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ORIGINAL ARTICLE

Development and Evaluation of a Phospholipid-sterol-protein Membrane Resembling System

Olga Gortzi · Magdalini Rovoli · Stavros Lalas · George Kontopidis

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Abstract The development and evaluation of a phospholipid-sterol-protein membrane resembling system containing β-lactoglobulin (β-Lg) has been studied. Liposomes have been successfully employed for the protection, controlled release and site specific delivery of sensitive molecules, such as enzymes and vitamins. β-Lg (the major whey protein of ruminant species) was selected as model protein due to its ability to bind lipophilic molecules (i.e. phospholipids). Different techniques were used for the encapsulation studies (Differential Scanning Calorimetry, SDS polyacrylamide gel electrophoresis, Fourier Transform Infrared spectroscopy). Quantitation of encapsulation was estimated by Fluorescence spectroscopy. The stability study was carried out by turbidity measurements. The shape and size of the formulations prepared were assessed by Scanning Electron Microscopy and Dynamic Light Scattering. The developed system revealed a high encapsulation percentage of β-Lg, homogeneous and small shape and a very promising stability behavior. The characteristics obtained suggested that it is a very promising delivery system.

Keywords Liposomes · β-lactoglobulin · Stability · ?Carrier complex

Olga Gortzi and Magdalini Rovoli contributed equally to this work.

O. Gortzi · S. Lalas
Department of Food Technology, Technological Educational Institution of Thessaly, Terma N. Temponera str., 43100 Karditsa, Greece

M. Rovoli · G. Kontopidis (✉)
Laboratory of Biochemistry, Department of Veterinary Science, University of Thessaly, 224 Trikalon str., 43100 Karditsa, Greece
e-mail: gkontopidis@vet.uth.gr

Introduction

Liposomes are colloidal particles, consisting of a membraneous system formed mainly of lipid and/or phospholipid molecules (bilayers) [1], encapsulating aqueous space(s). They have advantages over other delivery systems, being biodegradable, non-toxic and non-immunogenic [2]. Therefore, pharmaceutical, cosmetic, and food industries have investigated and developed micro- and nanocarrier systems for the protection and delivery of bioactive agents.

Liposomes could entrap, deliver and release water-soluble, lipid-soluble, and amphiphilic materials [3], such as proteins (enzymes), carbohydrates, vitamins, antioxidants and flavors. As a result encapsulated materials are protected from environmental and chemical changes (e.g. temperature, enzymatic modification, pH) [4, 5]. Colloidal carriers can enhance the transport of the associated macromolecules by different mechanisms depending on the carrier composition [6].

Various types of liposome formulations have been made available, each differ in their dimensions, composition, surface charge and structure. Some of them are designed through a combination of specific types of liposomes [7–10].

Although, lipid vesicles have been widely used in the food industry for their emulsifying and emulsion stabilizing properties [11], the need of a more stable and efficient carrier is still a challenge. The ability of liposomes to encapsulate lipids and vitamins can be further improved by the presence of a second carrier added to the system [12].

A protein which possesses carrier properties of its own is β-lactoglobulin (β-Lg). β-Lg is the major whey protein of ruminant species, and its properties have been regularly reviewed [13]. It can interact with phospholipids and lipophilic molecules and produce highly stable structures which

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improve the emulsifying properties of the protein alone [14]. Additionally, β-Lg can produce highly stable dispersions in foods that contain other surfactants (phospholipids, lipids, fatty acids) present in natural products (milk). Kontopidis et al. [15] indicated that β-Lg has the property of binding lipophilic molecules specifically (since it possesses a hydrophophic calyx similar to those found in other lipocalins and some membrane proteins) (Fig. 1).

In order to control β-Lg -phospholipids interactions, previous studies have been conducted using phospholipid monolayers and/or bilayers as model membrane systems [16] by mixing β-Lg with phospholipids. Many efforts have been made to design and synthesize supra molecular systems consisting of an amphiphilic block copolymer [12] or lipids and proteins that have properties similar to "natural' membranes and acting as mimics of biological membranes. However, the interaction between proteins and biological membranes as well as the kinetic mechanisms of any structural changes may vary from case to case. Distinct roles of hydrophobic and electrostatic interactions in various peptide or protein lipid binding processes remain elusive [11]. The specific role of hydrophobic and electrostatic interactions for protein adsorption, binding, and insertion into lipid membranes as well as for the stability of the β-Lg/lipid complex is still not clear.

Therefore, a liposome-protein membrane mimicking system consisting of phospholipid, sterol and protein was developed and evaluated as a more efficient carrier. The preparation method used was Dehydrated Rehydrated Vehicles (DRVs) technique. A full characterization, stability and particle morphology determination was carried out using various methods (Fluorescence Spectroscopy, Turbidity measurements, Scanning Electron Microscopy, Differential Scanning Calorimetry, Fourier Transform Infrared Spectroscopy and Dynamic Light Scattering).

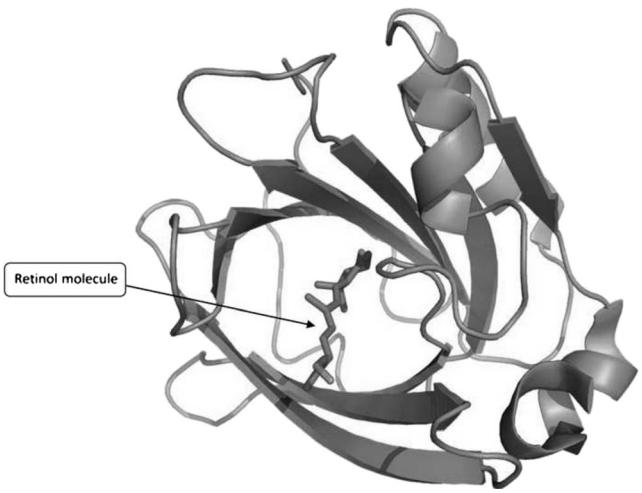

Fig. 1 Retinol molecule bound to β-Lg central calyx (PDB ID: 1GX8)

Materials and Methods

Liposome Preparation

Phosphatidylcholine (PC) and cholesterol (CH) (both from Sigma-Aldrich, Hohenbrunn, Germany) were used.

The DRVs entrapping β-Lg were prepared according to the method of Kim et al. (1999) [17] with some modifications. Specifically, mixtures of lipids (with molar ratios of PC/CH: 2/1 or 3/1) were dissolved in 10 mL chloroform which was then evaporated (using a rotary evaporator) until a thin film on the walls of a 50-mL round-bottom flask was formed. Residual organic solvents were removed by flushing with nitrogen, and the film was hydrated with 10 mL of Tris Buffered Saline, pH 7.4 (TBS, Sigma-Aldrich). Small DRV liposomes were prepared by probe sonication for 0.5 h (1 mL of this dispersion was centrifuged (in order to remove aggregates) and then mixed with 1 mL of a β-Lg solution (1 mg/mL) (90 % purity, Sigma-Aldrich, Hohenbrunn, Germany). The mixture was freeze-dried and then rehydrated.

Annealing of structural defects was carried out by incubation of liposome dispersions for 0.5 h at a temperature above the lipid T_m (35 °C), and the non-encapsulated β-Lg was separated by centrifugation (24 000 g for 30 min, at 25 °C). The liposomal pellets were re-suspended in TBS buffer at pH 7.4 to a final volume of 10 mL. The samples were finally freeze-dried and kept as powder at −80 °C.

Determination of β-Lg Encapsulation

The encapsulation of β-Lg was determined using four methods: Fluorescence Spectroscopy, SDS polyacrylamide gel electrophoresis (SDS-PAGE), Differential Scanning Calorimetry (DSC), and Fourier Transform Infrared Spectroscopy (FT-IR).

Fluorescence Spectroscopy

Encapsulation efficiency (EE%) was calculated by the determination of non-entrapped β-Lg in the supernatant of liposome suspensions, using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan).

The fluorescence intensity of β-Lg solutions (Excitation wavelength: 295 nm, Emission wavelength: 336 nm, Excitation slit: 5 nm, Emission slit: 5 nm) is linear at concentrations between 0.25 and 20 μM. A representative correlation curve for β-Lg in TBS buffer pH 7.4, in the range 0.25 to 20 μM were created and the unencapsulated β-Lg were measured and its concentration was estimated by the equation produced from the correlation curve. Before samples measurement, a protein standard solution (β-Lg in TBS) was measured, in order to confirm that the correlation curve used was still valid

for determination of β-Lg concentrations in the samples. All β-Lg standards gave similar results, reassuring that the correlation curve was meaningful for each sample, along with the fact that on the day of measurement, the instrument was operating in the same way as on the day the calibration curve was generated.

EE% was defined as the percentage amount of β-Lg entrapped in the prepared DRVs in relation to the total amount of β-Lg present during the vesicle formation and entrapment procedure [18] using the following equation:

$$[\mathrm{EE\%}] = \frac{[\text{Total protein-Unencapsulated protein}]\ (\mathrm{mg})}{\text{Total protein}\ (\mathrm{mg})} \times 100$$

SDS-PAGE

The presence of β-Lg in the liposome fractions (after disrupting the liposomes with methanol) was evaluated using the method of Badiee et al. [19]. Initially, samples (empty liposome dispersions with both PC/CH molar ratios and liposomes containing β-Lg) were all disrupted with methanol solution. After evaporation of methanol, the samples were subjected to SDS-PAGE in order to evaluate the presence and the integrity of protein. Liposomes, a sample of pure β-Lg and molecular weight markers were loaded into a 12 % SDS acrylamide gel and run using a Mini-Protean II (Bio-Rad Laboratories Inc., Hercules, CA) system. Electrophoresis was carried out at a constant voltage of 120 V for 45 min. Protein bands were visualized by Coomassie Brilliant Blue R-250 (Sigma-Aldrich) as described by Sambrook et al. [20].

DSC

Lyophilized β-Lg, empty liposomes (blanks), and liposomes containing β-Lg were analyzed by DSC in order to verify protein encapsulation. The samples (~3 mg) were placed in sealed aluminum pans. A Perkin-Elmer Diamond-DSC (Perkin Elmer Corp., Norwalk, USA) was used. The analysis was carried out under nitrogen flow (20 mL/min) on a temperature range of 25–250 °C [21].

FT-IR

The interaction between β-Lg and liposomes was determined using a Shimadzu IR Prestige-21 (Shimadzu, Tokyo, Japan).

Each sample was mixed separately with infrared grade KBr in the ratio of 1:100 and corresponding pellets were prepared using a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4 000–1 000 cm^{-1} with a speed of 2 mm/s at a resolution of 4 cm^{-1} at room temperature [22].

Stability Evaluation of Liposomes Containing β-Lg

The evaluation of the stability of β-Lg loaded liposomes was determined using two methods: Turbidity, and DSC.

Turbidity Measurements

The lyophilized liposomes (rehydrated in ddH_2O or TBS) were stored at 4 °C in the dark. Then, they were equilibrated for 30 min in 25 °C just before turbidity determination. Turbidity was measured at 450 nm using an HI 83414 turbidity meter (Hanna Instruments, USA) [23] at predetermined time intervals (0 h, 24 h, 48 h and 72 h).

DSC

The antioxidant activity of empty, β-Lg loaded liposomes, and pure β-Lg was estimated using DSC under oxidative conditions with the same equipment reported above. The method used was adapted from [24]. The analysis was carried out under an oxygen flow (20 mL/min) in a temperature range of 180–580 °C. The starting temperature of oxidation was determined as the onset temperature of the oxidation peak.

Particle Morphology

The evaluation of the particle morphology was determined using two methods: Scanning Electron Microscopy (SEM), and Dynamic Light Scattering (DLS).

SEM

The morphology of prepared liposomes was observed using a Jeol JSM 5600 SEM (Jeol Ltd, Tokyo, Japan). The samples were lyophilized, coated with gold using a vacuum evaporator, and examined using SEM at 20 kV accelerating voltage [2].

DLS

Dynamic light scattering (measurement angle, 173°) measurements were performed using a Malvern Nanosizer ZS (Malvern Instruments, UK), equipped with a 4 mW He-Ne laser operating at a wavelength of 633 nm and further with an avalanche photodiode detector. Reported polydispersity index (PDI) values, ranging from 0 (for an ideally monodisperse sample) to 1 (for very large size distributions) was derived from the formula $PDI=\sigma^2/D_h^2$ where σ is the standard deviation of the distribution (nm) and D_h is the volume-weighted mean hydrodynamic diameter.

Statistical Analysis

All determinations were carried out in triplicate and values are shown as mean±SD (apart from the case of FT-IR and DLS). Unpaired two tailed *t*-test and ANOVA (one-way layout and randomized blocks designs) were used for statistical comparison. For all these purposes $P<0.05$ was considered as significant.

Results and Discussion

Encapsulation Efficiency

Four different techniques have been used for encapsulation studies, three to confirm the encapsulation of protein (DSC, SDS-PAGE, and FT-IR) and one for estimation of the quantitative encapsulation efficiency (fluorescence spectroscopy).

Fluorescence Spectroscopy

Two different phospholipid compositions were used (2/1 PC/CH and 3/1 PC/CH molar ratios) for the formulation of liposomes loaded with β-Lg. The PC/CH mixture is a commonly used combination for liposome preparations [25], and the selected ratios are the most common working ratios according to the literature [18, 26].

The results on the encapsulation efficiencies of β-Lg in DRV liposomes are displayed in Table 1. In both preparations, the percentage of protein encapsulation was higher than those reported in the literature for other encapsulated proteins [2, 18]. This may be partially explained by the ability of β-Lg to bind to a variety of hydrophobic molecules [14].

As indicated by the results, the encapsulation percentage of β-Lg into DRV liposomes was similar (no significant difference) to both PC/CH ratios. It is known that the encapsulation efficiency of protein depends on interaction between the protein and the lipid bilayer and the entrapment can be increased by manipulation of the liposomal lipid composition or by increasing the lipid concentration, in order to favor electrostatic interactions [27]. This means that a higher lipid concentration leads to higher bioactive compound encapsulation efficiency, with the correlation reaching a plateau above an exact value [28]. This could explain our results, according to which, the 3/1 molar ratio of PC/CH has already a very high encapsulation efficiency (97.1 %) of protein and changing the lipid molar ratio to 2/1 PC/ CH could not further increase the encapsulation ability (97.9 %).

Table 1 Encapsulation efficiency (%) of β-Lg loaded PC/CH liposomes ($n=3$)

Lipid composition	Mean encapsulation percentage
2/1 PC/CH	97.93±0.35 %
3/1 PC/CH	97.10±1.30 %

SDS-PAGE

SDS-PAGE is a technique widely used to identify proteins in a variety of samples. The presence of protein in liposomes was detected by this technique. The gels obtained by this method are presented in Fig. 2.

A pure form of β-Lg was ran on a different SDS-PAGE gel (Fig. 2a, lane 1) and showed, as expected, a protein band of MW approximately 18.4 kDa. As expected, in lanes 1 and 3 (empty liposomes) in Fig. 2b, no protein was detected.

In lanes 2 and 4, a clear protein band with a MW above 17 kDa was observed and identified as β-Lg (MW of approximately 18.4 kDa) a clear indication of protein integrity.

DSC

DSC evaluation can determine whether the protein is entrapped in the bilayer or in the aqueous compartment of the liposomes by the melting and re-crystallization behavior of materials like lipid nanoparticles. Figure 3 shows the thermograms of empty liposomes and liposomes encapsulating β-Lg along with a thermogram of pure β-Lg.

The endotherms describing the pre- and main-phase transitions are good indicators of the quality of the liposomal lipid and of the presence of a bilayer interacting compound [29].

The main T_m of empty 2/1 and 3/1 PC/CH liposomes was about 147 °C. The main T_m showed minor changes for all liposomal samples. This thermotropic behaviour of liposomes showed that the main transition temperature remained almost unchanged after the incorporation of β-Lg. In summary, these experimental calorimetric results show that no fundamental structural changes, e.g. the formation of hexagonal arrangements, were observed in the structure of the liposomes when β-Lg was incorporated into them. This is in agreement with the results of [30] who stated that the thermodynamic parameters were only slightly modified in the protein presence.

The pre-transition temperature peak of empty liposomes disappeared at the thermograms of the liposomes encapsulating β-Lg. This reveals that β-Lg interacts with the polar head group of the phospholipids, in agreement with the results of [31]. The disappearance of the pre-transition is a sensitive criterion for the incorporation of substances into lipid bilayers [32]. This fact is interpreted as a consequence of the protein insertion into the bilayer of the membrane disturbing the hydrophobic interactions between the lipid molecules. The insertion of the protein between the polar heads of PC/CH liposomes could favor the development of a liquid crystalline

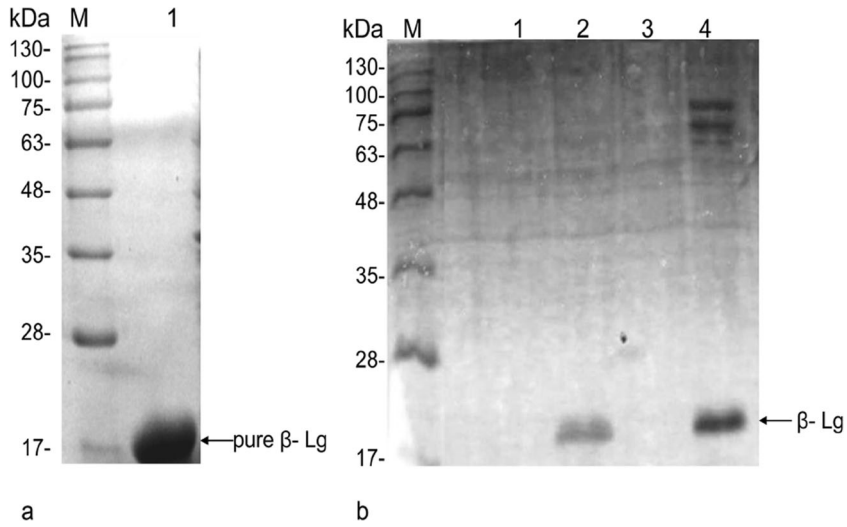

Fig. 2 SDS-PAGE of β-Lg extracted from PC/ CH=2/1 and PC/ CH=3/1 liposomes. **a** *Lanes*: M. Molecular Weight Markers, 1. β-Lg in pure form. **b** *Lanes*: M. Molecular Weight Markers, 1. PC/ CH=2/1 empty liposomes, 2. β-Lg after extraction with methanol from PC/ CH=2/1 liposomes, 3.PC/ CH=3/1 empty liposomes, 4. β-Lg after extraction with methanol from PC/ CH=3/1 liposomes)

phase less ordered than the gel phase and slightly increases the gel-to-liquid crystal phase transition temperature as observed by DSC [33]. The above observation agrees with previous findings [22] regarding the fact that interaction of the encapsulated substance with the lipid components of liposomes may alter the physicochemical properties of liposomes.

Additionally, pure β-Lg thermogram presents a melting plateau at about 83 °C. This melting plateau of β-Lg could not be observed in β-Lg loaded liposome thermograms, indicating good interaction of all components. Since β-Lg is lipophilic, it is possible that it might be entrapped in the bilayer compartment of liposome [21].

FT-IR

As indicated by [34], transitions in both protein secondary structure and PC vesicle structure can be studied by this particular technique. Figure 4 shows the FT-IR spectra of all samples (Fig. 4a: empty 2/1 PC/CH liposomes, loaded with β-Lg, and pure β-Lg; Fig. 4b: empty 3/1 PC/CH liposomes, loaded with β-Lg, and pure β-Lg). The symmetric and antisymmetric P=O stretching vibrations at 1051 and 1233 cm^{-1} of 2/1 PC/CH and β-Lg loaded liposomes, have been shifted to 1043 and 1234 cm^{-1}, respectively.

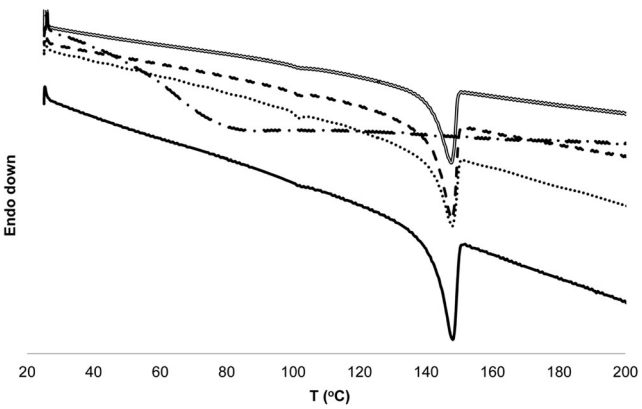

Fig. 3 DSC thermograms of liposomes suspensions in the absence and presence of β-Lg under a nitrogen atmosphere (▬ ▬) PC/CH (2/1) empty liposomes, (═══) PC/CH (2/1) + β-Lg liposomes, (• • • • • •) PC/CH (3/1) empty liposomes, (▬▬▬) PC/CH (3/1) + β-Lg liposomes, (— · · —) β-Lg

The phosphate group is part of the PC molecule and neither β-Lg nor CH have a phosphate group attached (Fig. 4c). The above shifts in spectrum indicate interactions between the charged phosphate group of PC and loaded β-Lg. These findings are in good agreement with the data reported by [22].

There is also additional evidence that interactions between loaded β-Lg and liposome come from the O-H stretching (3354 cm^{-1}) vibrations shifts. That wavenumber is characteristic for OH group of CH molecule, when that of the protein (β-Lg) is observed at 3289 cm^{-1}. The particular wavenumber has shifted from 3354 to 3360 cm^{-1} upon addition of β-Lg into the system.

Both, the phosphate group of PC and the OH group of CH interactions are located in the polar head of both molecules, which implies that β-Lg interacts with the hydrophilic part (polar head) of PC [35]. This is also suggested from the fact (Fig. 4a and b: β-Lg loaded 2/1 and 3/1 PC/CH liposomes spectra, respectively) that there were no changes in the frequency of the $(-CH_2-)_n$ chain bands (symmetric 2857 cm^{-1}, antisymmetric 2926 cm^{-1} stretch or bending 1463 cm^{-1}) which indicate a lack of interactions of β -Lg with alkyl chains. This also implies that β-Lg has not changed the structure of the bilayer membrane as no change was observed in the lipophilic part of liposome (alkyl chains of PC).

Those interactions between β-Lg and PC/CH liposome support the formation of a new structure (PC/CH/β-Lg) and thus the improved stability observed for the PC/CH liposome after addition of β-Lg.

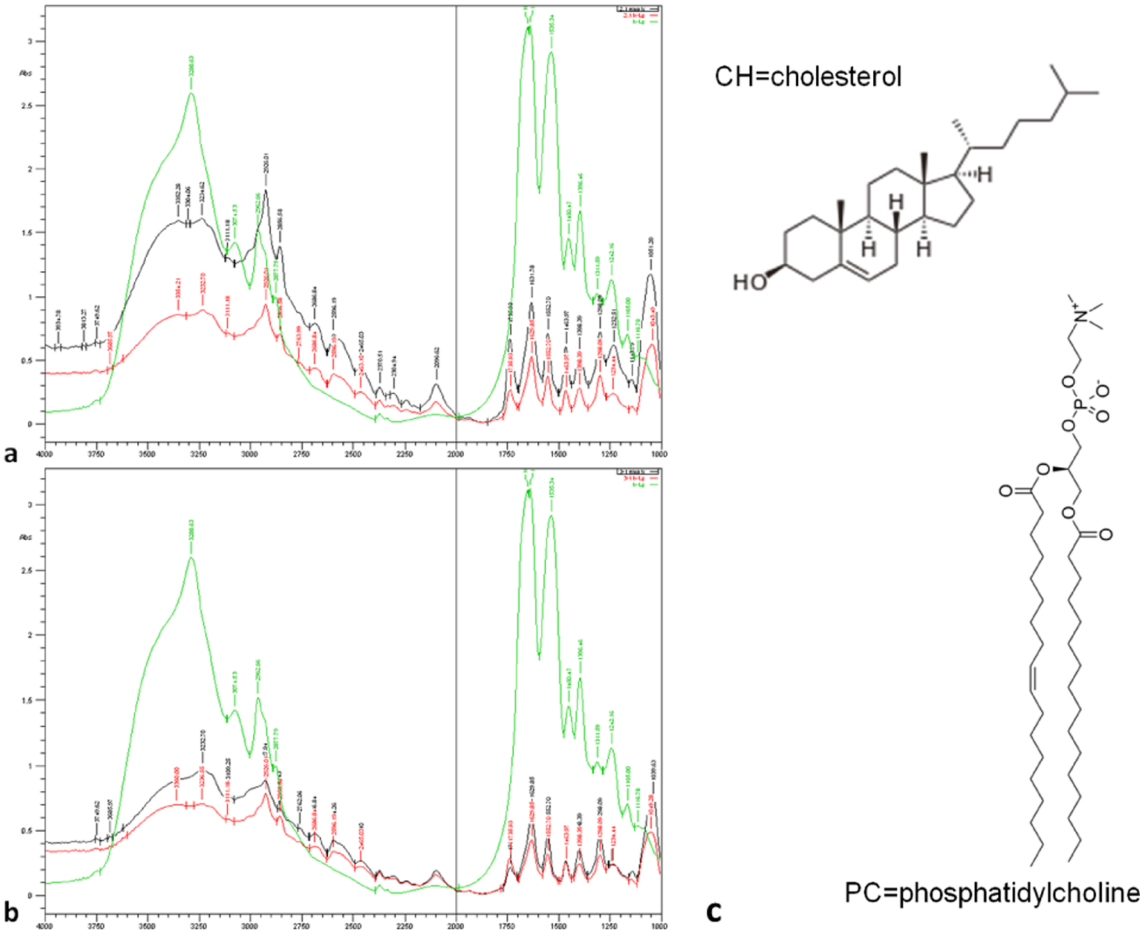

Fig. 4 **a**. FTIR spectra of: Pure β-Lg (*green*), 2/1 PC/CH empty liposomes (*black*) and 2/1 PC/CH + β-Lg liposomes (*red*). **b**. FTIR spectra of: Pure β-Lg (*green*), 3/1 PC/CH empty liposomes (*black*) and 3/1 PC/CH + β-Lg liposomes (*red*). **c**. Cholesterol and phosphatidylcholine molecules

Evaluation of Liposome/β-Lg Complex Stability

The stability behavior of liposomes during storage was examined, as an important factor for determining the ability of these systems to protect the encapsulated molecule [2]. Two different techniques have been used for the determination of stability of the liposomes encapsulating β-Lg: Changes in turbidity and antioxidant activity under oxidative conditions using DSC.

Turbidity

Turbidity measurements are often used as a crude tool to determine the size and stability of the scattering particles [23]. In the case of liposomes, an increase in turbidity is interpreted as an increase in the aggregation of vesicles due to decrease of their stability. An increase in the optical density is tightly correlated with an increase in the turbidity and as a consequence can be used to monitor changes in the sample's stability.

The scattering values of empty liposomes indicate that the size of the PC vesicle depends on the initial cholesterol level. The results (Fig. 4) showed that liposomes with a molar ratio 2/1 PC/CH had an improved stability behavior compared to those with a molar ratio 3/1 PC/CH in each of the two aqueous systems used [ddH_2O (Fig. 5a) and TBS (Fig. 5b)].

This could be explained by the fact that the 2/1 PC/CH formulation contains a higher relative amount of cholesterol. As it is well established, cholesterol incorporated into lipid bilayers improves membrane rigidity and hydrophobicity, and reduces permeability and polypeptide affinity [25].

Addition of cholesterol increases the stability of liposomes in ddH_2O and TBS medium. The minimum size of the vesicle formed in the presence of β-Lg is found to be independent of the initial cholesterol concentration in TBS medium. Turbidity results also suggest that the presence of β-Lg induces structural changes in the membrane and improves stability behavior of the system, especially in ddH_2O (Fig. 5a).

According to the literature, whey proteins are well known for their ability to stabilize emulsions and foams in food formulation [36]. Additionally, β-Lg has an affinity for cholesterol as published crystallographic data have already revealed [14]. Binding of cholesterol within a protein pocket or calyx could further stabilize the overall structure.

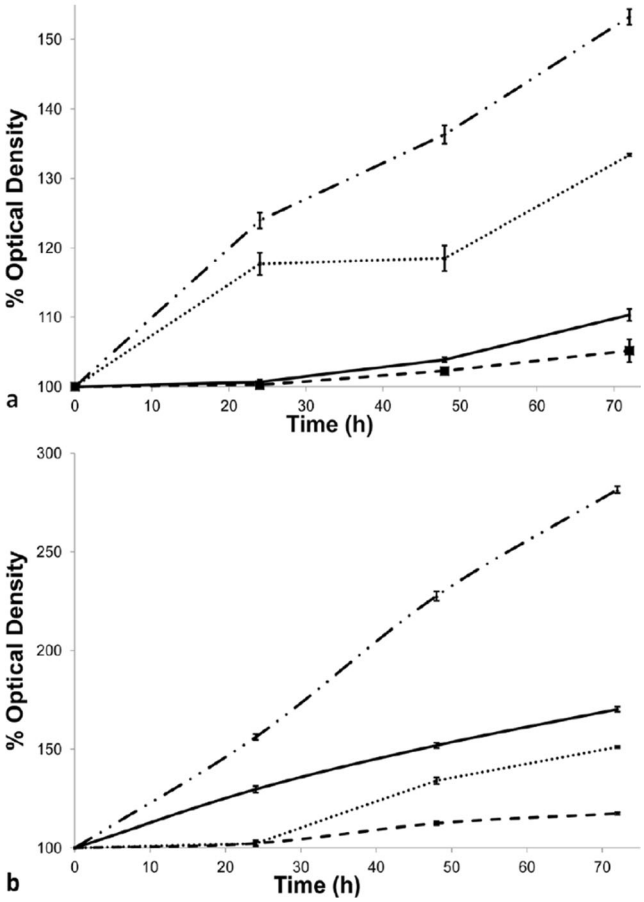

Fig. 5 Stability behavior of liposomes during storage after predetermined time intervals: Turbidity measurements at 25 °C using absorbance at 450 nm over a 72 h period of storage at 4 °C. **a**. Turbidity measurements of liposomes after dispersion in ddH_2O. **b**. Turbidity measurements of liposomes after dispersion in TBS. (▬▬) PC/CH (2/1) empty liposomes, (▬ ▬) PC/CH (2/1) + β-Lg liposomes, (— · · —) PC/CH (3/1) empty liposomes, (• • • • • •) PC/CH (3/1) + β-Lg liposomes

The liposome system with 2/1 PC/CH after β-Lg encapsulation was found to be the more promising complex regarding stability. The protein presence in the liposomes results in a stable system [12].

Determination of Antioxidant Activity Using DSC

Auto-oxidation of fats, fatty acids and lipids is a well-established exothermic process and methods of thermal analysis, such as DSC, are valuable for the study of thermostability and thermo-oxidation [37], while kinetic parameters of the fatty acid oxidation can also be determined [38]. The antioxidant activity is evaluated by the extrapolated temperature at the start of the oxidation process based on the measurements of the incubation period [24]. In Fig. 6 the DSC curves of liposome suspensions in the absence and in the presence of β-Lg under oxygen atmosphere are given. An exothermic peak of pure β-Lg was observed between 250 and 310 °C, while the auto-oxidation process of the liposome samples, displayed an exothermic peak in the range 320–350 °C.

As shown in Fig. 6, the addition of β-Lg encapsulated in liposomes had a more intense antioxidant action (significant at $P<0.05$) than itself in pure form. At the same heating rate, the temperature of the start of the oxidation reaction is significantly ($P<0.05$) higher. The modified antioxidant action of β-Lg during its encapsulation was expected since the complex (liposome membrane / β-Lg) constitutes a new system. Also, the antioxidant action of empty liposomes of the same lipid composition appeared lower (temp of the oxidation onset: pure β-Lg=286 °C, 3/1 empty liposomes = 330 °C, 3/1+ β-Lg liposomes = 332 °C, 2/1 empty liposomes = 334 °C, 2/1+ β-Lg liposomes = 335 °C) (significant at $P<0.05$) compared to those with β-Lg, implying that the encapsulation of protein in the aqueous part of liposomes stabilized the liposome membranes.

Particle Morphology

SEM

Electron microscopy is a well known technique to visualize the surface of a membrane and to directly reveal important features, such as morphology, homogeneity and size. Most microscopy techniques are fast, easy, and provide relatively straightforward specimen visualization [12].

The morphology (shape and structure) of lyophilized liposomes was examined by SEM since it is related to the physicochemical properties of liposomes.

Microscopic observations showed that the particles mostly had a spherical shape (Fig. 7).

Empty liposomes, obtained by the DRV method, seem to be heterogeneous with a wider particle size distribution than those of the β-Lg/liposome complex (Fig. 7a). Micrographs

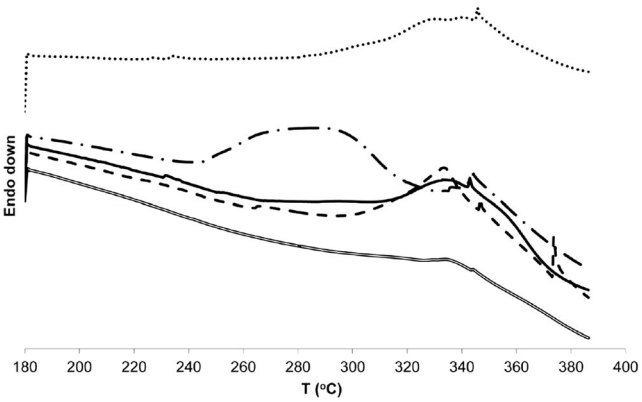

Fig. 6 DSC thermograms of liposomes suspensions in the absence and presence of β-Lg under oxygen atmosphere.(▬ ▬) PC/CH (2/1) empty liposomes, (═══) PC/CH (2/1) + β-Lg liposomes, (• • • • • •) PC/CH (3/1) empty liposomes, (▬▬) PC/CH (3/1) + β-Lg liposomes, (— · · —) β-Lg

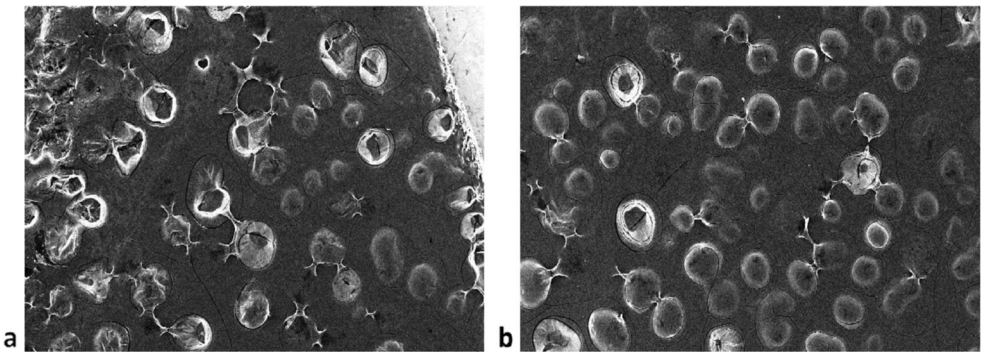

Fig. 7 Scanning electron micrographs of DRV liposomes before and after encapsulation of β-Lg obtained by thin film method (pH 7.4, room temperature). **a**. empty liposomes and **b**. β-Lg - loaded liposomes

reveal that the majority of liposomes containing β-Lg (Fig. 7b) have a more spherical shape compared to those without β-Lg.

Based upon the turbidity results, we proposed that presence of β-Lg in the membrane forms more stable PC/CH vesicles. This proposal correlates well with the SEM observation of smaller and more homogeneous vesicles.

DLS

The effect of the incorporation of β-Lg on the mean size of DRV preparations was also investigated by DLS. It was found that the empty 2/1 and 3/1 PC/CH liposomes had a mean diameter in the range of 922.00±9.16 nm and 1296.66± 289.36 nm, respectively, whereas liposomes with encapsulated β-Lg were smaller with a mean diameter around 501.66± 67.15 nm for 2/1 PC/CH liposomes and 447.33±59.18 nm for the 3/1 PC/CH liposomes. However, polydispersity values were high (2/1 PC/CH liposomes: 0.790±0.010, 2/1 PC/CH + β-Lg liposomes: 0.522±0.030, 3/1 PC/CH liposomes: 0.831±0.095, 3/1 PC/CH + β-Lg liposomes: 0.492±0.009) signifying highly heterogeneous populations of vesicles.

The above results are in good agreement with SEM images and turbidity results obtained for the same samples, according to which it is confirmed that the presence of β-Lg in liposomes induces formation of more stable PC/CH vesicles (smaller mean diameter and lower polydispersity values for the β-Lg loaded liposomes). The fact that the 3/1 PC/CH liposomes (with and without β-Lg) present higher mean diameter and polydispersity index compared to 2/1 PC/CH liposomes, confirms that higher cholesterol molar ratio leads to more stable, smaller and homogeneous vesicles.

The large mean diameters which were observed could be attributed to the fact that the liposomes have formed aggregations before measurements, as they have a tendency to undergo aggregation and fusion and freeze-drying and rehydration induces fusion between adjacent membranes resulting finally in DRVs (0.1 to 2.0 μm diameter) [6, 39]

Conclusions

The preparation and characterization of a β-Lg/liposome formulation and the determination of the β-Lg encapsulation efficiency were studied in order to develop a new, more efficient and stable liposome-based carrier. Liposomes encapsulating β-Lg, with two different lipid compositions, were prepared using the DRV method, and the retention of the protein in the vesicles was measured. The results of the FT-IR confirmed the creation of the liposome/β-Lg complex with the trapping efficiency for β-Lg to be higher compared with other encapsulated proteins mentioned in the literature. As seen in the SEM images and DLS results, DRVs containing β-Lg are smaller and more homogeneous, indicating that the protein molecules stabilize the lipid bilayer. Both lipid molar ratios that were used had very high encapsulation efficiency for β-Lg. Furthermore, β-Lg in liposomes stabilized the liposome formulation as demonstrated by DSC oxidation curves and turbidity measurements.

As a result, the liposome/β-Lg complex possesses all required properties to be used as an efficient carrier for delivery of bioactive molecule (drugs, vitamins, lipophilic molecules). An application of this carrier have been published with one lipophilic vitamin [40]. The improved system generated by this study may pave the way for its utility as a carrier for numerous molecules.

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Declaration of Interest The authors declare that they have no conflict of interest.

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