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Enhancing stability and bioactivity of *Chondrus ocellatus* polyphenols through nanoparticle fabrication

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ORIGINAL ARTICLE

Abstract

This article aimed to fabricate *Chondrus ocellatus* polyphenols (COPs)-gelatin-chitosan nanoparticles to enhance their stability and bioactivity. Different preparation conditions were tested to investigate the effects of formulation on nanoparticle fabrication. Free radical scavenging activity of COPs and their nanoparticles were compared. The consequences revealed that optimal preparation was obtained with a chitosan (CS) concentration of 0.5 mg/mL, gelatin (Gel) concentration of 1.0 mg/mL, COPs concentration of 5.0 mg/mL, and Gel-CS-COPs mass ratio of 2:1:1. The resultant nanoparticles had the particle size of 39.79 ± 5.15 nm and encapsulation efficiency of 60.95 % ± 1.86 %. The COPs-Gel-CS nanoparticles were distributed uniformly, and no obvious aggregation was observed by transmission electron microscopy. Nanoencapsulation of the COPs significantly improved their antioxidative stability. This study provided a potential formulation for the application of *Chondrus ocellatus* polyphenols in antioxidant activities.

Keywords: antioxidative stability; characterization; *Chondrus ocellatus*; nanoparticle; polyphenols; preparation

Introduction

A distinctive place is reserved for phenolic compounds in marine algae, as these algae have been recognized as a rich source of biologically active phenolic compounds (Thomas and Kim, 2011). Owing to their broad spectrum of antioxidant activities, these compounds have been recognized as having protective effects against many diseases, including cardiovascular diseases, diabetes, cancer, atherosclerosis, aging, and other degenerative diseases (Fernando *et al.*, 2016; Jayawardhana *et al.*, 2021). However, phenolic compounds are sensitive to oxidation and photolysis during utilization and storage and have low boiling points, volatility, and poor stability. Therefore, increasing the stability of phenolic compounds is expected to enhance their biological activities significantly (Carbonaro *et al.*, 2001; Chen *et al.*, 2023; Zou *et al.*, 2012).

Nanoparticles were first reported in the 1980s and defined as particulate dispersions or solid particles with a size range of 1–1000 nm (Mohanraj and Chen, 2006). Nanoparticles are not only small in size, and easy to be taken up by cells, but also have good biocompatibility and targeting properties (Stark *et al.*, 2015). Therefore, drugs or other bioactive compounds encapsulated in nanoparticles exhibit higher stability, bioavailability, and bioactivity (Guo *et al.*, 2021; Roger *et al.*, 2010). Gelatin (Gel)

is a natural protein-based biopolymer with relatively low antigenicity. Its biodegradability, biocompatibility, chemical modification potential, and cross-linking possibilities make Gel nanoparticles a promising drug delivery carrier system (Young *et al*., 2005). Chitosan (CS) is a nontoxic, safe, and nonantigenic natural cationic polysaccharide that has been widely used as a carrier for nanoparticles (Liang *et al.*, 2017; Pillai *et al.*, 2009). Nanoparticles have been attempted as drug delivery systems for some phenolic compounds. In one study, the encapsulation of cocoa procyanidins in Gel-CS nanoparticles significantly improved their stability (Zou *et al*., 2012). Encapsulation of catechins in CS nanoparticles significantly enhanced their intestinal absorption by 1.5 fold (Dube *et al*., 2010).

Chondrus ocellatus is a kind of red algae with clusters, and it has been reported as a potential source of antioxidant, antitumor, antiviral, anticoagulant, and immunomodulatory active substances (Zhou *et al*., 2005). In our previous studies, polyphenols were extracted from *C. ocellatus* and found to have antioxidant and inhibitory activities of α-amylase and α-glycosidase (Zhu *et al*., 2022). However, a suitable drug delivery system still needs to be developed for further development and utilization of *C. ocellatus* polyphenols (COPs). The COPs-Gel-CS nanoparticle system was planned to improve the stability of COPs, expand the application scope, and provide a reference for the development and utilization of other natural polyphenols. Thus, the objective of this study was to fabricate COPs-Gel-CS nanoparticles and to evaluate their advantages.

Material and Methods

Materials

Chondrus ocellatus was collected from the seaside of Xiapu, Fujian, China. Gel (250 bloom) and CS (deacetylation degree of 95 %, viscosity of 100–200 mpa.s) were purchased from Macklin Biochemical Technology Co., Ltd. All other chemicals and solvents were of analytical grade and were obtained from Macklin Biochemical Technology Co., Ltd unless otherwise indicated.

Extraction of COPs

COPs were extracted according to the procedure from Zhu *et al*. (2022). *C. ocellatus* was washed with running tap water for several minutes and fully dried. The dried *C. ocellatus* was pulverized by a laboratory blender for several minutes and sieved to produce fine powder for further use. The thin powder was dissolved in 61%(v/v) hydroethanolic solution at the ratio of material to liquid of 1:25 (w/v), extracted by ultrasonic at 60°C for 41 min, and the extract was dried in a vacuum to obtain polyphenol solid.

Preparation of the COPs-Gel-CS nanoparticles

The COPs-Gel-CS nanoparticles were prepared according to the methods reported by Sun *et al.* (2020) with some modifications. Previous studies showed that the solubility and fluidity of CS are good at pH 5.4 (Zhang *et al*., 2014). The specific preparation method was determined by single factor experiment and orthogonal experiment. CS was dissolved in 0.5 % acetate solution (v/v) to produce a concentration of 0.5 mg/mL. Gel solution (1.0 mg/mL) was obtained in distilled water by stirring at 40°C. COPs were dissolved in 40% ethanol solution (v/v) at a concentration of 5.0 mg/mL. Subsequently, the COPs solution was completely dissolved into the CS solution. Then, the mixture was added to the gel solution at a slow rate. The gel-CS-COPs mass ratio was 2:1:1. The mixture was magnetically stirred at room temperature for 10 min at 550 rpm/min, adjusted pH to 5.4 with 0.2 M NaOH, and finally mixed for another 30 min to obtain a suspension of nanoparticles.

Characterization and antioxidation of the COPs-Gel-CS nanoparticles

Particle size analysis

Mean particle size and polydispersion index (PDI) of the COPs-Gel-CS nanoparticles was performed by Zetasizer Nano-ZS (Malvern Instruments, UK). An appropriate amount of COPs-Gel-CS nanoparticles was pipetted out into the measuring dish, with the height of the sample controlled within 1–1.5 cm. Measurements were made according to the computer program. Samples were measured in triplicate to calculate the average particle size.

Encapsulation efficiency

For the prepared nanoparticles, the encapsulation efficiency was measured using the supernatant of the suspension obtained by centrifugation according to references (Ma *et al*., 2020). The suspension of the COPs-Gel-CS nanoparticles was separated by centrifugation at 12,000 rpm/min for 30 min at 4°C. The mass of COPs used for nanoparticle synthesis and the mass of COPs in the supernatant were measured using UV-Vis spectrophotometry at a detection wavelength of 760 nm. The following formula was used to calculate the encapsulation efficiency:

$$\text{Encapsulation efficiency (\%)} = \frac{M_t - M_n}{M_t} \times 100$$

where M_t was the mass of COPs used for nanoparticle synthesis. M_n was the mass of COPs in the supernatant.

Transmission electron microscopy (TEM) analysis

The morphology of nanoparticles was analyzed using TEM according to the methods reported by Chanphai and Tajmir-Riahi (2018) with some modifications. The appropriately prepared samples were placed on carbon-coated copper grids after dilution. After standing for a while, the samples were negatively dyed with phosphotungstic acid for several minutes and dried naturally. Then, the morphology and distribution of nanoparticles were imaged using TEM (Model JEM-1400 plus, JEOL Ltd, Tokyo, Japan).

Antioxidative stability

The absorbance of the nanoparticles was measured using the 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) method to determine their antioxidant properties according to the method reported by Sun *et al.* with some modifications (Sun *et al.*, 2021). ABTS kit (BC4770, Solarbio) was used according to the protocol of the kit. In this kit, the degree of decrease in absorbance was measured to reflect the ability of the sample to scavenge ABTS-free radicals.

For the stability study, 5 mg/mL of COPs and COPs-Gel-CS nanoparticles with an equivalent quantity of COPs were stored at 4°C and 37°C, respectively, to determine the change of free radical scavenging for a set period (1, 2, 3, 4, and 5 h).

Statistical analysis

Samples were analyzed in triplicate, and the average values were used. All the experimental data were expressed as mean ± standard deviation. Statistical analyses were performed using Student's t-test, and $P < 0.05$ was considered to be significantly different (Lee *et al.*, 2019).

Results and Discussion

Preparation of the COPs-Gel-CS nanoparticles

Effect of gel and CS concentration on encapsulation efficiency

COPs nanoparticles were prepared by following the coacervation method. The rupture of hydrogen bonds between water and Gel molecules brought the complementary charged segments of gelatin closer (coacervates) (Lee *et al.*, 2011). Consequently, negatively charged gelatin and positively charged chitosan self-assembled and encapsulated the polyphenols to form COPs nanoparticles. Turbidity and particle size changes of the solution system prepared by different mass ratios of CS and Gel are shown in Figure 1.

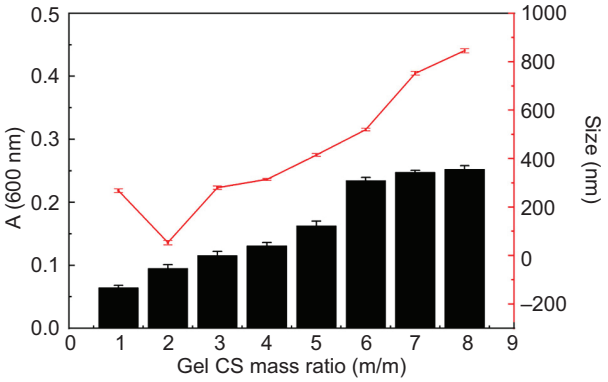

Figure 1. Effect of Gel-CS mass ratio on turbidity and particle size. The numbers in the abscissa denote the gelatin to chitosan ratio, and 1–9 represent gelatin: chitosan ratios of 1: 1, 2: 1, 3: 1, 4: 1, 5: 1, 6: 1, 7: 1, 8: 1, and 9: 1, respectively.

The results showed that when the mass ratio was 1:1, the content of protonated CS in the system was low. Its hydrophilicity was not affected after mixing with Gel, and it could be evenly dispersed in water. With the increase of the molecular weight ratio of Gel, the complex structure was compact, and the particle size reached the minimum at 2:1. A pale blue opalescence with the gender effect can be seen from the surface. With the increase in gel mass ratio, more free molecules were generated, which affected the stable crosslinking of molecules. Blue opalescence disappeared and white flocculent condensates precipitated.

Investigation of factors affecting particle size

We investigated the effects of prescription factors (CS concentration, Gel concentration, COPs concentration, and CS-COPs mass ratio) on the particle size of COPs-Gel-CS nanoparticles. The results are shown in Figure 2. The results showed that nanoparticles with larger particle sizes could be formed with a higher concentration of nanocarrier and nanodrug, and the mass ratio of carrier to the drug was close to equal. The study also examined the impact of manufacturing processes (preparation time and rotation speed) on nanoparticle size, as shown in Figures 2E and 2F. Both time and rotation speed were found to significantly affect particle size. A preparation time of 20 min and a stirring speed of 550 r/min were chosen accordingly.

To further reflect the effects of prescription factors on the particle size, the results of the orthogonal test are shown in Tables 1–3. The order of influence was A (CS concentration) > B (Gel concentration) > C (COPs concentration) > D (CS-COPs mass ratio). The optimal conditions were $A_2B_1C_2D_2$. CS, Gel, and COPs concentrations and CS-COPs mass ratio were 0.5 mg/mL, 1.0 mg mL, 5.0 mg/mL, and 1:1, respectively. The verification results

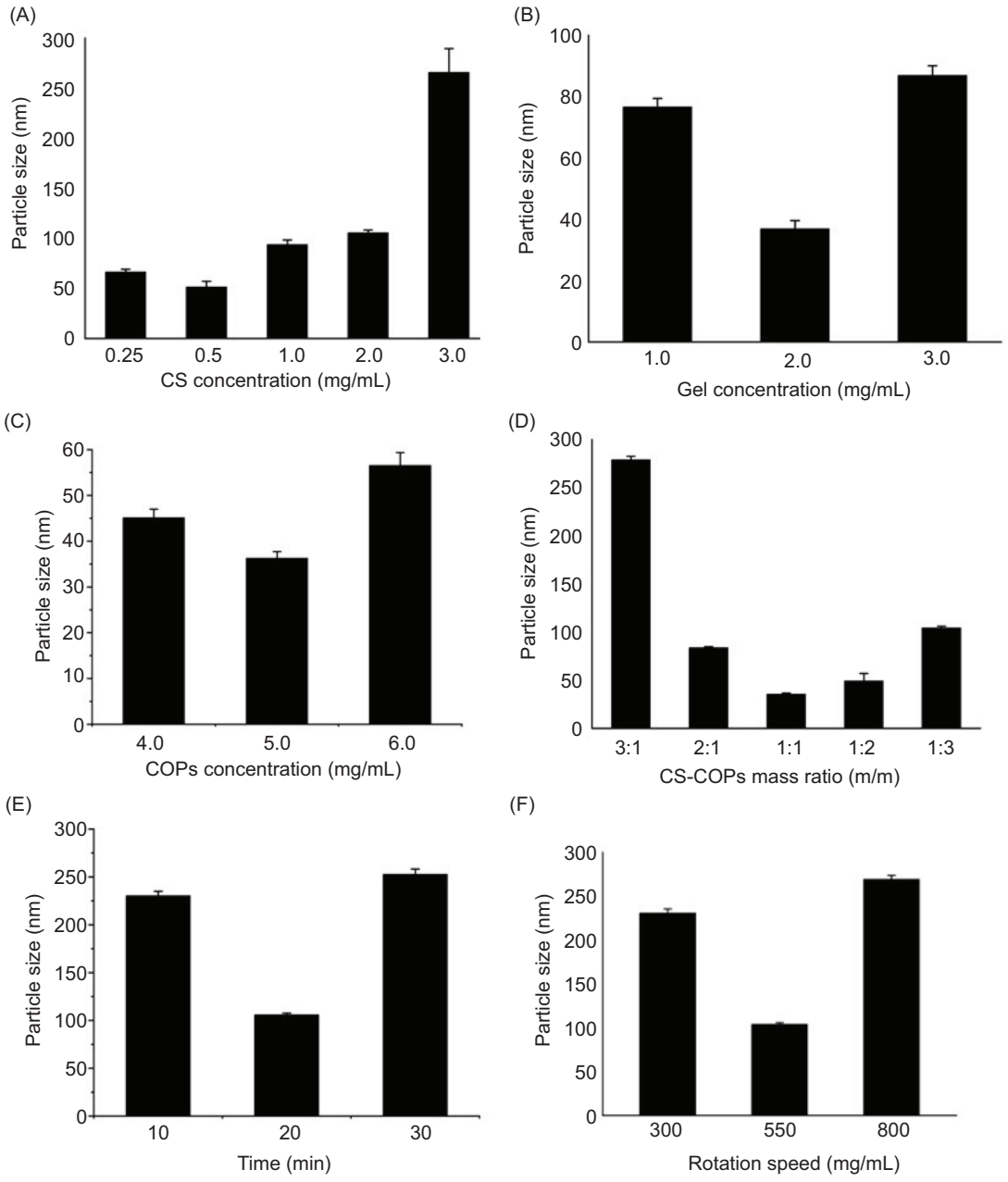

Figure 2. Effect of each factor on particle size.

showed that the particle size of COPs-Gel-CS nanoparticles was 39.79 ± 5.15 nm.

Particle size, Polymer dispersity index (PDI), and encapsulation efficiency

As shown in Figures 3 and 4, under the optimal preparation process, the particle size and zeta potential of Gel-Cs-COPs nanoparticles were 39.79 nm and 32.9 ± 7.08 mV, respectively.

Table 1. Orthogonal test program.

Symbol	Independent variables	levels		
		1	2	3
A	CS concentration (mg/mL)	0.25	0.5	1.0
B	Gel concentration (mg/mL)	1.0	2.0	3.0
C	COPs concentration (mg/mL)	4.0	5.0	6.0
D	CS-COPs mass ratio (m/m)	2:1	1:1	1:2

Table 2. Orthogonal test results.

No.	CS concentration (mg/mL)	Gel concentration (mg/mL)	COPs concentration (mg/mL)	CS-COPs mass ratio (m/m)	Size (nm)
1	0.25	1.0	4.0	2:1	74.59
2	0.25	2.0	5.0	1:1	67.84
3	0.25	3.0	6.0	1:2	68.70
4	0.5	1.0	5.0	1:2	39.72
5	0.5	2.0	6.0	2:1	56.39
6	0.5	3.0	4.0	1:1	57.20
7	1.0	1.0	6.0	1:1	71.70
8	1.0	2.0	4.0	1:2	92.20
9	1.0	3.0	5.0	2:1	87.82
Mean 1	70.377	62.003	74.663	72.933	
Mean 2	51.103	72.143	65.127	65.580	
Mean 3	83.907	71.240	65.597	66.873	
Range	32.804	10.140	9.536	7.353	
Sequence			A>B>C>D		
Optimal Combination			$A_2B_1C_2D_2$		

Table 3. Orthogonal test variance analysis table.

Source of variance	Sums of squared deviations	Degree of freedom	Mean square	F	P	Significance
A	1630.5810	2	543.52700	17.63404	0.020762	*
B	188.9516	2	62.98387	2.043431	0.286092	No significant difference
C	173.3734	2	57.79112	1.874959	0.309286	No significant difference
D	92.46782	2	30.82261	1.000000	0.500000	No significant difference

*P < 0.05.

PDI was 0.236, which indicated that the nanoparticle has good dispersion and the system was stable (Gaumet *et al*., 2008). The encapsulation efficiency of COPs in the Gel-Cs-COPs nanoparticles was 60.95 ± 1.86%. Encapsulation efficiency is an important index of plant polyphenol nanoparticles. Similar to Gel-Cs-COPs nanoparticles in this study, the encapsulation efficiency of litchi polyphenol-loaded chitosan nanoparticles reported by Cheng *et al*. (2023) is 45.9%, and the kaempferol-loaded silk fibroin nanoparticles reported by Yang *et al*. (2022) is 53.8%, which is lower than the encapsulation efficiency prepared in our investigation, demonstrating the benefits of chitosan–gelatin composite capsules in this study. However, the encapsulation efficiency of sodium alginate composite cross-linked corn starch tea polyphenols nanoparticles prepared by Bu *et al*. (2023) can reach 78.4%, which is higher than the encapsulation efficiency of the nanoparticles in this study, indicating that sodium alginate-cross-linked corn starch composite capsule material may have a better encapsulation effect.

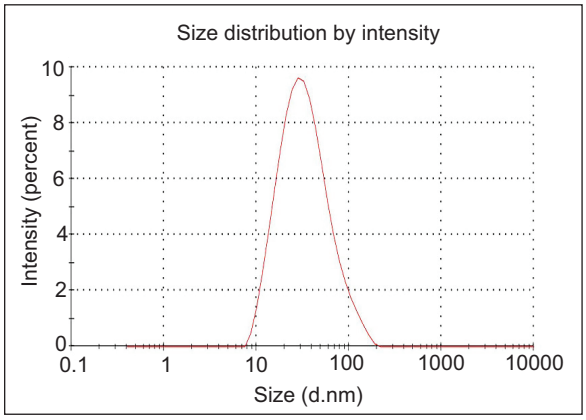

Figure 3. Size distribution of Gel-Cs-COPs nanoparticles.

TEM analysis

As shown in Figure 5, the formation of nanoparticles was confirmed by the TEM images. It should be noted that when preparing TEM samples, the shell structure of

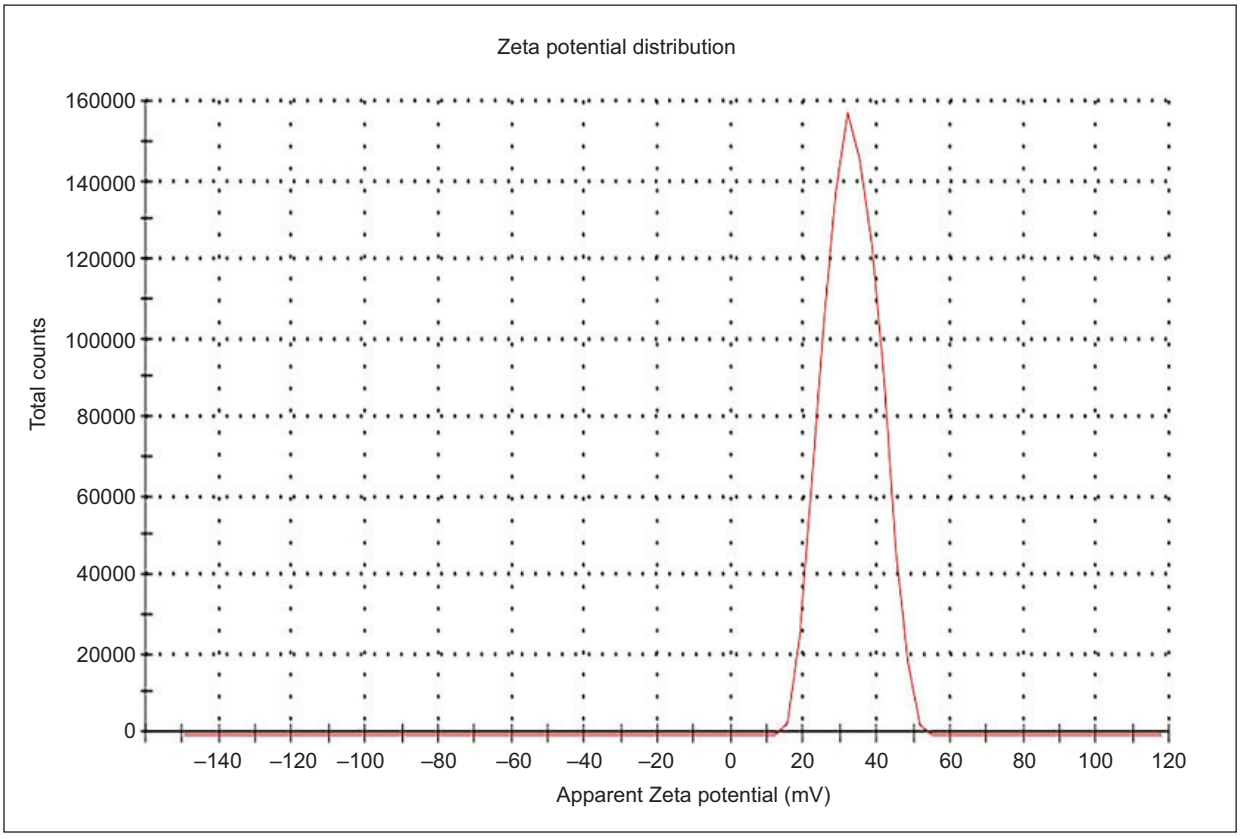

Figure 4. Zeta potential distribution of Gel-Cs-COPs nanoparticles.

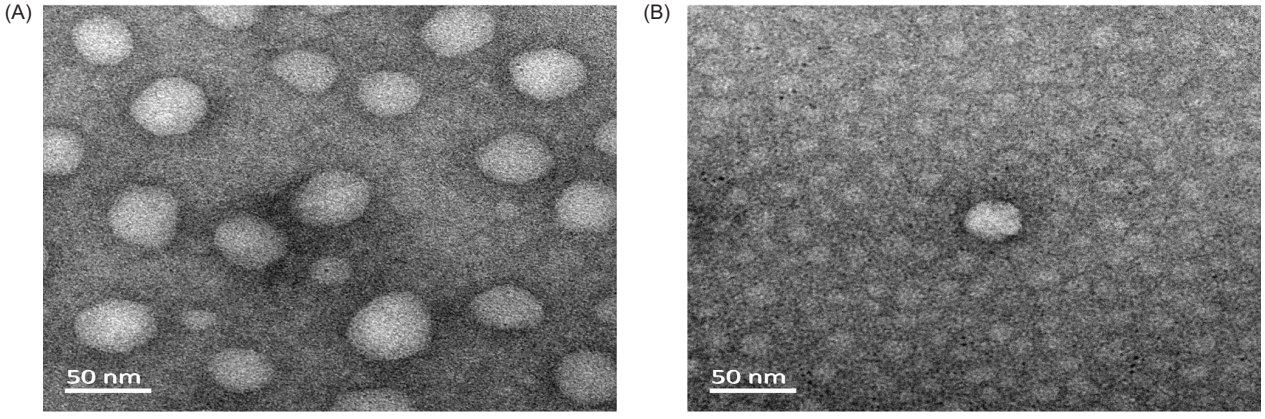

Figure 5. TEM micrograph of COPs-Gel-CS nanoparticles. The two images were observed at different sites of the nanoparticle solution on a slide at the same magnification.

phosphotungstic acid was loose and the core layer was dense, which made it difficult for phosphotungstic acid to penetrate the core layer during the red staining process, so the color of the core layer was relatively light. It could be seen that the Gel-Cs-COPs nanoparticles were spherical with uniform distribution, and no obvious aggregation was observed. The diameter of most particles was less than 50 nm. Compared to other nanoparticles, such as the biosynthesized silver nanoparticles (AgNPs) with the aid of a combination of chitosan and seaweed-derived polyphenols reported by Rezazadeh *et al*. (2020), our Gel-Cs-COPs nanoparticles had similar particle size, uniform distribution, and composite nanoparticle standard.

Antioxidative stability

The free radical scavenging activity of free COPs and Gel-Cs-COPs nanoparticles was studied by 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt, ABTS assay (Figure 6). Under the same condition, compared with free COPs, COPs coated with gelatin–chitosan were more stable. Even at 5 h, COPs-Gel-Cs nanoparticles still had higher stability and stronger free radical scavenging activity. Meanwhile, the free radical scavenging activity of those stored at 4°C was higher than that stored at 37°C. Due to the higher temperature, COPs were more susceptible to oxidative degradation by oxidation reactions. The Gel-Cs-COPs nanoparticles maintained high scavenging activity at different temperatures and times, while the scavenging activity of COPs decreased significantly with the increase of time. The results confirmed that the preparation of COPs into nanoparticles increased their antioxidant stability by avoiding their volatile and poor stability properties.

Discussion

Dosage forms of plant polyphenols have been studied intensively. Due to the instability of polyphenol properties, raw materials will be consumed under the conventional dosage form preparation conditions (Li *et al*., 2020; Yang *et al*., 2020). Similarly, the properties of *C. ocellatus* polyphenols are unstable and prone to oxidation and photolysis. To improve the utilization rate of polyphenols, new dosage forms such as microcapsules and clathrates were gradually used by researchers to improve the physical properties of plant polyphenols. For example, Alizadeh and Nazari (2022) prepared thymol (TH)/β-cyclodextrin (β-CD)-inclusion complex (ICs) clathrate using β-CD and CS as nanocarriers to reduce its oxidation and photolysis and effectively improved the storage and utilization of thymol. In addition to β-CD and CS, sodium alginate, pectin (Sun *et al*., 2022), gelatin, and so on were commonly used as carriers for the preparation of microcapsules. The *Camellia chrysantha* polyphenols nanoparticles prepared by Luong *et al*. (2021) used sodium alginate and CS as carriers to improve the solubility of tea polyphenols. In our study, to reduce the loss of *C. ocellatus* polyphenols due to their oxidation and photolysis, the nanoparticles with antioxidant and photolytic activity were prepared using chitosan and gelatin as carriers. Mathew and Arumainathan (2022) demonstrated that the microcapsules prepared by chitosan had pretty biological properties, but chitosan had pH-responsive drug release, which needed to be effectively released under acidic conditions. However, the compounding of chitosan and gelatin facilitated continuous release in a neutral medium, and the chitosan–gelatin microcapsules had plummy solubility and content release properties. Therefore, the use of polyphenols was enhanced to a certain extent by using chitosan and gelatin to prepare composite microcapsule materials, which could alleviate the disadvantages caused by the unstable properties of *C. ocellatus* polyphenols. (Lv *et al*., 2018). Menezes *et al*. (2016) prepared βCD carvacrol microcapsules using physical mixture (PM), paste completion

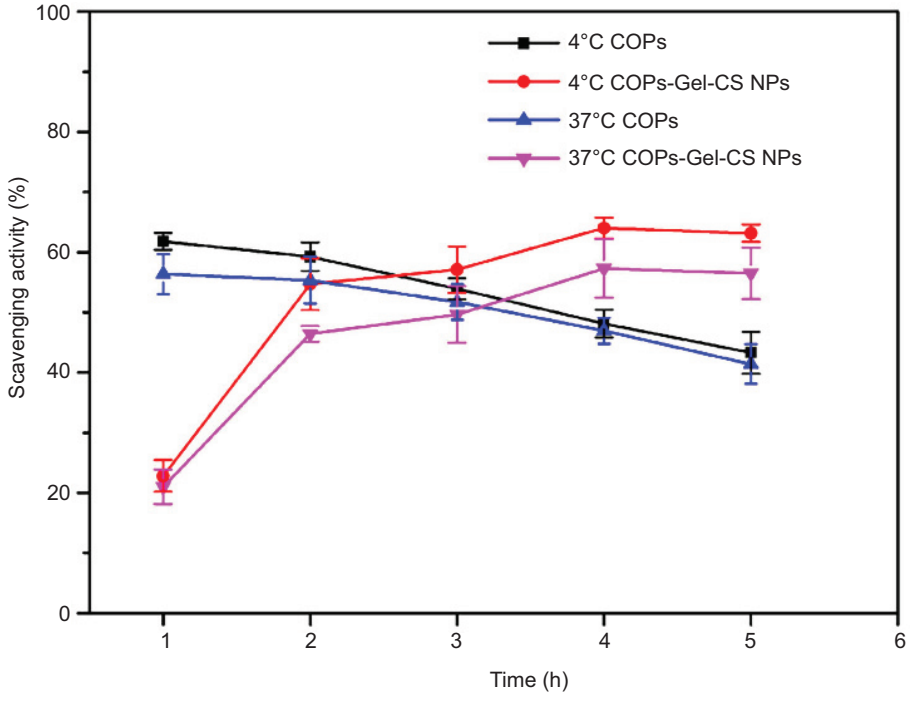

Figure 6. Free radical scavenging activity of free COPs and Gel-Cs-COPs nanoparticles as measured by ABTS assay.

(PC), and slurry completion (SC) methods. The encapsulate efficiency of the microcapsules prepared by PM and PC methods was 2.96 ± 0.015 and 34.30 ± 0.24%, respectively, indicating a low entrapment efficiency. The encapsulation efficiency of the microcapsules prepared by SC method was improved to 71.68 ± 0.062%. However, the process of preparing the composite dry powder requires additional experimental steps and is relatively time-consuming. Furthermore, compared with a single carrier, composite carriers have drawn more and more attention due to the stable interaction between them without the need to add crosslinkers as well as their good drug-loading properties (Sethi *et al*., 2022). In our study, the positive charge of chitosan after dissolution could form a stable interaction with the negative charge of gelatin. After adding *C. ocellatus* polyphenols, gelatin, and chitosan could encapsulate the polyphenols to form the *C. ocellatus* nanoparticles in the process of synthesis and formation of nanoparticles due to electrostatic interaction. The components of the polyphenols were relatively complex and other components might influence the formation of the nanoparticles to a certain extent. The encapsulation efficiency was 60.95 ± 1.86%, and the preparation process was simple physical mixing. The formed nanoparticles were clear in appearance. The particles were regular and round, and the antioxidant performance of the seaweed polyphenol was improved (Tao *et al*., 2022).

In this study, the gelatin–chitosan complex was prepared by complex coacervation. Gelatin was a protein and molecular chains contained -NH_2 and -COOH and their corresponding dissociation groups -NH^{3+} and -COO^-, but the amount of ions containing-NH^{3+} and -COO^- was affected by the pH of the medium. When pH was lower than the isoelectric point of gelatin, the number of -NH^{3+} was more than that of -COO^-, and the solution had a positive charge. When the pH of the solution was higher than that of the gelatin isoelectric point, -NH^{3+} was less than that of -COO^-, and the solution became negatively charged (Sethi *et al*., 2022). The negative charge of the gelatin solution was the highest at pH 5. Chitosan was a positively charged polyelectrolyte in a solution with strong adsorption. In this study, chitosan dissolved in 0.5% acetic acid formed a positively charged cationic group in the solution. The *C. ocellatus* polyphenols alcohol solution was injected into the chitosan solution in advance, and the composite solution was added into the gelatin solution according to proportion. After pH was adjusted, the chitosan and the gelatin were mutually aggregated due to electrostatic interaction between charges, and the *C. ocellatus* polyphenols were encapsulated to form nanoparticles. The microcapsule formed a closed protective layer which was not reacted with the closed protective layer and was stably combined with the polyphenol outer layer, so that the gel-CS microcapsule could contact with oxygen and light, thus reducing its oxidation and photolysis.

Conclusion

In conclusion, we developed an optimal preparation method for *C. ocellatus* polyphenols nanoparticles. Under these conditions, the encapsulation efficiency could reach 60.95 ± 1.86%. TEM analysis demonstrated the formation of uniform spherical nanoparticles. The antioxidant experiments showed that the nanoparticles could effectively alleviate the problem of polyphenol oxidation and enhance its stability. This study provided ideas for further application of polyphenols. However, this study mainly focused on the optimization of the preparation conditions of nanoparticles and the determination of antioxidant activity in vitro. It is also necessary to further study the performance of polyphenol nanoparticles prepared by this technology in the human body.

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Author Contributions

Conceptualization: J.S. and Y.Z.; Data curation: X.L., L.C., and F.Z.; Formal analysis: X.Z., and C.L.; Funding acquisition: J.S., and Y.Z.; Investigation: S.Z., Y.Z., and K.Q.; Methodology: J.S.; Project administration: J.S., and Y.Z.; Resources: J.S., and Y.Z.; Writing: J.S.

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Conflict of Interest

The authors have declared no conflicts of interest for this article.

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