Preparation and Characterization of Liposomes Encapsulating Chitosan Nanoparticles

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Liposomes are an important colloidal carrier system for controlled drug delivery. However some highly hydrophilic small molecules are difficult to entrap into liposomes and store stably, resulting in poor encapsulation efficiency and fast leakage. In the present work, fluorescein sodium (FS) was used as a model drug that was loaded into chitosan nanoparticles and then encapsulated into liposomes by reverse-phase evaporation (RPV). The encapsulation efficiency, particle size, zeta potential, release *in vitro* and pharmacokinetics in rats were determined in order to characterize the novel drug delivery system. The entrapment efficiency was above 80% in nanoparticles (Np) and 95% in liposomes encapsulating the nanoparticles (Lip-Np). The Lip-Np was composed of soybean phospholipids, cholesterol and chitosan, which the average diameter was 202.6 nm and zeta potential was −34.8 mV. The release rate of fluorescein sodium from Lip-Np was slower than from Np and liposomes. FS in Lip-Np administered to rats exhibited prolonged circulation and higher bioavailability than FS in Np. The results indicated that liposomal release kinetics can be controlled by encapsulating nanoparticles and thus solid-cored liposomes can be used as a potential drug delivery system.

Key words liposome; chitosan nanoparticle; solid core; reverse-phase evaporation; prolonged release; pharmacokinetics

Liposomes are one of the most widely used drug delivery systems, having been investigated for delivery of chemotherapeutic agents for cancer,[1] vaccines for immunological protection,[2] radiopharmaceuticals for diagnostic imaging,[3] and nucleic acid-based medicines for gene therapy.[4] As is observed for colloidal drug delivery systems, however, the physico-chemical stability of liposomes varies with their compositions and structures.[5] In most cases liposomes tend to aggregate and fuse with or leak entrapped drugs, especially highly hydrophilic small molecules. Likewise, during storage, the phase state transition of lipids induces the release of their content. We supposed that if the intraliposomal segment was solid instead of liquid, leakage from the liposome would decrease dramatically for drug must be released from the solid-core before diffusing across the lipid membrane. Additionally, the solid core could facilitate controlled or sustained-release.

In the present study, chitosan and the oppositely charged, highly hydrophilic small molecule, fluorescein sodium (FS) were selected to form the solid cores, which were encapsulated in liposomes. Preparation, size and zeta potential of this nanocombination were investigated and the biopharmaceutical applicability and pharmacokinetics of this system was assessed *in vitro* and in rats.

MATERIALS AND METHODS

Materials and Animals Soybean phospholipids (SPL) (Lipoid GmbH, Germany), cholesterol (Sigma, U.S.A.), fluorescein sodium (Shanghai SSS Reagent Co., Ltd., China), and chitosan (MW 60 kDa, Yuhuan Biochemistry Co., Ltd., China) were purchased from the indicated manufacturers. All other chemicals were of analytical grade. Sprague–Dawley rats (male, weighting 250±20 g) were purchased from Laboratory Animal Center of Zhejiang University.

Preparation of FS Chitosan Nanoparticles (FS-Np) FS-Np were prepared *via* a modification of the ionic cross-linking technique.[6] FS was dispersed in deionized water and added to an acetic acid solution containing chitosan, while stirring. We tested several chitosan/FS ratios: 1.6, 1, 0.8 (w/w). Sodium tripolyphosphate (TPP) (0.1%, w/v) was then added to the above solution, while stirring. After 30 min, the cross-linking solution containing FS-Np was obtained. Then this dispersions were filtrated through 0.45 μm filter membrane after sonicated by ultrasound probe (400w).

Preparation of Liposome-Encapsulating Chitosan Nanoparticle (Lip-Np) Lip-Np was prepared by RPV method as follows. A lipid mixture of SPL–cholesterol (2 : 1, w/w) was dissolved in hexane–chloroform (1 : 1, w/w) in a pear-shaped flask. The FS-Np suspension was added to the lipid solution to form an emulsion. The flask was then attached to a rotary evaporator to dry the organic solvent under vacuum, and the liposomal suspension was obtained with the final lipid concentration of 30 mg/ml. Sample was extruded through 0.45 μm filter menbrance after sonicated by ultrasound probe (400w). FS-liposomes with the final lipid concentration of 50 mg/ml were prepared using the same method. Then ultracentrifugation (80000×g, 1 h) was used to separate the free drug and FS-liposomes. The FS concentration of liposomes was determined before used for *in vitro* and *in vivo* experiments.

Encapsulation Efficiency Analysis A Microcon centrifugal filter device containing an ultrafiltration membranes (MWCO 50,000, Millipore, U.S.A.) was used to determine encapsulation efficiency. An aliquot of the above suspension was added to the sample reservoir and centrifuged for 10 min at 12000×g. The filtrate was assayed to determine the concentration of the unencapsulated drug. Another aliquot of the suspension was added to methanol (1 : 10, v/v) and sonicated for 10 min in a bath sonicator to release the encapsulated molecules, which were quantitated to determine the total drug concentration. Encapsulation efficiency (%) was calculated by the following formula:

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encapsulation efficiency (%)=[1−(unencapsulated drug/total drug)]×100

Zeta Potential and Particle Size Measurements Zeta potential and particle sizes of Lip-Np, FS-Np, liposomes, and mixture thereof were determined by laser diffraction spectrometry using the Malvern Zetasizer 3000HS (Malvern, U.K.).

***In Vitro* Release of Drug** The release trial was performed in a modified Franz vertical diffusion assembly (Shanghai KaiKai Science & Technology Company, China). The dialysis membranes (MWCO 14 kDa) were mounted between the receptor and donor compartments. The available area of the modified Franz cell was 5 cm^2. The contents were stirred at 300 rpm and maintained at 37 °C. At appropriate intervals, all of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution (PBS). The accumulation of released drug was calculated.

Pharmacokinetics in Rats The pharmacokinetics were evaluated in male Sprague–Dawley rats weighting 250±20 g. Rats were anaesthetized by an i.p. injection of 40 mg/kg body weight of sodium pentobarbital. The Lip-Np, FS-Np suspension and FS aqueous solution were introduced intravenously at a dose of 1.5 mg/kg. Blood samples (0.45 ml) were collected from the left femoral artery. An equivalent volume of saline was injected into the animals after each blood sample was taken to maintain constant blood volume.

The plasma (200 μl) was acidulated with 20 μl of 1 mol/l hydrochloric acid solution, and 1 ml ethyl acetate was added. The solution was agitated on a vortex mixer for 2 min and centrifuged at 3000×***g*** for 5 min. The supernatant was transferred to a clean tube and dried under nitrogen gas in a 60 °C water bath. The residues were reconstituted into the mobile phase. Aliquots (20 μl) of the extracts were subjected to HPLC (Agilent 1100, U.S.A.) analysis using as the following chromatographic conditions: Diamonsil C_{18} column (150×4.6 mm, 5 μm), flow rate of 1 ml/min, column temperature of 30 °C, mobile phase: Acetonitrile–50 mM KH_2PO_4 adjusted to pH 2.5 with phosphoric acid (55 : 45, v/v) and a detection wavelength of 225 nm.

RESULTS AND DISCUSSION

Preparation of Lip-Np by RPV Several methods have been developed for preparation of Lip-Np including thin-film hydration, ethanol infection and RPV techniques. However, the Lip-Np suspension prepared by the former two techniques was found to be unstable, and depositions were formed a few minutes after completion of the process. A stable and macroscopically homogeneous suspension of Lip-Np was obtained by the RPV method. Because SPL and chitosan are oppositely charged, in the thin-film hydration and ethanol injection methods, SPL is surrounded by the aqueous medium containing FS-Np. These constituents are absorbed together and neutralized, causing particle aggregation and formation of the deposition. In the RPV, a stable w/o emulsion was formed and the FS-Np suspensions were separated from the continuous phase as the aqueous cores. Gradual liposome formation was followed by the evaporation of organic solvent. Thereby agglutination was avoided.

High Encapsulation Efficiency Obtained by Lip-Np Using RPV The encapsulation efficiencies of different ratio of chitosan/FS were near. Because chitosan and FS are oppositely charged, their electrostatic interactions may be advantageous for the formation of chitosan nanoparticles that exhibit high encapsulation efficiencies. Lower encapsulation efficiency was performed in FS-liposomes. As phospholipid concentration is more than 30 mg/ml, multilamellar vesicles (MLV) can be prepared by RPV with high entrapment values near 65% for many aqueous soluble solutes, and even higher for a optimized process.[7] In this study as the lipid concentration increased to 50 mg/ml, the encapsulation efficiency was achieved 67%. The encapsulation efficiency of FS-Np, Lip-Np and FS-liposomes are showed in Table 1.

Characterization by Zeta Potential and Particle Size Measurements The mean particle sizes were 153.5, 139.7 and 576.6 nm for chitosan/FS ratios of 1.6, 1 and 0.8 (w/w), respectively. Therefore, a 1 : 1 ratio of chitosan/FS was selected as the test formula for the smaller particle size could facilitate to entrap into liposomes.

Zeta potential has often been used for characterizing colloidal drug delivery systems,[8] and these measurements facilitate the understanding of the dispersion and aggregation processes.[5] therefore, we studied the electrophoretic behavior of FS-Np, Lip-Np, blank liposomes and mixtures of FS-Np and liposomes by comparing their zeta potential. We also measured particle sizes. The size of Lip-Np was slightly increased but without significant difference to Np. Lipid bilayer was reported that only about 3.9 nm in thickness.[9] Therefore, lipid coating brought out little effect on the vesicles volume. Results from both analyses are shown in Table 2.

The zeta potential of Lip-Np encapsulating FS-Np was higher than the blank liposomes. This difference may be attributed to the influence of the positively charged cores. Charge of the core and the lipid layer may neutralize, which results in the increasing of zeta potential. Mixtures of FS-Np and blank liposomes, oppositely charged, neutralize each other and cause aggregation and fusion. This results in increased particle size and neutralized zeta potential.

Prolonged Release of Drug in Lip-Np *in Vitro* As

Table 1. The Encapsulation Efficiency of FS-Np, Lip-Np and FS-Liposomes (n=3)

Sample	Encapsulation efficiency (%)
FS-Np (chitosan/FS 1.6, w/w)	84.5±0.7
FS-Np (chitosan/FS 1.0, w/w)	86.1±1.9
FS-Np (chitosan/FS 0.8, w/w)	87.9±1.6
Lip-Np	96.2±2.5
FS-liposomes	67.3±3.2

Table 2. Particles Size and Zeta Potential Measurements of FS-Np, Lip-Np, Liposomes and Mixtures of Np and Liposomes

Sample	Size (nm)	Pot Z (mV)
FS-Np	139±30	32.7±0.6
Lip-Np	144±37	−34.8±1.1
Liposomes	145±47	−40.6±6.8
FS-Np+liposomes	1088±212	7.0±1.1

Table 3. Pharmacokinetic Parameters of FS in Rats ($n=4$) after Single i.v. Dose of Different Preparations

Parameter	Lip-Np	FS-Np	FS
A (μg/ml)	4.99±1.06*	3.68±0.8	2.88±1.06
α (min^{-1})	0.030±0.009*	0.045±0.011	0.052±0.02
B (μg/ml)	1.36±0.41	0.90±0.3	0.69±0.24
β (min^{-1})	3.0±0.9	4.0±0.7	4.1±1.1
$T_{1/2}\alpha$ (min)	28.08±9.3*	17.79±5.5	16.17±5.4
$T_{1/2}\beta$ (min)	297.05±58.91	272.71±27.86	262.75±73.46
AUC (μg·min/ml)	677.29±139.18*	329.99±90.55	286.99±108.22
CL (ml/min)	0.51±0.11*	1.03±0.33	1.27±0.50
Vd (ml)	53.08±4.18*	86.31±7.06	92.43±14.79

* $p<0.05$, the parameters with asterisks in Lip-Np column show significant difference comparing with the other two groups.

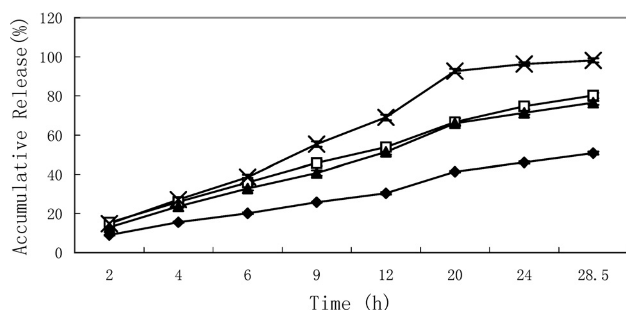

Fig. 1. Release of FS from (a) Lip-Np (◆); (b) FS-Np (▲); (c) FS-liposomes (□) and (d) FS Aqueous Solution (×) ($n=3$)

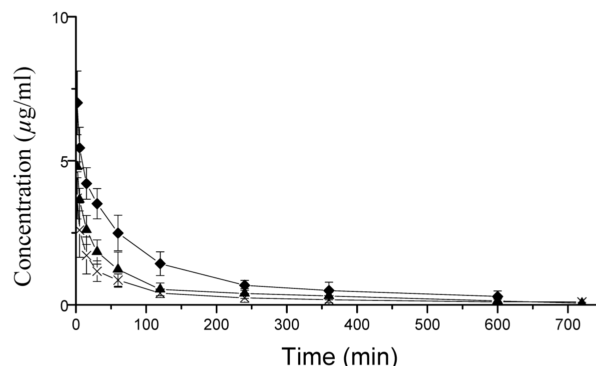

Fig. 2. Concentration–Time Curve in Plasma after i.v. Dosing in Rats ($n=4$) of Lip-Np (◆), FS-Np (▲) and FS Aqueous Solution (×)

showed in Fig. 1, the release of FS from the Lip-Np encapsulating FS-Np was delayed. We determined that the intraliposomal solid core played a key role in the controlled or sustained-release, and the lipid bilayer provided an additional barrier to the release of FS. Diffusion of the drug blocked by a negatively charged nitrocellulose dialysis membrane was found. Free drug needed more than 12 h to dialyse the 80% of the total drug in the FS aqueous solution group. Although the *in vitro* release trial was not completely successful for the blocking effect, the data provide supplementary evidence to our conclusion that liposomal release kinetics can be controlled by encapsulating nanoparticles.

Prolonged Circulation and Higher Bioavailability The FS concentrations in plasma were determined by HPLC. Standard curves ranged from 0.075—12.5 μg/ml and absolute recovery was determined by spiking drug-free plasma with the compound at the following concentration: 0.15, 3.75 and 12.5 μg/ml. The range of absolute recovery was 66.2—69.7% with satisfactory stability. Inter-day and intra-day precision at low, middle and high concentration were below 5.2 and 13.1%.

Pharmacokinetic calculations were performed using the 3p87 program (Chinese Pharmacological Association: Beijing, China). The concentration in plasma after i.v. dosing were analyzed by 3p87 and best fit by two compartments with a weight of 1/C. Plasma *AUC* values were estimated by the statistical moment method. Several of the parameters are listed in Table 3. Statistical analyses were performed using one-sided Student's *t*-test.

The absolute bioavailability of Lip-Np, as indicated by the *AUC*, was substantially improved relative to that for FS-Np and FS. Likewise, Lip-Np exhibited a longer half-life of α. These results can be attributed to the charge variations on the surface of the carriers. Because positively charged particles exhibit a higher tendency to bind opsonin in the blood, they are taken up at a faster rate by cells of the reticuloendothelial system (RES) after systemic administration and eventual clearance by the liver, or spleen.[10] Therefore, the negatively charged surface of the lipid bilayer of Lip-Np reduces interactions with plasma proteins and prolongs its circulation compared to FS-Np (Fig. 2). In addition, the prolonged release of drug from Lip-Np also attributes this result.

CONCLUSIONS

Liposomes encapsulating a solid core exhibit excellent potential both *in vitro* and *in vivo* for drug delivery. The use of an intraliposomal solid core would promote efficient drug loading and control its release. Drug need to be released from the intraliposomal solid-core before diffusing across the lipid bilayer membrane. The drug release to medium must pass through the two barriers. Therefore, there may be alternative ways to controlled release. In addition, the solid-cored liposomes are more stable than the Np or liposomes due to the lower drug leakage. Chitosan used as core material is advantageous to achieve high drug entrapment efficiency due to oppositely charged to lipid layer as shown in this work. The solid-cored liposomes, encapsulating chitosan nanoparticles, that we described in the present study exhibit these attributes and thus show great potential for development as a new drug carrier.

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REFERENCES

1) Harrington K. J., Syrigos K. N., Vile R. G., *J. Pharm. Pharmacol.*, **54**, 1573—1600 (2002).
2) Zho F., Neutra M. R., *Biosci. Rep.*, **22**, 355—369 (2002).
3) Matteucci M. L., Thrall D. E., *Vet. Radiol. Ultrasound*, **41**, 100—107 (2000).
4) Pedroso de Lima M. C., Neves S., Filipe A., Duzgunes N., Simoes S., *Curr. Med. Chem.*, **10**, 1221—1231 (2003).
5) Heurtault B., Saulnier P., Pech B., Proust J. E., Benoit J. P., *Biomaterials*, **24**, 4283—4300 (2003).
6) Shu X. Z., Zhu K. J., *Int. J. Pharm.*, **201**, 51—58 (2000).
7) Pidgeon C., "Preparation of MLV by the Method: Vesicle Structure and Optimum Solute Entrapment," Vol. I, Chap. 6. ed. by Gregoriadia G., CRC Press, FL, U.S.A., 1993, pp. 99—110.
8) Barratt G., *Pharm. Tech. Europe*, **11**, 25—32 (1999).
9) Radler J. O., Koltover I., Salditt T., Safinya C. R., *Science*, **275**, 810—814 (1997).
10) Lian T., Ho R. J., *J. Pharm. Sci.*, **90**, 667—680 (2001).