188.2 ± 5.7	50.3 ± 2.2

Table 3
Effect of hydration solution on particle size and encapsulation efficiency of insulin-loaded cationic liposomes ($n = 3$).

Hydration solution	Particle size (nm)	Encapsulation efficiency (%)
PBS	202.4 ± 3.3	50.3 ± 2.2
5% Dextrose	258.5 ± 9.3	56.7 ± 0.5
10% Sucrose	222.5 ± 9.0	73.7 ± 1.3

Here, a brief centrifugation was used to remove unencapsulated insulin. While incomplete separation is expected with this method (Park et al., 2010), brief centrifugation is far more convenient than exhausted dialysis techniques (Mokhtar et al., 2008). The prepared liposomes were large unilamellar vesicles, PEG chains might be located on both sides of the liposomal membranes.

3.1.3. Effect of aqueous phase on particle size and encapsulation efficiency of liposomes

The particle size of insulin-loaded cationic liposomes differed depending on the hydration media used (Table 3). Liposomes hydrated in PBS, 5% dextrose, or 10% sucrose were about 202 nm, 258 nm, and 222 nm, respectively.

Furthermore, the encapsulation efficiency of insulin was dependent on the hydration media. The encapsulation efficiency of insulin in liposomes decreased in the following order: 10% sucrose, 5% dextrose, PBS. This corresponds to the results of our previous study of BSA-loaded liposomes (Park et al., 2010). The highest encapsulation efficiency (74%) was obtained by hydration with 10% sucrose, whereas PBS, generally used as an aqueous phase, showed the lowest encapsulation efficiency (50%). It is assumed that free insulin hydrated in 10% sucrose or 5% dextrose can better be encapsulated in liposomes. In practice, carbohydrate excipients such as sucrose and dextrose have been commonly used in protein formulations 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, preventing disruption of the membrane bilayer during the process (Crowe et al., 2006; van Winden, 2003). Moreover, sucrose has been reported to be effective in reducing the size of nanoparticles (Vandana and Sahoo, 2009). Thus, a suitable aqueous phase for hydration as well as PEG addition should be selected to increase the encapsulation efficiency of liposomes.

3.2. Particle morphology

The morphology of lyophilized cationic liposomes was examined by scanning electron microscopy (SEM). Micrographs showed that the particles had a spherical shape. If transmission electron microscopy (TEM) is available to observe the morphology of liposomes, the clear observation of liposomal lamellarity could be obtained. PEGylated liposomes presented smaller particle sizes than plain liposomes (Fig. 1). Micrographs of unloaded cationic

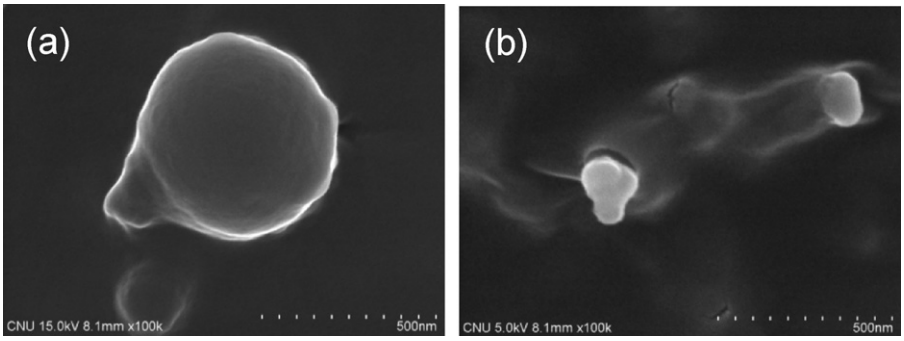

Fig. 1. SEM images of (a) blank plain liposomes and (b) blank PEGylated liposomes.

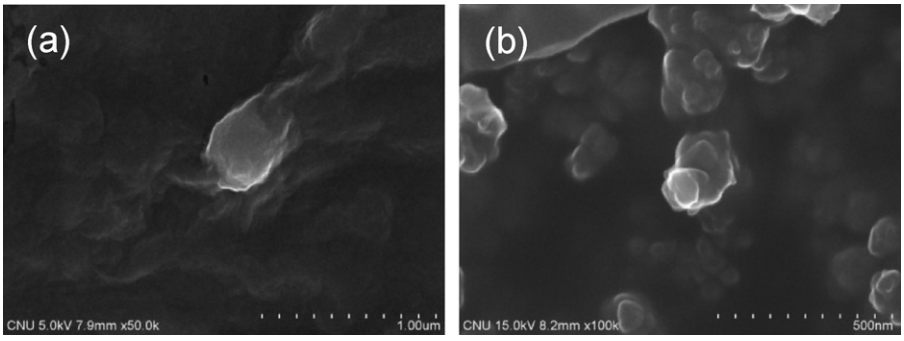

Fig. 2. SEM images of (a) insulin-loaded PEGylated liposomes in PBS and (b) insulin-loaded PEGylated liposomes in 10% sucrose.

liposomes (Fig. 1a) clearly showed their spherical shape, and show the effect of DSPE-PEG on mean particle size (Fig. 1b). Moreover, the particle size-reducing effect of PEG was clear even in insulin-loaded liposomes (Fig. 2). The size distribution of insulin containing cationic liposomes was 100–200 nm (Fig. 2). Additionally, there were shape differences between lyophilized insulin-loaded cationic liposomes in PBS (Fig. 2a) and insulin-loaded cationic liposomes in 10% sucrose (Fig. 2b). The shape of cationic liposomes in 10% sucrose was unclear, however, due to interference from sucrose crystallization.

3.3. CD spectra of insulin loaded cationic liposomes

Fig. 3 shows the circular dichroism spectra of insulin-loaded cationic liposomes. The CD spectra, both in solution and in cationic liposomes, with minima at 222 and 208 nm (Fig. 3), were typical of signals for insulin structure (Rasmussen et al., 2010). Peaks observed for insulin encapsulated into cationic liposomes or free in solution were seen at similar wavelengths, suggesting that there was no difference in protein structure between the two states.

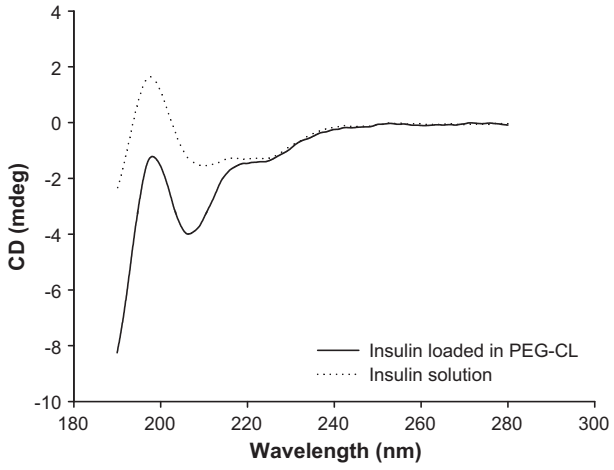

Fig. 3. Circular dichroism spectra of insulin loaded PEGylated cationic liposomes (PEG-CL) (line) and insulin solution (dot).

Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light, which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum, which can contain both positive and negative signals. The effect of forming a protein-protein complex on the thermal stability of the individual proteins in the complex can also be determined. This works best if the individual proteins have CD spectra which are quite different from each other, such that changes at specific wavelengths can be monitored to follow changes in the corresponding protein.

3.4. In vitro release profiles

In vitro release of insulin from liposomes was monitored over 72 h at 37 °C (Fig. 4). An initial burst release of 34% of loaded insulin from PEGylated liposomes was observed at 30 min, followed by a continuous release of 48–73% insulin over 72 h. However, the plain liposomes provided a much faster release than PEGylated liposomes, suggesting that about 46% of insulin from the plain liposomes was released at 0.5 h, followed by a continuous release of 55–100% for up to 72 h. It is thought that the burst release in 30 min resulted from insulin remaining on the surface of liposomes even after the centrifugation process. Additional experimentation is needed to investigate ways to prevent this initial burst release, although it should be noted that this initial release of insulin would be sufficient to avoid a delayed onset in plasma insulin elevation. *In vitro* release tests clearly illustrated that cationic liposomes can release insulin in a sustained manner. There is a potential advantage in the propensity of insulin to partition favorably into lipid vesicles,

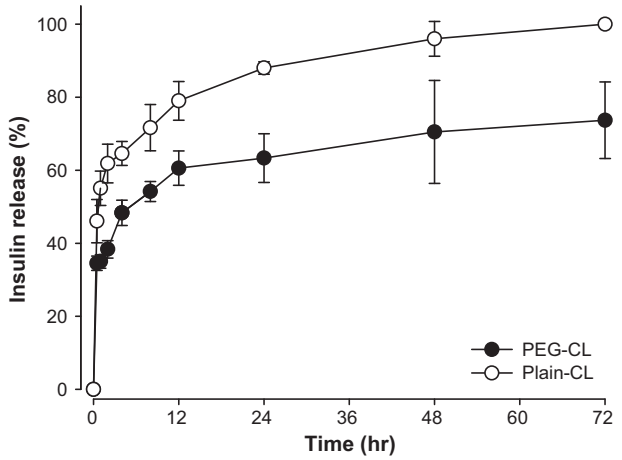

Fig. 4. Insulin release (%) from PEGylated cationic liposomes (PEG-CL) and plain cationic liposomes (Plain-CL) in insulin-loaded cationic liposome in PBS at 37 ± 0.5 °C. Data represent the mean ± S.D. (n = 3).

and, more importantly, results suggest that the release of insulin from cationic liposomes can be controlled. This result corresponds with those of a previous study showing that the release rates of tPA and BSA from PEGylated liposomes were slower than from plain liposomes (Kim et al., 2009; Park et al., 2010). 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 should contribute to the sustained release of encapsulated drugs.

3.5. Stability test of the insulin loaded cationic liposomes

One of the major problems limiting the widespread use of liposomes is stability, both physical and chemical. Cationic liposomes with entrapped insulin were examined repeatedly over time. Because of aggregation or fusion of vesicles, particle size of insulin-loaded cationic liposomes increased (Fig. 5). Insulin-loaded cationic liposomes had larger mean particle sizes than unloaded liposomes, although the difference was small. Also, while monitoring the physical appearance of the insulin-loaded liposomes, the formation of sediment and an increase in the turbidity of supernatants were observed after 1 week. Some of the stability problems may be overcome by lyophilization, where the final liposome product is freeze-dried with a cryoprotectant and reconstituted with vehicle immediately prior to administration.

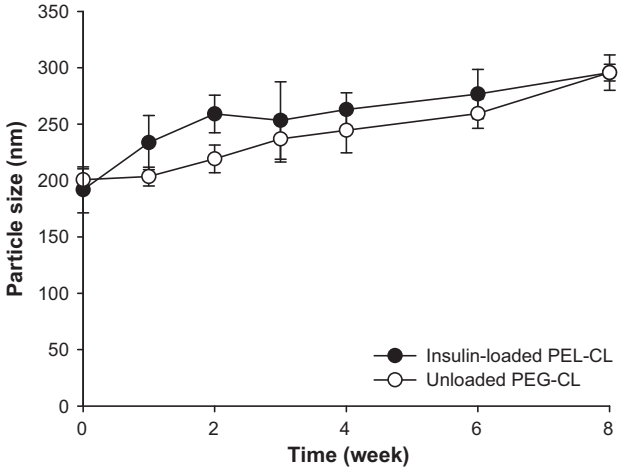

Fig. 5. Physical stability of insulin-loaded or unloaded PEG-CL at 4 °C. Changes of particle size were monitored at predetermined interval. Data represent the mean ± S.D. (n = 3).

Table 4
Cytotoxicity of blank vehicles and insulin-loaded liposomes (n = 4).

Loading and vehicles	Hydration solution	Cell viability (%)
None	10% Sucrose	74.4 ± 5.4
Blank cationic liposomes	10% Sucrose	83.2 ± 10.1
Free insulin	10% Sucrose	78.8 ± 3.9
Free insulin	PBS	84.0 ± 10.8
Insulin-loaded cationic liposomes	PBS	91.0 ± 8.6
PEGylated insulin-loaded cationic liposomes	PBS	101.1 ± 2.2
PEGylated insulin-loaded cationic liposomes	5% dextrose	96.2 ± 11.5
PEGylated insulin-loaded cationic liposomes	10% sucrose	91.8 ± 7.6

3.6. Cytotoxicity assay

The cytotoxicity of insulin-loaded cationic liposomes was monitored using MTT assays (Table 4). Cell viability after exposure to 10% sucrose, unloaded cationic liposomes in 10% sucrose, or an insulin solution in 10% sucrose was 74.4%, 83.2%, and 78.8%, respectively. When liposomes were hydrated in PBS, viability after exposure to insulin solution, insulin-loaded cationic liposomes, or PEGylated insulin-loaded cationic liposomes was 84.0%, 91.0%, and 101.1%, respectively. For PEGylated insulin-loaded cationic liposomes, all hydration solutions resulted in high cell viability (>90%) and samples were less toxic to HepG2 cells than free insulin.

4. Conclusions

Insulin-loaded cationic liposomes were formulated by optimizing the lipid ratio and adding DSPE-PEG. The addition of PEG reduced the particle size of liposomes, as well as improving encapsulated efficiency. Moreover, altering the aqueous phase enhanced encapsulation efficiency. The molecular states of insulin in liposomes were also investigated. We found that the hydration solution can be an important factor in the preparation of liposomes and can increase their encapsulation efficiency and stability.

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