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1. Introduction

Fish and other seafood sources have gained much interest in recent years as potential reservoirs of novel bioactive peptides with diverse biological functions like antioxidant, antimicrobial, anti-diabetic, anti-inflammatory, antitumor, neuroprotective, and anti-hypertensive activities (Hosseini, Rezaei, & McClements, 2022). In this sense, fish-derived antioxidant peptides are a famous group of interest, whose antioxidant potentialities have been reported in an increasing number of studies in recent years (Hosseini et al., 2022). Antioxidant peptides normally contain 20 amino acid residues that possess molecular masses ranging between 500 and 1800 Da, which are inactive within the sequence of the parent proteins but may be released when they are hydrolyzed using suitable proteolytic enzymes (Samaranayaka & Li-Chan, 2011).

Today, small fish species (sardines, anchovies, etc.) are actively caught for non-food purposes such as conversion into fishmeal and fish oil (FAO, 2016). Orangefin ponyfish (Photopectoralis bindus) is a schooling fish most frequently observed in near-shore and estuarine in Indo-Pacific regions (Deyrestani, Alavi-Yeganeh, & Sadeghizadeh, 2015). Most ponyfish species are small in size (max. length is 11 cm) and they are mostly commonly fished as by-catch (55% of the quantity is by-catch) in the Persian Gulf, and generally used for fishmeal production (Deyrestani et al., 2015). Thus, production of high-value added ingredients (e.g., protein hydrolysates and peptides) can pave the way for better use of this nutrient-rich fish. However, despite the interest in using fish-originated peptides as a new generation of antioxidants, their practical utilization remains restricted due to their bitter taste, low bioavailability, short half-lives, and likelihood of interacting with the other food substances (Ramezanzade, Hosseini, & Nihkhah, 2017).

Nanoscale delivery systems can be designed to enhance water-dispersibility, modulate release profiles, and enhance the bioavailability of bioactive substances encapsulated inside them. Previous studies have demonstrated the encapsulation of fish peptides in different nanocarriers e.g., polymeric nanoparticles (Hosseini, Soleimani, & Nihkhah, 2018), electrospun nanofibers (Hosseini, Nahvi, & Zandi, 2019), and nanoliposomes (Ramezanzade et al., 2017). Recently, non-lamellar liquid lyotropic crystalline (LLC) nanoparticles, known also as ISAsomes,
are gaining increasing attention for delivery of bioactive compounds, because of their unique structural features and relatively high capacity for loading hydrophilic, hydrophobic, and amphiphilic molecules (Gontsarik, Yaghmur, & Salentinig, 2021; Tan, Hosseini, & Jafari, 2022). The size characteristics of these LLC nanoparticles are similar to liposomes but their interiors have different structural features: two-dimensional (2D) and three-dimensional (3D) inverse bicontinuous cubic (Q2) and hexagonal (H2) phases, respectively (Tan et al., 2022; Wibroe, Mat Azmi, Nilsson, Yaghmur, & Moghim, 2015). Phytantrol (PHYT) and glycerol monooleate (GMO) are the major lipid constituents of ISAsomes, which are usually stabilized with an efficient non-food grade emulsifiers e.g., Pluronic F108 and Pluronic F127 (also called Poloxamer 407, P407) (Tan et al., 2022). However, there are concerns with the biosafety (e.g., enhanced cytoxicity and low hemocompatibility) of these formulations (Fornasier et al., 2020; Wibroe et al., 2015). For example, P407-stabilized PHYT hexosomes and cubosomes have been shown to induce cytoxicity in various cell lines (Fornasier et al., 2020). To address these limitations, we present for the first time to our knowledge hexosomes acceptable for use for delivery of peptides or functional foods by using the food-grade negatively charged emulsifier citrem, which is composed of citric acid esters of monoglycerides and diglycerides, as a safe stabilizer. Citrem has been shown to efficiently stabilize and regulate the internal architectures of hexosomes and cubosomes in a concentration-dependent manner (Hedegaard, Nilsson, Laurinmaki, Butcher, Urtti, & Yaghmur, 2013; Prajapati, Salentinig, & Yaghmur, 2018; Wibroe et al., 2015). However, the pH sensitivity of citrem-stabilized nanodispersions has not been investigated.

The present study, therefore, focuses on the development and in-depth characterization of pH-responsive HEXs based on binary lipid mixture of Dimodan U (commercial distilled monoglyceride product) and citrem, and loaded with the fish-derived peptide fraction (PF10). These nano-self-assemblies are attractive for use as a functional food (including peptide) nanocarriers. However, they are rarely investigated in the food nanotechnology area as most reported investigations were on nano-self-assemblies (including peptide) nanocarriers. However, they are rarely investigated in the food nanotechnology area as most reported investigations were on ISAsomes, which are usually stabilized with an efficient non-food grade emulsifiers e.g., Pluronic F108 and Pluronic F127 (also called Poloxamer 407, P407) (Tan et al., 2022). However, there are concerns with the biosafety (e.g., enhanced cytoxicity and low hemocompatibility) of these formulations (Fornasier et al., 2020; Wibroe et al., 2015). For example, P407-stabilized PHYT hexosomes and cubosomes have been shown to induce cytoxicity in various cell lines (Fornasier et al., 2020). To address these limitations, we present for the first time to our knowledge hexosomes acceptable for use for delivery of peptides or functional foods by using the food-grade negatively charged emulsifier citrem, which is composed of citric acid esters of monoglycerides and diglycerides, as a safe stabilizer. Citrem has been shown to efficiently stabilize and regulate the internal architectures of hexosomes and cubosomes in a concentration-dependent manner (Hedegaard, Nilsson, Laurinmaki, Butcher, Urtti, & Yaghmur, 2013; Prajapati, Salentinig, & Yaghmur, 2018; Wibroe et al., 2015). However, the pH sensitivity of citrem-stabilized nanodispersions has not been investigated.

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2. Materials and methods

2.1. Materials and chemicals

Orangefin ponyfishes (10 ± 1 cm length) were obtained from the fishing harbor of Khuzestan province in Iran and delivered to the laboratory on ice. Whole ungutted fish was first washed, minced, and frozen at −20 °C until use. Alcalase enzyme with activity of 2.4 U/g, DPPH, and panceratin from porcine pancreas (activity equivalent to 8xUSP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Potassium persul-fate, bovine serum albumin (BSA), and Folin-Ciocalteu reagent (1.9–2.1 N) were supplied by Merck Chemicals Co. (Darmstadt, Germany). ABTS and pepin from porcine gastric mucosa (activity 250 U/mg) were purchased from Biobasic Inc., (Ontario, Canada), and sodium hydroxide was supplied by AppliChem (Darmstadt, Germany). Dimodan U, which is a distilled monoglyceride product from refined sunflower oil, and grinsted citrem LR10 were kindly gifted by Danisco A/S (Copenhagen, Denmark).

2.2. Preparation and fractionation of fish protein hydrolysate

50 g of the defrosted fish mince was placed in distilled water, and homogenized for 2 min. The obtained homogenates of mince were then treated with Alcalase (1%) at pH 8.5 and temperature of 55 °C. The reaction was stopped by placing the solution into 90 °C water for 10 min, followed by keeping the obtained mixture at room temperature for cooling, and separation through centrifugation at 7000g for 20 min. The resulting fish hydrolysate was kept at −20 °C prior to analysis, and was sequentially fractionated at room temperature via three ultrafiltration membranes with molecular weights of 3, 10, and 30 kDa (Merck-Millipore, Germany), leading to the production of three fractions; 10–30 kDa (PF30), 3–10 kDa (PF10), and < 3 kDa (PF3) (Ramezanazade et al., 2017).

2.3. Preparation of Dimodan U/citrem/PF10 nano-self-assemblies

At a constant total lipid (Dimodan U and citrem) content of 5.0 wt%, and Dimodan U/citrem ratio of 1:1 (w/w), the PF10-free aqueous nanodispersions (HEXs) were prepared by vortexing the binary lipid in excess PBS (pH 7.4) for 1 min, and emulsification through use of microtip probe sonicators (Dr Hielscher, Teltow, Germany) for 15 min in pulse mode (8 s pulse, 2 s break) until obtaining colloidally stable milky solutions (Prajapati et al., 2018). For preparing the peptide fraction (PF10)-loaded nanodispersions at PF10 concentrations in the range of 1–5 mg/mL, the post-application method was used. In this method, an appropriate amount of PF10 was added to already prepared aqueous nanodispersions according to the aforementioned emulsification procedure. It is worth noting that the peptidic fraction PF10 was chosen according to preliminary experiments in our laboratory dealing with antioxidant assays.

2.4. Dynamic light scattering (DLS) and ζ-potential measurements

The mean nanoparticle diameters, polydispersity index (PDI), and ζ-potential were determined with a Zetasizer Nano ZS instrument (Malvern Instruments, UK) equipped with a He-Ne laser and a detection angle of 173° at 25 °C. To avoid multiple scattering phenomena, the nanodispersions were diluted 10 folds with PBS (pH 7.4).

2.5. Small-angle X-ray scattering (SAXS)

SAXS experiments were conducted at two different temperatures (25 and 37 °C) on a Xenocs BioXolver L equipped with a GenIX3D micro-focus X-ray source at a wavelength of 1.54 Å, and a STD (sample-to-detector distance) of 571 mm. The detector covered a q-range (q = (4π/ λ) sin(θ), where λ is the wavelength and θ is the scattering angle) from about 0.003 to 0.500 Å−1. To calibrate the angular scale of the measured intensity, silver behenate [CH3(CH2)20-COOAg with a d-spacing value of 58.38 Å] was applied. Sample handling was done through an automatic loading by using robot from a 96-well tray. The SAXS measurements (10–12 frames) were done with an exposure time of 60 s for each frame. Before adjusting pH, SAXS investigations were conducted at 25 °C and the measured pH of the continuous aqueous medium was around 5.0. SAXS investigations were also conducted at 25 and 37 °C on nanodispersions prepared at pH of 6.0 and 7.0. The two-dimensional (2D) SAXS patterns were unified into one-dimensional (1-D) scattering function I(q) employing BIOXTAS RAW 1.6.0. The scattering from the corresponding buffer was utilized as a background and subtracted from the SAXS data of the samples prior to additional analysis. Lorentzian
fitting was applied to measure the \( q \)-values of the identified Bragg reflections. The lattice parameters, \( a \), of the \( \text{H}_2 \) phase were gleaned from SAXS reflections by calculating the distinctive distance \( (d = 2\pi/q) \) for every reflection and using a standard method for calculation of lattice parameters.

### 2.6. Cryogenic transmission electron microscopy (cryo-TEM)

The morphological features of PF10-free and PF10-loaded Dimodan U/citrem aqueous nanodispersions prepared at pH 7.0 were examined in a frozen-hydrated state as recently described (Bor et al., 2022). Briefly, lacey carbon 300 mesh copper grid (Ted Pella Inc., California, USA) was treated with 3–4 \( \mu \)L of the sample and blotted with filter paper for 5 s, and the sample was rapidly plunged into liquid-nitrogen-cooled ethane (-180 °C). The vitrified samples were transferred with a Gatan 626 cryoholder (Gatan, UK) and observed with a Tecnai G2 20 transmission electron microscope (FEI, Holland) at a voltage of 200 kV under a low-dose condition (~5 e/Å²). Further details are given in (Bor et al., 2022).

### 2.7. FT-IR spectroscopy

FT-IR spectra of pure PF10, PF10-free and PF10-loaded nanodispersions were done at 25 °C using an infrared spectrophotometer (Thermo Nicolet, USA). The samples were prepared by blending with KBr and pressing them into circular disks, and scanned from 400 to 4000 cm\(^{-1}\) at a resolution of 2 cm\(^{-1}\).

### 2.8. Differential scanning calorimetry (DSC)

Thermal analysis was conducted with the instrument DSC-214 Polyma (Netzsch, Germany) under \( \text{N}_2 \) atmosphere (10 mL/min). About 3.0 mg sample was placed in a sealed aluminum pan and heated from 40 to 450 °C at a heating rate of 10 °C/min.

### 2.9. PF10 encapsulation efficiency (EE)

The EE, i.e., the mass of PF10-loaded Dimodan U/citrem nanocarriers was quantified using the centrifugal method (Yaghmur, Tran, & Moghimi, 2020). Briefly, the free PF10 was removed from the nanodispersion by centrifugation at 9600g, 10 min and its concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using BSA as a standard within the range 1–1000 μg/mL. The EE was calculated following Eq. (1):

\[
EE(\%) = \frac{C_{\text{total}} - C_{\text{free}}}{C_{\text{total}}} \times 100
\]

where \( C_{\text{total}} \) and \( C_{\text{free}} \) are the concentration of initial and free PF10 in the nanodispersion.

### 2.10. In vitro release assay

The release study of PF10 from Dimodan U/citrem nanocarriers was investigated at 37 °C using the dynamic dialysis method (Li et al., 2019). A freshly prepared nanodispersion was put into a dialysis membrane of 12 kDa MW cutoff, and then placed in 100 mL release medium of PBS, pH 7.4, at 37 °C in a rotary shaking incubator. At designated time intervals, 2 mL of sample was removed from the release medium and an equal volume of fresh medium was replaced. Afterwards, the PF10 content was determined using a UV–vis spectrophotometer at a wavelength of 220 nm. The release rate was calculated as:

\[
\text{Cumulative release (\%) = } \frac{C_n}{C_t} \times 100
\]

where \( C_n \) is the released PF10 content in the solution and \( C_t \) is the total volume of release solution.

#### 2.11. Determination of antioxidant activities

#### 2.11.1. DPPH assay

DPPH radical scavenging assay was done based on the method described by Ramezanzade et al. (2017). A 1 mL aliquot of the sample was blended with 1 mL of 0.16 mM ethanolic solution of DPPH. After vigorous shaking, the mixture was left for 30 min in the dark followed by reading its absorbance at 517 nm. The scavenging capacity was determined by Eq. (3):

\[
\text{DPPH scavenging activity (\%) = } \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

#### 2.11.2. ABTS assay

ABTS radical scavenging capacity was measured as previously described (Ramezanzade et al., 2017). ABTS stock solution was prepared by blending 7 mM ABTS solution with 2.45 mM \( \text{K}_2\text{S}_2\text{O}_8 \) (potassium persulfate) and letting the mixture stand in the dark for 16 h. A 20 μL of the sample was blended with 980 μL of the ABTS working solution and the absorbance at 734 nm was read. The scavenging capacity was determined from Eq. (4):

\[
\text{ABTS scavenging activity (\%) = } \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

#### 2.12. In vitro simulated gastrointestinal digestion

In vitro pepsin-pancreatin digestion of PF10 alone and PF10 loaded to Dimodan U/citrem nano-self- assemblies was studied in SGF (or SIF) by adopting a method described by Ansari et al. (2022) with a slight modification. The SGF was comprised of 0.2% NaCl, pepsin (activity 250 U/mg), and its pH was adjusted to 1.2 with 1 M HCl, whereas SIF was comprised of 0.685 M potassium phosphate monobasic, 1% NaOH, and pancreatin (activity 8xUSP), and its pH was adjusted to 6.8 with NaOH. Briefly, 1 mL of the samples were reconstituted in 20 mL of SGF (or SIF) medium, suspended in a dialysis bag, and incubated at 37 °C for 2 and 4 h for SGF and SIF, respectively. Stability of the loaded PF10 to Dimodan U/citrem nanocarriers was assessed by exploring the difference in the antioxidant activities (i.e., DPPH and ABTS assays) initially and after incubation in SGF (or SIF).

#### 2.13. Statistical analysis

Statistical differences were assessed by one-way ANOVA test, followed by Duncan’s multiple range tests (\( p \leq 0.05 \)). OriginPro 2018 (v9.5.0.193) software was applied for plotting the graphs (OriginLab Corporation, USA).

### 3. Results and discussion

#### 3.1. Particle size and \( \zeta \)-potential measurements

DLS was applied to determine the mean nanoparticle sizes and size distributions and zeta potentials of Dimodan U/citrem nano-self-assemblies containing various PF10 concentrations, and the results are presented in Table 1. The size distribution profiles of the PF10-free and PF10-loaded nanodispersions are depicted in Fig. 1A and B of Supplementary material, respectively. In absence of peptide, the nanoparticles had mean nanoparticle size (a hydrodynamic diameter) of 191.1 nm (Fig. 1A of Supplementary material), whereas loading PF10 at a concentration of 2 mg/mL led to a significant increase in the mean nanoparticle size (415.5 nm) (Fig. 1B of Supplementary material). As shown in Table 1, the control samples (i.e., PF10-free nanodispersion) had a mean nanoparticle size (diameter) of 202.7 ± 14.1 nm, with a PDI of 0.20 ± 0.003. Nevertheless, the mean particle size and PDI were gradually increased upon increasing PF10 concentration (size range, 304.0–569.8 nm; PDI, 0.42–0.55).

Next, \( \zeta \)-potentials of all Dimodan U/citrem/PF10 nanoparticles were
Table 1

<table>
<thead>
<tr>
<th>PF10 (mg/mL)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>202.7 ± 14.10b</td>
<td>0.20 ±</td>
<td>-5.93 ± 1.38a</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>304.0 ± 24.46b</td>
<td>0.28 ±</td>
<td>-6.68 ± 0.72b</td>
<td>89.9 ± 0.5b</td>
</tr>
<tr>
<td>2</td>
<td>367.3 ± 25.66c</td>
<td>0.42 ±</td>
<td>-6.81 ± 1.24bc</td>
<td>83.5 ± 0.9b</td>
</tr>
<tr>
<td>3</td>
<td>416.1 ± 41.79d</td>
<td>0.45 ±</td>
<td>-7.18 ± 0.41c</td>
<td>80.5 ± 0.2b</td>
</tr>
<tr>
<td>4</td>
<td>534.7 ± 63.63c</td>
<td>0.46 ±</td>
<td>-8.98 ± 1.30c</td>
<td>78.9 ± 0.3b</td>
</tr>
<tr>
<td>5</td>
<td>569.8 ± 57.98f</td>
<td>0.47 ±</td>
<td>-9.52 ± 1.10d</td>
<td>64.6 ± 0.1b</td>
</tr>
</tbody>
</table>

Different letters in each column (a, b, c) represents a significant difference between treatments. (p < 0.05). Data are presented as mean ± SD (n = 3).

Table 2

<table>
<thead>
<tr>
<th>S2</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample S2</td>
<td>61.86 ± 0.04 Å</td>
<td>0.00b</td>
<td>-2.67 ± 0.72b</td>
<td>83.5 ± 0.9b</td>
</tr>
<tr>
<td>S2</td>
<td>62.81 ± 0.02 Å</td>
<td>0.00b</td>
<td>-2.67 ± 0.72b</td>
<td>83.5 ± 0.9b</td>
</tr>
<tr>
<td>S2</td>
<td>62.81 ± 0.06 Å</td>
<td>0.00b</td>
<td>-2.67 ± 0.72b</td>
<td>83.5 ± 0.9b</td>
</tr>
</tbody>
</table>

Different letters in each column (a, b, c) represents a significant difference between treatments. (p < 0.05). Data are presented as mean ± SD (n = 3).

3.2. SAXS studies

SAXS investigations were performed at two temperatures (25 and 37 °C) to obtain insight into the effect of PF10 loading (2 mg/mL) on the structural characteristics of Dimodan U/citrem nanoparticles at pH of 6.0 and 7.0, and the results are shown in Fig. 1A. These temperatures were selected for investigating the samples under normal storage conditions (at 25 °C) and at body temperature (37 °C). The latter condition is important when combining SAXS experiments with in vivo or in vitro evaluation studies. At Dimodan U/citrem weight ratio of 1:1, SAXS studies were carried at 25 °C on two nanodispersions (samples S1 and S2: PF10-free and PF10-loaded nanodispersions, respectively) prepared by dispersing the binary mixture of Dimodan U/citrem in excess buffer with pH of 7.4. It was evident from SAXS findings (black and red colored SAXS patterns, Fig. 1A) that the two nanodispersions are hexosomes (HEXs) as indicated from the detected first three Bragg peaks [(1 0 0), (1 1 0), and (2 0 0)] characteristic for the H2 phase in both SAXS patterns. Clearly, PF10 loading was associated with an increase in the lattice parameter of the H2 phase from 61.89 ± 0.06 Å to 62.81 ± 0.06 Å (Table 2). This is probably due to penetration of certain amount of the loaded peptide to the hydrophilic domains (water nanochannels) of the H2 phase.

In absence of PF10, the formation of HEXs is in agreement with previous reports on citrem-stabilized nanoparticles prepared from an unsaturated monoglyceride (Myverol® 18–99 K, monoolein, or poly unsaturated omega-3 fatty acid monoglyceride), PHYT, or soybean phospholipid (Azmi et al., 2016; Hedegaard et al., 2013; Yaghmur, Al-Hosayni, Amenitsch, & Salentini, 2017). The lattice parameter of the internal H2 phase in these reported HEX preparations was in the range of 46–65.6 Å, and was dependent on the used lipid type, lipid composition, citrem concentration, and temperature. It is also worth noting that Dimodan U alone tends to form inverse bicontinuous cubic Ph3m phase in excess buffer at ambient temperatures (Mertz, Østergaard, Yaghmur, & Larsen, 2019). Thus, the formation of HEXs on dispersing binary Dimodan U/citrem mixture in excess buffer is most likely attributed to the localization of a certain amount of the latter negatively charged oil-soluble food-grade emulsifier in the hydrophobic domains of the nanoparticle inner nanostructure, leading to a cubic Ph3m-H2 phase transition.

Depending on citrem concentration, a similar phase transition was also detected on dispersing binary mixtures of citrem and Myverol® 18–99 K (or GMO, or PHYT) in excess water (Hedegaard et al., 2013). In these nanodispersions, it was also reported on colloidal transformation from HEXs to nanoparticles with an internal inverse micellar (L2) phase (emulsified L2 phase nanoparticles, known as ELPs) at relatively high citrem concentrations (Hedegaard et al., 2013). The following order phase transition was also noted upon increasing concentration of citrem in soybean phospholipid/citrem nanodispersions: lamellar (L0) → inverse bicontinuous cubic Pn3m phase → H2 phase → L2 phase (Azmi et al., 2016; Prajapati et al., 2018). Clearly, citrem is suitable for application as a safe stabilizing agent for the production of different colloidally stable lamellar and non-lamellar LLC nanoparticles through electrostatic stabilization mechanism (Wilbroe et al., 2015). However, its incorporation into the nanoparticles’ interiors leads to a significant impact on their structural features.

In both PF10-free and PF10-loaded nanodispersions, it is worth noting that dispersing the binary mixture of Dimodan U/citrem in excess buffer with pH of 7.4 was associated with a decrease in pH of the continuous aqueous medium. In both nanodispersions, the obtained pH was about 5.0. It is worth noting that Dimodan U is commercial product mainly composed of neutral monoglycerides. Thus, the pH-responsiveness is primarily originated from citrem and the charged impurities within (most likely free fatty acids). This pH decrease is most likely attributed to citrem and the charged impurities within (most likely free fatty acids). Citrem is emulsifier having two citric acid moieties with two pKa values of 3.1 and 4.8 (Kaasgaard & Keller, 2010). It is therefore negatively charged lipid under different basic and moderately acidic conditions. As presented in Table 1 of Supplementary material, the ζ-potentials for the PF10-free nanodispersion was increased from –4.62 ± 0.66 mV to –4.76 ± 1.03 mV and –5.93 ± 1.38 mV upon adjusting pH from about 5.0 to 6.0 and 7.0, respectively. To obtain more insight into the pH sensitivity of the structural features of Dimodan U/citrem nanodispersions, SAXS investigations were also performed on samples prepared at pH of 6.0 and 7.0. All nanodispersions were HEXs (Fig. 1A) at 25 and 37 °C. At 25 °C and pH ~ 5.0, loading the peptide fraction PF10 (sample S2) was associated with an increase in the lattice parameter from 61.86 ± 0.04 Å for the sample S1 (PF10-free nanodispersion) to 62.81 ± 0.02 Å for the sample S2 (corresponding PF10-loaded nanodispersion), indicating most likely penetration of the loaded peptide in the latter sample into the hydrophilic domains of the nanoparticles’ H2 interiors. This is in agreement with previous studies on loading peptides and lipopeptides to non-lamellar LLC nanoparticles (Castelletto et al., 2019; Gontsarik et al., 2016). It was reported on enlargement of the hydrophilic domains of the internal inverse cubic phases in cubosomes and the involved structural transitions upon augmenting their concentrations (Castelletto et al., 2019; Gontsarik et al., 2016).

It is interesting that increasing pH to 6.0 and 7.0 led to a different behaviour at 25 and 37 °C. At both pH values, the SAXS results indicated that the internal H2 phase was maintained and loading the PF10 did not affect its lattice parameter (samples S3-S6, Table 2). This is most likely indicating that the loaded peptide is mainly localized in the continuous aqueous medium without an indication on its localization in the nanoparticles’ H2 interiors. However, we do not exclude the adsorption of certain amount of the peptide to the outer surfaces of the nanoparticles. In this respect, it was important for understanding the influence of peptide loading on the structural attributes of the nanoparticles to compare the ζ-potentials before and after loading PF10 at the given pH values. We found that loading the peptide was associated with a decrease in the ζ-potentials. For instance, the ζ-potentials at pH of 7.0 was decreased from –5.93 ± 1.38 mV to –9.52 ± 1.10 mV upon loading the peptide (Table 1 of Supplementary material). A similar decrease in ζ-potentials was also reported upon loading fish-derived peptide to citrem-stabilized fish oil-in-water emulsion (Farvin et al., 2014).
Adsorption of the peptide molecules at the nanoparticles’ outer surfaces led most likely to the observed slight decrease in ζ-potentials (Table 1 of Supplementary material).

It is also worth mentioning that loading PF10 in all nanodispersions prepared at different pH values was correlated with a dramatic increase in the average particle sizes (Table 1 of Supplementary material) and the observation of lipid lumps in the PF10-loaded nanodispersions after two days of their preparation. The main reasons behind the formation of these lumps (non-dispersed lipid forms) in the milky samples is still not clear. However, they are most likely start to appear due to release of Dimodan U alone to excess buffer and/or presence of citrem-peptide complexes. At the different pH values, it seems from the obtained results that complexation of citrem with the loaded peptide through most likely hydrogen bonding and electrostatic and hydrophobic interactions play a significant role in modulating the colloidal stability of citrem-stabilized nanoparticles. This is in line with former studies reporting on the tendency of citrem to form complexes with proteins and peptides in aqueous media that occur mainly through hydrophobic interactions: interactions of the hydrophobic moieties of citrem with the non-polar moieties of the proteins or the peptides (Amagliani, O’Regan, Kelly, & O’Mahony, 2022; Fernández et al., 2020). However, changes in pH values can enhance also the interactions through electrostatic forces and hydrogen bonding (Fernández et al., 2020). In our study, such possible complex formation of citrem with the loaded peptide is most likely the main reason behind the decrease in citrem’s stabilization efficiency as indicated from the significant increase in the mean nanoparticle sizes in all prepared nanodispersions at different pH values upon loading the peptide. Further investigations (including additional SAXS experiments on the observed lipid lumps) are required to investigate the nature of the observed lipid lumps in PF10-loaded nanodispersions and shed further light on interactions of citrem alone with the peptide. It is also worth improving the colloidal stability of the PF10-loaded nanodispersions in future investigations by using a different stabilizer than citrem or combining citrem with an additional stabilizing agent (such as Tween 80) for stabilizing the dispersed nanoparticles through steric stabilization alone or combination of steric and electrostatic effects.

Fig. 1. (A) Effect of PF10 loading on the structural features of Dimodan U/citrem nanodispersions (samples S1-S6, Table 2). Before adjusting pH (samples S1 and S2), SAXS experiments were performed at 25 °C and the measured pH of the continuous aqueous medium was around 5.0. SAXS experiments were also conducted at 25 and 37 °C on nanodispersions prepared at pH of 6.0 (samples S3-S4) and 7.0 (samples S5-S6). All nanodispersions are hexosomes, and the Bragg peaks are represented with blue arrows for the corresponding Miller indices of the H2 phases. In all panels, the scattering intensity was shifted by a constant arbitrary for better visibility. (B) ATR-FTIR spectra, and (C) DSC thermograms of (a) free peptidic fraction PF10, (b) empty HEXs, and (c) PF10-loaded HEXs prepared at PF10 concentration of 2 mg/mL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. Cryo-TEM observations

The formation of HEXs was also verified by cryo-TEM observations on PF10-free (Fig. 2A-C) and PF10-loaded nanodispersions (Fig. 3A-D) prepared at pH 7.0. In line with DLS findings, the sizes of the detected HEXs were in the range of 80–250 and 75–390 nm, respectively. Thus, an increase in nanoparticle sizes upon loading the peptide was also confirmed by cryo-TEM imaging. Similar to other reported citrem-stabilized non-lamellar LLC nanoparticles (Hedegaard et al., 2013; Wibroe et al., 2015), these HEXs were covered with surface phases (vesicular structures, marked with orange dashed arrows). Such surface phases (vesicular or sponge structures) covering hexosomes, cubosomes, and related nano-self-assemblies were also detected in nanodispersions stabilized by other stabilizing agents (including PEGylated lipids) (Azmi et al. 2016; Bor et al., 2022).

3.4. FT-IR spectroscopy

FT-IR spectrometry was applied to monitor the host (i.e., PF10)-guest (i.e., HEX) interactions. The spectrum of plain PF10 displayed distinctive peaks of O–H and C–H stretching at 3421 and 2963 cm\(^{-1}\), C–O (amide I) stretching at 1634 cm\(^{-1}\), C–N stretching (amide III) and N–H bending at 1388 cm\(^{-1}\), C–N and C–O stretching at 1110 and 1041 cm\(^{-1}\), and C–C stretching vibrations of amino acids at 875 cm\(^{-1}\) (Fig. 1B-a) (Hosseini, Nahvi, & Zandi, 2019). As shown in Fig. 1B-b, according to the spectrum of HEXs, 3466 and 3419 cm\(^{-1}\) were the stretching vibration peaks of –OH; 2926 and 2854 cm\(^{-1}\) were –CH\(_2\) stretching vibration bands; 1736 and 1634 cm\(^{-1}\) were C–O stretching of the ester bond; 1382 cm\(^{-1}\) was –CH\(_2\) shear vibration peaks; 1174 cm\(^{-1}\) was bending vibrations of –CH\(_3\), and 1115 cm\(^{-1}\) was C–O stretching (Wang et al., 2021). In Fig. 1B-c, it could be seen from the spectrum of PF10-loaded HEXs that the –OH stretching vibration peak

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>PF10 concentration (mg/mL)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>LP(a) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(_1)</td>
<td>0(^a)</td>
<td>25</td>
<td>5.0</td>
<td>61.86 ± 0.04</td>
</tr>
<tr>
<td>S(_2)</td>
<td>2(^b)</td>
<td>25</td>
<td>5.0</td>
<td>62.81 ± 0.02</td>
</tr>
<tr>
<td>S(_3)</td>
<td>0</td>
<td>25</td>
<td>6.0</td>
<td>64.37 ± 0.02</td>
</tr>
<tr>
<td>S(_4)</td>
<td>2</td>
<td>37</td>
<td>6.0</td>
<td>64.47 ± 0.01</td>
</tr>
<tr>
<td>S(_5)</td>
<td>2</td>
<td>37</td>
<td>6.0</td>
<td>64.42 ± 0.06</td>
</tr>
<tr>
<td>S(_6)</td>
<td>0</td>
<td>25</td>
<td>7.0</td>
<td>66.05 ± 0.03</td>
</tr>
<tr>
<td>S(_7)</td>
<td>0</td>
<td>37</td>
<td>7.0</td>
<td>65.88 ± 0.03</td>
</tr>
<tr>
<td>S(_8)</td>
<td>2</td>
<td>25</td>
<td>7.0</td>
<td>66.04 ± 0.03</td>
</tr>
<tr>
<td>S(_9)</td>
<td>2</td>
<td>37</td>
<td>7.0</td>
<td>65.88 ± 0.03</td>
</tr>
</tbody>
</table>

\(a\) LP: lattice parameter of the inverse hexagonal (H\(_2\)) phase as derived from SAXS data analysis of SAXS patterns presented in Fig. 1.

\(b\) The PF10-free and PF10-loaded nanodispersions were prepared by dispersing the binary Dimodan U/citrem mixture in excess buffer with pH 7.4.

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**Fig. 2.** Cryo-TEM images of PF10-free Dimodan U/citrem nanodispersion prepared at pH 7.0 and Dimodan U/citrem weight ratio of 1:1 (sample S\(_3\)). In panels (A)-(C), the detected hexosomes (80–250 nm) covered with a surface phase consisting of a lipidic bilayer (vesicular structure, denoted with orange dashed arrows). In all images (including the enlarged area in panel (A)), the scale bar is 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
has down-shifted and moved to 3423 cm\(^{-1}\), revealing most likely the formation of hydrogen-bonds among PF10 molecules and the molecules of the lipids (citrem and Dimodan U) (Wang et al., 2021). The C=O characteristic peak seen for HEXs also moved from 1736 cm\(^{-1}\) to 1743 cm\(^{-1}\) after loading PF10, indicating the formation of hydrogen bond between the carbonyl group of Dimodan U and peptide (Sarabandi & Jafari, 2020). Furthermore, downshifts from 1634 and 1174 cm\(^{-1}\) in empty HEXs towards 1627 and 1167 cm\(^{-1}\) were recorded upon loading PF10. The band at 1115 cm\(^{-1}\), attributed to the C–O stretching, also showed a minor but seeable shift of 2 cm\(^{-1}\) towards lower wavelengths (from 1115 cm\(^{-1}\) in the empty HEXs to 1113 cm\(^{-1}\)). These changes demonstrate that loading the peptide fraction PF10 to be corresponded with its localization within the hydrophilic domains (water nanochannels) of the H\(_2\) phase (Bitan-Cherbakovsky et al., 2014).

### 3.5. DSC analysis

The DSC thermograms of PF10, empty HEXs, and PF10-loaded HEXs are shown in Fig. 1C. The PF10 thermogram indicates an endothermic peak at 46.8 °C (Fig. 1C-a) attributing to its glass transition temperature (\(T_g\)), revealing its semi-crystalline nature, as discussed previously (Hosseini et al., 2019). The absence of the PF10 endothermic peak in the thermogram of PF10-loaded HEXs revealed most likely its loading to HEXs (Nasr, Ghorab, & Abdelazem, 2015). Moreover, the thermograms of empty HEXs and PF10-loaded HEXs show endotherms at 83.6 and 89.7 °C, respectively (Fig. 1C-b and c), which are most likely attributed to the phase transition from H\(_2\) phase to L\(_2\) phase (Abdel-Bar, Khater, Ghorab, & Al-Mahallawi, 2020; Yaghmur, Laggner, Zhang, & Rappolt, 2007). Under full hydration conditions, it was reported that pure monoolein (glycerol monooleate) tends to induce phase transitions from inverse cubic \(Pn3m\) phase to \(H_2\) and \(L_2\) phases at temperatures of \(\sim 90\) °C and \(> 100\) °C, respectively (Yaghmur et al., 2007). In the present study, the detection of \(H_2\) and \(L_2\) phases at significantly lower temperatures is attributed to the use of commercially distilled lipid and the significant impact of citrem on the structure as discussed above.

### 3.6. Encapsulation efficiency (EE)

To confirm the integration of PF10 into HEXs, the EE of the peptide was studied. As shown in Table 1, the EE was in the range of 64.6–89.3%, which was in very good agreement with the previously reported EE of 68.3–97.5% for phenytoin (an anti-seizure drug) loaded in either selachyl alcohol or PHYT cubosomes and hexosomes stabilized with P407 or Tween 80 (Mohammad, Prentice, Boyd, & Rizwan, 2022). We found also that the EE is dependent on the loaded concentration of PF10. For instance, the EE of PF10 was decreased from 89.3% to 64.6% upon increasing PF10 concentration from 1 mg/mL to 5 mg/mL. This might be attributed to the saturation degree of nanolipidic carrier which do not support an increased load of peptide (Mohammad et al., 2022). This is also probably due to complex formation of citrem with the loaded peptide, which is most likely the main reason behind the decrease in peptide’s EE as discussed by SAXS results (see section 3.2.).

### 3.7. In vitro release assay

The \textit{in vitro} release assay was conducted in PBS (pH 7.4) and the results are presented in Fig. 2 of Supplementary material. Free PF10 dissolved in buffer diffused totally via the dialysis bag into the release medium within 12 h (92.8 ± 0.53%) owing to its hydrophilic nature. On the other hand, the release of PF10 from HEXs exhibited a sustained release behavior: only 32.2 ± 0.62% of PF10 was released after 24 h. A sustained release behavior was also reported for fluoxetine hydrochloride (antidepressant drug) loaded in either selachyl alcohol or PHYT cubosomes and hexosomes stabilized with P407 or Tween 80 (Mohammad et al., 2022). This is also probably due to complex formation of citrem with the loaded peptide, which is most likely the main reason behind the decrease in peptide’s EE as discussed by SAXS results (see section 3.2.). Such diffusion pattern enables a sustained release of drugs in vivo, improves their health-giving properties, and reduces their side effects.
3.8. Antioxidant activities of PF10-loaded HEXs

3.8.1. DPPH radical scavenging activity

Recently, marine-derived peptides have been drawing increasing attention because of their diverse biological functions like antihypertensive, anti-diabetic, anti-inflammatory, immunomodulatory, and antioxidant activities (Hosseini et al., 2022). Here, we measured the antioxidant capacity of PF10 to evaluate the effect of its encapsulation in HEXs on its intrinsic antioxidant activity. As shown in Fig. 4A, the DPPH scavenging capacity of plain PF10 increased from 65.13 to 80.58% when its concentration was increased from 1 to 5 mg/mL; this was in a good agreement with the previous findings of Zamorano-Apodaca et al. (2020) for collagen hydrolysates extracted from the by-products of various fish species. Here, it is worth noting that the antioxidant activity is attributed to the presence of relatively high number of hydrophobic amino acids (50.6% in this study, Table 2 of Supplementary material), which might increase solubility in lipid matrices and, thus, enhance antioxidant capability (Ramezanzade et al., 2017). The DPPH scavenging activity of the PF10-loaded HEXs varied from 59.47 to 73.57% at concentrations of 1 to 5 mg/mL; the slight difference seen in the DPPH results of the PF10 before and after its encapsulation, may be connected to the interactions that happened between peptide and Dimodan U/citrem during the process of encapsulation. Thus, the encapsulation of PF10 into the lipid nanocarrier could retain its antioxidant activity.

3.8.2. ABTS radical scavenging activity

The ABTS assay is an appropriate method to determine the antioxidant capacity of hydrogen-donating or chain-breaking antioxidants (Sarabandi & Jafari, 2020). The scavenging activity of PF10-loaded HEXs on ABTS radical was basically consistent with the scavenging activity of DPPH radical. The ABTS scavenging activity in HEXs was also influenced by PF10 concentration as the scavenging activity was increased on increasing PF10 concentration from 1 to 5 mg/mL (Fig. 4B). Likewise, except at low amounts of PF10 (i.e., HEXs loaded with PF10 at concentrations of 1 and 2 mg/mL), there was no remarkable difference in the scavenging capacity of peptidic fraction after hexosomal encapsulation ($p > 0.05$). So, it is worth mentioning that encapsulation of PF10 in HEXs resulted not only in no remarkable differences in its antioxidant capacity, but also led to its sustained release as discussed above (see section 3.9.).

3.9. Gastrointestinal fate of PF10-loaded HEXs

The stability of PF10-loaded HEXs in gastric fluids is an utmost important attribute, because it reflects the resistance of bioactive peptides to hydrolysis within the GI tract. The DPPH and ABTS assays of the plain and loaded-PF10 to HEXs were evaluated during simulated GI fluid conditions (Fig. 4C and D). Both free- and nanoencapsulated-PF10 showed a similar evolution pattern during the simulated GI digestion. The DPPH and ABTS radical scavenging activities in both samples...
decreased significantly after exposure to the gastric environments (SGF/ SIF) \( (p < 0.05) \). This effect was attributed to the enzymatic hydrolysis of the original peptide, which resulted in the production of smaller species (smaller peptidic fractions) with lower antioxidant capacities (Sepúlveda, Aleman, Zapata, Montero, & Gomez-Guillen, 2021). Furthermore, the chemical transformations of peptides as a result of different mechanisms during the GI digestion may have also influenced their antioxidant activity. These results are in accordance with the findings of Mohammadi, Hamishehkcar, McClements, Shahvalizadeh, and Barri (2023) in their study on the antioxidant activity in the *Spirulina* protein hydrolysates after simulated GI transit. Nonetheless, the antioxidant properties of both encapsulated and nonencapsulated peptidic fraction PF10 was substantially higher in the gastric phase than intestinal phase. This might be attributed to higher release of bioactives, with free radical-scavenging activity, from the samples under the acidic environments of gastric digestion (Gillon, Pintado, Fernández-López, Pérez-Alvarez, & Viuda-Martos, 2015). Furthermore, the pH value of a material is understood to influence the racemization of molecules, which possibly leads to two chiral enantiomers having different bioactivities; by that, during digestion process, antioxidants might be more reactive especially at gastric acid medium than neutral medium (i.e., intestinal phase), as racemization would increase with pH in other bioactive agents (Wootton-Beard, Moran, & Ryan, 2011). Notably, remarkable differences in retention rate of antioxidant activity between free- and nonencapsulated-PF10 were observed at SIF/SGF media, which is in line with the hypothesis that was proposed in the 'Introduction'. This discrepancy may arise from the fact that loading PF10 to HEKS contributes to the chemical stability of peptide by separating it from the GI tract digestible liquids (Feng et al., 2019). Improved enzymsolyis stability of hispolon, a phenol molecule derived from mushroom, by liquid nanoencapsulated-PF10 were observed at SGF/SIF media, which is in line with the hypothesis that was proposed in the 'Introduction'. This might be attributed to higher release of bioactives, with free radical-scavenging activity, from the samples under the acidic environments of gastric digestion (Gillon, Pintado, Fernández-López, Pérez-Alvarez, & Viuda-Martos, 2015). Furthermore, the pH value of a material is understood to influence the racemization of molecules, which possibly leads to two chiral enantiomers having different bioactivities; by that, during digestion process, antioxidants might be more reactive especially at gastric acid medium than neutral medium (i.e., intestinal phase), as racemization would increase with pH in other bioactive agents (Wootton-Beard, Moran, & Ryan, 2011). Notably, remarkable differences in retention rate of antioxidant activity between free- and nonencapsulated-PF10 were observed at SIF/SGF media, which is in line with the hypothesis that was proposed in the 'Introduction'. This discrepancy may arise from the fact that loading PF10 to HEKS contributes to the chemical stability of peptide by separating it from the GI tract digestible liquids (Feng et al., 2019). Improved enzymsolyis stability of hispolon, a phenol molecule derived from mushroom, by liquid nanoencapsulated-PF10 has also been reported by Ansari et al. (2022).

4. Conclusions

The production and characterization of food-grade HEKSs based on binary mixtures of Dimodan U and citrem are presented. DLS, cryo-TEM, SAXS were used for investigating the effect of loading the fish peptide fraction PF10 on the size characteristics, morphological features and internal architectures of the nanoparticles. Further, the nanoparticle-peptide interactions were evaluated through use of FTR and DSC. Our experimental findings showed that the prepared HEKSs were able to prolong PF10 release as only 32.2% of the loaded PF10 was released in the release medium (PBS with pH 7.4) after 24 h. Depending on the loaded peptide concentration to HEKSs, the encapsulation efficiency of PF10, was in the range of 64.6–89.3%. PF10-loaded HEKSs are attractive options and can be considered as functional food ingredients owing to the detected significant antioxidant capacity after in vitro simulated gastrointestinal digestion. Collectively, our findings on the designed Dimodan U/citrem nano-self-assemblies might open a new horizon in the development of advanced lipidic nanovehicles for efficient delivery of antioxidant peptides for pharmaceutical and food applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary material

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References


