Journal of Liposome Research

http://informahealthcare.com/lpr
ISSN: 0898-2104 (print), 1532-2394 (electronic)

J Liposome Res, Early Online: 1–8
 DOI: 10.3109/08982104.2013.839701

informa healthcare

RESEARCH ARTICLE

β-Lactoglobulin improves liposome's encapsulation properties for vitamin E delivery

Magdalini Rovoli[1], Olga Gortzi[2], Stavros Lalas[2], and George Kontopidis[1]

[1]*Biochemistry Laboratory, Veterinary Department, University of Thessaly, Karditsa, Greece and* [2]*Department of Food Technology, Technological Educational Institution of Thessaly, Karditsa, Greece*

Abstract

Vitamin E (VE) or α-tocopherol is the major fat-soluble antioxidant in the human body. It is a sensitive, easily oxidized in the air, molecule, so it must be protected from pro-oxidant elements which could affect its physiological benefits. Encapsulation constitutes a promising approach to maintain VE native properties over time and increase its concentration in aqueous media. Liposomes have been studied as sustained delivery systems, being biodegradable, non-toxic and non-immunogenic. A new liposome/β-lactoglobulin (β-Lg) formulation has been developed and characterized as a possible stable delivery system for VE. β-Lg has been selected due to its property to bind a variety of hydrophobic molecules. The aim of this study was the preparation of β-Lg-liposome formulation and the determination of VE encapsulation efficiency, in order to develop a new more efficient carrier for VE in aqueous media.

Keywords

β-Lactoglobulin, carrier, encapsulation, liposome, stability, vitamin E

History

Received 15 May 2013
Revised 23 August 2013
Accepted 28 August 2013
Published online 7 October 2013

Introduction

Vitamins possess important functions in certain metabolic processes in the human body. Vitamin E (VE) in particular is known as a membrane stabilizer due to its strong affinity to biomembranes through interactions with membrane phospholipids (Neunert et al., 2010). VE is a generic name for a mixture of different types of α-, β-, γ-, and δ-tocopherols, found in foods in four main forms (Dwiecki et al., 2007), which differ in the number and position of the methyl groups on the chroman ring (Raneva et al., 2003). The α-tocopherol which is the most active VE compound, accounts for 90% of VE in tissues (Cohn, 1997) and is related to the most important biological activity (Raneva et al., 2003).

VE is an example of a phenolic antioxidant. Such molecules readily donate the hydrogen from the hydroxyl (-OH) group on the ring structure to free radicals, which then become non-reactive. On donating the hydrogen, the phenolic compound itself becomes a relatively non-reactive free radical because the unpaired electron on the oxygen atom is usually delocalized into the aromatic ring structure thereby increasing its stability (Scott, 1997).

The major biologic role of VE is to protect polyunsaturated fatty acids (PUFAs) and other components of cell membranes and low-density lipoproteins (LDLs) from oxidation by free radicals. Elevated levels of lipid peroxidation products are associated with numerous diseases and clinical conditions. VE is primarily located in cell and organelle membranes, where it can exert its maximum protective effect, in concentration that may only be one molecule for every 2000 phospholipid molecules. This suggests that after its reaction with free radicals it is rapidly regenerated, possibly by other antioxidants (WHO, 2004).

In addition to oxidation problem, VE also has poor absorbability and bioavailability which limits its clinical application (Colletier et al., 2002; Gonnet et al., 2010).

Encapsulation systems is a promising approach to preserve encapsulated compound native properties over time and increase concentration in aqueous media but also enhance absorption and bioavailability (Nacka et al., 2001). Encapsulation reduces reactivity with the environment (water, oxygen, light), toxicity and degradation and enhances bioavailability and protection (Mozafari et al., 2008b; Perrie et al., 2001).

Encapsulation in a form of liposomes (mainly lipid and/or phospholipid molecules-bilayers) presents advantages over other delivery systems, being biodegradable, non-toxic and non-immunogenic (Mozafari et al., 2002; Park et al., 2011). The fact that they consist of natural ingredients or indigenous biomolecules enables their use in order to prepare biocompatible and acceptable dispersions for human consumption (Mozafari et al., 2008b).

Liposomes can efficiently entrap and deliver-release lipid-soluble materials (Mozafari et al., 2008a), such as VE, and as a result, the encapsulated materials are protected from

Address for correspondence: George Kontopidis, Biochemistry Laboratory, Veterinary Department, University of Thessaly, 224 Trikalon str., GR-43100, Karditsa, Greece. Tel: +30 24410 66017. Fax: +30 24410 66041. E-mail: gkontopidis@vet.uth.gr

environmental and chemical changes (e.g. temperature, enzymatic modification, pH) (da Silva Malheiros et al., 2010; Hwang et al., 2012).

Various types of liposome formulations have been made available for encapsulation of sensitive molecules, each differing in their dimensions (Muthu et al., 2011), composition, surface charge and structure, some of which are designed through a combination of specific types of liposomes (Balkina et al., 2009; Edwards & Baeumner, 2006; Hădărugă et al., 2010; Zhang & Granick, 2006).

One type of liposomes, the dehydrated rehydrated vehicles (DRVs), have been reported to possess many advantages including relatively higher encapsulation efficiency (EE) and little potential damage to the encapsulated substance (Chen et al., 2010).

Physicochemical characteristics of liposomes are affected by the method of preparation and lipids composition used. Cholesterol can modulate the physical properties of the plasma membrane (Ohtake et al., 2005) and improve stability characteristics (Vemuri & Rhodes, 1995).

The Phosphatidylcholine/Cholesterol (PC/CH) mixture is commonly used for liposomes preparation according to literature (Kawano et al., 2003; Rodríguez-Nogales & López, 2006; Zaru et al., 2007).

Encapsulation in liposomes has also been reported to enhance absorption and bioavailability of VE (Nacka et al., 2001).

The addition of β-lactoglobulin (β-Lg) to liposome preparation has shown an improvement of liposomes stability (under publication). Bovine β-Lg is one of the most extensively studied proteins (Chanasattru et al., 2007; Sawyer et al., 1999), being the most significant whey protein of ruminant species, its properties have been regularly reviewed (Hansted et al., 2011; Ge et al., 2010). This protein belongs to the family of lipocalins or lipocalycins, which are widely valued by the food industry for their emulsifying and emulsion stabilizing properties (Kontopidis et al., 2004). The β-Lg is selected as a model protein for liposome system preparation under its property to bind lipophilic molecules (i.e. fatty acid and vitamins; Kontopidis et al., 2002).

The preparation and characterization of β-Lg/liposome formulation and the determination of β-Lg EE were studied, in order to develop a new more efficient carrier for lipophilic molecules in aqueous media (results under publication).

The main objectives of this study were the preparation, characterization, and determination of VE encapsulation in two different liposome complexes DRV. PC/CH and PC/CH/β-Lg-liposomes were compared in order to develop a new, more efficient carrier for VE in aqueous media.

Materials and methods

Liposome preparation

DRV liposomes encapsulating β-Lg (>90%, Sigma-Aldrich Chemie Gmbh, Munich, Germany) and α-tocopherol (VE) (>98%, Alfa Aesar) were prepared according to the method of (Marsanasco et al., 2011) with some modifications. The following lipid composition was used: Phosphatidylcholine (PC, >99% from egg yolk, Sigma Aldrich)/Cholesterol (CH, >90%, Sigma Aldrich) with the molar ratios: PC/CH (2/1) where PC = 83.35 mg, CH = 12.56 mg.

Lipids and VE (1 mg) were mixed in ethanol solution (5 mL) and then evaporated until a thin film was formed on the walls of a 50-mL round-bottom flask. Residual organic solvents were removed by nitrogen, and the film was hydrated with Tris Buffered Saline (TBS), pH 7.4. Small unilamellar vehicles (SUVs) liposomes were then prepared by probe sonication (using a Misonix Sonicator S3000, Misonix Inc., Farmingdale, NY) and 1 mL of SUV dispersion (after centrifugation to remove aggregates and titanium particles) was mixed with 1 mL of a β-Lg solution (1 mg/mL). The mixture was lyophilized (freeze-dryer, Telstar Cryodos 80, Telstar Industrial, SL Spain) and then rehydrated with a controlled stepwise protocol.

Annealing of liposome dispersions took place with incubation for 0.5 h at a temperature above lipid phase transition temperature (Tm) −35 °C (Koynova & Caffrey, 1998). The liposomal entrapped β-Lg and VE were separated from free (supernatant) β-Lg and VE by centrifugation at 25 000g for 30 min, at 25 °C, at least 3 times. The liposomal pellets were subsequently resuspended in the TBS buffer at pH 7.4 to a final volume of 5 mL.

All other chemicals used were of analytical grade and were purchased by Sigma-Aldrich.

Encapsulation efficiency

Evaluation of α-tocopherol encapsulation

High performance liquid chromatography (HPLC) provides a convenient method for quantification and characterization of VE (Lalas et al., 2011). The analysis was carried out on a Shimadzu CBM-20A liquid chromatograph. Detection was performed using a Shimadzu RF-10AXL fluorescence detector, the column used was a Waters μ-Porasil, (125 Å, 10 μm, 3.9 × 300 mm; Waters Corp., Milford, MA). The samples were dissolved in *n*-hexane before injection, so as the liposomes to be disrupted and VE to be released. Before the analysis, the samples were filtered by inorganic membrane filter (Anotop 10 IC, 0.2 μm, 10 mm) and finally a 1 μL of the sample was injected into the HPLC. 2-Propanol:*n*-hexane: absolute ethanol (2/97.5/0.5 v/v/v) was used as a mobile phase.

As a result, the EE was calculated as:

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

Evaluation of β-Lg encapsulation

Sodium dodecyl sulfate polyaAcrylamide gel electrophoresis (SDS-PAGE) describes a technique widely used to identify proteins (Jagannadham & Chowdhury, 2012). In liposome fractions, protein presence can also be detected by this method (Badiee et al., 2009). The β-Lg was evaluated by this technique after disrupting the liposomes with methanol.

Samples of the lyophilized liposome dispersions (PC/CH (2/1, mol/mol) empty liposomes, VE-loaded PC/CH (2/1, mol/mol) liposomes, PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, and VE-loaded PC/CH/β-Lg (2/1/0.004,

mol/mol/mol) liposomes) were all disrupted with methanol solution (1 mg of sample per 2 mL of methanol) and were subjected to an 12% SDS-PAGE (after evaporation of methanol) in order to evaluate β-Lg presence. Samples were run using electrophoresis system Mini-Proteans II (Bio-Rad Laboratories Inc., Hercules, CA) system. Electrophoresis was carried out at 120 V constant voltages for 45 min. The molecular weight markers which were used ranged from 180 KDa to 17 KDa and protein bands were visualized by Coomassie Brilliant Blue R-250 (Sambrook & Russell, 2001).

Stability of VE-loaded liposome suspension during storage

In order to determine the physical stability of vesicles, lyophilized samples of PC/CH (2/1, mol/mol) empty liposomes, VE-loaded PC/CH (2/1, mol/mol) liposomes, PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, and VE-loaded PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, were rehydrated in TBS or ddH_2O and were stored at 4 °C for 72 h under light protection. Stability was evaluated by turbidity measurements in predetermined time intervals (0 h, 24 h, 48 h, and 72 h), after equilibration for 30 min in 25 °C. During the measurement, the temperature of the sample was maintained stable at 25 °C. Turbidity was measured at 450 nm using a turbidity meter (HI 83414, Hanna Instruments, Smithfield, RI; Dwiecki et al., 2007).

Differential scanning calorimetry (DSC) for determination of antioxidant activity

The oxidation activity of samples: PC/CH empty liposomes (2/1, mol/mol), VE-loaded PC/CH (2/1, mol/mol) liposomes, PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, and VE-loaded PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, β-Lg and VE in pure forms were evaluated by DSC (Ghatnur et al., 2012). A Perkin–Elmer Diamond-DSC calorimeter (Perkin Elmer Corp., Norwalk, CT) was employed to study the samples oxidation stability. The method used was adopted from Gortzi et al. (2006). The starting temperature of oxidation was determined as the onset temperature of the oxidation peak. The temperature program was: hold for 1 min at 180 °C, heat from 180 to 580 °C (with heat rate 10 °C/min), hold for 1 min at 580 °C.

Vehicles morphology (scanning electron microscopy – SEM)

The morphology of the prepared liposomes was observed by scanning electronic microscopy (SEM, JEOL type JSM 5600). Samples were lyophilized, coated with gold using a vacuum evaporator, and examined using SEM at 20 kV accelerating voltage (Park et al., 2011).

Statistical analysis

All measurements were conducted at 25 °C in triplicate, values are shown as mean ± SD. 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 significant.

Results and discussion

Liposome preparation

To the best of our knowledge, this is the first time that DRV liposomes have been used for VE encapsulation.

DRV liposomes have been selected because of their ability to encapsulate sensitive molecules (e.g. vitamins, proteins), under conditions that do not result in decomposition or the loss of activity of active substances (Antimisiaris, 2010; Rodríguez-Nogales & López, 2006). By the dehydration–rehydration method, the EE and storage stability of liposomes are improved (Hsieh et al., 2002; Ntimenou et al., 2006). The addition of VE has also been shown to increase the stability of liposomes but only in relative low concentration (mol% VE/PC ratio; Dwiecki et al., 2007; Nacka et al., 2001). As a consequence, a low level of VE/total lipids molar ratio, was selected for the current study (2/1/0.035 PC/CH/VE molar ratio or 1.15 mol%).

The ability of liposome to encapsulate lipids molecules and vitamins could be further improved due to the presence of a second carrier added to the system (Zhang et al., 2012).

The β-Lg is a model protein to assess the above idea by its property to bind lipophilic molecules (i.e. fatty acid and vitamins; Kontopidis et al., 2002). The addition of β-Lg in liposome preparation could result in a new more efficient carrier for VE in aqueous media.

Encapsulation efficiency

Two different techniques have been used for encapsulation studies, one to confirm qualitatively the encapsulated protein (SDS-PAGE) and one for determination of VE EE (HPLC).

Evaluation of VE encapsulation

Lipid concentration affects the liposomal EE of biomolecules. Higher lipid concentration have been shown to increase encapsulation of bioactive compounds (Xu et al., 2012).

Trapping efficiencies for VE in DRVs liposomes with and without β-Lg were determined by HPLC (Figure 1) and the results are recorded below (Table 1).

The %EE of VE in liposomes in absence and presence of β-Lg was: 59.42% (± 2.31%) and 96.59% (± 1.52%), respectively.

Other groups (Marsanasco et al., 2011; Nacka et al., 2001) using different (than the current work) types of liposome formulation (MLV or GOL), have reported VE EE in a range of 50% to 99%. Those large differences are dependent on lipid composition, method of preparation and the lipids/VE molar ratio. The percentage of encapsulation of VE in liposomes/β-Lg formulation appeared elevated, compared to the percentage of VE encapsulated in PC/CH liposomes. The above mentioned % (w/w) ratios of VE encapsulation in liposome converts to a molar ratio of 2/1/0.021 or 2/1/0.035 for PC/CH/VE or PC/CH/β-Lg/VE, respectively. As previously shown (Kontopidis et al., 2002), β-Lg binds to one molecule of VE (molar ratio 1:1). The encapsulation molar ratio of β-Lg in liposome was determined at 0.004 moles (Table 1). The encapsulated VE moles were increased from 0.021 for PC/CH/VE to 0.035 for PC/CH/β-Lg. This difference of 0.014 moles (0.035 minus 0.021) in VE encapsulation cannot be

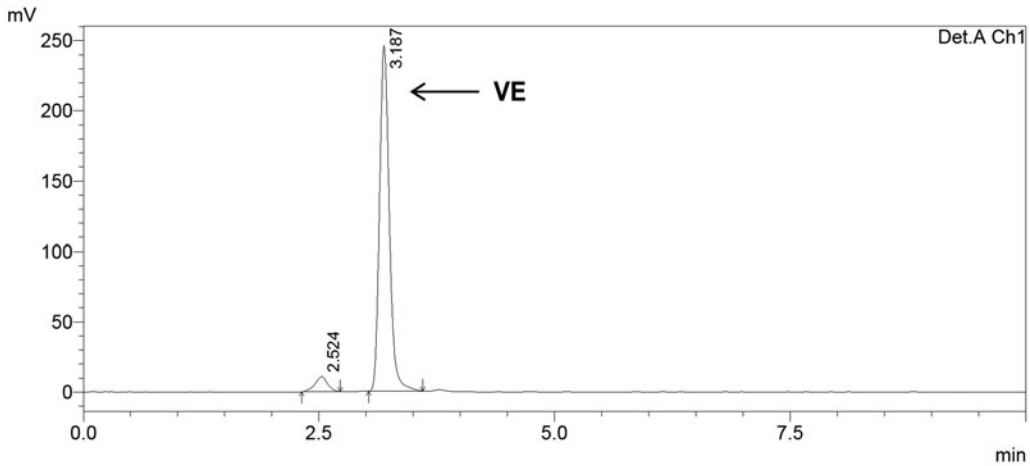

Figure 1. HPLC chromatogram of a sample containing VE (VE was released after liposomes disruption with *n*-hexane).

Table 1. Encapsulation efficiency (EE) (%) of VE loaded in PC/CH (2/1, mol/mol) and PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes ($n = 3$).

Sample	EE (%) of VE
PC/CH (2/1, mol/mol)	59.42 ± 2.31
PC/CH/β-Lg (2/1/0.004, mol/mol/mol)	96.59 ± 1.52

The initial amount of VE added during liposome preparation was 1 mg.

solely explained by the equimolar (1:1) binding of VE to β-Lg. The excess encapsulated molar ratio of VE possesses an additional property for PC/CH/β-Lg system. This, along with the improved stability of the PC/CH/β-Lg system, presents different physicochemical properties and thus supports the argument of physicochemical alterations of the newly synthesized liposome system.

In order to fully appreciate the behavior of VE in membranes, we should accept that the side chain is able to occupy many different conformers other than the fully extended one. The whole molecule can rotate about its own long axis, and it is able to diffuse in the two-dimensional fluid of one leaflet of the bilayer. The reasons for any preferential location of VE in membranes are very phospholipid dependent (Atkinson et al., 2008; Atkinson et al., 2010; Wang & Quinn, 2000).

SDS polyacrylamide gel electrophoresis

The presence of β-Lg in liposomes was detected by SDS-PAGE after disrupting the liposomes with methanol. After distaining, the gel is presented in Figure 2.

SDS-PAGE could identify the molecular weight (MW) of proteins in the sample. Bands with MW higher than 18 kDa (molecular weight of β-Lg: 18.4 kDa) would indicate that β-Lg has been aggregated and formed oligomers.

As it was expected, in lanes 2 and 4, where empty liposomes were loaded, no presence of protein was detected. Regarding lanes 3 and 5, a clear protein band with a molecular weight around 18 kDa was observed and identified as β-Lg. No presence of bands at higher or lower MWs has been observed. The above results qualitatively confirm the presence of β-Lg in liposomes. Modifications in protein structure (Lefèvre & Subirade, 2001) are not expected in the experimental conditions used (30 °C and lipophilic environment) for encapsulation of β-Lg in PC/CH liposomes.

Stability of VE-loaded liposome suspension during storage

Turbidity measurements can be used as an alternative tool for determination (scattering particles) of size modifications and thus stability of liposomes (Dwiecki et al., 2007). In the case of liposomes, an increase in turbidity is interpreted as an increase in the aggregation of the vesicles due to the decrease of their stability (Nacka et al., 2001). As a consequence, an increase in the percentage of optical density is tightly related to an increase in the turbidity and finally a decrease in the samples stability. The presented turbidity data (Figure 3) are based on the results of three different sets of experiments. Results show an improvement stability of liposomes containing β-Lg and VE for both of aqueous medium tested (TBS and ddH_2O).

Improved stability could be explained by VE's properties as membrane-stabilizing agent and protector of the peroxidation of membrane lipids. This occurred by the formation of complexes with unsaturated fatty acids, or by restricting the molecular mobility of the membrane components (Atkinson et al., 2008; Ortiz et al., 1987). The molecular explanation of reduced mobility should be attributed to the hydrogen bonding (α-tocopherol hydroxyl group and oxygen of the phosphate group of phosphatidylcholine) (Srivastava et al., 1983) and hydrophobic interactions (α-tocopherol and phosphatidocholine fatty chain).

The presence of protein in liposomes formulations also seems to improve system stability (Figure 3). Studies have shown that β-Lg plays an important role in the morphology and structure of the phospholipid bilayers (Junghans et al., 2011; Lefèvre & Subirade, 2000, 2001; Zhang & Keiderling, 2006). According to literature, milk proteins are well known for their ability to stabilize emulsions and foams in food formulations (Ron et al., 2010; Semo et al., 2007).

Our findings for liposome stability over time correlate well with those of other studies (Park et al., 2011). The optical density (OD) measurements have shown a significant increase of stability for the new carrier PC/CH/β-Lg (Figure 3).

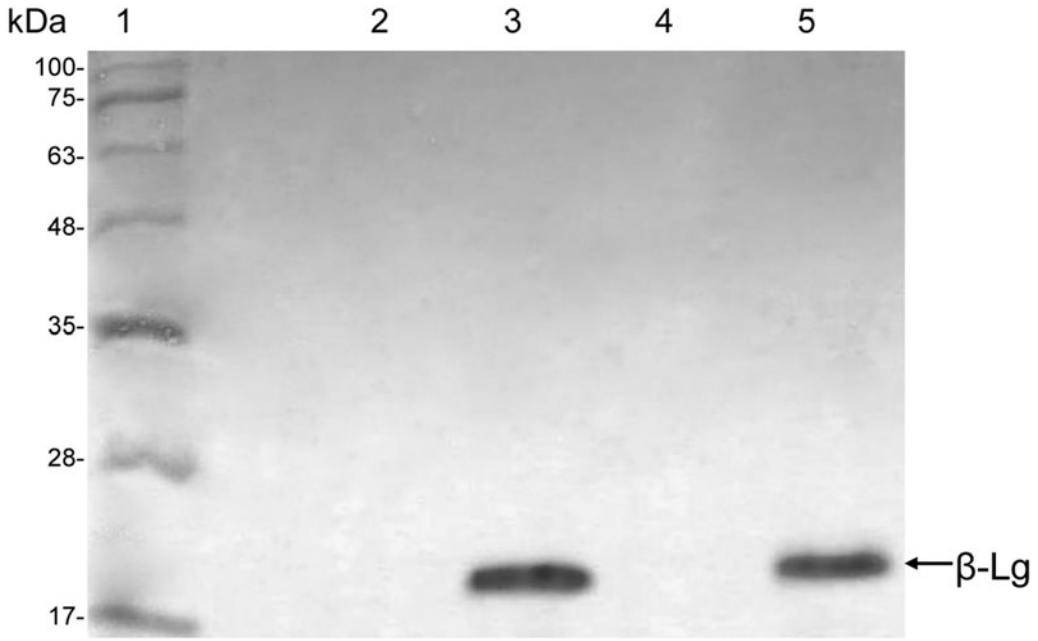

Figure 2. SDS-PAGE of different liposome fractions. Lanes: 1. Molecular weight markers (ranged from 180 kDa to 17 kDa), 2. PC/CH (2/1, mol/mol) empty liposomes, 3. PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, 4. PC/CH (2/1, mol/mol) VE-loaded liposomes, 5. PC/CH/β-Lg (2/1/0.004, mol/mol/mol) VE-loaded liposomes.

Observation of ddH_2O data in a time course indicates that the most stable system is PC/CH/β-Lg for the first 24 h but after 48 h (storage period) PC/CH/β-Lg/VE proved more stable.

Conformational changes of lipids induced by the presence of β-Lg can be very different depending on the nature of lipids (Lefèvre & Subirade, 2000). For the type of lipids been used (PC) in the current study, the most possible explanation for this could be that β-Lg molecules or some of its residues are incorporated into the bilayers, facilitating hydrophobic interactions with lipophilic chain.

Differential scanning calorimetry (DSC) for determination of antioxidant activity

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 the thermostability and thermo-oxidation (Chiavaro et al., 2011) while the kinetic parameters of the non-inhibited and inhibited fatty acid oxidation can also be determined (Liolios et al., 2009). The antioxidant activity is evaluated by the extrapolated temperature at the start of the oxidation process based on the measurements of the incubation period (Gortzi et al., 2006).

As it is demonstrated in Figure 4, all samples display different DSC curves. Differences in shape reflect differences in physicochemical characteristics of the system (Samuni et al., 1998).

Thermal oxidative decomposition of pure substances (pure β-Lg and pure VE) and that of liposome preparations (which contained or did not contain these molecules) were studied, using as onset temperature (T_o) of curves, the temperature at which the auto-oxidation process begins. Data indicate that the addition of β-Lg encapsulated in liposomes results in a more intense ($T_o = 330\,^{\circ}C$) antioxidant action (significant at $p < 0.05$) than itself ($T_o = 285\,^{\circ}C$), in pure form. This shift also implies an additional protection for the protein, due to encapsulation.

VE oxidative stability (pure or encapsulated) also presents similar behavior, where a shift from $T_o = 320\,^{\circ}C$ (pure VE) to $T_o = 330\,^{\circ}C$ for the encapsulated in liposomes was observed.

The PC/CH/β-Lg/VE system has a higher oxidation peak (around $T_o = 335\,^{\circ}C$) than those of VE ($T_o = 320\,^{\circ}C$) and β-Lg ($T_o = 280\,^{\circ}C$) in pure forms. It has also the highest oxidation peak of all liposome preparations (Figure 4).

In the case of liposomes containing β-Lg+VE, there is an increase of the antioxidant activity and thereby of the stability of the β-Lg/liposome/VE complex. VE is well accepted as naturally effective, lipid-soluble, and chain-breaking antioxidant (Zhao et al., 2011). The modified antioxidant action of VE after its encapsulation in β-Lg/liposome formulation was expected, since this system possesses new physicochemical characteristics and bioactivity depended on the structure and size of the system.

Additionally the protein existence in the liposomes improves their stability and results in a very stable structure, due to the possible interactions between the lipids and protein (Zhang et al., 2012).

Vehicles morphology (SEM)

Microscopy is a well-known tool to visualize the surface of a membrane and to immediately reveal important aspects, such as morphology, homogeneity, or size. Most microscopy techniques are fast, easy, and provide relatively straightforward specimen visualization (Zhang et al., 2012).

The morphology (shape and structure) of lyophilized liposomes have been regularly studied with SEM (Ntimenou et al., 2006). Microscopic observations showed that the

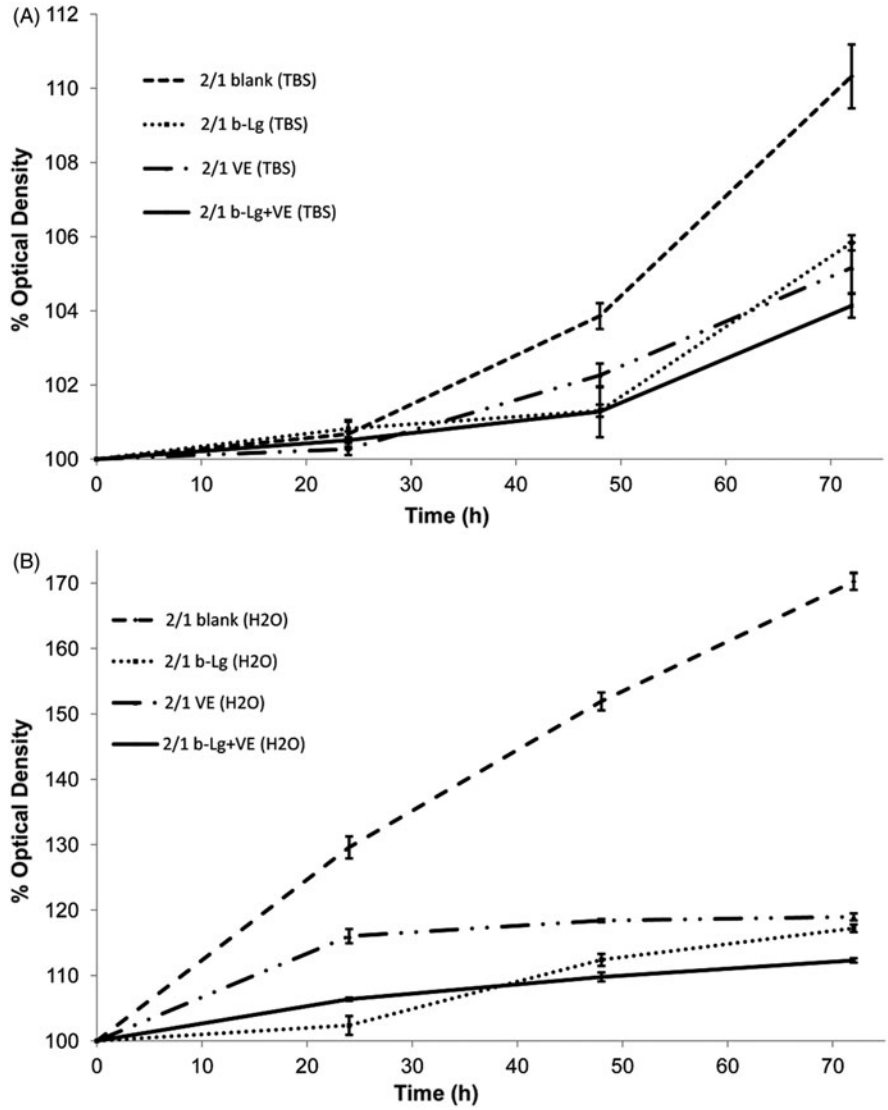

Figure 3. Stability behavior of liposomes during storage after predetermined time intervals: Turbidity measurements at 25 °C using absorbance at 450 nm over a 72 h period storage at 4 °C. A. Turbidity measurements of liposomes after dispersion in ddH_2O, B. Turbidity measurements of liposomes after dispersion in TBS. (▬ ▬) PC/CH (2/1, mol/mol) empty liposomes, (• • • • • •) PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, (— · · —) PC/CH (2/1, mol/mol) VE-loaded liposomes, (▬▬) PC/CH/β-Lg (2/1/0.004, mol/mol/mol) VE-loaded liposomes.

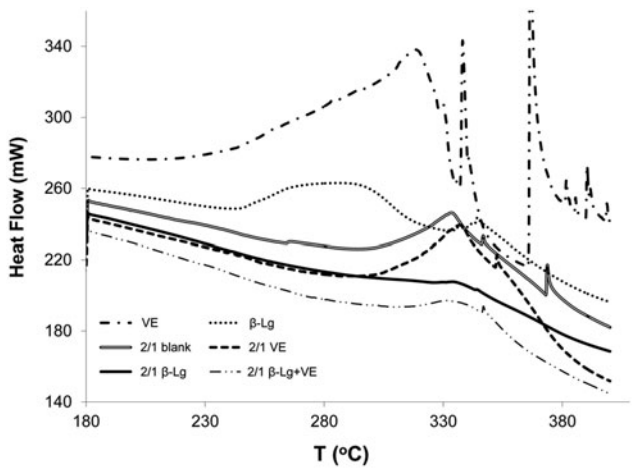

Figure 4. DSC oxidation curves of liposomes suspensions in the absence and presence of β-Lg and/or VE. (═══) PC/CH (2/1, mol/mol) empty liposomes, (▬▬) PC/CH/β-Lg (2/1/0.004, mol/mol/mol) liposomes, (▬ ▬ ▬) PC/CH (2/1, mol/mol) VE-loaded liposomes, (— · · — · ·) PC/CH/β-Lg (2/1/0.004, mol/mol/mol) VE-loaded liposomes, (• • • • • •) β-Lg, (▬ ▪ ▬) VE.

liposomes particles had a mostly spherical shape. All pictures in Figure 5 are presented in the same magnification for direct comparison.

Empty liposomes (Figure 5A), obtained by the thin film method, presented larger mean particle sizes, heterogeneous and with a wide particle size distribution compared to those containing β-Lg only (Figure 5B) or those containing β-Lg+ VE (Figure 5C).

Encapsulation of VE in a PC/CH membrane seems to decrease vesicle diameter, probably due to the formation of new structures. This observation correlates with those of other researchers (Afri et al., 2004), suggesting that the inclusion of VE acetate within the liposomal bilayer lead to the formation of smaller liposomes.

Images of liposomes containing β-Lg are smaller than empty and those containing β-Lg+ VE are even smaller. So, both β-Lg and VE reduce particle side during preparation. Also the addition of β-Lg and VE lead to a formation of bridges between neighbored liposome particles.

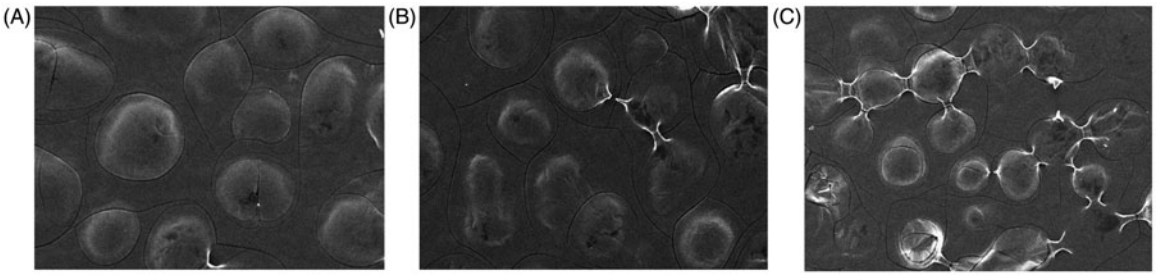

Figure 5. Scanning electron micrographs of DRV liposomes before and after encapsulation of β-Lg and VE obtained by thin film method (pH 7.4, room temperature). (A) Empty liposomes, (B) β-Lg loaded liposomes, (C) β-Lg+VE -loaded liposomes.

Conclusion

VE, a lipid-soluble and chain-breaking antioxidant, with a significant role in food preservation and nutrient supplements is a very sensitive molecule. As a result, the need for it to be effectively protected and delivered is of great importance. A beneficial effect of therapeutic supplementation with VE is well documented for several malabsorption states in humans.

Liposomes+β-Lg encapsulating VE were prepared using the DRV method. The EE of VE in DRV liposomes loaded with β-Lg was studied. The results of this study show that the trapping efficiency of VE is higher in the liposome containing β-Lg compared to the trapping efficiency of VE in liposome without the presence of the protein. The ability of β-Lg to bind hydrophobic molecules such as VE could not solely explain the above. Physicochemical alterations of lipids bilayer should also play an important role.

Furthermore, a promising stability behavior of the liposomes containing β-Lg/VE was observed, which might prove to be a very important factor, regarding the use of the liposomes/β-Lg complex as a carrier of VE. The system stability can also be attributed to the fact that VE binds and stabilizes the lipids bilayer.

Summarizing, in the present study, we demonstrate that the VE could be efficiently entrapped and delivered in liposomes/β-Lg formulation, a newly synthesized promising carrier. Future studies will evaluate the pharmacokinetics and biodistribution of the VE/β-LG -loaded liposomes as well as their efficacy to release the vitamin at an appropriate rate.

Acknowledgements

We would like to thank Prof. Philippos J. Pomonis for the availability of the Scanning Electronic Microscopy at his laboratory. We also thank Mrs Vivien Buchanan-Lappas for help with the English.

Declaration of interest

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program ''Education and Lifelong Learning'' of the National Strategic Reference Framework (NSRF) Research Funding Program: Heracleitus II: Investing in knowledge society through the European Social Fund. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

References

Afri M, Ehrenberg B, Talmon Y, et al. (2004). Active oxygen chemistry within the liposomal bilayer: Part III: Locating Vitamin E, ubiquinol and ubiquinone and their derivatives in the lipid bilayer. Chem Phys Lipids 131:107–21.

Antimisiaris SG. (2010). Preparation of DRV liposomes. Meth Mol Biol (Clifton, N.J.) 605:51–75.

Atkinson J, Epand RF, Epand RM. (2008). Tocopherols and tocotrienols in membranes: a critical review. Free Radic Biol Med 44:739–64.

Atkinson J, Harroun T, Wassall SR, et al. (2010). The location and behavior of α-Tocopherol in membranes. Mol Nutr Food Res 54: 641–51.

Badiee A, Jaafari MR, Khamesipour A, et al. (2009). The role of liposome charge on immune response generated in BALB/c mice immunized with recombinant major surface glycoprotein of Leishmania (rgp63). Exp Parasitol 121:362–9.

Balkina AS, Selischeva AA, Larionova NI. (2009). Liposomal formulations of protein proteinase inhibitors: preparation and specific activity. Biochem (Moscow) Suppl B: Biomed Chem 3:48–53.

Chanasattru W, Decker EA, Mcclements DJ. (2007). Modulation of thermal stability and heat-induced gelation of β-lactoglobulin by high glycerol and sorbitol levels. Food Chem 103:512–20.

Chen C, Han D, Cai C, Tang X. (2010). An overview of liposome lyophilization and its future potential. J Control Rel 142:299–311.

Chiavaro E, Rodriguez-Estrada MT, Bendini A, et al. (2011). Differential scanning calorimetry thermal properties and oxidative stability indices of microwave heated extra virgin olive oils. J Sci Food Agric 91: 198–206.

Cohn W. (1997). Bioavailability of vitamin E. Eur J Clin Nutr 51:S80–5.

Colletier JP, Chaize B, Winterhalter M, Fournier D. (2002). Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. BMC Biotechnol 2:9.

Da Silva Malheiros P, Daroit DJ, Brandelli A. (2010). Food applications of liposome-encapsulated antimicrobial peptides. Trends Food Sci Technol 21:284–92.

Dwiecki K, Górnaś P, Wilk A, et al. (2007). Spectroscopic studies of D-α-tocopherol concentration-induced transformation in egg phosphatidylcholne vesicles. Cell Mol Biol Lett 12:51–69.

Edwards KA, Baeumner AJ. (2006). Analysis of liposomes. Talanta 68: 1432–41.

Ge N, Zhang X, Keiderling TA. (2010). Kinetic studies of the interaction of β-Lactoglobulin with model membranes: stopped-flow CD and fluorescence studies. Biochemistry 49:8831–8.

Ghatnur SM, Sonale RS, Balaraman M, Kadimi US. (2012). Engineering liposomes of leaf extract of seabuckthorn (SBT) by supercritical carbon dioxide ($SCCO_2$)-mediated process. J Lipos Res 22:215–23.

Gonnet M, Lethuaut L, Boury F. (2010). New trends in encapsulation of liposoluble vitamins. J Control Rel 146:276–90.

Gortzi O, Lalas S, Chinou I, Tsaknis J. (2006). Reevaluation of antimicrobial and antioxidant activity of Thymus and spp. extracts before and after encapsulation in liposomes. J Food Prot 69: 2998–3005.

Hădărugă DI, Hădărugă NG, Lăzău C, et al. (2010). Liposomes containing undoped and Au^{+}/Ag^{+} doped titanium dioxide nanoparticles. Digest J Nanomater Biostruct 5:919–25.

Hansted JG, Wejse PL, Bertelsen H, Otzen DE. (2011). Effect of protein-surfactant interactions on aggregation of β-lactoglobulin. Biochim Biophys Acta – Proteins Proteomics 1814:713–23.
Hsieh YF, Chen TL, Wang YT, et al. (2002). Properties of liposomes prepared with various lipids. J Food Sci 67:2808–13.
Hwang SY, Kim HK, Choo J, et al. (2012). Effects of operating parameters on the efficiency of liposomal encapsulation of enzymes. Coll Surf B: Biointerfaces 94:296–303.
Jagannadham MV, Chowdhury C. (2012). Differential expression of membrane proteins helps Antarctic *Pseudomonas syringae* to acclimatize upon temperature variations. J Proteomics 75:2488–99.
Junghans A, Champagne C, Cayot P, et al. (2011). Probing protein-membrane interactions using solid supported membranes. Langmuir 27:2709–16.
Kawano K, Takayama K, Nagai T, Maitani Y. (2003). Preparation and pharmacokinetics of pirarubicin loaded dehydration–rehydration vesicles. Int J Pharmaceut 252:73–9.
Kontopidis G, Holt C, Sawyer L. (2002). The ligand-binding site of bovine β-lactoglobulin: evidence for a function? J Mol Biol 318: 1043–55.
Kontopidis G, Holt C, Sawyer L. (2004). Invited review: β-lactoglobulin: Binding properties, structure, and function. J Dairy Sci 87:785–96.
Koynova R, Caffrey M. (1998). Phases and phase transitions of the phosphatidylcholines. Biochim Biophys Acta (BBA) – Rev Biomembranes 1376:91–145.
Lalas S, Athanasiadis V, Gortzi O, et al. (2011). Enrichment of table olives with polyphenols extracted from olive leaves. Food Chem 127: 1521–5.
Lefèvre T, Subirade M. (2000). Interaction of β-lactoglobulin with phospholipid bilayers: a molecular level elucidation as revealed by infrared spectroscopy. Int J Biol Macromol 28:59–67.
Lefèvre T, Subirade M. (2001). Conformational rearrangement of β-lactoglobulin upon interaction with an anionic membrane. Biochim Biophys Acta – Protein Str Mol Enzymol 1549:37–50.
Liolios CC, Gortzi O, Lalas S, et al. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. Food Chem 112: 77–83.
Marsanasco M, Márquez AL, Wagner JR, et al. (2011). Liposomes as vehicles for vitamins E and C: an alternative to fortify orange juice and offer vitamin C protection after heat treatment. Food Res Int 44: 3039–46.
Mozafari MR, Johnson C, Hatziantoniou S, Demetzos C. (2008a). Nanoliposomes and their applications in food nanotechnology. J Lipos Res 18:309–27.
Mozafari MR, Khosravi-Darani K, Borazan GG, et al. (2008b). Encapsulation of food ingredients using nanoliposome technology. Int J Food Proper 11:833–44.
Mozafari MR, Reed CJ, Rostron C, et al. (2002). Formation and characterisation of non-toxic anionic liposomes for delivery of therapeutic agents to the pulmonary airways. Cell Mol Biol Lett 7: 243–4.
Muthu MS, Kulkarni SA, Xiong J, Feng SS. (2011). Vitamin e TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells. Int J Pharmaceut 421:332–40.
Nacka F, Cansell M, Méléardb P, Combe N. (2001). Incorporation of α-tocopherol in marine lipid-based liposomes: *in vitro* and *in vivo* studies. Lipids 36:1313–20.
Neunert G, Polewski P, Markiewicz M, et al. (2010). Partition of tocopheryl glucopyranoside into liposome membranes studied by fluorescence methods. Biophys Chem 146:92–7.
Ntimenou V, Mourtas S, Christodoulakis EV, et al. (2006). Stability of protein-encapsulating DRV liposomes after freeze-drying: a study with BSA and t-PA. J Lipos Res 16:403–16.
Ohtake S, Schebor C, Palecek SP, De Pablo JJ. (2005). Phase behavior of freeze-dried phospholipid-cholesterol mixtures stabilized with trehalose. Biochim Biophys Acta – Biomembranes 1713:57–64.
Ortiz A, Aranda FJ, Gomez-Fernandez JC. (1987). A differential scanning calorimetry study of the interaction of α-tocopherol with mixtures of phospholipids. Biochim Biophys Acta – Biomembranes 898:214–22.
Park SJ, Choi SG, Davaa E, Park JS. (2011). Encapsulation enhancement and stabilization of insulin in cationic liposomes. Int J Pharmaceut 415:267–72.
Perrie Y, Frederik PM, Gregoriadis G. (2001). Liposome-mediated DNA vaccination: the effect of vesicle composition. Vaccine 19: 3301–10.
Raneva GV, Shimasaki H, Ueta N, Takahashi J. (2003). Interaction between α-tocopherol, tocotrienols and astaxanthin in liposomes, subjected to lipid peroxidation. J Oleo Sci 52:347–52.
Rodríguez-Nogales JM, López AD. (2006). A novel approach to develop β-galactosidase entrapped in liposomes in order to prevent an immediate hydrolysis of lactose in milk. Int Dairy J 16:354–60.
Ron N, Zimet P, Bargarum J, Livney YD. (2010). Beta-lactoglobulin–polysaccharide complexes as nanovehicles for hydrophobic nutraceuticals in non-fat foods and clear beverages. Int Dairy J 20:686–93.
Sambrook J, Russell DW. (2001). Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.
Samuni AM, Crommelin DJA, Zuidam NJ, Barenholz Y. (1998). Differential scanning calorimetry. A tool to assess physical and chemical alterations in liposomes. J Thermal Anal Calorimetr 51: 37–48.
Sawyer L, Kontopidis G, Wu SY. (1999). β-Lactoglobulin – a three-dimensional perspective. Int J Food Sci Technol 34:409–18.
Scott G. (1997). Antioxidants: in science, technology, medicine & nutrition. Chichester: Albion Pub.
Semo E, Kesselman E, Danino D, Livney YD. (2007). Casein micelle as a natural nano-capsular vehicle for nutraceuticals. Food Hydrocolloids 21:936–42.
Srivastava S, Phadke RS, Govil G, Rao CNR. (1983). Fluidity, permeability and antioxidant behaviour of model membranes incorporated with α-tocopherol and vitamin E acetate. Biochim Biophys Acta – Biomembranes 734:353–62.
Vemuri S, Rhodes CT. (1995). Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharmaceutica Acta Helvetiae 70:95–111.
Wang X, Quinn PJ. (2000). The location and function of vitamin E in membranes (Review). Mol Membr Biol 17:143–56.
WHO (2004). Vitamin and mineral requirements in human nutrition: report of a joint FAO/WHO expert consultation, Bangkok, Thailand, 21–30 September 1998. Stylus Pub Llc.
Xu X, Costa A, Burgess DJ. (2012). Protein encapsulation in unilamellar liposomes: high encapsulation efficiency and a novel technique to assess lipid–protein interaction. Pharmaceut Res 29:1919–31.
Zaru M, Mourtas S, Klepetsanis P, et al. (2007). Liposomes for drug delivery to the lungs by nebulization. Eur J Pharmaceut Biopharmaceut 67:655–66.
Zhang L, Granick S. (2006). How to stabilize phospholipid liposomes (Using nanoparticles). Nano Lett 6:694–8.
Zhang X, Keiderling TA. (2006). Lipid-induced conformational transitions of β-lactoglobulin. Biochemistry 45:8444–52.
Zhang X, Tanner P, Graff A, et al. (2012). Mimicking the cell membrane with block copolymer membranes. J Polym Sci A: Polym Chem 50: 2293–318.
Zhao LP, Xiong H, Peng H, et al. (2011). PEG-coated lyophilized proliposomes: preparation, characterizations and in vitro release evaluation of vitamin E. Eur Food Res Technol 232:647–54.