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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Abstract

Microbial infections are considered as one of the most important concerns of the world community. Developing drug delivery systems based on formulation of nanoparticles with antimicrobial agents have shown beneficial effectiveness against microbial infections and related antimicrobial resistance. In this study we prepared and characterized a chitosan based hydrogel loaded with zinc oxide nanoparticles for controlling the release of vancomycin and also improving its antibacterial effect. Characterization studies demonstrated that the developed biopolymeric hydrogel was able to sustained and controlled the release of vancomycin in response to acidic media for 96 hours. Furthermore, antimicrobial studies showed siginificant and efficient antibacterial activity of prepared hydrogel against S. aureus and P. aeruginosa. Based on obtained results, it can be concluded that the prepared chitosan hydrogel containing ZnO nanoparticles has a desirable activity for controlling the release of vancomycin and improving its antibacterial properties.

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Manuscript statement

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Abstract

Microbial infections are considered as one of the most important concerns of the world community. Developing drug delivery systems based on formulation of nanoparticles with antimicrobial agents have shown beneficial effectiveness against microbial infections and related antimicrobial resistance. In this study we prepared and characterized a chitosan based hydrogel loaded with zinc oxide nanoparticles for controlling the release of vancomycin and also improving its antibacterial effect. Characterization studies demonstrated that the developed biopolymeric hydrogel was able to sustained and controlled the release of vancomycin in response to acidic media for 96 hours. Furthermore, antimicrobial studies showed siginificant and efficient antibacterial activity of prepared hydrogel against *S. aureus* and*P. aeruginosa.* Based on obtained results, it can be concluded that the prepared chitosan hydrogel containing ZnO nanoparticles has a desirable activity for controlling the release of vancomycin and improving its antibacterial properties.

Keywords: Hydrogel, Chitosan, Nanoparticle, Zinc oxide, Vancomycin, Antibacterial activity

Introduction

In the early twentieth 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 as 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 administartion and prolonged therapy with vancomycin, enhancing the risks of toxicity and aggravation of deletrious 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). Formulattion of NPs with antibiotics exert synergistic effects against bacteria, inhibit biofilm formation and have 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 oixde (ZnO) nanoparticles in infection disease have been considered due to their potential biocompatibility over other metal oxides and also their remarcable antibacterial activities over a wide spectrum of bacterial species (16-19).

For treatment of microbial infections, it is crucial that antimicrobial agents can be released in a sustained manner to efficiently treatment and prevent biofilm formation (20). Hydrogels have been used as carriers for antimicrobial agents and also instruments for co-delivery of antmicrobial agents to achieve synergistic effects. This codelivery approach significantly reduce 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 can be achieved by using genipin as a natural crosslinkers which its cytotoxicity is approximately 10,000 times less than of glutaraldehyde (27, 28). According to the above mentioned facts, the main aim of present study is preparation and characterization of chitosan hydrogl loaded with ZnO NPs for controlling release of vancomycin and also improving its antibacterial activity.

Materials and Methods

Materials

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

Bacterial *strains:* Standard strains of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 have been used from iranian biological resource center.

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 hours. Then NaOH 0.1M was added to the prepared solution drop wise until the pH of solution reach to 9 by color change and precipitation. The solution was stirred for 24 hours to complete sedimentation. The solution was centriguged with 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 dried precipitate was calcined in a furnace at 400°C for 1 hour which the color was changed from white to grey.

Nanoparticles characterization

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

Hydrogel synthesis

Hydrogel were synthetized with genipin at different concentations. Firstly, a 2% (w/v) chitosan solution was prepared by dissolving chitosan powder into acetic acid 1% and let it to dissolve for 24 hours under stirring. 100 mg of vancomycin was added into the prepared solution. After that, 10 mg of ZnO nanoparticles were added and let to disperse completely for 24 hours. Solution of genipin was then prepared by dissolving genipin powder in ethanol with different concentrations of 2 and 4 mg/mL. This soluion was added to the chitosan solution and mixed for 30 minutes 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 oven for 24 hours at 50 $^{\circ}$C. The same method was used For preparations of hydrogels without ZnO nanoparticles, except adding ZnO nanoparticles. Table 1 summerized details of hydogel formulations.

Hydrogel characterization

The conjugation between amine group of chitosan polymer and C-OH group of genipin was confirmed by FTIR. The samples of chitosan polymer and chitosan hydrogel 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 chitosan hydrogel prepared with 2 mg genipin (as a selected formulation). Sample were mounted to the sample stub using double-sided carbon tape, and images were obtained quickly to prevent sample shrinkage from drying.

Hydrogel swelling analysis

The swelling profiles of different formulation of hydrgels were investigated in PBS (pH 7.4) and Citrate buffer (pH 5.8) at 37degC during 24 hours. At each time interval (30 minutes,1, 2, 3, 4, 24 hours) hydrogel sample mass was recorded. Swelling behavior 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.

Equation 1: Swelling (%) = ((Wf - Wi)/Wi) x 100

In vitro **release study**

In vitro release of vancomycin from different hydrogels was investigated at 37 degC in two different pHs (PBS with pH 7.4 and citrate buffer with pH 5.8) under stirring. An appropriate amount of hydrogel (50mg) was dispersed in 2 ml of buffer solution then the obtained suspension was poured in a dialysis bag (molecular weight cutoff 12 kDa) and then it was plunged in 50 ml buffer solution. At specified time intervals (0.5, 1, 2, 3, 4, 24, 48, 72 and 96 hours), 2ml of media was withdraw 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 nanoparticles 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 then the obtained suspension was poured in a dialysis bag (molecular weight cutoff 12 kDa) and then it was plunged in 50 ml buffer solution. At specified time intervals (2, 12, 24 and 48 hours), 2ml of media was withdraw and replaced by 2 ml of fresh media. The concentration of released ZnO nanoparticles was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectro Arcos, Germany) (29). All experiments were carried out in triplicates.

Antimicrobial studies

The agar disc diffusion method was employed to test the antibacterial activity of chitosan hydrogel with 2 mg genipin. 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 CFU.mL-1), and then equally dispersed onto Muller-Hinton agar. 5 mm side squares of the hydrogel samples inclduing chitosan hydrogel (CH), chitosan hydrogel contained ZnO NPs (CNH) and chitosan hydrogel 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 37degC 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 concentartion in all experiments was 6.5 and 12.5 µg/ml for antibacteril study against of *S. aureus* and *P. aeruginosa* respectively.

Furthernore, bacterial kiling assay (CFU assay) was used to assess the bacteria absolute load reduction values of prepared hydrogels. The hydrogel samples at concentrations of 0.5 mg/mL 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 in 150 rpm. At the end of 24 h incubation, a series of ten-fold dilutions of the bacteria from each tube were made in phosphate-buffered saline (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 logarithmic scale (31). All tests were perfomred in triplicate and reported by mean average values.

Statistical analysis

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

Results and discussion

ZnO nanoparticle characetrization

According to the Figure 1, the synthesized ZnO nanoparticles were spherical and homogenous with the average size of 33 nm. Furthermore, crystallinity of ZnO was studied using an X-ray diffractometer (XRD) and results showed the hexagonal structure which peaks were indexed according to JCPDS card No. 96-900-4180. The crystallite size ZnO nanoparticles determined 41.59 nm by Scherrer equation.

Hydrogel characetrization

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 (32, 33). Furthermore, obtained image from FESEM showed a highly porous hydrogel network with approximately 200-400 nm hydrogel pore size. (Figure 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 prepraed by 4 mg genipin and without ZnO nanopartilces in compared with CNH4 formulation which loaded by ZnO nanopartilces showed higher swelling ratio that could be related to the presence of ZnO nanoparticles in CNH4 formulation. Kumar *et al* . also observed the presence of ZnO nanopartilces could decrease the swelling ratio of prepared chitosan hydrogel (34). However, chitosan hydrogel formulation prepared with 2 mg genipin did not show significant difference between swelling ratio in the presence or abesence of ZnO nanopartilces. This observation could be related to the lower amount of crosslinker in formultion that cause to increase the pore size of hydrogel and therefore ZnO nanopartilces could easily pass from the pores and not making hindrance. According to the obtained results, the pore size of prepared chitosan hydrogels with 2 mg genipin was 200 nm and the synthtized ZnO nanoparticles also showed 33 nm size. Furthermore, highest swelling ratio was observed in CNH2 formulation in acidic citrate buffer medium which could be related to the electrostatic repulsion between amine groups of chitosan in the acidic medium (35).

In vitro **release study**

The *in vitro* release of different formualtions were investigated for 96 hours. According to the results in Figure 4, the presence of ZnO nanoparticles affected the release profile of vancomysin in CH4 formulatio which prepared using high amount of genipin as crosslinker agents. In this formulatiln the presence of nanoparticles decreased the release rate of vancomycin. However, the CH2 formulation with or without nanoparticles which prepared by lower amount of genipin did not show any differnce in vancomycine release profile. The effect of ZnO nanoparticles in decreasing swelling ratio of chitosan hydrogel with high amounts of crosslinker and therfore lower drug releas was also previously observed by PT et al. (34). On the other hand, in CH2 formulation , the lower amount of crosslinker caused to increase the pore size of hydrogel and therefore ZnO nanopartilces could easily pass from the pores and didn't able to make hindrance. Therefore, the study was continued 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 degradation rate of prepared hydrogel was decreased by increasing the genipin concentration (36).

According to the Figure 4B, the mentioned hyrogel 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 (37). Lui *et al,* also observed a pH responsive release behaviour for genipin crosslinked chitosan hydrogel. 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 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 (35).

Furthermore, the released quantity of ZnO nanoparticles from the hydrogel formulations was investigated in two different pHs (pH 7.4 and pH 5.8). According to the Figure 4C, high amount of genipin as crosslinker agent in CNH4 formulation caused to decrease relase of ZnO NPs in comparrison with CNH2 formulation which showed higher ZnO NPs release. Additionally, in CNH2 formulation observed controlled release behaviour 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.

Antimicrobial studies

The antimicrobial studies were performed on formulation with 2 mg genipin since better results of swelling ratio and release profile was 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 5).

Bacterial kiling assay was used to assess the bacteria absolute load reduction values of prepared hydrogel. The results demonstarted that chitosan hydrogel contained ZnO NPs and vancomycin significantely reduced the growth of both *S. aureus* and *P. aeruginosa* in comparrison with vancomyicn (Figure 6). 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 animicrobial agent which could help vancomycine to generate higher antimicrobial activity and the second, controlled release property of this system. The antmicrobial mechanism of ZnO nanoparticles is not completely understood however direct contact of ZnO-NPs with cell walls and ROS formation has been proposed (38). Vasile *et al.* also observed developing gentamicin controlled release system using chitosan and ZnO could efficinetly increase antibacetrial activity againts bacteria (39).

Conclusion

In conclusion, in this study we developed a chitosan based hydrogelloaded with zinc oxide nanoparticles for controlled release of vancomycin and analyzed its antimicrobial effectivness. Furthermore, the effect

of genipin concentration on hydrogel properties were evaluated to optimize the formulation. The results were encouraging since chitosan hydrogel prepared by 2 mg genipin showed pH responsive controlled release behaviour and also significantely enhanced antimicrobial effevtivenss against *S. aureus* and *P. aeruginosa* in comparrison with free vancomyicn. 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. .

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Tables:

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

Figure legends:

Figure 1. ZnO nanoparticles characetrixation. FESEM image (A) and XRD pattern (B) of ZnO nanoparticles.

Figure 2. Chitosan hydrogel characterization. FTIR spectrums of chitosan polymer (A) and chitoan hudrogel (B). FESEM image of chitosan hydrogel (C).

Figure 3. Swelling ratio of different hydrogel formulations in PBS and citarte buffer mediums.

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 forulation 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.

Figure 5 . 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 $p<0.0001$.

Figure 6 . 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 $p<0.0001$

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