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The use of cholesteryl pullulan for the preparation of stable vincristine liposomes

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Abstract

An improved method for the synthesis of cholesteryl pullulan has been developed. This method involves 1,4-diazabicyclo(2,2,2)octane as a catalyst which greatly reduced the reaction time. The synthesized cholesteryl pullulan (CHP) was characterized using FT-IR and FT-NMR and used for coating the vincristine liposomes. Phosphatidylcholine liposomes with and without vincristine sulfate were prepared by a sonication method. The percentage of vincristine encapsulated in the liposomes was determined by solubilizing the bilayers using Triton-X 100. The liposomes were coated with two different concentrations of CHP solution (PC/CHP weight ratio of 3:0.5 [CHP-CL1] and PC/CHP weight ratio of 3:1 [CHP-CL2]). Both the CHP coated liposomes were characterized by transmission electron microscopy and turbidity measurement. Transmission electron micrograph showed the spherical shape of the CHP-CL1 and CHP-CL2 liposomes. The micrograph further showed the complete coat of CHP at the outermost surface of the CHP-CL2 liposomes. The average sizes of CHP-CL1 and CHP-CL2 liposomes are 340 and 350 nm, respectively. The turbidity measurement clearly indicated that the vincristine liposomes coated with higher concentration of CHP (CHP-CL2) are more stable than the liposomes coated with low concentration of CHP (CHP-CL1). The in vitro release of the encapsulated vincristine was carried out in phosphate buffered saline, pH 7.4. In phosphate buffered saline, the liposomes coated with higher concentration of CHP (CHP-CL2) released the drug for prolonged period of time compared to the CHP-CL1 liposomes. These experiments demonstrated that the CHP could be synthesized in a shorter reaction time and the vincristine liposomes coated with PC/CHP weight ratio of 3:1 could be the optimum concentration to achieve the more stable liposomes. These CHP coated liposomes are ideal carriers for the targeted delivery of therapeutic molecules.

Keywords: Cholesteryl pullulan; Turbidity; Vincristine; Coating; In vitro release

1. Introduction

Development of stable liposomes is a prerequisite for its exploitation in the delivery of therapeutic molecules. For many years, attempts have been made to improve the stability of liposomes by several methods, such as, including additional compounds in the bilayer (Gregoriadis, 1991; New, 1990), freeze drying (Weiner, Martin, & Riaz, 1989), charge modification (Park, Maruyama, & Huang, 1992), and polymerization (Regen, Czech, & Singh, 1980; Sivakumar & Panduranga Rao, 2001). Sunamoto and co-workers (Sunamoto, Iwamoto, Takada, Yuzuriha, & Katayama, 1984) have developed a strategy that consisted of coating the outermost surface of liposomal bilayers with polysaccharides, such as amylopectin, pullulan, mannan and chitin derivatives (Mobed & Chang, 1998) with the aim of improving the physical and biochemical stability of liposomes and the ability to target the liposomes to specific organs and cells. Sugar moieties on the liposomal surface have been shown to possess the capability of recognizing specific cells/tissues and this may have many applications in chemo and immunotherapy (Baszkin, Rosilio, Albrecht, & Sunamoto, 1991; Sunamoto & Iwamoto, 1986). Pullulans and amylopectins were modified with palmitoyl or cholesteryl moieties as hydrophobic side groups to anchor the bilayers to improve the stability of liposomes (Moellerfeld, Prass, Ringsdorf, Hamazaki, & Sunamoto, 1986; Moghimi, Hunter, & Murray, 2001).

Pullulan, a biodegradable polysaccharide first described in 1959 (McCarthy, 1993) is a water-soluble extra cellular neutral glucan synthesized by a fungus *Aureobasidium pullulans*. The structure of pullulan predominantly consists of maltotriose units linked via α-1,6 glycosidic bonds. Pullulan is known to protect plasma membranes against

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physico-chemical stimuli. However, when adsorbed to the liposomal surface it is easily removed by dilution. Therefore, chemical modification of pullulan is required and this can be made by conjugating a hydrophobic group with pullulan, which will allow the polysaccharide to tightly interact with the liposomal membrane. The hydrophobic part of the derivatized pullulan will tightly bind to the liposomal bilayers thereby preventing the removal of pullulan from the liposomal membranes. Several researchers (Kang, Akiyoshi, & Sunamoto, 1997; Moreira, Almeida, Geraldes, Madeiva, & Costa, 1997; Sunamoto et al., 1987) have modified the pullulan with palmitoyl or cholesteryl moieties, however, the reactions take a long time to synthesize the pullulan derivative. In the present investigation, pullulan was modified with cholesterol moiety and in this reaction 1,4-diazabicyclo(2,2,2)octane was used as a catalyst to appreciably reduce the reaction time. The synthesized cholesteryl pullulan (CHP) was used for coating the vincristine containing liposomes. Vincristine sulphate, a potent anticancer drug, was chosen for incorporation in the liposomes. Vincristine is widely used for the treatment of several types of cancer. The liposomal vincristine formulation was investigated by many researchers (Mayer, Masin, Nayar, Boman, & Bally, 1995; Tokudome, Oku, Doi, Namba, & Okada, 1996). Vincristine liposomes coated with CHP was characterized and the in vitro release of the encapsulated vincristine was investigated.

2. Experimental

2.1. Materials

Egg phosphatidylcholine (PC) and vincristine sulphate (VCR) was purchased from Sigma Chemicals, USA. Pullulan (M_w 55,000) was obtained as a gift sample from Central Food Technological Research Institute, Mysore, India. Hexamethylene diisocynate was purchased from Fluka, Ag Germany. 1,4-diazabicyclo(2,2,2)octane (DABCO) was obtained from Aldrich, USA. All other chemicals used were of analytical grade. Solvents were distilled before carrying out the experimental work.

2.2. Methods

2.2.1. Synthesis of cholesteryl N-(6-isocynatohexyl)carbamate

Cholesterol derivative was synthesized using Akiyoshi method which was (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993) modified in our laboratory. Cholesterol (7.80 g), hexamethylene diisocynate (6.73 g) and 1,4-diazabicyclo(2,2,2)octane (0.13 g) were added to 100 ml of dry toluene in a 250 ml round bottomed flask and heated at reflux in an oil bath at 80 °C with continuous stirring for 8 h. The proceeding of the reaction was monitored by thin layer chromatography (TLC), (appearance of a single spot of cholesteryl *N*-(6-isocynatohexyl)-carbamate at R_f value 0.5 developed by chloroform). After completion of reaction, the reactant was poured in to 800 ml of petroleum ether and it was stored overnight at −10 °C. The precipitate obtained was separated and again washed twice with petroleum ether and dried in a vacuum desiccator. The cholesteryl *N*-(6-isocynatohexyl)carbamate was obtained as a white powder. 65% of yield was obtained by this method.

The cholesterol derivative was characterized by FT-IR and FT-NMR. FT-IR: (KBr, cm^{-1}): 1680 (C=O), 1130 (OCO), 2320 (NC=O), 3350 (NH). FT-NMR ($CDCl_3$ solvent): δ 0.7–2.30 (cholesterol-H), 1.30–1.5 (C_4H_8), 3.10 (CH_2N), 3.20 (CH_2CNO), 3.50 (cholesterol-H), 5.30 (cholesterol-H).

2.2.2. Synthesis of cholesteryl pullulan

Cholesteryl *N*-(6-isocynatohexyl)carbamate (0.34 g), pullulan (4.00 g) and 1,4-diazabicyclo(2.2.2)octane (0.01 g) were added to 100 ml of dry dimethyl sulphoxide (DMSO) in a 250 ml round bottomed flask and heated under reflux in an oil bath at 80 °C for 8 h with continuous stirring. The disappearance of cholesteryl *N*-(6-isocynatohexyl)carbamate was checked by TLC (disappearance of R_f value, 0.5, developed by chloroform). After completion of the reaction, 500 ml of ethanol was added to the reaction mixture and stored overnight at 4 °C. The precipitate obtained was separated, purified by dialysis against distilled water and lyophilized. The yield was 87%. The degree of substitution of cholesterol was determined by elemental analysis, and was found to be 1.4 cholesterol groups per 100 glucose units for pullulan.

The synthesized cholesteryl pullulan was characterized by FT-IR and FT-NMR.

FT-IR (KBr, cm^{-1}); 1676 (C=O), 1120–900 (COC). Absence of NCO peak at 2320 cm^{-1} indicated that the unreacted free NCO groups of the cholesteryl derivative reacted with OH groups of the pullulan and formed the cholesteryl pullulan. FT-NMR (DMSO-d6/D_2O) δ 0.60–2.40 (cholesterol-H), 2.60–4.60 (glucose-H), 4.60 (glucose-H), 5.03 (glucose-H).

2.2.3. Preparation of CHP coated liposomes

2.2.3.1. Liposomes coated with CHP in the weight ratio of 3:0.5 (PC/CHP) [CHP-CL1]. The parent pullulan easily dissolves in water without sonication and gives a clear solution. The cholesteryl pullulan dissolves in dimethyl formamide and DMSO, but not so easily in water. Therefore sonication was used to prepare an optically clear solution of CHP. 12.5 mg of CHP was suspended and swelled in 20 ml of phosphate buffer under stirring for 24 h at 50–60 °C to give a milky suspension. It was further sonicated using a probe sonicator (Sonics and Materials Inc., USA) at room temperature for 5 min. The sonication procedure was

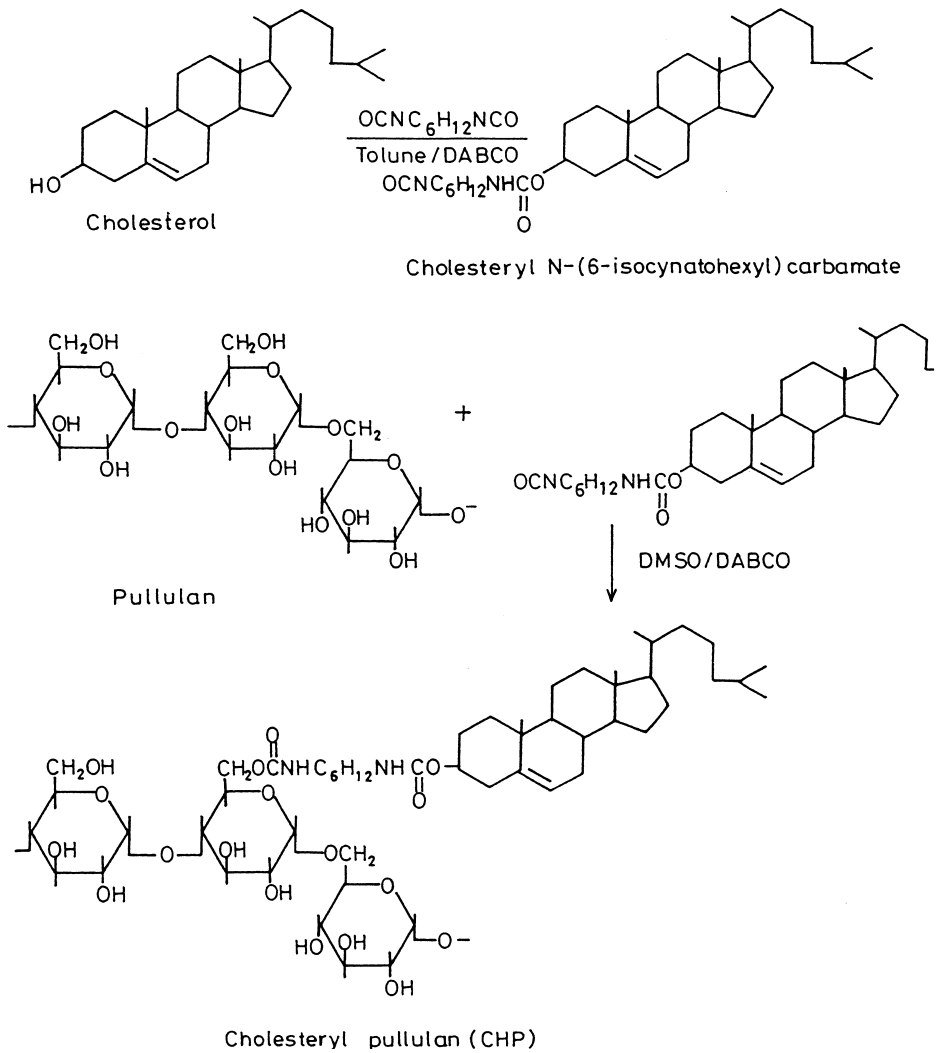

Fig. 1. Synthesis of cholesteryl pullulan.

repeated several times until an optically clear solution of CHP was obtained.

Liposomes with and without VCR were also prepared by sonication. 75 mg of PC in chloroform was taken in a round bottomed flask and the solvent was removed through rotary evaporation to form a thin film and dried in a vacuum desiccator. The dried thin film was hydrated with phosphate buffer (pH 7.4) and sonicated for 30 min intermittently at 25 °C in a probe sonicator. 20 mg of VCR was added along with the phosphate buffer to prepare the VCR encapsulated liposomes and the rest of the procedure followed was similar as described above. Liposomes were purified by a dialysis method before coating with CHP solution. Liposomal preparations were taken in a dialysis sac (12 kD MWCO, Sigma, USA) and dialyzed against 250 ml of phosphate buffer (pH 7.4) for 24 h to remove the unencapsulated drug.

Coating of liposomes with CHP was carried out according to the procedure described by Takada et al. CHP solution was added to the previously formed liposomes under stirring for 2 h at below 20 °C. Takada et al. have reported that the presence of free polysaccharide in the liposome suspended buffer medium did not exert any relevant effect on the liposomes size variation therefore, separation of any free polysaccharide that might exist in the medium was not carried out. In the case of VCR encapsulated liposomes, coating was carried out after purifying the liposomes.

2.2.3.2. Liposomes coated with CHP in the weight ratio of 3:1 (PC/CHP) [CHP-CL2]. The CHP coated liposomes were prepared exactly the same manner as stated above except that 25 mg of CHP was dissolved in 20 ml of phosphate buffer.

2.2.4. Characterization of CHP coated liposomes

2.2.4.1. Transmission electron microscopy. To observe the liposomes under a transmission electron microscope, the samples were placed on a farmvar coated copper grid #400 and 2 min was allowed for some of the liposomes to attach to the grid. The excess sample from the grid surface was

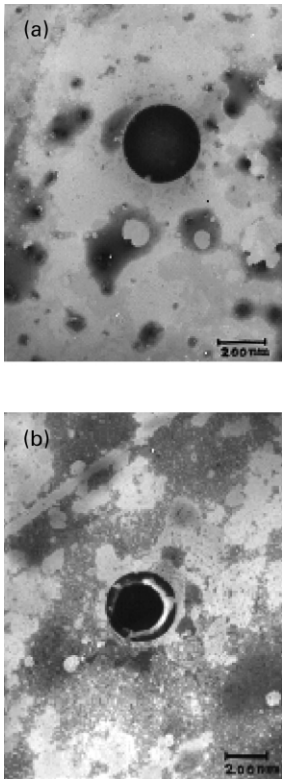

Fig. 2. Transmission electron micrograph of (a) CHP-CL 1 liposomes; (b) CHP-CL 2 liposomes.

removed with a filter paper (Whatman No. 1) and stained with a drop of 1% phosphotungstic acid. After 1 min, excess fluid was removed and the grid was air-dried. The dried grids were screened in a transmission electron microscope (JOEL-JEM 1200 EX II) at an accelerating voltage of 60 kV.

2.2.4.2. Turbidity measurement. Turbidity measurement was carried out according to the procedure reported by Regen et al. (1980). The CHP coated liposomes were appropriately diluted with phosphate buffer (pH 7.4). An increasing concentration of ethanol (0–30% v/v) was added to the diluted liposome preparations, mixed well and kept aside at room temperature for 1 h. Then the absorbance was measured at 400 nm using an UV–Vis spectrophotometer (Shimadzu 2100 S). All the spectrophotometric analyses were carried out in triplicate and the values averaged. The measured absorbance was corrected for the volume change as given below.

$$\text{Corrected absorbance} = \frac{\text{Observed absorbance} \times \text{Volume of aqueous vesicle solution} + \text{Volume of ethanol added}}{\text{Volume of aqueous vesicle solution}}.$$

2.2.4.3. Percentage encapsulation of VCR in liposomes. The percentage of VCR encapsulated in the CHP-CL1 and CHP-CL2 liposomes was determined before coating the liposomes with CHP solution. 10% Triton X-100 (0.1 ml) was added to the purified liposome preparations (0.1 ml) to completely break the liposomal bilayers and to release the encapsulated drug. The volume was made up to 3 ml with phosphate buffer and vortexed. The blank was prepared in a similar manner using liposomes without VCR for the spectrophotometric determinations. The amount of VCR was measured at 297 nm using an UV–Vis spectrophotometer.

2.2.4.4. In vitro release of VCR in phosphate buffered saline. A 2 ml of CHP coated liposomes were taken separately in a dialysis sac (12 kD MWCO, Sigma, USA) and placed in 50 ml of PBS (pH 7.4) and incubated at 37 °C. Periodically, 3 ml of the samples were withdrawn and the same volume of PBS was replaced. The samples were analyzed for VCR content at 297 nm in an UV–Vis spectrophotometer. CHP coated liposomes without VCR was used as controls.

3. Results and discussion

Coating the outermost surface of liposomes with a suitable polysaccharide such as pullulan could considerably reduce the permeability of the encapsulated water-soluble drug. But the pullulan is not tightly bound to the liposomal bilayers, hence pullulan is easily removed by dilution. In order to prevent the removal of pullulan from the liposomal surface and to make it tightly interact with the liposomal membrane it was conjugated with cholesterol. Several researchers have established the role of cholesterol in improving liposomal stability. The synthesis of cholesteryl pullulan is schematically given in Fig. 1. The hydroxyl groups of cholesterol were reacted with NCO groups of hexamethylene diisocyanate to form *N*-(6-isocyanotohexyl)carbamate. The free NCO groups present in the cholesterol derivative were further reacted with hydroxyl groups of pullulan to obtain cholesteryl pullulan. In both the reactions, DABCO was used as a catalyst. DABCO is a hygroscopic crystal, soluble in water and organic solvents and it is expected that the use of DABCO will considerably reduce the reaction time of both the cholesterol derivative and CHP synthesis.

3.1. Preparation of CHP coated liposomes

Coating of liposomes with CHP is a potential strategy for the improvement of liposomal stability. The PC/CHP weight ratio is the crucial factor that determines the stability and permeability of the CHP coated liposomes. In the present investigation the PC liposomes were prepared by a sonication method and coated with two different

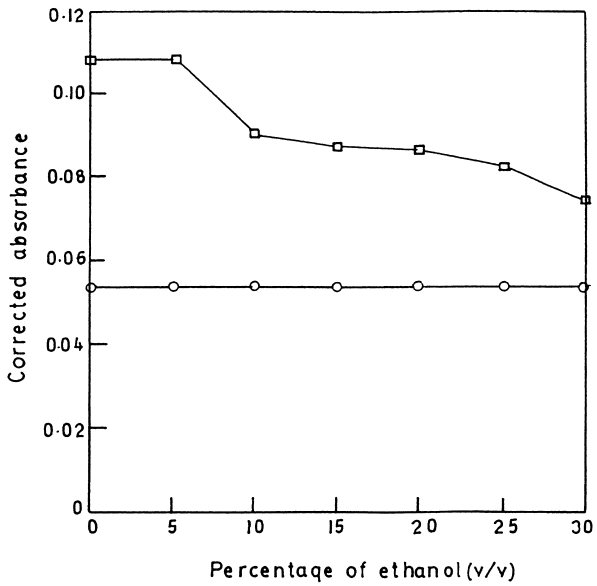

Fig. 3. Effect of ethanol on the stability of CHP coated liposomes (□) CHP-CL 1 liposomes; (○) CHP-CL 2 liposomes.

concentrations of CHP. In the preparation of CHP coated liposomes the PC/CHP weight ratio of 3:0.5 and 3:1 were employed.

3.2. Characterization of CHP coated liposomes

3.2.1. Transmission electron microscopy

The morphology and size of the two different concentrations of CHP coated liposomes were observed under transmission electron microscopy (TEM). Fig. 2a and b shows the representative transmission electron micrographs of CHP-CL1 and CHP-CL2 liposomes, respectively. Both the liposomes were found to be spherical in shape. The average sizes of CHP-CL1 and CHP-CL2 are 340 and 350 nm, respectively. The complete coating of CHP at the outermost surface of CHP-CL2 is clearly distinguishable from the TEM picture. The TEM observation indicated that coating the liposomes with PC/CHP ratio of 3:0.5 and 3:1 had not significantly altered the size of the liposomes.

3.2.2. Stability of CHP coated liposomes

The effect of structural stabilization of liposomes by coating with CHP was examined by a turbidity method. Decrease in turbidity on the addition of alcohol/surfactant is an indication of poor stability. Ethanol is known for its disintegrating property of liposomal bilayers. The CHP-CL1 liposomes exhibited stable turbidity value only up to 5% v/v ethanol addition and beyond that the turbidity value gradually decreased upon addition of an increasing concentration of ethanol (Fig. 3). On the other hand, the turbidity value of CHP-CL2 remained constant with the addition of 30% v/v ethanol. In the CHP-CL1 liposomes, the concentration of CHP is not adequate to completely protect the liposomes whereas in CHP-CL2, the concentration of CHP is sufficient to protect the liposomes. The turbidity data indicated that the CHP-CL2 liposomes are the most stable liposomes among the two liposomal preparations.

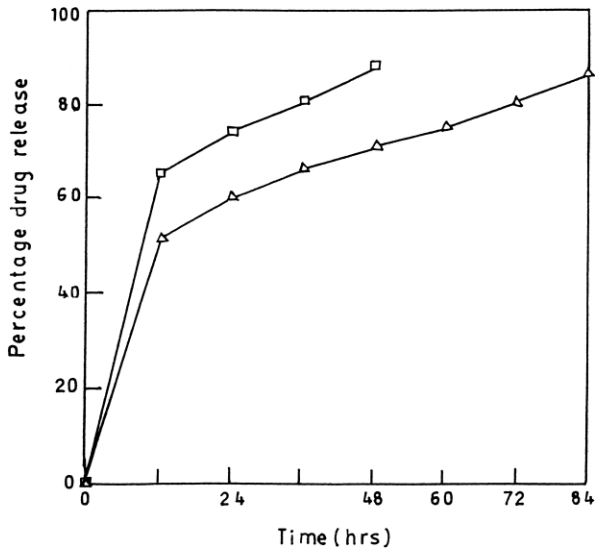

Fig. 4. In vitro release of VCR in PBS, pH 7.4 at 37 °C from (□) CHP-CL 1 liposomes; (△) CHP-CL 2 liposomes.

3.2.3. VCR encapsulation in liposomes

A direct method was employed for the determination of percentage encapsulation of VCR in liposomes. The uncoated liposomes were completely solubilized using 10% v/v Triton X-100 and the drug was measured spectrophotometrically. Both the liposomal preparations produced for CHP coating encapsulated almost similar percentage of VCR (CHP-CL1: 39.6% and CHP-CL2: 38.8%).

3.2.4. In vitro release of VCR from CHP coated liposomes in PBS

In vitro release of the encapsulated VCR from CHP-CL1 and CHP-CL2 liposomes was carried out in PBS, pH 7.4 at 37 °C and the release profiles are shown in Fig. 4. The CHP-CL1 liposomes released 88% of the encapsulated VCR in 48 h. On the other hand, CHP-CL2 liposomes released 86% of the VCR in 84 h. Both the CHP coated liposomes prolonged the release of the encapsulated VCR in PBS. However, the CHP-CL2 liposomes prolonged the release of VCR for almost twice the time of CHP-CL1 liposomes. The cholesterol moiety of CHP anchored onto the lipid bilayer and tightly bound to the liposome membrane and the formation of stable coat by the pullulan part of the CHP around the outermost surface of liposomes are the reasons attributed for the prolonged duration of release of VCR from the CHP-CL2 liposomes. In the case of CHP-CL1 liposomes, the amount of CHP utilized to coat the liposomes was less hence, the amount of hydrophobic part (cholesterol) of CHP anchored onto the bilayers and the coating efficiency of pullulan might have been inadequate to

increase the stability of liposomes. The faster release of VCR from the CHP-CL1 could be due to the lesser stability of the liposomes.

4. Conclusion

A significant decrease in the reaction time for the synthesis of CHP was achieved using DABCO as a catalyst. A very stable vincristine containing liposomes were prepared by coating the outermost surface of the liposomes with higher amount of CHP. In vitro release of the encapsulated VCR in PBS indicated that the CHP-CL2 liposome are more stable and releases the drug in a sustained manner for prolonged period of time. The above results clearly suggests that, it is possible to prepare highly stable liposomes coated with polysaccharides in appropriate weight ratio and it may have potential applications in the controlled and targeted delivery of therapeutic molecules.

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