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# Interaction of amphiphilic derivatives of chitosan with DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine)

**Vera Aparecida de Oliveira Tiera · Françoise M. Winnik · Márcio José Tiera**

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**Abstract** The interaction of chitosan and its *N*-dodecyl and poly(ethylene glycol) derivatives with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) vesicles was studied to evaluate the influence of molecular architecture of the polymers on the liposomes. The study was carried out in aqueous solution using differential scanning microcalorimetry (DSC) and dynamic light scattering. The interaction of these polymers with DPPC vesicles altered the gel–liquid crystalline phase transition temperature and decreased both the enthalpy ($\Delta H$) and cooperativity of the phase transition. The results obtained indicate that perturbations in the vesicles surface and the incorporation of chitosan and its derivatives into the lipid bilayer upon polysaccharides interaction are responsible for the formation of large vesicles.

**Keywords** Microcalorimetry · Liposomes · Modified chitosans · Coating · Vesicle fusion

## Introduction

Liposomes have been recognized for a long time as good candidates for drug delivery due to the possibility of using these systems as carriers for oral and intravenous administration [1]. Their structures mimic that of cell membranes making them good models for physical and biochemical studies of biomembranes. However in vitro and in vivo instability of liposomes has limited their potential advantages since they may be degraded by bile salts and pancreatic lipases in the gastrointestinal tract. On the other hand they may be recognized by the reticuloenthelial system when administered by intravenous injection. In this aspect a great effort has been made to inhibit the interaction with proteins and cells responsible for their clearance from circulation [2]. Modification of the liposomes surfaces with hydrophilic polymers as poly(ethylene glycol) [3] and polysaccharides [4, 5] proved to inhibit these interactions prolonging the blood time circulation. Recently the interest for natural polymers as chitosan and dextran has increased due to their ability to interact with the liposome surface leading to new systems with potential applications for drug targeting and delivery [6, 7]. Chitosan is a cationic natural biopolymer produced by alkaline N-deacetylation of chitin, the most abundant natural polymer after cellulose. It is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine, which behaves as a weak base. It shows some biological activities such as immunological, antibacterial, and wound healing activity. More recently chitosan has been used to coat liposomes aiming to obtain new drug delivery systems [8]. The interaction between *o*-carboxylmethylchitosan (OCMCS), a chitosan derivative, and dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was shown to induce the fusion of small DPPC multilamellar vesicles (MLV) to form large lamellar structures [9]. The interaction of chitosan with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) also showed vesicles with higher stability against salt (NaCl) and pH shocks, revealing strong interactions between chitosan and DOPC, which were credited to electrostatic and hydrophobic interactions [10].

V. A. de Oliveira Tiera (✉) · M. J. Tiera
Departamento de Química e Ciências Ambientais,
Instituto de Biociências Letras e Ciências Exatas, UNESP,
São José do Rio Preto, SP, Brazil
e-mail: verapoli@ibilce.unesp.br

F. M. Winnik
Faculty of Pharmacy and Department of Chemistry,
Université de Montréal, Montreal, QC, Canada

In this work we have used chitosan and its derivatives aiming to evaluate how these amphiphilic derivatives having hydrophobic *N*-dodecyl and hydrophilic poly(ethylene glycol) attached to the chitosan backbone interact with dipalmitoyl-sn-glycero-3-phosphocholine. DPPC was chosen as our model system because DPPC is a major component of cell membranes and it has been widely used as a standard for characterizing of cholesterol and drugs induced membrane perturbations [11–13]. The fundamental interaction between chitosan and their derivatives with DPPC membrane bilayer was probed by differential scanning calorimetry (DSC) and light scattering.

## Experimental

Chitosan ($M = 3.58 \times 10^4$ g/mol, Wako, degree of acetylation DA = 0.14), sodium cyanohydroborate (Sigma), acid acetic, methanol, dodecylaldeyde (Aldrich), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) from Avanti Polar Lipids, Sodium phosphate monobasic (ACS reagent—Anachemia chemicals Ltd.), Pyrene (Sigma, recrystallized twice from ethanol). All solutions were prepared by weight using a Precisa balance with sensitivity of $5 \times 10^{-5}$ g. Chitosan derivatives containing poly (ethylene glycol) (Mn = 2,000 g mol$^{-1}$) $PEG_8CH$ and dodecylated derivative ($PEG_{15}$–$C_{12}$–CH) were prepared as described previously by Muslim et al. [14] and Desbrières et al. [15]. The chemical structures of chitosan and its derivatives are shown in Fig. 1.

We obtained small liposomes by the reverse phase evaporation technique. The lipid (60 mg) was placed in a round-bottomed flask and dissolved in 30 mL of diethyl ether–chloroform (1:1) mixture. Buffer (1 mL) was rapidly injected into the lipid solution using a syringe fitted with a 23 gauge needle. The system was sealed with a glass stopper, placed in a sonicator and sonicated for 3 min. Then the flask was quickly transferred to a rotary evaporator and the solvent was removed under a low vacuum until a gel was formed. The gel was converted into a fluid suspension of giant unilamellar vesicles (GUVs) through vigorous mechanical agitation on a vortex mixer. The evaporation was continued for 5 min and 15 mL of buffer was added and the sample was sonicated for 1 h at 333 K and thereafter the volume was adjusted to 30 mL.

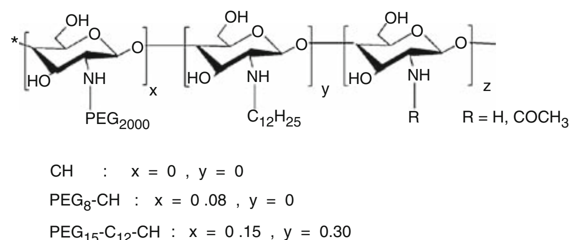


**Fig. 1** Structures of the chitosans used in this study

The liposome–chitosan mixtures were prepared by the method described by Yaroslavov et al. [16]. Appropriate volumes of liposomes and polymers solutions previously dissolved in the buffer (phosphate buffer pH 6.2, 50 mM) were mixed, preheated above phase transition temperature (323 K) and kept under stirring for 1 h. Thereafter the solution was permitted to cool at room temperature and kept under gentle magnetic stirring for 1 h. The final liposome concentration in all experiments was 1.0 g/L and the polymers concentration was varied from 0.01 to 3.0 g/L. Differential Scanning Calorimetry (DSC) measurements were performed on a VP-DSC microcalorimeter (MicroCal Inc.) at an external pressure of ca. 180 kPa. The cell volume was 0.517 mL. The heating rate was 1.0 K min$^{-1}$. Data from five scans were corrected for instrument response time and analyzed using the software supplied by the manufacturer. The DPPC concentration was 1.0 g/L and the polymer concentration was varied from 0.01 to 5.0 g/L and the enthalpy values are the average of two independent preparations using the same liposome stock solution. Dynamic (DLS) light scattering experiments were performed on a CGS-3 goniometer (ALV GmbH) equipped with an ALV/LSE-5003 multiple-$\tau$ digital correlator (ALV GmbH), a He–Ne laser ($\lambda = 632$ nm), and a C25P circulating water bath (Thermo Haake). The temperature was $298.0 \pm 0.5$ K unless otherwise stated. The polymer concentration was varied from 0.025 to 5 g/L. All solutions were prepared using previously filtered buffer using 0.45 μm Millex Millipore PVDF filters.

## Results and discussion

The phase transition of small unilamellar vesicles (SUVs) of DPPC was monitored by DSC microcalorimetry technique at pH 6.2. At this pH chitosan is partially ionized but it is still soluble in the buffer solution. As shown in Fig. 2 (curve a) the original SUVs ($R_h$ of 60 nm measured by DLS) was characterized by a small pre-transition at 306.0 K and a narrow peak corresponding to the main transition with a maximum at 314.3 K. For pure DPPC the main transition enthalpy was found 39.8 kJ/mol, in good agreement with previously reported values [17]. The phase transition temperature and enthalpy ($\Delta H$) for DPPC vesicle bilayers were slightly affected by the non-modified chitosan (CH, 2.0 g/L). The DPPC:CH mixtures exhibited narrower curves when compared to that of pure DPPC and a small decrease in the enthalpy transition to 37.2 kJ/mol was observed (Fig. 2a). This result indicates that interaction of chitosan with DPPC prepared by this procedure is dominated by interactions occurring mainly on the vesicles

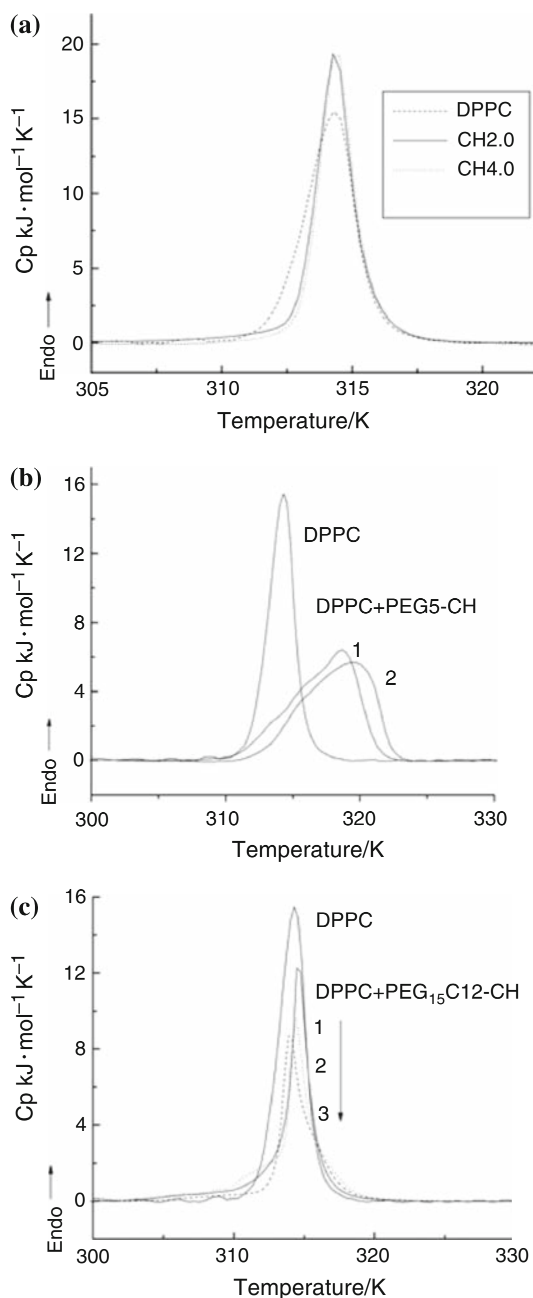


**Fig. 2** DSC curves of DPPC in the presence of **a** chitosan 2.0 g/L (CH 2.0) and 4.0 g/L (CH 4.0); **b** $PEG_8$–CH: 1—1.0 and 2—2.5 g/L; and **c** $PEG_{15}$–$C_{12}$–CH: 1—0.1, 2—0.2 and 3—3.0 g/L, at pH 6.2 in phosphate buffer (50 mM)

surface by electrostatic interaction with negative charges of DPPC (phosphate heads) [18]. However hydrophobic interactions may induce the chitosan incorporation into the lipid bilayer [19]. The study of the interaction of non modified chitosan with phospholipids vesicles by Fang et al. [19] and more recently by Quemeneur et al. [10] showed that independently of the molecular mass, chitosan adsorption may also take place on the vesicle surface.

A similar result was obtained for DPPC:$PEG_8$–CH mixtures however a more pronounced decrease in $\Delta H$ was obtained. This polymer is more hydrophilic than chitosan due to PEG chains and a lower tendency to interact with the vesicles surface could be expected. The $PEG_8$–CH concentration was increased from 0.01 to 2.5 mg/mL and the peak height of the main transition decreased and the transition temperature shifted to 318.6 K. The line shapes in the thermal curves were also distorted and broadened relative to the rather sharp, bell-shaped curve observed for pure DPPC (Fig. 2b). Accordingly, this result indicated incorporation of PEG chains into the DPPC bilayer which may lead to a more tight packing induced by the $PEG_8$–CH association, thus reducing the hydration of the zwitterionic groups [20]. Nonaka et al. [21] showed by means of ESR measurements that interaction of amphiphilic chitosan with DPPC resulted in a highly ordered membrane in contrast to the low-molecular-mass surfactants. Savva and Huang [22] have also observed that PEG interacts with the phospholipid acyl chains deep in the bilayer of DPPC which would explain the smaller enthalpy obtained for this derivative when compared to non-modified chitosan (CH). Meyuhas and Lichtenberg [23] also showed that polyethylene glycols (PEGs) of different molecular masses induced reversible aggregation of phospholipid vesicles, mostly due to dehydration of the vesicle surface and depletion forces. On the other hand Wetterau and Jonas [24] studying DPPC vesicles found that the phase transition temperature shifts to higher temperatures when the vesicles size is increased. Therefore these increasing phase transition temperatures for DPPC:$PEG_8$–CH mixtures may be interpreted as increasing vesicles sizes associated with a more tight packing of the lipidic bilayer.

For the more amphiphilic derivative $PEG_{15}$–$C_{12}$–CH the interaction with DPPC may take place on the vesicle surface as well as by anchoring of the dodecyl chains into the bilayer. As it can be seen from Fig. 2c, the addition of $PEG_{15}$–$C_{12}$–CH to DPPC solution showed a higher effect on $\Delta H$ and for the highest concentration used (3.0 g/L) the enthalpy decreased to 23.0 kJ/mol. Figure 3 shows a comparison of the effects of chitosan and its derivatives on the phase transition temperature as a function of the polymer concentration. Clearly the $PEG_{15}$–$C_{12}$–CH derivative affects more the phase transition enthalpy indicating a more effective interaction of this derivative with the DPPC bilayer, what can be attributed to alkyl chain incorporation [25, 26].

A light scattering study was performed to evaluate the vesicles sizes as a function of the $PEG_8$–CH concentration. The correlation functions for pure DPPC in the presence of increasing $PEG_8$–CH concentration shift to longer times as $PEG_8$–CH increases (Fig. 4a), indicating that vesicles fusion starts to occur at very low polymer concentration.

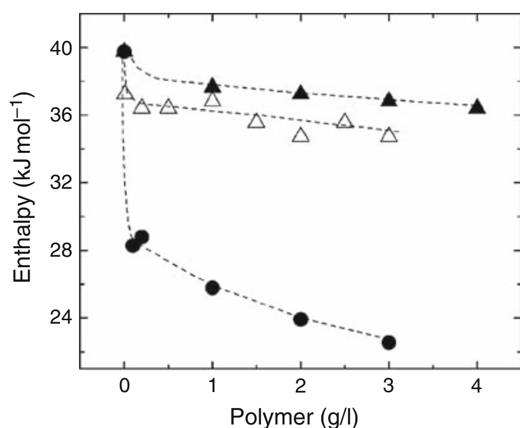


**Fig. 3** Phase transition enthalpy($\Delta H$) for DPPC in the presence of increasing polymer concentration of CH (*filled triangles*); $PEG_8$–CH (*open triangles*) and $PEG_{15}$–$C_{12}$–CH (*filled circles*) at pH 6.2 (phosphate buffer 50 mM)

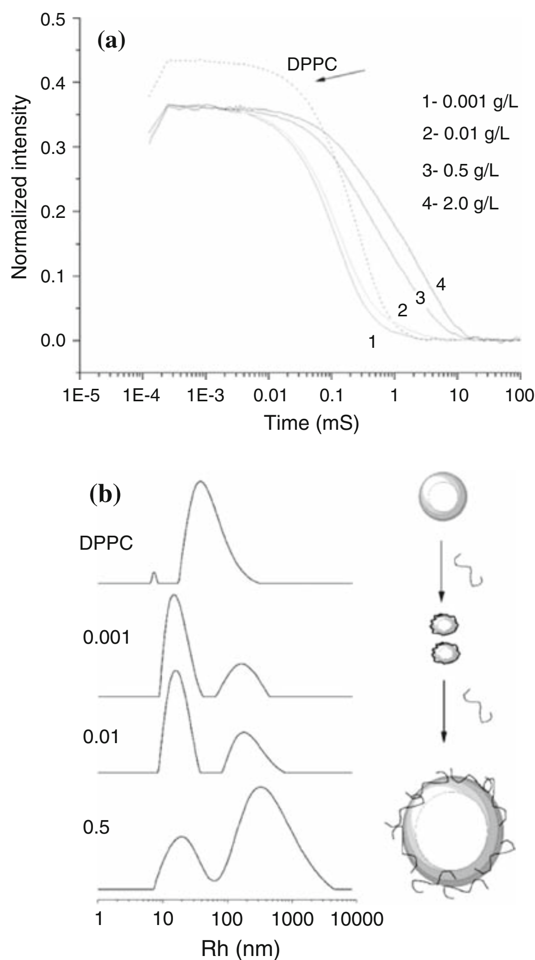


**Fig. 4** **a** Correlation functions for pure DPPC and in the presence of increasing $PEG_8$–CH concentration and **b** hydrodynamic radius (Rh) and structural changes suggested for DPPC vesicles as function of $PEG_8$–CH concentration

A similar result has been observed by Sabín et al. [26] working on liposomes formed by DPPC with the semifluorinated diblock F6H10, in the presence of $Gd^{3+}$, $Ca^{2+}$. These correlation functions were adjusted to bimodal distributions and as can be seen from Fig. 4b two peaks were obtained. The first peak, centered at 20 nm, is more prominent at low polymer concentration. It may be attributed to small vesicles (Rh ~20 nm) or bilayer fragments. The second peak is shifted to higher hydrodynamic radius and it becomes more prominent as the polymer concentration increases, indicating that this distribution corresponds to large vesicles (Rh ~400 to 500 nm) induced by the interaction with $PEG_8$–CH.

These results confirm that the addition of small amounts of chitosan induces the fragmentation of DPPC vesicles and the formation of smaller structures (Rh ~20 nm). As the polymer concentration increases these smaller structures reorganize to form large vesicles. This trend is clearly observed in Fig. 4b by comparing the relative proportion of the two distributions. A plot of Rh as a function of polymer concentration showed that the vesicles sizes remained constant (Rh ~450 nm) for $PEG_8$–CH concentration higher than 0.5 g/L (data not shown).

## Conclusions

The interaction of unilamellar DPPC vesicles with charged chitosan and its derivatives resulted in formation of large coated liposomes. The interaction was examined by differential scanning calorimetry and dynamic light scattering and the results showed that the interaction may take place by a partial penetration of chitosan backbone into the lipid bilayer. Chitosan derivative containing poly(ethylene glycol) attached to main chain showed a stronger interaction when compared to pure chitosan and more pronounced decreases in $\Delta H$ were obtained. The chitosan derivative containing dodecyl groups attached to chitosan chain was easily anchored into the vesicle bilayer decreasing the phase transition enthalpy by almost 50% as observed for the highest polymer concentration studied (3.0 g/L). The light scattering study indicated that at very low polymer concentration DPPC vesicles may be fragmented forming smaller structures. As the polymer concentration increases these structures start to grow resulting in formation of large vesicles, whose hydrodynamic sizes were around 500 nm. These results indicate that chitosan structure may be tailored to obtain coated liposomes of different sizes and surface properties.

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