Preparation and Characterization of Chitosan/ZnO/Ag Nanocomposites as Antibacterial Hydrogel for Wound Dressing

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Abstract. The aim of this study is the preparation of chitosan/Ag/ZnO (CS/Ag/ZnO) composite to investigate the antibacterial activity of the prepared nanocomposites. Chitosan is a linear biodegradable natural polymer having excellent characteristics, such as biocompatibility. Hydrogel as a wound dressing could be made to act as antibacterial, antimicrobial, and anti-inflammatory agents in the presents of ZnO and Ag Nanoparticles. In this research, pure chitosan (Cs) and Cs/ZnO/Ag nanocomposites were papered to evaluate the antimicrobial effect against gram-positive and gram-negative microorganisms. The results show that the pure chitosan with (6mM) concentration has the best antibacterial effect against K-pneumoniae bacteria as compared to S.aureus and E. coli bacteria with an Inhibition Zone of (20.66-65.33) mm at pH value in the range of (5-6), while the prepared nanocomposite of Cs/Ag/ZnO (30/10) nanocomposites shows that the antibacterial behavior against S-aureus bacteria is better than the antibacterial effect against K-pneumoniaibacteria. The results illustrated that the bi-nanocomposites shows a higher antibacterial effect due to the combined effect of Cs and nanoparticles (NPs).

Keywords: Antibacterial; Nanocomposites; Chitosan; Nanoparticles; Wound dressing
1. Introduction.
Nanocomposite has been used for many applications like wound healing, tissue engineering, thermal therapy, and drug delivery. Nanocomposite has various properties make it appropriate for wound healing process such as mechanical and thermal characteristics, antimicrobial properties, balance, and structure with the porosity to allow absorbance, and capability to provide perfect humidity in the wound bandage [Shabunin et al., 2019].

The most important properties of wound dressing are, biocompatible, biodegradable, infection protection, stable shape, good mechanical characteristics, non-toxic, and cost effective [Noori et al., 2018]. Chitosan is a biocompatible polymer consist of polysaccharide which has antibacterial properties that have been added to wound dressings [Cremar et al., 2018].

Chitosan is a bio-copolymer including N-acetylglucosamine and glucosamine. It has many characteristics such as non-toxic, biocompatibility, biodegradability, moisture retentive and low cost [Lu et al., 2017]. In the wound healing process, chitosan work as a painkiller drug and anti-provocative factor, which can show a good effect when used to an open wound [Cremar et al., 2018]. In vivo examinations have revealed that the pain is decreased when chitosan fabric/membrane is used as wound dressings to the burns, skin abrasions, skin grafts and, ulcers [Ohshima et al., 1987]. The mechanism of chitosan as a wound has been studied by using a suspension of chitosan/acetic-acid composites on mice, which showed a decrease in the inflammatory pain because of an increase in the pH from protonation of –NH2 to –NH3+ [Okamoto et al., 2002]. Various shapes of chitosan employed for wound dressing have been studied, like hydrogels, films, scaffolds, and sponges [Ma et al., 2014, Zakhem et al., 2012].

Nano-sized ZnO exhibits varying morphologies and shows significant antibacterial activity over a wide spectrum of bacterial species explored by a large body of researchers [Buzea et al., 2007, Brayner et al., 2006, Raghupathi et al., 2011]. ZnO NPs in the micro and the nano scale are used as antibacterial material especially when their size is reduced into the nanometer scale, ZnO NPs can interact with the surface of the bacteria or enter into the cell wall of the bacteria, as a result shows its antibacterial mechanisms. The interactions between NPs and bacteria are typically toxic, which have been subjugated for antimicrobial applications like food industries and wound healing [Seil et al., 2012].

Metal NPs like Ag, ZnO, TiO, Cu, CuO, and Au have revealed wonderful antimicrobial activity to antibiotics, particularly versus antimicrobial resistant. These metal NPs create reactive oxygen species (ROS) which destroy microbial DNA and interrelate with proteins and organic constituents of the microbe cause electrolyte inequity leading to killing of microbes [Dizaj et al., 2014].

For instance, ZnO inhibits the adhesion and internalization of enterotoxigenic E. coli (ETEC) into enterocytes (Roselli et al., 2003). In addition, ZnO nanoparticles (ZnO-NPs) exhibit antibacterial activity and can reduce the attachment and viability of microbes on biomedical surfaces (Brayner et al., 2006; Yamamoto, 2001). Interestingly, several results suggest a selective toxicity of ZnO-NPs preferentially targeting prokaryotic systems, although killing of cancer cells has also been demonstrated (Hanley et al., 2008; Reddy et al., 2007; Taccola et al., 2011).

Furthermore, Ag+ ions and Ag-based compounds are highly toxic to several microorganisms, which make them interesting candidates for multiple applications in the medical field (Furno et al., 2004; Prakash et al., 2013). Ag is generally used as nitrate salt, but in the form of Ag nanoparticles (Ag-NPs) the surface area is increased and thereby antimicrobial efficacy is greatly enhanced.

In this study, we produced an antibacterial wound dressing by loading ZnO/Ag nanocomposite to chitosan. The antibacterial activity was investigated against three types of microorganisms gram-positive (S-aureus and K-pneumonia) and gram-negative (E-coli).

2. Materials and methods
Chitosan powder (Molecular weight of 161.16 g/mol - Sigma Aldrich-UK), Acetic acid with chemical
formula CH₃COOH was made in India with purity equal to 99.5%. ZnO was made in USA with purity equal to 99.8% and the particle size is in the range of 10-30nm. AgNPs were made in India with purity equal to 99% and the particle size is in the range of 20nm. Three different types of bacteria S-aureus, K-pneumonia and E-coli were gently supplied from laboratories of biology department/college of science/ university of kufa.

2.1 Preparation of chitosan/ acetic acid solution

In order to prepare solutions of chitosan, the chitosan in powdered form was weighed using a sensitive electronic balance device (type HT series- Japan) Vibra company and added into (100 ml) of acetic acid to prepare a three different viscous polymeric solution as illustrated in table (1).

| Solution no. | Chitosan weight (g) | AA (ml) | Concentrations (mM) |
|--------------|---------------------|---------|---------------------|
| 1            | 0.1                 | 100     | 6                   |
| 2            | 2                   | 100     | 80                  |
| 3            | 1.62                | 100     | 100                 |

2.2 Preparation of Cs/ZnO and Cs/Ag Nanocomposites

To prepare the nanocomposites, ZnO NPs and Silver nanoparticles were incorporated with two different weight percentages (0.05wt%, 0.10wt %) and mixed by sonicator for 10 minutes, see table (2). The following equation was used to evaluate the weight of the NPs required to be added into the solution of chitosan to obtain the nanocomposites. (William. D., 2007):

\[
V_f = \frac{W_m}{\rho_m + \frac{W_p}{\rho_p}} 
\]

Where:
Wm: weight of matrix
Wp: weight of particles
ρm: density of matrix
ρp: density of particles
Vf: volume fraction

The NPs were added into chitosan and the nanocomposite solution was subjected to ultrasonic device (1200W ultrasonic processor) from MTI company in USA to homogenizing NPs in the polymeric solution.
Antibacterial activities of the prepared nanocomposite solutions were tested versus Gram-positive and Gram-negative germs with S. aureus and E. coli and K. pneumoniae correspondingly. The procedure for culturing the prepared nanocomposites and nanofibers can be explained as follows:

1. All the used Petri dishes were autoclaved by using autoclave before the experimental work.
2. To prepare the medium for culturing bacteria, five to six different colonies were allocated in petri dishes and incubated for 24 hr at 37°C.
3. Three different types of bacteria were sprayed on the prepared medium.
4. For culturing nanocomposites, many holes are created in the medium with (6mm) in diameter, after that the nanocomposites are added to the holes using micropipette (10µl).
5. For culturing nanofibers obtained from Electrospinning process using Electrospinning system that laboratory manufactured in Nanotechnology and Advanced Materials Research Unit (NAMRU) at Faculty of Engineering in University of Kufa/IRAQ, the prepared nanofibers were shaped into circular having diameter of (6mm), and attached to the surface of the bacteria.
6. All the prepared petri dishes were incubated for 24hr.

### Table 2. Weight percentages of Nano composite formulations

| Sample No. | Concentrations            |
|------------|---------------------------|
| 1          | Cs/Ag=20ml/0.10wt%        |
| 2          | Cs/ZnO=20ml/0.05wt%       |
| 3          | Cs/Ag=60ml/0.10wt%        |
| 4          | Cs/ZnO=60ml/0.05wt%       |
| 5          | Cs/Ag/ZnO=10/10           |
| 6          | Cs/Ag/ZnO=30/10           |
| 7          | Cs/Ag/ZnO=10/30           |

### Results and Discussion

When the pH of Cs decreases, the antibacterial activity of Cs is increased. This is likely due to the fact that the amino groups of chitosan become positively ionized below pH 6 [Erdem et al., 2016].

From table (3) can be observed that the maximum inhibition zone is for the concentration of (0.1M), as shown in fig.1. The mechanism which explain the antibacterial behavior of chitosan can be describe by interaction of positive charge in chitosan such as protonated NH3 and the negative charge present in the cell wall of bacteria through electrostatic forces. These interactions destroying the function of microbial cell membrane such as escaping of intracellular compounds and also prevent transformation of nutrients which leading to kill the bacteria [Pasaribu et al., 2018, Liu et al., 2006]. Srinath et al., 2019 observed that the (IZ) of the Gram-negative bacteria (E. coli) is larger than the Gram-positive bacteria (S. aureus).
Figure 1. Shows how the inhibition zone effected by antibacterial activity of chitosan concentrations on the gram positive and gram negative bacteria.

Table 3. Illustrate the diameters of the Inhibition Zone (IZ) of chitosan.

| Concentration (mM) | S-aureus IZ (mm) | E-coli IZ (mm) | K-pneumoniae IZ (mm) |
|------------------|-----------------|----------------|----------------------|
| 100              | 38.33           | 49.33          | 65.33                |
| 80               | 25              | 38.33          | 64                   |
| 6                | 20.66           | 44             | 61.33                |

The cell walls of the bacteria containing many layers of protein and lipids (give the hydrophobic properties) in specific structure known as bi-layer [Haider et al., 2015]. Table (4) shows the concentrations of prepared Cs/Ag/ZnO nanocomposites and the diameters of the inhibition zone of three types of bacteria.

Table 4. Illustrate the diameters of the Inhibition Zone (IZ) of chitosan nanocomposites.

| Sample No. | Concentrations    | S-aureus IZ (mm) | E-coli IZ (mm) | K-pneumoniae IZ (mm) |
|------------|------------------|------------------|----------------|----------------------|
| 1          | Cs/Ag=20ml/0.10wt% | 42.33            | 37.66          | 31                   |
| 2          | Cs/ZnO=20ml/0.05wt% | 42              | 36             | 29.66                |
| 3          | Cs/Ag=60ml/0.10wt% | 45.33            | 39             | 32.66                |
| 4          | Cs/ZnO=60ml/0.05wt% | 40.33            | 32.33          | 34.33                |
| 5          | Cs/Ag/ZnO=10/10   | 37.33            | 34.33          | 32                   |
| 6          | Cs/Ag/ZnO=30/10   | 45.66            | 36.66          | 40                   |
| 7          | Cs/Ag/ZnO=10/30   | 40.33            | 31.66          | 35.33                |

From table (4) the Cs/Ag/ZnO nanocomposites shows that the best antibacterial behavior was against S-aureus. The addition of ZnO NPs into chitosan/acetic acid aqueous solution leading to formation of different types of ROS like (•OH), (O2• −), and (H2O2). (•OH) and (O2• −), have negative charge so they cannot go through the cell wall due to the repulsion of negative charges, while (H2O2) has positive charge and due to attraction between the different charges, the (H2O2) enter the cell membrane. Li-Hua
et al., 2010, reported that the CS/ZnO nanocomposite showed antibacterial activity against Bacillus subtilis, Escherichia 317 coli, and Staphylococcus aureus. The formation of ROS relies on the surface area of the nanoparticles (higher surface area produces higher concentration of ROS) [Shi et al., 2014]. Cs has strong tendency towards metal ions since the Cs possess many amine and hydroxyl groups [Sanpui et al., 2008].

**Figure 2.** Shows the antibacterial behavior of Cs/Ag/ZnO nanocomposite against (a), (d) K-pneumonia, (b), (e) S-aureus, and (c), (f) E-coli.

Fig. 2 shows that the Cs/Ag/ZnO nanocomposite has the best inhibition zone against S-aureus bacteria in all prepared compositions, and the lowest antibacterial effect was against K-pneumonia. The combined effect of chitosan and nanoparticles can be explained by various mechanisms such as changing the chemistry of the surface area of the particles, consequently, changing their biological characteristics. Li et al., 2010 reported that the increase of Ag and ZnO contents causing increase in the diameter of (IZ). Claudia et al., 2014 reported that the systems CS/Ag/ZnO have higher antimicrobial activity that of the chitosan indicating that ZnO and Ag enhance the antimicrobial activities of chitosan. The most possible methods are (i) as the chitosan wrapper the surface of the NPs causing "steric stabilization" and reduced the agglomeration, that consequently rising the effectual concentration of NPs; (ii) improving the binding forces between the positive charges of chitosan and the negative charges from the bacteria [Potara et al., 2011].

4. Conclusions

To conclude, we have produced nanocomposites of chitosan loaded with different weight percentages of silver and zinc oxide nanoparticles to enhance the antimicrobial efficiency against both grams positive and gram negative pathogens. All the prepared compositions showed a range of bactericidal behavior and the bimetallic formulations exhibited a synergistic antibacterial effect due to the high antibacterial activity of ZnO and Ag nanoparticles which improve the antibacterial activity of the bi-nanocomposites (ZnO/Ag/Cs).

Acknowledgments

The authors would like to acknowledge the assistance offered by Nanotechnology and Advanced Materials Research Unit (NAMRU) at Faculty of Engineering in University of Kufa/IRAQ. The authors would like to express their appreciation to the University of Kufa/College of Science/ Department of biology for its support of this work.
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