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Chitosan-coated liposomes: characterization and interaction with leuprolide

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Abstract

The objective of the present work was to investigate the effect of chitosan concentration and lipid type on the characteristics of chitosan-coated liposomes and their interactions with leuprolide. Liposomes from lipid of high purity and low purity were prepared and coated by chitosan. Physical properties, drug entrapment efficiency, and stability upon dilution were respectively compared. Results showed that the particle size increment of liposomes from low purity lipid was larger than that from high purity lipid, indicating a thicker coating layer. The high zeta potential of particles from low purity lipid was thought to play an important role in the resistance to flocculation. As to particles from high purity lipid, polymer bridging caused flocculation at low polymer concentration while at high concentration, the adsorbed chitosan molecule led to steric stabilization. Drug entrapment efficiency decreased as chitosan was added to liposomes, showing the disturbance of bilayers. Upon dilution, the leakage of leuprolide from low purity liposomes was larger than that from high purity liposomes. In conclusion, low purity lipid possessed more negative charge and formed thicker adsorptive layer by stronger electrostatic attraction with chitosan. The interaction between chitosan and the polar head groups on the surface of phospholipid bilayers may interfere with leuprolide entrapped in liposomes and result in the leakage of leuprolide.

Keywords: Chitosan-coated liposomes; Leuprolide; Characterization; Flocculation; Interaction

1. Introduction

Most of the peptide and protein drugs can be administered only by injection due to their instability in the gastrointestinal (GI) tract and poor absorption. Particulate systems, such as liposomes, micro- or nanoparticles, have attracted a great deal of attention as possible oral dosage forms for such peptide drugs (Damge et al., 1995). Among these, liposomes possess the advantages that they are composed of physiological materials, e.g. phospholipid (Fukunaga et al., 1991). However, liposomes are liable to be destructed by the pH, bile salts, and pancreatic lipase in the GI tract (Kato et al., 1993). To minimize the disruptive influences, the formation of a polymeric membrane around the liposome has been studied (Iwanaga et al., 1999). Since the discovery of polysaccharides on cell surfaces and the high affinity of chitosan to cell membranes, several investigators have utilized chitosan derivatives as coating materials for liposome (Janes et al., 2001).

Chitosan is a hydrophilic, biocompatible, and biodegradable polymer of low toxicity. Several studies have highlighted the potential use of chitosan as an absorption-enhancing agent. Moreover, because

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of its bioadhesive properties, chitosan has also received substantial attention in novel bioadhesive drug delivery systems (Filipovic-grcic et al., 2001).

By combining chitosan and liposomal characteristics, specific, prolonged, and controlled release may be achieved (Takeuchi et al., 1996). Takeuchi et al. showed that the chitosan-coated liposomes were formed via ionic interaction between the positively charged chitosan and negatively charged diacetyl phosphate on the surface of the liposomes. However, the authors do not show the hydrogen bonding and hydrophobic interaction between chitosan and neutral lipid.

In order to compare the effect of the electrostatic interaction with that of the hydrophobic interaction, we chose lipid of high purity (hydrophobic interaction dominates) and lipid of low purity (electrostatic interaction dominates) to prepare liposomes. We tried to reveal the effect of lipid type and chitosan concentration on the characteristics of chitosan-coated liposomes and their interactions with leuprolide. Furthermore, in the preliminary study, we found liposomes from low purity lipid formed thicker coating layer than those from high purity lipid while delivered less leuprolide to systemic circulation after intragastric administration to rat (unpublished data). Therefore, it is necessary to compare the physicochemical properties of chitosan-coated liposomes from different lipids. The model drug leuprolide acetate is a synthetic superpotent agonist of luteinizing hormone releasing hormone (LHRH) receptor. It is a nonapeptide chemically defined as 5-Oxo-Pro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-ProNHEt acetate with a molecular weight of 1209. Like most other peptide or protein drugs, leuprolide has a very low oral bioavailability.

2. Materials and methods

2.1. Materials

Epikuron 170 (composed of phosphatidylcholine (min. 70%), phosphatidylethanolamine (min. 10%), phosphatidylinositol (max. 3%), phosphatidic acid (max. 3%), lyso-phosphatidylcholine (max. 4%), and fatty acids approximately 10%) and Epikuron 200 (composed of phosphatidylcholine (min. 92%), lyso-phosphatidylcholine (max. 3%), other phospholipids (max. 2%), and fatty acid approximately 1%) were purchased from Lucas Meyer (Hamburg, Germany). Leuprolide acetate (lot number: 011015) was obtained from Lizhu Pharmaceutical Factory (China). Cholesterol was from Sigma. Chitosan (1000 kDa, >90% deacetylation, 2.45 cps for a 0.1% solution at 25 °C) was supplied by Jiangshu Xinxing Pharmaceutical Factory (China). Methanol, chloroform, sodium chloride, and other reagents, all p.a., were products of Nanjing Chemical Corporation (China).

2.2. Preparation of chitosan-coated liposomes

Liposomes were prepared from lipids of different purity to compare the effect of lipid composition. Conventional rotary evaporation–sonication method was developed. Appropriate amounts of lecithin (600 mg) and leuprolide (5 mg) were dissolved in co-solvent of methanol and chloroform (1:1). Cholestrol (100 mg) was also added. The mixture was dried to a thin film under vacuum. The film was then hydrated with phosphate buffer (0.1 M, pH 5.0) to make a 10 ml of lipid coarse suspension. Sonication was carried out at 4 °C (JY 92-II ultrasonic processor, China) to obtain small liposomes.

For the preparation of chitosan-coated liposomes, chitosan of various concentrations (0.1, 0.2, 0.5, and 1%) was added dropwise to liposomes of equal volume under magnetic stirring at room temperature (20 °C) for 2 h.

2.3. Characterization of chitosan-coated liposomes

Particle size and zeta potential of chitosan-coated liposomes were measured by a Zetamaster 3000H instrument (Malvern Instruments, Malvern, UK).

Samples for transmission electron microscopy (TEM) were prepared at room temperature by conventional negative staining methods using 0.3% phosphotungstic acid buffer (pH 6). Samples were viewed on an H-7000 model transmission electron microscope (Hitachi, Japan).

2.4. Content and entrapment efficiency of drug

Concentration of leuprolide was determined by HPLC. The HPLC system consisted of a pump (Model LC-10A, Shimadzu, Japan), a shim-pack CLC-ODS

column (150 mm × 6 mm i.d., Shimadzu) maintained at 25 °C, an UV detector (Model SPD-10A, Shimadzu) at 220 nm, and a data station (Model SCL-10A, Shimadzu). The composition of the mobile phase was acetonitrile and 0.1% trifluoroacetic acid (30:70). The mobile phase was delivered at a flow rate of 1 ml/min. The injection volume was 20 μl and the relative retention time was found to be 11.0 min.

Total drug content of suspensions was determined by dissolving chitosan-coated liposomes in methanol and measured by HPLC.

The entrapment efficiencies of the various liposomes were determined by ultracentrifugation at 50,000 × *g* for 5 h. The free drug in the supernatant was detected by HPLC. Total drug content of the suspensions was also determined. The entrapment efficiency was calculated as the ratio of drug content within the liposomes to the total drug content of the suspension. The drug content within the liposomes was calculated as the total drug content of the suspension minus the free drug.

2.5. Determination of sedimentation volume

The chitosan-coated liposomes were transferred to a 5-ml measuring cylinder incubated at 20 °C. The sediment volume was recorded at 1 day after preparation. The value of the sedimentation volume (*F*) was calculated from the equation:

$$F = \frac{V_u}{V_0}$$

where V_u is the volume of sedimented solid at fixed time and V_0 is the total volume of the suspension.

2.6. Coating efficiency of liposomes

The coating efficiency of liposomes by chitosan was determined as the phosphor content within the chitosan-coated liposomes versus the total phosphor content of liposomes. The chitosan-coated liposomes were subjected to centrifugation at 3000 rpm for 10 min until the sedimentation of coated liposomes. Then, chloroform (2 ml) was added to solubilize the precipitate and the phosphor content was subsequently determined by Stewart's method (Stewart, 1974). The total content of phosphor was determined by the same method without centrifugation.

2.7. Leuprolide leakage from liposomes upon dilution

The changes of entrapment efficiencies upon dilution in HCl solution (0.1 M, pH 1.2) and phosphate buffer (30 mM, pH 6.9) were recorded. Leuprolide release was evaluated by incubating the various liposome suspensions (1 ml) in 9 ml of media for 2 h at 37 °C. After incubation, the suspensions were subjected to ultracentrifugation at 50,000 × *g* for 5 h. The free drug in the supernatant was detected by HPLC.

3. Results and discussion

3.1. Morphological shape

Liposomes were spherical particles while the existence of polymer layers surrounding the liposomes was well visualized on the surface of chitosan-coated liposomes (Fig. 1).

3.2. Particle size

Liposomes obtained from lipid of low purity (Epikuron 170) were transparent colloidal dispersions with a mean diameter of 15 nm, while the mean diameter of liposomes from high purity lipid (Epikuron 200) was 54 nm. Epikuron 170 was composed of low phosphatidylcholine content (>70%) and high fatty acid content (approximately 10%). Fatty acid increased the flexibility of liposomes, making the large liposomes easier to break into smaller ones (Cevc et al., 1998). As stated in Fig. 2, the particle size of chitosan-coated liposomes in each formulation was increased with the increasing concentration of polymer solution, suggesting the formation of coating layer on the surface of the liposomes. Liposomes obtained from low purity lipid adsorbed more chitosan than those from high purity liposomes. As to low purity lipid, the mean size of chitosan (0.05%)-coated liposomes was four times more than that of the free liposomes.

3.3. Zeta potential

The zeta potential of liposomes from Epikuron 170 was negative (−29.6 mV), while that from Epikuron 200 was positive (+5 mV). Components of phos-

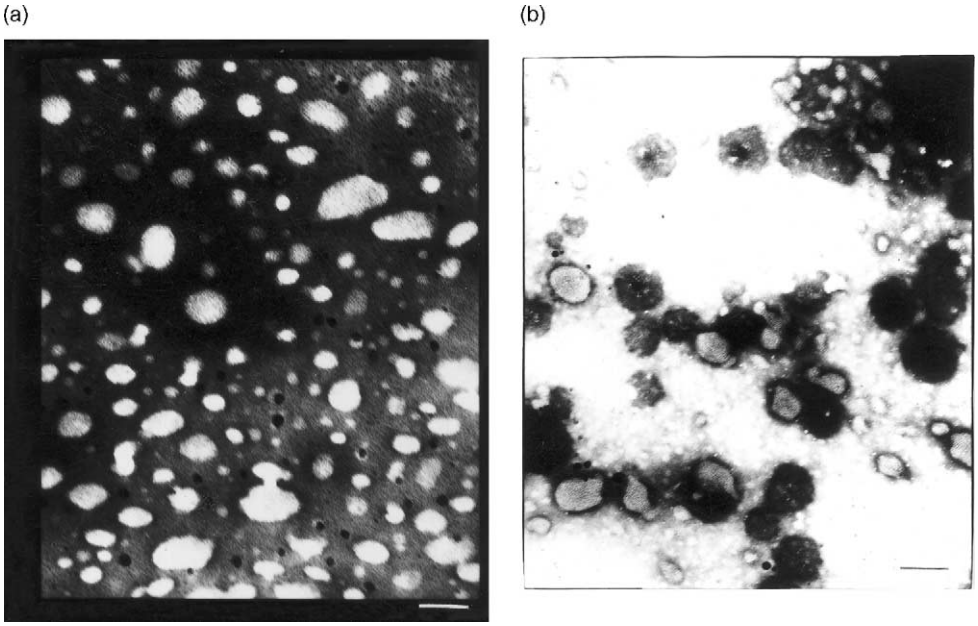

Fig. 1. Transmission electron micrographs: (a) liposomes; (b) chitosan-coated liposomes. Bar is 100 nm.

phatidylinositol (max. 3%), phosphatidic acid (max. 3%), and fatty acids (approximately 10%) in Epikuron 170 contributed to the negative zeta potential of liposomes. Therefore, the main interaction between Epikuron 170 and chitosan was electrostatic attraction. Epikuron 200 contained only approximately 1% fatty acids and zeta potential was influenced by the kinds of buffer used. Epikuron 200 was nearly neutral and the mechanism of coating neutral phosphatidylcholine liposomes by chitosan probably involved hydrogen bonding between the polysaccharide and the phospholipid head groups (Perugini et al., 2000).

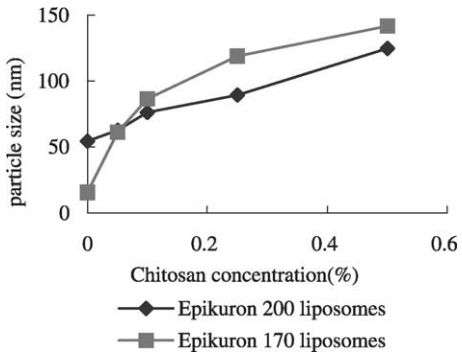

Fig. 2. The influence of chitosan concentration on sizes of chitosan-coated liposomes. The results are the mean of three experiments.

All the zeta potentials of chitosan-coated liposomes were positive. Since chitosan carried high positive charge, the adsorption of chitosan increased the density of positive charge and made the zeta potential positive. Chitosan-coated liposomes from Epikuron 170 adsorbed more chitosan and possessed higher zeta potential. The zeta potential increased as the chitosan concentration increased to 0.25%, then it came to a relatively constant value (Fig. 3).

3.4. Sedimentation volume

The sedimentation volumes of all chitosan-coated liposomes from Epikuron 170 were zero (Fig. 4). Since all such liposomes possessed high positive zeta potential (from 9.1 to 43.9 mV), the zeta potential of particles is thought to play an important role in the resistance to flocculation and coagulation. As to liposomes from Epikuron 200, the zeta potentials were small at low polymer concentrations. Thus, polymer bridge resulted in flocculation. At relatively high polymer concentrations (>0.1%), surface coverage by

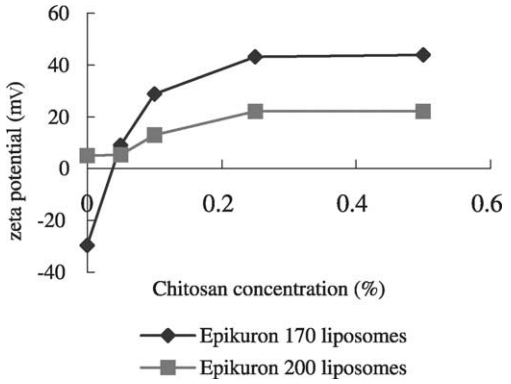

Fig. 3. The influence of chitosan concentration on zeta potential of chitosan-coated liposomes. The results are the mean of three experiments.

the adsorbed polymer is sufficiently high to prevent polymer bridge flocculation. The structured adsorbed polymer film now serves to stabilize the particles against particle–particle interaction presumably by the mechanism of steric stabilization (Kellaway and Najib, 1981). On the other hand, the thickness of adsorptive layer is equally important. When the concentration of chitosan was 0.05%, the zeta potential of Epikuron 170 liposomes was 9.1 mV, similar to that of Epikuron 200 liposomes (5.4 mV). However, the adsorptive layer in the former was 45.6 nm, much larger than the latter (8.2 nm). The thick adsorptive layer on the liposomes kept the particles apart and led to stabilization.

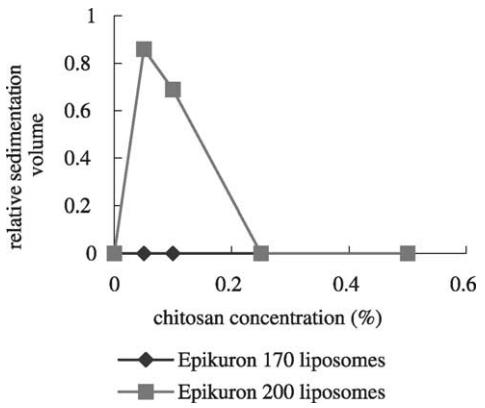

Fig. 4. The influence of chitosan concentration on sedimentation volume of liposomes. The results are the mean of three experiments.

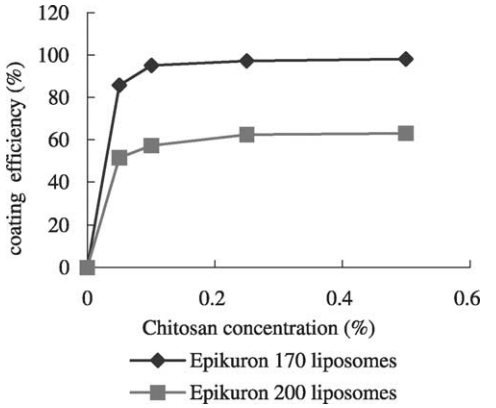

Fig. 5. The influence of chitosan concentration on coating efficiency of chitosan-coated liposomes. The results are the mean of three experiments.

3.5. Coating efficiency of liposomes

Fig. 5 showed coating efficiency of liposomes by chitosan increased as the concentration of chitosan increased from 0.05 to 0.1%. Then, it came to a saturated state. Coating efficiency was 98 and 63% with respect to Epikuron 170 and 200. Epikuron 170 liposomes attracted more chitosan than Epikuron 200 liposomes.

3.6. Leuprolide entrapment efficiency

The leuprolide entrapment efficiency was 73.16 and 58.59% with respect to Epikuron 170 and 200. However, the addition of chitosan decreased the entrapment efficiency to 62.48 and 49.19%, respectively. Leuprolide possessed positive charge in the normal buffer (pI = 10.5), and it has been reported that the extent of interaction of positively charged peptides with lipid membranes depends both on electrostatic attraction at the head group level and the apolar part of the membrane (Lo and Rahman, 1995). Epikuron 170 carried more negative charge and possessed stronger interaction with leuprolide by electrostatic attraction. Therefore, it had higher entrapment efficiency. On the other hand, chitosan has strong affinity for the phospholipid. Since leuprolide and chitosan both bear positive charge, they compete with each other for the affinity to phospholipid, which subsequently decreased the leuprolide entrapment efficiency. As the concentration of chitosan increased from 0.1%, there was no more

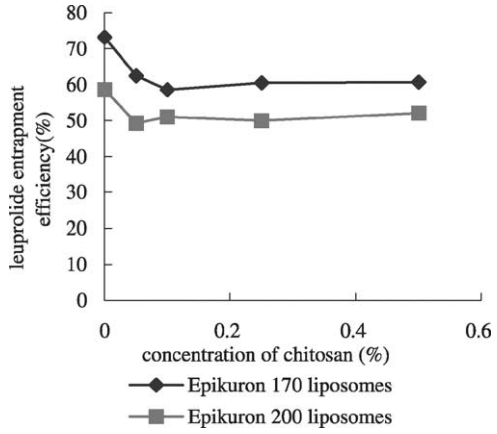

Fig. 6. The effect of chitosan concentration on the entrapment efficiency of leuprolide. The results are the mean of three experiments.

leuprolide released (Fig. 6). Since adsorptive layer has already formed, chitosans could only surround from outside and were unable to interfere with the bilayer.

3.7. Leakage in HCl solution and phosphate buffer

From Fig. 7, there was a little leakage of leuprolide from Epikuron 200 liposomes upon dilution in HCl solution and phosphate buffer no matter whether the liposomes were coated by chitosan. However, the leakage from Epikuron 170 liposomes was much larger. The electrostatic interaction between leuprolide and Epikuron 170 is labile to the changes of pH and buffer.

4. Conclusion

Both chitosan and leuprolide may interact with the polar head groups on the surface of phospholipid bilayers via electrostatic attraction and hydrophobic interaction. Such interactions were influenced by the lipids of different purity. A stronger electrostatic attraction existing between leuprolide and lipid of low purity led to higher entrapment efficiency. In the mean time, chitosan coating possibly formed by electrostatic interaction between chitosan and lipid resulted in particle size increment and more positive zeta potential, and these changes were more substantial in case of low purity lipid. However, a too thick coating of chitosan may prevent the delivery of leuprolide to the intestinal mucosa and reduce the absorption (unpublished data). Hydrophobic forces dominated the interactions between chitosan, leuprolide, and lipid of high purity and, therefore, such an interaction is more stable and affected negligibly by the changes of pH and buffer upon dilution. The medium extent of coating and stable interaction may contribute to the better in vivo effect of liposomes from high purity lipid.

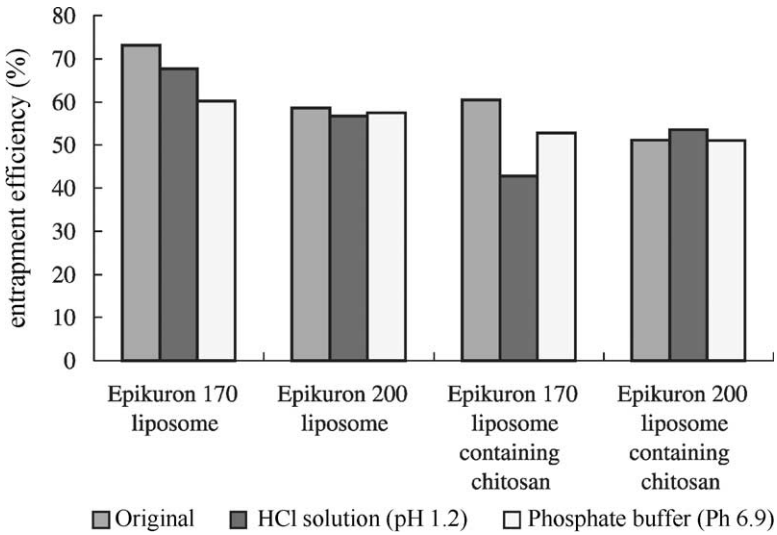

Fig. 7. The leakage of leuprolide from liposomes upon dilution in HCl solution (pH 1.2) and phosphate buffer (pH 6.9). The results are the mean of three experiments.

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