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IMMOBILIZATION OF ZINC OXIDE NANOPARTICLES IN CHITOSAN-GELATIN COMPOSITE MEMBRANE FOR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Novel chitosan-gelatin/Zinc Oxide nanoparticles (C-G/ZnO NPs) composites with enhanced anti bacterial activity is prepared by casting method at room temperature. In the present work, the combination of chitosan gelatin with ZnO for antibacterial activity is reported. Antibacterial activity of each nanocomposite is observed for Gram negative *E. coli* and Gram positive *S. aureus*. Prepared samples were characterized by XRD to study the morphology and to determine the crystallite size of ZnO NPs. The crystallite size is calculated to be 27nm by Debye-Scherrer formula. The UV-Vis spectroscopy was used to study the absorbance patterns of ZnO NPs in composite membranes. UTM was used to calculate the tensile strength and % elongation. The results showed the excellent strength value of 93.8 MPa for 1% ZnO NPs compositions. The tensile strength and antibacterial activity studies showed the potential of the nanocomposites to be used in biomedical applications.

KEYWORDS: **Chitosan, Gelatin, ZnO nanoparticles, Mechanical properties, Anti bacterial activities.**

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1. INTRODUCTION

In past few decades, organic-inorganic composite materials have gathered colossal attention among scientific community. Each component in the composite has its own property which enhances on combining. Chitosan, biomedically valuable material, is a linear polysaccharide derived from the deacetylation of chitin. It consists of varying amount of β-(1-4)-linked 2-acetamido-2-deoxy-β-D- glucopyranose (GlcNAc) and 2-amino-2-deoxy- β-D-glucopyranose (GlcN) units[1]. It has great economic value because of its versatile biological properties such as non-toxicity, biodegradability, nonantigenicity, biocompatibility with bio-adhesive, antimicrobial and wound healing property[2]. Protein possesses the property of synthetic polymer with good absorbability and low toxicity[3]. Gelatin is a partial degradation product of collagen. It is a biopolymer having multifunctional properties and a unique sequence of amino acids useful for biomedical applications. Gelatin plays vital role in wound healing by promoting cell adhesion, differentiation and proliferation[4].

A lot of work has been done on chitosan-gelatin composite materials. Such composites membranes have gained much attention due to improved mechanical and biological properties. This composite membrane enhances cell adhesion, migration, differentiation and proliferation properties essential for wound healing and tissue engineering[5-9]. Chitosan and chitosan- gelatin composites additionally blended with inorganic nanoparticles have improved mechanical and antibacterial activities have been reported by several researchers[10-14]. Also, there are few reports on different orientations of chitin and gelatin along with inorganic nanoparticles composites for their use in various biomedical applications[15-18]. Such composites mimic the extracellular matrix for bone tissue engineering, skin tissue engineering and cartilage regeneration. Number of research articles is available on chitosan-gelatin composites and its combination with inorganic nanoparticles for biomedical applications. However, in the present investigation, an attempt has been made to synthesize C-G/ZnO NPs composites without using any cross linking agent. To the best of our knowledge, this is the first time chitosan-gelatin composite especially with ZnO NPs combination has been prepared. The composite shows excellent mechanical and antibacterial properties which can be used as a promosing material for wound dressing application.

2. EXPERIMENTAL

2.1 MATERIALS

Chitosan with medium molecular weight and 75-85% degree of deacetylation was purchased from Sigma-Aldrich. Gelatin, Zinc nitrate [Zn $(NO_3)_2.6H_2O$], Sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and glacial acetic acid (CH_3COOH) were purchased from Merck specialties Pvt. Ltd, Mumbai. All the chemicals were of analytical grade (AR grade) and used without further purification. The media components were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Double distilled water was used throughout the experiments.The two microorganisms, Gram positive (*Staphylococcus aureus* NCIM 2654) and Gram negative (*Escherichia coli* NCIM 2066) were used for antibacterial test. The *S. aureus* and *E. coli* were purchased from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India.

2.2 PREPARATION OF ZnO NPs

ZnO nanoparticles were prepared by precipitation method reported previously with slight modification[19]. 0.1 M [Zn $(NO_3)_2.6H_2O$] and 0.2 M NaOH were mixed with continuous stirring for 2 h maintaining temperature at 75ºC. Then, 1 mol H_2O_2 was added to the precipitate to make a translucent sol. The sol was filtered and washed several times with distilled water and allowed to dry at room temperature for 24 h.

2.3 MECHANISM OF FORMATION OF ZnO NPs

The choice of the synthesis of NPs depends upon its applications. Different synthesis methods yield different structures and particle size, recommended for biomedical application. As the particle size attends nanometer scale, surface to volume ratio increases. This phenomenon plays a vital role in biomedical applications. In this study, precipitation method with some changes for the synthesis of ZnO NPs is used as explained above. Reaction Mechanism for the formation of ZnO NPs[19]:

$$ZnO(NO_3).6H_2O + 2NaOH \xrightarrow{\Delta} Zn(OH)_2 + NaNO_3 \quad (1)$$

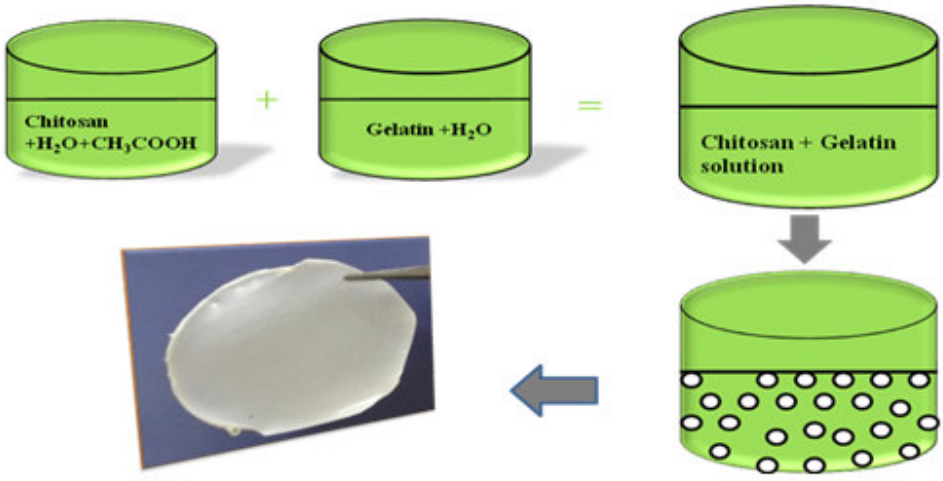
$$Zn(OH)_2 + H_2O_2 \xrightarrow{\Delta} ZnO_2 + 2H_2O \xrightarrow{\Delta} ZnO \quad (2)$$

In the above reaction, the ratio of zinc nitrate to sodium hydroxide was taken as 1:2. Both the precursors were allowed to dissolve completely in separate beakers. After complete dissolution, both the solutions were mixed to precipitate zinc hydroxide at 75°C as shown in (1). Then after, 1 mol of H_2O_2 was added to mixture so that all the impurities are completely dissolved. The reaction mixture is washed several times with double distilled water till all impurities are removed, resulting into the formation of pure ZnO NPs from equation (2).

2.4PREPARATIONOFCHITOSAN/GELATIN/ZnO NPs (C-G/ZnO) COMPOSITE

0.5 g chitosan was dissolved in 19 mL distilled water containing 0.45 g CH_3OOH and continuously stirred for 6 h till transparent solution is formed[20]. After that, 20 g of gelatin was dissolved in an equal amount of water. The solution mixture was agitated at 40°C so that gelatin dissolves completely. Both the solutions were mixed together and appropriate amount of distilled water was added to make up 100 mL solution.

Figure 1
Schematic diagram of Chitosan-Gelatin/ZnO NPs composite membrane



To this solution ZnO NPs of varying mass ratios were prepared. The whole solution was stirred continuously in order to form a homogenous mixture. The prepared solution was transferred to Teflon moulds and dried at room temperature. The prepared membranes were immersed in 0.1M NaOH solution overnight to attain physiological pH value. After being removed from the NaOH solution membrane was washed several times with double distilled

water, again dried at room temperature for 24 h and used further for characterization. ZnO concentrations varied as 0%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% and the samples are designated as SA, SB, SC, SD, SE, SF, SG SH, SI and SJ respectively.

2.4 CHARACTERIZATIONS

The X-ray diffraction studies of ZnO NPs and composite membranes were carried out using Rigaku 600 Miniflex X-ray diffraction instrument (XRD) in the range 10^0-80^0. The UV-Vis absorption spectra were recorded from Shimadzu UV 1600 spectrophotometer in the range of 300 nm-700 nm. Surface morphology was studied by Scanning Electron Microscopy (SEM) of Jeol JSM-6360LV. The tensile strength (TS) and elongation at break (EB) of the composite films were measured by using Shimadzu AG-100 kN. The films were prepared with dimensions of 5cm × 4cm ×0.01cm. The films were clipped vertically on both the ends and the load was applied to check mechanical stress and % elongation of the samples. All the experiments were repeated thrice and the results were averaged.

2.5 ANTIBACTERIAL ACTIVITY

The well diffusion assay was used to study antimicrobial effects of samples against two different pathogens. The relative activities of composites of varying composition of ZnO NPs were investigated against both Gram positive *S. aureus* and Gram negative *E. coli* bacteria. In this method, nutrient agar media and all glassware were used after complete sterilization. 100 µl of cultured bacterial cell suspension was spread over the agar plate using glass spreader. The plates were refrigerated at 10°C for 10 min to diffuse the sample in the media. Then the plates were incubated at 37°C for 24 h for the growth of microorganism. After 24 h of incubation, plates were observed for zone of inhibition[21]. The zone of inhibition was measured for each sample in mm.

3. RESULTS AND DISCUSSION

3.1 XRD ANALYSIS

Fig. 2 illustrates the XRD patterns of C-G composite and C-G/ZnO composite with minimum and maximum concentration of NPs. The characteristic peaks of 31.72° (100), 34.39° (002), 36.23° (101) and 47.44° (102) corresponds to pure ZnO NPs (JCPDS NO. 36-1451) while peaks 20.00° and 28.39° corresponds to pure chitosan (both data not shown here). When gelatin component is incorporated into chitosan hydrogel, the peak intensity of chitosan decreased. This is due to the fact that the concentration of amorphous gelatin is much higher than chitosan. As a result, composite becomes virtually amorphous in nature. The crystallite size of ZnO NPs is found to be 27 nm, estimated by Debye-Scherrer formula.

$$D = \frac{0.9\lambda}{\beta \cos\theta} \qquad (3)$$

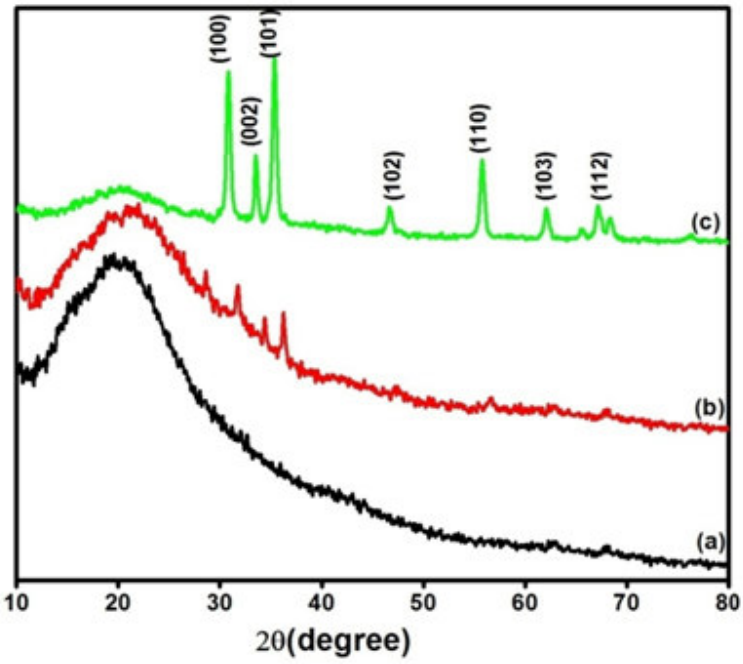


Figure 2
X-ray diffraction pattern of (a) C-G composite, (b) C-G/ 1%ZnO and (c) C-G/ 5%ZnO

As the concentration of inorganic NPs increases, peak intensity of ZnO in composite also increases. This amorphous composite again changes to crystalline structure as the ZnO concentration increases.

3.2 UV-VISIBLE ANALYSIS

Fig. 3 shows the UV-Visible absorption spectra of C-G composite and same composite with minimum and maximum concentration of ZnO NPs. Pure C-G composite does not show absorption peak due to the absence of conjugated double bonds in polymer components. Fig. (3c) shows that,

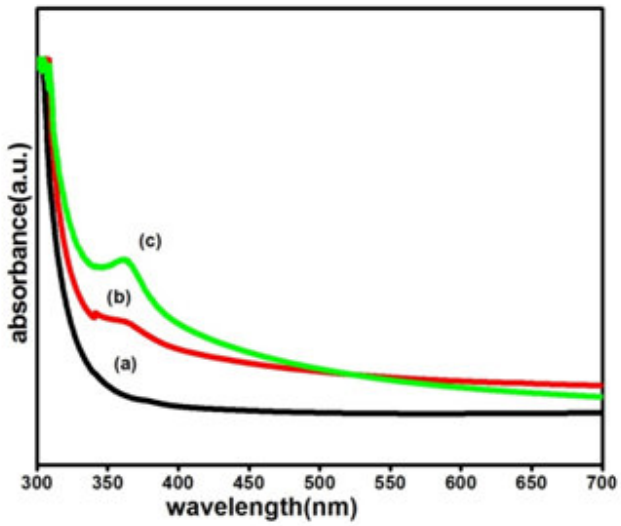


Figure 3
UV –Vis spectra of (a) C-G composite, (b) C-G/ 1%ZnO and (c) C-G/ 5%ZnO

the absorption peak at maximum concentration of NPs is 361 nm lower than that of macrocrystalline ZnO NPs (372 nm). Whereby, absorption spectrum is in blue shift region. This renders information that ZnO NPs are in nanometer range in C-G composite.

3.3 SURFACE MORPHOLOGICAL STUDIES

Fig. 4 presents the micrograph images of composites with minimum and maximum concentration of NPs. Fig. 4(a) shows that C- G composite have smooth surface morphology. This is due to the adequate mixing of chitosan and gelatin component forming smooth area. Fig. (b) depicts the minimum concentration of NPs in composite showing slightly disturbed surface morphology.

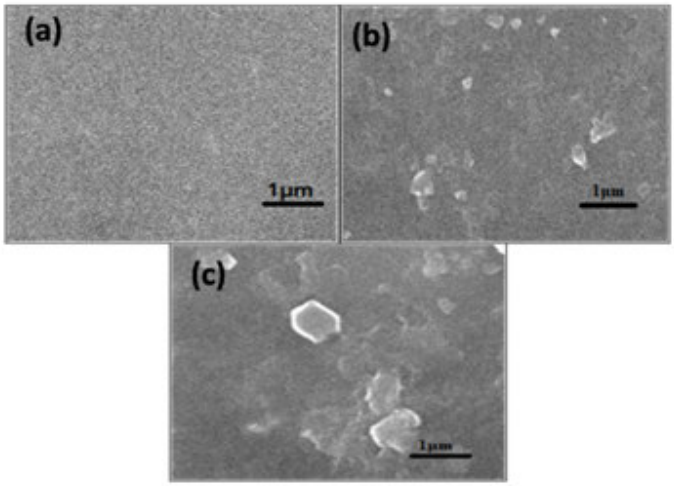


Figure 4
SEM images of (a) C-G composite, (b) C-G/ 1%ZnO and (c) C-G/ 5%ZnO

Interestingly, the crystal structure of NPs could be observed clearly in composite. At maximum concentration of NPs, agglomeration can be seen in Fig. 4(c). Even though at high concentration of NPs, crystallite structure of ZnO can be seen in micrograph images. The smoothness decreases as the concentration of ZnO increases in composite.

3.6 MECHANICAL PROPERTIES

Fig. 5 (a and b) shows tensile strength and % elongation of the samples. The tensile strength of C-G composite (SA) is measured to be 27.5 MPa as shown in table 1. After incorporating 1% ZnO NPs into C-G matrix, significant increase in tensile strength of the composite is observed. It reaches the

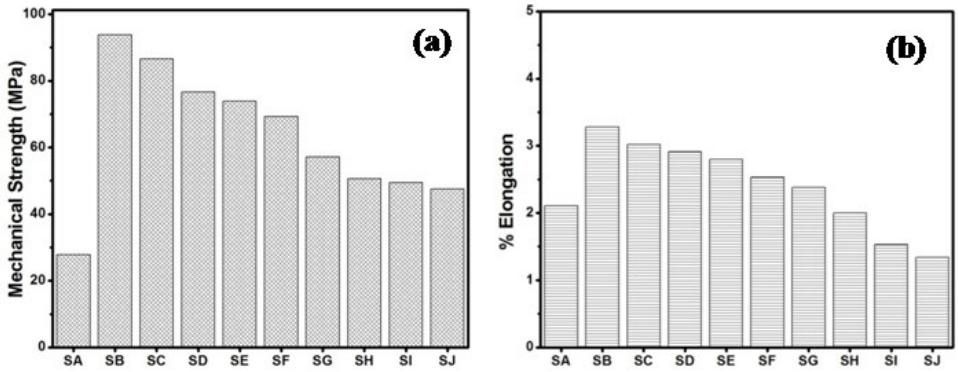


Figure 5
(a) Mechanical property of composites with varying concentrations of ZnO and (b) % Elongation of composites with varying concentrations of ZnO

value of 93.8 MPa. As the concentration of dopant increases upto 5 % variation in mechanical behavior of the samples is observed. However, this small change in strength is also beneficial for composites to be used as biomedical materials. The variation in mechanical behavior of the composites is due to disturbance of excess dopant between two components. The hydrogen bonds are formed between -OH and $–NH_2$ groups from chitosan and -COOH group from gelatin. These inter and intra molecular bonding is disturbed with addition of NPs into composite matrix, resulting in the fluctuations of mechanical strength of samples with increasing NPs

Table 1
Mechanical properties of C-G composites and varying concentration of ZnO nanoparticles

Sample % of ZnO nanoparticles in films	Tensile strength (MPa)	Elongation at break %
SA (0%)	27.8	2.11
SB (1%)	93.8	3.2
SC (1.5%)	86.562	3.0
SD (2%)	76.562	2.9
SE (2.5%)	73.81	2.8
SF (3%)	69.312	2.5
SG (3.5%)	57.187	2.3
SH (4%)	50.625	2.0
SI (4.5%)	49.5	1.5
SJ (5%)	47.562	1.3

concentrations. In C-G composite, amorphous gelatin concentration is much higher than crystalline chitosan. Due to this reason, composite become stiff. The % elongation of samples SB and SC is calculated to be nearly equal.

3.7 ANTIBACTERIAL ACTIVITY

Table 2 elaborates the results for antibacterial activity of *E. coli and S. aureus* bacteria. Here, a very facile and economic well diffusion method is used to assess the antibacterial effect of prepared composites materials. C-G composite is used as control at neutral pH. From table 2, it is observed that, as the mass ratio of ZnO NPs to C-G increases, significant enhancement in the zone of inhibition occurs. The Gram negative *E. coli* bacterium is more susceptible to composites whereas Gram positive bacterium shows little resistance. This is attributed to the outer cell membrane structure of *S. aureus* which is firm, owing to higher amount of peptidoglycan. The antibacterial activity of ZnO NPs generally explained on the basis of oxidative stress mechanism. ZnO NPs generates reactive oxygen species upon irradiation with light. The ROS generated (·OH, $O\cdot^{-}_{2}$, H_2O_2) causes cell death. Free hydroxyl radicals bind on the negative surface of the cell membrane and hydrogen peroxide penetrates inside the cell leading to the leakage of minerals, proteins and denaturing of DNA ultimately leads to the cell death[22, 23]. Additionally, ZnO NPs may produce Zn^{2+} ions which penetrate inside the cell and physical contact occurs between cell membrane and ions due to electrostatic force of attraction leading to cellular death [24-26].

Table 2
Antibacterial activities of composite membranes

Composites	Zone of inhibition in mm	
	E. Coli	*S. aureus*
SA	0	0
SB	18	1.2
SC	18	1.5
SE	19	1.8
SF	20	1.9
SG	21	2
SH	22	2.1
SI	23	2
SJ	23	2

Chitosan-Gelatin/ZnO composite membrane have been successfully prepared by casting method and reported for the first time. The membrane shows favorable antibacterial and excellent mechanical properties with the value of 93.8MPa at 1% ZnO NPs concentration. But the % elongation seems little less due to the high concentration of gelatin making it stiff membrane. The composite shows excellent mechanical strength and enhanced anti bacterial activity for all composites. The antibacterial activity shows enhancement with increase in NPs concentration in composite membranes. Hence, it is revealed that the chitosan-gelatin/ZnO NPs composites possess potential to be used in biomedical applications.

4. CONCLUSION

Chitosan-Gelatin/ZnO composite membrane have been successfully prepared by casting method and reported for the first time. The membrane shows favorable antibacterial and excellent mechanical properties with the value of 93.8MPa at 1% ZnO NPs concentration. But the % elongation seems little less due to the high concentration of gelatin making it stiff membrane. The composite shows excellent mechanical strength and enhanced anti bacterial activity for all composites. The antibacterial activity shows enhancement with increase in NPs concentration in composite membranes. Hence, it is revealed that the

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