**Changes in structural integrity of sodium caseinate films by the addition of nanoliposomes
encapsulating an active shrimp peptide fraction**

Pilar Montero, Mauricio Mosquera, Daniel Marín-Peñalver, Ailén Alemán, Óscar Martínez-
Álvarez and M. Carmen Gómez-Guillén *

Institute of Food Science, Technology and Nutrition (ICTAN-CSIC). José Antonio Novais 10,
Ciudad Universitaria, 28040 Madrid (Spain)

*Author for correspondence: mc.gomez@csic.es

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ABSTRACT

A shrimp peptide fraction (ST1) with biological activity (ABTS radical scavenging capacity, angiotensin-converting enzyme and dipeptidyl-peptidase-IV inhibitory activities), was encapsulated in partially purified soy phosphatidylcholine nanoliposomes (L-ST1) using glycerol to preserve the bilayer during the film-forming dehydration step. The z-average, zeta potential and encapsulation efficiency of L-ST1 were, respectively, 99.98 nm, -53.87 mV and 52.37 %. Transmission Electron Microscopy images showed that the liposomes incorporated in sodium caseinate films caused film matrix disruption, but vesicle structure was well preserved and uniformly distributed along the film matrix. The films with liposomes became more water soluble, adhesive and mucoadhesive, with no changes in thickness or transparency. The film showed a very favourable taste perception, regardless the presence or type of liposomes, while the buccal dissolution was faster with the films carrying the

23 liposomes. The film could be used in different food designs without distorting the sensory
24 acceptance of the final product.

25 **Key words:** shrimp peptide fraction, nanoliposomes, caseinate film, film structure, sensory
26 properties, mucoadhesion.

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29 **1. INTRODUCTION**

30 In the food sector there is currently a growing demand for products of natural origin, with
31 bioactive capacity, low toxicity and preferably environmentally friendly. To prepare this
32 functional food made to meet specific health requirements, a wide variety of biologically
33 active molecules can be used as ingredients. Great part of these molecules could derive from
34 industrial food by-products or waste. Valorisation of this material would result in an
35 economic and environmental benefit, and at the same time, value-added products or even
36 co-products would be obtained (López-Caballero et al., 2016).

37 Obtaining protein hydrolysates from industrial fish waste has received considerable
38 attention in the last decade (López-Caballero et al., 2013). Such hydrolysates are an
39 important source of bioactive peptides with diverse properties such as antioxidant,
40 antihypertensive or hypoglycaemic activity (López-Caballero et al., 2013, Ketnawa et al.,
41 2016, Ji et al., 2017). Bioactive peptides could be used as potential ingredients in functional
42 food. However, during storage and/or food processing, peptides can be subjected to
43 proteolytic degradation, show instability under extreme conditions (pH, temperature,
44 oxygen...) and/or interact with other components (divalent cations, lipids, proteins...) (Aasen
45 et al., 2003; Chollet et al., 2008; Mozafari et al., 2008). All these factors will greatly limit their
46 potential health benefits, and make necessary the search for protective mechanisms to
47 maintain the peptides in active forms until consumption or even to directly deliver them to
48 the physiological targets. Liposomes have the ability to trap water-soluble, lipid or
49 amphiphilic materials, therefore the encapsulation in liposomes has been reported to be a
50 good way to protect and transport bioactive peptides (Mozafari et al., 2008; Malheiros,
51 Daroit and Brandelli, 2010). The high polyunsaturated fatty acid composition and residual
52 tocopherol content in natural soy lecithin or partially purified phosphatidylcholine provides
53 extra nutritional value for food-grade liposome production (Taladrid et al., 2017).

Furthermore, the use of a film matrix as vehicle of peptide-loaded liposomes could represent a versatile strategy to introduce them into a functional food, without the need of restructuring or greatly affecting the food presentation. At the same time, the undesirable effects derived from strong flavour or excessive interactions of bioactive peptides with other constituents present in the film or in the food matrix could be greatly avoided. It should be noted that the film by itself could also be considered as a model system of food with low water content. However, free peptides or liposome-encapsulated peptides could induce physical changes in the film structure that should be taken into account. To this respect, Giménez et al. (2009) observed that the incorporation of peptide hydrolysates induced strong plasticization in gelatin films. The addition of liposomes was also found to change the molecular structure in chitosan films, leading to the decrease in density and tensile strength, with concomitant increase in water solubility (Cui et al., 2017). Casein films have been reported to be an excellent matrix to incorporate nisin-loaded liposomes and halloysite (nanoclay), without altering the film mechanical resistance and thermal degradation properties (Boelter and Brandelli, 2016).

The use of mucoadhesive edible films carrying bioactive peptides-loaded liposomes (either as a quick-dissolving edible strip or as a food wrapping) could be an interesting way for buccal delivery of these bioactive molecules. The buccal and sublingual mucosae are highly permeable, and it would allow the nanocapsules loading bioactive compounds to pass directly to the systemic circulation and further to penetrate into tissues through fine capillaries (Patel et al., 2011). Recent research indicates that these mucoadhesive systems must provide fast release in the oral cavity (Silva et al., 2015; Mašek et al., 2017). Regardless the mode of presentation, the response in mouth of such mucoadhesive films requires being easy-dissolving, avoiding the impression to ingest an artificial plastic.

The objective of the present work was the preparation of a functional sodium caseinate film with mucoadhesive and good sensory properties by introducing nanoliposomes containing an active peptide fraction from discarded shrimp. The peptide fraction was characterised in terms of molecular weight, aminoacid composition and in vitro biological activity (antioxidant, antihypertensive and hypoglycaemic capacity).

2.Materials & Methods

2.1.Preparation of the peptide fraction

Shrimp (*Penaeus notialis*) frozen during 6 years at -20 °C (non-commercial value) were unfrozen and washed in cold water. Endogenous enzymes were firstly inactivated by heating shrimp (previously vacuum packed) in a water bath at 80 °C for 20 min. Boiled shrimp were then grounded in an Osterix blender (Osterizer, Par Sunbeam, Mod. 867 50 E, USA) and the resulted homogenate was used as raw material. The sample was then mixed with 0.07 M phosphate buffer at pH 8 (1:5, w/v) and the slurry was heated to 38 °C. The enzymatic hydrolysis was started by adding 1% (w/w) of Pancreas trypsin (7 Units/mg, Novozymes, Bagsvaerd, Denmark). During hydrolysis, temperature was controlled, and the pH was kept constant by addition of 1 N NaOH solution using a pH-stat (TIM 856, Radiometer analytical, Villeurbanne, France). After the required digestion time (90 min), the reaction was stopped by heating the sample at 90 °C for 20 min. The hydrolysate was sieved to remove the solid material and the liquid fraction was clarified by centrifuging at 12000 g (Sorvall RT60008 centrifuge, DuPont Co., Delaware, USA) at room temperature for 20 min.

The degree of hydrolysis (DH), determined using the pH-stat method (Adler-Nissen, 1986), was 17.77%.

The hydrolysate was filtered through a 1 kDa MW cut-off (MWCO) membrane of 1 kDa (Amicon Ultra-15 centrifugal filter, Merck Millipore, Billerica, MA, USA). Permeate was freeze-dried and stored at – 80 °C. The peptide fraction obtained was designated as ST1.

2.2. Characterisation of the peptide fraction

The molecular weight distribution of ST1 was determined by SEC-HPLC (SPE-MA10AVP, Shimadzu, Kyoto, Japan) as described by Martínez-Álvarez et al. (2012). Aprotinin (6,511 Da), Vitamin B12 (1,345 Da), Hippuryl histidyl leucine (429 Da) and glycine (75 Da) were used as molecular weight standards.

The amino acid composition of the peptide fraction was determined following the procedure described by Alemán et al. (2011).

The Angiotensin converting enzyme (ACE) inhibitory activity and the ABTS radical (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging capacity of ST1 were determined according to Alemán et al (2011). The IC₅₀ value was defined as the concentration of protein (µg protein/mL) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE activity). The results of the ABTS radical scavenging capacity were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g protein, based on a standard curve of vitamin C. The amount of protein was calculated from the amino acid analysis. Determinations were carried out in triplicate.

The Dipeptidyl peptidase-IV inhibitory activity (DPP-IV) inhibiting activity of the ST1 was measured according to Tulipano, et al. (2011) with slight modifications. The assay was performed in a 96-well plate. Briefly, 10 µL of human DPP-IV (previously diluted in 100 mM Tris HCl buffer, pH 8) were incubated in the absence or in the presence of different concentrations of sample (final volume 300 µL per well) at 37 °C for 15 min. The sample was previously diluted in 100 mM Tris HCl buffer at pH 8. The reaction started after addition of

100 μ L of assay buffer containing the chromogenic substrate (H-Gly-Pro-AMC·HBr) at final concentration of 25 μ M. The change in fluorescence at 355 nm/460 nm was monitored at 1-min intervals for 15 min in an Appliskan Multimode Microplate Reader (Thermo Fisher Scientific, MA, USA). Recorded data were plotted versus time. The inhibitory activity was calculated from the initial (maximal) slopes obtained in the absence or in the presence of the test sample at different concentrations. Logarithmic regression was used to calculate the IC_{50} value, or concentration of hydrolysate needed to inhibit 50% of DPP-IV activity.

2.3. Purification of crude soybean lecithin

Samples of crude soybean lecithin were provided by Manuel Riesgo, S.A. (Madrid, Spain). Partially purified phosphatidylcholine (PC) was obtained by performing two washes with acetone, following the procedure described by Taladrid et al. (2017).

2.4. Liposome production

PC was milled with a mortar in a porcelain pestle and filtered through a sieve. Five (5) g of PC were then mixed with 0.5 g of ST1. The mixture was dissolved in 20 mL of phosphate buffer (10 mM) at pH 7 and kept in a bath at 80 °C for 1 h. The solution obtained was diluted in 15 mL of phosphate buffer (v/v) containing 5 mL of glycerol; the mixture was kept in a bath at 80 °C for 1 h and then vortexed at 60 °C to produce multilamellar lipid vesicles (MLVs), after to complete the solution with 60 mL of phosphate buffer. Sonication of the preparation was carried out in an ultrasonic cell disrupter (Model Q700, Qsonica sonicators, Newton, CT, USA) with a power rating of 700 W (90% amplitude), where the MLV were exposed to five cycles of sonication for 1 min, followed by 1 min stopped to allow sample cooling. The peptide-loaded liposome suspension (L-ST1) was then filtered through 0.22 μ m membrane (Millex-GP Syringe Filter Unit, Millipore, USA). For comparison purposes, empty liposomes

(L-E) were prepared in the same way, dissolving PC directly into the phosphate buffer without the peptide fraction.

2.5.Characterization of liposomes

The mean particle size (z-average in intensity), size distribution (polydispersity index) and ζ -potential of liposome dispersions were measured using a Nanosizer (Nano-ZS, Nanoseries, Malvern Instruments Ltd, Worcestershire, UK). Measurements were performed by triplicate immediately after diluting the formulations in 10 mM phosphate buffer, to avoid particle aggregation.

2.6. Entrapment efficiency

Sephadex filtration method was performed to separate free ST1 and encapsulated (L-ST1). A glass-column (350 mm high x 7 mm diameter) was packed with 8 mL of drained resin Sephadex® G-75 (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mmol/L phosphate buffer pH 7.0 as mobile phase. Fractions of 0.5 mL were collected. Entrapment efficiency (EE) was evaluated by determining the amount of protein in each one of the collected fractions by the Lowry test (Lowry et al. 1951). Data was analyzed with Origin 8SR0 data analysis software version 8.0724 (OriginLab Corporation, Northampton, MA 01060 USA). Data obtained were plotted and areas of curves representing ST1 and L-ST1 concentration were determined (output timing of each of them were determined in a previous analysis). The EE was calculated using the following equation:

$$EE = (L-ST1 \text{ area} / \text{free ST1 area} + L-ST1 \text{ area}) \times 100.$$

2.7. Film preparation

For film preparation, 8 g of sodium caseinate were firstly dissolved in 100 mL of phosphate buffer 10 mM pH 7. The mixture was subjected to 5 cycles of sonication for one minute

each, with intervals of 1 min rest in an ultrasonic cell disruptor (Model Q700, Qsonica sonicators, Newton, CT, USA). Then, 12.5 mL of this solution were mixed with 7.5 mL of the dispersion of L-ST1. The obtained mixture was placed on a plate (11.5 x 11.5 cm) and dried at 35 °C in a forced air oven (Binder, model FD 240, Tuttlingen, Germany) for 12 h. Films were conditioned at 58% RH (with saturated KBr solution) and 22 °C for 4 days prior to analysis.

2.8. Film properties

The moisture content was determined according to the official AOAC method 934.01 (1995).

The water solubility was determined by a modification of the technique described by Alemán et al. (2016). Film pieces of 2x2 cm were placed in aluminum capsules (7 cm diameter) with 15 mL of distilled water for 15 h at 22 °C. The contents were filtered through Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, UK) to recover the non-solubilized film, that was dried at 105 °C for 24 h. The film water solubility was calculated according to the following equation:

Where: W_o is the initial weight of the film (dry matter) and W_f is the dry weight of the remaining dried film.

The film thickness was determined according to the technique described by Alemán et al. (2016). Six measurements on each film were taken in different random areas using a micrometer (MDC- 25M, Mitutoyo, Kanagawa, Japan). The results were expressed as the average of 6 measurements.

The film transparency was determined according to the technique described by Alemán et al (2016). The measurements were performed at 600 nm using a spectrophotometer (Model

SPC -240, Shimadzu, Kyoto, Japan). The opacity index was calculated by the equation $O = \text{Abs}_{600}/x$, where Abs 600 is the value of absorbance at 600 nm and x is the thickness (mm).

2.9. Transmission Electron Microscopy (TEM)

TEM was used to study the morphology of liposomes and film structure. The microscope used was a JEOL JEM -1010 (JEOL, Tokyo, Japan), with a working potential of 80 kV. The photographs were taken with a CCD camera and processed by the software "Soft Imaging Viewer". Liposomes were analyzed by negative staining technique. One hundred microliters of the suspension of liposomes were diluted in 10 mL of distilled water. A drop of this solution was placed on parafilm paper. Above the drop, a copper grid (200 mesh) covered graphite was placed for 5 min. Finally, the grid was placed in a 2% solution of uranyl acetate for 20 s.

The films were fixed in a solution of 2.5% glutaraldehyde and 4% paraformaldehyde dissolved in phosphate buffer 0.1M pH 7 for 2 h. The samples were washed with distilled water. The post-fixation was performed with osmium tetroxide to 1%. The samples were dehydrated with acetone, embedded in Epon resin 812 and dried at 200 °C. Ultrathin sections of sample were cut using an ultramicrotome (LKB Bromma 2088, Switzerland) and stained with uranyl acetate 2%.

2.10. Adhesion and Mucoadhesion test

The tests were performed using a texture analyser TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) based on the method described by Ivarsson & Wahlgren (2012) with some modifications. The method describes the tensile strength measurement as the maximum force necessary to detach the film (placed on the bottom platform of the texturometer) of a layer of commercial mucin porcine (Sigma Aldrich, St. MO. USA) solution

(20% w/v), spread on the underside of a cylindrical metal probe (20 mm diameter). Under these conditions, the probe moves downwards at a constant speed of 0.2 mm/s until to contact the film. To standardize the measurement, a constant preload of 0.5 N is applied for 15 s. The probe is then removed at a constant speed of 2 mm/s, and the maximum detachment force (N) is recorded. The determinations were made as a minimum of 8 measurements. An adhesion test without using mucin was carried out under identical conditions.

2.11. Sensory properties

The sensory evaluation was carried out by 12 trained panellists. Nevertheless, they showed previous experience in sensorial analysis of edible films. The films were cut in strips of 5 cm x 2 cm and used as samples. The strips were put in the mouth and allowed to dissolve. The parameters selected as descriptors were: salivation intensity, adherence to the teeth, flavour and time to perceptible dissolving. The descriptive analysis was carried out using a scale from 1 to 10 for the attributes: salivation intensity (0 = null, 10 = excessive), adherence (0 = null, 10 = excessive) and flavour (0 = strange or unpleasant flavour, 10 = very pleasant flavour). For the last descriptor (dissolving time), it was established on a scale of 3 intervals: short time (between 0-40 seconds), medium (between 40-80 s) and long-time (greater than 80 s).

2.12. Statistical analysis

Statistical analysis was performed using a STATGRAPHICS Centurion XV version 15.2.06 program (StatPoint, Inc. Warrenton, VA, USA). One-way ANOVA was followed by the Tukey HSD test for the statistical treatment. The level of significance was set at $p \leq 0.05$.

3. Results & Discussion

3.1. Properties of the peptide fraction

The amino acid composition of the peptide fraction ST1 is shown in Table 1. The most abundant amino acid residues were Gly and Glu (18.5 and 11.5 % respectively), with also a relatively high amount of Pro and Ala (4.7 and 9% respectively) and 52% of total hydrophobic residues. Although the amino acid composition may vary depending on species, age and type of feed, relatively high amounts of Gly, Pro and Ala have been also reported in different crustacean species (Cobb et al., 1974, Mente et al., 2002; Espinosa et al., 2013). It is worth noting the contribution of Hyp and Hyl residues, specific collagen amino acids, attributed to the hydrolysis of intramuscular connective tissue.

ST1 presented a molecular weight profile at 214 nm with two differentiated fractions with molecular weight average around 550 Da and 200 Da (Figure 1). The molecular weight profile was different when signal was recorded at 280 nm. Thus, three different peaks rich in aromatic residues were observed, with molecular weight averages around 490, 240 and 150 Da. This indicates the presence of different peptide populations. The fractions with molecular weight average around 200 and 240 Da would correspond to small dipeptides, while that around 150 Da may correspond to free aromatic residues. By taking into account the relative abundance of the different amino acids observed in Table 1, it could be expected a high frequency of dipeptides including Ala or Gly in the fraction around 200 Da. The fraction around 240 Da should contain dipeptides including one residue of Tyr, Trp or Phe in their composition. Moreover, the fractions around 490 and 550 Da could correspond to peptides containing 4 to 5 residues. Cao et al. (2009) found a relatively similar range (915-207 Da) in shrimp hydrolysates made with Alcalase.

263 The antioxidant activity of ST1, determined by the ABTS radical scavenging ability, was 69.13
264 ± 1.39 mg VCAC/g. This activity was significantly lower than that of a peptide fraction (< 10
265 kDa) obtained by hydrolysis of shrimp muscle with Alcalase (122.16 ± 4.95 mg VitC Eq/g)
266 (Alemán et al., 2016). The radical scavenging ability of ST1 may be related to the
267 considerable amount of hydrophobic amino acids, and more specifically, to the abundance
268 of Pro, Ala, Val and Leu (Mendis et al., 2005).

269 The peptide fraction ST1 also showed noticeable ACE-inhibitory capacity ($IC_{50} = 74 \pm 7.1$
270 $\mu\text{g/mL}$). Similarly, Cheung & Li-Chan (2010) determined an IC_{50} value of $70 \mu\text{g/mL}$ for a
271 shrimp (*Pandalopsis dispar*) waste Protamex hydrolysate. The considerable ACE-inhibitory
272 activity of ST1 could have been favoured by the abundance of Pro residues and hydrophobic
273 residues in the peptide chain (Contreras et al., 2009). The ACE-inhibitory capacity of protein
274 hydrolysates has been also associated with the presence of small peptides in their
275 composition (Kim et al., 2012). Some of these peptides, contained in the BIOPEP DATABASE
276 created by Minkiewicz et al. (2008), could be Leu-Val-Tyr-Pro (490 Da) or Tyr-Ala (252 Da).

277 The DPP-IV inhibitory activity showed an $IC_{50} = 0.46 \pm 0.05$ mg/mL. This activity is slightly
278 higher than that reported for protein hydrolysates from Atlantic salmon gelatin ($IC_{50} = 1.35$
279 mg/mL, (Li-Chan, Hunag, Jao, Ho & Hsu, 2012), krill protein ($IC_{50} = 0.833$ mg/mL fraction III)
280 (Ji et al 2017) or barbell protein (1.94 mg/mL) (Sila et al., 2015). Most of the DPP-IV
281 inhibiting peptides described contain between 2-8 amino acid residues (Li-Chan et al., 2012;
282 Sila et al., 2015). The peptide sequence seems to be the most determinant factor, since the
283 DPP-IV enzyme presents a great specificity for substrates type X-Ala-Y or X-Pro-Y in the N-
284 terminal side of the peptide chain (Yazbeck et al., 2009). The presence of hydrophobic amino
285 acids at the N-terminal position seems also to be important for the enzymatic inhibition (Sila
286 et al., 2015). Our shrimp hydrolysate presented a high content of short peptides that may

include hydrophobic residues and/or Ala that could be responsible for the important DPP-IV-inhibiting activity showed in this work.

3.2. Liposome properties

The peptide fraction ST1 was encapsulated in liposomes of phosphatidylcholine (L-ST1). The mean particle size of liposomes was 99.98 ± 4.0 nm and 94.4 ± 2.0 nm for L-ST1 and L-E (empty liposome), respectively. The incorporation of the peptide fraction led to a slight increase in the liposomal average size. A similar effect was observed by Taylor et al. (2007) in nisin-loaded PC liposomes, with no relationship between nisin concentration and liposomal average size. In the present work, both the peptide and phosphatidylcholine concentrations were selected according to previous studies (Mosquera et al., 2014). The polydispersity index (PDI) for L-ST1 and L-E were, respectively, 0.186 ± 0.006 and 0.192 ± 0.007 . The PDI measures the degree of particle sizes variation in the liposome dispersion. A value of ≈ 0.2 would indicate relatively low polydispersity of the liposomes formed (Da Silva Malheiros et al., 2011). Liposomes loaded with peptides (<6.5 KDa) from collagen squid tunic, which were prepared under similar conditions, had lower z-average (75.5 ± 0.5 nm), but slightly higher polydispersity (0.231 ± 0.010) (Marín et al., 2018). The slight difference in particle size could be attributed to the different amino acid profile, which could influence the liposomal membrane conformation.

The zeta potential was -53.86 ± 2.91 mV and -44.37 ± 2.85 mV for L-ST1 and L-E, respectively. The zeta potential measures the electric charge intensity of the liposomal surface, and is commonly used as a stability index of a colloidal dispersion. Based on this assumption, the presence of ST1 contributed slightly to increase the liposomal stability, since higher electronegative value was recorded for L-ST1. The composition of phospholipids and their interactions with the encapsulated compound can determine the surface charge of liposomes (Da Silva Malheiros et al., 2011). According to Taylor et al. (2007), the possible

chemical interaction of peptides with the phospholipid membrane may exert a stabilizing effect on nanovesicles. PC liposomes normally show properties of uncharged or slightly anionic monolayers at near neutral pH values (Bailey and Sullivan, 2000). Regarding the zeta potential of L-E, there is a big difference when compared with empty liposomes obtained by Taylor et al. (2007), who reported a zeta potential of -8 mV for PC liposomes prepared by the film hydration method. The liposomes prepared in the present study without peptide entrapment showed zeta potential of -44.37 mV, strongly suggesting that other lipids or impurities in the partially purified PC may have influenced the membrane charge (Taladrid et al., 2017). As reported in previous work by Taladrid et al. (2017), glycerol included in the production of the liposomes was not the main responsible for the high electronegative zeta potential. Glycerol has been used to maintain vesicle integrity, prevent vesicle sedimentation and loss of the entrapped peptides in the filmogenic solution, which should be subsequently dehydrated to produce the edible sodium caseinate film. Lipid vesicles dehydrated or freeze-dried in the absence of a protective solute may undergo massive fusion with concomitant release of the entrapped material. To this respect, the bilayer of PC vesicles has been shown to present high permeability to glycerol. It results in its equilibration on both sides of the vesicle membrane at the appropriate concentration, leading to an increased protection against freeze-thaw induced leakage (Mozafari, 2005). On the other hand, vesicles composed of phospholipids, glycerol and water, the so called glycosomes, have been reported to increase the liposomal bilayer fluidity (Manca et al., 2013).

The encapsulation efficiency of L-ST1 was $52.37 \pm 2.38\%$, this being an intermediate value among different peptide encapsulation efficiencies described in the literature, where it is possible to find values $< 20\%$ and $> 90\%$ (Were et al., 2018; Da Silva Malheiros et al., 2011; Mosquera et al., 2014; Marín et al., 2018). The encapsulation efficiency depends greatly on the electrostatic interactions between the peptides and the phospholipid (Were et al.,

2003). The peptide fraction ST1 has a balanced content of hydrophobic and hydrophilic amino acids, which means that about half of the polar peptide material may be trapped in the core of the liposome and / or associated with the polar head groups exposed to the outside of PC, while the non-polar material would be preferably interacting with the available hydrocarbon tails forming the bilayer (Mozafari et al., 2008).

The micrographs shown in Figure 2 depict liposomes with well-defined spherical morphology, which appeared more uniformly dispersed in the case of L-E suspension. Moreover, the bilayer formed by the hydrophobic tails of phosphatidylcholine in L-E was more clearly visible than with L-ST1. This effect was attributed to the interference of the more hydrophobic peptide material into the lipid bilayer, being likely responsible for the slight increase in the average particle size, as well as greater liposomal stability, as described above. The L-ST1 dispersion exhibited some areas with apparently cross-linked material forming a network around the nanovesicles, which would correspond to peptide material interacting with the polar heads of phosphatidylcholine.

3.3. Film properties

The dispersion with the encapsulated peptide fraction was incorporated into the formulation of edible films made with sodium caseinate (FL-ST1). In order to ascertain the effect of peptide-containing liposome addition in the film properties, analogue films with empty liposomes (FL-E) and without liposomes (F) were also prepared.

The structural integrity of the liposomes in the film was verified by TEM analysis (Figure 3). Both types of liposomes showed an even distribution and were well integrated in the film protein matrix despite provoking an evident discontinuity (Fig. 3 a,d). Regarding the liposomal morphology, it is generally observed a tendency to lose the spherical shape, yielding more irregular and oval perimeter structures, while retaining in general the

structural integrity and original size. No evident vesicular fusion or aggregation was observed, regardless the type of liposome. The lipid bilayer was clearly visible in both the empty liposomes and the peptide-containing liposomes.

The physical properties are important characteristics to evaluate the film and its potential application. Physical properties of films are presented in Table 2. Films with liposomes showed lower moisture content than films without them, especially those with empty liposomes. The discontinuous matrix induced by the presence of liposomes might have favoured water removal during the film dehydration step. Incorporating nanovesicles slightly increased the film density, especially those incorporating the peptide fraction; however, thickness did not significantly ($p > 0.05$) change in any case. No significant differences ($p > 0.05$) in the level of transparency was found, which is attributed to the small size (range nano) of added liposomes. However, the incorporation of emulsified lipids in sodium caseinate films has been described to produce a marked decrease in transparency, with average particle sizes in a range between 0.8 and 2.5 microns (Fabra et al., 2010).

Both films with liposomes were significantly more soluble in water than the film without the nanovesicles. The increased solubility could be attributed to the discontinuity producing the liposomes in the aggregated caseinate matrix, more pronouncedly when including the empty liposomes. The non-encapsulated peptide fraction in FL-ST1 would likely contribute to reduce the protein matrix discontinuity by favouring peptide interactions leading to noticeable lower water solubility with respect to those matrix that contain empty liposomes.

Since these films have to be dissolved in the mouth, either with the intention of palatability improving, or with the intention that the liposomes pass through the sublingual and buccal mucosa to the blood capillaries, the property of easy and rapid dissolution is of great interest. According to Souza et al. (2009), the desired solubility in an edible film depends on the required function, so that less soluble films could be used to protect products with high

water activity, whereas highly water soluble films could serve as bioactive carriers and allow absorption at the oral cavity level (Heinemann et al., 2013).

A sensory evaluation of the different films was carried out (Figure 4). None of the three films showed significant differences ($p < 0.05$) in the salivation intensity during the dissolution in the mouth, or in the uneasy feeling of adherence to the teeth, which were low. There were also no significant differences in flavor, which was described by the judges as a pleasant taste and reminded of milk and / or toffee, which was attributed to the presence of casein and moderate heat treatment for its preparation. There were no signs of bitterness or astringency that the presence of free amino acids or peptides could cause. Regarding the dissolving time of the film, all the judges considered that the time was in general "short" for the 3 samples. However remarkable differences were noticed between those that contained liposomes (loaded or empty), which delayed no more than 40 s, and those without liposomes, which took longer time to dissolve, between 40 s up to 80 s ($p \leq 0.05$). As discussed before, the liposomes produced some discontinuity in the film matrix, which makes it easier to disintegrate and became more water-soluble.

All of them are factors that can contribute to this prototype of loaded liposome carrier film, being a good candidate for the elaboration of a functional film destined for the active compounds to be absorbed by the mucous membranes of the oral cavity, or to be a wrap as a new design for functional foods with enhanced palatability. In the last years, some authors have indicated that these characteristics are suitable for absorption via oral and sublingual mucosa (Patel et al., 2011; Mašek et al., 2017).

The adhesion and *in vitro* mucoadhesion capacity of the films is indicated in Table 3. Different authors describe that the mechanism of mucoadhesion requires three successive stages: contact (adhesion), interpenetration and consolidation (Ivarsson and Wahlgren, 2012; Sandri et al., 2015). For the first stage of adhesion, the physical state is fundamentally

influenced by hydration, in the second the contact between the highly adhesive polymer and the mucosal epithelium is established, producing an interaction at the level of the mucus layer (with high content in mucin), and in the third, after a while, mechanical and chemical bonds are established (Ivarsson and Wahlgren, 2012; Sandri et al., 2015).

Assuming the importance of hydration, and given that at the oral level is practically instantaneous, and the film dissolves very quickly as it has been observed in the sensory analysis, the *in vitro* instrumental measurement has been carried out by conditioning the films at two relative humidity levels: 58% RH, which is the humidity at which the films are normally stabilized, and 75% RH, which is a considerably higher humidity but still allows these films to be easily handled.

Mucosadhesion values are much higher than those shown for the adhesion test, which is indicative of a mucoadhesive effect of the polymer film (Ivarsson and Wahlgren, 2012). This effect is maintained at both relative moisture levels, although the differences are lower at 75% RH (the ratio mucoadhesion / adhesion values at 58% RH ranged from 74.59 to 38.97, and at 75% RH from 33.34 to 18.32). In both, adhesion and mucoadhesion test at 58% HR, a significant increase can be seen with the inclusion of liposomes inside the film, and even more when these are loaded with peptides. This effect is attributed to the plasticizing effect of the liposomes favoring the discontinuity of the matrix and greater solubilization of the biopolymer matrix. However, at 75% HR in mucoadhesion test there are not significant differences, probably because at this humidity the matrix is quite lax itself.

Conclusion

A low molecular weight peptide fraction from a shrimp hydrolysate, with antioxidant activity, ACE- and DPP-IV inhibiting activity, was successfully encapsulated in soy phosphatidylcholine nanoliposomes. These liposomes showed high stability, an average size

of ≈ 100 nm, and a relatively high encapsulation efficiency ($\approx 52\%$). The results of the present study indicate that soy phosphatidylcholine liposomes can be incorporated in edible films of sodium caseinate without apparent loss of vesicle structural integrity, but causing internal discontinuities in the film matrix that increased the water solubility. The resulting films have adequate sensory characteristics to dissolve rapidly in the mouth, especially those containing liposomes, with a very pleasant taste. The film mucoadhesion capacity was favored by the increase of film moisture and the presence of liposomes, especially when they were loaded with the peptide fraction. The addition of liposomes enhanced the palatability of the film in the mouth and therefore it could be used in a variety of food designs without distorting the sensory acceptance of the final product.

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Legend to Figures:

Figure 1.- Molecular weight distribution of ST1 peptide fraction by SE-HPLC.

Figure 2.- TEM microscopy of liposomal dispersions at different magnifications: (a and b) with empty liposomes (L-E); (c and d) with ST1-loaded liposomes (L-ST1).

Figure 3.- TEM microscopy of sodium caseinate films at different magnifications: (a and b) with empty liposomes (L-E); (c and d) with ST1-loaded liposomes (L-ST1).

Figure 4.- Sensorial attributes of films without liposomes (F), film with empty liposomes (FL-E), and film with liposomes loaded with ST1 (FL-ST1).

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Table 1. Aminoacid composition of the peptide fraction ST1

Aminoacid residue	Number of residues (%)
Asp	82
Thr	40
Ser	49
Glu	115
Gly	185
Ala	90
Cys	4
Val	44
Met	22
Ile	35
Leu	69
Tyr	24
Phe	33
Hyl	2
His	17
Lys	75
Arg	60
Hyp	7
Pro	47
Σ Hidrophobic*	524

* Gly, Pro, Ala, Val, Ile, Met, Leu, Phe

Table 2. Physical properties of films: FL-ST1 film with liposomes loaded with ST1, FL-E film with empty liposomes, and F: film without liposomes.

	F	FL-E	FL-ST1
Moisture (%)	37.58 ± 1.39 ^a	21.96 ± 1.94 ^b	28.49 ± 1.26 ^c
Thickness (mm)	0.097 ± 0.02 ^a	0.090 ± 0.02 ^a	0.097 ± 0.03 ^a
Density (g/cm ³)	1.07 ± 0.045 ^a	1.15 ± 0.023 ^b	1.32 ± 0.013 ^c
Solubility (%)	37.58 ± 1.4 ^a	91.52 ± 1.2 ^b	58.39 ± 2.4 ^c
Transparency	1.07 ± 0.37 ^a	1.00 ± 0.16 ^a	1.22 ± 0.02 ^a

Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among samples for each property.

Table 3.- Adhesion and mucoadhesion, expressed as maximum force (N), of films conditioned at 58% and 75% relative humidity (RH). F: film without liposomes; FL-E: film with empty liposomes; FL-ST1: film with liposomes loaded with ST1.

	Adhesion	Mucoadhesion	Adhesion	Mucoadhesion
	58% RH		75% RH	
F	1.18 ± 0.00 ^a	87.64 ± 1.95 ^a	4.23 ± 0.04 ^a	140.92 ± 12.70 ^a
FL-E	2.34 ± 0.20 ^b	127.45 ± 6.85 ^b	6.58 ± 0.21 ^b	170.27 ± 5.01 ^a
FL-ST1	3.55 ± 0.30 ^c	138.32 ± 10.24 ^c	9.58 ± 0.88 ^c	175.48 ± 4.10 ^a

Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among samples for each determination