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Preparation and properties of poly(vinyl alcohol)-stabilized liposomes

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Abstract

The purpose of this work is to evaluate the improvement in physical stability of poly(vinyl alcohol) (PVA) modified liposomes. Liposomes composed of soya phosphatidylcholile (SPC) and cholesterol (1:1 molar ratio) were prepared by reverse phase evaporation method. Two types of interaction between liposome and PVA were investigated: PVA addition into lipid bilayer during liposome preparation and coating of already formed liposomes with PVA. The microparticles system was morphologically characterized by transmission electron microscopy (TEM) and particles analysis. Changes in particles size and zeta potential confirmed the existence of a thick polymer layer on the surface of liposomes. The amount of PVA adsorbing to liposomes and the encapsulation efficiency increased with increasing polymer concentration. The physical stability was evaluated by measuring the release rate of contents at 20 and 37 °C, the PVA modified liposomes were more stable than the conventional liposomes. Comparing with PVA-coated liposomes, the liposomes with PVA addition to the bilayer were more stable, and had higher entrapment efficiency.

Keywords: Liposome; Poly(vinyl alcohol); Polymer modifying; Stability; Zeta potential

1. Introduction

Liposomes have been first described by Bangham (Bangham et al., 1965). Due to their biocompatibility and capability of incorporating both hydrophilic and lipophilic drugs, liposomes have been investigated as effective drug carrier (Gregoriadis, 1995; Amamath and Umas, 1997; Lasic and Papahadjopoulos, 1996; Lasic, 1998).

However, the limited stability of liposomes during storage and administration restricts their application and development (in vitro and in vivo). Many attempts have been made to enhance the stability of liposomes. The initial research showed dramatically improved stability of liposomes composed of cholesterol and neutral long chain saturated phospholipids (Kerby et al., 1980). However, the modification of bilayer composition alone is not sufficient to get stable liposomes. Successful results were obtained by the modification of liposome with several substances, such as, poly(ethylene glycol) (PEG) (Klibanov et al., 1990; Allen et al., 1991; Lebanon et al., 1990) poloxamer (Jamshaid et al., 1988), polysorbate 80 (Kronberg et al., 1990; Jorg, 2001), carboxymethyl chitin (Dong and Rogers, 1991), chitosan (Guo et al., 2003; Rengel and Barisic, 2002) and dextran derivatives (Elferink et al., 1992) have been used for preparation of polymer-coated liposomes.

Apart from these polymers, Poly(vinyl alcohol) (PVA) and PVA-R are also used to improve liposomal physical stability (Takeuchi et al., 1998, 1999, 2000), which was made by simply mixing the liposomal suspension with the polymer solution.

In this study, liposomes were modified with PVA in two different methods, the morphological property of liposome were investigated with transmission electron microscopy (TEM). Properties of coatings were evaluated by measuring the particle size and zeta potential. The physical stability of the polymer-coated liposomes was investigated by evaluating the change in the retention of entrapped calcein after incubation at 20 and 37 °C.

2. Materials and methods

2.1. Materials

Soya phosphatidylcholine (SPC) (self-made, ⩾98%), poly-(vinyl alcohol) (95%, hydrolysis M.W.9500) was purchased

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from ACROS, Calcein and cholesterol were purchased from Sigma, water used as distilled twice. All other chemicals were reagent grade and used as received.

2.2. Preparation of liposomes

2.2.1. PVA-coated liposome

Liposomes composed of SPC and cholesterol were prepared by reverse phase evaporation method. SPC and cholesterol (1:1 molar ratio) dissolved in chloroform (3 ml) were taken in round-bottomed flask. It was then attached directly to the rotary evaporator under reduced pressure to form a thin lipid film. After keeping under vacuum overnight to ensure complete removal of solvent, the film was hydrated in calcein solution (63 mmol/l in phosphate buffer, isosmolar to PBS, pH 7.4) and ultrasonicated for 10 min in a bath sonicator (DL180, 50 Hz, Haitian Electrical Instrument Works, Zhejiang, China) and then reduced to a semi-solid gel by evaporation under vacuum for 1 h. The next step was to subject the gel to vigorous mechanical shaking with a vortex mixer. The resultant multilamellar liposomal suspension was submitted to extrusion process.

For preparation of polymer-coated liposomes, an appropriate amount of PVA was dissolved in PBS (1–4%, w/v). About 0.5 ml liposomal suspension was added to 2 ml PVA solution under continuous magnetic stirring, afterwards they were incubated at 10 °C for 1 h. To adjust the liposomal concentration of non-coated liposomes to coated ones, the same amount of buffer was added to the liposomes under continuous magnetic stirring, followed by incubation under the same condition.

2.2.2. PVA-loaded liposomes

For the preparation of polymer-loaded liposomes, PVA was encapsulated into liposomes during vesicle preparation process as follows: the W_1/O emulsion, obtained by emulsifying a calcein solution into SPC: cholesterol (1:1 molar ratio) lipid mixture was dropped into PVA solution (W_2) containing the same concentration of the calcein volume ratio among the three phases (1:6:1.5) and a W_1/O/W_2 emulsion was obtained by ultrasonication. The subsequent steps to produce the vesicle were same as described above.

2.3. Characterization

2.3.1. Transmission electron microscopy (TEM)

Liposomes were analyzed on negative stain electron microscopy using a JEM 1010 electron microscopy (Joel, Japan). A drop of liposome suspension (5 μmol/ml) was applied to carbon-coated grids and after 2 min, the excess was drown off with filter paper; uranyl acetate aqueous solution was used as a staining agent. The excess was eliminated with distilled water and the sample was analyzed by TEM.

2.3.2. PVA adsorbing amount

To calculate the amount of polymer adsorbing with liposomes, an aliquot of liposomal suspension (0.3 ml) was ultracentrifuged at 75,000 rpm for 120 min. After adding 3 ml of boric acid solution (0.65 M) and 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) to 0.05 ml of supernatant and diluting to 10 ml with distilled water. The polymer concentration was measured spectrophotometrically at a wavelength of 690 nm after 15 min incubation. The amount of adsorbing was calculated from the reduced polymer concentration in the solution.

2.3.3. Zeta potential and size distribution

The liposome size distribution and Zeta potential were determined by a Zetameter (BI200SM, Brookhaven). The samples (liposome suspension) were diluted with a large amount of PBS. All measurements were preformed at 298 K.

2.3.4. Entrapment efficiency

In this work, calcein was used as an aqueous marker entrapped in the liposomes. The entrapment efficiency (%) was calculated by the following formula:

$$\text{entrapment efficiency}\,(\%) = \frac{(C_{\text{total}} - C_{\text{out}})}{C_{\text{total}}} \times 100\%$$

where C_{out} is the liposome suspension was ultracentrifuged at 40,000 rpm for 2 h to remove the liposomes (Heeremans, 1995). The fluorescent intensity of calcein was measured on a spectrophotofluorometer (960MC Shanghai Lengguang Corporation) at 490 and 520 nm for excitation and emission wavelengths, respectively.

C_{total} is the liposome suspension was diluted to four-fold of the volume with 10% Triton X-100 solution and heated at 60 °C for a few minutes in order to disrupt the liposomes completely and release the calcein encapsulated within them. The Triton X-100 solution was cooled and ultracentrifuged. The fluorescent intensity of calcein was measured according to the procedure described above.

2.3.5. Calcein release from liposome

The permeability of the liposome was determined by the release of calcein. This was run immediately after separation of the non-encapsulated calcein from the liposomes. The liposomal suspension diluted ten-fold with PBS was incubated in buffer at 20 (room temperature) and 37 °C under mild agitation. After incubation under the condition for appropriate time periods, liposomes were separated from free calcein by ultracentrifugation as described above. Generally, the percentage release of calcein from liposome was defined as

$$\text{release}(\%) = (C_{\text{f}} - C_0)/(C_{\text{total}} - C_0) \times 100\%$$

where C_0 and C_{f} were the fluorescence intensities of calcein at the beginning and at different times after preparation of the liposome suspension, respectively.

C_{total} was the fluorescence intensity of calcein after addition of Triton X-100 to destroy the liposome membrane completely.

3. Results and discussion

3.1. Morphology

Fig. 1 shows the morphological characterization of liposomes and PVA modified liposomes. In all cases, the presence

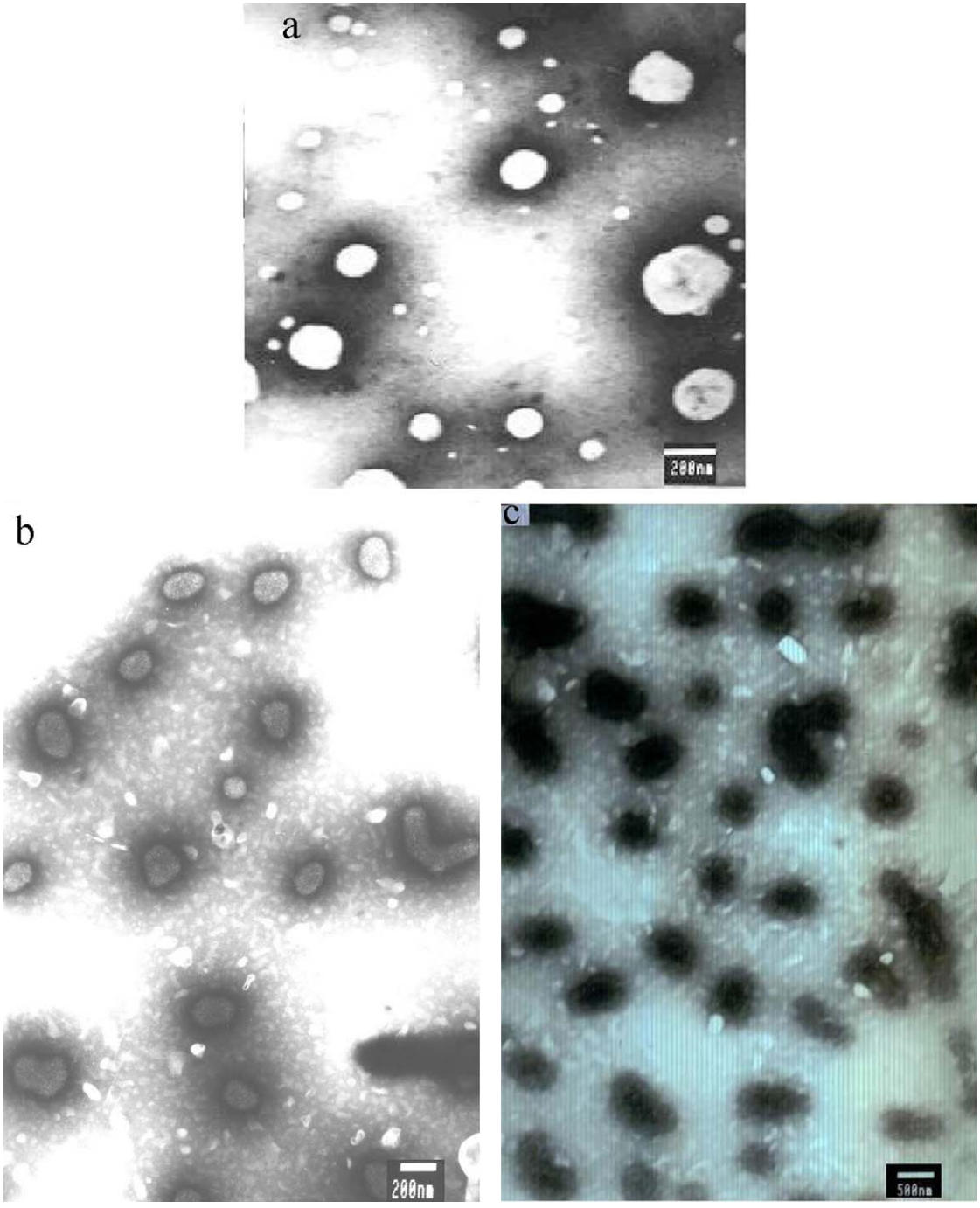

Fig. 1. Transmission electron micrographs: (a) SPC liposomes; (b) PVA coated-liposomes; (c) PVA loaded-liposomes.

of spherical-shaped vesicles were predominant. When PVA is introduced to liposomes, a different trend in morphological change is observed. In contrast to Fig. 1a, much bigger vesicles (Fig. 1b and c) exist, resulting from the reorganization of SPC liposome with PVA modified.

3.2. Liposome size distribution

The particles sizes distribution for all liposome is shown in Fig. 2. We used liposomes without calcein in this experiment because the calcein that leaked out from the liposomes could interfere with the result. The SPC liposome showed a peak with the mean diameter of about 168 nm (Fig. 2a). When liposomes were coated with PVA, the liposomes appeared to be two populations with the mean diameter of 148 and 421 nm (Fig. 2b). Two different peaks were also observed when liposomes were loaded with PVA; the two diameters were 162 and 632 nm (Fig. 2c). The PVA-loaded liposomes have broad size distribution range between 121 and 956 nm, whereas the range of coated liposome is between 119 and 625 nm.

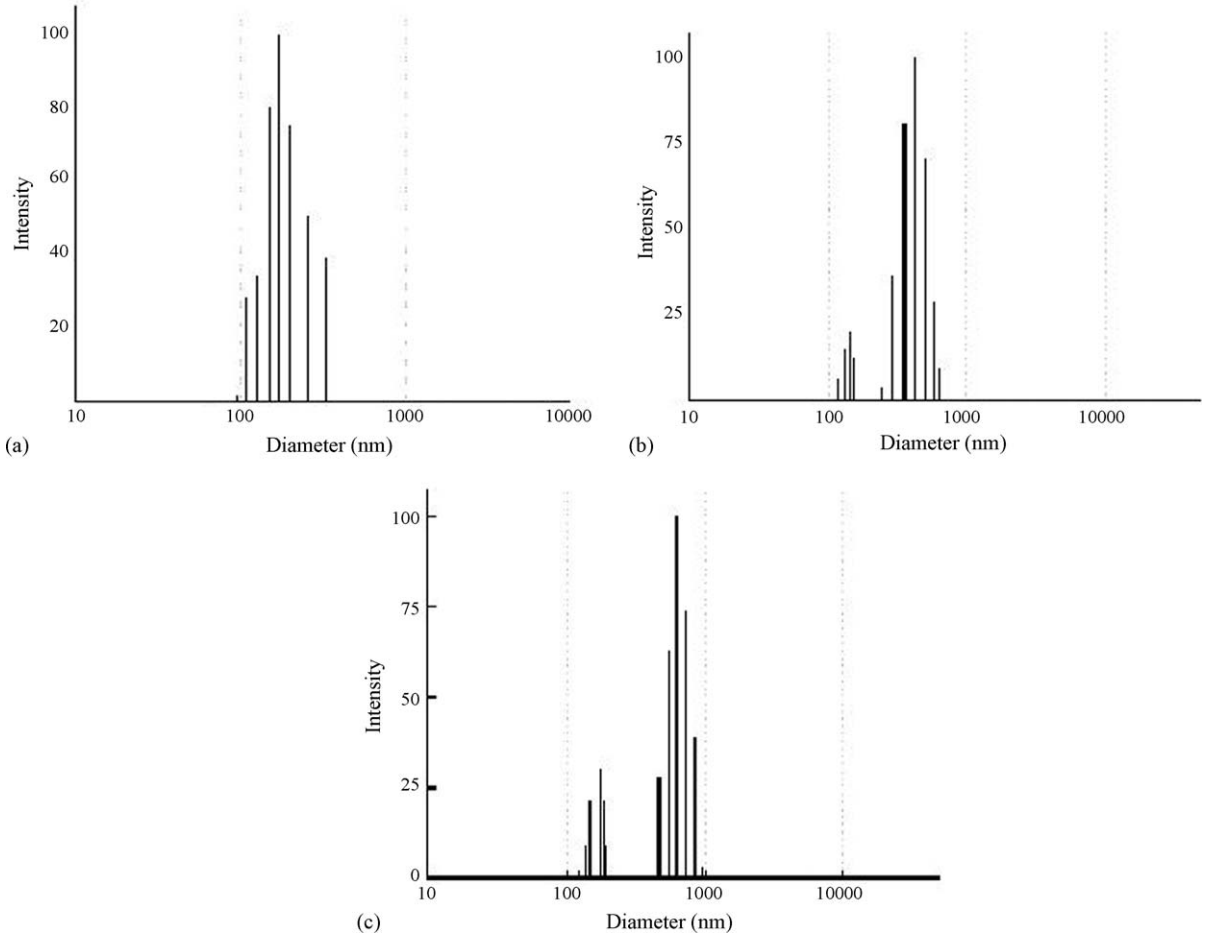

Fig. 2. Liposome size distribution: (a) SPC liposomes; (b) PVA coated-liposomes; (c) PVA loaded-liposomes.

According to Masayukl Hara's (Masayukl et al., 1989) proposal, there were three states in the polymer-coated liposome (1) liposomes were partially coated with polymer; (2) cross-linked by the polymer to form aggregation; (3) fully coated with liposome. We think that the population of smaller size liposomes were partially polymerized ones, The population of the larger liposomes were fully coated liposomes and aggregation of liposomes is caused by PVA chains.

Comparing with SPC liposomes, the increase is due to a steric repulsion among PVA chains exposed from the outer leaflet of the layer membrane, which will increase the liposome particles curvature, where the PVA chains exposure to the inner leaflet do the opposite. To the coated liposomes, PVA is supposed to be mainly on outer leaflet of the layer, whereas there were more in the inner leaflet of the liposomal bilayer membrane to the loaded liposomes. So the size of loaded liposomes is larger than the coated liposomes.

As stated in Fig. 3, the particle size of liposome in each formulation were increasing with the concentration of polymer solution, suggesting the formation of coating layer on the surface of the liposomes. When liposome was loaded with PVA, the extent of change in particle size was bigger than liposome coated with PVA.

3.3. Zeta potential

Fig. 4 depicts the relation between zeta potential and the concentration of PVA. Experimental results reveal that the SPC liposomes possess negative charges at pH 7.4, indicating that

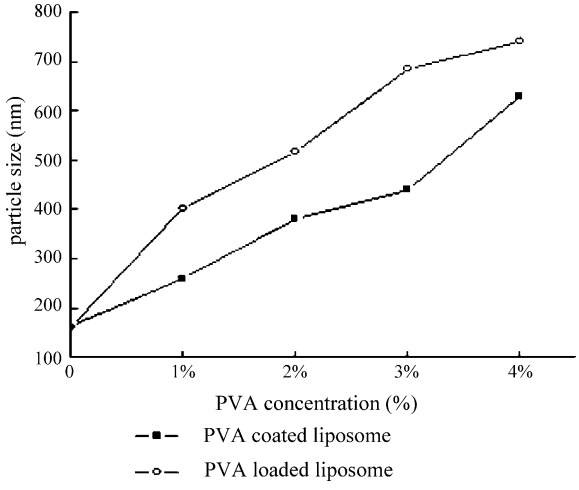

Fig. 3. The influence of PVA concentration on the liposomal size. The results are the mean diameter of three experiments.

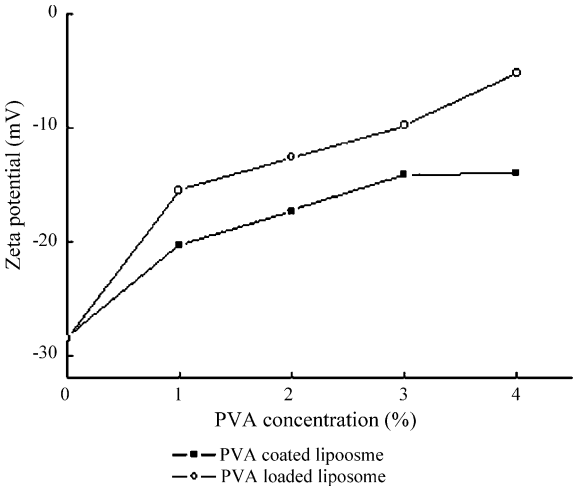

Fig. 4. The influence of PVA concentration on the liposomal zeta potential. The results are the mean of three experiments.

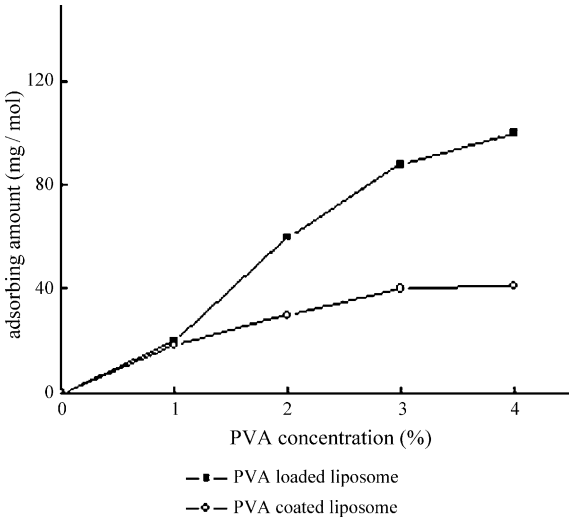

Fig. 5. The influence of PVA concentration on the amount of polymer adsorbing. The results are the mean of three experiments.

there is a weak electrostatic repulsive force between the SPC liposomes at pH 7.4. This experiment also demonstrates that the incorporation of PVA into liposome bilayer decreased the negative zeta potential significantly. The zeta potential of the PVA-coated liposome decreased from −28.5 to −13.9 mV as the PVA concentration increased to 3% then it came to a relatively constant value. Where the zeta potential of PVA-loaded liposomes decreased from −28.5 to −5.2 mV as the concentration of PVA increasing. The results show the main interaction between lipid and PVA was electrostatic attraction. The change in zeta potential may attributed to the increase thickness of polymer layer, which PVA formed on the surface of liposome shield the negatively charged liposomal surface, and this corresponds to the results of particles size measurements.

3.4. The amount of PVA adsorbing to liposomes

The amount of polymer adsorbing to the liposomes was estimated by measuring the polymer concentration of the supernatant in the centrifuge liposomal suspension.

The adsorbing amount of PVA increased with increasing concentration of PVA used in the experiment (Fig. 5). Although the amount of PVA coated increased with concentration of the polymer solution, it was much smaller than PVA loaded when compared at the same PVA concentration.

3.5. Encapsulation efficiency

Encapsulation of calcein in liposomes was characterized with regard to the effects of PVA amounts (Fig. 6). The addition of PVA increased the entrapment efficiency. Both the PVA modified liposomes encapsulated higher amount of calcein when comparing with SPC liposomes. The encapsulation efficiency of coated liposomes increased with the increasing polymer concentration from 0% to 3%, then it came to saturate. And the highest encapsulation occurred in PVA-loaded liposomes, which increased with increasing from 0% to 4%. This attributed to the formation of polymer in the liposomal bilayer. Addition of polymer could increase rigidity of the liposomal bilayer thereby reducing the leakage of the encapsulated calcein from the liposomes during the ultracentrifuge. In the loaded liposomes, more amount of the encapsulation efficiency could be obtained.

3.6. Release of calcein

Fig. 7 depicts profiles of calcein release from all liposomes and modified liposome at 20 and 37 °C. At 20 °C, coated and loaded liposomes released 45% and 30% of the encapsulate calcein by 24 h, respectively. But an intense release was induced at 37 °C, both of the liposomes released 72 and 60%. And all polymer modified liposomes release less entrapped calcein than the SPC liposomes. The PVA-loaded liposome exhibit a lower extent of release than the PVA-coated liposomes. Fig. 8 shows the release rate of calcein decreased with increasing PVA concentration.

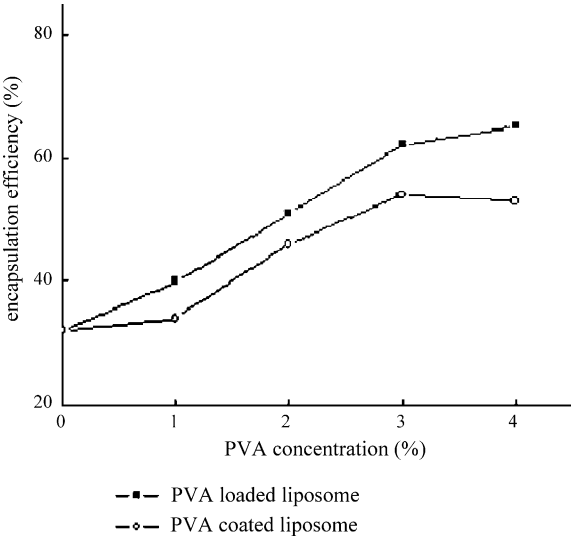

Fig. 6. The influence of PVA concentration on the entrapment efficiency of liposome. The results are the mean of three experiments.

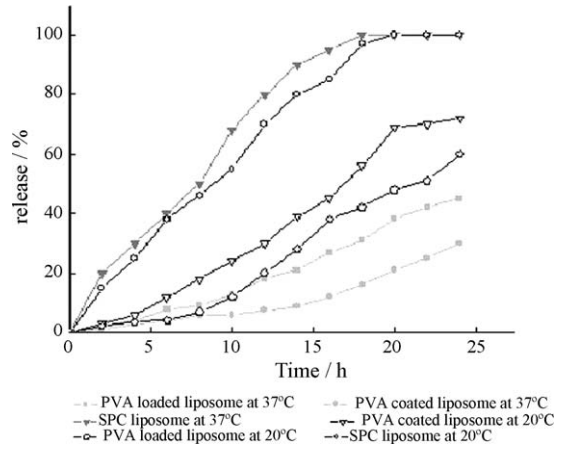

Fig. 7. The realease of calcein form liposoments. The results are the mean of three experiments.

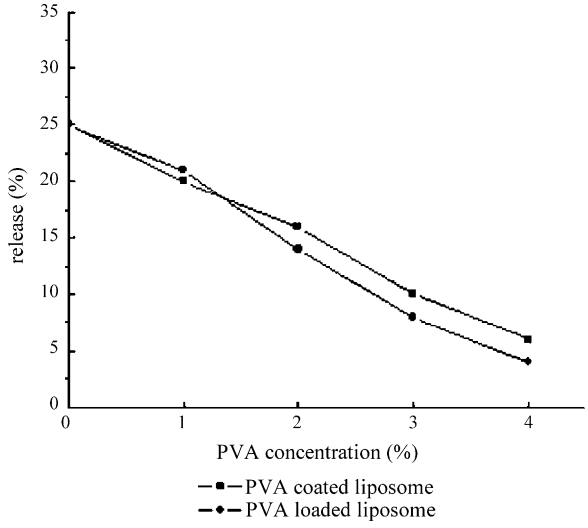

Fig. 8. The influence of PVA concentration on the release of calcein. The results are the mean of three experiments.

The results show that the polymer is positive in modulating the calcein release from liposomes. This could be due to the PVA aqueous layer around the liposome, which enhances the stability of liposome. Soya phospholipid is a zwitterionic lipid at the pH of the measurements (7.4). Hence, as generally assumed there is not a strong electrostatic repulsion force between the amphoteric phospholipid liposomes, so it is not stable. Incorporation of PVA into the bilayer can protect the liposomes. Among all the liposomes, the loaded liposomes release calcein for prolonged period of times. Figs. 7 and 8 show that PVA loaded released slowly than the coated one. This may be responsible for the difference in the manner by which PVA bound to liposomes and these difference in adsorbing amount partly explained this fewer protective efficiency of liposomes.

4. Conclusion

The micrograph clearly showed the spherical shape of SPC liposomes and polymerized liposomes, the size, zeta potential and the adsorbing amount of polymer confirmed the thicker layer on the surface of the liposome. PVA-coated liposome and loaded liposome have larger entrapment efficiency and a prolonged period of release. Based on these results, a thicker polymer layer on the surface of liposomes is necessary to improve the physical stability.

Comparing with PVA-loaded liposomes, the coated liposomes were less effective to improving the physical stability of liposomes. This may attribute to the difference in the manner by which PVA bound to liposomes. Loading liposome with PVA is a favorite method to prepare polymer-modified liposomes.

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