Carboxymethyl cellulose films containing nanoliposomes loaded with an angiotensin-converting enzyme inhibitory collagen hydrolysate

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Abstract

A collagen hydrolysate (HC) obtained from giant squid (*Dosidicus gigas*) tunics was encapsulated in soy phosphatidylcholine liposomes in the presence and absence of glycerol. The z-average and ζ -potential of empty and loaded liposomes ranged from 92.4 to 96.0 nm and from -30.4 to -34.5 mV, respectively. The HC was encapsulated with an entrapment efficiency of 80–83%, and the resulting liposomal dispersions showed $\approx 50\%$ of angiotensin-converting enzyme (ACE) inhibitory capacity. An *in vitro* process of gastrointestinal digestion of the liposomes increased the antihypertensive potential, regardless of the presence or absence of glycerol. The liposomal dispersions were incorporated into carboxymethyl cellulose films, in which two ways of adding glycerol were compared: adding it directly to the film-forming dispersion or incorporating it previously into the liposomes. The presence of intact liposomes in the films, which was corroborated by transmission electronic microscopy at cryogenic temperature, decreased the water solubility and increased adhesiveness. The results demonstrated that glycerol-containing liposomes were less affected by the film drying step and also the subsequent simulated gastrointestinal digestion, with greater preservation of vesicle size and morphology, than if glycerol was added directly to the film-forming solution.

Keywords: carboxymethyl cellulose films, glycerol, collagen hydrolysate, phosphatidylcholine liposomes, gastrointestinal digestion, ACE inhibition.

1. Introduction

The attempt to take advantage of available resources in a more comprehensive manner is a challenge with a double benefit, environmental and economic. The use of waste from the food industry to obtain raw materials is one of these cases, and frequently the product thus obtained can constitute a food ingredient, acquiring an added value (Sayari et al., 2016). Protein hydrolysates and peptides from seafood are one of these cases which has been studied in the last few years (López-Caballero, Giménez, Gómez-Guillén, and Montero, 2013), since they are an excellent source of bioactive peptides with various properties, such as antioxidant and antihypertensive properties (Cudennec, Ravallec-Plé, Courtois, and Fouchereau-Peron, 2008), providing attractive material for the manufacture of functional food products. Peptides can undergo modifications, either degradation or interactions with the supporting matrix during food processing or storage, or during gastrointestinal digestion, which can lead to a decrease in functionality. It may be also necessary to mask bitter or unpleasant flavours that would negatively affect the sensory acceptability of the food product. Various strategies have been developed to protect peptides, one of them being encapsulation in nanoliposomes.

Liposomes are amphipathic colloids whose wall consists mainly of a double layer of phospholipids (Mozafari, Johnson, Hatziantoniou, and Demetzos, 2008). Both hydrophilic and hydrophobic peptides can be encapsulated in liposomes, maintaining their activity (Malheiros, Daroit, and Brandelli, 2010). There are previous studies with similar liposomes encapsulating peptide fractions with ACE-inhibitory capacity using the same sonication procedure, such as Montero et al. (2019) using a <1 kDa shrimp peptide fraction (99.98 nm, -53.87 mV and 52.37% entrapment efficiency) or Mosquera et al. (2014) with a <3 kDa fraction obtained from sea bream scale collagen (90.3 nm, -40.8 mV and entrapment efficiency of 74.6%). However, the behaviour of liposomes, their stability and their potential antihypertensive capacity after *in vitro* simulated gastrointestinal digestion have not yet been evaluated.

The use of natural soybean lecithin as an alternative to synthetic phospholipids has no drawbacks in food legislation and provides nutritional value owing to the high profile of polyunsaturated fatty acids (Laye, McClements, and Weiss, 2008). In addition, the use of this underutilized material confers additional value. The instability of colloidal vesicles is one of the main drawbacks of this encapsulation system, which is otherwise very versatile because of its amphipathic nature. Furthermore, the entrapment of liposomes in edible films can be a way of providing or favouring system stability; however, they can behave in different ways, depending on the biopolymer that constitutes the filmogenic matrix. Thus, for example, it has been observed that liposomes encapsulating phages embedded in chitosan films show evident activity against *E. coli* on cherry tomato, indicating that the liposomes gradually release the phages (Cui, Yuan, Li, and Lin, 2017a). Other authors found that interactions between chitosan and soybean lecithin liposomes in a composite chitosan film improved film stiffness but reduced the antimicrobial capacity of the encapsulated nettle extract (Haghju, Beigzadeh, Almasi, and Hamishehkar, 2016).

On the other hand, Montero et al. (2019) observed that the inclusion of liposomes loaded with peptide fractions (<1 kDa) in sodium caseinate films caused matrix disruption, increasing its solubility and adhesive and mucoadhesive properties. From a sensory point of view, these effects resulted in greater or more rapid flavour perception, and greater availability of liposomes in the lingual mucosa.

Carboxymethyl cellulose (CMC) is an edible inexpensive anionic cellulose derivative which is attracting increasing interest due to its nontoxicity, biodegradability and high filmogenic capacity. The main drawback of the resulting films is the relatively low water resistance, which limits their use to protect foods with relatively high water content (Abdollahi, Damirchi, Shafafi, Rezaei, and Ariaei, 2019). A rice grass phenolic extract microencapsulated with maltodextrin was used to increase both water barrier and antioxidant properties of the resulting composite CMC film (Rodsamran and Sothornvit, 2018). Little work has been published about CMC films containing liposomes, and none could be found including liposomes loaded with ACE-inhibitory peptides. Silva-Weiss et al. (2018) studied the release mechanism and kinetics of single antioxidant polyphenols (quercetin and rutin) from dipalmitoyl lecithin liposomes

embedded in CMC films to improve food shelf life, whereas Imran et al. (2012) reported on hydroxypropyl methylcellulose films incorporating nisin-loaded liposomes as antimicrobial food packaging. In the present work, the film with antihypertensive liposomes is intended to be ingested together with the food to which it is attached. In this sense, the functional CMC film may act also as a source of low-fermentable, high-viscous dietary fibre (Smits, Moughan, and Beynen, 2000) that, besides providing additional protection to the embedded liposomes, can meet the benefits associated with dietary fibre intake.

Glycerol is frequently used as a plasticizer in edible films to avoid breaking or cracking. In the context of liposome preparation, the addition of glycerol is known to increase the fluidity of the bilayer (Manca et al., 2013) and provide protection during the dehydration process (Stark, Pabst, and Prassl, 2010).

The presence of liposomes and glycerol in the film formulation has been shown to confer adhesive and mucoadhesive properties (Montero et al., 2019), which, in the intestinal tract, could help to favour film adhesion to the intestinal membrane and its mucus during gastrointestinal digestion, and thus presumably favour intestinal absorption of the liposomes. Moreover, this complex system permits versatility in food design, since the film can act as a cover or wrapping by adhesion to the food surface or even constitute a food by itself. There are several commercialized products that transport the bioactive compound in a film as a release and stabilizer system. Some are for buccal dosing and others have to be ingested, perhaps the best known being caffeine strips (Patel, Liu, and Brown, 2011) and oral insulin administration strips (Modi, Mihic, and Lewin, 2002). The films thus formed have certain peculiarities; they must be consistent for easy handling, with adhesive properties, but at the same time they must disintegrate in the mouth easily; in both cases, whether they act in the mouth, passing the nanoparticles to the sublingual tract (Patel et al., 2011), or pass them to the digestive tract, they must be sensorily acceptable (Montero et al., 2019).

The objective of the present work was to evaluate the stability and bioaccessibility of partially purified phosphatidylcholine liposomes loaded with collagen hydrolysate, which in turn were embedded in CMC films. The protective role of glycerol added to the liposome bilayer or to the film-forming solution was evaluated, in particular the

properties that this plasticizer could provide to the film through indirect application by prior incorporation into the nanoparticles. *In vitro* gastrointestinal digestions of liposomal systems and complex films were performed to evaluate liposome stability in terms of residual ACE-inhibitory activity and structural integrity.

2. Materials and methods

2.1. Materials

Frozen tunics of giant squid *Dosidicus gigas* were purchased from PSK Océanos S.A. (Madrid, Spain). Soybean lecithin (*Glycine max*) and sodium carboxymethyl cellulose were obtained from Manuel Riesgo S.A. (Madrid, Spain). The pepsin, pancreatin and ACE (EC 3.4.15.1) enzymes and norleucine were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Collagen hydrolysate (HC)

The preparation procedure and chemical properties of the collagen hydrolysate used in the present study were reported in a previous work (Marín, Alemán, Sánchez-Faure, Montero, and Gómez-Guillén, 2018a). Briefly, the acid-soluble collagen from giant squid (*Dosidicus gigas*) tunics was subjected to enzymatic hydrolysis with Alcalase and subsequently stabilized by freeze-drying. The predominant fraction (89%) of HC peptides was smaller than 1.3 kDa. Glycine residues were the most abundant (25.3%), followed by glutamic acid (11.7%) and alanine (9.8%). The total proportion of hydrophobic residues was 28% (Marín et al., 2018a).

2.3. Encapsulation in liposomes

2.3.1. Purification of phosphatidylcholine

Partially purified phosphatidylcholine was obtained, following the method described by Taladrid et al. (2017), by performing five consecutive washes with acetone. The dried powder (PC) was ground in an Osterizer (Sunbeam Par, 4153-50, Mexico) and stored at −20 °C in darkness.

2.3.2. Preparation of liposomes

Empty and HC-loaded liposomes were prepared according to Marín et al. (2018a). The partially purified phosphatidylcholine was dispersed in pH 7.0 phosphate buffer and subjected to two incubations at 80 °C, each lasting 1 h. In the required formulations, the peptide extract (0.04 g per g of PC) and glycerol (0.6 mL per g of PC) were added before the first and second incubations, respectively. Finally, the liposomal dispersions were sonicated and stored at 4 °C. In this way, four types of liposomal dispersions were obtained: empty (LE), empty with glycerol (LEG), loaded with HC (LHC), and loaded with HC and glycerol (LHCG).

2.4. Characterization of liposomes

2.4.1. Particle characterization

The determination of mean particle size (z-average, in intensity), polydispersity index (PDI) and ζ-potential (mV) of the liposomes (previously diluted with phosphate buffer 1:10) was performed using a Nano ZS Zetasizer (Malvern Instruments Ltd, Worcestershire, UK), according to Mosquera, Giménez, Montero, and Gómez-Guillén (2016). The results were determined in triplicate.

2.4.2. Entrapment efficiency

The entrapment efficiency (EE) of the liposome-encapsulated collagen hydrolysate (LHC and LHCG) was determined by measuring the protein content with a LECO FP-2000 nitrogen/protein analyser (LECO Corp., St. Joseph, MI, USA), using a nitrogen-to-protein conversion factor of 6.25.

The encapsulated hydrolysate was calculated as the difference between the total and non-encapsulated material, considering all the free HC as non-encapsulated material. To separate the non-encapsulated HC, the liposomes were placed in an Amicon Ultra-15 centrifugal filter device with molecular weight cut-off of 10 kDa (10000 MWCO, Millipore Corp.) and were centrifuged at 4000 *g* for 30 min. The non-encapsulated hydrolysate penetrated through the membrane while the liposomes remained in the filter.

EE was calculated using the following equation:

$$\% \text{ EE} = \text{encapsulated HC in liposomes} / \text{total HC in liposomes} \times 100$$

2.5. Incorporation of liposomes into the films

Various formulations based on carboxymethyl cellulose (CMC) were tested to determine the influence of both HC-liposomes and glycerol on the film properties. First, CMC was dissolved (4%) in distilled water, employing an Ultra-Turrax homogenizer (T 25 basic, IKA-WERKE, Staufen, Germany). The various film components were added as follows: phosphate buffer or liposome dispersion in a proportion of 1:1 with respect to the CMC solution to obtain a total volume of 320 mL; whether inside the liposome (forming part of the liposome conformation) or outside (freely added to the film-forming dispersion), the total amounts of glycerol and HC added were 4.8 mL and 0.320 g, respectively. The mixtures were homogenized with the help of an ultrasonic bath (Ultrasons, P-Selecta). Next, the dispersions (80 mL) were poured onto rectangular Petri plates and dried in an oven (FD 240, Binder, Tuttlingen, Germany) at 45 °C for 24 h. Finally, the films were conditioned for at least 3 days (maximum 1 week) in a desiccator containing NaBr solution, which kept the relative humidity of the films around 58% at room temperature. The films obtained were: (i) CMC + Glycerol (F+G); (ii) CMC + Glycerol + HC (F+G+HC); (iii) CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer) (F+LHC+G) and (iv) CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome) (F+LHCG).

2.6. Film Characterization

2.6.1. Colour

The L (lightness), a* (redness/greenness) and b* (yellowness/blueness) parameters were determined with a Konica Minolta CM-3500d colorimeter (Konica Minolta, Madrid, Spain), using D65 as illuminant (daylight) and D10 as standard observer. At least eight replicates were carried out per film. The total colour difference (ΔE) and whiteness (W) parameters were calculated according to the following formulas:

$$\Delta E = [\Delta L^*^2 + \Delta a^*^2 + \Delta b^*^2]^{1/2}$$

$$\text{Whiteness} = L^* - 3b^*$$

The F+G film (CMC + Glycerol) was used as reference film in total colour difference calculation.

2.6.2. Moisture content

The water content was determined gravimetrically in triplicate according to method 950.46 (A.O.A.C., 2005).

2.6.3. Water solubility

Film pieces measuring 2 × 2 cm were solubilized without agitation in distilled water (15 mL) at room temperature overnight. Then the solutions were filtered through previously weighed Whatman No. 1 filters. Finally, the filters were dried at 105 °C overnight and weighed again. The solubility, expressed as a percentage, was determined by weight differences and the results were the mean of three replicates.

2.6.4. Thickness

A micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan) was used to determine the film thickness. The results were the mean of 10 replicates.

2.6.5. Opacity

Pieces 8 cm in length and 1 cm wide were cut from each film. Then they were put into quartz cuvettes and an absorbance sweep test from 800 to 190 nm was performed in a spectrophotometer (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Then the opacity index was calculated by the following formula:

$O = A / x$, where O is the degree of opacity, A is absorbance at 600 nm, and x is the thickness of the film (mm). The value was calculated in triplicate for each film.

2.6.6. Adhesiveness test

It was carried out according to the method described by Ivarsson and Wahlgren (2012) with slight modifications. The method is based on lowering a cylindrical metal probe (20 mm diameter) coupled to a TA.XT plus TA-XT2 texture analyser at a velocity of 0.2 mm/s until it makes contact with the film, which is placed on the bottom platform of the texturometer. Then the probe is raised at a velocity of 2.0 mm/s until the detachment of the film, and the maximum force (N) needed to detach the film from the cylindrical probe is quantified. To standardize the measurements, a preload of 500 mN was applied for 15 s. The measurements were the mean of at least eight replicates.

2.6.7. Tensile test

A tensile test was run at least in triplicate using a TA.XT plus TA-XT2 texture analyser as described by Blanco-Pascual et al. (2013). A 5 kg load cell was used and the specimens were cut into rectangular pieces (100 mm × 20 mm), leaving an initial grip separation (l_0) of 60 mm, with a cross-head speed of 100 mm/min. The tensile strength (TS, MPa) and elongation at break, $[(l_{\text{break}} - l_0)/l_0] \times 100$, (EAB, %), were determined from the stress vs. strain curves at the breaking point.

2.6.8. Transmission Electronic Microscopy at cryogenic temperature (Cryo-TEM)

Films dissolved in water (10–20 mg/mL) were vitrified (4 μ L) by cryo-preservation in Quantifoil R2/2 carbon grids previously prepared employing a Gatan cryo-plunge and liquid ethane. The observation was carried out in a JEOL JEM-1230 transmission electron microscope (TEM) operating at 100 kV with a nominal magnification of 30 K, using a Gatan 626 cryo-specimen holder. The micrographs were recorded by a 4 k × 4 k TVIPS CMOS camera detector (TemCam-F416) at -180°C .

2.7. In vitro gastrointestinal digestion (GID)

The simulated digestion of collagen hydrolysate, liposomes and films was performed according to Alemán, Gómez-Guillén, and Montero (2013). The samples (2 mg/mL for HC and 50 mg/mL for films, both dissolved in water, and the liposomes directly as liposomal dispersion) were incubated for 2 h at 37°C with agitation (100 strokes/min) employing the enzyme pepsin (pH 2.0), and for 2 h again with pancreatin and bile salts (pH 6.5). Finally, the mixes were adjusted to pH 7.2 with 0.5 M NaOH and stored at 4°C until use. Aliquots of sample were collected at each hour of digestion, leading to four controls for each sample (1 h, 2 h, 3 h and 4 h). The digested films were tested by transmission electronic microscopy at cryogenic temperature (Cryo-TEM) according to section 2.6.8.

2.8. Angiotensin converting enzyme (ACE) inhibitory capacity

This activity was carried out following the method of Alemán, Giménez, Pérez-Santín, Gómez-Guillén, and Montero (2011). Briefly, the samples (HC and liposomes) were mixed with a 5 mM HHL (hippuryl histidyl lysine) solution and with angiotensin-

converting enzyme (ACE), and incubated at 37 °C and 160 rpm/min for 2 h. Then HCl was used to inactivate the enzyme. The samples were injected into a HPLC together with HA (hippuric acid) and HHL controls, which enabled the quantification (percentage, %) of the ACE-inhibitory capacity by differences between them. The measurements for each sample were performed in triplicate.

2.9. Statistical analysis

The statistical analysis was performed using the SPSS computer program (SPSS Statistics 22 software, IBM, Inc., Chicago, IL, USA). Differences among samples were assessed by the Tukey test, based on confidence intervals with a significance level set at $p \leq 0.05$. Analysis of variance (ANOVA) and tests of related samples were carried out.

3. Results and discussion

3.1. Liposome properties

The particle properties (z-average, polydispersity index and ζ -potential) of the liposomes that were studied are compared in Table 1. All of them kept the same range of sizes ($p > 0.05$), between 92.4 and 96.0 nm, and similar electronegative charge, with values from -30.4 to -34.5 mV. Marín, Alemán, Montero, and Gómez-Guillén (2018b) reported similar results for liposomes loaded with the same hydrolysate in the presence of glycerol. All the liposomes had a nanometric size and acceptable monomodal size distribution with a well-defined single population. More specifically, PDI values ranged from 0.235 in LHC to 0.319 in LEG. In this respect, Malheiros, Sant'Anna, Micheletto, Da Silveira, and Brandelli (2011) established a good polydispersity between 0.2 and 0.3 for systems derived from biological materials. The addition of glycerol contributed to a slight increase in polydispersity only in the case of empty liposomes. The four liposomes had great membrane stability, according to Müller, Jacobs, and Kayser (2001), who established that liposomal suspensions with values more electronegative than -30 mV have good stability. The loaded liposomes showed high entrapment efficiency, 80.0% for LHC and 82.9% for LHCG. In general, no notable differences were found as a function of addition of glycerol or HC, probably

because glycerol is well associated with the headgroups of phospholipids and the highly hydrophilic hydrolysate is efficiently encapsulated in the inner core of the liposome and also associated to the polar heads at the external membrane surface.

3.2. Film properties

The liposomal dispersion encapsulating the collagen hydrolysate was incorporated into carboxymethyl cellulose films; glycerol was added to the film directly as a plasticizer (F+LHC+G) or previously to the liposomes (F+LHCG). For comparison purposes, CMC films without liposomal dispersions were also prepared with addition of glycerol (F+G) or glycerol plus collagen hydrolysate (F+G+HC). Glycerol was present in all the film formulations because of its plasticizing capacity. Furthermore, it can have an additional role, protecting the liposomal membrane during the film drying step by avoiding vesicle aggregation.

3.2.1. Cryo-TEM micrographs

Films containing liposomes were first dissolved in water and subjected to Cryo-TEM examination in order to ensure that the morphology of the liposomes that were embedded inside the biopolymer matrix was mostly preserved. The images shown in Figure 1 revealed a predominance of spherical unilamellar vesicles, with some cases of invagination and bi- and multi-lamellarity, indicating that they resisted the drying step in the process of making the films quite well. However, the liposomes in both films presented highly polydispersed sizes, and were characterized by the appearance of large vesicles, many of them consisting of multivesicular structures. In films containing glycerol-free liposomes (F+LHC+G) (top micrographs), a greater proportion of large vesicles (above 200 nm) was observed, while in the films in which glycerol was incorporated into the liposomes (F+LHCG) (bottom micrographs), smaller vesicles (less than 200 nm) predominated, indicating that the liposomal structure was more efficiently protected during drying.

3.2.2. Physical properties

All the films with and without liposomes showed close values for luminosity and for the a^* parameter (red–green range), although with some significant differences ($p \leq 0.05$) (Table 2), with the films that did not contain liposomes showing a greater

tendency to green coloration and slightly lower luminosity. With respect to the b^* parameter, the films with liposomes showed an evident tendency to yellowness. These differences were fundamental to substantially modify the whiteness index, which was similar in the two films with liposomes ($p > 0.05$) but much lower than in the films without liposomes. The total colour difference (ΔE) resulting from the incorporation of the hydrolysate in the film formulation was quite low (0.76 ± 0.09); in contrast, when liposomes were incorporated the ΔE was considerably higher, i.e. 8.38 ± 0.54 for F+LHC+G and 8.63 ± 0.59 for F+LHCG, with no significant differences between them ($p > 0.05$).

The moisture content (Table 3) was significantly lower ($p \leq 0.05$) in the liposome-containing films, probably owing to the higher dry matter in formulations with liposomal dispersions. Valencia-Sullca et al. (2016) obtained similar moisture results (24%) for chitosan films with lecithin liposomes. Montero et al. (2019), who observed similar behaviour when they included liposomes in sodium caseinate edible films, indicated that the presence of liposomes in the film produces discontinuities in the matrix that favour the elimination of water during film drying.

Regardless of whether the glycerol was added separately or incorporated in the liposome, the films containing liposomes were quite similar ($p > 0.05$) in terms of moisture, water solubility, thickness and opacity. They were ≈ 30 and 20% less water soluble ($p \leq 0.05$) than the F+G and F+G+HC films, respectively (Table 3). It should be noted that no differences were found between the films with liposomes with regard to the way in which glycerol was added. In the control films (without liposomes), the addition of the hydrolysate caused a significant increase ($p \leq 0.05$) only in the opacity level, but not in water solubility. It has been observed that the effect of the presence of liposomes on the solubility of films depends greatly on the nature of the filmogenic polymer, and how soluble the film itself is. Thus, in films made with chitosan and lecithin liposomes, Valencia-Sullca et al. (2016) obtained the same solubility in water (37%) as in the present work. However, in sodium caseinate films that were much more insoluble, the presence of phosphatidylcholine liposomes increased the solubility (Montero et al., 2019). According to Souza et al. (2009), the film solubility that is desired depends on the function that is required. Thus the presence of liposomes in

CMC films favours a decrease in solubility, which would make it possible to maintain their integrity when they are applied to coat the wet surface of a great variety of foods.

The film thickness did not increase significantly with the addition of peptides, while the presence of liposomes led to considerably thicker films ($p \leq 0.05$), probably owing to the higher content of dry matter. It should be noted that relatively thick films ($\approx 200 \mu\text{m}$) were produced intentionally by using a high CMC concentration (4%) in the film-forming solution in order to make it possible to load a high amount of liposomes with potential biological activity after oral ingestion without compromising the film integrity and handling. Montero et al. (2019) found no significant differences in the thickness of liposome-containing sodium caseinate films, both empty and filled with a <1 kDa peptide fraction, but they observed a slight increase in film density as a result of the incorporation of liposomes. In cooked shrimp myofibrillar protein film containing liposomes filled with protein hydrolysate (<10 kDa), Alemán et al. (2016) did not find differences in thickness due to the presence of liposomes. Both cases were protein films, although the molecular weights of the biopolymers were very different, and in the second case with a high degree of aggregation. Cui, Yuan, and Lin (2017b) indicated that the thickness of the film depends on the film composition and the interactions that are established; they observed that the thickness increased in chitosan films containing lecithin-cholesterol liposomes as a result of the increase in the amount of polymer that was used. On the other hand, lower opacity (greater transparency) (Table 3) ($p \leq 0.05$) was observed in the two types of films that contained liposomes, which in turn were also considerably thicker. Rodsamran and Sothornvit (2018) obtained a degree of transparency of 3.2 for CMC films incorporating microcapsules loaded with rice extract, values within a range similar to that obtained for CMC films without liposomes in the present work. In general, at least at the high CMC concentration used in the present work, the films were not very transparent, and despite the increase in thickness, the inclusion of liposomes enhanced their transparency, favouring the versatility of their application in foods, since it is often desired that the product can be seen without being masked by the cover.

The adhesive capacity of the films, determined as the maximum force required to detach the film from a surface, is shown in Table 4. Adhesiveness was similar ($p < 0.05$) in the two films without liposomes; therefore, the hydrolysate did not influence this film property. However, the presence of liposomes (both types) in the films greatly increased the adhesiveness, probably attributable to a certain plasticizing effect, since the water content was lower. The film containing liposomes that were prepared with glycerol (F+LHCG) showed lower ($p \leq 0.05$) adhesive capacity than the film with glycerol added separately, in which it should be more available to the matrix, thus helping to increase film adhesiveness by increasing hygroscopicity. In films and hydrogels, adhesiveness is often related initially with the ability to hydrate the biopolymer matrix (Ivarsson and Wahlgren, 2012). In work performed under similar relative humidity conditions (58% RH), sodium caseinate films also showed an increase in adhesive capacity owing to the presence of liposomes (Montero et al., 2019), although to a much lesser extent than in the present case, probably because adhesiveness in the sodium caseinate film was much higher than in the CMC film. The ease of adhering to and contacting food and other types of products is an interesting and necessary property in many applications in which films are used as a cover.

The tensile strength and elongation at break values were very close in all the film formulations (Table 4), despite the great difference in thickness between films with and without liposomes. Tensile strength was not modified by the presence of free hydrolysate in the film ($p > 0.05$), but it decreased slightly in the presence of liposomes, being significantly ($p \leq 0.05$) only when glycerol was added freely as film plasticizer (F+LHC+G). The elongation was quite high, around 50%, and there were no significant differences ($p > 0.05$) among films, irrespective of whether they had free hydrolysate or embedded liposomes. These properties provide films with great versatility to make and/or coat products. Ebrahimi et al. (2018) found much higher values for tensile strength (14 MPa), and much lower values for elongation (25%) in CMC films made with glycerol that were thinner than in the present work. Such a great difference could be likely attributed to different molecular properties of the CMC used. In terms of mechanical properties, their films were not modified by the incorporation of microorganisms. In contrast, the tensile strength and elongation at break values in

the present work were higher than those found in hydroxypropyl methylcellulose films containing phospholipid and cholesterol liposomes (Zhu et al., 2018). In films made with another polysaccharide, such as chitosan, with the addition of glycerol and liposomes (Cui et al., 2017b), similar behaviour was observed, although with higher tensile strength values ($\approx 11\text{--}13$ MPa): the initial elongation of their film without liposomes (55%) dropped to around 45% in the liposome-containing film, a value that was very close to the one in the present work. The behaviour of different film matrices as a result of the addition of liposomes may depend on the nature of the biopolymer, making it possible to select the most suitable one according to the need for protection, release or biological activity of the encapsulated compound.

3.3. *In vitro gastrointestinal digestion*

Peptides can undergo changes not only as a result of interaction with food constituents during processing but also after oral ingestion, when they undergo the action of digestive proteases. Similarly, liposomes can also be subjected to alterations that could compromise their integrity and the bioaccessibility of the active compound. For this reason, in the case of films entrapping bioactive liposomes intended for oral intake, it should be first necessary to ascertain the behaviour of the liposomes, in terms of residual activity and structural integrity, after a simulated gastrointestinal digestion.

3.3.1. *ACE-inhibitory activity*

The ACE-inhibitory activity of the collagen hydrolysate (HC), at the tested concentration of 0.8 mg/mL, was $43.96\% \pm 0.84$ (undigested). The *in vitro* GID of HC induced progressively increasing activity for 3 hours and then declined ($p \leq 0.05$) (Table 5). An increase in activity after digestion has been observed on other occasions and is attributed to further hydrolysis during digestion, with the production of smaller peptides with greater activity (Alemán et al., 2013), but up to a limit where hydrolysis may be excessive (Gong et al., 2016). Table 5 also shows the ACE-inhibitory activity of the HC-liposomes, without glycerol (LHC) and with glycerol (LHCG), before and after *in vitro* GID for 1–2 h (gastric phase) and 3–4 h (intestinal phase). The presence of glycerol in the liposomes (LHCG) did not significantly modify ($p > 0.05$) the activity

compared to that without glycerol (LHC). Both liposomes had higher ($p \leq 0.05$) antihypertensive potential than the HC hydrolysate, probably as a consequence of interaction of the bioactive compound with the liposomal membrane, which might favour a more active peptide configuration. It can be seen that both undigested liposomes had lower values than their digested counterparts ($p \leq 0.05$), and that no significant differences were found in the course of the *in vitro* digestion. The same effect was observed by Gong et al. (2016) in liposomes made with soybean phospholipids and cholesterol subjected to 2 h of *in vitro* digestion. They reported a greater % of ACE inhibition in nanoliposomes than in liposomes, and more in liposomes than in free hydrolysate, concluding that the nanoliposomes showed more bioavailability after *in vitro* GID, partly owing to the effect that pepsin and pancreatin may have on ACE-inhibitory activity. In addition, it should be emphasized that the significant drop in ACE inhibition shown by HC after 4 h of digestion was not found in either of the liposomal preparations, indicating the protective role of liposomes.

The ACE-inhibitory activity of digested films with loaded liposomes could not be determined, owing to the strong interference caused by the highly viscous nature of CMC acting as fibre. Also, when an attempt was made to separate the CMC by centrifugation, no activity was detected in the supernatant, suggesting that the liposomes were mostly dragged with CMC into the precipitate, hampering a reliable measurement. To evaluate the extent to which the liposomes are wrapped by the fibre after the GID, and the degree to which they would be available to cross the epithelial membrane, a series of experiments would be needed that would be the object of another study. To this respect, there is previous knowledge that demonstrates the transport capacity of CMC matrices, which release the bioactive compound in the intestinal phase of the *in vitro* GID, suggesting that it is absorbed predominantly in the small intestine (Feng et al., 2013). They observed that when doxorubicin was administered loaded in nanochitosan-CMC matrices the bioavailability increased with respect to its free form. In the context of the present work, the prevalence and integrity of liposomes in the film matrix after simulated gastrointestinal digestion was confined to visualization by Cryo-TEM.

3.3.2. Cryo-TEM of digested films

The images of the digested films obtained by Cryo-TEM showed that liposomes were abundantly present with no visual signs of rupture (Figure 2), but apparently with fewer cases of bi- or multi-lamellarity than in their undigested counterparts (Figure 1). On the other hand, there were a considerable number of multivesicular structures.

As in the undigested films, the digested F+LHC+G film presented a greater number of large vesicles than the digested F+LHCG film, in which small vesicles predominated, either in the free form or included in large multivesicular structures. These observations indicate the more efficient protecting role of glycerol in vesicle integrity, both during film drying and after subsequent *in vitro* GID, when included during liposome preparation rather than being added directly to the film-forming solution.

4. Conclusions

The particle properties (z-average and ζ -potential) of the liposomes were not affected either by collagen hydrolysate (HC) or by glycerol. The presence of glycerol did not hinder the ACE-inhibitory capacity of the liposomes loaded with HC, which in fact were slightly more potent inhibitors than the hydrolysate *per se*, and the activity was protected and even enhanced during the *in vitro* gastrointestinal digestion.

The addition of liposomes to the CMC films greatly increased their adhesiveness, which could be of interest to favour interactions with intestinal membranes for liposome delivery. Prior incorporation of glycerol to the liposome structure helped to preserve the morphology and size of the liposomes embedded in the CMC films to a greater extent than when added in the free form to the film-forming solution, and the same effect was also observed after the simulated gastrointestinal digestion of the corresponding films.

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Figure captions

Figure 1. Cryo-Transmission Electron Microscopy (Cryo-TEM) of liposome-containing films dissolved in water. A-B: F+LHC+G film; C-D: F+LHCG film.

HC: collagen hydrolysate; F+LHC+G: CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer); F+LHCG: CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome).

Figure 2. Cryo-Transmission Electron Microscopy (Cryo-TEM) of liposome-containing films after *in vitro* gastrointestinal digestion. A-B: F+LHC+G film; C-D: F+LHCG film.

HC: collagen hydrolysate; F+LHC+G: CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer); F+LHCG: CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome).

Table 1. Z-average (nm), polydispersity index (PDI) and ζ -potential (mV) of freshly empty and loaded liposomes.

	Z-average (nm)	Polydispersity index (PDI)	ζ -potential (mV)
LE	96.0 $\pm$ 1.5 ^A	0.271 $\pm$ 0.030 ^{A,B}	-30.4 $\pm$ 1.9 ^C
LEG	94.2 $\pm$ 0.8 ^A	0.319 $\pm$ 0.039 ^B	-33.4 $\pm$ 0.6 ^{A,B}
LHC	95.4 $\pm$ 2.2 ^A	0.235 $\pm$ 0.006 ^A	-34.5 $\pm$ 0.4 ^A
LHCG	92.4 $\pm$ 0.4 ^A	0.255 $\pm$ 0.005 ^A	-31.5 $\pm$ 0.4 ^{B,C}

Different letters (A, B, C) indicate significant differences ($p \leq 0.05$) among samples. LE: empty liposomes; LEG: empty liposomes with glycerol; LHC: liposomes loaded with HC (collagen hydrolysate); LHCG: liposomes loaded with HC and glycerol.

Table 2. Colour parameters of films: L*, a*, b* and whiteness.

	L*	a*	b*	Whiteness
F+G	30.38 $\pm$ 0.59 ^A	-0.43 $\pm$ 0.07 ^B	-0.74 $\pm$ 0.12 ^A	32.60 $\pm$ 0.95 ^A
F+G+HC	30.16 $\pm$ 0.25 ^A	-0.60 $\pm$ 0.05 ^A	-0.04 $\pm$ 0.13 ^B	30.27 $\pm$ 0.42 ^B
F+LHC+G	31.85 $\pm$ 0.22 ^B	-0.34 $\pm$ 0.18 ^B	7.48 $\pm$ 0.56 ^C	9.41 $\pm$ 1.80 ^C
F+LHCG	31.86 $\pm$ 0.21 ^B	-0.13 $\pm$ 0.20 ^C	7.73 $\pm$ 0.56 ^C	8.66 $\pm$ 1.67 ^C

Different letters (A, B, C) indicate significance differences ($p \leq 0.05$) among samples for each colour parameter. F+G: CMC + Glycerol; F+G+HC: CMC + Glycerol + HC (collagen hydrolysate); F+LHC+G: CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer); F+LHCG: CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome).

Table 3. Physico-chemical properties of films.

	Moisture (%)	Solubility (%)	Thickness (μ m)	Opacity
F+G	31.8 $\pm$ 3.9 ^B	66.1 $\pm$ 9.5 ^B	207 $\pm$ 50 ^A	3.58 $\pm$ 0.25 ^B
F+G+HC	32.8 $\pm$ 2.8 ^B	59.1 $\pm$ 5.1 ^B	215 $\pm$ 31 ^A	4.78 $\pm$ 0.19 ^C
F+LHC+G	24.2 $\pm$ 2.0 ^A	37.6 $\pm$ 3.8 ^A	342 $\pm$ 25 ^B	1.89 $\pm$ 0.21 ^A
F+LHCG	21.7 $\pm$ 2.9 ^A	37.6 $\pm$ 5.1 ^A	328 $\pm$ 39 ^B	1.29 $\pm$ 0.46 ^A

Different letters (A, B) indicate significance differences ($p \leq 0.05$) among samples for each parameter. F+G: CMC + Glycerol; F+G+HC: CMC + Glycerol + HC (collagen hydrolysate); F+LHC+G: CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer); F+LHCG: CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome).

Table 4. Mechanical properties of films.

	Adhesiveness (mN)	Tensile strength (N/mm ²)	Elongation at break (%)
F+G	37.8 ± 6.7 ^A	6.0 ± 0.7 ^B	51.2 ± 8.6 ^A
F+G+HC	37.6 ± 3.3 ^A	5.8 ± 0.8 ^B	53.2 ± 9.3 ^A
F+LHC+G	429.3 ± 26.4 ^C	4.2 ± 0.8 ^A	45.6 ± 7.2 ^A
F+LHCG	392.1 ± 25.1 ^B	5.2 ± 0.6 ^{AB}	51.0 ± 7.7 ^A

Different letters (A, B, C) indicate significance differences ($p \leq 0.05$) among samples for each parameter. F+G: CMC + Glycerol; F+G+HC: CMC + Glycerol + HC (collagen hydrolysate); F+LHC+G: CMC + HC-Liposomes + Glycerol (outside the liposome, as film plasticizer); F+LHCG: CMC + HC-Liposome-Glycerol (glycerol forming part of the liposome).

Table 5. ACE-inhibitory activity (expressed as percentage of ACE inhibition) of hydrolysate and liposomes without and with glycerol, undigested and after being subjected to an *in vitro* gastrointestinal digestion for 1 h and 2 h (gastric phase), and 3 h and 4 h (intestinal phase).

Hydrolysate	Inhibition (%)	Liposome	Inhibition (%)	Liposome	Inhibition (%)
HC	43.96 ± 0.84 ^{A/X}	LHC	49.88 ± 1.42 ^{A/Y}	LHCG	51.18 ± 1.29 ^{A/Y}
HC 1 h	55.23 ± 0.24 ^{C/X}	LHC 1 h	63.03 ± 2.80 ^{B/Y}	LHCG 1 h	63.98 ± 2.09 ^{B/Y}
HC 2 h	58.56 ± 0.22 ^{D/X}	LHC 2 h	64.11 ± 2.48 ^{B/X}	LHCG 2 h	65.15 ± 1.64 ^{B/X}
HC 3 h	60.11 ± 0.99 ^{D/X}	LHC 3 h	64.02 ± 2.56 ^{B/X}	LHCG 3 h	63.12 ± 2.28 ^{B/X}
HC 4 h	51.12 ± 0.12 ^{B/X}	LHC 4 h	68.90 ± 2.02 ^{B/Y}	LHCG 4 h	66.22 ± 0.55 ^{B/Y}

Different letters (A, B) in the same column indicate significance differences ($p \leq 0.05$) among digestion times for the same sample; (X, Y) between samples for the same digestion time. HC: collagen hydrolysate; LHC: liposomes loaded with HC; LHCG: liposomes loaded with HC and glycerol.

Figure 1

Figure 2