

Article

Eco-Friendly In Situ Fabrication of Silver Nanoparticle-Loaded Chitosan Nanogels for Antibacterial Applications

Tianji Li ^{1,2,†}, Minghui Zhao ^{1,†}, Luohui Wang ¹, Delong Dai ¹, Youming Dong ³ , Fei Xiao ^{4,*}, Cheng Li ¹ , Xiuhong Zhu ^{1,*} and Jianwei Zhang ^{1,*}

¹ College of Forestry, Henan Agricultural University, Zhengzhou 450046, China

² School of Earth and Space Science and Technology, Wuhan University, Wuhan 430072, China

³ College of Materials Science and Engineering, Nanjing Forestry University, Nanjing 210037, China

⁴ Hunan Academy of Forestry, Changsha 410018, China

* Correspondence: xiaofly2030@163.com (F.X.); zhuxiuhong@henau.edu.cn (X.Z.); zhangjianwei@henau.edu.cn (J.Z.)

† These authors contributed equally to this work.

Abstract

Eco-friendly chitosan nanogels (CSNG) with an average diameter of 48.5 nm were synthesized via alkali/urea dissolution and employed as templates for in situ silver nanoparticle fabrication. Silver nanoparticle size was controlled by adjusting CSNG to AgNO₃ mass ratios, with the optimal ratio of 18:1 producing ultrasmall particles of 3.72 nm, uniformly dispersed in the matrix. The nanocomposites demonstrated superior antibacterial activity, with inhibition zones of 14.3 mm against *E. coli* and 12.1 mm against *S. aureus*, significantly exceeding pure CSNGs at 7.4 mm and 6.9 mm, respectively. Rheological analysis revealed shear-thinning behavior, with viscosity decreasing from 450 Pa·s to 0.1 Pa·s, confirming excellent injectability. Cytotoxicity evaluation showed cell viability exceeding 82.3% at 100 µg/mL, which was substantially superior to conventional silver formulations. Thermogravimetric analysis and FTIR spectroscopy verified enhanced thermal stability and coordination interactions between chitosan and silver species. This green synthesis approach yields injectable, size-tunable nanocomposites with combined antibacterial efficacy and biocompatibility for biomedical applications.

Keywords: Chitin; nano-hydrogel; antibacterial property; silver nanoparticles

1. Introduction

Antibiotics have played a pivotal role in the early control and treatment of bacterial infections and are widely regarded as one of the most significant breakthroughs in modern medicine. However, their widespread misuse and overuse have led to the rapid emergence of antibiotic-resistant pathogens, posing a serious threat to global health. The rise in superbugs—bacteria resistant to multiple antibiotics—has become an unprecedented challenge to humanity [1,2]. A UK government report predicts that by 2050, antimicrobial resistance could cause up to 10 million deaths annually worldwide. Since the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported, bacteria that are resistant to nearly all existing antibiotics have continued to emerge [3]. To combat this escalating crisis, researchers have intensified efforts to develop new and effective antimicrobial agents. Among various alternatives, Silver (Ag) has garnered considerable attention due to its broad-spectrum antimicrobial agent and strong efficacy against drug-resistant microorganisms [4]. Currently, silver-based materials are extensively employed



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in medical devices, wound dressings, surgical tools, water filtration systems, food packaging, and antimicrobial coatings [5,6]. Particularly, silver nanomaterials exhibit unique physicochemical properties arising from their nanoscale dimensions and distinct atomic arrangements, which lie between the molecular and solid states. These characteristics confer a quantum size effect, a large specific surface area, and enhanced reactivity, leading to superior antimicrobial performance compared to conventional silver compounds [7,8]. Consequently, silver nanoparticles (AgNPs) demonstrate potent activity against a broad range of pathogens, including bacteria, fungi, and mycoplasma [9,10], making them a promising solution for combatting antibiotic-resistant infections.

Silver nanoparticles can be synthesized through various physical and chemical methods [11]. The most commonly used approach involves the chemical reduction of Ag ion solutions with reducing agents, such as sodium borohydride, sodium citrate, or ascorbic acid [12]. In this process, silver precursors are first reduced to silver atoms, which then form clusters and eventually grow into nanoparticles. However, due to their inherently high surface energy, AgNPs are prone to aggregation [13]. To prevent this, stabilizers are typically added to the reaction system. Nonetheless, the simultaneous use of stabilizers and reducing agents can introduce toxicity and environmental pollution [14]. In contrast, green synthesis methods offer an environmentally benign alternative, avoiding hazardous reagents while maintaining good control over particle formation. These methods include mixed polymetallic oxide reduction, polysaccharide-mediated reduction, radiation reduction and bioreduction. In the mixed polymetallic oxide method, polymetallic oxide derivatives serve simultaneously as reducing agents for silver ions and as stabilizers for the resulting nanoparticles [15]. Michalcova et al. [16], for example, utilized plant extracts that were rich in proteins and bioactive compounds as both reducing and stabilizing agents to produce stable, shape-controlled AgNPs. Similarly, the Tollens method utilizes the reduction of $\text{Ag}(\text{NH}_3)_2^+$ by sugars to produce AgNPs with particle sizes ranging from 50 to 200 nm, including smaller Ag particles of 20–50 nm and colloids of various shapes [17].

In recent decades, chitosan-based materials, derived from the natural polysaccharide chitosan, have emerged as a major focus in antimicrobial research [18]. Chitosan exhibits broad-spectrum antimicrobial activity, effectively inhibiting numerous bacterial and fungal species [19]. To enhance its antimicrobial efficacy, chitosan can be utilized as both a reducing and stabilizing agent for the synthesis of AgNPs, thereby combining the antimicrobial mechanisms of both components. In this study, chitosan was dissolved in an alkali/urea system and regenerated through high-speed stirring to form chitosan nanogels (CSNGs). These nanogels were then employed as templates for the in situ synthesis of AgNPs. The incorporation of silver endowed the CSNGs with enhanced bactericidal properties, while the chelating effect of chitosan moderated the release of silver ions, thereby reducing their potential cytotoxicity [20]. During synthesis, the amino groups in chitosan reduced Ag ions to AgNPs, while the nanogel acted as a stabilizer to prevent aggregation [21–23]. The resulting CSNGs@AgNPs were comprehensively characterized using Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), and transmission electron microscopy (TEM). Their cytocompatibility was evaluated through MTT assays, and their antimicrobial performance was also systematically examined. The primary objective of this work is to develop an eco-friendly, injectable chitosan nanogel platform for in situ synthesis of size-tunable silver nanoparticles with enhanced antibacterial activity and good biocompatibility. Specific objectives include the following: (1) establishing a green synthesis method without external crosslinkers or organic solvents; (2) achieving in situ controlled synthesis of AgNPs, utilizing chitosan amino groups as both reducing agents and stabilizers; (3) systematically examining the influence of the CSNG/AgNO₃ mass ratio on AgNPs properties; (4) evaluating antibacterial efficacy against Gram-negative and

Gram-positive bacteria; (5) assessing biocompatibility through in vitro cytotoxicity experiments; and (6) exploring application potential for wound dressings, tissue engineering, and biomedical fields.

2. Results and Discussion

2.1. Characterization of Chitosan Nanogels

After dissolving chitosan in a KOH/LiOH/urea solution, the heat generated during mechanical stirring induced gelation. In this process, without adding any cross-linking agents, chitosan molecules self-assembled into nanoscale gels driven by thermal energy, while high-speed shear forces ensured uniform dispersion.

The microstructure of the resulting chitosan nanogels was observed by high-resolution transmission electron microscopy. As shown in Figure 1a, the nanogels exhibited a spherical morphology with diameters on the order of several tens of nanometers, although partial aggregation was also observed. The particle size distribution (Figure 1b) revealed sizes ranging from 25 to 70 nm, with an average diameter of approximately 46 nm.

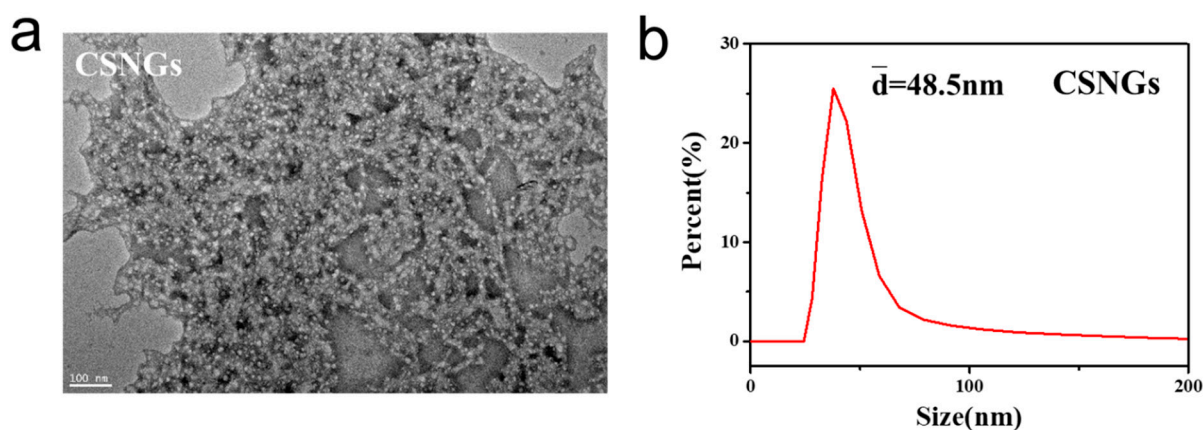


Figure 1. (a) Transmission electron microscopy (TEM) image and (b) particle size distribution of chitosan nanogels.

In order to investigate whether the preparation process altered the chemical structure, crystallinity, and thermal stability of chitosan, XRD, FTIR, and TGA were carried out on both the pristine chitosan powder and the prepared chitosan nanogels, as shown in Figure 2. The XRD patterns revealed that the pristine chitosan powder exhibited two distinct diffraction peaks at 12° and 21° [24]. The chitosan nanogels displayed similar diffraction peaks without any noticeable shift, indicating that the dissolution and regeneration process did not change the crystalline structure of chitosan. As shown in Figure 2b, the FTIR spectra of the chitosan raw material displayed characteristic peaks at 3480 cm^{-1} and 3264 cm^{-1} , corresponding to O-H and N-H stretching vibrations [25,26], respectively; a C-O stretching vibration at 1062 cm^{-1} [27]; an N-H bending vibration at 1598 cm^{-1} [28]; and polysaccharide absorption bands at 1065 cm^{-1} and 1153 cm^{-1} [29]. A comparison between the spectra of chitosan powder and chitosan nanogels revealed no new peaks, suggesting that the chemical structure of chitosan remained unchanged during nanogel preparation. However, broadening of the O-H and N-H stretching bands indicated modifications in the intermolecular hydrogen-bonding network. These results collectively indicate that the regeneration process primarily affected the physical interactions while preserving the intrinsic chemical and crystalline structures of chitosan.

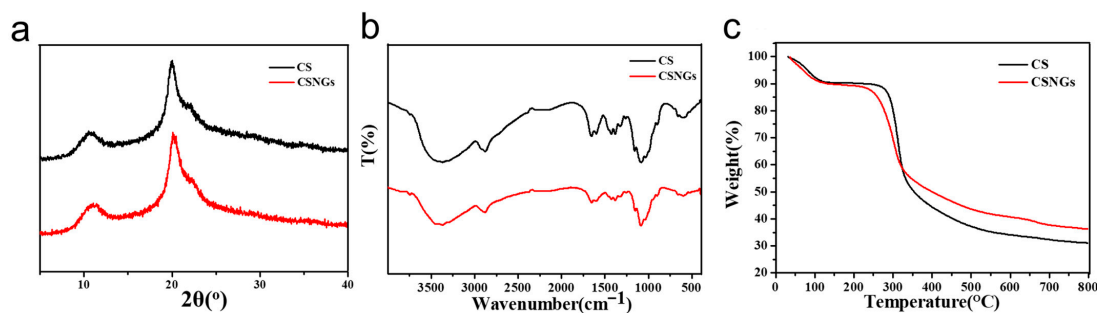


Figure 2. (a) XRD, (b) FTIR and (c) TG analyses of chitosan powder and nanogels.

The thermal stability of pristine chitosan powder and chitosan nanogels was evaluated using thermogravimetric (heat loss) analysis, as shown in Figure 2c. Both samples exhibited a slight weight loss between 30 and 130 °C, which was mainly due to the evaporation of adsorbed and intercalated water, without affecting the molecular structure of chitosan [30]. The second stage represents the main thermal decomposition phase, occurring between 271 and 432 °C for pristine chitosan and 218–412 °C for chitosan nanogels, corresponding to the breakdown of polymeric chains and a pronounced reduction in mass. At higher temperatures (550–800 °C), the residual masses of pristine chitosan and chitosan nanogels were approximately 36% and 33%, respectively, indicating a slightly lower char yield and thermal stability for the nanogels.

The rheological behavior of chitosan nanogels is shown in Figure 3. Dynamic rheological testing was used to evaluate the linear viscoelasticity of the nanogels through a strain sweep, as presented in Figure 3a. Within the linear viscoelastic region, the storage modulus (G') and loss modulus (G'') remained nearly constant, with G' being significantly higher than G'' . This indicates that the nanogels exhibit dominant elastic (gel-like) behavior under small deformations (strain < 2%). However, once the strain exceeded 2%, G' decreases sharply, suggesting the breakdown of the gel network structure [31]. At larger strains (above 40%), G' became lower than G'' , reflecting a transition from solid-like to liquid-like behavior [32,33]. Figure 3b shows the shear viscosity of chitosan nanogels as a function of shear rates (0–100 s^{-1}). As the shear rate increases from 0.1 s^{-1} to 100 s^{-1} , the viscosity decreases from approximately 450 Pa·s to approximately 0.1 Pa·s, corresponding to a reduction of about 99%. This significant shear-thinning facilitates administration via syringe, with the material flowing easily under high shear conditions while rapidly recovering its viscosity after injection. The nanogels displayed a pronounced shear-thinning behavior due to the disruption of the internal gel network. The shear-thinning property imparts good injectability to chitosan nanogels, broadening their potential applications in biomedical and pharmaceutical fields [34].

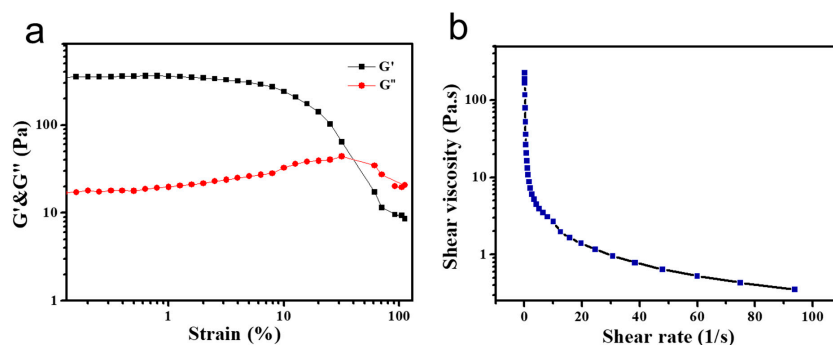


Figure 3. (a) Storage modulus (G') and loss modulus (G'') versus angular frequency of CSNGs and (b) viscosity as a function of shear rate for CSNGs dispersions.

2.2. Effect of Synthesis Conditions on the Morphology of AgNPs in CSNGs@AgNPs

The amino groups in chitosan possess an inherent reducing ability, enabling the formation of chitosan nanogel–silver nanoparticle (CSNGs@AgNPs) composites through the reaction of AgNO_3 with chitosan nanogels at different ratios [35]. The microscopic morphology of the resulting composites was examined using high-resolution transmission microscopy. As shown in Figure 4, the CSAg31 sample exhibited non-uniform AgNPs particle sizes, which were primarily spherical, with a noticeable agglomeration of larger particles. In comparison, the CSAg61 sample exhibited fewer large particles and a higher proportion of smaller, more uniformly distributed AgNPs. With a further decrease in AgNO_3 concentration, the CSAg121 sample primarily contained AgNPs with particle sizes within 20 nm. Notably, the CSAg181 sample demonstrated uniformly dispersed, well-defined, and homogeneously distributed spherical AgNPs of small size, indicating that a lower AgNO_3 concentration favored the formation of finer and more stable nanoparticles within the chitosan nanogel matrix.

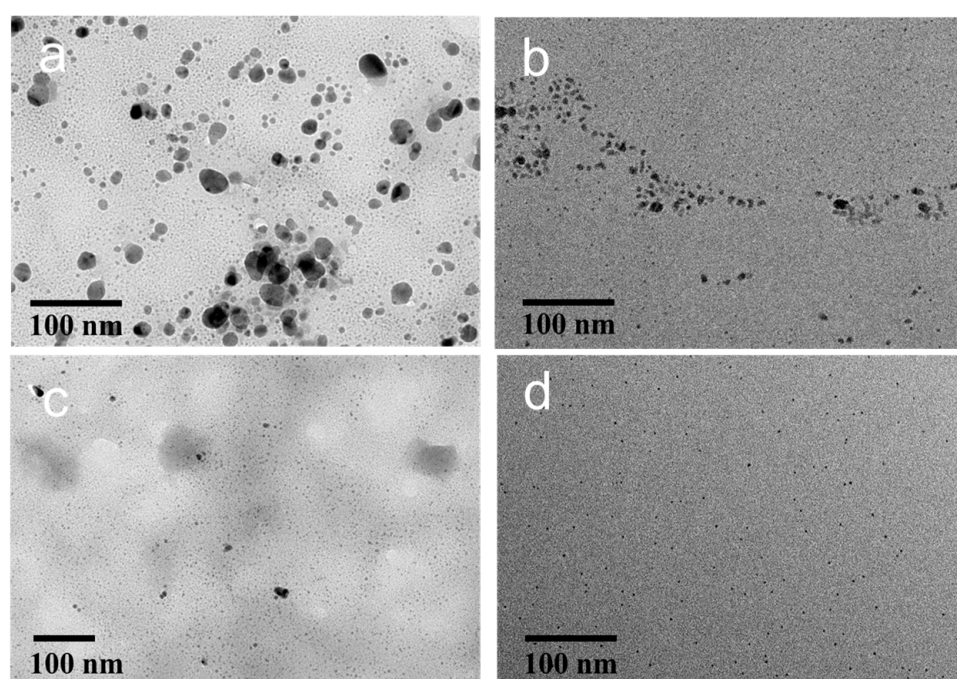


Figure 4. TEM images and photographs of CSNGs@AgNPs obtained from CSNGs with different AgNO_3 ratios: (a) CSAg31, (b) CSAg61, (c) CSAg121, and (d) CSAg181.

The particle size distributions of AgNPs in CSAg31, CSAg61, CSAg121, and CSAg181 are shown in Figure 5. The AgNPs in CSAg31 exhibited a wide particle size range of 2–34 nm, containing both small and relatively large nanoparticles, resulting in an average particle size of approximately 15 nm. In CSAg61, the size distribution narrowed to 3–19 nm with an average size of 9 nm, while CSAg121 showed particles that were mainly within 1–17 nm, most of which were smaller than 10 nm. The CSAg181 sample displayed the smallest and most uniform particles, ranging from 1 to 7 nm with an average size of about 4 nm. A comparative analysis of the four samples revealed that the AgNPs synthesized via chitosan nanogel reduction were predominantly spherical, and the AgNO_3 concentration significantly affected the particle size. As the AgNO_3 concentration decreased, the average AgNP size became smaller, and their dispersion improved, reducing aggregation tendencies [36].

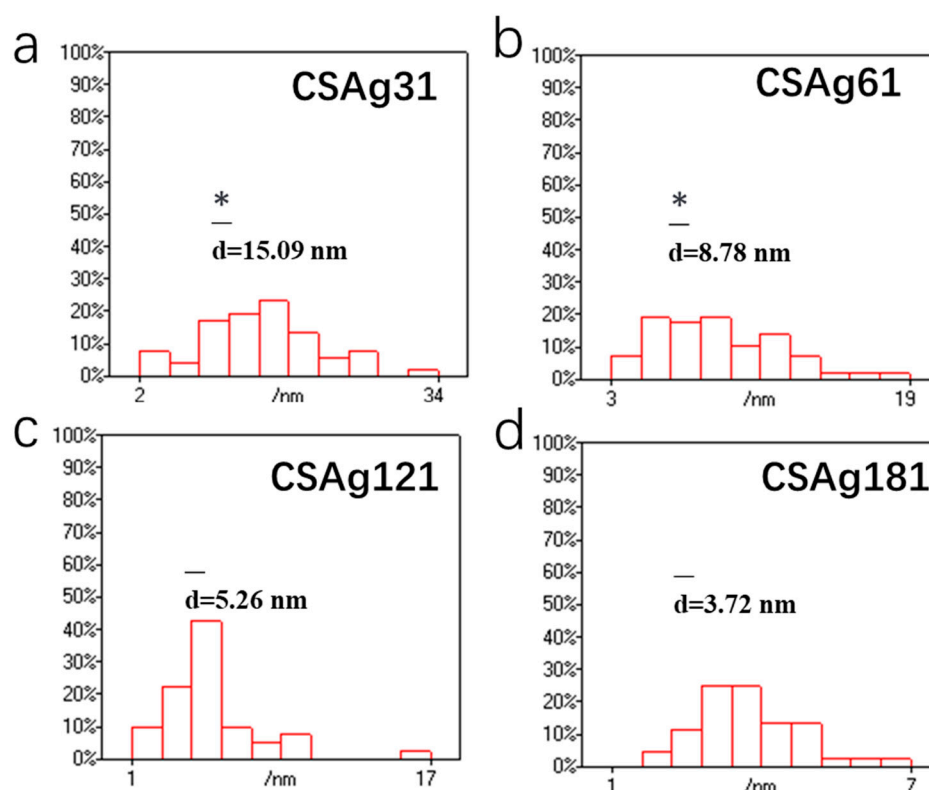


Figure 5. Statistical size distributions of AgNPs obtained from CSNGs with different AgNO_3 ratios: (a) CSAg31, (b) CSAg61, (c) CSAg121, and (d) CSAg181. Statistical significance was determined using one-way ANOVA with a post hoc Tukey test. Asterisks (*) denote significant differences from the CSAg181 at $p < 0.05$.

2.3. Structural and Thermal Characterization of CSNGs@AgNPs

Fourier transform infrared spectroscopy and thermogravimetric analyses were performed on the prepared CSNGs and the CSAg181, CSAg121, CSAg61, and CSAg31 composites, as shown in Figure 6. In the FTIR spectra, characteristic absorption peaks of CSNGs at 3480 cm^{-1} and 3264 cm^{-1} correspond to the stretching vibrations of O-H and N-H, respectively. Compared with CSNGs, the N-H stretching peaks in the CSNGs@AgNPs weakened or even disappeared, while the deformation vibration band of the amino group at 1593 cm^{-1} also diminished with an increasing AgNO_3 concentration. These observations indicate that the amino groups in chitosan participated in the reduction in Ag ions [37]. Moreover, the gradually broadening of the O-H absorption peak with increasing amounts of reduced AgNO_3 suggests that hydroxyl groups between the chitosan sugar units were also involved in the chelation of Ag^+ [38]. These FTIR results confirm that both reduction and chelation reactions occurred during the formation of AgNPs within the chitosan nanogel matrix. As shown in Figure 6b, TG analysis revealed two main stages of weight loss for all samples: $70\text{--}130^\circ\text{C}$, attributed to water evaporation, and $200\text{--}600^\circ\text{C}$, corresponding to chitosan decomposition. The CSNGs@ AgNO_3 composites exhibited lower total mass loss compared with pure CSNGs, owing to the presence of AgNPs. Furthermore, the residual mass increased with a higher AgNO_3 content, further verifying the successful formation of chitosan nanogel–silver nanoparticle composites. DTG curves (Figure 6c) showed that pure CSNGs exhibited a maximum degradation rate at 300°C ($-0.6\%/^\circ\text{C}$). Silver nanoparticle incorporation significantly enhanced thermal stability, with CS/Ag31 displaying the most gradual decomposition profile. The reduced weight loss rates indicate strong interactions between the silver and chitosan matrix, effectively suppressing thermal

degradation. Higher silver content corresponded to improved thermal stability, suggesting that Ag-chitosan coordination interactions provide thermal protection.

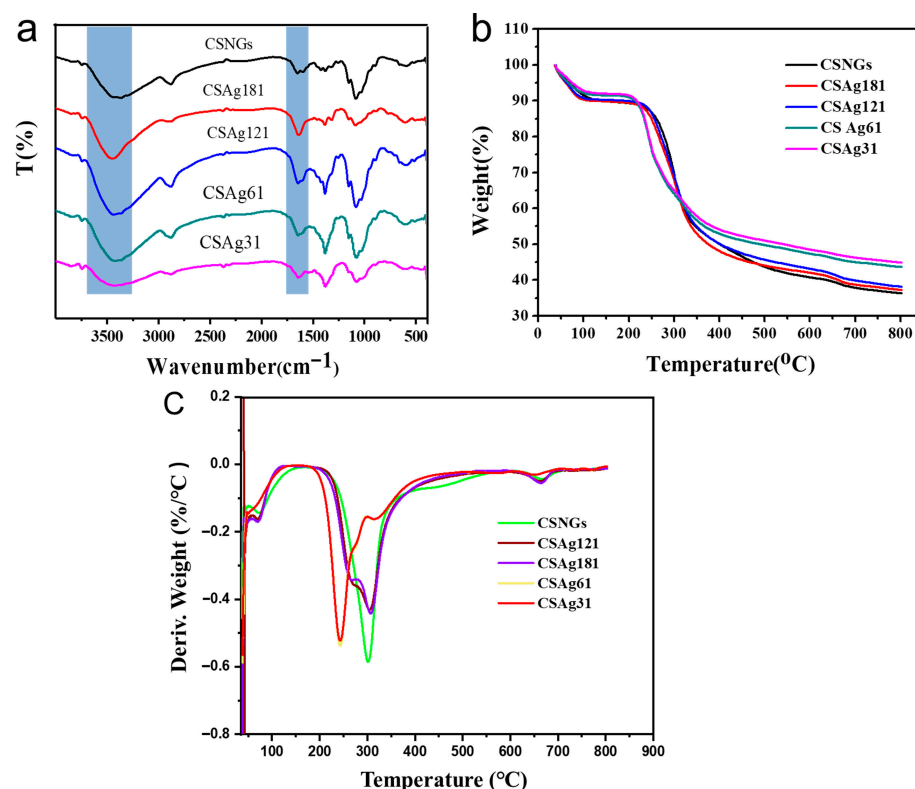


Figure 6. (a) FTIR spectra, (b) TG, and (c) DTG of CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31.

2.4. In Vitro Antimicrobial Properties of Chitosan Nanogels and CSNGs@AgNPs

Chitosan exhibits antibacterial activity primarily by disrupting bacterial cell membranes, resulting in the altered permeability and leakage of intracellular contents, such as DNA and other substances [39]. Ag nanoparticles mainly exert their bacteriostatic effect through the release of Ag ions, which readily penetrate bacterial cells and interfere with vital intracellular processes, including the inhibition of protein synthesis and suppression of DNA translation and transcription [40].

In this study, the antimicrobial activities of the samples were evaluated against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*, using the zone of inhibition method. As shown in Figure 7a, the CSNGs@AgNPs samples exhibited distinct inhibition zones against both bacteria, and the corresponding diameters measured using a micrometer are presented in Figure 7b. Pure chitosan nanogels exhibited inhibitory effects against both bacteria; however, their antimicrobial efficacy significantly increased upon incorporation of AgNPs. Interestingly, the bacteriostatic effect of the composites did not show a positive correlation with the AgNP content. The order of antibacterial activity was CSAg31 > CSAg181 > CSAg61 > CSAg121. The non-linear relationship between the Ag content, particle size, and antibacterial activity can be explained by multiple factors. CSAg181 contains the smallest and most uniformly dispersed AgNPs, resulting in the largest specific surface area. Despite its total Ag content being the lowest, the increased specific surface area facilitates more rapid and effective Ag⁺ release, and smaller nanoparticles have higher surface energy and more surface active sites, enhancing interaction with bacterial cell walls [41]. In contrast, TEM images show that AgNPs in CSAg31 and CSAg61 exhibit larger particle sizes and partial aggregation; such aggregation reduces the effective surface area and limits the number of active sites available for Ag⁺ release and direct bacterial contact. Consequently, CSAg31, despite having the

highest silver content, does not exhibit a proportionally enhanced antibacterial activity. Furthermore, FTIR analysis shows interactions between chitosan amino and hydroxyl groups with silver ions, indicating that chitosan modulates Ag^+ release kinetics through chelation, providing a sustained antibacterial effect rather than a burst release. Beyond a certain silver content threshold, the antibacterial efficacy plateaus, which is possibly due to limited sites on bacterial cell surface available for Ag^+ binding. CSAg31 and CSAg181 show similar inhibition zone diameters against *E. coli*, supporting this saturation hypothesis. Overall, these findings emphasize the importance of optimizing antibacterial performance through precise control of AgNPs size and dispersion, rather than simply increasing the silver content.

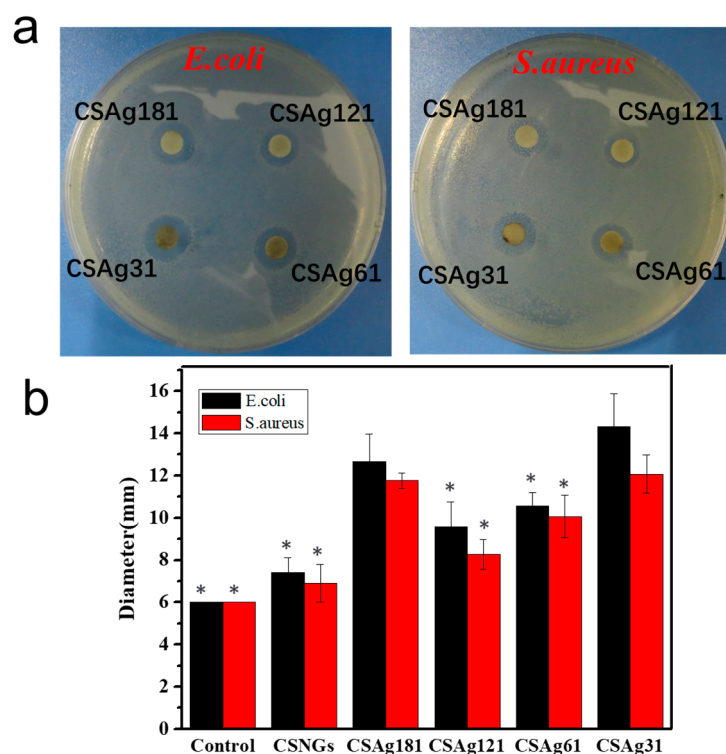


Figure 7. (a) Photographs and (b) bacterial inhibition zones of CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31 against *E. coli* and *S. aureus*. Error bars represent standard deviations (SD) from triplicate experiments. Statistical significance was determined using one-way ANOVA with a post hoc Tukey test. Asterisks (*) denote significant differences from the CSAg31 at $p < 0.05$.

In addition, the inhibitory effects varied between *S. aureus* and *E. coli*. All samples exhibited stronger inhibition against *E. coli* than against *S. aureus*. This difference can be attributed to the distinct structural characteristics of the bacterial cell walls: *S. aureus* possesses a thicker peptidoglycan layer that hinders the penetration of antimicrobial agents, whereas *E. coli* has a thinner outer membrane that allows for faster diffusion and more effective interaction with Ag^+ ions [42,43]. Notably, many previously reported silver nanoparticle formulations show relatively limited antimicrobial activity under comparable conditions. For example, Kora et al. [44] reported that silver nanoparticles synthesized by chemical reduction exhibited inhibition zones of only 7–9 mm against *E. coli* at similar test concentrations, representing a twofold lower efficacy compared to our system. Fan et al. [35] reported chitosan nanogels with in situ AgNPs but without quantitative size characterization. The advantages of our system include the following: small and uniformly dispersed AgNPs (average 4 nm in CSAg181), providing high surface area for Ag^+ release; green synthesis without external reducing agents or toxic chemicals; tunable AgNPs size through simple adjustment of CSNG/ AgNO_3 ratio; and an injectable gel formulation that

is suitable for localized antimicrobial therapy. These advantages position our system as a promising candidate for biomedical applications.

2.5. Cytocompatibility Studies of Chitosan Nanogels and CSNGs@AgNPs

The strong antimicrobial activity of AgNPs makes them highly attractive as antimicrobial agents; however, their potential cytotoxicity remains a key consideration for biomedical applications. Therefore, the cytocompatibility of chitosan nanogels and CSAG181 composites was preliminarily evaluated by using an MTT assay. L929 fibroblast cells were co-cultured with CSNGs and CSAG181 at concentrations of 0, 10, 25, and 100 $\mu\text{g/mL}$ for 24, 48, and 72 h, respectively. The corresponding cell viability results are shown in Figure 8. Across all tested concentrations (0–100 $\mu\text{g/mL}$), cell survival exceeded 80% for both CSNGs and CSAG181, indicating minimal cytotoxic effects. Overall, CSNGs exhibited slightly higher cell viability than CSAG181.

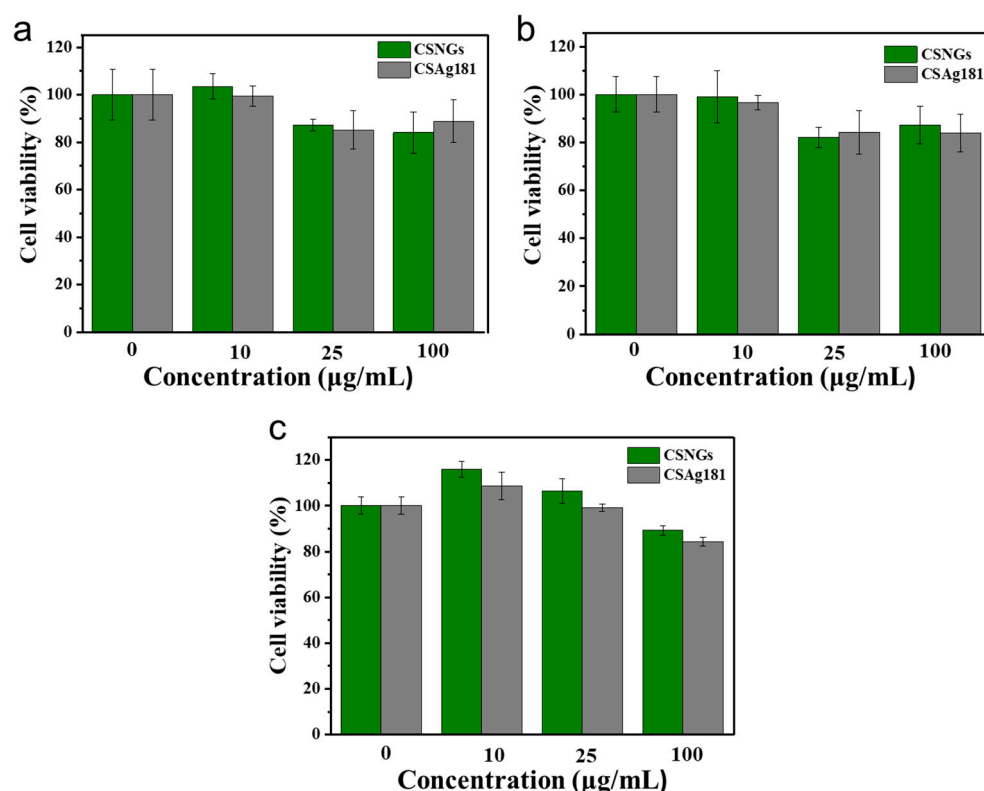


Figure 8. Effect of different concentrations of CSNGs and CSAG181 on the viability of L929 cells after incubation for (a) 24 h, (b) 48 h, and (c) 72 h.

CSAG181 demonstrated excellent cytocompatibility, with cell viability of $82.3 \pm 3.5\%$ at 100 $\mu\text{g/mL}$ against L929 fibroblasts after 24 h incubation, which is significantly superior to most conventional AgNP formulations. For comparison, AshaRani et al. [45] reported that free silver nanoparticles (6–20 nm) exhibited only 40–50% cell viability at 100 $\mu\text{g/mL}$ against human fibroblasts, indicating severe cytotoxicity.

The markedly superior cytocompatibility of the present nanogel system can be attributed to several critical factors. First, the chitosan nanogel matrix provides a controlled, sustained Ag^+ release, preventing the burst release phenomenon that is commonly observed with free AgNPs that causes acute cytotoxicity. Second, the ultrasmall AgNPs (~ 4 nm) are strongly stabilized within the chitosan network, preventing the particle aggregation and uncontrolled dissolution that contribute to cellular damage [46]. Third, the green synthesis approach eliminates the residual toxic chemicals (e.g., reducing agents, surfactants) that are present in conventional AgNP preparations. Fourth, chitosan itself ex-

hibits excellent biocompatibility and can chelate excess Ag^+ ions, further reducing cytotoxic effects. This combination of potent antimicrobial activity with excellent biocompatibility positions our system as a significantly safer alternative to conventional silver formulations for clinical applications.

3. Conclusions

In this study, chitosan was successfully regenerated for the first time, using an alkali/urea dissolution system to produce nanogels (CSNG), with an average particle size of about 48 nm. Rheological analyses revealed that the nanogels exhibited pronounced shear-thinning behavior and excellent injectability. Silver nanoparticles (AgNPs) were synthesized *in situ*, using the chitosan nanogels as both reducing and stabilizing agents, with a particle size that was controllable via the CSNG-to- AgNO_3 ratio. The particle size of AgNPs decreased with a decreasing AgNO_3 concentration, with the CSAG181 sample (CSNGs-to- AgNO_3 mass ratio of 18:1) exhibiting an average particle size of only 4 nm. The cytotoxicity tests show that the chitosan nanogel–AgNPs composites possessed excellent biocompatibility. This study establishes a simple and green approach for producing injectable, size-tunable chitosan–AgNPs composites with strong antibacterial efficacy and potential biomedical applications.

4. Materials and Methods

4.1. Experimental Materials

The main materials and reagents used in this study are summarized in Table 1. All chemicals were of analytical grade and used as received, unless otherwise stated. Deionized water was employed throughout the experiments as the solvent for solution preparation and rinsing processes.

Table 1. Main experimental materials and reagents.

Reagent Name	Specification	Manufacturer
Chitosan	Industrial grade	Qingdao Yunzhou Biotechnology Co., Ltd., Qingdao, China
NaOH	AR	Sinopharm Group Chemical Reagent Co., Ltd., Shanghai, China
Urea	AR	Sinopharm Group Chemical Reagent Co., Ltd.
LiOH	AR	Sinopharm Group Chemical Reagent Co., Ltd.
HCl	35%	Sinopharm Group Chemical Reagent Co., Ltd.
NaClO	98%	Sinopharm Group Chemical Reagent Co., Ltd.
AgNO_3	AR	Sinopharm Group Chemical Reagent Co., Ltd.
Durboco modified Eagle medium	--	Yingjie Company, Shanghai, China
Penicillin	--	Yingjie Company, Shanghai, China
Streptomycin	--	Yingjie Company, Shanghai, China
Fetal bovine serum	--	Yingjie Company, Shanghai, China
Thiazolyl blue (MTT)	--	Yingjie Company, Shanghai, China
AGAR	--	Sinopharm Group Chemical Reagent Co., Ltd.
Beef paste	--	Sinopharm Group Chemical Reagent Co., Ltd.
Peptone	--	Sinopharm Group Chemical Reagent Co., Ltd.

4.2. Preparation of Chitosan Nanogels

A total of 2 wt% chitosan powder was dispersed in an aqueous solution containing 7 wt% KOH, 4.5 wt% LiOH and 8 wt% urea. After achieving a homogeneous dispersion, the mixture was frozen at $-30\text{ }^\circ\text{C}$ for 4 h in an ultra-low temperature freezer. The frozen sample was then thawed at room temperature and stirred thoroughly to ensure complete dissolution. This freeze–thaw process was repeated three times, resulting in a transparent

chitosan solution. The chitosan solution was centrifuged at $8000 \times g$ rpm for 10 min at room temperature, using a high-speed refrigerated centrifuge to remove any undissolved chitosan residues. The transparent chitosan solution was subsequently stirred at $12,000 \times g$ rpm for 30 min, using a high-speed homogenizer to produce chitosan nanogels (CSNGs) dispersion. The resulting CSNGs were dialyzed against ultrapure water for 7 days until a neutral pH was achieved.

4.3. Preparation of Chitosan–Silver Nanocomposites (CSNGs@AgNPs)

A suspension of CSNGs was mixed with varying amounts of a 10 mg/mL AgNO_3 solution to achieve a final CSNGs concentration of 10 mg/mL. The resulting mixtures were magnetically stirred continuously for 48 h at room temperature to allow for the in situ reduction and formation of silver nanoparticles (AgNPs) within the nanogel matrix. The 48 h reaction time was optimized through preliminary experiments. The reduction of Ag^+ by the chitosan amino groups reached completion after approximately 48 h, as evidenced by the color change from colorless to yellow-brown and subsequent stabilization of the suspension. Shorter reaction times (e.g., 24 h) resulted in incomplete reduction and inconsistent particle sizes, whereas extending the reaction time to 72 h did not result in any significant changes in particle size or distribution. After completion of the reaction, the resulting CSNGs@AgNPs suspensions were purified by centrifugation at $10,000 \times g$ rpm for 15 min. This purification process was repeated three times with deionized water washing to remove unreacted AgNO_3 . The purified products were stored at 4°C for subsequent use. The mass ratios of CSNGs to AgNO_3 were adjusted to 1:0, 18:1, 12:1, 6:1, and 3:1, and the corresponding samples were designated as CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31, respectively.

4.4. Characterization of CSNGs@AgNPs

Solutions of CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31 were each diluted to 0.01%, and 10 μL of each sample was dropped onto a copper grid and allowed to air-dry naturally. The morphology of the resulting samples was examined using high-resolution transmission electron microscopy (HR-TEM, JEOL JEM-2100, Tokyo, Japan). The chemical structures of pristine chitosan powder, CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31 were analyzed by Fourier transform infrared spectroscopy (Nicolet iS10, Thermo Fisher Scientific, Waltham, MA, USA), using the potassium bromide pellet method. Spectra were recorded with a resolution of 4 cm^{-1} over the range of $400\text{--}4000\text{ cm}^{-1}$. The thermal stability of the samples was evaluated using a thermogravimetric analyzer (SDT Q 600, TA Instruments, New Castle, DE, USA) with a heating rate of $10^\circ\text{C}/\text{min}$ from room temperature to 800°C under nitrogen atmosphere with a flow rate of $100\text{ mL}/\text{min}$ [22]. Approximately 5 mg of the freeze-dried sample was placed in alumina crucibles for each measurement. The nitrogen atmosphere was used to prevent oxidative decomposition and ensure thermal degradation behavior reflected the intrinsic stability of the materials. The crystalline structures were characterized by X-ray diffraction (XRD) using a SmartLab 9 diffractometer (Rigaku, Akishima-shi, Tokyo, Japan) with $\text{Cu K}\alpha$ radiation ($\lambda = 1.5406\text{ \AA}$). The diffraction patterns were recorded over a 2θ range of $5\text{--}80^\circ$, with a scan rate of $5^\circ/\text{min}$ and a step size of 0.02° . The operating voltage and current were 40 kV and 40 mA, respectively. Freeze-dried powder samples were used for XRD analysis. Additionally, the particle size distribution of the chitosan nanogels was measured using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, Worcestershire, UK).

4.5. Rheological Analysis of Chitosan Nanogels

The steady and dynamic rheological properties of the chitosan nanogel dispersions were analyzed using a DHR rheometer (TA Instruments, New Castle, DE, USA). The mold

for the rheometer was a circular vertebra (40 mm in diameter), and the gap between the plates was maintained at 1 mm. The samples were initially subjected to a strain sweep test to determine the linear viscoelastic region. Subsequently, at an angular frequency of 1 rad s^{-1} , the shear viscosity of the chitosan nanogels was measured across a shear rate range of $0\text{--}100 \text{ s}^{-1}$.

4.6. Antibacterial Experiment

The antibacterial properties of CSNGs and CSNGs@AgNPs were determined by the inhibition zones of the samples against Gram-positive bacteria, *Staphylococcus aureus*, and Gram-negative bacteria, *Escherichia coli*, at 37°C . Firstly, the two types of bacteria were activated on the agar medium and diluted with physiological saline to a concentration of $1 \times 10^5 \text{ CFU/mL}$. Then, $100 \mu\text{L}$ of bacterial suspensions were evenly spread onto beef-peptone agar medium. Then, circular filter paper disks with a diameter of 6 mm were prepared using a puncher and immersed in PBS, CSNGs, CSNGs, CSAg181, CSAg121, CSAg61, and CSAg31 solutions for 30 s. Each sample had a CSNGs concentration of 10 mg/mL but different CSNG/AgNO₃ mass ratios (18:1, 12:1, 6:1, 3:1), resulting in different total Ag contents. The disks were then removed and sterilized by ultraviolet irradiation for 30 min. After sterilization, the disks were placed onto the agar surfaces inoculated with the bacterial suspension and incubated at 37°C for 24 h. The diameters of the inhibition zone were measured using a micrometer and recorded. All experiments were conducted in triplicate.

4.7. Cytotoxicity Evaluation

The cytotoxicity of CSNGs and CSAg181 was assessed by using the standard MTT assay with rat fibroblasts (L929) [23]. Briefly, $200 \mu\text{L}$ of the cell suspension was inoculated into 96-well plates at a density of $3 \times 10^4 \text{ cells/mL}$ and incubated at 37°C for 24 h. Subsequently, different concentrations of the sample suspensions were added to the wells, while PBS served as the control group. The cells were then cultured for 24, 48, and 72 h, respectively. After each incubation period, $10 \mu\text{L}$ of MTT reagent was added to each well and incubated for 4 h. The supernatant was carefully removed, and the wells were rinsed twice with PBS. Then, $100 \mu\text{L}$ of DMSO was added to each well, and the plates were shaken in the dark for 10–15 min. The optical density (OD) of each well, which is directly proportional to the number of viable cells, was measured at 490 nm, using an ELISA microplate reader (DSX, Dynex Technologies, Chantilly, VA, USA). The relative cell proliferation rate was calculated as the cell survival rate compared to the control:

$$\text{Cell survival (\%)} = \frac{(\text{OD value for each concentration group} - 0 - \text{well OD value})}{\text{Negative control OD value} - 0 \text{ hole OD value}} * 100\%$$

Cytotoxicity was evaluated according to the toxicity grading criteria, where cell viability between 80 and 100% or above 100% was classified as Grade 0, 60–80% as Grade 1, 40–60% as Grade 2, 20–40% as Grade 3, and 0–20 as Grade 4. The negative control group consisted of cells cultured without any sample, while the blank (zero) well contained only the culture medium.

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