Biophysical Chemistry 152 (2010) 74–79

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

The effect of cholesterol on the reconstitution of alkaline phosphatase into liposomes

M. Bolean [a], A.M.S Simão [a], B.Z. Favarin [a], J.L. Millán [b], P. Ciancaglini [a,*]

[a] Depto. Química, FFCLRP-USP, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[b] Sanford Children's Health Research Center, Sanford–Burnham Medical Research Institute, La Jolla, CA, USA

ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form 28 July 2010
Accepted 6 August 2010
Available online 14 August 2010

Keywords:
Matrix vesicles
GPI-anchored
Alkaline phosphatase
Cholesterol
Biomimetic system
Differential scanning calorimetry

ABSTRACT

Tissue-nonspecific alkaline phosphatase (TNAP), present on the surface of chondrocyte- and osteoblast-derived matrix vesicles (MVs), plays key enzymatic functions during endochondral ossification. Many studies have shown that MVs are enriched in TNAP and also in cholesterol compared to the plasma membrane. Here we have studied the influence of cholesterol on the reconstitution of TNAP into dipalmitoylphosphatidylcholine (DPPC)-liposomes, monitoring the changes in lipid critical transition temperature (T_c) and enthalpy variation (ΔH) using differential scanning calorimetry (DSC). DPPC-liposomes revealed a T_c of 41.5 °C and ΔH of 7.63 Kcal mol^{-1}. The gradual increase in cholesterol concentration decrease ΔH values, reaching a ΔH of 0.87 Kcal mol^{-1} for DPPC: cholesterol system with 36 mol% of cholesterol. An increase in T_c, up to 47 °C for the DPPC:cholesterol liposomes (36 mol% of Chol), resulted from the increase in the area per molecule in the gel phase. TNAP (0.02 mg/mL) reconstitution was done with protein:lipid 1:10,000 (molar ratio), resulting in 85% of the added enzyme being incorporated. The presence of cholesterol reduced the incorporation of TNAP to 42% of the added enzyme when a lipid composition of 36 mol% of Chol was used. Furthermore, the presence of TNAP in proteoliposomes resulted in a reduction in ΔH. The gradual proportional increase of cholesterol in liposomes results in broadening of the phase transition peak and eventually eliminates the cooperative gel-to-liquid-crystalline phase transition of phospholipids bilayers. Thus, the formation of microdomains may facilitate the clustering of enzymes and transporters known to be functional in MVs during endochondral ossification.

1. Introduction

Skeletal mineralization is a tightly regulated process in which specialized tissues, cells and organelles participate in the coordination and regulation of the metabolic events involved in the deposition of hydroxyapatite via the production of inducers and inhibitors of mineralization [1–3].

Chondrocytes and osteoblasts control the composition of the extracellular matrix and also release matrix vesicles (MVs), considered by some investigators to serve as the initial sites of hydroxyapatite (HA) mineral formation [4–7]. MVs are small (20–200 nm) spherical bodies observed in the pre-mineralized matrix of dentin, cartilage and bone. They appear to be bound by a lipid bilayer and are often found associated with small crystals of calcium phosphate mineral [4–6,8].

MVs are enriched in membrane microdomains containing high levels of cholesterol [9], glycosylphosphatidylinositol (GPI)-anchored enzymes, e.g., tissue-nonspecific alkaline phosphatase (TNAP) [10–14], metaloproteinases [15], caveolin-1 [16], and contain a number of other enzymes and ion transporters functional during mineralization, including adenosine-5′-triphosphatase (ATPase), adenosine-5′-monophosphatase (AMPase), inorganic pyrophosphatase (PPiase), nucleoside triphosphate pyrophosphohydrolase (NPP1), [17–23], Pi-transporters [9,24], and Anexins [25]. TNAP is localized on the external surface of MVs in direct contact with extracellular cartilage fluid, where it can act on its natural substrates, ATP and PPi, present at nanomolar/micromolar concentrations [10–12,26–28]. The GPI structure is a phosphatidylinositol-glycolipid anchor that is covalently attached to the carboxyl terminus (C-terminus) of TNAP through an amide linkage. This anchor structure results in lateral mobility in the membrane and allows the release of the enzyme from the membrane through the action of phospholipases [10–13,23,28–34].

The organization of biological membranes into microdomains is believed to play a key role in several cellular processes such as protein targeting and signal transduction. The existence of these microdomains, is explained mainly by the lateral phase separation of membrane lipids in a fluid liquid-crystalline phase (L_α) and a liquid ordered phase (Lo) rich in cholesterol and sphingolipids [35,36]. When cholesterol was mixed with lipids containing saturated acyl chains having high T_c such as dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SM) in model membranes, the formation of a phase state similar to gel state, tightly packed and ordered, was observed but the lateral motion was almost as rapid as in the disordered liquid (Lα) state. This cholesterol-containing state was eventually named liquid ordered (Lo) state [37].

Sterols are essential components in the membranes of eukaryotic cells. In mammalian cell membranes, cholesterol is the main sterol. It often constitutes around one third of the total lipid mass of the plasma membrane [38]. Detergent-resistant plasma membranes (DRMs)

* Corresponding author. Depto Química, FFCLRP-USP, Av. Bandeirantes, 3900, 14040-901, Ribeirão Preto, SP, Brazil. Fax: +55 16 36024838.
E-mail address: pietro@ffclrp.usp.br (P. Ciancaglini).

0301-4622/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2010.08.002

from chondrocytes are enriched in Ganglioside I (GM1) and cholesterol, as well as in GPI-anchored TNAP [9,14]. Furthermore, these membrane domains are enriched in phosphatidylserine (PS), localized to the external leaflet of the bilayer, and have significantly higher TNAP activity than non-cholesterol enriched domains [4,8].

A number of studies have been performed to determine the effects of cholesterol on the structural and dynamic properties of synthetic and natural membranes [39–42]. These studies revealed that even small quantities of cholesterol can affect membrane dynamics. Cholesterol, besides being present in microdomains, acts as a modulator of the physical properties and lateral organization of the plasma membrane lipid bilayer [43–45]. Milhiet et al. [46], using atomic force microscopy, studied the preferential localization of bovine intestinal alkaline phosphatase through its GPI anchor inside lipid ordered domains constituted by dioleoylphosphatidylcholine (DOPC) and sphingomyelin (SM), with and without cholesterol, and found that its localization was markedly increased in the microdomains rich in cholesterol.

Here we report the production of a cholesterol-containing proteoliposome harboring TNAP and the biophysical characterization of this vesicular MV biomimetic system by differential scanning calorimetry (DSC).

2. Materials and methods

2.1. Materials

All aqueous solutions were made using Millipore DirectQ ultra pure apyrogenic water. Bovine serum albumin (BSA), Tris hydroxymethyl-amino-methane (Tris), 2-amino-2-methyl-propan-1-ol (AMPOL), trichloroacetic acid (TCA), adenosine 5′-triphosphate disodium salt (ATP), sodium dodecylsulfate (SDS), p-nitrophenyl phosphate disodium salt (PNPP), dexamethasone, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, β-glycerophosphate, polyoxyethylene-9-lauryl ether (polidocanol), α-naphthyl phosphate, Fast Blue RR were from Sigma Chemical Co. (St Louis, MO, USA); sodium chloride and magnesium chloride were from Merck (São Paulo, SP, Brazil). 75 cm^2 plastic culture flasks were from Corning (Cambridge, MA, USA). α-MEM, fetal bovine serum, ascorbic acid, gentamicin and fungizone were from Gibco-Life Technologies (Grand Island, NY, USA). Phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus thuringiensis* was obtained from Oxford University (UK). Analytical grade reagents were used without further purification.

2.2. Rat bone marrow cell isolation and culture

Cells were prepared and cultured according to Simão et al. [11]. Bone marrow was obtained from young adult male rats of the Wistar strain weighing 110–120 g. The femora were excised aseptically, cleaned of soft tissues, and washed 3 times, 15 min each in culture medium containing 10 times the usual concentration of antibiotics (see below). The epiphyses of femora were cut off and the marrow flushed out with 20 mL of culture medium. Bone marrow cells released were collected in a 75 cm^2 plastic culture flask containing 10 mL of culture medium composed by α-MEM, supplemented with 15% fetal bovine serum, 50 μg/mL gentamicin, 0.3 μg/mL fungizone, 10^{-7} M dexamethasone, 5 μg/mL ascorbic acid and 2.16 mg/mL β-glycerophosphate. Cells were cultured for 14 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. The cultures were observed and evaluated under an inverted phase microscope after 24 h, 4 days, 10 days and 14 days.

2.3. Preparation of membrane-bound alkaline phosphatase

Membrane-bound alkaline phosphatase, an osteoblast-specific marker, was prepared from cell culture as described by Simão et al. [11,12]. The cells were washed with 50 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, removed with a spatula and resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgSO_4$ and 0.8 M NaCl (osmotic buffer).

The cell suspension was homogenized using a "potter system" for gentle cell disruption, at 4 °C for 15 min, centrifuged at 1000×*g* for 3 min and finally the supernatant was centrifuged at 100,000×*g* for 1 h at 4 °C. The pellet corresponding to membrane-bound alkaline phosphatase, was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, frozen in liquid nitrogen and stored at −20 °C.

2.4. Estimation of protein

Protein concentrations were estimated according to Hartree [47] in the presence of 2% (w/v) SDS. Bovine serum albumin was used as standard.

2.5. Enzymatic activity measurements

p-Nitrophenylphosphatase (p-NPPase) activity was assayed discontinuously at 37 °C in a Spectronic (Genesys 2) spectrophotometer by following the liberation of p-nitrophenolate ion (1 M, pH 13 = 17,600 M^{-1} cm^{-1}), at 410 nm. Standard conditions were 50 mmol/L AMPOL buffer, pH 10.0, containing 2 mmol/L $MgCl_2$ and 1 mmol/L p-NPP in a final volume of 1.0 mL, as previously described [11,12].

All determinations were carried out in duplicate and the initial velocities were constant for at least 90 min provided that less than 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to allow for the non-enzymatic hydrolysis of substrate. One enzyme unit (1 U) is defined as the amount of enzyme hydrolyzing 1.0 nmol of substrate per min at 37 °C per mL or mg of protein.

2.6. Solubilization of alkaline phosphatase with polyoxyethylene 9-lauryl ether

Membrane-bound alkaline phosphatase (0.02 mg/mL of total protein) was solubilized with 1% polidocanol (w/v) (final concentration) for 1 h with constant stirring at 25 °C. After centrifugation at 100,000×*g* for 1 h at 4 °C, the solubilized enzyme was concentrated as described by Ciancaglini et al. [13]. To remove excess detergent, 1 mL of polidocanol-solubilized enzyme (~0.05 mg protein/mL) was added to 200 mg of Calbiosorb resin as described by Camolezi et al. [48] and Simão et al. [23] and the suspension was mixed for 2 h at 4 °C. The supernatant is the source of detergent-free, solubilized enzyme.

2.7. Liposome preparation

Dipalmitoylphosphatidylcholine (DPPC) and cholesterol were dissolved in chloroform and dried under nitrogen flow. The resulting lipid film was kept under vacuum overnight and resuspended in 50 mmol/L Tris–HCl buffer, pH 7.5, containing 2 mmol/L $MgCl_2$. The mixture was incubated for 1 h at 60 °C, above the critical phase transition temperature of the lipid, and vortexed for each 10 min. Large unilamellar vesicles (LUVs) were prepared by submitting the suspension to extrusion (eleven times) through two 100 nm polycarbonate membranes in a LiposoFast extrusion system (Liposofast, Sigma-Aldrich). Alternatively, when necessary, LUVs were prepared with different diameters using 200 or 400 nm polycarbonate membranes. LUVs constituted by DPPC:cholesterol in a 10 to 36 mol% of cholesterol were prepared and used in the same day.

2.8. Incorporation of alkaline phosphatase into liposomes

Equal volumes of liposomes (10 mg/mL) and TNAP (0.02 mg/mL) resulting in a 1:10,000 protein:lipid ratio, in 50 mmol/L Tris–HCl buffer, pH 7.5, containing 2 mmol/L $MgCl_2$, were mixed and incubated at 25 °C

during 1 h and the sample was centrifuged at 150,000×*g* for 20 min. The pellet was resuspended in 50 mmol/L Tris–HCl buffer, pH 7.5, containing 2 mmol/L $MgCl_2$, to the original volume. Alkaline phosphatase activity of the supernatant and the resuspended pellet was assayed and used to calculate the percent of protein incorporation [23].

2.9. Dynamic light scattering measurements

The determination of liposomes size distribution was performed by dynamic light scattering, using a N5 Submicron Particle Size Analyser (Beckman Coulter, Inc., Fullerton, CA, USA). Average value (n = 5) of the liposomes diameters was obtained at 25 °C by unimodal distribution, previously filtered (0.8 μm).

2.10. Differential scanning calorimetry

Transition phase temperatures (Tc) of the LUVs membranes prepared with different lipid compositions were studied by differential scanning calorimetry (DSC). All LUVs suspensions and reference buffer employed in the experiment were previously degasified under vacuum (140 mbar) during 15 min.

The samples were scanned from 10 °C to 90 °C at an average heating rate of 0.5 °C/min and the recorded thermograms were analyzed using a Nano-DSC II — Calorimetry Sciences Corporation, CSC (Lindon, Utah, USA). A minimum of at least three heating and cooling scans were performed for each analysis and all thermograms were reproducible. In order to ensure homogeneity in the analysis of the effect of the insertion of the enzyme and presence of cholesterol on the lipid phase transitions, we have chosen the simplest baseline correction to introduce the least amount of variability when comparing thermograms from different sets of experiments.

2.11. Enzymatic release of alkaline phosphatase

Proteoliposomes harboring TNAP were incubated in 50 mmol/L Tris–HCl buffer, pH 7.5 with specific phosphatidylinositol phospholipase C (0.1 U of PIPLC from *B. thuringiensis*) for 1 h under constant rotary shaking, at 37 °C. The incubation mixture was centrifuged at 100,000×*g* for 1 h, at 4 °C. The supernatant was the source of enzymatically released TNAP that was concentrated as previously described [13].

3. Results and discussion

3.1. Effect of cholesterol concentration in the thermotropic behavior of DPPC-liposomes

Liposomes constituted by DPPC alone or by DPPC:cholesterol mixtures, with increasing proportions of cholesterol (mol%), were prepared by the extrusion method using a polycarbonate membrane of 100 nm. For the preparation of the liposomes, proportions of cholesterol ranging from 10 to 36 mol% were used, with a tendency for separation of cholesterol monohydrate microcrystals at higher cholesterol concentrations. A study showed that the maximum solubility of cholesterol in bilayers of various saturated and monounsaturated phosphatidylcholines was 67 mol%, as compared to only 51 mol% in phosphatidylethanolamine bilayers [49]. Furthermore, microdomains with cholesterol concentrations higher than 50% (molar ratio) are not detectable by DSC [39].

The different liposomes preparations were analyzed by DSC. As shown on the thermograms of Fig. 1, there is a progressive broadening of the transition peaks due to the cholesterol concentration increase in the vesicular systems. These broadening occur due to a decrease of the phase transition cooperativity caused by the presence of high concentrations of this sterol. This behavior was also observed by Halling and Slotte [50] when using cholesterol concentrations up to 25% (molar ratio). Furthermore, at the concentration of 18 mol% of cholesterol, one can observe a phase lateral segregation evidenced by a large shoulder to the right of the main transition peak, which suggests the formation of cholesterol-rich microdomains.

One of the major effects of cholesterol incorporation into phospholipid monolayer and bilayer model membranes is a broadening and eventual elimination of the cooperative gel-to-liquid-crystalline phase transition and its replacement by a phase with an intermediate degree of organization. Thus, in the liquid-crystalline phase, which would exist at physiological temperatures in the absence of sterols in biological membranes, the presence of cholesterol significantly increases the orientational order of the phospholipid hydrocarbon chains and decreases the cross-sectional area occupied by the phospholipid molecules. In addition, the presence of cholesterol increases both the thickness and the mechanical strength while decreasing the permeability of the phospholipid bilayer in the physiologically relevant liquid-crystalline phase [51].

The effects of increasing cholesterol concentrations on the thermodynamic parameters of the liposomes are shown in Fig. 2, where one can observe an increase in the average diameter of the liposomes constituted by DPPC:cholesterol (Fig. 2A), when compared to DPPC-only vesicles. It is important to note that low polydispersion index (PI) values were obtained for all liposome preparation indicating that the samples were homogeneous. An increase in the transition temperature of the liposomes with increasing cholesterol concentrations is observed in Fig. 2B. Addition of cholesterol causes an increase in the area occupied per molecule in monolayers in gel phase, thus increasing lipid/lipid interactions, which results in the increase of the transition temperature of the binary system. Furthermore, DPPC has a cylindrical geometry and, due to its curvature radius, DPPC-liposomes present a spherical vesicular shape. This shape is appropriate for cholesterol insertion in the spaces between DPPC molecules, since cholesterol has a conical geometry. We observed a decrease of the enthalpy values with increasing cholesterol concentrations in the liposomes (Fig. 2C), suggesting that cholesterol makes the lipid systems more fluid [50].

Other effects of cholesterol are: increase of the area occupied per molecule in monolayers in gel phase and the inverse effect for the fluid phase; decrease of the orientational order of the phospholipid hydrocarbon chains in gel phase (increase in the number of *gauche* conformations) and the inverse effect for the fluid phase; increase of the

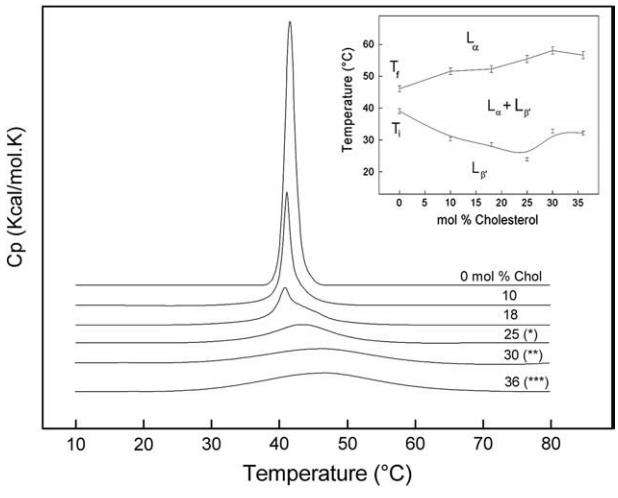

Fig. 1. DSC thermograms of liposomes 10 mg/mL. Differential scanning calorimetry thermograms were registered using a Nano-DSC II calorimeter, and processed in Excess heat capacity, Cp (kcal/K mol) as function of temperature (°C) of liposomes constituted by DPPC and cholesterol in different mol% proportion. The exothermic flow values are normalized and are multiplied by a factor of 1.7 (*), 3 (**) or 4 (***) where indicated, in order for small exothermic peaks to be visible. Inset: phase diagram representing the initial (T_i) and final (T_f) temperature of phase transition. With increasing cholesterol concentration there is a greater range where both phases coexist: gel phase ($L_{\beta'}$) and liquid-crystalline phase (L_α).

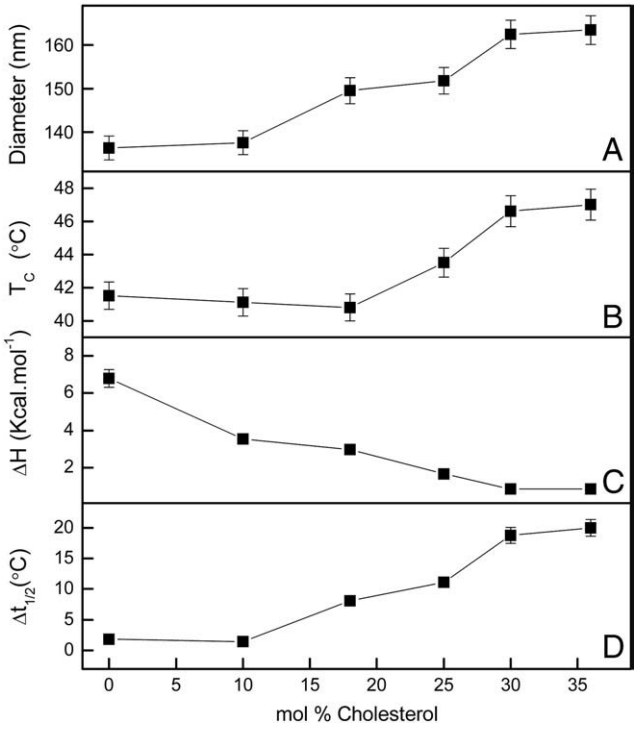

Fig. 2. Effect of cholesterol (Chol) concentration in the thermodynamic parameters of DPPC-liposome: (A) vesicle diameters; (B) transition temperature (T_c); (C) enthalpy variation (ΔH) and (D) cooperativity ($\Delta t_{1/2}$).

passive permeability of the phospholipid bilayer in the gel phase and the inverse effect for the fluid phase. It is clear that these effects are correlated with the thermotropic changes described above [52].

When the cholesterol concentration was restricted to 10 mol%, we observed a slight increase in the cooperativity of the phase transition, shown as a decrease in $\Delta t_{1/2}$ values, from 1.81 °C to 1.48 °C (Fig. 2D), suggesting that, in low concentrations, cholesterol is able to stabilize the vesicles due to its conical geometry that is appropriate for its interaction with lipid bilayers. For concentrations above 10 mol%, $\Delta t_{1/2}$ values

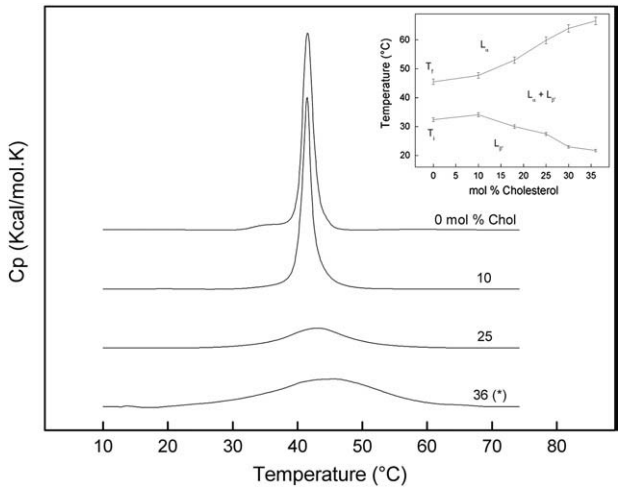

Fig. 3. DSC thermograms of proteoliposomes 10 mg/mL. Differential scanning calorimetry thermograms were registered using a Nano-DSC II calorimeter, and processed in Excess heat capacity, Cp (kcal/K mol) as function of temperature (°C) of liposomes constituted by DPPC and cholesterol in different mol% proportion. The exothermic flow values are normalized and are multiplied by a factor of 5 (*) where indicated, in order for small exothermic peaks to be visible. Inset: phase diagram representing the initial (T_i) and final (T_f) temperature of phase transition. With increasing cholesterol concentration and the presence of the enzyme there is a greater range where both phases coexist: gel phase ($L_{\beta'}$) and liquid-crystalline phase (L_α).

increased significantly, pointing to a decrease in the cooperativity of the phase transition with the increase of the sterol concentration in the vesicular systems.

The gradual proportional increase of cholesterol in liposomes results in broadening of the phase transition peak and the formation of microdomains [39].

3.2. Effect of cholesterol concentration in the thermotropic behavior of DPPC-proteoliposomes

The thermotropic behavior of TNAP proteoliposomes systems (Fig. 3) is similar to that of liposomes alone (Fig. 1), showing similar broadening of phase transitions with the increase in the cholesterol concentration. However, the presence of the enzyme in the vesicular system causes significant changes in the thermodynamic behavior of the systems. We also studied the influence of cholesterol on the incorporation of TNAP into the liposomes, as shown in Fig. 4. In the absence of cholesterol, we observed an incorporation of 85% of enzyme activity for DPPC-liposomes. With the increase on cholesterol concentration in the vesicles (Fig. 4A), we observed a negative effect on the incorporation of the enzyme activity; incorporation reached only 42% of the added enzyme when a lipid composition of 36 mol% of Chol was used.

Furthermore, we observed an increase in the average of the diameter (Fig. 4B), an increase in T_C (Fig. 4C) similar to that found for the liposomes system, a strong decrease in ΔH values (Fig. 4D) and a significant increase in $\Delta t_{1/2}$ values (Fig. 4E) with increasing cholesterol concentrations (Fig. 4A).

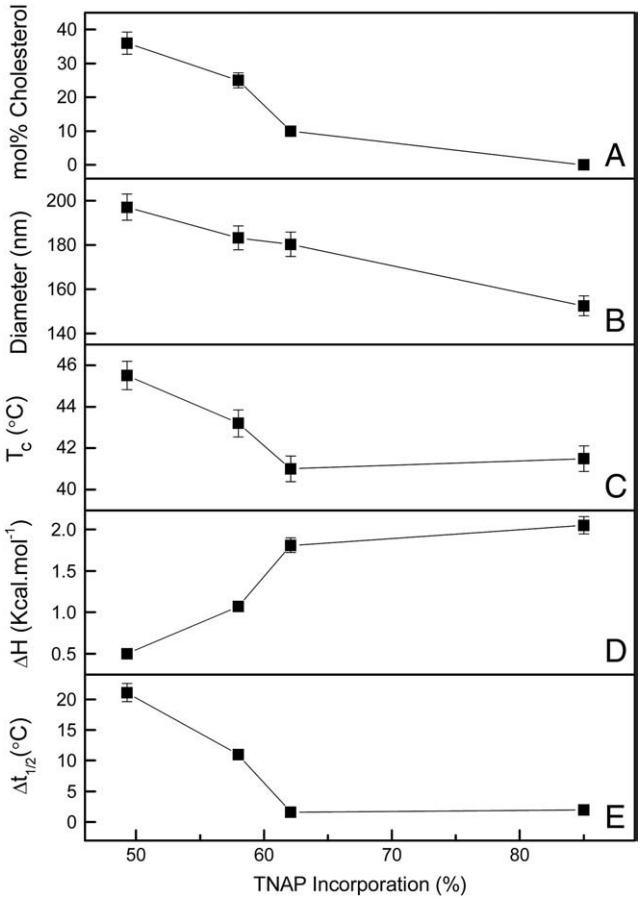

Fig. 4. Effect of the TNAP incorporation in the thermodynamic parameters of DPPC-proteoliposomes in: (A) concentration cholesterol (mol%); (B) diameter (nm); (C) transition temperature (T_c); (D) enthalpy variation (ΔH) and (E) cooperativity ($\Delta t_{1/2}$). 0% cholesterol corresponds to DPPC-proteoliposomes prepared in the absence of cholesterol. For this system, an enzyme incorporation of 85% was observed. The amount of TNAP corresponding to 100% is 0.02 mg/mL.

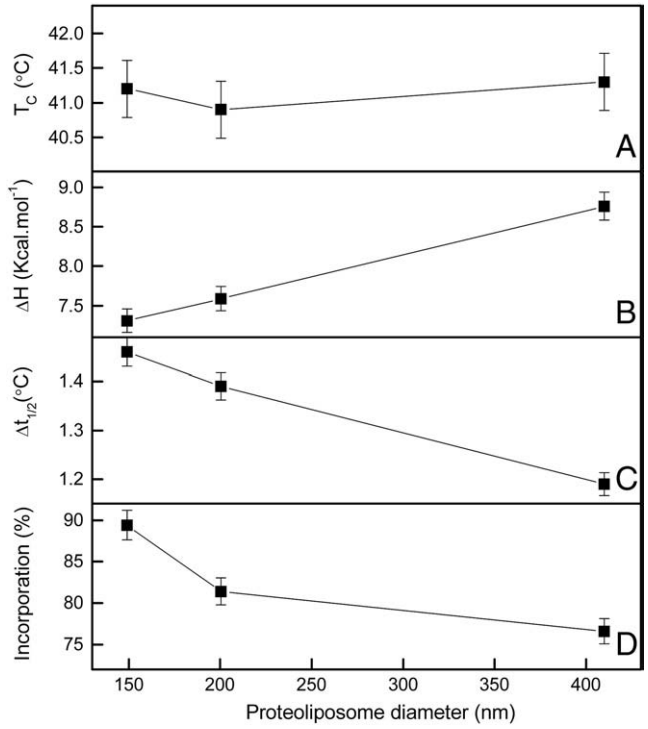

Fig. 5. Effect of DPPC-proteoliposome diameter in the thermodynamic parameters: (A) transition temperature (T_c); (B) enthalpy variation (ΔH); (C) cooperativity ($\Delta t_{1/2}$) and (D) percentage of alkaline phosphatase incorporation.

It is important to note that the presence of TNAP causes extensive changes in the absolute values of the thermodynamic parameters of the proteoliposomes compared to the respective liposomes. Comparison of the values obtained in Figs. 2 and 4 for the DPPC-only vesicular systems (liposome and proteoliposome, respectively) indicates a considerable reduction in ΔH values (from 7.63 to 2.05 kcal mol^{-1}) and an increase in $\Delta t_{1/2}$ values (from 1.81 to 2.00 °C). These data show that the presence of the enzyme makes the phase transitions of the vesicular systems less cooperative. A decrease in ΔH values and an increase in $\Delta t_{1/2}$ values were seen upon increasing the cholesterol concentrations, and these effects were even more pronounced in the presence of TNAP. Thus, enzyme incorporation influences cooperativity and induces further changes in Tc and calorimetric enthalpy by affecting lipid–lipid interactions.

The incorporation of TNAP into the liposomes caused an increase in the average diameter of the vesicles (Fig. 4B). To verify if this change in the average diameters of the vesicles might influence the incorporation of GPI-anchored TNAP into the liposomes, we carried out experiments where the diameter of DPPC-only vesicles was varied. The different sized proteoliposomes were analyzed by DSC and, as shown in Fig. 5, present the same thermodynamic profile when compared to the liposomes. We observed a small decrease (about 15%) in the incorporation of the enzyme activity with the increase of the diameter of the vesicles formed (Fig. 5D), probably related to the increase in the packing of the system, resulting in the increase of ΔH (reduction of the area occupied by each lipid), suggested by a more cooperative phase transition (reduction in $\Delta t_{1/2}$, see Fig. 5C). Thus, it is evident that the increase of vesicles size, caused by the gradual increase in the cholesterol concentration in the liposome systems, results in a slight effect in the incorporation of the enzyme, only partly explaining the reduction in TNAP incorporation. This reduction is attributed mainly to the presence of cholesterol in the DPPC vesicles, which results in a reduction in ΔH values and broadening of the phase transition peak, although with higher steric hindrance and mechanic strength for GPI insertion, resulting in the reduction of enzyme incorporation (Fig. 4).

Furthermore, membrane features, such as curvature and lipid composition, can affect enzymatic activity in different ways, depending on the substrate used [48,53]. Sesana et al. [53], using cosolubilization method in the formation of proteoliposomes harboring human placental alkaline phosphatase, demonstrated a strong inverse correlation between activity and palmitoyloleoylphosphatidylcholine (POPC)-liposome diameter. The activity-membrane curvature relationship was further confirmed by comparing the activity of proteoliposomes having different sizes but identical lipid compositions [53].

Table 1
Thermodynamic parameters of DPPC (10 mg/mL) liposomes and proteoliposomes containing TNAP after PIPLC treatment.

Vesicles	PIPLC treatment	ΔH (Kcal mol^{-1})	T_c (°C)	$\Delta t_{1/2}$ (°C)
Liposome	–	7.63 ± 0.4	41.5 ± 1.5	1.81 ± 0.1
Proteoliposome	No	2.42 ± 0.1	41.3 ± 1.0	2.21 ± 0.1
	Yes	1.88 ± 0.2	41.3 ± 0.9	1.88 ± 0.2

3.3. Thermotropic behavior of DPPC-proteoliposomes after treatment with PIPLC

So far we have shown that the insertion of the enzyme in the liposome increases its fluidity, however it is not clear what provokes this sharp decrease in ΔH's values and increase in $\Delta T_{1/2}$ values; if it is the presence of the enzyme or simply the insertion of its anchor into the vesicular system. Basically, the GPI anchor is covalently bonded to the C-terminal portion of the protein and consists of a glycan chain linked to a phosphatidylinositol with two acyl chains anchored into the bilayer [28,30,32,54].

In order to examine this issue, the DPPC constituted proteoliposomes were incubated with PIPLC from *B. thuringiensis*, which cleaves specifically GPI anchors, allowing a selective release of the TNAP protein chain to the solution [13,23]. This treatment results in vesicular systems where the GPI anchor remains inserted. The three distinct DPPC constituted systems (liposome, proteoliposome and vesicles containing the inserted GPI anchor) were analyzed by DSC. Table 1 shows a pronounced decrease in ΔH value when liposomes systems (7.63 Kcal mol^{-1}) are compared with the proteoliposomes (2.42 Kcal mol^{-1}). However, the ΔH value was not significantly changed after treatment of the proteoliposomes with PIPLC (1.88 Kcal mol^{-1}).

Hence we conclude that the intense decrease observed for ΔH value of proteoliposomes when compared to liposomes is related mainly to the GPI anchor insertion. The presence of the TNAP polypeptide chain does not have a significant effect on the ΔH. The formation of microdomains may facilitate the clustering of enzymes and transporters known to be functional in MVs during endochondral ossification.

Acknowledgements

The authors thank Priscila Cerviglieri for the linguistic advice. We also thank FAPESP, CAPES and CNPq for the financial support given to our laboratory. MB and BZF received a CAPES and CNPq scholarship, respectively.

References

[1] C.M. Giachelli, Inducers and inhibitors of biomineralization: lessons from pathological calcification, Orthod. Craniofac. Res. 8 (2005) 229–231.
[2] H.C. Anderson, R. Garimella, S.E. Tague, The role of matrix vesicles in growth plate development and biomineralization, Front. Biosci. 10 (2005) 822–837.
[3] M.J. Glimcher, Bone: nature of the calcium phosphate crystals and cellular, structural, and physical chemical mechanisms in their formation, Rev. Mineral. Geochem. 64 (2006) 223–282.
[4] R.E. Wuthier, Lipids of matrix vesicles, Fed. Proc. 35 (1976) 117–121.
[5] H.C. Anderson, Mineralization by matrix vesicles, Scan. Electron Microsc. (Pt 2) (1984) 953–964.
[6] H.C. Anderson, Matrix vesicles and calcification, Curr. Rheumatol. Rep. 5 (2003) 222–226.

[7] C. Thouverey, A. Strzelecka-Kiliszek, M. Belcerzak, R. Buchet, S. Pikula, Matrix vesicles originate from apical membrane microvilli of mineralizing osteoblast-like Saos-2 cells, J. Cel. Biochem. 106 (2009) 127–138.
[8] E.E. Golub, Role of matrix vesicles in biomineralization, Biochen. at Biophys. Acta. 1790 (2009) 1592–1598.
[9] M. Damek-Poprawa, E.E. Golub, L. Otis, G. Harrison, C. Phillips, K. Boesze-Battaglia, Chondrocytes utilize a cholesterol-dependent lipid translocator to externalize phosphatidylserine, Biochem. 45 (2006) 3325–3336.
[10] J.L. Millán, Mammalian Alkaline Phosphatase: From Biology to Applications in Medicine and Biotechnology, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2006.
[11] A.M.S. Simão, M.M. Beloti, R.M. Cezarino, A.L. Rosa, J.M. Pizauro, P. Ciancaglini, Membrane-bound alkaline phosphatase from ectopic mineralization and rat bone marrow cell culture, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146 (2007) 679–687.
[12] A.M.S. Simão, M.M. Beloti, A.L. Rosa, P.T. de Oliveira, J.M. Granjeiro, J.M. Pizauro, P. Ciancaglini, Culture of osteogenic cells from human alveolar bone: a useful source of alkaline phosphatase, Cell Biol. Int. 31 (2007) 1405–1413.
[13] P. Ciancaglini, A.M.S. Simão, F.L. Camolezi, J.L. Millán, J.M. Pizauro, Contribution of matrix vesicles and alkaline phosphatase to ectopic bone formation, Braz. J. Med. Biol. Res. 39 (2006) 603–610.
[14] D.C. Morris, K. Masuhara, K. Takaoka, K. Ono, H.C. Anderson, Immunolocalization of alkaline phosphatase in osteoblasts and matrix vesicles of human fetal bone, Bone. Min. 19 (1992) 287–298.
[15] D.D. Dean, Z. Schwartz, O.E. Muniz, R. Gomez, L.D. Swain, D.S. Howell, B.D. Boyan, Matrix vesicles are enriched in metalloproteinases that degrade proteoglycans, Calcif. Tissue Int. 50 (1992) 342–349.
[16] N. Sawada, Y. Taketani, N. Amizuka, M. Ichikawa, C. Ogawa, K. Nomoto, K. Nashiki, T. Sato, H. Arai, M. Isshiki, H. Segawa, H. Yamamoto, K. Miyamoto, E. Takeda, Caveolin-1 in extracellular matrix vesicles secreted from osteoblasts, Bone 41 (2007) 52–58.
[17] H.C. Anderson, Molecular biology of matrix vesicles, Clin. Orthop. Rel. Research. 314 (1995) 266–280.
[18] H.H.T. Hsu, H.C. Anderson, A role for ATPase in the mechanisms of ATP-dependent Ca and phosphate deposition by isolated rachictic matrix vesicles, Int. J. Biochem. 27 (1995) 1349–1356.
[19] H.H.T. Hsu, H.C. Anderson, Evidence of the presence of a specific ATPase responsible for ATP-initiated calcification by matrix vesicles isolated from cartilage and bone, J. Biol. Chem. 271 (1996) 26383–26388.
[20] H.C. Anderson, J.B. Sipe, L. Hessle, R. Dhamyamraju, E. Atti, N.P. Camacho, J.L. Millán, Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice, Am. J. Pathol. 164 (2004) 841–847.
[21] H.C. Anderson, R. Garimella, S.E. Tague, The role of matrix vesicles in growth plate development and biomineralization, Front. Biosci. 10 (2005) 822–837.
[22] P. Ciancaglini, M.C. Yadav, A.M.S. Simão, S. Narisawa, J.M. Pizauro, C. Farquharson, M.F. Hoylaerts, J.L. Millán, Kinetic analysis of substrate utilization by native and TNAP-, NPP1- or PHOSPHO1-deficient matrix vesicles, J. Bone. Miner. Research. 25 (2010) 716–726.
[23] A.M.S. Simão, M.C. Yadav, S. Narisawa, M. Bolean, J.M. Pizauro, M.F. Hoylaerts, P. Ciancaglini, J.L. Millán, Proteoliposomes harboring alkaline phosphatase and nucleotide pyrophosphatase as matrix vesicles' biomimetics, J. Biol. Chem. 285 (2010) 7598–7609.
[24] D.H. Solomon, R.J. Wilkins, D. Meredith, J.A. Browning, Characterization of inorganic phosphate transport in bovine articular chondrocytes, Cell. Physiol. Biochem. 20 (2007) 99–108.
[25] R.E. Wuthier, L.N. Wu, G.R. Sauer, B.R. Genge, T. Yoshimori, Y. Ishikawa, Mechanism of matrix vesicle calcification: characterization of ion channels and the nucleational core of growth plate vesicles, Bone Miner. 17 (1992) 290–295.
[26] S.A. Siegel, C.F. Hummel, R.P. Carty, The role of nucleoside triphosphate pyrophosphohydrolase in in vitro nucleoside triphosphate-dependent matrix vesicle calcification, J. Biol. Chem. 258 (1983) 8601–8607.
[27] T. Matsuzawa, H.C. Anderson, Phosphatases of epiphyseal cartilage studied by electron microscopic cytochemical methods, J. Histochem. Cytochem. 12 (1971) 801–808.
[28] J.M. Pizauro, P. Ciancaglini, F.A. Leone, Characterization of the phosphatidylinositol-specific phospholipase C-released form of rat osseous plate alkaline phosphatase and its possible significance on endochondral ossification, Mol. Cel. Biochem. 152 (1995) 121–129.
[29] M.G. Low, J.B. Finean, Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C, Biochim. Biophys. Acta 508 (1978) 565–570.
[30] M.G. Low, M.A.J. Ferguson, A.H. Futerman, I. Silman, Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins, Trends Biochem. Sci. 11 (1986) 212–215.
[31] M.G. Low, A.H. Futerman, K.E. Ackermann, W.R. Sherman, I. Silman, Removal of covalently bound inositol from torpedo acetylcholinesterase and mammalian alkaline phosphatases by deamination with nitrous acid. Evidence for a common membrane-anchoring structure, Biochem. J. 241 (1987) 615–619.
[32] M.G. Low, A.R. Saltiel, Structural and functional roles of glycosylphosphatidylinositol in membranes, Science 239 (1988) 268–275.
[33] G. Harrison, I.M. Shapiro, E.E. Golub, The phosphatidylinositol-glycolipid anchor on alkaline phosphatase facilitates mineralization initiation in vitro, J. Bone Miner. Res. 4 (1995) 568–573.
[34] F.A. Leone, J.M. Pizauro, P. Ciancaglini, Rat osseous plate alkaline phosphatase: a search for its role in biomineralization, Trends in Comp. Biochem. Physiol. 3 (1997) 57–73.
[35] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221–17224.
[36] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[37] E. London, How principles of domain formation in model membranes may explain ambiguities concerning lipid raft formation in cells, Biochim. Biophys. Acta 1746 (2005) 203–220.
[38] Y. Lange, M.H. Swaisgood, B.V. Ramos, T.L. Steck, Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts, J. Biol. Chem. 264 (1989) 3786–3793.
[39] T.P. McMullen, R.N. Lewis, R.N. McElhaney, Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines, Biochem. 32 (1993) 516–522.
[40] T.P. McMullen, C. Vilchèze, R.N. McElhaney, R. Bittman, Differential scanning calorimetric study of the effect of sterol side chain length and structure on dipalmitoylphosphatidylcholine thermotropic phase behavior, Biophys. J. 69 (1995) 169–176.
[41] S.A. Pandit, D. Bostick, M.L. Berkowitz, Complexation of phosphatidylcholine lipids with cholesterol, Biophys. J. 86 (2004) 1345–1356.
[42] F. de Meyer, B. Smit, Effect of cholesterol on the structure of a phospholipid bilayer, Proc. Natl Acad. Sci. USA 106 (2009) 3654–3658.
[43] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[44] Y. Barenholz, Cholesterol and other membrane active sterols: from membrane evolution to "rafts", Prog. Lipid Res. 41 (2002) 1–5.
[45] Z. Korade, A.K. Kenworthy, Lipid rafts, cholesterol, and the brain, Neuropharmacology 55 (2008) 1265–1273.
[46] P.E. Milhiet, M.C. Giocondi, O. Baghdadi, F. Ronzon, B. Roux, C. Le Grimellec, Spontaneous insertion and partitioning of alkaline phosphatase into model lipid rafts, EMBO report 3 (2002) 485–490.
[47] E.F. Hartree, Determination of protein: a modification of the Lowry method that gives a linear photometric response, Anal. Biochem. 48 (1972) 422–427.
[48] F.L. Camolezi, K.P.R. Daghastanli, P.P. Magalhães, J.M. Pizauro, P. Ciancaglini, Construction of an alkaline phosphatase-liposome system: a tool for biomineralization study, Int. J. Biochem. Cell Biol. 1282 (2002) 1–11.
[49] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[50] K.K. Halling, J.P. Slotte, Membrane properties of plant sterols in phospholipid bilayers as determined by differential scanning calorimetry, resonance energy transfer and detergent-induced solubilization, Biochim. Biophys. Acta 1664 (2004) 161–171.
[51] D.A. Mannock, R.A.H. Lewis, R.N. McElhaney, Comparative calorimetric and spectroscopic studies of the effects of lanosterol and cholesterol on the thewrmotropic phase behavior and organization of dipalmitoylphosphatidylcholine bilayer membranes, Biophys. J. 91 (2006) 3327–3340.
[52] R.B. Gennis, Biomembranes: Molecular Structure and Function, Springer-Verlag, New York, 1989.
[53] S. Sesana, F. Re, A. Bulbarelli, D. Salerno, E. Cazzaniga, M. Masserini, Membrane features and activity of GPI-anchored enzymes: alkaline phosphatase reconstituted in model membranes, Biochem. 47 (2008) 5433–5440.
[54] F. Ronzon, S. Morandat, B. Roux, M. Bortolato, Insertion of a glycosylphosphatidylinositol-anchored enzyme into liposomes, J. Membr. Biol. 197 (2004) 169–177.