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Synthesis and Antimicrobial Activity of Chitosan Nanoparticles Incorporating Copper Ions

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Chitosan nanoparticles are a naturally derived substance with exceptional physicochemical properties and non-toxic bioactivity, making them suitable for extensive applications. This study evaluates and compares chitosan nanoparticles and chitosan nanoparticles incorporating antimicrobial agents. Copper ions were incorporated into chitosan matrices using a modified ionic gelation method to form a nano-coating solution. The particle size of chitosan nanoparticles (CN) was 118.23 ± 0.58 nm, and the particle size of chitosan nanoparticles loaded copper ions (CNCu) was 166.65 ± 7.36 nm, with low polydispersity index values and moderate to high zeta potential values. Scanning Electron Microscope (SEM) and Transmission Electron Microscopy (TEM) were used to determine their particle size and morphology. The antioxidant activity of chitosan nanoparticles and chitosan nanoparticles loaded with copper ions was also evaluated. CNCu exhibited superior antimicrobial activity against yeasts and molds. The results suggest CN and CNCu have great pre and post-harvest fruit preservation potential.

1. Introduction

Active coating technology has been gaining increased attention due to its numerous advantageous properties, including sustainability, antioxidant activity, and antibacterial activity, making it a promising solution for food preservation (Viscusi et al., 2021). Biopolymers have been used to develop films and coatings with various antimicrobial agents, including essential oils, metallic nanoparticles, probiotics, and prebiotics. These agents can be released gradually over time, resulting in improved quality of food products (Phan et al., 2022). Chitosan (CS) is a polysaccharide obtained from the deacetylation of chitin, which is a natural polymer found in various biological sources (Tran et al., 2022). CS possesses several desirable properties, such as biodegradability, film-forming capabilities, non-toxicity, and broad-spectrum antimicrobial activity with a wide range of applications (Tran et al., 2022).

Metal ions have gained interest in their antimicrobial functions in the past century. Due to its chelating ability, chitosan can form complexes with transition and heavy metals (Azmana et al., 2021). Copper ions have gained prominence relative to other metals owing to their advantageous attributes, such as antimicrobial activity (Tóth et al., 2020), cost-saving, and infrequent use compared to silver or gold (Pham et al., 2019). Chitosan-copper (Cu/Ch) exhibits promising antifungal activity with potential applications in various industries (Kaur et al., 2015). Combining chitosan with antimicrobial agents and bringing them to the nanoscale offers a promising direction for improving their characteristics and efficacy in various applications. Nanostructured coatings have emerged as a positive solution for preserving fruit's freshness and bioactive components (Viscusi et al., 2021). This study aims to provide valuable insights into the potential of chitosan and metal ions in developing nanostructured coatings for antifungal applications. This study employs the ion gelation method to develop chitosan nanoparticles with and without loaded copper ions, which is considered effective in determining the optimal loading capacity of chitosan nanoparticles with copper ions.

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2. Materials and Methods

2.1 Materials and chemicals

Low molecular weight chitosan (80 % deacetylation) was supplied by Vietnam Food Joint Stock Company, Vietnam. All chemicals were purchased from Chemsol with the pure grade, including sodium tripolyphosphate (STPP), acetic acid (CH_3COOH), distilled water, copper sulfate pentahydrate ($CuSO_4.5H_2O$), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH).

2.2 Preparation of chitosan nanoparticles

Chitosan nanoparticles (CN) were produced using the ionic gelation technique, as outlined in Table 1. Chitosan was dissolved in 1 % (v/v) acid acetic to prepare the chitosan solutions. A crosslinked solution was prepared by mixing STPP (0.1 % wt/vol) in Deionized water. Preparing chitosan nanoparticles involved adding 40 mL of TPP solution dropwise to 100 mL of CS solution and mixing at room temperature for 1 h.

2.3 Preparation of chitosan nanoparticles loaded copper ions

Copper ions–loaded chitosan nanoparticles (CNCu) were involved in the electrostatic binding of chitosan with TPP anions (Saharan et al., 2015) (Table 1). 50 mL of chitosan was prepared by dissolving chitosan in acetic acid solution (1 %) and stirring for 30 min at room temperature. And this mixture was added to 50 mL $CuSO_4.5H_2O$ to form the cross-linking blended solution. Later, 40 mL TPP solution was dropped wise into this solution and stirred at room temperature for 1 h.

Table 1: The preparation of chitosan nanoparticles loaded copper ions

Sample codes	CS:TPP mass ratio	CS:TPP:Cu^{2+} mass ratio
CN1	5:0.8	5:0.8:0.00
CN2	5:1.6	5:1.6:0.00
CN3	5:2.0	5:2.0:0.00
CN4	5:3.0	5:3.0:0.00
CNCu1	5:2.0	5:2.0:0.25
CNCu2		5:2.0:0.30
CNCu3		5:2.0:0.50
CNCu4		5:2.0:1.00

2.4 Fourier transform infrared spectroscopy (FT-IR)

The spectra of chitosan-based nanoparticles solution were discovered with an FT-IR spectrometer (Alpha II, Bruker, Germany) at a resolution of 4 cm^{-1} with a range of 400-4,000 cm^{-1}.

2.5 Field-Emission Scanning Electron microscope (FE SEM)

The chitosan-based nanoparticles were observed and analyzed by FE-SEM. The FE-SEM images were captured using S4800, Hitachi, Japan High Resolution, at an accelerating voltage of 10 kV.

2.6 Dynamic Light Scattering (DLS)

The mean particle size, zeta potential (ζ) of nanoparticles, and polydispersity index (PDI) were measured by dynamic light scattering (DLS) experiments at 25 °C using Malvern Instruments Zetasizer Nano ZS90.

2.7 Antioxidant activity

DPPH radicals were used to determine the antioxidant activity of various treatments. 10 mL of each sample was reacted with 100 mL of radical solution (0.1 M) and sonication ins the dark for 30 min at room temperature. The absorbance of the resulting mixtures was determined using UV-Visible Spectroscopy 754 (Stech International) at 517 nm, following the method described by (Hasheminejad and Khodaiyan, 2020). The number of free radicals scavenged (% DPPHsc) was measured by the formula:

$$DPPHsc\ (\%) = \frac{Ac - As}{Ac} \times 100 \quad (1)$$

where Ac is the absorbance of the control sample, and As is the absorbance of the nanoparticle sample.

2.8 Antifungal activity

Yeasts and molds are the most prevalent and can significantly impact the quality of food products (Vella et al., 2023). The microbial enumeration was conducted by spreading 0.1 mL of serial dilutions of homogenate

chitosan-based nanoparticles on a Rose Bengal Chloramphenicol (RBC) agar plate and incubating at 25 °C for 5 days. The data were reported as the number of colony-forming units per gram of the product (CFU/g) (Bazargani-Gilani et al., 2015).

3. Results

3.1 Synthesis and Characterization

The spectra of the characteristic absorption bands of chitosan are shown in Figure 1, such as a broad band between 3000 and 3600 cm^{-1} (−OH and $-NH_2$ stretching), 1580 cm^{-1} (amide I), and 1031 cm^{-1} (C−O−C stretching) (Hosseini et al., 2013). With chitosan nanoparticles, the amide I peak ($-NH_2$ bending) shifted from 1631 to 1539 cm^{-1}, while new peaks emerged at 1251 cm^{-1} (C−O−C stretch) and 1539 cm^{-1} (amide II), indicating the formation of an ionic complex between the NH_3^+ group and the phosphate groups (Hosseini et al., 2013). The spectra of CNCu revealed the bending peaking at 1539 and 1621 cm^{-1} (N−H) were overlapping and shifted to 1638 cm^{-1} (Mekahlia and Bouzid, 2009). With the wavelength range of 3000 and 3600 cm^{-1}, the stretching vibrations of $-NH_2$ and -OH displayed a broader peak attributed to a reduction in hydrogen bonding (Qi et al., 2005). The findings implied that the N−H groups of chitosan bond with copper ions.

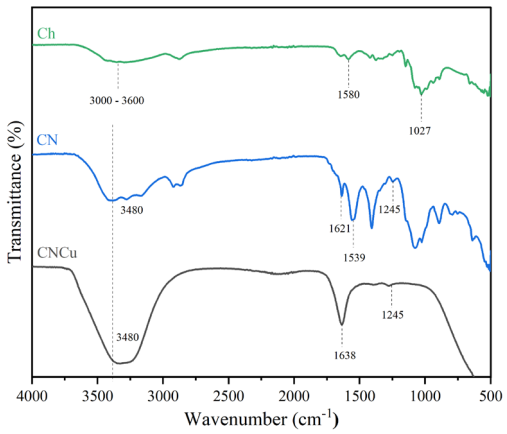

Figure 1: FT-IR spectra of chitosan, chitosan nanoparticles, and chitosan loaded copper ions

The SEM images of CN and CNCu are presented in Figure 2. CN can form nanostructures in isolated nanoparticles. When adjusting the ratios of the components, both CN and CNCu exhibit a smoother and more spherical morphology. This uniform distribution may allow for a more controlled release of copper ions, resulting in more effective and stable agent delivery. The smoother surface may reduce the likelihood of agglomeration and improve the stability of the nanoparticles.

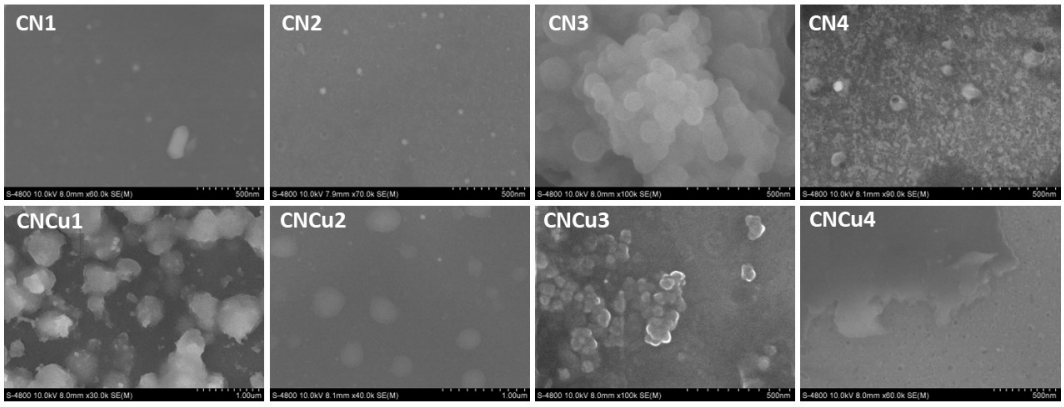

Figure 2: FE-SEM images of chitosan nanoparticles and chitosan-loaded copper ions

Figure 3 shows the TEM image of chitosan nanoparticles. TEM facilitated the determination of the size and surface morphology of chitosan nanoparticles in solution, with results consistent with the mean diameter

obtained through DLS analysis. The observed nanoparticles exhibited a spherical shape and a narrow size distribution, ranging from 5 to 100 nm. The findings highlight the similarity between TEM and DLS measurements when analyzing liquid samples.

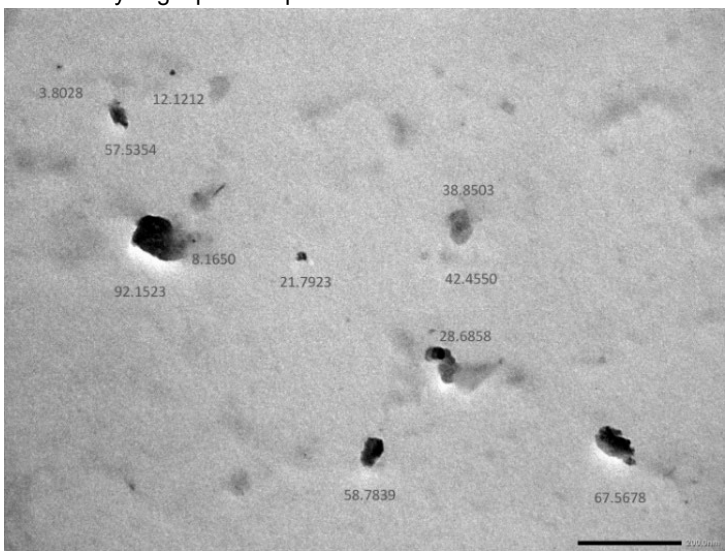

Figure 3: TEM image of chitosan nanoparticles

The results of average particle size, zeta potential, and PDI of chitosan-base nanoparticles are presented in Table 2. These parameters are crucial indicators for assessing the stability of nanoparticles in the solution. The optimized ratio of Ch/TPP is 5:2, resulting in a particle size of CN measuring 118.23 ± 0.58 nm. With this ratio, the particle size of CNCu exhibited a decreasing trend upon the addition of copper ions. The mean particle size of CNCu4 was 166.65 ± 7.36 nm with moderate zeta potential (22.6 mV). The zeta potential value of CNCu indicates greater dramatically high stability. According to (Li et al., 2015), colloidal systems with zeta potential values above 30 mV are stable due to the strong repulsive forces that prevent particle agglomeration. The concentrations of active agents with CN are inversely proportional to the zeta-potential values due to the higher forming agglomerates or decreasing availability of free amine groups on the surface of nanoparticles resulting from cross-linking interaction (Granata et al., 2021). The monodisperse distribution of CNCu, indicated by a PDI ranging from 0.3 to 0.45 (< 0.5), demonstrates the stability of the nanoparticles (Choudhary et al., 2017).

Table 2: The average size, ζ potential, and PDI of chitosan nanoparticles and chitosan-loaded copper ions

Sample codes	Mean particle size (nm)	Zeta potential (mV)	PDI
CN1	153.15 ± 18.51^{e}	41.05 ± 4.53^{a}	0.698 ± 0.104^{a}
CN2	139.67 ± 2.62^{ef}	39.20 ± 0.10^{ab}	0.421 ± 0.049^{b}
CN3	118.23 ± 0.58^{f}	40.56 ± 1.88^{ab}	0.459 ± 0.041^{b}
CN4	302.50 ± 14.05^{a}	26.50 ± 1.06^{de}	0.321 ± 0.056^{b}
CNCu1	214.67 ± 4.98^{bc}	34.33 ± 0.38^{bc}	0.379 ± 0.042^{b}
CNCu2	216.23 ± 13.04^{b}	31.16 ± 0.90^{cd}	0.430 ± 0.060^{b}
CNCu3	187.96 ± 4.66^{cd}	28.03 ± 0.91^{cd}	0.393 ± 0.047^{b}
CNCu4	166.65 ± 7.36^{de}	20.93 ± 1.75^{e}	0.337 ± 0.060^{b}

Note: The statistical difference between mean values with different lowercase superscripts within a column was determined using Tukey's test ($P < 0.05$), n = 3.

3.2 Antioxidation activity

The DPPH radical scavenging activity of CN and CNCu is shown in Figure 4. The free amino group of chitosan was attributed to the antioxidant properties of CNCu. With chitosan nanoparticles, the scavenging activity increased considerably as the STPP concentration decreased. The highest scavenging activity recorded was 75.89 ± 1.00 %, which closely aligns with the findings of MubarakAli et al. (2018), with a value of 76.2 %. The DPPH scavenging activity of the solutions increased markedly with increasing copper concentrations to the ratios CS:TPP:Cu^{2+} = 5:2:0.3 and gradually reduced. The antioxidant activity of chitosan nanoparticles with and without copper loaded was strongly related to the free amino group of chitosan (Divya et al., 2018).

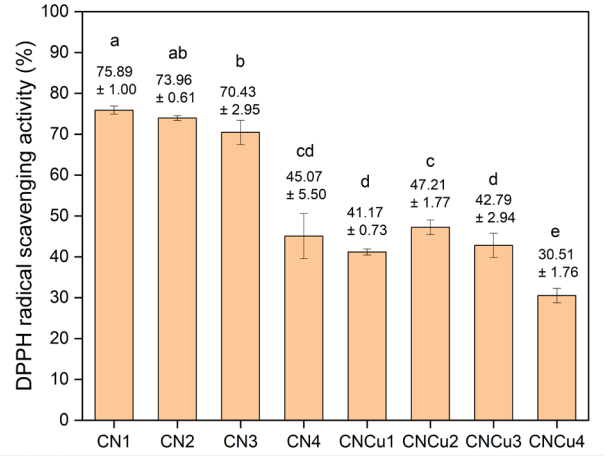

Figure 4: DPPH results of chitosan nanoparticles and chitosan-loaded copper ions

3.3 Antimicrobial activity

The impact of chitosan-based coatings on the inhibition of microbial stored at 4 ± 0.5 °C is presented in Table 3. Combining chitosan and copper ions is expected to display high results due to the individual antifungal properties of these agents. CNCu4 exhibited significantly enhanced antifungal activity without molds and yeasts (0 CFU/g). The fungal control can be ascribed to chitosan, which intervenes in modulating cell membrane permeability. This could be initiated by electrostatic interactions between positively charged chitosan and the fungal plasma membrane phospholipids carrying negative charges (Wu et al., 2019). CNCu is predicted to have high antifungal activity due to the properties of copper ions (Brunel et al., 2013). Copper ions can disrupt fungal cell membranes, generating reactive oxygen species and causing oxidative damage (Borkow and Gabbay, 2005). Positive zeta potential boosts antifungal activity by enhancing electrostatic interactions with membranes (Saharan et al., 2013).

Table 3: Inhibitory effect of chitosan-based nanoparticles

Sample codes	Molds and yeasts (CFU/g)
CN3	10^4
CNCu4	0

4. Conclusions

This study synthesized chitosan-based nano-coatings with copper via modified ionic gelation. Characterization of the nanoparticles revealed low polydispersity index values and moderate to high zeta potential values. The morphology of the nanoparticles was found to be spherical. The solutions of CN1 (75.89 ± 1.00 %) and CNCu2 (47.2 ± 1.77 %) showed the best antioxidant capacity in the tested samples. CNCu demonstrated superior antimicrobial activity against yeasts and molds with 0 CFU/g. These chitosan-based nano-coatings offer a promising solution for sustainable preservation strategies in the food industry. In future studies, it will be necessary to investigate the potential application of copper ions in fruit preservation, including examining their effects on fruit quality, toxicity and determining optimal release concentrations.

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