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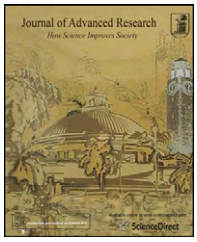

ORIGINAL ARTICLE

Effect of chitosan coating on the characteristics of DPPC liposomes

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Abstract Because it is both biocompatible and biodegradable, chitosan has been used to provide a protective capsule in new drug formulations. The present work reports on investigations into some of the physicochemical properties of chitosan-coated liposomes, including drug release rate, transmission electron microscopy (TEM), zeta potential and turbidity measurement. It was found that chitosan increases liposome stability during drug release. The coating of DPPC liposomes with a chitosan layer was confirmed by electron microscopy and the zeta potential of liposomes. The coating of liposomes by chitosan resulted in a marginal increase in the size of the liposomes, adding a layer of (92 ± 27.1 nm). The liposomal zeta potential was found to be increasingly positive as chitosan concentration increased from 0.1% to 0.3% (w/v), before stabilising at a relatively constant value. Turbidity studies revealed that the coating of DPPC liposomes with chitosan did not significantly modify the main phase transition temperature of DPPC at examined chitosan concentrations. The appropriate combination of liposomal and chitosan characteristics may produce liposomes with specific, prolonged and controlled release.

Introduction

Chitosan is a typical biological macromolecule derived from crustacean shells. It has several emerging applications, including in drug development, obesity control and tissue engineering [1]. It has been used to provide a protective capsule in new drug formulation because it is both biocompatible and biodegradable [2,3]. Owing to its properties, chitosan can be used in a variety of areas, including medicine, pharmacy, biotechnology, agriculture and the food industry [4,5].

Recently, a number of studies have shown that chitosan forms a complex nanoparticle with recombinant DNA plasmids that provides an effective means of delivering genes into cells [6–9]. For instance, chitosan–DNA nanoparticles successfully delivered a dominant peanut allergen gene to the intestine of a murine model of peanut allergy and substantially reduced the allergen-induced anaphylaxis [7].

Meanwhile, liposomes have been a visible feature in innovative drug delivery systems for a number of years [10]. They have been investigated for the delivery of chemotherapeutic agents for cancer [11], vaccines for immunological protection [12], radiopharmaceuticals for diagnostic imaging [13], and nucleic acid-based medicines for gene therapy [14].

However, liposomes also have some limitations. First, they generally show a short circulation half-life after intravenous administration [15]. Second, they are prone to adhering to each other and

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fusing to form larger vesicles in suspension, which may result in inclusion leakage [16,17]. Therefore, stability is a general problem with lipid vesicles [18,19].

Several authors have used chitin or chitosan related polymers as a liposome coating in order to increase their stability towards drug release [20,21], to stabilise haemosomes 'Artificial Red Blood Cells' [22,23], and for targeting purposes [24].

We realised that an appropriate combination of the polymer-based and lipid-based systems could integrate the advantages and mitigate the disadvantages of each system, and thus lead to new applications [25,26].

In our previous study the interaction between chitosan and DPPC liposomes was studied using a number of biophysical techniques (including FTIR spectroscopy, viscometry and liposomes solubilisation) in an attempt to understand the overall behaviour of the chitosan–liposomes system [27].

The present work uses drug release rate, transmission electron microscopy, zeta potential and turbidity measurements at 400 nm to investigate the characteristics of chitosan-coated liposomes to develop and further optimise liposomes that are directed for topical release in systemic pharmacological applications.

Material and methods

Materials

L-α-Dipalmitoyl phosphatidylcholine (DPPC) specified 99% pure and Triton X-100 were purchased from Sigma (St. Louis, Mo, USA). Chitosan (from crab shells) was purchased from Fluka with molecular weight of 150 kD and was used as received. Chloroform was of analytical grade and obtained from Merck. Double distilled deionised water was used. Doxorubicin hydrochloride (MW = 579.98) was manufactured by CIPLA Ltd. (India) as freeze dried powder on a 50 mg vial and was used without further purification. The chemical structure of DPPC and chitosan are shown in Fig. 1.

Preparation of chitosan-coated liposomes

For DPPC liposomal preparation, the lipids were first dissolved and mixed in chloroform to ensure a homogeneous mixture of lipids. The organic solvent was then removed by rotary evaporation to obtain a thin lipid film, formed on the sides of a round bottom flask. The lipid film was thoroughly dried to remove residual organic solvent by placing the flask on a vacuum pump for nearly 90 min. Hydration of the dry lipid film was accomplished by adding an aqueous solution to the container of the dry lipid film and agitating at a temperature above the phase transition temperature of the lipid [28].

For liposomes encapsulated doxorubicin, the resulting thin film was hydrated with an appropriate amount of doxorubicin solution. The non-encapsulated drug was separated by centrifugation at 9000 rpm for 20 min. The formed pellet was washed with sterile double distilled deionised water and re-centrifuged; this step was repeated four times and the pellet then re-suspended in an appropriate amount of sterile double distilled deionised water.

For chitosan-coated liposomes, an appropriate amount of 0.5% (w/v) chitosan solution was added drop wise to the liposomal suspension under magnetic stirring at room temperature [29]. After addition of chitosan, the mixture was left to stir for approximately 1 h and then incubated overnight at 4 °C.

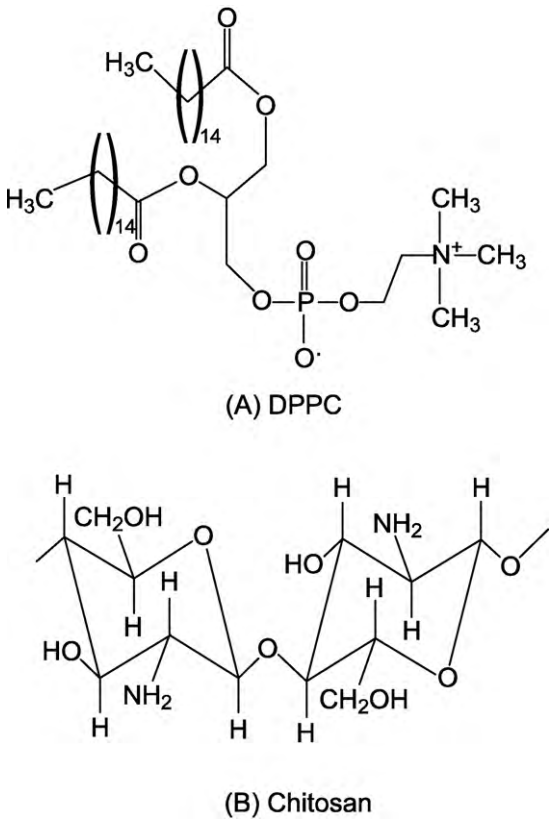

Fig. 1 Chemical structure of (A) DPPC and (B) chitosan.

Release experiment

The release experiments were run immediately after the separation of the free doxorubicin from that encapsulated in liposomes. To avoid erroneous results due to sudden temperature changes, the purified liposome preparations were gradually warmed to 37 °C, at which most of the in vitro experiments were performed. Both liposomes and chitosan-coated liposomes samples (encapsulating doxorubicin) were incubated at 37 °C for different periods of time. An appropriate amount from each sample was taken after incubation and then the fluorescence intensity (F_i, excitation at 470 nm, emission at 585 nm) was measured using a Perkin Elmer Spectrofluorometer LS 55 B (U.K.). To lyse liposomes completely, 100 μl of Triton X-100 was added and the total fluorescence F_{total} (corresponding to 100% release) was measured. The percentage of doxorubicin release was calculated by dividing F_i by F_{total}. The percentage increase of drug release was plotted as a function of time.

Transmission electron microscopy

DPPC liposomes and chitosan-coated liposomes were analysed via negative stain electron microscopy using a JEM 1230 Electron Microscope (Jeol LTD, Tokyo, Japan). A drop of each liposomal suspension was applied to copper coated with a carbon grid. The excess was drawn off with filter paper. An aqueous solution of ammonium molybdate (1%, w/v) was used as a negative staining agent. After waiting for 2 min at room temperature, the excess solution was removed with a filter paper and then examined under the electron microscope. The particle size was measured by the

software (Gatan program) accompanying the transmission electron microscope.

Zeta potential measurements

Zeta potential of DPPC liposomes and chitosan-coated liposomes with different chitosan concentrations (0.1–0.5%, w/v) were determined using the Malvern Zetasizer 2000 (Malvern Instruments, U.K.) after samples centrifugation at 13,000 rpm for 20 min. Pellets were then re-suspended in double distilled deionised water. The zeta potential of the chitosan-coated liposomes was measured after centrifugation to confirm that the liposomes were coated.

Turbidity measurements

Turbidity measurements were monitored as a function of temperature by continuous recording of optical density at 400 nm using a UV/VIS Spectrophotometer (Jenway 6405; Barloworld Scientific, Essex, UK) at 400 nm. The samples were heated by a temperature-controlled bath. Turbidity profiles were plotted for DPPC liposomes and chitosan-coated liposomes after coating with two different amounts (0.5 and 0.75 ml) of 0.5% (w/v) chitosan solution.

Results and discussion

Fig. 2 shows the effect of chitosan coating of DPPC liposomes on the drug release rate from liposomes at different time intervals. It is clear that the percentage increase of drug release from liposomes was reduced after coating with chitosan at all examined time intervals. For example, after 2.5 h, the percentage increases of drug release were 34.5% and 29.6% for DPPC liposomes and chitosan-coated liposomes respectively. Our results are in agreement with those of previous investigations into the effect of surface coating with polymers to preserve liposome stability [30–32]. The protective effect of hydrophilic polymer coating depends on the ability of the polymer to adhere to the lipid bilayers [29]. Mady et al. [27] found that the interaction between chitosan and DPPC liposomes contributed to an improvement in the stability of lipid vesicles.

The coating of DPPC liposomes by a chitosan layer was confirmed by electron microscope images and the zeta potential of liposomes [33].

The coating of liposomes by chitosan resulted in a marginal increase in the size of the liposomes by a coating layer of (92 ± 27.1 nm). The chitosan layer thickness was measured by the

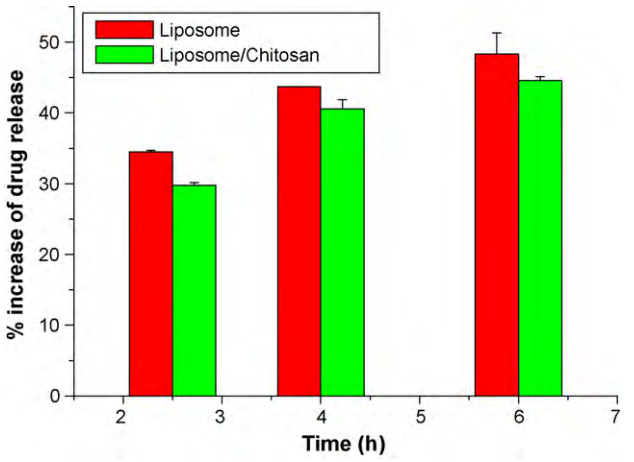

Fig. 2 Percentage increase of drug release from liposomes and chitosan-coated liposomes incubated at 37 °C ($n = 3$).

software (Gatan) accompanying the transmission electron microscope. Surface morphological studies on the shape of the prepared systems using transmission electron microscopy indicated that the systems were almost spherical (Fig. 3a). Further, the existence of chitosan surrounding the liposomes was well visualised on the surface of chitosan-coated liposomes (Fig. 3b). *t*-Test was made for the liposomal size values before and after chitosan coating. *P*-value was <0.01, indicating that the difference in liposomal size between those with and without the chitosan coating was highly significant. The interaction between chitosan and liposomes appears due to a combination of adsorption coagulation and bridging between them [34].

Zeta potential (ζ potential) is a measure of the surface electrical charge of particles, and has often been used to characterise colloidal drug delivery systems. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. As the zeta potential increases, repulsion between particles will be greater, leading to a more stable colloidal dispersion. If all particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to come together [10].

Information on the overall charge of chitosan-coated liposomes by zeta potential measurements can speed up the development of liposomes with specific, prolonged and controlled release.

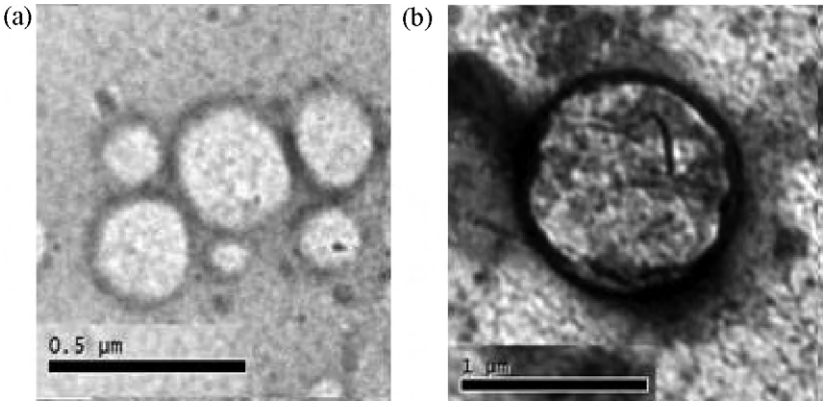

Fig. 3 Transmission electron micrographs of: (a) liposomes and (b) chitosan-coated liposomes ($n = 3$).

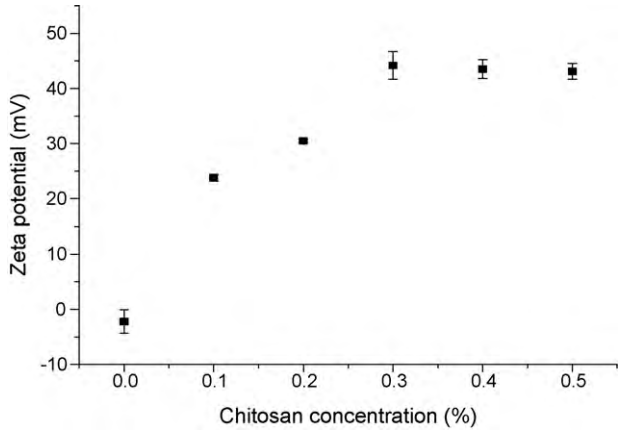

Fig. 4 Effect of chitosan at different concentrations on ζ potential of DPPC liposomes ($n = 3$).

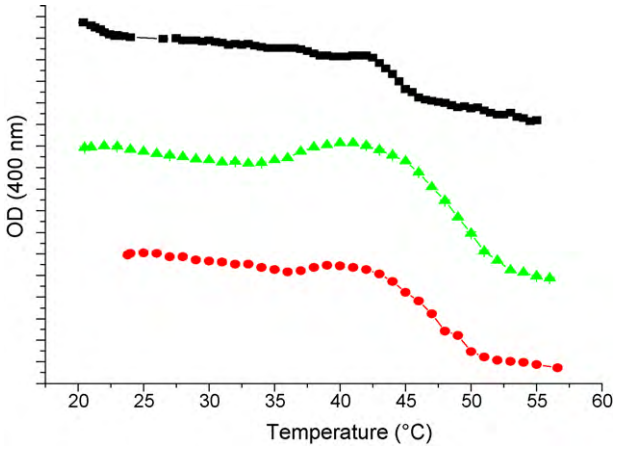

Fig. 5 Temperature dependence of optical density for: DPPC liposomes (■) and chitosan-coated liposomes with 0.5 ml chitosan (●) and 0.75 ml chitosan (▲).

DPPC liposomes showed a slight negative zeta potential, in agreement with the observations of previous studies [35–39]. It is clear from Fig. 4 that the coating of liposomes by chitosan shifted the zeta potential from slightly negative to positive values. The results show that DPPC liposomes had positive ζ values after their coating with (0.1–0.5%, w/v) chitosan solutions. The liposome zeta potential was found to be increasingly positive as chitosan concentration increased from 0.1% to 0.3%, before coming to a relatively constant value [40]. The increase of ζ potential can be attributed to more cationic polymers adsorbed to the liposomal surface. Since chitosan carries a high positive charge, the adsorption of chitosan appears to have increased the density of positive charge and hence made the zeta potential positive. DPPC liposomes are typically nearly neutral and the mechanism of coating neutral DPPC liposomes by chitosan probably involved hydrogen bonding between the polysaccharide and the phospholipid head groups [40,41].

Chitosan-coated liposomes have been used as a mucoadhesive delivery system; their positively charged surface favours adhesion to the cells membranes, which are normally negatively charged [29,30,40]. The adhesive ability has been shown to be an important factor in prolonging retention in the gastro-intestinal tract and promoting penetration into the mucus layer [33].

The turbidity technique at visible range is a spectroscopic technique that provides valuable information about membrane phase transition temperatures and membrane order [42,43]. Lipid turbidity study has been previously utilised in membrane research [42,44,45].

In the present study, the effect of chitosan was investigated on lipid-phase transition, order and dynamics, and hydration states of the head and near the aqueous region of zwitterionic DPPC MLVs as a function of temperature and amount of chitosan. The chitosan alone showed no peaks of heat absorption below 100 °C and therefore no phase transition occurred in this temperature range [46].

Fig. 5 represents the variation in optical density at 400 nm as a function of temperature for DPPC liposomes in the absence and presence of different amounts of chitosan. As can be seen in the figure, for pure DPPC liposomes absorbance values decrease as a function of increasing temperature and show two transitions: a pre-transition at nearly 36 °C, and a main transition around 41 °C of DPPC. These temperatures are very close to the values that have been reported by calorimetric [47,48] and turbidity studies [43,44]. Turbidity studies revealed that coating of DPPC liposomes by chitosan did not significantly modify the main phase transition temperature of DPPC at examined chitosan concentrations.

Conclusion

Chitosan coating resulted in a particle size increase and a more positive zeta potential of liposomes, forming a more stable system. Chitosan coating has a significant effect on drug release behaviour, but has no significant effect on the phase transition temperature of DPPC liposomes. Appropriate combinations of the liposomal and chitosan characteristics may produce liposomes with specific, prolonged and controlled release. The results indicate that chitosan-coated liposomes may be used either in cosmetology or pharmacology as an effective drug delivery system.

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