

## The Effects of Storage Conditions on the Stability of Porcine Placenta Extract-loaded Liposome Formulations

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**ABSTRACT** – We aimed to evaluate the effect of temperature, pH, and light conditions on the stability of porcine placental extract (PPE)-loaded liposomes with different surface charges. The size distribution profiles and *in vitro* release patterns were investigated by dynamic light scattering method and spectrophotometry. The stability of PPE-loaded liposomes was affected by the surface charges of the liposomes. As compared to neutral and anionic liposomes, cationic liposome formulations showed significantly lower physical stability. At the test storage conditions of different temperatures and pHs, the mean sizes of cationic PPE-loaded liposomes substantially increased. In contrast, neutral and anionic liposomes did not reveal significant changes in mean sizes upon various storage conditions. The neutral and anionic liposomes showed no significant differences in the release profiles of PPE after storage at various temperatures and pHs. Our results indicate that anionic and neutral liposome compositions might be more suitable for the formulations of PPE providing the higher stability.

**Key words** – Surface charges, Stability of liposome, Placenta extracts

Liposomal formulations have been studied for sustained delivery of small chemicals and peptide/protein drugs. (Kaasgaard et al., 2010; Marcato et al., 2008). To develop liposomes as carriers of bioactive substances, the stability at various temperature and pH must be considered. The stability of liposomes during storage mainly depends on the physic-chemical properties of the lipid membrane composition. (Eliyahou et al., 2007; Ntimenou et al., 2006; Sulkowski et al., 2005). Lipid-bilayer and membrane-surface characteristics, and particle sizes are the critical factors that affect the liposomal behavior and drug release dynamics. (Li et al., 2010). The stability of liposomes has been also reported to be affected by the storage conditions. (Pietzyk et al., 2000).

Emulsions and liposomes have been studied as topical formulations of porcine placenta extracts (PPE). (Kim et al., 2007). However, the effects of surface charges on the stability of the PPE-loaded liposome formulations have not been elucidated. In this study, we tested whether the surface charges of various PPE-loaded liposomes could affect the physical stability of the liposomes and *in vitro* release rate of PPE. The stability of liposomes was evaluated under storage at various temperature, pH, and light conditions. Here, we report that the liposomal surface charges may govern the physical stability of

liposomes during storage, indicating the importance of liposome composition for the placental extract formulation.

## Materials and Methods

### Materials

l- $\alpha$ -Egg phosphatidylglycerol (PG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), l- $\alpha$ -egg phosphatidylcholine (PC) and cholesterol (Chol) were purchased from Avani Polar Lipids. Inc. (Birmingham, AL, USA). Porcine placenta extracts (PPE) was kindly provided from Dr. Jeong Ho Kim (Seogang University, Seoul, South Korea).

### Liposome Preparation

To prepare liposomes of different surface charges, lipids were dissolved in chloroform with various compositions. The compositions of liposomes were PC/Chol (1/1, mole/mole) for neutral, DOTAP/DOPE (1/1, mole/mole) for cationic, and PG/PC/Chol (1/1/1, mole/mole) for anionic liposomes, respectively. The lipid mixtures were placed in a rotary evaporator to remove the chloroform and obtain thin lipid films. For the entrapment of PPE into the liposomes, lipid films were hydrated with 1 mL of PPE in phosphate buffered saline solution (PBS, pH 7.4, containing PPE at a concentration of 5 mg/mL). After vortexing, the liposomes were extruded three times through 0.2  $\mu$ m polycarbonate membrane filter using an

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extruder (Northern Lipids, British Columbia, Canada). The PPE-loaded liposomes were passed through PD-10 column to remove the unentrapped PPE.

### Size Measurement of Liposomes

The analysis of size distribution of PPE-loaded liposomes was determined by dynamic light scattering technique. The samples were diluted in PBS and/or 0.1 M sodium phosphate buffer and placed into a four sided cuvette in an ELS-8000 light scattering instrument (Photal, Osaka, Japan). The diameters of the liposomes were measured via He-Ne laser (10 mW) light scattering at an angle of 90° at 25°C. An ELS-8000 software package supplied by the manufacturer was used to analyze the data.

### *In vitro* Release Test

For *in vitro* release study, the PPE-loaded liposomes (1 mL) were added to a dialysis bag having a pore size of 2.4 nm and molecular weight cutoff between 12,000 and 14,000 kD. The dialysis tubing with PPE-loaded liposomes was placed in 5 mL of PBS and maintained at 25°C. At various time intervals of 0.5, 1, 2, 3, 6, 12 and 24 hrs, 200 µL of the sample was collected. The amounts of PPE in each sample were measured by bicinchoninic acid assay (BCA) method. An aliquot (150 µL) of the samples were added to 100 µL of BCA solution. The samples were mixed while shaking at 300 rpm at 37°C for 30 min. The optical density values were measured at 570 nm using a spectrophotometer Zenyth 3100 (Anthos Labtec., Salzburg, Austria). The calibration curves of protein concentrations were established using bovine serum albumin (BSA) solutions.

### Liposome Stability Test

To examine the stability of liposomes by temperature, pH, and light conditions, the PPE-loaded liposomes (1 mL) were divided into three aliquots and incubated at different conditions. To evaluate the thermal stability of liposomes, aliquots were incubated at 4 and 25°C for 7 days, respectively. For examination of pH stability, liposomes aliquots were stored at 0.1 M sodium phosphate buffer with different pH values (pH 5, 6, 7, and 8) at room temperature for 7 days. Furthermore, aliquots of liposomes were placed at with or without light conditions for 7 days to evaluate the light stability of liposomes at room temperature.

### Statistic Analysis

Statistical analysis of data was performed using ANOVA, with the Student-Newman-Keuls test employed as a post-hoc

test. SigmaStat software (version 3.5, Systat Software, Richmond, CA, USA) was used for all analyses. A *p* value of less than 0.05 was considered significant.

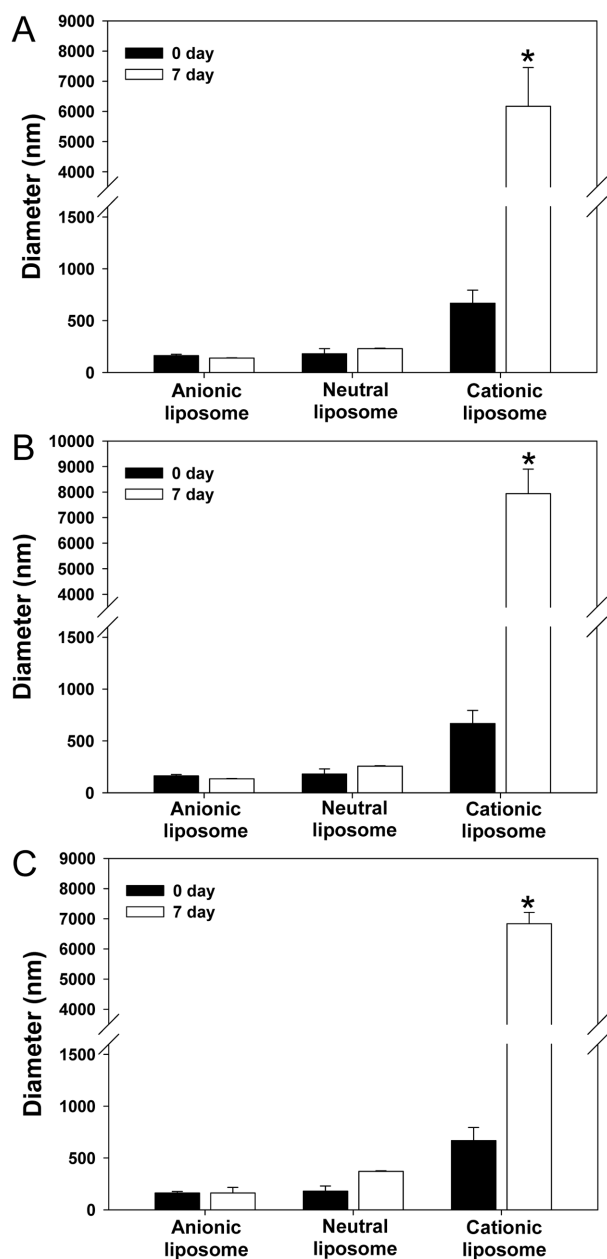
## Results and Discussions

### Effect of Temperatures and Light on the Stability of PPE-loaded Liposomes

The storage temperature of liposomes affected the stability of PPE-loaded liposomes in composition-dependent manner. The effect of storage temperatures on the stability of different surface charged PPE loaded-liposomes was examined. Anionic and neutral surface charges of PPE loaded-liposome did not show significant differences in size distribution patterns after 7-day storage at 4 (Fig. 1A) and 25°C (Fig. 1B). The mean size of anionic PPE-loaded liposomes was 162.5±15.7 nm on the preparation day. At 7 days after storage at 4°C, the mean size of anionic liposomes was 142.3±5.9 nm. The mean sizes of neutral liposomes ranged from 180 to 230 nm upon storage at 4 and 25°C for 7 days. In contrast, the mean sizes of cationic PPE-loaded liposomes increased 8.9- and 11.8-fold after storage at 4 and 25°C for 7 days, respectively. Similarly, 10.2-fold increase was observed in the mean size of cationic PPE-loaded liposomes after storage under dark condition at 25°C for 7 days (Fig. 1C).

The instability of cationic PPE-loaded liposomes during storage at light (Fig. 1B) and dark (Fig. 1C) conditions suggest that the instability of the liposomes might be not due to the light. Rather, it is more probable that the instability of the cationic PPE-loaded liposomes might be caused by the interaction of the components of PPE with cationic moieties of liposomes. In this study, all the liposomes were extruded using a polycarbonate filter of 200 nm. Notably, the cationic PPE-loaded liposomes showed higher mean sizes than neutral and anionic liposomes from the preparation day. Given the higher mean size of cationic PPE liposomes on day 0, we can't exclude the possibility that the components of PPE interacting with cationic parts may be involved in the more than 10-fold of abrupt increase at 25°C for 7 days. It is unlikely that empty cationic liposome per se is unstable as compared to the anionic and neutral liposomes. In case of liposomes containing other chemical drug, temoporin, cationic liposomes have been reported to show the higher stability than anionic liposomes. (Dragicevic-Curic et al., 2010). Moreover, we observed that empty cationic liposomes did not show significant increase in sizes even after 7 day storage (data not shown).

The liposomal formulations have been optimized for the properties of bioactive substances. In this study, we aimed to



**Figure 1.** Effects of temperature and light on the mean sizes of PPE-loaded liposomes.

The mean sizes of various PPE-loaded liposomes were measured at day 0 and day 7 after storage at 4°C (A), 25°C (B), and 25°C (C) under dark conditions. The pH of liposomal solution was fixed as 7.4. The results are expressed as the mean±SD (n=3). \*: significantly different from anionic and neutral liposomes (ANOVA and Student-Newman-Keuls test,  $p < 0.05$ ).

figure out the optimal formulations for liposomes entrapping PPE. Due to the nature of complex components of PPE, we speculated that the charges of liposomal formulations may influence the stability of liposomes in different pHs and during storage periods. Indeed, we observed that cationic liposomes

may not be suitable formulations for PPE in terms of physical stability due to the huge increase of sizes during storage.

Seven days are not sufficient time to test the stability of formulations. However, we think that this period can be used to screen out the most unstable formulations in relatively short periods. Indeed, we figured out that cationic liposomal formulations may have stability problems even after 7 day storage. Longer period of storage test should be done further in the near future.

#### Effect of pH on the Stability of PPE-loaded Liposomes

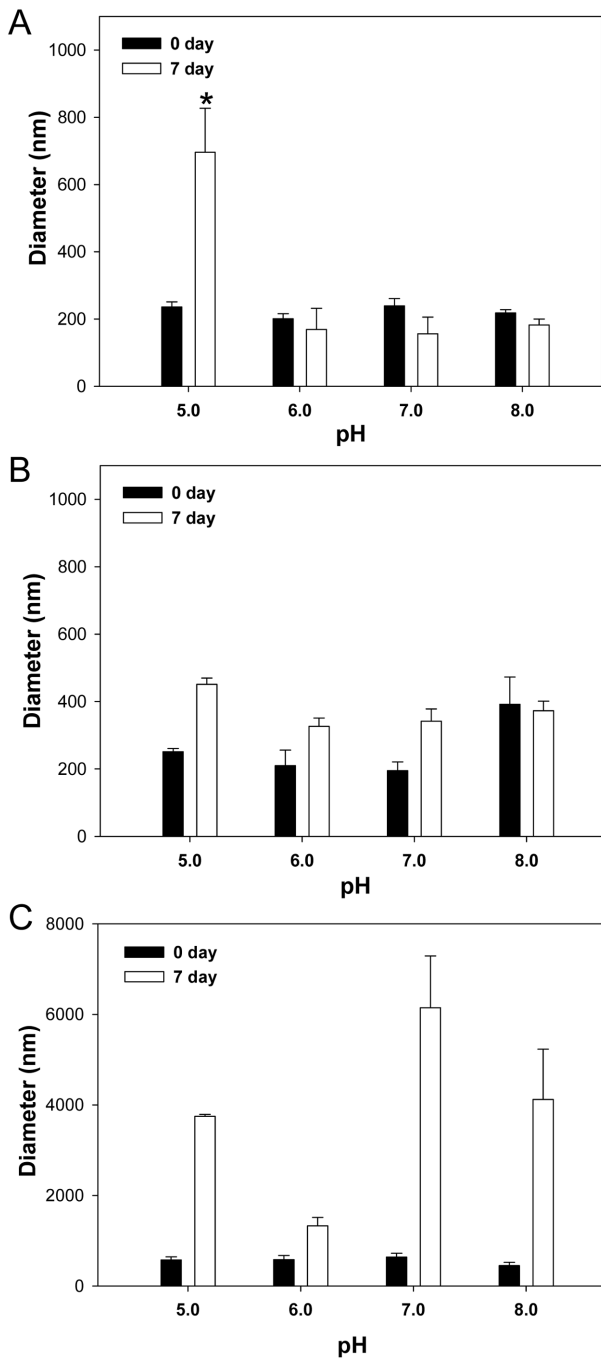
Although the anionic and neutral liposomes did not show significant differences in mean sizes upon storage at different temperature and light conditions, the anionic and neutral liposomes showed the increases in the mean sizes at acidic pH conditions. At pH 5, the mean sizes of anionic liposomes increased from  $236 \pm 14.8$  nm at day 0 to  $696 \pm 131.4$  nm after storage at pH 5 for 7 days (Fig. 2A). In neutral liposomes, the mean size was the highest after storage at pH 5 for 7 days (Fig. 2B). However, at other pH conditions the increase of the liposomal mean sizes was not significant or minimal for both anionic (Fig. 2A) and neutral (Fig. 2B) liposomes.

Meanwhile, the instability of cationic PPE-loaded liposomes was consistently observed at all the pH conditions tested in this study (Fig. 2C). The mean sizes of cationic PPE-loaded liposomes were the highest after storage at pH 7.0 for 7 days, showing 9.6-fold increase. The lowest change in the mean sizes of cationic liposomes was observed after storage at pH 6.0.

#### *In vitro* Release Profile of PPE from Liposomes

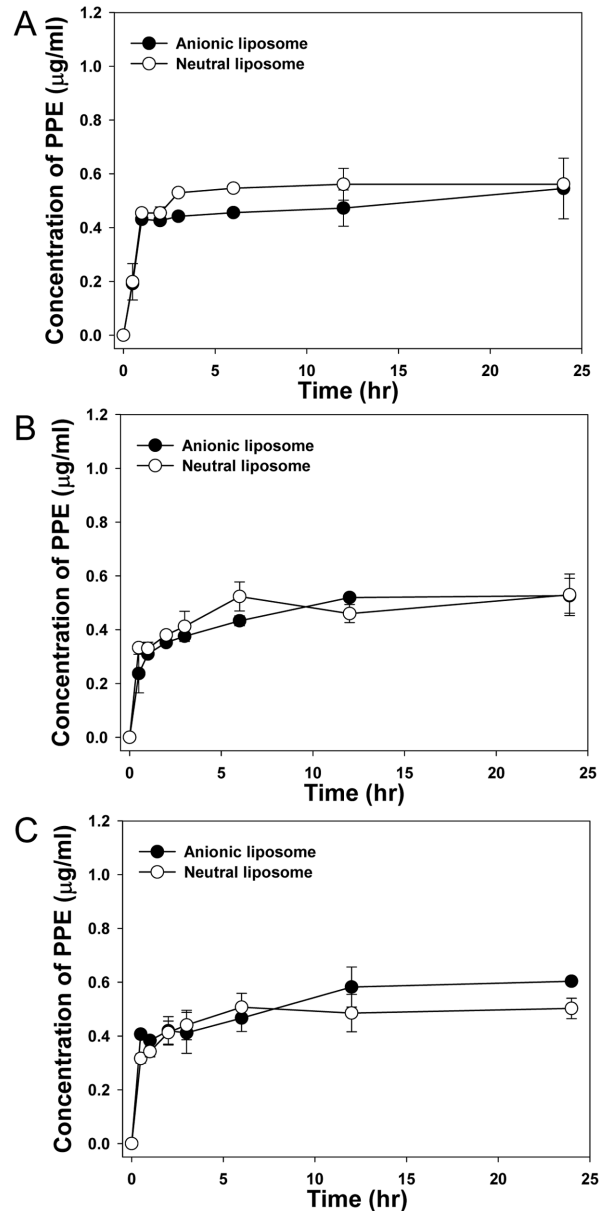
Since the cationic PPE-loaded liposomes showed substantial instability during storage, we tested the *in vitro* release profile of PPE only from neutral and anionic liposomes stored at different temperatures. To test whether anionic and neutral liposomes show different patterns of PPE release regardless of stability, we first measured the *in vitro* profile of PPE from those two types of freshly prepared liposomes. Fig. 3A shows that there was no significant difference in the release pattern of PPE from neutral and anionic liposomes at day 0. After storage for 7 days at 4°C, anionic liposomes revealed similar patterns of PPE release to neutral liposomes (Fig. 3B). Even after the storage at 25°C, anionic and neutral liposomes exhibited the similar rates of PPE release (Fig. 3C).

The pH of storage conditions affected the release of PPE from anionic liposomes. When anionic PPE-loaded liposomes were stored at pH values of 6 or 7, the release patterns of PPE



**Figure 2.** Effect of pH on the mean sizes of PPE-loaded liposomes. The mean sizes of PPE-loaded anionic (A), neutral (B), and cationic (C) liposomes were measured at day 0 and day 7 after storage at various pH conditions. All the liposomes were stored at 25°C. The results are expressed as the mean±SD (n=3). \*: significantly different other pH conditions (ANOVA and Student-Newman-Keuls test, p<0.05).

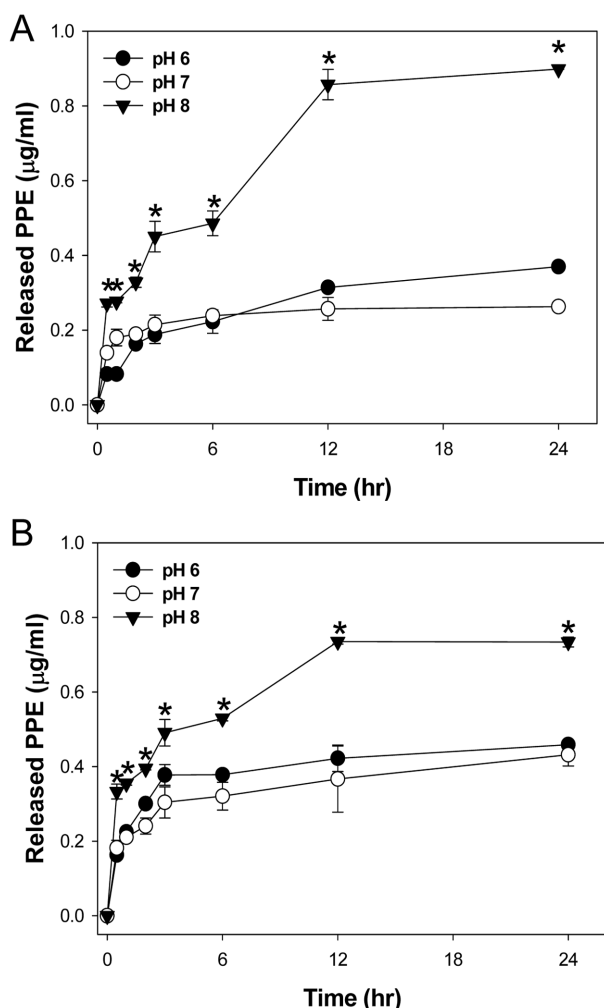
did not differ. The amounts of PPE from anionic liposomes were higher after storage at pH 8 than at other pH values (Fig. 4A). Similar to anionic liposomes, neutral liposomes showed



**Figure 3.** *In vitro* release of PPE from liposomes stored at various temperature and light conditions. The *in vitro* release of PPE from liposomes was measured at day 0 (A) and 7 after storage at 4°C (B) and 25°C (C). The pH of liposomal solution was fixed as 7.4. The results are expressed as the mean±SD (n=3).

the highest release of PPE after storage at pH 8 (Fig. 4B). Given that the major components of PPE extracts are considered to be peptides and amino acids, the charges of major PPE components may have affected the stability and release behaviors from liposomal formulations.

Although the mean sizes of neutral and anionic liposomes did not significantly change (Fig. 2), we observed the highest increase of PPE release after storage at pH 8 (Fig. 4). The dis-



**Figure 4.** *In vitro* release of PPE from liposomes stored at various pH conditions.

The *in vitro* release of PPE from anionic (A) and neutral (B) liposomes was measured at day 7 after storage at various pH conditions. All the liposomes were stored at 25°C. The results are expressed as the mean±SD (n=3). \*: significantly different other pH conditions (ANOVA and Student-Newman-Keuls test,  $p < 0.05$ ).

crepancy between the similar mean sizes and different release pattern might be partly due to the chemical instability of PPE-loaded liposomal membranes stored at pH 8.

Our study on the stability of PPE-loaded liposomes at various pH conditions might be applied to the design of topical gels containing PPE-loaded liposomes. Previously, chlorophylline-loaded liposomes or temoporin-loaded liposomes have been formulated in carbomer-based hydrophilic gels for topical delivery. (Dragicevic-Curic et al., 2009; Dragicevic-Curic et al., 2005). Our observation on the physical stability of liposomes implies that pH ranges of 6-7 would be proper for the gel base of neutral or anionic PPE-loaded liposomes.

## Conclusion

The physical stability of PPE-loaded liposomes was significantly affected by the surface charges of the liposomes. Unlike anionic and neutral liposomes, cationic PPE-loaded liposomes formed large aggregates upon storage at various temperatures. The size distribution and *in vitro* release study revealed that neutral liposomes composed of PC/Chol and negatively charged PG/PC/Chol-based liposomes might be suitable formulations for prolonged storage of PPE. Moreover, this study indicates that the surface charges can play an important role for determining the physical stability of PPE-loaded liposomes.

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