Received: 2 February 2023 | Revised: 27 May 2023 | Accepted: 19 June 2023

DOI: 10.1049/mna2.12172

Micro & Nano Letters

The Institution of Engineering and Technology WILEY

LETTER

A chitosan-based hydrogel containing zinc oxide nanoparticles as a carrier for improving antibacterial activity and controlling the release of antibiotics

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Funding information
Iran University of Medical Sciences, Grant/Award Number: 99-2-99-18890

Abstract

Microbial infections are considered one of the most important concerns of the world community. Developing drug delivery systems based on formulation of nanoparticles (NPs) with antimicrobial agents has shown beneficial effectiveness against microbial infections and related antimicrobial resistance. In this study, the authors prepared and characterized a chitosan-based hydrogel loaded with zinc oxide NPs for controlling the release of vancomycin and also improving its antibacterial effect. Characterization studies demonstrated that the developed biopolymeric hydrogel was able to sustain and control the release of vancomycin in response to acidic media for 96 h. Furthermore, antimicrobial studies showed significant and efficient antibacterial activity of prepared hydrogel against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Based on the obtained results, it can be concluded that the prepared chitosan hydrogel (CH) containing zinc oixde (ZnO) NPs has a desirable activity for controlling the release of vancomycin and improving its antibacterial properties.

1 | INTRODUCTION

In the early 20th century, the discovery of antimicrobial or antibiotic agents was a milestone in the field of pharmacy that led to a remarkable decline in mortality and morbidity [1]. However, long-time therapy or treating infections with high doses leads to drug resistance [2, 3]. Today, microbial resistance is considered one of the most important concerns of the world. The World Health Organization has declared that microbial resistance is one of the top 10 global public health threats for humanity [4]. Vancomycin as a complex tricyclic glycopeptide antibacterial agent is extensively used to treat gram-positive infections like meticillin-resistant *Staphylococcus Aureus* (MRSA) [5, 6]. High-dose administration and prolonged therapy with vancomycin enhance the risks of toxicity and aggravation of deleterious impacts [7–10]. Due to the undeniable beneficial effects of antibiotics, development of new drug delivery systems to maintain the beneficial effects of antibiotics and reduce their side effects is essential.

Inorganic nanoparticles (NPs) have shown antibacterial effects due to their distinctive physical and chemical properties and can interact with bacterial cells, modifying cell membrane penetration and impeding with molecular pathways [11–13]. Formulation of NPs with antibiotics exerts synergistic effects against bacteria, inhibits biofilm formation, and has been used to prevent multidrug-resistant organisms and combination of NPs and antimicrobial agents may be useful in fighting the current crisis of antimicrobial resistance [14, 15]. Recently, application of zinc oxide (ZnO) NPs in infection disease has been considered due to their potential biocompatibility over other metal oxides and also their remarkable antibacterial activities over a wide spectrum of bacterial species [16–19].

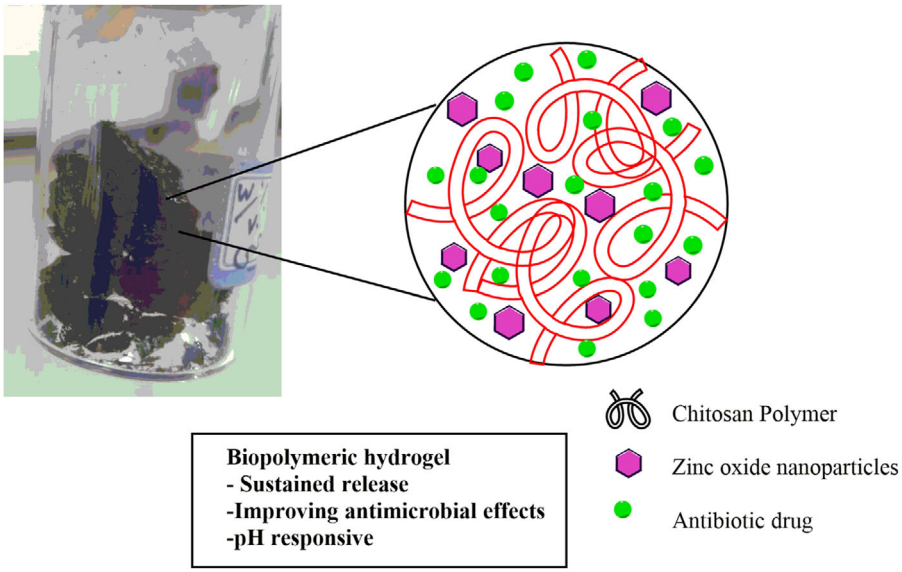

FIGURE 1 Preparation of chitosan hydrogel loaded with ZnO NPs for controlling release of vancomycin. NPs, nanoparticles; ZnO, zinc oxide.

For treatment of microbial infections, it is crucial that antimicrobial agents can be released in a sustained manner to efficiently treat and prevent biofilm formation [20]. Hydrogels have been used as carriers for antimicrobial agents and also instruments for co-delivery of antimicrobial agents to achieve synergistic effects. This co-delivery approach significantly reduces antibiotic toxicity by decreasing the required doses and administration intervals [21–24].

Chitosan is a biodegradable and biocompatible polymeric material from a natural source with high efficiency for preparing hydrogel carriers [25, 26]. Chemically crosslinked chitosan hydrogels (CHs) can be achieved by using genipin as a natural crosslinker whichits cytotoxicity is approximately 10,000 times less than of glutaraldehyde [27, 28]. According to the above mentioned facts, the main aim of the present study is the preparation and characterization of CH loaded with ZnO NPs and vancomycin for improvement of antibacterial effect and making pH-responsive hydrogel to avoid extra use of antibiotic (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Materials

Chitosan (MW: 127 kD, deacetylation degree: 97%) was purchased from Primex Co. Genipin and triton-100 were obtained from Sigma-Aldrich. Zinc acetate was purchased from Samchun pure chemical. Vancomycin was obtained from Afachemi company. All the other analytical grade reagents were obtained from Merck.

Bacterial *strains*: Standard strains of *Staphylococcus aureus* American type culture collection (ATCC) 6538 and *Pseudomonas aeruginosa* ATCC 15442 have been used from Iranian biological resource centre.

2.2 | ZnO nanoparticles preparation

25 mL of 0.2 N zinc acetate solution was prepared and poured into a flask and placed on a magnetic stirrer. 4.5-mL triton x-100 was added to the solution and incubated at room temperature for 24 h. Then NaOH 0.1 M was added to the prepared solution drop wise until the pH of solution reaches 9 by colour change and precipitation. The solution was stirred for 24 h to complete sedimentation. The solution was centrifuged with a rate of 8000 rpm for 30 min. The sediment was washed twice with deionised water and absolute ethanol, respectively, and dried in 60°C in the oven. Then the dried precipitate was calcined in a furnace at 400°C for 1 h whose colour was changed from white to grey.

2.3 | Nanoparticles characterization

Field emission scanning electron microscope (Hitachi S-4160, Germany) was used to investigate the size and surface morphology of prepared ZnO NPs. Furthermore, crystallinity of ZnO was studied using an X-ray diffractometer (XRD) (D4-BRUKER) fitted with a Cu-K*a* source.

2.4 | Hydrogel synthesis

Hydrogel were synthesized with genipin at different concentrations. Firstly, a 2% (w/v) chitosan solution was prepared by dissolving chitosan powder into 10 mL of acetic acid 1% and let it dissolve for 24 h under stirring. 100 mg of vancomycin was added into the prepared solution. After that, 10 mg of ZnO NPs were added and let it disperse completely for 24 h. Solution of genipin was then prepared by dissolving genipin powder in ethanol with different concentrations of

TABLE 1 Different hydrogel formulations.

Formulation names	Genipin (mg/mL)	Chitosan (mg)	ZnO nanoparticles (mg)	Vancomycin (mg)
CH2	2	200	–	100
CNH2	2	200	10	100
CH4	4	200	–	100
CNH4	4	200	10	100

CH, chitosan hydrogel; CNH, chitosan hydrogel contained ZnO NPs.

2 and 4 mg/mL. This solution was added to the chitosan solution and mixed for 30 min to form the hydrogel precursor solution. The precursor solution was sonicated for 30 min in the ultrasonic bath (Backer vClean) and then was dried in an oven for 24 h at 50°C. The same method was used for preparations of hydrogels without ZnO NPs, except adding ZnO NPs. Table 1 summarizes the details of hydrogel formulations.

2.5 | Hydrogel swelling analysis

The swelling profiles of different formulations of hydrogels were investigated in phosphate-buffered saline (PBS) (pH 7.4) and citrate buffer (pH 5.8) at 37°C during 24 h. At each time interval (30 min, 1, 2, 3, 4, and 24 h) hydrogel sample mass was recorded. Swelling behaviour of prepared hydrogels was calculated as a percentage using Equation (1), where Wf is the weight of the hydrogel at each time point and Wi is the initial dry weight of the hydrogel.

$$\text{Swelling}\,(\%) = ((\text{Wf} - \text{Wi})/\text{Wi}) \times 100 \quad (1)$$

2.6 | In vitro release study

In vitro release of vancomycin from different hydrogels was investigated at 37°C in two different pHs (PBS with pH 7.4 and citrate buffer with pH 5.8) under stirring. An appropriate amount of hydrogel (50 mg) was dispersed in 2 mL of buffer solution and then the obtained suspension was poured in a dialysis bag (molecular weight cutoff 12 kDa) and it was plunged in 50-mL buffer solution. At specified time intervals (0.5, 1, 2, 3, 4, 24, 48, 72, and 96 h), 2 mL of media was withdrawn and replaced by 2 mL of fresh media. The concentration of released vancomycin was measured by reading amount of UV absorption at 280 nm.

Furthermore, the released quantity of ZnO NPs from the hydrogel formulations was investigated in two different pHs (PBS with pH 7.4 and citrate buffer with pH 5.8). For this, 50 mg of hydrogel was dispersed in 2 mL of buffer solution and then the obtained suspension was poured in a dialysis bag (molecular weight cutoff 12 kDa) and it was plunged in 50-mL buffer solution. At specified time intervals (2, 12, 24, and 48 h), 2 mL of media was withdrawn and replaced by 2 mL of fresh media. The concentration of released ZnO NPs was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectro Arcos) [29]. All experiments were carried out in triplicates.

2.7 | Hydrogel characterization

The conjugation between amine group of chitosan polymer and C-OH group of genipin was confirmed by Fourier transform infrared (FTIR). The samples of chitosan polymer and CH were mixed with dried potassium bromide (KBr) separately and FTIR was carried out in the spectral range of 400 to 4000 cm^{-1} for each one.

Field-emission scanning electron microscopy (FESEM) (Tescan Mira) was used to evaluate the morphology of CH prepared with 2-mg genipin (as a selected formulation). The sample was mounted to the sample stub using double-sided carbon tape, and the images were obtained quickly to prevent sample shrinkage from drying.

2.8 | Antimicrobial studies

The agar disc diffusion method was employed to test the antibacterial activity of CH with 2-mg genipin [30]. Briefly, from an overnight culture of *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853, a suspension of the bacteria was prepared, diluted in nutrient broth (NB) (about 10^7 colony forming unit (CFU)·mL^{-1}), and then equally dispersed onto Müller-Hinton agar. 5-mm side squares of the hydrogel samples including CH, chitosan hydrogel contained ZnO NPs (CNH) and CH loaded by vancomycin and ZnO NPs (CNH-loaded vancomycin) were cut out and carefully arranged on agar petri dishes. The agar plates were incubated at 37°C and the diameters of the inhibition zones were measured after 24 h according to the Kirby–Bauer method [30]. Vancomycin was used as control and its concentration in all experiments was 6.5 and 12.5 μg/mL for antibacterial study against of *S. aureus* and *P. aeruginosa*, respectively.

Furthermore, bacterial killing assay (CFU assay) was used to assess the bacteria absolute load reduction values of prepared hydrogels. The hydrogel samples at concentrations of 0.5 and 0.7 mg/mL were prepared in NB to further explore the antimicrobial ability on *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853, respectively. The prepared tubes were inoculated with bacteria stock suspensions (10^7 CFU mL^{-1}) and incubated for 24 h at 37°C in 150 rpm. At the end of 24-h incubation, a series of 10-fold dilutions of the bacteria from each tube were made in PBS and equally dispersed onto nutrient agar plates in order to acquire viable counts. After overnight incubation, the CFU was finally calculated and expressed in a logarithmic scale [31]. All tests were performed in triplicate and reported by mean average values.

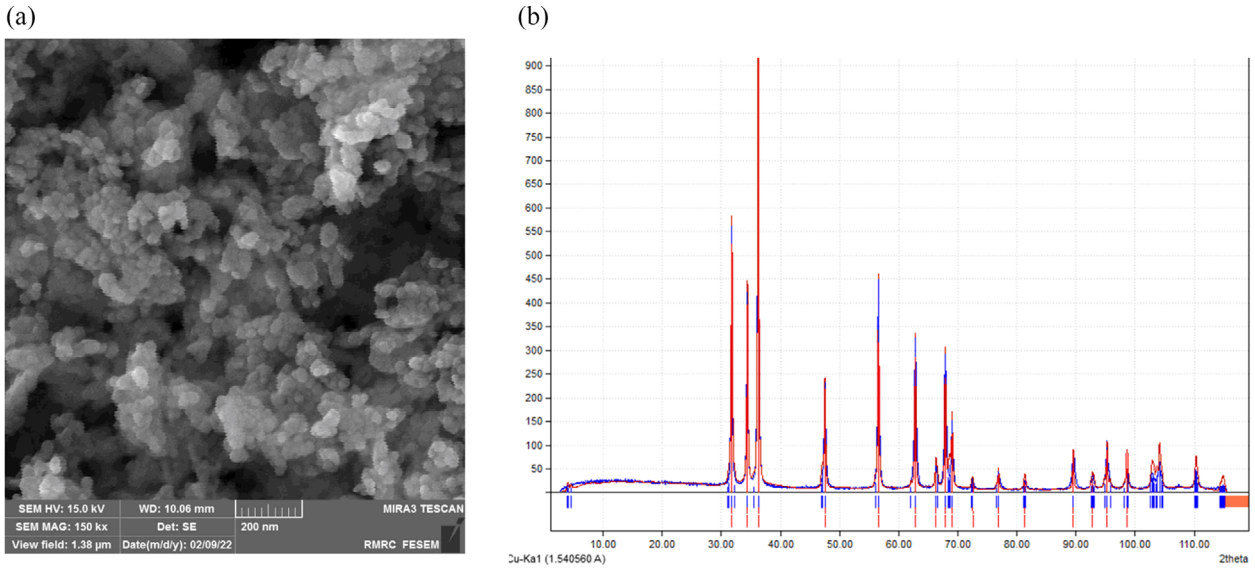

FIGURE 2 ZnO nanoparticles characterization. FESEM image (A) and XRD pattern (B) of ZnO nanoparticles. FESEM, field-emission scanning electron microscopy; XRD, X-ray diffractometer; ZnO, zinc oxide.

2.9 | Statistical analysis

The statistical analysis was performed using GraphPad Prism version 8. Multiple comparison tests were performed by the ANOVA test. Data are presented as the mean ± standard deviation (SD). The levels of $P < 0.05$ were defined to be statistically significant difference.

3 | RESULTS AND DISCUSSION

3.1 | ZnO nanoparticle characterization

According to Figure 2, the synthesized ZnO NPs were spherical and homogenous with the average size of 33 nm. Furthermore, crystallinity of ZnO was studied using an XRD and the results showed the hexagonal structure whose peaks were indexed according to JCPDS card No. 96-900-4180. The crystallite size ZnO NPs was determined 41.59 nm by the Scherrer equation.

3.2 | Hydrogel swelling analysis

The swelling ratio of different hydrogels was studied by using media with different pH values (Figure 3). According to the results the swelling ratio of CH4 formulation which was prepared by 4-mg genipin and without ZnO nanoparticles as compared with CNH4 formulation which was loaded by ZnO NPs showed a higher swelling ratio that could be related to the presence of ZnO NPs in CNH4 formulation. Kumar et al. also observed the presence of ZnO nanoparticles could decrease the swelling ratio of prepared CH [32]. However, CH formulation prepared with 2-mg genipin did not show significant difference between swelling ratio in the presence or absence of ZnO

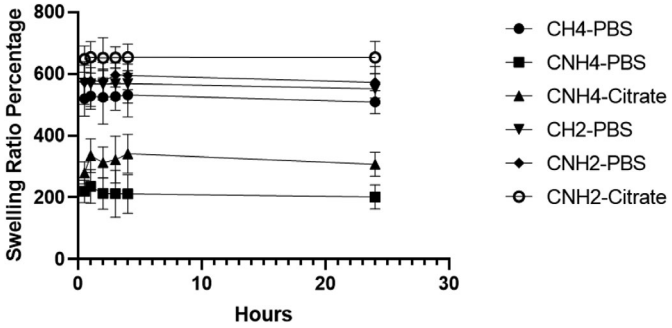

FIGURE 3 Swelling ratio of different hydrogel formulations in PBS and citrate buffer mediums. PBS, phosphate-buffered saline.

NPs. This observation could be related to the lower amount of crosslinker in the formulation that causes to increase the pore size of hydrogel and therefore ZnO NPs could easily pass from the pores without making hindrance. According to the obtained results, the pore size of prepared CHs with 2-mg genipin was 200 nm and the synthesized ZnO NPs also showed 33-nm size. Furthermore, a highest swelling ratio was observed in CNH2 formulation in the acidic citrate buffer medium which could be related to the electrostatic repulsion between amine groups of chitosan in the acidic medium [33].

3.3 | In vitro release study

The in vitro release of different formulations was investigated for 96 h. According to the results in Figure 4, the presence of ZnO NPs affected the release profile of vancomycin in CH4 formulation which was prepared using high amount of genipin as crosslinker agents. In this formulation the presence of NPs

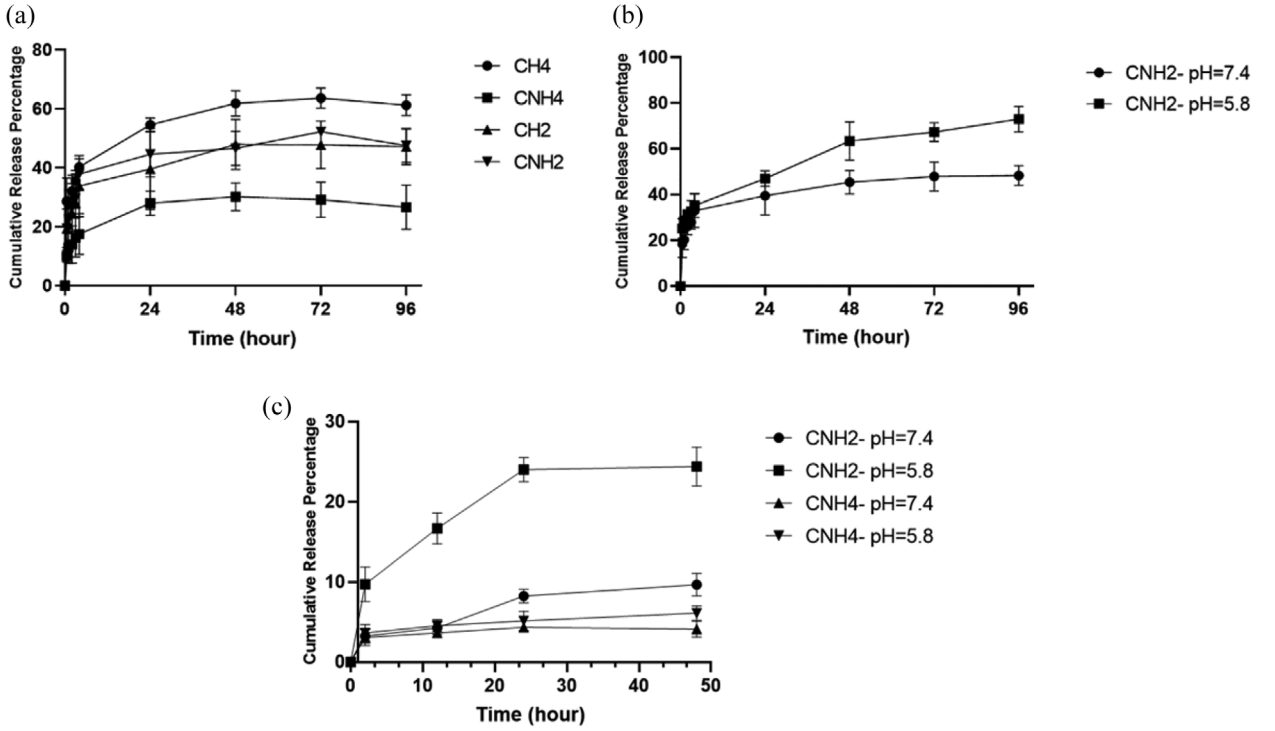

FIGURE 4 A) Drug release profiles of different hydrogel formulations at pH 7.4 in the presence and absence of ZnO nanoparticles. (B) Drug release profiles of hydrogel formulation with 2-mg genipin and ZnO nanoparticles at different pHs 7.4 and 5.8. (C) The release profile of ZnO nanoparticles from the different hydrogel formulations in two different pH 7.4 and pH 5.8. ZnO, zinc oxide.

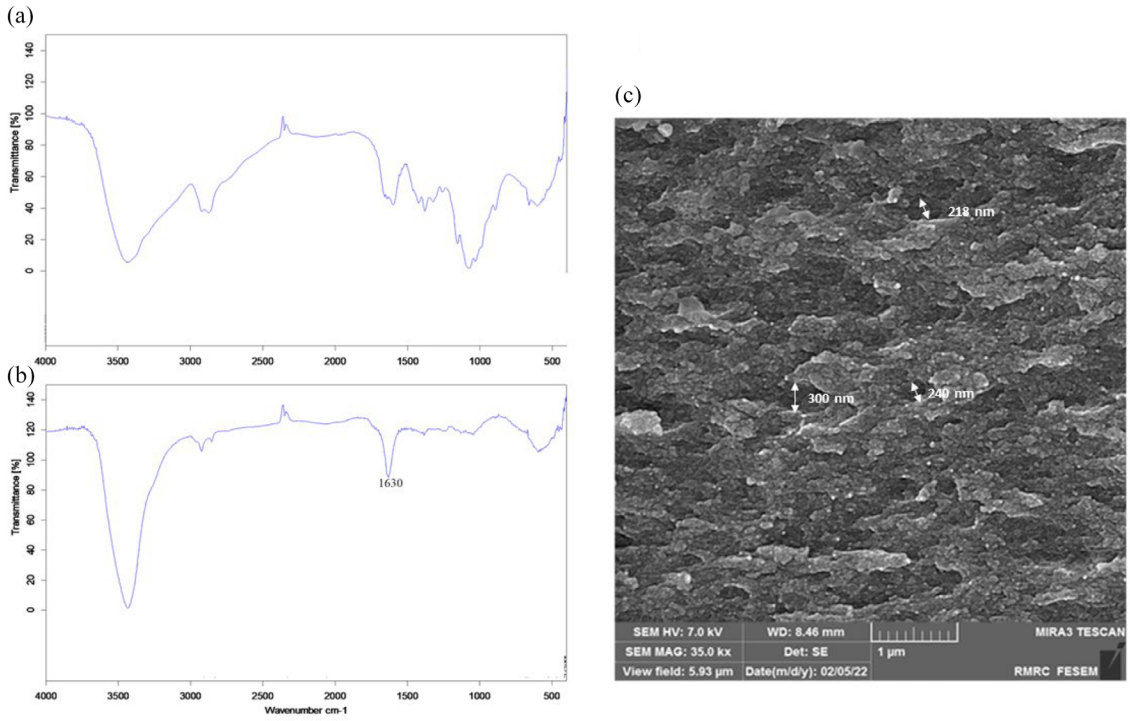

FIGURE 5 Characterization of chitosan hydrogel CNH2. FTIR spectrums of chitosan polymer (A) and chitosan hydrogel (B). FESEM image of chitosan hydrogel (C). CNH2, chitosan hydrogel contained ZnO NPs; FESEM, field-emission scanning electron microscopy.

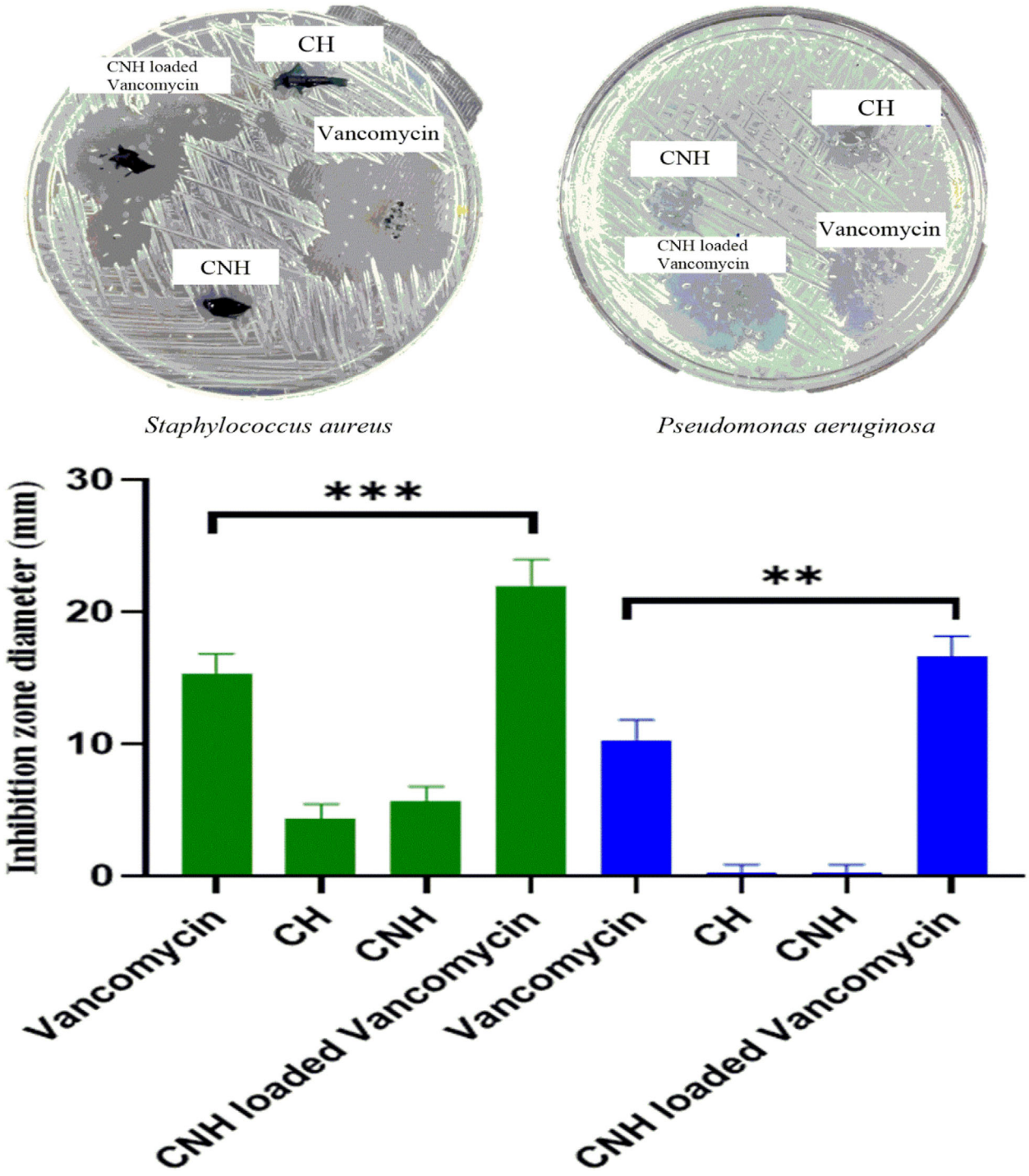

FIGURE 6 Inhibition zone diameters (mm) of *S. aureus* (green) and *P. aeruginosa* (blue) grown in the presence of vancomycin, chitosan hydrogel (CH), chitosan hydrogel contained ZnO NPs (CNH) and chitosan hydrogel loaded by vancomycin and ZnO NPs (CNH loaded vancomycin). Data are represented as mean ± SD (n = 3). ** Denotes significant differences with $p < 0.0002$ and *** Denotes significant differences with $p < 0.0001$. NPs, nanoparticles; SD, standard deviation; ZnO, zinc oxide.

decreased the release rate of vancomycin. However, the CH2 formulation with or without NPs which was prepared by lower amount of genipin did not show any difference in vancomycin release profile. The effect of ZnO NPs in decreasing swelling ratio of CH with high amounts of crosslinker and lowering drug release was also previously observed by PT et al. [32]. On the other hand, in CH2 formulation, the lower amount of crosslinker caused to increase the pore size of hydrogel and therefore ZnO NPs could easily pass from the pores and was not able to make hindrance. Therefore, the study was con-

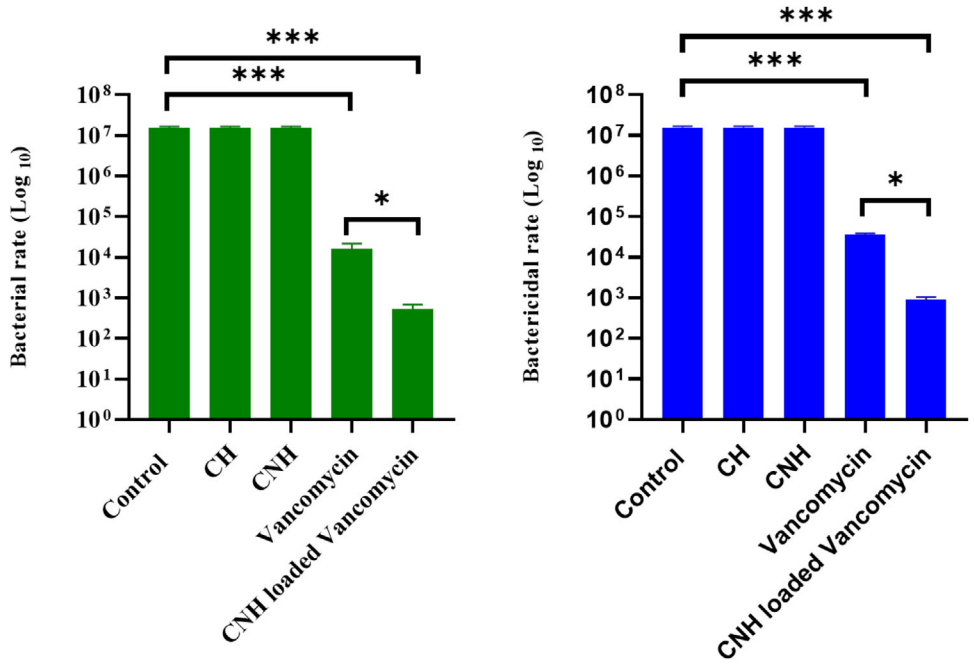

FIGURE 7 Bacterial number of *S. aureus* (green) and *P. aeruginosa* (blue) after treatment with chitosan hydrogel (CH), chitosan hydrogel contained ZnO NPs (CNH) and chitosan hydrogel loaded by vancomycin and ZnO NPs (CNH loaded vancomycin). Data are represented as mean ± SD ($n = 3$). * Denotes significant differences with $p < 0.05$ and *** Denotes significant differences with $p < 0.0001$. NPs, nanoparticles; SD, standard deviation; ZnO, zinc oxide.

tinued by CNH2 formulation which showed more desirable swelling ratio and release profile. This finding was in agreement with the previous studies. Oustadi et al. indicated that the swelling ratio and the degradation rate of prepared hydrogel were decreased by increasing the genipin concentration [34]. Furthermore, based on the obtained results, 30% of drug was released after 2 h which could be related to the burst release phenomenon. Number of mechanisms suggested for burst releasing including surface desorption and pore diffusion of drug [35].

According to Figure 4B, the mentioned hydrogel showed a controlled release behaviour in the response of pH. In fact, in acidic pH the release of vancomycin was increased due to the electrostatic repulsion between amine groups of chitosan in the acidic medium. This controlled release profile is very applicable for antibiotic delivery to the infection sites which are more acidic and cause to have high concentration of antibiotic in the infection site against pathogens [36]. Huang et al. developed smart release self-healing hydrogel for wound healing and showed that the bacterial growth caused acidic condition which induces Tobramycin release in the site of infection and avoids the abuse of antibiotics [37]. Lui et al. also observed a pH responsive release behaviour for genipin crosslinked CH. In their study, the drug release decreased by increasing the amount of genipin as a crosslinker and they showed drug release could increase in acidic media. These findings are related to the impeding of the diffusion of drug from the hydrogel matrix by increasing the amount of crosslinker agents and also related to the protonation of amine groups in chitosan chains in the acidic media [33].

Furthermore, the released quantity of ZnO NPs from the hydrogel formulations was investigated in two different pHs (pH 7.4 and pH 5.8). According to Figure 4C, high amount of genipin as a crosslinker agent in CNH4 formulation caused to decrease release of ZnO NPs in comparison with CNH2 formulation which showed higher ZnO NPs release. Additionally, the release behaviour of CNH2 formulation in response to pH is similar to vancomycin release in acidic pH which could be related to the electrostatic repulsion between amine groups of chitosan in the acidic medium.

3.4 | Hydrogel characterization

The conjugation between amine group of chitosan polymer and C-OH group of genipin was confirmed by FTIR. An amine group of chitosan polymer undergoes nucleophilic attack at the C–OH group of genipin and resulting formation of an amid bond which is indicated by absorption band at 1630 cm^{-1}. This result is in good agreement with other studies [38, 39]. Furthermore, the obtained image from FESEM showed a highly porous hydrogel network with approximately 200 to 400 nm hydrogel pore size (Figure 5).

3.5 | Antimicrobial studies

The antimicrobial studies were performed on formulation with 2-mg genipin since better results of swelling ratio and release profile were observed from this formulation. According to

the obtained results, inhibition zone of prepared hydrogel contained both ZnO NPs and vancomycin was significantly broader than free vancomycin against *S. aureus* and *P. aeruginosa* (Figure 6).

Bacterial killing assay was used to assess the bacteria's absolute load reduction values of prepared hydrogel. The results demonstrated that CH contained ZnO NPs and vancomycin significantly reduced the growth of both *S. aureus* and *P. aeruginosa* in comparison with vancomycin (Figure 7). The obtained results suggest that prepared hydrogel (CNH2 formulation) has a valuable potential to use as an instrument for enhancing antibacterial activity of vancomycin. This efficiency could be related to both characteristics of this hydrogel. First, the loaded ZnO NPs that can act as an antimicrobial agent which could help vancomycin to generate higher antimicrobial activity and the second, controlled release property of this system. ZnO NP is a biosafe material which showed bactericidal and bacteriostatic mechanisms by the generation of reactive oxygen species (ROS) including hydrogen peroxide (H2O2), OH- (hydroxyl radicals), and O2 (peroxide) [40, 41]. Vasile et al. also observed developing gentamicin controlled release system using chitosan and ZnO could efficiently increase antibacterial activity against bacteria [42].

4 | CONCLUSION

In conclusion, in this study we developed a chitosan-based hydrogel loaded with zinc oxide NPs for controlled release of vancomycin and analyzed its antimicrobial effectiveness. Furthermore, the effect of genipin concentration on hydrogel properties was evaluated to optimize the formulation. The results were encouraging since CH prepared by 2-mg genipin showed pH responsive controlled release behaviour and also significantly enhanced antimicrobial effectiveness against *S. aureus* and *P. aeruginosa* in comparison with free vancomycin. Finally, this prepared hydrogel could be an attractive and low-cost option for developing an efficient antibiotic controlled delivery system for different skin, bone or other tissues microbial complications.

AUTHOR CONTRIBUTIONS

Ali Rastegari: Conceptualization, Formal analysis, Methodology, Supervision and Writing. Fatemeh Hasanshakir: Investigation. Zohreh Mohammadi: Conceptualization, Methodology, Supervision and Writing. Fatemeh Saadatpor: Investigation and Methodology. Homa Faghihi: Formal Analysis and writing. Fatemeh Moraffah: Conceptualization and Methodology.

ACKNOWLEDGEMENTS

This work would not have been possible without the invaluable excellent technical assistance of Ms. Sanaz Rezaie. This research has been supported by Iran University of Medical Science.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FUNDING INFORMATION

Research grant of Iran University of Medical Sciences, Grant Number: 99-2-99-18890.

DATA AVAILABILITY STATEMENT

The authors confirm that the data of this study is available on request.

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How to cite this article: Rastegari, A., Hasanshakir, F., Mohammadi, Z., Saadatpor, F., Faghihi, H., Moraffah, F.: A chitosan-based hydrogel containing zinc oxide nanoparticles as a carrier for improving antibacterial activity and controlling the release of antibiotics. Micro Nano Lett. 18, 1–9 (2023).
https://doi.org/10.1049/mna2.12172