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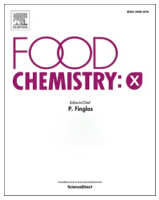

Preparation of soybean oil-based emulsions stabilized by shiitake mushroom chitosan modified in both enzymatic and non-enzymatic systems and their application in β-carotene delivery

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ABSTRACT

This study investigates the laccase-catalyzed grafting of gallic acid (GA) onto shiitake mushroom chitosan to enhance its emulsifying properties and improve β-carotene delivery. Structural characterization using FTIR, XPS, and ^{1}H NMR revealed that laccase catalysis promoted the formation of amide bonds, disrupted the crystalline structure of chitosan, and enhanced both its hydration and interfacial activity. The modified chitosan emulsions exhibited significantly improved emulsification capacity and stability, with GA-grafted chitosan achieving emulsification activity of 3.34 L/g·cm and stability of 97.6 %. The β-carotene encapsulation efficiency increased to 82.1 %, with enhanced resistance to UV light and H_2O_2-induced degradation. In vitro digestion experiments demonstrated that the modified chitosan emulsion improved β-carotene bioaccessibility (75.8 %) and cellular uptake (55.3 %), significantly improving delivery efficiency. This study provides a novel approach for the development of functional emulsion carriers and lays the foundation for their application in food and drug delivery systems.

1. Introduction

Chitosan is a natural polysaccharide widely used in food, pharmaceuticals, and cosmetics, primarily due to its excellent biodegradability, biocompatibility, and multifunctionality (Khalaf et al., 2023). Traditionally, chitosan is extracted from marine organisms such as shrimp and crab shells; however, with increasing environmental concerns, fungal-derived chitosan, such as shiitake mushroom chitosan, has gained significant attention (Chien et al., 2016; Yen & Mau, 2007). Shiitake mushroom chitosan not only retains the functional properties of traditional chitosan but also offers advantages such as better solubility, environmental sustainability, and vegan compatibility, making it a promising material for various applications.

However, the use of native chitosan is limited by its poor solubility, weak emulsifying properties, and unstable bioactivity, which restrict its direct application in certain food and pharmaceutical systems (Abd El-Hack et al., 2020). Therefore, the modification of chitosan has become a crucial research direction to enhance its functionality. Therefore, the modification of chitosan has become a crucial research direction to enhance its functionality. Various modification strategies including chemical methods (carboxymethylation, quaternization), physical treatments (irradiation, microwave), and biological approaches have been developed, each with distinct advantages and limitations. Among these, enzymatic modification stands out as a highly efficient, selective, and mild method that provides an innovative and environmentally friendly approach (Wang et al., 2021). Laccase, an enzyme that catalyzes the oxidation of phenolic compounds, has garnered particular attention for chitosan modification as it operates under mild reaction conditions (aqueous medium, moderate temperature, near-neutral pH), demonstrates high substrate specificity, and produces minimal environmental impact compared to chemical modification methods. The enzyme effectively enhances the physicochemical properties of chitosan by promoting the oxidation of phenolic hydroxyl groups to generate reactive phenoxy radicals that readily couple with chitosan's amino groups,

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achieving efficient grafting without hazardous coupling reagents (N. Liu et al., 2021).

Gallic acid (GA), a naturally occurring phenolic compound, is widely found in plants and exhibits remarkable antioxidant, antimicrobial, and anti-inflammatory activities. Compared to other phenolic acids, gallic acid contains multiple phenolic hydroxyl groups and a hydrophobic benzene ring, which provides unique advantages in promoting emulsification, enhancing molecular interactions, and improving chitosan's functionality (Meng et al., 2023; Sharkawy et al., 2020). Gallic acid not only forms hydrogen and covalent bonds with chitosan molecules to improve its stability and functionality but also enhances the antioxidant capacity of modified chitosan, which is crucial for protecting active ingredients. Therefore, the use of gallic acid as a modifying agent can not only enhance the antioxidant and antimicrobial properties of chitosan but also improve its emulsifying ability through the hydrophobic structure of gallic acid, making it more suitable for food and drug delivery systems (T. Yang et al., 2017; Zarandona et al., 2020).

Emulsion systems, as common delivery carriers, play a significant role in food formulations by stabilizing hydrophobic nutrients and serving as carriers for bioactive compounds. However, traditional emulsifiers often rely on synthetic surfactants or proteins, which may pose stability or health safety concerns (Fu et al., 2025). As a result, the development of natural, biodegradable emulsifiers has become a key research direction in food science. Laccase-catalyzed grafting of gallic acid onto shiitake mushroom chitosan not only significantly enhances its emulsifying properties but also boosts its antioxidant and antimicrobial activities, thereby improving the stability of emulsions and the delivery efficiency of active ingredients such as β-carotene.

β-Carotene, a lipophilic antioxidant and precursor to vitamin A, is widely used in functional foods and nutraceuticals. However, the poor water solubility and susceptibility to oxidation of β-carotene limit its application in food systems (Tabatabaei et al., 2024). The development of emulsion delivery systems aims to improve the stability and bioavailability of β-carotene, with the choice of emulsifier playing a crucial role in delivery effectiveness (Xu et al., 2024). Soybean oil-based emulsions, combined with gallic acid-modified shiitake mushroom chitosan as an emulsifier, offer a promising solution for encapsulating and protecting β-carotene, significantly enhancing its functional efficacy in food applications.

This study aims to develop a soybean oil-based emulsion system stabilized by laccase-modified shiitake mushroom chitosan and investigate its application in β-carotene delivery. By adjusting the grafting ratio of gallic acid, the emulsifying performance, stability, β-carotene encapsulation efficiency, and bioaccessibility of the modified chitosan emulsions will be evaluated. The results will provide new insights into the development of natural emulsifiers and lay the foundation for their application in functional food and drug delivery systems.

2. Materials and methods

2.1. Materials

The soybean oil and shiitake mushrooms (*Lentinus edodes*) used in this study were purchased from a local supermarket. Shiitake mushroom chitosan (Cs, deacetylation degree 92.7 %) was extracted and prepared in-house. Laccase (EC 1.10.3.2, activity 20 U/mg) was obtained from Solarbio Life Sciences, Beijing, China. Gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, β-carotene, Tween 80, and hydrogen peroxide (H_2O_2) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were obtained from the Microbiological Culture Collection Centre, Guangdong, China. All other reagents were of analytical grade and were supplied by Xilong Chemical Co., Ltd. (Shantou, China).

2.2. Extraction of shiitake mushroom chitosan

The extraction of shiitake mushroom chitosan (Cs) followed an improved deproteinization and deacetylation process. Fresh shiitake mushrooms were purchased from a local supermarket, cleaned, sliced, and dried at 50 °C to constant weight. The dried mushrooms were ground and sieved through a 60-mesh sieve to obtain mushroom powder. A certain amount of mushroom powder was mixed with 1.5 % (*w*/*v*) NaOH solution at a solid-to-liquid ratio of 1:20 (g/mL), and the mixture was stirred at 90 °C for 2 h to remove proteins and some polysaccharides. The resulting precipitate was washed with deionized water until neutral, centrifuged (8000 rpm, 10 min), and the solid was collected and dried at 60 °C for further use. Subsequently, the deproteinized sample was treated with 40 % (w/*v*) NaOH solution at 120 °C for 4 h to increase the deacetylation degree. The obtained solid was washed with deionized water to pH 7.0, centrifuged (8000 rpm, 10 min), and the precipitate was collected. The deacetylated product was dissolved in 2 % (*v*/v) acetic acid solution and stirred at 50 °C for 3 h. The resulting solution was filtered through a 0.45 μm filter membrane, and 95 % (v/v) ethanol was slowly added until the ethanol volume fraction reached 70 %. The mixture was allowed to stand for 12 h for complete chitosan precipitation. Finally, the precipitate was collected by centrifugation (8000 rpm, 10 min), washed twice with anhydrous ethanol, and dried at 50 °C to constant weight.

From 100 g of dried shiitake mushroom powder, approximately 3.2 g of purified chitosan was obtained, representing an extraction yield of 3.2 % (*w*/w). The resulting shiitake mushroom chitosan powder had a deacetylation degree of 92.7 % (determined by potentiometric titration), an average molecular weight of 122,770 Da (measured by GPC), and exhibited a white to pale yellow appearance.

2.3. Preparation of modified chitosan

The preparation of modified chitosan was based on previous studies with certain modifications (Božič et al., 2013). Accurately weigh 10 mmol of gallic acid, add it to a flask, and then add 100 mL of deionized water, stirring with a magnetic stirrer until dissolved. Next, add 1 g of laccase and continue stirring until completely dissolved. Accurately weigh 10 g of chitosan (Na—Cs), add 10 mL of absolute ethanol to wet it, and then disperse it into 500 mL of deionized water, stirring with a magnetic stirrer to achieve uniform dispersion. Afterward, slowly add 5 mL of glacial acetic acid, and stir for 2 h to ensure complete dissolution of chitosan. Mix the gallic acid-laccase solution prepared in the previous step with the chitosan solution, then place them on a 50 °C water bath magnetic stirrer, and allow the reaction to occur while supplying oxygen (2 L/min) for 4 h. After the reaction, add 300 mL of 1 % acetic acid solution to the mixture, stir for 20 min to dissolve the modified chitosan. After centrifugation at 4000 rpm for 10 min to remove the precipitated laccase, dialyze (7–14 kDa MWCO membrane against deionized water) the mixture for 48 h to remove any unreacted phenolic acid. Finally, remove the water from the modified chitosan solution by freeze-drying and collect the solid powder, which is the gallic acid-chitosan copolymer.

The samples prepared by heating for 4 h and 8 h are labeled as GaE1-Cs and GaE2-Cs, respectively, while the sample prepared without laccase is labeled as GaN-Cs.

2.4. Structural characterization

To confirm the successful grafting of gallic acid onto chitosan and the structural changes, various analytical techniques were employed.

UV–Visible spectra were recorded using a T9 UV–Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China) over a wavelength range of 200–400 nm to observe the characteristic absorption peak shifts after gallic acid grafting.

Proton nuclear magnetic resonance (^{1}HNMR) spectra were obtained

using a BRUKER 400 M NMR spectrometer (Bruker Co., Ltd., Germany) with D_2O as the solvent. Chemical shift changes were analyzed to further confirm the formation of amide bonds.

Fourier-transform infrared (FTIR) spectra were recorded on a NICOLET IS 10 FTIR spectrometer (Thermo Fisher Co., Ltd., USA) with a scanning range of 4000–400 cm^{-1} using the KBr pellet method to detect functional group changes.

X-ray photoelectron spectroscopy (XPS) was performed using an EscaLab 250Xi XPS spectrometer (Thermo Fisher Co., Ltd., USA) to analyze elemental composition and chemical environment changes.

X-ray diffraction (XRD) was conducted using a Rigaku Ultima IV X-ray diffractometer (Rigaku Co., Ltd., Japan) with Cu-Kα radiation (λ = 0.154 nm) in the scanning range of 5–50° and a step size of 0.02° to analyze the crystallinity changes of chitosan.

Gel permeation chromatography (GPC) was used to determine the molecular weight (Mw) distribution, using a Waters 1525 GPC system (Waters, USA). The mobile phase was deionized water, with a flow rate of 1.0 mL/min and column temperature at 35 °C. Polyethylene glycol (PEG) was used as the standard to calculate the weight-average molecular weight of the samples.

2.5. Dynamic viscosity test

To investigate the effect of laccase-catalyzed gallic acid grafting on the rheological properties of chitosan solutions, dynamic viscosity measurements were performed using an LVDV-II + P viscometer (Bolefield Co., Ltd., America).

The samples were dissolved in 1.0 % (*w/v*) acetic acid solution at a concentration of 2 % (w/v) and stirred at 4000 rpm for 4 h to achieve complete swelling. Viscosity measurements were taken at different shear rates to analyze the shear-thinning behavior of the samples. Additionally, a temperature scanning experiment was conducted to record the viscosity changes with temperature, assessing the temperature sensitivity of the modified chitosan.

2.6. Antioxidant activity testing

To evaluate the antioxidant capacity of laccase-catalyzed gallic acid grafted chitosan, two methods were employed: the DPPH radical scavenging test and β-carotene bleaching inhibition assay (Cheng et al., 2024).

2.6.1. DPPH radical scavenging test

In this experiment, 5 mL of 0.1 mM DPPH ethanol solution was mixed with 50 μL of 0.01 g/mL chitosan solution. After reacting for 30 min at room temperature in the dark, the absorbance was measured at 517 nm. Absolute ethanol (50 μL) was used as the control. The DPPH radical scavenging rate was calculated using the following formula:

$$DPPH\ clearance = \frac{A_0 - A_1}{A_0} \times 100\% \quad (1)$$

Where A_0 is the absorbance of the control group, and A_1 is the absorbance of the sample group after 30 min of reaction.

2.6.2. B-carotene bleaching inhibition rate

The β-carotene bleaching inhibition test was used to assess the sample's ability to inhibit lipid oxidation, which was performed according to the method described by previous study (Fei et al., 2022). β-Carotene, linoleic acid, and Tween 80 were emulsified in water, and 100 μL of 0.01 g/mL modified chitosan solution was added to 5 mL of β-carotene emulsion. After mixing thoroughly using a vortex mixer at 2000 rpm for 30 s, followed by gentle inversion 10 times to ensure homogeneous distribution without excessive foam formation, the mixture was incubated at 50 °C for 2 h. Absorbance changes were recorded at 470 nm both initially and after 2 h of incubation, with 95 % ethanol as the control group. The β-carotene bleaching inhibition rate was calculated using the following formula:

$$Y = \left(1 - \frac{A_{s0} - A_{s2}}{A_{c0} - A_{c2}}\right) \times 100\% \quad (2)$$

where A_{s0} and A_{s2} are the absorbances of the sample group at the initial time and after 2 h, respectively, and A_{c0} and A_{c2} are the absorbances of the control group at the initial time and after 2 h.

2.7. Antibacterial activity test

To evaluate the antibacterial activity of laccase-catalyzed gallic acid grafted chitosan, the agar diffusion method (inhibition zone method) was used to study the inhibition of *E. coli* and *S. aureus*. The bacterial strains were pre-cultured at 37 °C for 12 h, and the bacterial suspension was adjusted to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). A 100 μL bacterial suspension was evenly spread on the surface of nutrient agar plates and allowed to dry. Afterward, wells were punched in the agar, and 100 μL of sample solution (2 mg/mL) was added. Deionized water was used as the negative control. The plates were incubated at 37 °C for 24 h, and the inhibition zone diameter (mm) was measured (Fei et al., 2022).

2.8. Emulsifying properties analysis

2.8.1. Emulsion preparation

The soybean oil-water emulsion system was prepared using chitosan as the emulsifier. Specifically, 1 % (*w/v*) chitosan was dissolved in 16 mL of 0.1 M acetic acid solution and stirred at room temperature (25 °C) for 2 h to ensure complete dissolution. Then, 4 mL of soybean oil was slowly added, and the mixture was homogenized at 10,000 rpm for 3 min using a high-speed homogenizer to prepare the emulsion.

2.8.2. Microscopic observation of emulsion droplets

The microscopic morphology of the emulsion was observed using an ECLIPSE CI upright fluorescence microscope (Nikon Co., Ltd., Japan). A small amount of emulsion was placed on a glass slide, covered with a cover slip, and the droplet images were captured.

2.8.3. Zeta potential measurement

The Zeta potential of the emulsion was measured using a Malvern Zetasizer Nano ZS nanoparticle size and Zeta potential analyzer (Malvern Co., Ltd., UK) to evaluate the adsorption ability of modified chitosan at the oil-water interface and the emulsion stability.

2.8.4. Emulsifying activity and emulsion stability

Emulsifying activity (EA) and emulsion stability (ES) were determined using the turbidity method. After the emulsions were prepared by the above-mentioned emulsification steps, the absorbance at 510 nm was measured. The emulsifying activity was calculated using the following formula:

$$Emulsion\ activity = \frac{A_1 \times V}{L \times \varnothing \times C} \quad (3)$$

where A_1 is the absorbance of the diluted emulsion at 510 nm, V is the dilution factor, L is the path length of the cuvette (mm), ϕ is the volume fraction of the dispersed phase, and C is the chitosan solution concentration (g/L).

Emulsion stability was measured by recording the absorbance change after the emulsion was left to stand for 3 h. The formula for emulsion stability is as follows:

$$Emusion\ stability = \frac{A_2}{A_1} \times 100\% \quad (4)$$

where A_1 is the initial absorbance of the emulsion, and A_2 is the absorbance after 3 h.

2.8.5. Storage stability

The emulsion was stored at room temperature (25 °C) and observed every 24 h for phase separation. The storage stability of the emulsion was evaluated by documenting the separation process using a camera.

2.9. Emulsion loading of β-carotene

To evaluate the loading capacity of laccase-catalyzed gallic acid grafted chitosan emulsions for β-carotene, encapsulation efficiency (EE %) and loading capacity (LC%) were determined.

To prepare β-carotene-loaded emulsions, β-carotene was first dissolved in soybean oil (0.1 % *w/v*) with gentle heating (40 °C) and stirring for 30 min until completely dissolved. This β-carotene-containing oil phase was then used in the emulsion preparation procedure described in Section 2.8.1, following the same homogenization conditions. The final emulsions contained β-carotene at a concentration of 0.02 % w/v based on the total emulsion volume. Then, 2 mL of the emulsion was centrifuged at 10,000 rpm for 15 min, and both the supernatant and precipitate were collected. After ultrafiltration, the free β-carotene concentration (C_free) in the supernatant was measured using a UV–Visible spectrophotometer (T9, Beijing Purkinje General Instrument Co., Ltd.) at 450 nm, and the concentration was calculated using a standard curve. The encapsulation efficiency was calculated using the following formula (Guo et al., 2022):

$$EE\ (\%) = \left(1 - \frac{C_{free}}{C_{total}}\right) \times 100\% \tag{5}$$

where C_{free} is the free β-carotene concentration in the supernatant and C_{total} is the total β-carotene concentration in the emulsion.

The loading capacity is used to assess the amount of β-carotene contained in a unit mass of the emulsion. The formula is as follows:

$$LC\ (\%) = \left(\frac{W_{total} - W_{free}}{W_{emulsion}}\right) \times 100\% \tag{6}$$

where W_{free} is the mass of free β-carotene in the supernatant, W_{total} is the total mass of β-carotene in the emulsion, and $W_{emulsion}$ is the total mass of the emulsion.

2.10. Protective effect of emulsion on β-carotene

To evaluate the protective effect of laccase-catalyzed gallic acid grafted chitosan emulsions on β-carotene, UV degradation and H_2O_2 oxidation degradation experiments were performed to study their stability.

2.10.1. UV degradation test

Emulsion samples were exposed to a 365 nm UV light source (6 W) at room temperature (25 °C) in the dark. Samples were taken at 0, 12, 24, 36, and 48 h. The residual β-carotene content (R%) was measured using a UV–Visible spectrophotometer (T9, Beijing Purkinje General Instrument Co., Ltd.) at 450 nm. The residual amount was calculated using the following formula:

$$Residual\ amount\ (\%) = \frac{A_t}{A_0} \times 100\% \tag{7}$$

where A_t is the absorbance after t hours, and A_0 is the initial absorbance.

2.10.2. H_2O_2 oxidation degradation test

5 mL of emulsion sample was mixed with 0.1 mM H_2O_2 and stored in the dark at 25 °C. Samples were taken at 0, 6, 12, 18, and 24 h. The residual β-carotene content was measured at 450 nm using a UV–Visible spectrophotometer, and the residual amount was calculated using the formula (7).

2.11. Bioaccessibility and cellular uptake of β-carotene

To evaluate the bioaccessibility of β-carotene and its absorption in cells, laccase-catalyzed gallic acid grafted chitosan emulsions were subjected to an in vitro digestion model to determine the bioaccessibility of β-carotene, and the cellular uptake was investigated using Caco-2 cell uptake experiments.

2.11.1. In vitro digestion model for bioaccessibility measurement

The in vitro digestion experiment used a simulated gastrointestinal digestion system, including the gastric phase (GP) and intestinal phase (IP). The composition and conditions of the digestion fluids were based on previous study (C. Zheng et al., 2023).

Gastric Phase Digestion: 5 mL of β-carotene emulsion was mixed with 5 mL of artificial gastric juice (containing 0.32 % (*w/v*) pepsin, pH 2.5) and incubated at 37 °C with shaking for 2 h to simulate gastric digestion.

Intestinal Phase Digestion: The gastric digestion products were mixed with 5 mL of artificial intestinal fluid (containing 0.4 % (w/v) pancreatin and bile salts, pH 7.4) and incubated at 37 °C for another 2 h. The mixture was then centrifuged at 10,000 rpm for 30 min. The supernatant was collected, and the β-carotene content was measured at 450 nm using a UV–Vis spectrophotometer. The bioaccessibility was calculated using the following formula:

$$Bioaccessibility\ (\%) = \frac{C_{micelle}}{C_{initial}} \times 100\% \tag{8}$$

where $C_{micelle}$ is the soluble β-carotene content after intestinal digestion, and $C_{initial}$ is the initial β-carotene content in the emulsion.

2.11.2. Caco-2 cell uptake experiment

Caco-2 cells (human colon adenocarcinoma cell line) were used to simulate the absorption of β-carotene by intestinal epithelial cells. The experimental procedure is as follows: Caco-2 cells were cultured in DMEM medium (containing 10 % FBS and 1 % penicillin-streptomycin) at 37 °C under 5 % CO_2 conditions until they reached 80 % confluence. The digested emulsion (IP product) was diluted to 0.1 mg/mL and transferred to a 6-well Caco-2 cell culture plate. The plate was incubated at 37 °C for 4 h, followed by washing with PBS three times. The cells were then collected, and the β-carotene uptake was measured. The cellular uptake rate was calculated using the following formula:

$$Cellular\ Uptake\ (\%) = \frac{C_{cell}}{C_{initial}} \times 100\% \tag{9}$$

where C_{cell} is the β-carotene content in the Caco-2 cells, and $C_{initial}$ is the initial β-carotene concentration in the culture medium.

2.12. Data analysis

All experiments were performed in triplicate, and the data are presented as mean ± standard deviation (Mean ± SD). Statistical analysis was performed using SPSS 26.0 software (IBM, USA). One-way ANOVA was used to compare the differences between different treatment groups, and Duncan's multiple comparison test was applied for post-hoc analysis to determine the significance of differences between groups. A significance level of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. UV–vis and ^{1}H NMR analysis

This section analyzes the molecular structure changes of shiitake mushroom chitosan after laccase-catalyzed grafting of gallic acid (GA) using UV–Vis and ^{1}H NMR spectroscopy. The experiment compared the spectra of native shiitake mushroom chitosan (Na—Cs), gallic acid-grafted chitosan under non-enzymatic conditions (GaN-Cs), and two

enzymatically modified samples (GaE1-Cs and GaE2-Cs). Fig. 1 presents the spectral data for different samples. Fig. 1(a) shows the UV–Vis absorption spectra. Na—Cs does not exhibit any distinct characteristic absorption peaks in the 240–400 nm range, whereas GaN-Cs, GaE1-Cs, and GaE2-Cs show a significant absorption peak at 263.5 nm, attributed to the $\pi \rightarrow \pi^*$ electronic transition of the aromatic ring in gallic acid, indicating that gallic acid has been successfully grafted onto the chitosan molecules (Xiao et al., 2022).

Additionally, Fig. 1(b) shows the grafting rates of the different samples. The grafting rates for GaN-Cs, GaE1-Cs, and GaE2-Cs were 8.3 %, 10.1 %, and 13.5 %, respectively, with laccase catalysis significantly improving the grafting efficiency. This suggests that while some grafting can occur under non-enzymatic conditions, laccase catalysis activates the phenolic hydroxyl groups of gallic acid through oxidation, promoting the formation of amide bonds with chitosan's amino groups, thus increasing the grafting efficiency. It's worth noting that Zhang et al. (2024) reported higher maximum grafting rates (up to 29.6 %) for phenolic acid-modified chitosan using carbodiimide chemistry. However, their approach requires coupling reagents that may pose toxicological concerns, while our laccase-catalyzed method offers a greener alternative with sufficient grafting efficiency to significantly enhance functional properties.

Fig. 1(c) presents the ^{1}H NMR spectra. Na—Cs exhibits characteristic peaks primarily between 1.8 and 4.2 ppm, corresponding to the hydrogen signals of the chitosan backbone. In contrast, GaN-Cs, GaE1-Cs, and GaE2-Cs show a new characteristic peak at 6.98 ppm, which is attributed to the ortho-hydrogen of the gallic acid aromatic ring (Fig. 1(d)), further confirming successful grafting. Additionally, some hydrogen signals of the chitosan backbone (3.83 ppm, 3.65 ppm, and 3.10 ppm) show slight chemical shifts after grafting, indicating that the grafting reaction altered the local electronic environment of chitosan and may have affected its molecular chain conformation (Xiao et al., 2022). These results are consistent with the UV–Vis analysis and further confirm that laccase catalysis promoted the formation of amide bonds, enhancing the structural modification efficiency of chitosan.

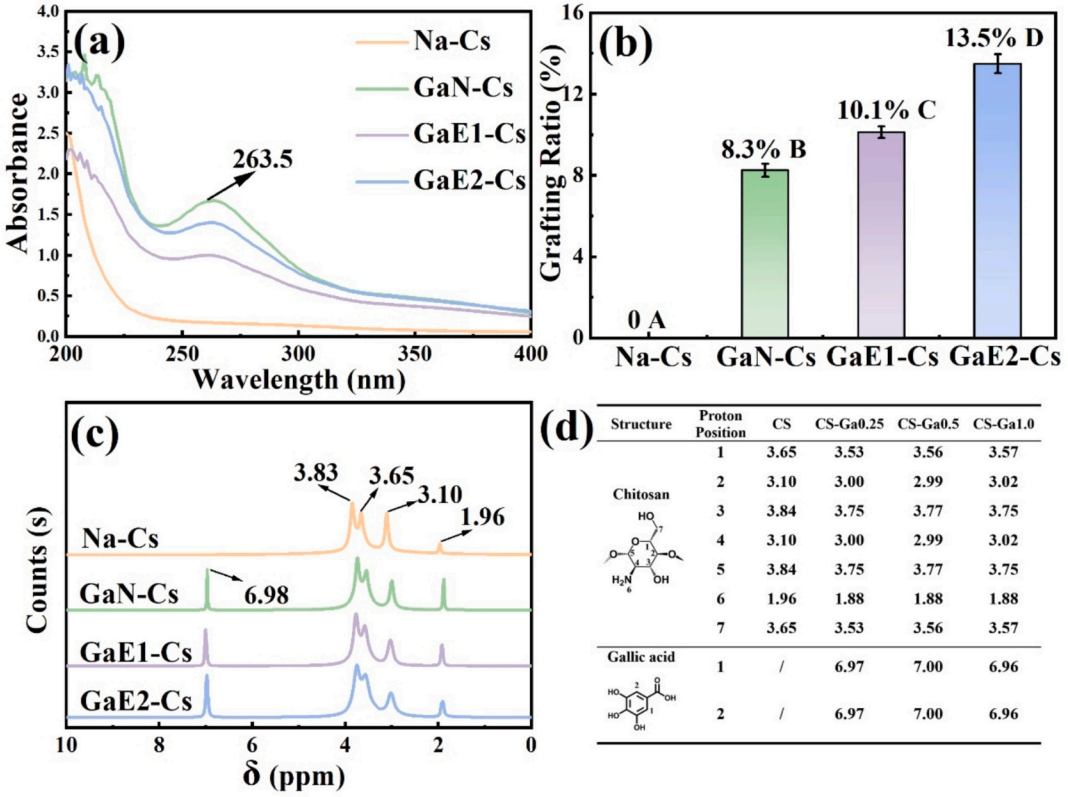
In summary, laccase catalysis promotes the covalent bonding of gallic acid to chitosan through oxidation, significantly increasing grafting efficiency, while altering the electronic environment and molecular structure of chitosan, thus laying the foundation for further enhancement of its functional properties.

3.2. FTIR analysis

In this experiment, FTIR and XPS were used to analyze the structural changes of chitosan after grafting with gallic acid to further verify the success of the grafting reaction and its impact on the molecular structure, as shown in Fig. 2. Fig. 2(a) displays the FTIR spectra in the range of 4000–400 cm^{-1}. All samples exhibit a broad and strong absorption peak at 3407 cm^{-1}, corresponding to the –OH and –NH_2 stretching vibrations in chitosan molecules. After grafting, this peak becomes broader and stronger, indicating that the introduction of gallic acid enhances the intramolecular hydrogen bonding. Additionally, the C—H stretching vibration peak at 2921 cm^{-1} does not show significant change, suggesting that the carbon-hydrogen backbone structure remains stable (Alimi et al., 2023).

Fig. 2(b) shows a zoomed-in FTIR spectrum in the range of 1900–900 cm^{-1}. Na—Cs exhibits characteristic peaks at 1648 cm^{-1} and 1598 cm^{-1}, corresponding to the –NH_2 and –OH bending vibrations. After modification, these peaks shift to lower wavenumbers, and new peaks appear at 1645 cm^{-1} and 1556 cm^{-1}, corresponding to the Amide *I* band (C═O stretching) and Amide II band (N—H bending), indicating that the –NH_2 group in chitosan has reacted with the –COOH group in gallic acid to form amide bonds. Furthermore, the C—N stretching vibration peak at 1376 cm^{-1} in Na—Cs shifts to 1410 cm^{-1} after modification, further confirming the formation of the amide bond.

Fig. 2(c) and (d) show the XPS elemental analysis results, where C, N,



Structure	Proton Position	CS	CS-Ga0.25	CS-Ga0.5	CS-Ga1.0
Chitosan	1	3.65	3.53	3.56	3.57
	2	3.10	3.00	2.99	3.02
	3	3.84	3.75	3.77	3.75
	4	3.10	3.00	2.99	3.02
	5	3.84	3.75	3.77	3.75
	6	1.96	1.88	1.88	1.88
	7	3.65	3.53	3.56	3.57
Gallic acid	1	/	6.97	7.00	6.96
	2	/	6.97	7.00	6.96

Fig. 1. UV spectra, grafting ratio, and ^{1}H NMR spectra of native and modified shiitake chitosan. (a): UV spectra; (b): Grafting ratio; (c): ^{1}H NMR spectra; (d): ^{1}H NMR related data.

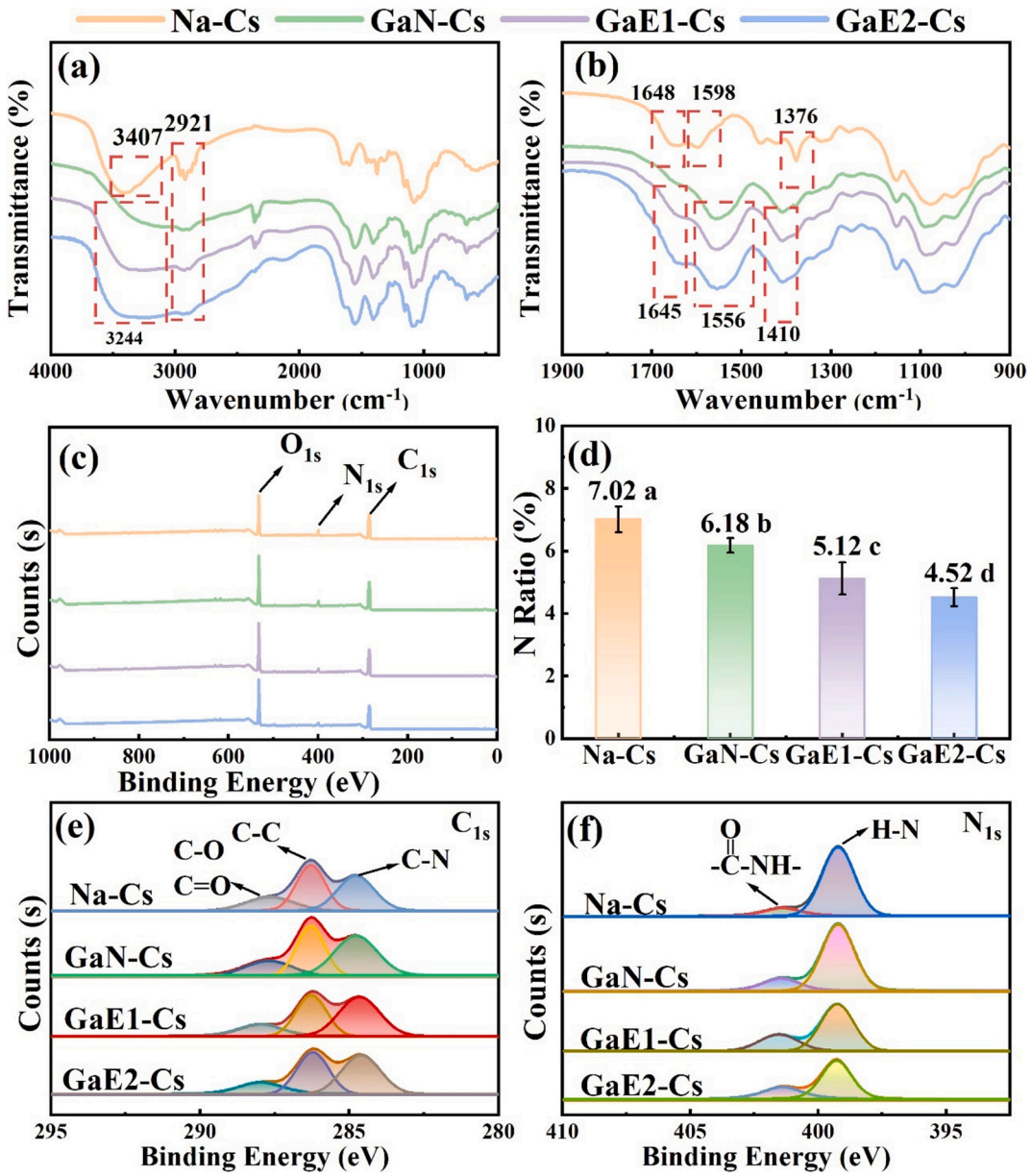


Fig. 2. FTIR and XPS spectra of native and modified shiitake chitosan. (a): FTIR spectra (4000–400 cm^{-1}); (b): FTIR spectra (1900–900 cm^{-1}); (c): Survey XPS spectra; (d): Nitrogen content obtained from XPS analysis; (e): High-resolution XPS spectra of C_{1s}; (f): High-resolution XPS spectra of N_{1s}.

and O are the main elements in all samples. The nitrogen content in Na—Cs is 7.02 %, whereas in GaN-Cs, GaE1-Cs, and GaE2-Cs, it decreases to 6.18 %, 5.12 %, and 4.52 %, respectively (Moreno-Vásquez et al., 2017). This indicates that as the grafting rate increases, the nitrogen content gradually decreases, likely due to the dilution effect of gallic acid grafting on the nitrogen content in chitosan. Fig. 2(e) shows the high-resolution C1s XPS spectrum, where the C1s peak of Na—Cs can be divided into C—N (284.6 eV), C—O (285.5 eV), and C═O, C—O (286.2 eV). As gallic acid is grafted, the C—N signal increases, indicating that the formation of amide bonds enhances the C—N component. Fig. 2 (f) shows the high-resolution N1s XPS spectrum, where the N_{1s} peak of Na—Cs is mainly composed of N—H (399.1 eV). In the modified samples, with the introduction of gallic acid, a N–C=O signal at 401.8 eV is observed, and its intensity increases with the degree of grafting, further proving the successful formation of amide bonds (Fei et al., 2022).

In summary, the FTIR and XPS results both indicate that laccase catalysis facilitates the formation of amide bonds between gallic acid and chitosan, enhancing intermolecular interactions through hydrogen bonds and covalent bonding, and altering the surface chemical structure of chitosan (N. Liu et al., 2021). This molecular modification is expected to improve the water solubility, interfacial activity, and biofunctional properties of chitosan, providing a foundation for its applications in food, drug delivery, and biomaterials.

3.3. XRD, molecular weight, and dynamic viscosity analysis

This section investigates the effects of laccase-catalyzed grafting of gallic acid on the molecular structure and physicochemical properties of chitosan using X-ray diffraction (XRD), molecular weight determination, and dynamic viscosity analysis, as shown in Fig. 3.

Fig. 3(a) displays the XRD spectra. Na—Cs shows a distinct crystallization peak at 19.90°, indicating that its molecular chains are arranged in an ordered stack. After grafting with gallic acid, GaN-Cs shows a shift of the crystallization peak to 20.96° and a decrease in peak intensity, suggesting an increase in the branching of the molecular chains, which weakens the ordered arrangement between the chains. For GaE1-Cs and

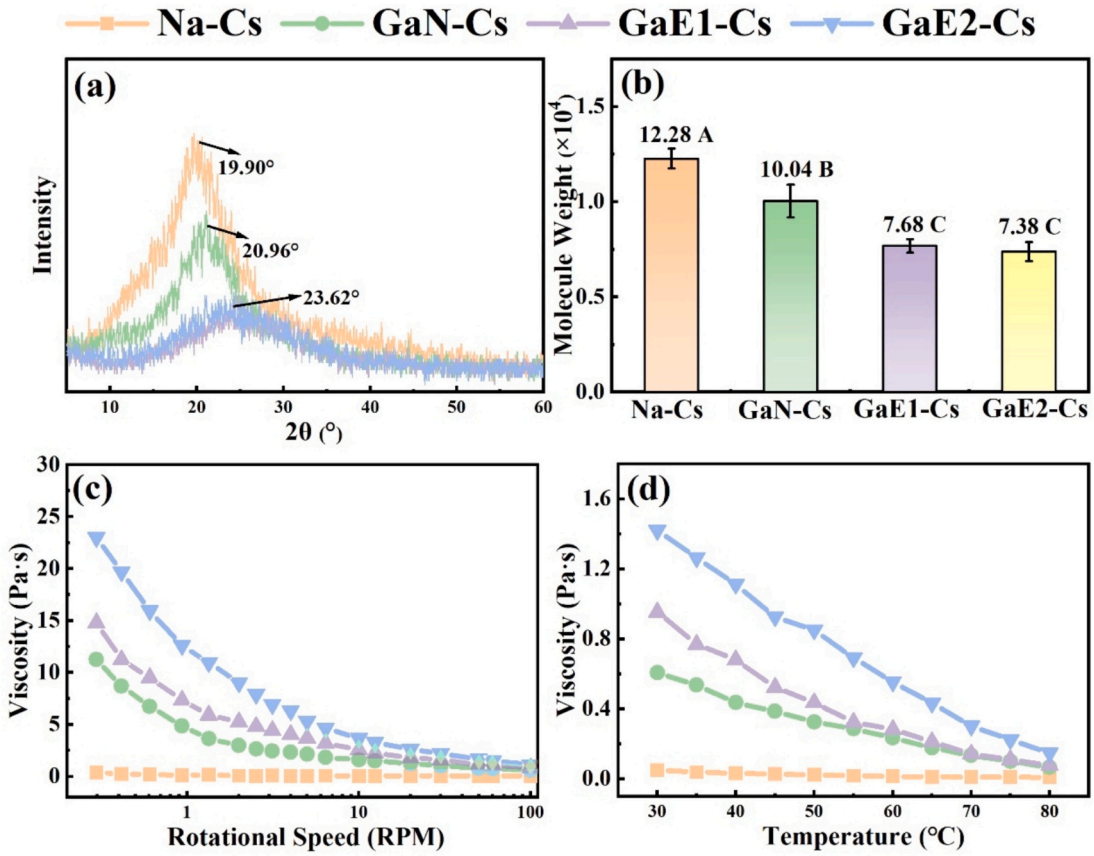


Fig. 3. XRD spectra, molecular weight, and dynamic viscosity analysis of native and modified shiitake chitosan. (a): XRD spectra; (b): Molecular weight data; (c): Shear rate vs. viscosity; (d): Temperature-dependent viscosity.

GaE2-Cs, the crystallization peaks almost disappear completely, possibly due to the phenoxy radicals formed during laccase catalysis, which promote the formation of amide bonds while disrupting the ordered stacking structure of chitosan, causing the molecular chains to become amorphous (Q. Hu et al., 2016; J. Liu et al., 2017).

Fig. 3(b) shows the molecular weight data of the different samples. The Mw of Na—Cs is 122,770, which decreases to 100,465 for GaN-Cs after grafting with gallic acid, indicating that the grafting reaction leads to some degradation of chitosan molecular chains. For the laccase-catalyzed GaE1-Cs and GaE2-Cs, the molecular weight further decreases to 76,786 and 73,873, respectively, suggesting that the phenoxy radicals generated during laccase oxidation have caused some degradation of the chitosan molecular chains.

Fig. 3(c) and (d) show the dynamic viscosity characteristics of the different samples. All samples exhibit shear-thinning behavior, with viscosity decreasing as shear rate and temperature increase. Compared to Na—Cs, the gallic acid-grafted samples exhibit higher viscosity under all tested conditions, indicating significant changes in the molecular structure. This increase in viscosity is likely due to the increase in molecular chain branching after grafting, while the formation of amide bonds enhances the intermolecular hydrogen bonding, increasing the internal friction of the system and thus raising the viscosity (Ferraris et al., 2020; Xie et al., 2016).

In summary, laccase-catalyzed grafting of gallic acid reduces the crystallinity of chitosan, making its molecular chains more amorphous. At the same time, partial chain degradation lowers the molecular weight, but the enhanced amide and hydrogen bonding increases the system's viscosity. These changes are crucial for the application of chitosan in emulsion delivery systems, as the lower crystallinity helps improve emulsion stability, while the higher viscosity enhances the rheological properties of the emulsion, thereby improving its loading capacity and controlled release of active ingredients. These improvements enhance its potential applications in the food, pharmaceutical, and cosmetics industries.

3.4. Antioxidant and antibacterial activity analysis

This experiment evaluates the antioxidant and antibacterial activities of gallic acid-grafted chitosan using the DPPH radical scavenging rate, β-carotene bleaching inhibition, and inhibition zone experiments. The results are shown in Fig. 4. Fig. 4(a) and (b) show the enhancement in antioxidant capacity. The DPPH radical scavenging rate of Na—Cs is only 3.34 %, whereas the rates for GaN-Cs, GaE1-Cs, and GaE2-Cs increase to 42.5 %, 69.7 %, and 75.9 %, respectively. The β-carotene bleaching inhibition follows the same trend, increasing from 16.4 % for Na—Cs to 49.3 %, 74.3 %, and 83.3 % for GaN-Cs, GaE1-Cs, and GaE2-Cs, respectively. This significant improvement is attributed to the phenolic hydroxyl group of gallic acid acting as a hydrogen donor to scavenge free radicals, thereby enhancing the antioxidant capacity. Similarly, Hu et al. (2024) reported comparable free radical scavenging effects when they prepared phenolic acid-modified chitosan through carbodiimide chemistry for facial mask applications. Additionally, the amide bonds formed through laccase catalysis reduce the interchain aggregation of chitosan, causing the molecular chains to expand in solution and increasing the exposure of antioxidant sites, further enhancing the free adical scavenging ability (Xie et al., 2014).

Fig. 4(c) and (d) show the enhanced antibacterial activity. The inhibition zone diameters of Na—Cs against *E. coli* and *S. aureus* were 12.2 mm and 11.3 mm, respectively, whereas the inhibition zone diameters for GaE2-Cs increased to 14.8 mm and 13.1 mm. The enhanced antibacterial activity may result from two factors: first, gallic acid itself has antimicrobial properties, and when grafted onto chitosan, it further

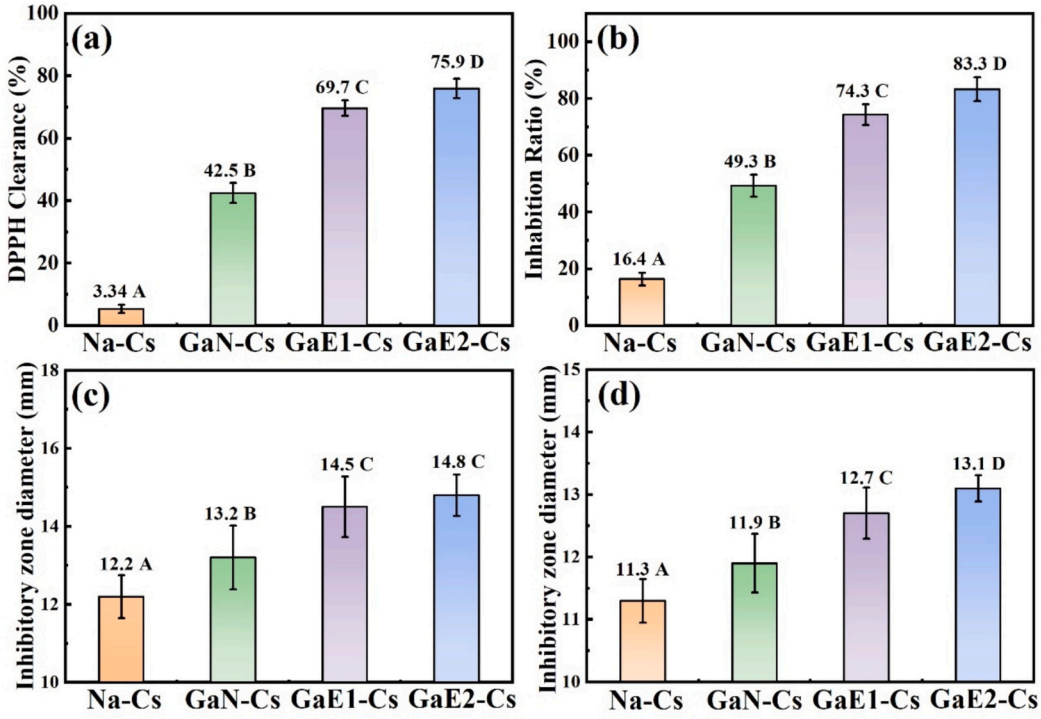


Fig. 4. Antioxidant and antibacterial activities of native and modified shiitake chitosan. (a): DPPH radical scavenging rate; (b): β-carotene bleaching inhibition rate; (c): Inhibitory zone diameter against *E. coli*; (d): Inhibitory zone diameter against *S. aureus*.

disrupts bacterial cell membranes, inhibiting bacterial growth; second, the solubility and surface charge distribution of the modified chitosan improve, enhancing its interaction with bacterial cells, thus improving its antibacterial effect (K. Li et al., 2019). These antimicrobial properties have led many researchers to apply such modified chitosan systems in the preservation of fruits, vegetables, fish, and meat products (Mou et al., 2023; X. Yang et al., 2023; Zhou et al., 2024).

In conclusion, laccase-catalyzed grafting of gallic acid effectively enhances the antioxidant and antibacterial activities of chitosan. This modification improves the stability of the emulsion system, effectively inhibits lipid oxidation and microbial contamination, reduces the degradation of active components such as β-carotene, and enhances its delivery efficiency and bioaccessibility, providing better protection and controlled release for food and drug delivery systems.

3.5. Emulsifying activity and stability analysis

This section investigates the changes in emulsifying properties of chitosan after modification by analyzing the microstructure of the emulsion, droplet size, emulsifying activity, emulsion stability, and Zeta potential. Fig. 5 presents the performance of native shiitake mushroom chitosan and its modified samples in terms of emulsifying properties.

Fig. 5(a)–(d) show the microscopic morphology of emulsions from different samples. The Na—Cs emulsion exhibits an uneven distribution of droplets, with a large droplet size (21.06 μm), while the droplet size in the GaN-Cs, GaE1-Cs, and GaE2-Cs emulsions decreases to 16.53 μm, 13.61 μm, and 11.96 μm, respectively, with more uniform and compact droplets. These results suggest that the grafting of gallic acid effectively improves the emulsifying ability of chitosan, allowing it to form a more stable emulsifying layer at the oil-water interface. The improved emulsification can be attributed to enhanced wettability and interfacial characteristics of modified chitosan particles, where the gallic acid's aromatic rings and hydroxyl groups create an amphiphilic structure that positions more effectively at the oil-water interface.

Fig. 5(f) and (g) show the emulsifying activity and emulsion stability, respectively. The emulsifying activity of Na—Cs is 0.76 L/g·cm, and the emulsion stability is 53.5 %. After grafting with gallic acid, both of these parameters significantly improve, with the emulsifying activity of GaE2-Cs increasing to 3.34 L/g·cm and the stability increasing to 97.6 %. This indicates that the modified chitosan can more effectively adsorb at the oil-water interface, improving emulsion stability and preventing droplet aggregation and phase separation.

Fig. 5(h) shows the change in Zeta potential. The Zeta potential of Na—Cs solution is relatively low (22.3 mV), but after modification, the Zeta potentials of GaN-Cs, GaE1-Cs, and GaE2-Cs increase to 38.6, 42.2, and 48.6 mV, respectively. This increased surface charge provides strong electrostatic stabilization that complements the steric barrier formed by the adsorbed chitosan layer, resulting in a dual stabilization mechanism that significantly enhances emulsion stability. This change is likely due to the introduction of gallic acid, which disrupts the crystalline structure of chitosan and enhances the chain extension, resulting in a higher surface charge on chitosan, which increases the electrostatic repulsion between particles. Moreover, the carboxyl and hydroxyl groups on gallic acid enhance the interaction between the molecules and water, while the benzene ring structure interacts hydrophobically with the oil phase, further improving the emulsifying properties (Y. Liu et al., 2023).

In summary, laccase-catalyzed grafting of gallic acid significantly improves the emulsifying properties of chitosan, reducing droplet size and enhancing emulsion stability. These improvements not only help in the effective encapsulation of hydrophobic active ingredients like β-carotene but also reduce phase separation and sedimentation in the emulsion system, thereby enhancing delivery efficiency and providing a more stable emulsifying carrier for food and drug delivery systems.

Additionally, the appearance changes of emulsions at different storage times were recorded to study the storage stability of both native and modified chitosan-based emulsions. Fig. 6 shows the appearance changes of Na—Cs, GaN-Cs, GaE1-Cs, and GaE2-Cs emulsions at different days. It is notable that the modified chitosan emulsions displayed a yellowish-brown coloration, attributable to the grafted gallic acid phenolic structures. While this coloration correlates with enhanced antioxidant activity, it presents minimal limitations for most food applications due to its mild intensity. In addition, the Na—Cs emulsion

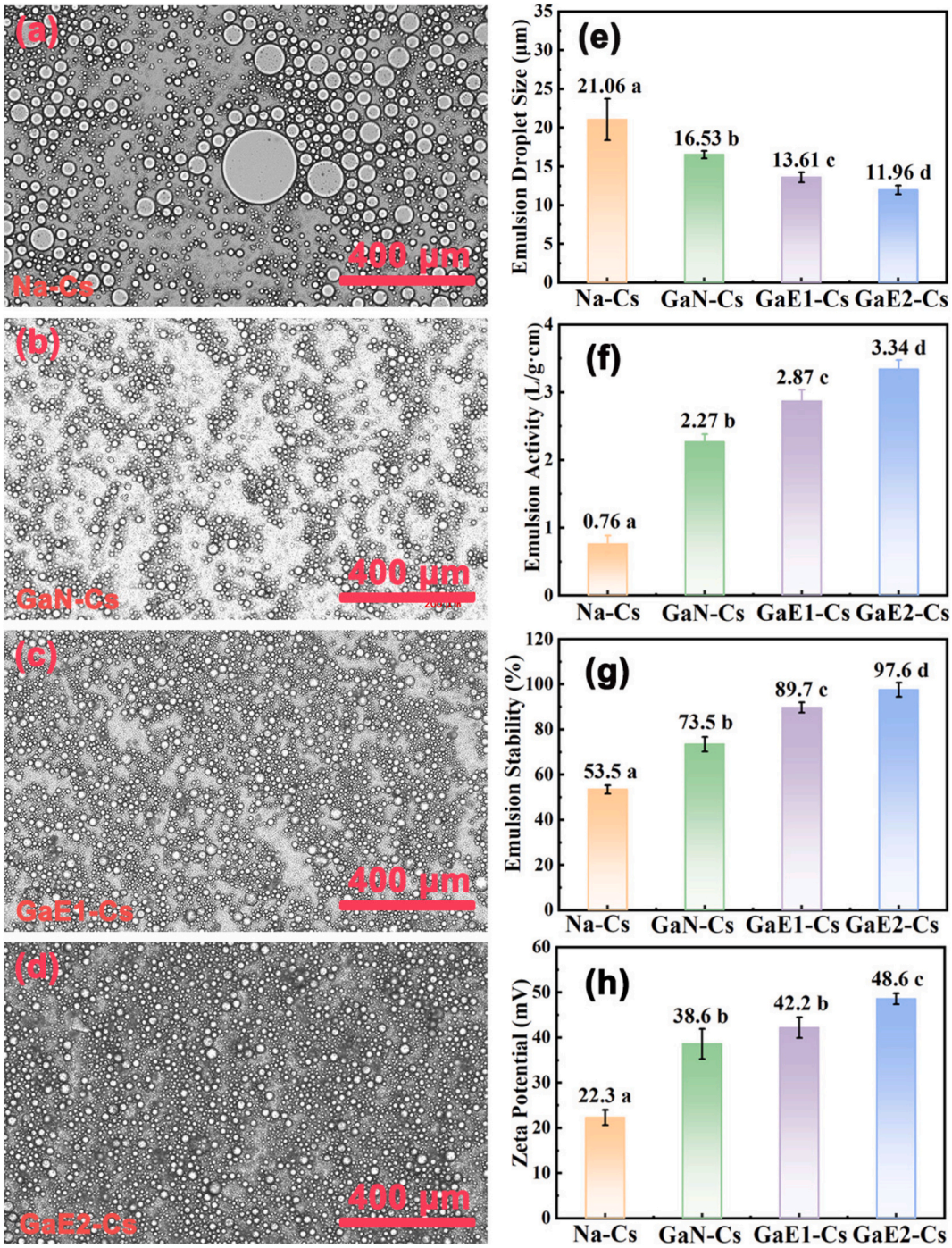


Fig. 5. Emulsification properties of native and modified shiitake chitosan-based emulsions. (a)–(d): Micromorphology; (e): Emulsion droplet size; (f): Emulsifying activity; (g): Emulsion stability; (h): Zeta potential.

exhibits noticeable phase separation after 1 day of storage, with the most severe oil-water separation occurring by day 3. This indicates that native chitosan has poor emulsifying stability and is unable to maintain emulsion uniformity over long periods. In contrast, the storage stability of modified chitosan emulsions is significantly improved. The GaN-Cs emulsion begins to show slight phase separation after 12 days, while the GaE1-Cs and GaE2-Cs emulsions only exhibit noticeable phase separation after 26 and 33 days, respectively. This suggests that the grafting of gallic acid effectively enhances the emulsifying stability of chitosan, and as the grafting ratio increases, the long-term storage stability of the emulsion is further improved (Y. Zhang et al., 2023).

The improvement in storage stability may be attributed to two factors: first, the introduction of gallic acid increases the surface activity of chitosan, enabling it to form a more stable interfacial film at the oil-water interface, which reduces droplet aggregation and coalescence; second, the increase in Zeta potential enhances the electrostatic repulsion between particles, further preventing droplet aggregation and sedimentation (T. Yang et al., 2017). Moreover, the enhanced long-term stability of the emulsion is of significant importance for the delivery of hydrophobic active ingredients such as β-carotene, as it effectively prevents degradation caused by droplet aggregation or sedimentation, improving its potential applications in food and drug delivery systems (Yi et al., 2021).

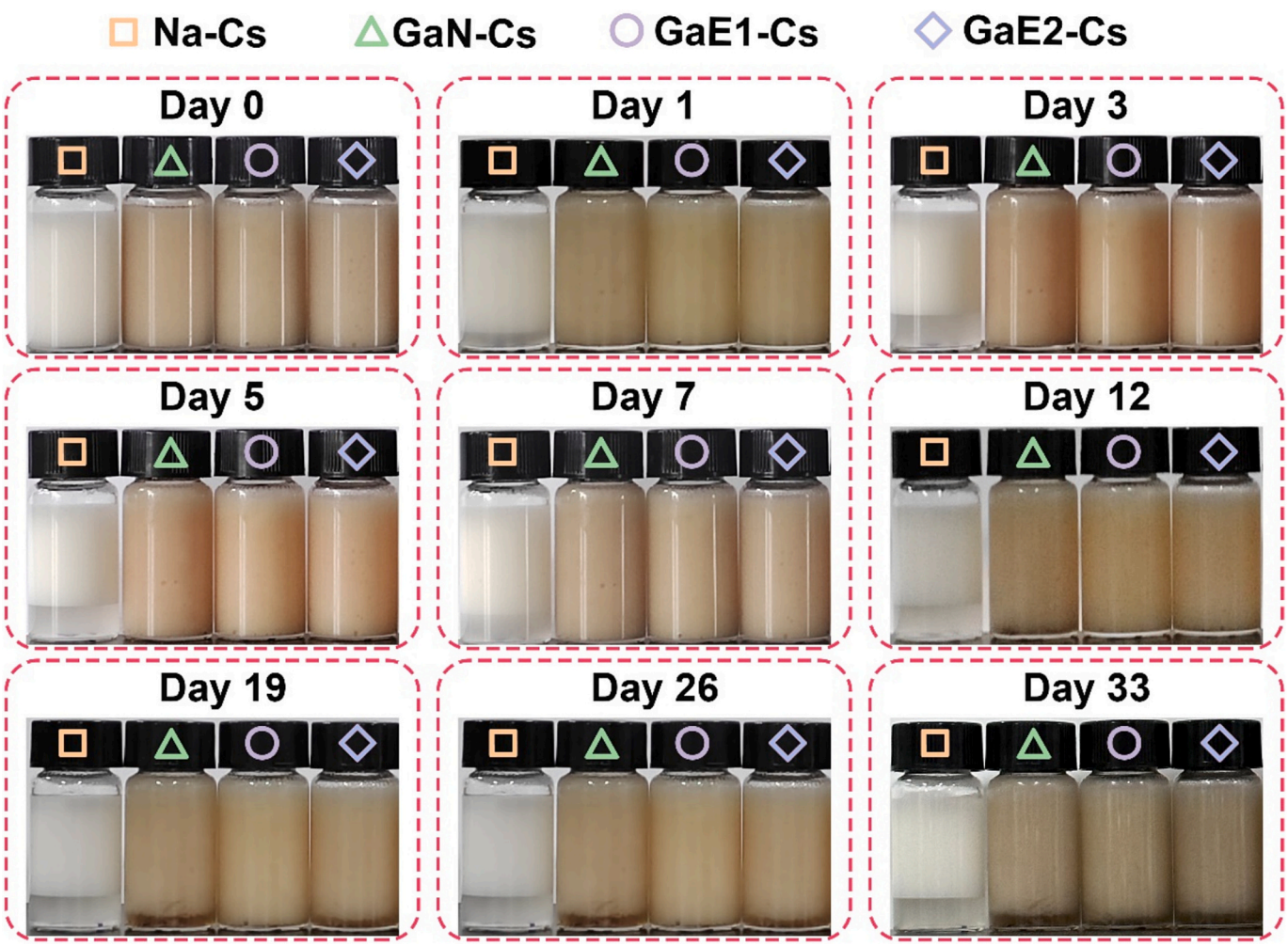


Fig. 6. Storage stability of native and modified shiitake chitosan -based emulsions.

3.6. Emulsion loading and protection of β-carotene

This section investigates the loading and protective ability of gallic acid-grafted chitosan emulsions for β-carotene through encapsulation efficiency (EE%), loading capacity (LC%), UV degradation experiments, and H_2O_2 oxidation degradation experiments. The results are shown in Fig. 7. Fig. 7(a) and (b) show the encapsulation efficiency and loading capacity of β-carotene. The encapsulation efficiency of Na—Cs emulsion is the lowest (45.2 %), while for GaN-Cs, GaE1-Cs, and GaE2-Cs, it increases to 60.3 %, 78.6 %, and 82.1 %, respectively. The loading capacity follows the same trend, with Na—Cs at 2.17 %, and GaE2-Cs increasing to 5.23 %. This indicates that laccase-catalyzed grafting of gallic acid disrupts the crystalline structure of chitosan, causing its molecular chains to stretch and improving the loading capacity of β-carotene through enhanced hydrophobic interactions (Xie et al., 2014; Xiong et al., 2018).

Fig. 7(c) and (d) show the degradation trends of β-carotene under UV light and H_2O_2 treatment. After 48 h of exposure to 365 nm UV light, the residual β-carotene content in Na—Cs emulsion is only 8.2 %, while in GaE2-Cs, it increases to 38.5 %. Similarly, after 24 h of exposure to H_2O_2, the residual β-carotene content in Na—Cs emulsion is 4.6 %, while in GaE2-Cs, it remains at 35.1 %. These results suggest that the modified chitosan emulsions effectively slow down the degradation of β-carotene and improve its stability.

In summary, laccase-catalyzed grafting of gallic acid enhances the encapsulation ability of chitosan, improving the stability of β-carotene and its antioxidant protection. Among the emulsions, GaE2-Cs exhibits the best overall performance, showing greater potential for applications in food and drug delivery systems.

3.7. Effect of emulsion on β-carotene bioaccessibility

This section analyzes the effect of chitosan modification on the delivery efficiency of β-carotene by measuring bioaccessibility and cellular uptake of β-carotene using an in vitro digestion model and Caco-2 cell uptake experiments, as shown in Fig. 8. Fig. 8(a) shows the bioaccessibility of β-carotene. The bioaccessibility of β-carotene in Na—Cs emulsion is the lowest (32.5 %), while in GaN-Cs, GaE1-Cs, and GaE2-Cs emulsions, it increases to 45.6 %, 62.3 %, and 75.8 %, respectively. This trend suggests that gallic acid grafting significantly improves the digestive stability and emulsification of chitosan emulsions, promoting the dissolution and release of β-carotene and enhancing its bioavailability in the in vitro digestion system (X. Li et al., 2020).

Fig. 7(b) shows the Caco-2 cell uptake rates. The β-carotene uptake rate in Na—Cs emulsion is 35.4 %, while in GaN-Cs, GaE1-Cs, and GaE2-Cs emulsions, it increases to 42.7 %, 53.1 %, and 55.3 %, respectively. GaE2-Cs emulsion shows the best cellular uptake efficiency, likely due to its higher emulsification stability, smaller droplet size, and stronger surface charge, which enhance the adsorption and endocytosis efficiency of the emulsion on the cell membrane (Chen et al., 2021; M. Zheng et al., 2022).

In conclusion, laccase-catalyzed grafting of gallic acid effectively enhances the bioaccessibility and cellular uptake of β-carotene, providing theoretical support for its application in food and drug

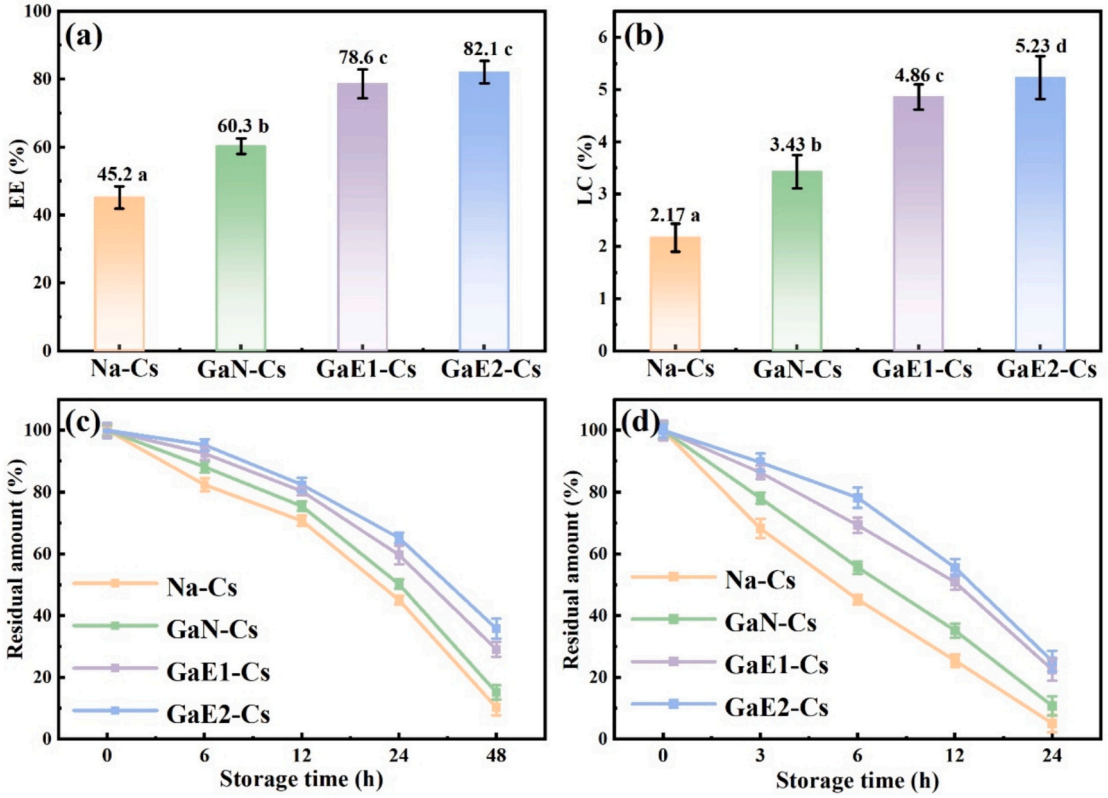


Fig. 7. Loading and protection of β-carotene by native and modified chitosan-based emulsions. (a) Encapsulation Efficiency; (b) Loading Capacity; (c) UV Degradation Experiment; (d) H_2O_2 Oxidation Degradation Experiment.

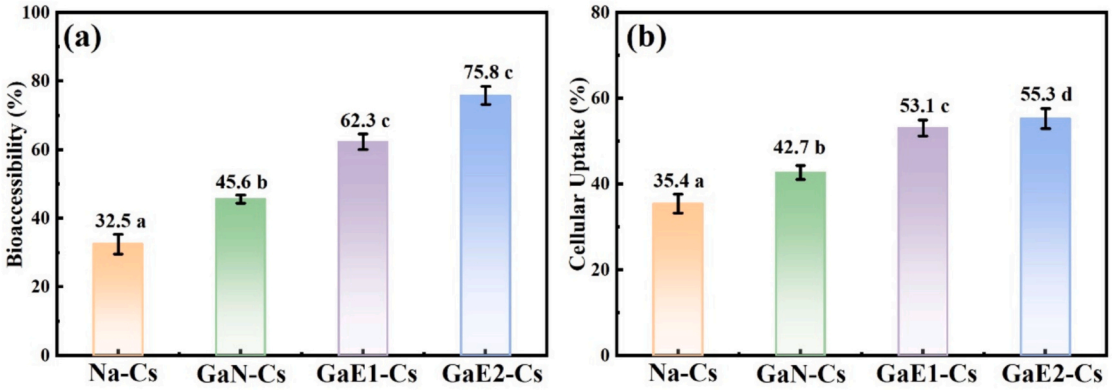


Fig. 8. Bioaccessibility (a) and cellular uptake (b) of β-carotene by native and modified chitosan-based emulsions.

delivery systems.

4. Conclusion

This study demonstrates that laccase-catalyzed grafting of gallic acid onto shiitake mushroom chitosan significantly enhances its emulsifying properties and β-carotene delivery capabilities. The modification disrupts chitosan's crystalline structure, increases surface activity, and improves interfacial adsorption at oil-water interfaces. The modified chitosan (GaE2-Cs) showed superior performance with emulsifying activity of 3.34 L/g·cm and stability of 97.6 %.

The β-carotene encapsulation efficiency and loading capacity of the modified chitosan emulsion were significantly improved, with GaE2-Cs emulsion reaching 82.1 % encapsulation efficiency. Moreover, the modified chitosan emulsion effectively protected β-carotene against UV light and H_2O_2 oxidation, improving its stability. In vitro experiments confirmed that the system significantly enhanced β-carotene bioaccessibility (75.8 %) and cellular uptake (55.3 %).

This approach provides an environmentally friendly strategy for developing natural emulsifiers with enhanced functionality. The laccase-catalyzed modification method has several advantages over traditional chemical modification approaches, including milder reaction conditions, higher specificity, and reduced environmental impact. However, challenges remain regarding the discoloration of emulsions during extended storage, suggesting that further optimization of the purification process may be necessary for commercial applications.

CRediT authorship contribution statement

Jiaofen Lin: Writing – original draft, Methodology, Data curation.

Jian Zeng: Formal analysis. **Guozong Shi:** Formal analysis. **Zesheng Zhuo:** Data curation. **Yanyun Guan:** Investigation, Formal analysis. **Zhipeng Li:** Investigation, Data curation. **Hui Ni:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Peng Fei:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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.

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Data availability

Data will be made available on request.

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