Iranian Journal of Fisheries Sciences 22(5) 1018-1038 2023
DOI: 10.22092/ijfs.2023.130549

Research Article

Encapsulation of the extracted phycobiliprotein from *Gracilaria gracilis* in nanoliposomes: Physicochemical, structural and stability properties

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Received: May 2023 Accepted: August 2023

Abstract

Nanoencapsulation of compounds in nanoliposomes can be used as a protective carrier system for bioactive compounds. Phycobiliproteins (PBPs) were extracted from algae with antioxidant, antimicrobial, anti-cancer, and anti-inflammatory properties during processing and storage under different conditions to produce raw materials for healthy foods. In the present study, phycobiliprotein was extracted from *Gracilaria gracilis* and the amount of pigments was investigated. Also, the physicochemical properties and thermal stability of nanoliposomes (0%, 0.5%, 1%, and 1.5% w/v chitosan) were evaluated. The nanoliposomes' particle size and polydispersity index (PDI) were from 336.9 to 577.7 nm and 0.25 to 0.28 with the zeta potential (-10.238 to +30.33 mV), respectively. in nanocarriers. The highest nanoencapsulation efficiency of PBPs (83.98%) was obtained under optimal conditions in nanoliposomes with 1.5% chitosan coating. Evaluation of *in-vitro* release of PBPs from nanoliposomes showed prolonged release of PBPs by increasing the ratio of chitosan in the wall formulation. Thermal behavior and spectroscopy of the samples confirmed the successful nanoencapsulation of the PBPs in the nanoliposome. The results show the use of lipid nanocarriers containing phycobiliprotein can be considered an effective method to protect it against undesirable chemical, sensory, microbial, and tissue changes in food products.

Keywords: *Gracilaria gracilis*, Nanoliposomes, Phycobiliprotein, Release, Thermal behavior

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Introduction

Regular use of functional products can be considered as a dietary supplement with no side effects on human health. Previous studies have shown that metabolites and phytochemicals extracted from seaweed had positive effects on human health, thus reducing the symptoms of chronic diseases such as cancer, arthritis, diabetes, and autoimmune, ocular, and cardiovascular diseases. Although their effects are mild compared to the drugs used, they are considered safe and secure and use through daily diet as a supplement will have long-term physiological health advantages (Tanna and Mishra, 2018). Today, due to the beneficial properties of bioactive compounds for health and consumer awareness, the interest in functional products is increasing (Alves *et al*., 2018). Seaweed is a rich source of ω-3 fatty acids, essential amino acids, vitamins A, B (B_1, B_2, B_9 and B_{12}), C, D, E, K, and essential minerals (calcium, iron, iodine, magnesium, phosphorus, potassium, zinc, copper, manganese, selenium and fluoride) as well as dietary fiber (roughage). Red algae are distinguished from other algal species by their presence of *phycobiliproteins* and carotenoids (Shannon and Abu-Ghannam, 2019). Phycobiliproteins (PBPs) are light-absorbing pigment-protein complexes that can efficiently transfer light energy to chlorophyll a and provide conditions for algae photosynthesis. PBPs are divided into four main groups due to their light-absorbing properties and types of bilins, including *phycoerythrin* (λmax=540-570 nm), phycocyanins (λmax=610-620 nm), *phycoerythrocyanin*s (λmax=560-600 nm) and *allophycocyanins* (λmax=650-695 nm). PBPs are the most important compounds in phycobilisomes of red algae. Phycobilins have medicinal potential due to their unique antioxidant and antimicrobial activity (Li *et al*., 2019). They can also be used as a nutrient in the food industry and as a colorant in the cosmetics industry. In general, high amounts of PBPs can be rapidly extracted from seaweed through the aqueous extraction method. PBPs have been used in foods in combination with gelatinous desserts, puddings, sourdoughs, and soft and dairy drinks, although their use should be further investigated by toxicity testing. In the future, further studies will be needed to focus on the interaction of PBPs with food matrices and stabilizers, and bioavailability. Today, The tendency of consumers to use natural ingredients and the reluctance to use artificial additives is necessary for natural alternatives with unique properties (Chentir *et al*., 2018). However, natural dyes are generally more chemically unstable and highly sensitive to heat, pH, light, oxygen, and processing conditions. Although PBPs are sensitive to adverse environmental conditions and decompose under conditions such as heat, light, and pH, they are still more adaptable than other widely used colors such as gardenia and indigo (Kannaujiya and Sinha, 2016). The use of natural pigments in food depends on their stability under natural conditions as well as their shelf life and compatibility with other substances.

PBPs are stable up to 60°C, in a wide range of pH (3.5-9.5) and desired conditions for use in medicine, cosmetics, and natural food colors. According to the above, the use of PBPs in food is associated with limitations due to their low stability and solubility and undesired taste. Therefore, efforts have been made to find new techniques that reduce the pigments needed so that both their biological properties and the taste of the food are generally accepted, including the encapsulation of bioactive compounds.

Encapsulation is the technology of trapping solids, liquids, or gases in capsules that release their contents at controlled rates and under special conditions, including the formation of a wall around the bioactive compound, ensure that these compounds do not leak out and do not cover undesired compounds in the capsule (Ghorbanzade *et al*., 2017). Liposomes are one of the types of lipid carriers used to coat bioactive substances and drugs. Liposomes are colloidal vesicles composed of polar lipids, especially phospholipids, which form bilayer spherical structures in the presence of water molecules. Due to their amphiphilic properties, these compounds can encapsulate a wide range of hydrophilic, lipophilic, and amphiphilic compounds. liposomes and nanoliposomes have the same structural, chemical, and thermodynamic properties. However, nanoliposomes provide more surface area than liposomes (Rasti *et al*., 2012). They also have higher colloidal stability and cause less turbidity. However, lipid-based delivery systems are not suitable in the acidic environment of bile salts and gastric lipase due to their instability. The use of mucosal adhesive polymer systems such as chitosan is the most important step in increasing the liposomal delivery of bioactive protein compounds orally. The stability of the liposomal carrier and the absorption efficiency in the gastrointestinal tract can be greatly increased by a layer of chitosan coating due to chitosan and liposome binding through electrostatic reactions between the cationic groups of chitosan and anionic phospholipid groups. For the chemical stability of nanoliposomes, the stability of phospholipid compounds used in the structure of nanoliposomes plays an important role. The most widely used phospholipid in the structure of liposomes and nanoliposomes is phosphatidylcholine, which has a hydrophilic polar head called phosphocholine, a glycerol bridge, and a hydrophobic acyl hydrocarbon chain. If these phospholipids are placed in the liposome membrane alone, the membrane becomes rigid. For this reason, they use another component called cholesterol to increase membrane flexibility. Nanoliposomes encapsulated with active polymers have good properties such as better adhesive mucosa and permeability of the gastrointestinal wall (Souzaa *et al*., 2014).

On the other hand, with the controlled release of the drug into the target tissues, more drugs will reach the target cells and

the healing process will be faster. Therefore, due to the high distribution of seaweed and the potential for the production of value-added products, this study considered the assessment of nanoliposomes containing phycobiliprotein with high stability, improved flavor, and controlled release in- vitro to overcome the challenges of using bioactive compounds in the food and pharmaceutical industries.

Materials and methods

Materials

The red algae *Gracilaria gracilis* was purchased from the Iranian National Algae Culture Collection (Tehran, Iran). The sunflower oil (Oil, Iran) was procured at a local market. Soy lecithin and glycerol were purchased from Merck Company (Darmstadt, Germany). The coating materials were chitosan with low molecular weight (75%–85% degree of deacetylation) (Sigma-Aldrich, St. Louis, MO, USA). All other chemicals and reagents were of analytical grade. Distilled and deionized water was used for the preparation of all solutions. The experiments were carried out in three replicates for each sample.

Extraction of phycobiliproteins (PBPs)

The dried *Gracilaria gracilis* was milled and then mixed with 0.05 M sodium phosphate buffer (pH 6.8) under continuous stirring. The algae cells were further broken using ultrasonic waves in the ice bath and then stirred continuously for 8 h at 4℃. Cell residue was removed by centrifugation (10,000×g, 15 min) and the supernatant was collected. Phycobiliproteins (PBPs) were separated from the supernatant ammonium sulfate precipitation in two steps. The supernatant was removed after the salting-in treatment of 20% saturation of ammonium sulfate at first. The solution was centrifuged at 10,000×g for 15 min and then the resulting supernatant was set to 45% saturation of ammonium sulfate. After centrifugation, the sedimentation between 20% and 45% saturation of ammonium sulfate was collected and dissolved in 100 mL sodium phosphate buffer (pH 6.8). The sample was lyophilized for future study (Moraes *et al*., 2011).

Assessment of phycobiliprotein pigments of proteins

phycobiliprotein pigments were recognized on the dry powder of *Gracillaria* according to Wyman and Fay (1986) method. For this purpose, 0.1 g of the dried sample was refrigerated (glycerol) under severe osmotic pressure for two hours (4°C). Then, the cells were broken down by 0.3 N sodium acetate and distilled water so that the final concentration of sodium acetate in the solution was 200 mM. The adsorption of the samples against the blanks prepared from the materials used in the extraction of phycobiliprotein at wavelengths of 562, 615, 526, and 750 nm was read using a spectrophotometer (6800 UV / VIS model, Jenway, Germany). The following equations were used to calculate the phycobiliprotein of proteins in mg/g:

allophycocyanins (AP)= [1000 (A_{652}-A_{750}) –208 (A_{615}-A_{750})) / 5.09] (1)
phycocyanin (PC)= [1000 (A_{615}-A_{750}) –474 (A_{625}-A_{750})) / 5.34] (2)
phycoerythrin (PE)= [1000 (A_{562}-A_{750}) –2.41 (PC) -0.948 (AP)) / 9.62] (3)

Emulsifying property

Emulsifying properties of PBPs samples with different pH treatments were determined based on Yin *et al*. (2008).

Preparation of nanoliposomes

The nano-liposomes containing macroalgae phycobiliprotein were prepared as slight modifications according to Rasti *et al*. (2012) method. First, lecithin and sunflower oil were placed in a heating bath (30℃) to ensure the dissolved lecithin in the oil completely. Then, the phycobiliprotein was added to the lecithin-oil mixture during constant stirring on a hotplate. The solution was hydrated by adding deionized water and glycerol (final concentration 2%, v/v and preheated to 30℃) and then homogenized for 15 minutes. Uncoated liposomes and coated liposomes with chitosan (0.5%, 1%, and 1.5% w/v) are homogenized under high pressure (chitosan solution was prepared by dissolving chitosan in 1% v/v in acetic acid). To have the smaller particle mean size of the liposomes, the liposomal suspension was subjected to a sonication process in an ice bath (7 min, 1 s on and 1 s off) using a probe (Sonicator, 200 UPS, Dr. Hieschler, Teltow, Germany). Samples were exposed to nitrogen gas and kept at room temperature for further analysis. Finally, lipid vesicles were obtained by freeze-drying.

Analysis of phycobiliproteins (PBPs) nanoliposome

Particle Size, Poly Dispersity Index (PDI), and Zeta Potential

The hydrodynamic particle size and zeta potential of nano-capsules were determined by Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Zeta potential provides information about the potential difference between the dispersion medium and the stationary layer of the fluid attached to the dispersed particle, which helps to know about the stability of the nanoformulations. Measurements were made using aqueous diluted samples (2:1 ratio). Using the principle of photon correlation spectrometry, this instrument also gives the measurement of particle-size distributions in the range (Ojagh and Hasani, 2018).

Determination of encapsulation efficiency (EE)

Encapsulation efficiency (EE) was obtained based on Fathi *et al*. (2013) method with slight modification. The filtration method using 50 cc filtered falcon tubes (Biologix, Jinan, China) will be used to determine the encapsulation efficiency of nanoliposomes containing phycobiliproteins. For this purpose, 1,000 µL of the nanoliposome is added to the upper part, then the tube is centrifuged at 12,000×g for 10 minutes. After centrifugation, the liquid passed

through the filter is separated and the amount of adsorption was read by a spectrometer (6800 UV/VIS model, Jenway, Germany) (Fathi *et al*., 2013). Percentage efficiency (EE) was determined according to the following equation:

$$EE\ (\%)=W_T–W_F\ /W_T \quad (4)$$

Where, W_T is the total weight of the phycobiliprotein (mg) used in the nanoliposome formulation and W_F is the amount of free phycobiliprotein (mg) in the filtered phase.

Scanning electron microscopy (SEM)

Morphology of the freeze-dried nanoliposomes was analyzed by scanning electron microscopy (SEM; Leo EVO–40 VPX, Carl Zeiss SMT, Cambridge, UK). The samples were glued onto an adhesive tape mounted on the specimen stub and particles were covered with gold palladium before analysis. Representative SEM images were reported.

FTIR (Fourier transform infrared spectrometer) characterization

FTIR analyses for pure phycobiliprotein and nanoliposomes were performed using a Bruker Equinox 55 spectrometer (Equinox 55-LSI 01, UK) from a wave number range of 4000–500 cm^{-1}.

Differential scanning calorimetric (DSC)

Differential scanning calorimetry (DSC) analysis was done to determine the thermal stability of the pure phycobiliprotein and nanoliposomes using a Mettler-Toledo DSC model 822 (Mettler Toledo AG, Switzerland) at a heating rate of 10°C/min from 30 to 300°C.

In-vitro release

To determine the phycobiliprotein release profile from nanoliposomes, 20 mg of nanoliposomes containing phycobiliprotein were added to 5 ml of the release medium (deionized water) and stirred continuously with a magnetic stirrer. The amount of phycobiliprotein released from the nanoliposomes, and the concentration of phycobiliprotein (mg/L) in the release medium at sampling intervals using a visible-ultraviolet spectrometer at a wavelength of 575 nm (maximum absorption) were measured. The amount released (µg) was converted according to the volume of the release medium (mL). The percentage of phycobiliprotein released was calculated by dividing the amount of pigment released at each time point in the sample by the initial weight of the nano-encapsulated phycobiliprotein in the sample (Hosseini *et al*., 2013).

Statistical analysis

One-way analysis of variance (ANOVA) procedure was performed using SPSS (ver.26) software (SPSS Inc., Chicago, USA). Duncan's test ($p\leq0.05$) significance level was considered to examine differences that exist among the means.

Results

PBPs pigments

The amount of PBPs pigments on the frozen dry powder of *Gracilaria* was

obtained in mg/g dry weight of algae. According to the study result, among the phycobilin pigments, the highest amount was related to phycoerythrin (1.03±0.27), and the lowest amount was related to allophycocyanins (0.23±0.01), and then phycocyanin (0.7±0.0). The results showed that there was a significant difference (p<0.05) between the treatments.

Emulsion of PBPs

As shown in Figure 1, the emulsion stability of PBPs extracted from *Gracilaria* first showed an increasing and then a decreasing trend with increasing pH. In general, the emulsion stability index (ESI) measures the ability of an emulsion to withstand structural changes over a while. The pH = 4 was considered as an optimum pH for emulsion stability.

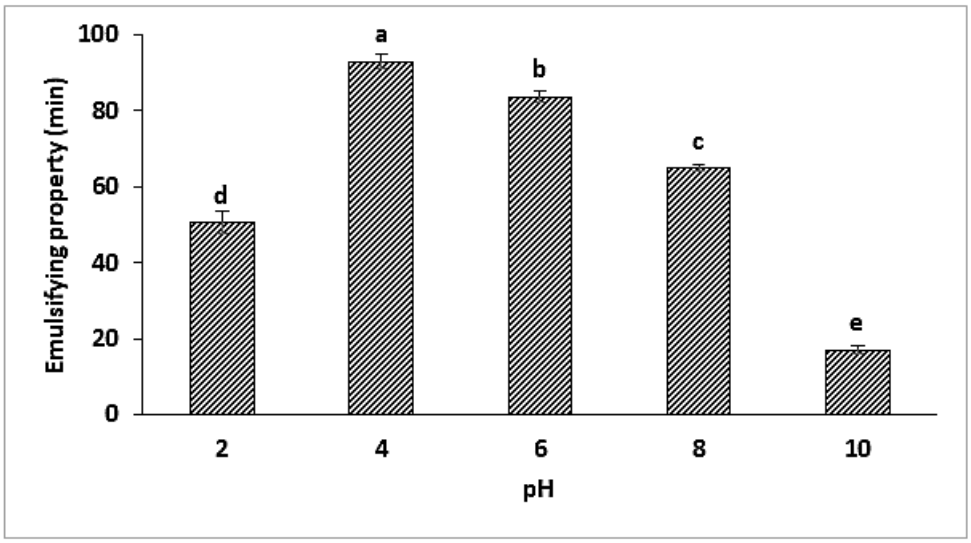


Figure 1: The emulsion stability of PBPs extracted from *Gracilaria* at different pH.

Physical, chemical, and structural properties of nanoliposomes

Particle size and poly dispersity index (PDI)

Mean particle size, particle polydispersity index (PDI), and nanoencapsulation efficiency in nano-encapsulated PBPs were assessed in liposomes with different formulations. The results of particle size and PDI are given in Table 1. Uncoated nanoliposomes with a mean of 557.7±25.9 nm and chitosan-encapsulated nanoliposomes (0.5%) with a mean of 336.9±21.30 nm have the highest and lowest particle sizes (p<0.05), respectively.

Table 1: Encapsulation efficiency (EE%), zeta potential (mV), particle size and polydispersity index (PDI) of phycobiliprotein nano-liposome.

Treatment	Particle size (nm)	EE%	Zeta potential	PDI
Uncoated Liposome (0% CH)	557.7 ± 25.90 [a]	70.14 ± 1.24 [d]	-10.8 ± 1.85 [c]	0.25 ± 0.00 [a]
Coated Liposome (0.5% CH)	336.9 ± 21.30 [c]	75.68 ± 1.20 [c]	22.6 ± 2.70 [b]	0.26 ± 0.02 [a]
Coated Liposome (1% CH)	406.8 ± 12.70 [b]	80.98 ± 1.12 [b]	30.33 ± 1.45 a	0.28 ± 0.00 [a]
Coated Liposome (1.5% CH)	515.5 ± 41.20 [a]	83.98 ± 0.73 [a]	21.31 ± 5.21 b	0.26 ± 0.02 [a]

Reported means (± standard deviations) derived from 3 replications with 3 samples per replication.
Means within a column followed by different superscripts are significantly different at $P \leq 0.05$.

Zeta potential of nanoliposomes

Zeta potential is one of the important factors in the stability of nanoparticles. The Zeta potential of nanoliposomes is shown in Table 1. The higher the zeta potential, the higher the surface charge of the nanoparticles and consequently the electrostatic repulsion. Low values of zeta potential reduce the stability and adhesion of nanoparticles. Zeta potential in the samples ranged from -10.8±1.85 to 30.33±1.45 mV.

Encapsulation efficiency (EE%)

The results showed that nanoliposomes prepared from 1.5% chitosan had the highest nanoencapsulation efficiency (83.98±0.72%) and nanoliposomes with 1% encapsulation (80.98±1.11%), 0.5% encapsulation (75.68±1.20%) and non-encapsulation (70.14±1.24%) showed lower encapsulation efficiency (Table 1).

Morphology of nanoliposomes

The surface structure of liposomes prepared using scanning electron microscopy was investigated. According to the images shown in Figure 2A-D, nanoliposomes containing *Gracilaria* PBPs with higher encapsulation efficiency, the plate-shaped images with smooth and non-cracked surfaces were observed and the removal of the nuclear substance from liposomes appeared less. However, some of the pores and cracks shown in Figure 2 are due to the residual of the encapsulated pigment active compounds caused by the removal of the active ingredient from the nucleus from the liposomes.

Differential scanning calorimetry (DSC)

As shown in Figure 3A, the DSC of PBPs pigment showed two endothermic and exothermic peaks related to the two processes of melting and decomposition of pigment compounds. The endothermic peak was found to be sharper and clearer. The exothermic peaks were also observed at 154 and 177°C and peaks above 200°C, which could be related to crystallization and oxidation processes.

The results of thermal analysis of uncoated and coated nanoliposomes (0.5, 1, and 1.5%) containing specific peak pigments showed no melting temperature of PBPs, possibly due to complete dissolution and amorphous crystalline structure of the pigment in a lipid structure.

DSC showed the displacement of the peaks after the process of

nanoencapsulation of PBPs in uncoated and coated nanoliposomes with different percentages of chitosan. The change in the peaks reflects the interaction between the liposome compounds, the encapsulation, and the bioactive pigment compounds, which led to the formation of new structures. According to the results of the present study, the nanoliposome pigment in chitosan encapsulation is a successful method to further protect sensitive PBP compounds against melting, evaporation, and oxidation processes during storage as well as thermal processes.

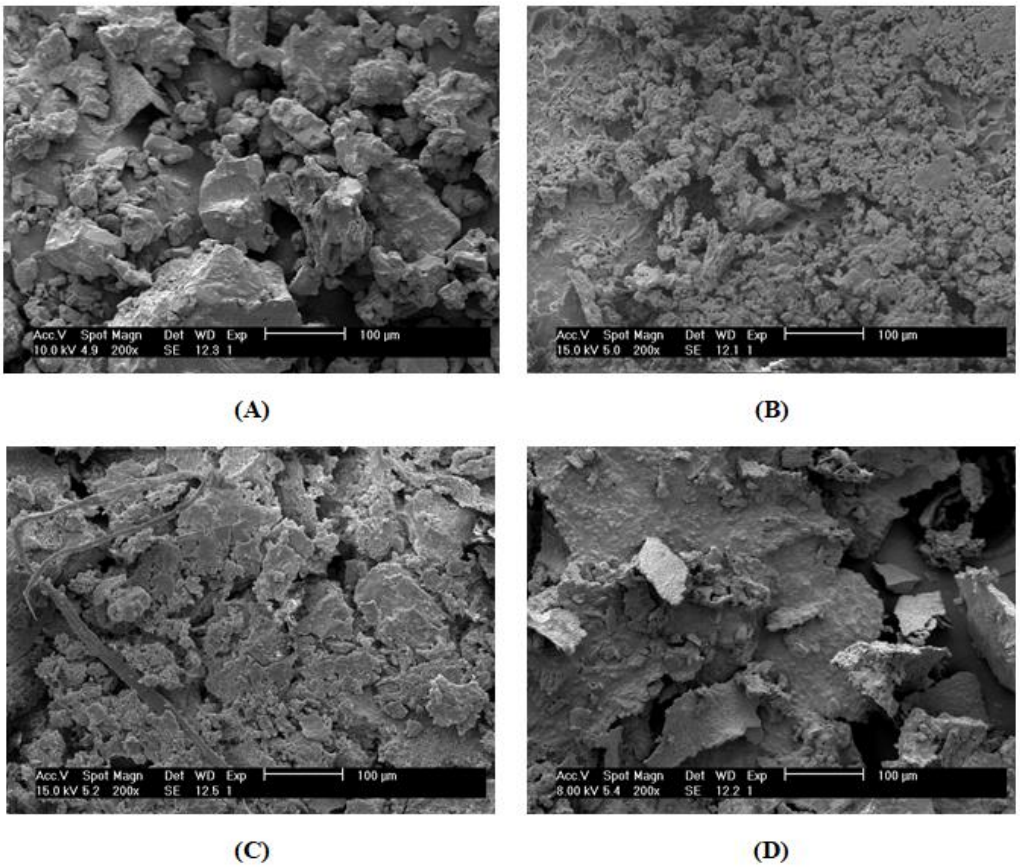
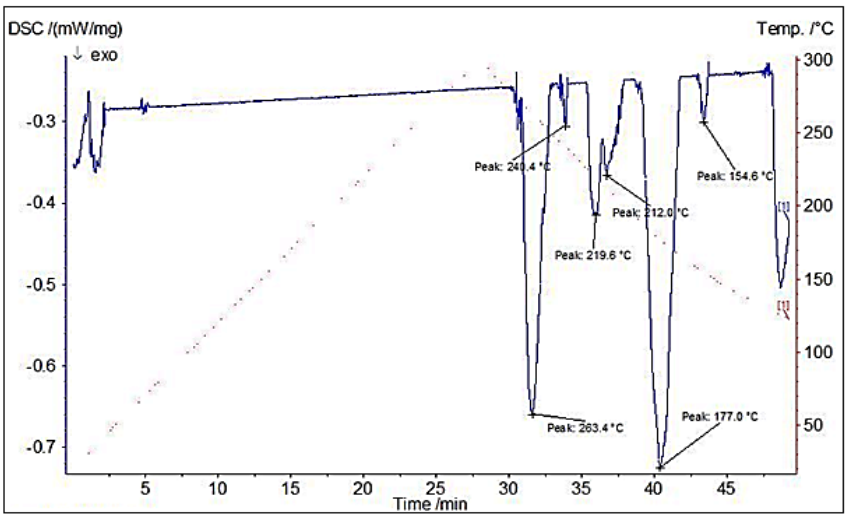
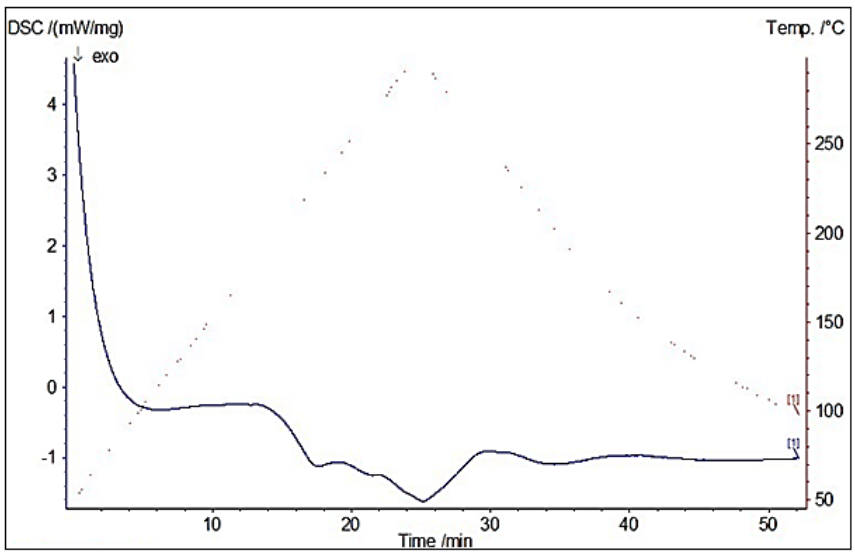


Figure 2: SEM images (×200) showing the morphology of dried phycobiliprotein nanoliposome (A) uncoated nanoliposome; (b) coated liposome (0.5% ch); (c) coated liposome (1% ch); (d) coated liposome (1.5% CH).



(A)



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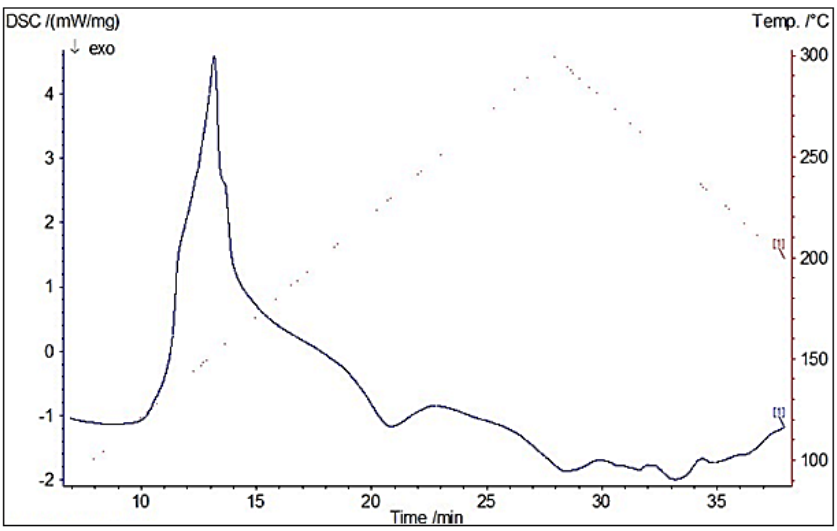

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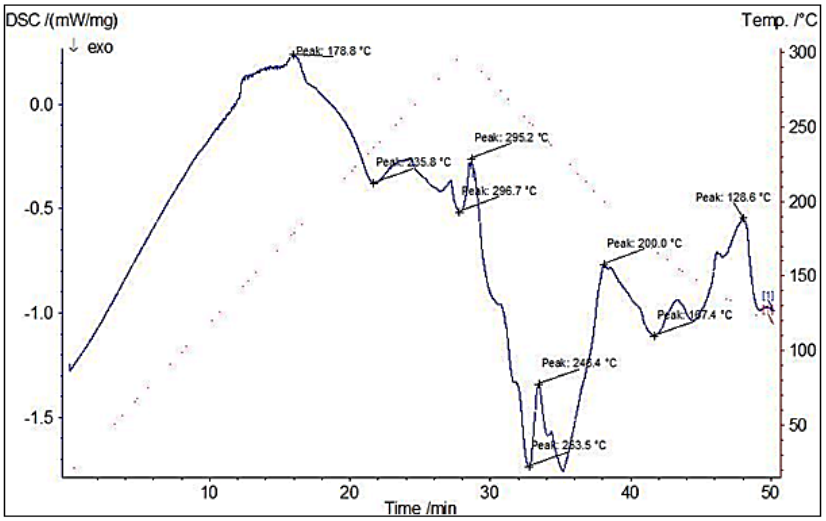
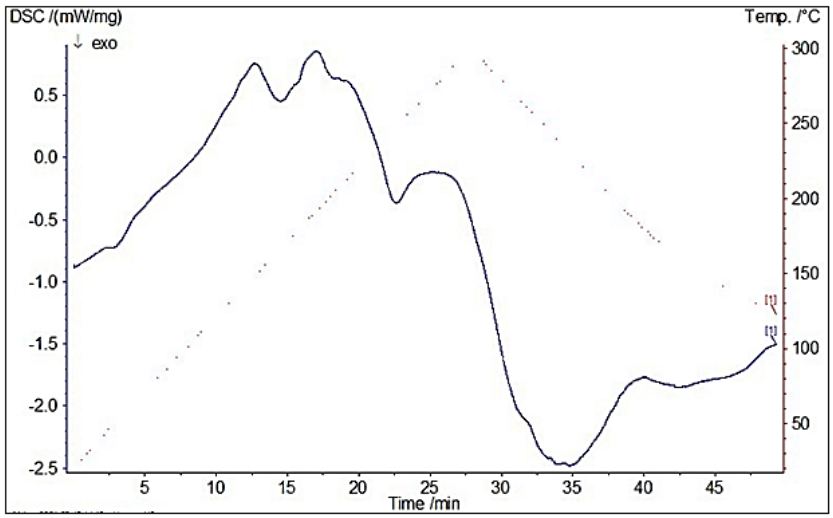


(D)



(E)

Figure 3: Differential Scanning Calorimetry (DSC) thermograms of (a) free phycobiliprotein; (b) uncoated liposome (0% ch); (c) coated liposome (0.5% ch); (d) coated liposome (1% ch) and (e) coated liposome (1.5% CH).

Fourier transform infrared spectroscopy (FTIR)

As shown in Figure 4, no significant difference was in the structural properties of the studied treatments by increasing the chitosan ratio in the complex. According to FTIR, no new peak was observed in the nanocapsules, indicating that PBPs, nanoliposomes, and chitosan physically bonded well with no chemical interaction between the compounds.

As shown in Figure 4, the band at the wavelength at 1400 cm^{-1} in the FTIR spectrum is assigned to symmetric tensile -COO-, symmetric deformation of the functional groups -CH_3 and CH_2, and deformation of the -OH group. In other encapsulated treatments, the peak is slower and transferred to slightly higher wavelengths. The other band observed at 1115 cm^{-1} is attributed to the vibration related to C-N tensile and N-H bending at amide III. According to the

result, it can be reported that the main bands at 1640 (amide I), ~1400 (C-N), and ~3120 (N-H) are associated with phycocyanin. According to Figure 4, specific peaks of chitosan such as (O-H stretching) (~3226- 3237 cm^{-1}), (C-H stretching) (2927 cm^{-1}), (C-O-C stretching) (1740 cm^{-1}), and pyranose ring vibrations It is observed in all formulations containing chitosan.

In-vitro release

In this study, the release of phycobiliprotein from nanoliposomes was assessed *in-vitro* over time (120 hours) at 25°C. According to the results (Fig. 5), the release did not happen at a stable level, and over time, the release rate was reduced.

The release of PBPs in the nanoliposome sample with no chitosan was faster than in other treatments and reached 95.57% after 120 hours, while 95.43%, 69.09%, and 53.10% in 0.5, 1, and 1.5% chitosan at the end of the period, respectively. In non-coated nanoliposomes, more than 70% of the PBPs were released from the nanoliposomes after 6 hours, which was followed by slow and steady release. The release pattern and rate of the pigment compounds were high for up to 12 hours and then reduced.

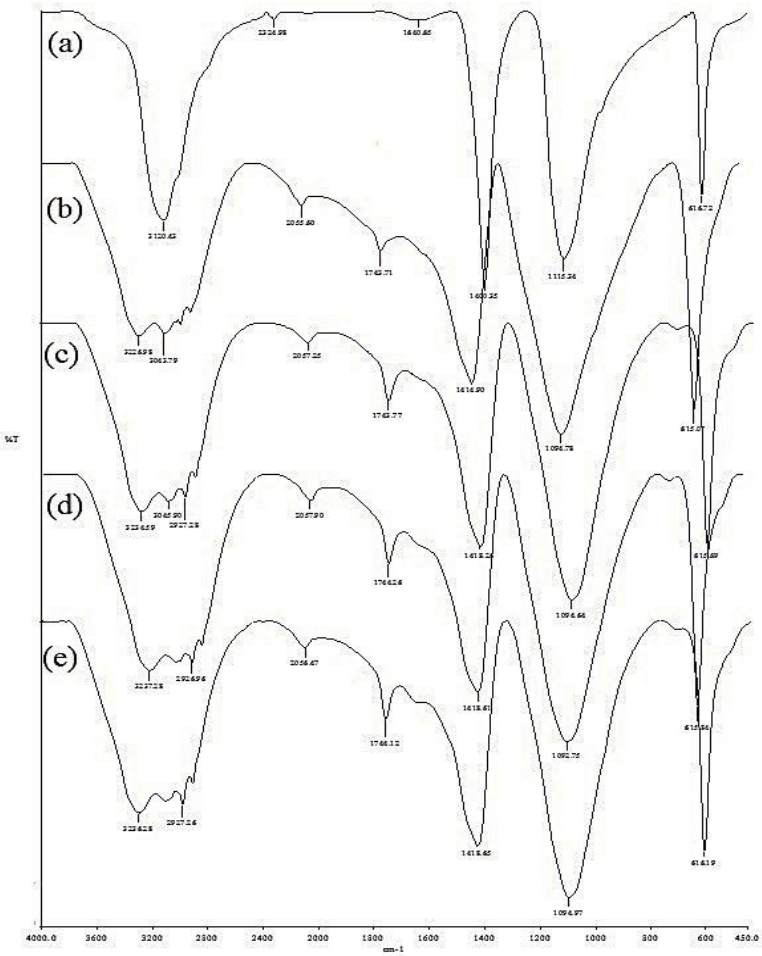


Figure 4: Fourier Transform Infrared Spectroscopy (FTIR) spectra of (A) Free phycobiliprotein; (B) Uncoated Liposome (0% CH); (C) Coated Liposome (0.5% CH); (D) Coated Liposome (1% CH) and (E) Coated Liposome (1.5% CH).

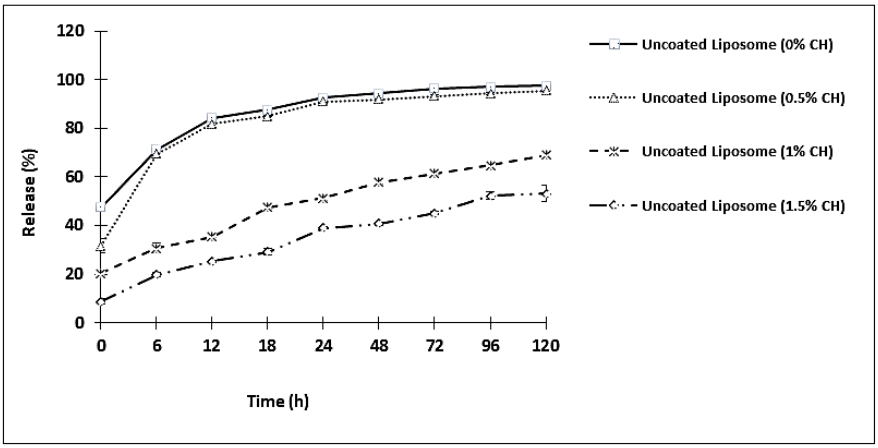


Figure 5: Profile of *in-vitro* release from the different formulations of phycobiliprotein nanoliposome.

Discussion

According to previous studies, the amounts of phycobilin pigments of ten species of red algae in the pigment phycoerythrin were in the range of 0.18-1.23 mg/g, 0.16-0.36 mg/g in allophycocyanins and 0.043-0.12 in allophycocyanins. The results of the present study were consistent with previous results. The results showed that the growing environment and different seasons of the year have significant effects on the concentration of pigments in algae (Kumar *et al*., 2014). Accordingly, the highest concentrations of phycobilin pigments were found in winter when the light beam and the concentration of nutrients in the area were suitable for the biosynthesis of these metabolites.

PBPs can be used as bioactive compounds and natural pigments in food. Since the use of processes such as heating, acid, or alkali for food processing is inevitable, it is necessary to study the effects of key indicators such as pH on the functional properties of PBPs. Proteins act as emulsifiers by forming a layer around oil droplets dispersed in an aqueous medium, thus preventing structural changes such as fusion, creaming, clotting, or settling (Chen *et al*., 2019). Thus, the emulsifying properties of proteins are affected by the hydrophobicity/hydrophilicity of the proteins and the structural constraints that determine the ease of opening of the proteins to form a layer around the dispersed oil droplets.

As shown in the particle size distribution diagram, the particle size distribution is uniform and thus the nanoliposome production process is successful with different percentages of chitosan encapsulation. Also, the PDI of nanoliposomes varied from 0.25 to 0.28, which indicates less dispersity and homogeneity of particle size. Chitosan-encapsulated liposomes, also known as chitosomes, have been reported by previous researchers, and their accuracy has been confirmed by a variety of microscopic methods (Katouzian and Jafari, 2016; Hasani *et al*., 2018) The mean particle size was reduced by

adding chitosan to the liposome and then increased by increasing chitosan concentration. The results of the present study showed that the use of chitosan up to 0.5% chitosan concentration during encapsulation led to the formation of smaller diameter liposomes by the contractile force caused by ionic tension and by increasing the amount of chitosan a thicker layer is caused that leads to the production of larger liposomes. Also, the stability of particles against adhesion and inhibition of sediment formation is due to the existence of electrostatic repulsion between particles (Salminen *et al*., 2016).

Particle size and PDI index in colloidal systems such as nanoliposomes are very important for determining their properties. The smaller the particle size implies the higher the surface-to-volume ratio. These two factors are inversely related. Therefore, it causes a decrease in size and an increase in solubility and bioavailability for substances that have less bioavailability. The stability of these two indicators in addition to the efficiency of encapsulation during a storage period indicates the colloidal stability of the system. The nature of the bioactive and carrier substance, formulation, and production method, the ratio of core substance to carrier substance, the solvent used in the process as well as environmental conditions such as temperature, humidity, pH, etc. are among the parameters affecting the particle size of vesicles. Liposomal membranes contain large amounts of phosphatidylcholine, and empty spaces are created in the structure due to the presence of large polar groups on the phospholipid surface (Savaghebi *et al*., 2020). The presence of cholesterol changes the structure of lipid placement and the orientation of acyl chains in the two lipid layers. With a hydrogen bond with the carbonyl ester groups, the hydroxyl groups of cholesterol keep the acyl chains tilted to one side in a straight line and fill the spaces between them. The presence of cholesterol in the bilayer structure leads to an increase in phospholipid molecule density (Hasani *et al*., 2018).

The electrical potential in the interfacial layer between the stationary immobile charged layer and the mobile layer around colloidal particles and droplets is called zeta potential. In other words, zeta potential is the potential difference between the stationary charged layer and the rest of the continuous phase (Mozafari *et al*., 2008). The reason for the negative surface charge of non-encapsulated nanoliposomes is the presence of lecithin as an ion emulsifier. By adding chitosan, negative and positive electrostatic forces are applied between the liposome and chitosan phospholipids encapsulated. Chitosan has a positive charge under acidic conditions due to the presence of free amine groups. After the process of encapsulation, ionic bonds between groups and the surface of liposomes with a negative charge change the zeta potential from negative to positive charge. In general, as the chitosan concentration increases, the intrinsic surface charge and the ionic reaction between the liposome and the

chitosan increase. Liu and Park (2009) observed that encapsulation of the surface of liposomes with chitosan leads to the surface hydrophilic property. The most important property of these lipid nanocarriers with chitosan encapsulation is the special effect on target cells. Previous studies found that values of zeta potential, mean particle size, and PDI of lipid nanocarriers could be significantly affected by the type of lipid, surfactant, and wall encapsulation composition. The shorter the fatty acid chain, the smaller the particle size. Previous studies have shown that an increase of more than 5% in the fat of nanoliposome formulation leads to an increase in particle size and micro-scale particle production.

Chitosan has a great ability to encapsulate bioactive compounds in phenolic compounds. In the structure of the liposome, there are two hydrophilic and hydrophobic parts, so hydrophilic compounds are trapped in the aqueous medium inside the liposome, and hydrophobic compounds between two phospholipid layers. Thus, the two phospholipid layers act as a reservoir for bioactive compounds.

The results of nonaencapsulation efficiency reported in the present study are similar to the results of previous studies (Katouzian and Jafari, 2016; Hasani *et al*., 2018) on nanoliposomes. In general, it was proved that the encapsulation efficiency of substances in liposome structure can be affected by specific size and surface. However, the present study showed a direct relationship between particle size and encapsulation efficiency for PBPs-containing liposomes. According to the results, chitosan molecules can be attached to the surface of the liposome, form a hard layer, and limit the fluidity of the lipid membrane, thus making the membrane harder and more resistant (Seyedabadi *et al*., 2020).

Morphological studies on powdered and dried nanocapsules provide valuable data on the formation and quality of nanoliposomes, the process and method of particle drying, as well as the physical and chemical factors affecting the shape and structure of nanoparticles. Based on the images obtained from scanning electron microscopy, the produced nanoliposomes, especially the chitosan-encapsulated liposomes, had a plate-like structure, which can be imagined in the powders obtained from freeze-drying. The presence of pores in the structure of nanocapsules is attributed to the formation of small ice crystals during the freezing process (Ezhilarasi *et al*., 2013).

The appearance and quality of liposomes also affect other factors such as particle size and encapsulation efficiency and depend on the constituents and the type and composition of the wall substance. Lim *et al*. (2011) observed a higher percentage of nanoencapsulation efficiency in particles with smooth surfaces, which led to the same results according to the images of nanoliposome powders and assessing and comparing the encapsulation efficiency of nanoliposomes containing PBPs in the present study.

Previous studies have reported that nanoparticles produced by spray-drying had spherical shapes with smooth surfaces compared to powder obtained from freeze-drying (Moayyedi *et al*., 2018). According to the images, the surface formation and deformation with high amounts of chitosan as liposome encapsulation can be justified.

Hundre *et al*. (2015) observed some agglomeration in the encapsulated treatments with the combined encapsulation, especially when carbohydrates were used in the wall composition formulation. Agglomeration and cohesive structure are associated with the glass transition of amorphous carbohydrate matrices (Bae and Lee, 2008) and low encapsulation efficiency.

The study results of Klinkesorn *et al*. (2006) showed cracks on the surface of the produced capsules due to mechanical stresses as a result of not drying the different parts of liquid particles uniformly at the early stages. Drying is due to the movement and displacement of moisture along the surface at this stage and the effect of viscous flow. The researchers observed no crack on the surface of the powders, but some pores may have formed at the final stage of drying due to uneven shrinkage of the substance.

Studies by Shah *et al*. (2017) attributed the lack of drug peaks in lipid nanocarrier formulations to drug dissolution, molecular dispersions, and drug structure changes.

The change in specific heat capacity depend on the moisture content, temperature, and constituents of products. An increase in melting temperature after encapsulation may be due to the interaction between hydrophobic bioactive compounds and unsaturated phospholipids in lecithin, which increased the lipid bilayer stiffness of nanoliposomes. Hydrogen bonds between the pigment compounds and the polar heads of phospholipids are effective in improving the melting temperature of liposomes. Other parameters affecting the thermal stability of liposomes are acyl chain length, degree of hydrocarbon chain saturation, particle size, and the nature of the encapsulated compound (Rafiee *et al*., 2017) Previous studies have shown that changes in melting point may be due to eutectic reactions between trapped bioactive compounds and liquid lipids. The above reactions will lead to a change in the melting enthalpy.

The presence of pigments in the nanoliposome did not increase or change the peaks, which could be due to the interaction between molecules without the formation of covalent and hydrogen bonds, hydrophobic forces, and electrostatic reactions. The results of the present study were consistent with the study results of (Hosseini *et al*., 2013) who reported no chemical interaction in FTIR between Origanum vulgare essential oil and chitosan nanoparticles. Previous studies showed that FTIR of phycocyanin extracted from Spirulina at the frequency of 1658 cm cm^{-1} indicates the presence of tensile vibrations from type 1 amide bands (C=O) and tensile vibrations from type II amide bands at

the frequency of 1546 cm^{-1}. They also reported a peak of 702 cm cm^{-1}, indicating tensile vibrations of S-O bands from sulfonic compounds, which was consistent with studies by Patel *et al*. (2005).

The behavior and function of bioactive compounds trapped in food model systems depend on their release pattern (Rodrigues *et al*., 2015). Among carrier systems, nanoliposomes have a unique ability to control and improve the targeted release of bioactive compounds. The initial higher release in the first 6 hours may be due to bioactive compounds trapped in the outer monolayer of the membrane, which can be released from the nanoliposomes at a higher rate (Hasani *et al*., 2018).

However, the slow release may be due to the release of trapped substances from the inner layers to the surface and then from the surface to the bulk of the release solution (Lopes *et al*., 2017). Previous studies have reported that the lipid bilayer structure and fluidity are controlled by ambient conditions so that the acidic pH reduces the surface charge of nanoliposomes and the repulsive forces between them. As a result, the size of the vesicles increases. Thus, the integrity of the two phospholipid layers reduces, and the release of trapped substances increases.

Stable and controlled release of bioactive compounds is very important for their use as food preservatives, especially during processing and storage. The study results of Wang *et al*. (2006) and Savaghebi *et al*. (2021) were consistent with the results of the present study on the mechanism of release of encapsulated effective compounds during storage.

In conclusion, the results of the present study showed that red alga (seaweed) *Gracilari gracilis* is rich in PBPs with unique properties. According to the results, liposomal nanocarrier can be considered one of the effective systems for the nanoencapsulation of bioactive compounds such as PBPs. High encapsulation efficiency, particle size distribution, and zeta potential of nanoliposomes showed high stability of chitosan-coated nanoliposome. The mechanism of PBPs release showed that nanoliposomes, especially chitosan-coated nanocarriers, well-controlled the release of PBPs over time. In addition, the results of DSC and FTIR of the samples showed that nanoliposomes can protect the trapped active substances against thermal processes. Therefore, it is possible to use coated nanoliposome system with chitosan due to the lack of any solvents and toxic substances as one of the new systems for stabilizing and improving the functional properties of protein pigments.

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