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Fabrication Of Wound Dressings Impregnated With Zinc Oxide Nanoparticles Synthesized From Honey: A Facile Approach For Antibacterial Application

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Abstract

The present study explores the fabrication of wound dressing materials impregnated with zinc oxide nanoparticles (ZnO NPs) using honey as a reducing and capping agent. Formation of Honey-zinc oxide nanoparticles (H-ZnO NPs) was confirmed by surface plasmon resonance absorption at 360 nm. Morphology and elemental composition of the synthesized H-ZnO NPs were analysed by scanning electron microscopy and energy dispersive X-ray spectroscopy. The wound dressing material was impregnated with H-ZnO NPs by pad-dry cure method. The biocompatibility of the ZnO NPs loaded dressing material was evaluated by hemolysis (1.18%) and whole blood clotting test. The antibacterial activity of the dressing materials was assessed qualitatively and quantitavely by AATCC technique. The ZnO NPs loaded dressing materials possessed a remarkable antibacterial activity compared to honey impregnated and untreated dressing materials. The results illustrate that the biofabricated wound dressing material can be efficiently exploited in therapeutic application.

Keywords: zinc oxide nanoparticles, honey, hemocompatibility, wound healing, biofabrication

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Introduction

Recently, there has been a tremendous surge in unraveling the potential application of metal oxide nanoparticles in biomedical application. Of the different metals, utilization of zinc in nanoparticle synthesis is meager. Nanosized ZnO is an highly preferred multitasking metal oxide due to its unique optical and electrical properties (Wang *et al.*, 2004). Furthermore, zinc oxide is

reported to be non-toxic, chemically stable and potentially safe for human use by the U.S. Food and Drug Administration (Premanathan *et al.,* 2011). The advantages of nanostructured ZnO in biomedical application over other metals are due to its lower cost; UV blocking properties, high catalytic activity, large surface area with greater dispersion (Kumar *et al.,* 2011). Eco-friendly and cost-effective procedures utilizing microorganisms and plants in the synthesis of nanoparticles have been reported extensively all over the world (Sangeetha *et al.,* 2011). Honey-the Food of Gods and one of the natural sweetener for centuries was subjected to extensive studies (Crane, 1975) all over the world related to its ingredients, physicochemical properties and mineral content. However, only a few reports are available on the synthesis of nanoparticles using natural honey. Furthermore, honey is reported to possess antibacterial and wound healing properties (Cooper *et al.,* 2002).

Commercially available wound dressings generally prevent the exposure of infected area to the microbial pathogens; allow free flow of air in order to establish a suitable milieu for natural healing (Paul and Sharma *et al.,* 2004). However lack of antimicrobial activity of wound dressings available in the market is of great concern.

Generally, wound dressings serve as a scaffold in the topological application of antimicrobial creams to the site of infection. Therefore, particular attention is oriented in biofabrication of wound dressings with inbuilt wound healing and antibacterial activity. This can be achieved by the impregnation of wound dressings with ZnO NPs by pad-dry cure method (Aljadi *et al.,* 2000). Utilization of honey as a reductant as well as capping material in the synthesis of zinc oxide nanoparticles will provide a two-fold advantage in wound dressing biofabrication technology. Therefore, in the present study an attempt has been made to biofabricate a wound dressing by the impregnation of the zinc oxide nanoparticle synthesized using honey as a reductant and capping agent. The biosynthesized nanoparticles were characterized by UV-Vis, SEM coupled with EDAX imaging. Therapeutic potential of the biofabricated wound dressing was illustrated by hemocompatibility and antibacterial activity.

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Materials and methods

Honey sample

Natural, untreated and unpasteurized honey was harvested by squeezing the comb of *Apis indica* collected from the foot hills of Papanasam, Tamil Nadu, India. The sample was filtered and stored under aseptic conditions at 5ºC until further analyses. The honey collected for analyses is of multifloral origin.

Physico-chemical properties of honey

The sample was analysed for pH, ash, moisture, acidity, sugars and hydroxyl methyl furfural (HMF) following Association of Official Analytical Chemists (AOAC) methods (1990). Electrical conductivity was measured as described by Bogdanov *et al.* (1997). Viscosity of honey was calculated using Ostwald's viscometer following the method of Akoh (1991).

Synthesis of zinc oxide nanoparticles

Zinc nitrate was mixed with 200 mL of honey to a final concentration of 0.05 mM. After complete dissolution, the mixture was kept under vigorous stirring at 150ºC for 6 h and allowed to cool at room temperature. The pale white precipitate was washed with sterile distilled water (2795xg, 20 minutes) followed by an ethanol wash for purification. The resultant white precipitate (H-ZnO NPs) was dried at 80ºC for 7-8 h. The synthesized ZnO NPs was characterized by UV-visible spectroscopy (Eppendorf Biospectrometer). The morphology and elemental composition of H-ZnO NPs was determined by scanning electron microscopy coupled with energy dispersive X-ray spectrum (HITACHI S-3000H).

Fabrication of wound dressings

For biofabrication studies, commercially available sterilized wound dressings (Surgicom® BP type 13) were purchased from the local medical shop. Wound dressings were impregnated with ZnO NPs (1% citric acid binder) for 5 minutes and then it was passed through a padding mangle (R.B. Electronic and Engineering, Mumbai), running at a speed of 15 m/min with a pressure of 1 Kgf/cm^2 to 100% wet pickup. After padding the samples with H-ZnO NPs dressings were dried at 70ºC for 3 minutes followed by curing at 150ºC for a brief period of 2 minutes. Untreated wound dressing was used as positive control. (El-Rafie *et al.*, 2014)

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Blood compatibility assay

A. Hemolysis assay

The fabricated nano dressing materials were cut into small pieces (approximately 2cm X 2cm) and equilibrated in 4 mL saline for 30 minutes at room temperature. Human blood mixed with acid citrate dextrose (ACD) (0.2 mL) was added to the suspension and incubated for 60 minutes. After incubation, 4 mL of saline was added to stop hemolysis. The solution was centrifuged (27595xg, 20 minutes) and optical density of the supernatant was measured at 545 nm. Positive and negative controls were maintained by adding 0.2 mL of ACD human blood to 4 mL of distilled water and saline respectively. Percent hemolysis was calculated as described by Dey and Ray (2003) as follows:

$$\%\ \text{hemolysis} = \left[\frac{\text{OD of test sample - OD (-) control}}{\text{OD (+) control-OD (-) control}}\right] \text{X } 100$$

B. Blood clotting index

Nano dressings were kept in beaker and prewarmed to 37ºC. Twenty five microlitre of fresh ACD human blood was dropped on to the dressing material followed by the addition of calcium chloride 0.02 mL (0.2 mol/l). The dressing material was incubated for 10 minutes at 37ºC with shaking. The red blood cells that were not trapped in the clot was measured at 540 nm at different time intervals. Blood clotting index was calculated as described by Archana *et al.* (2013)

$$\text{BCI} = \frac{\text{Absorbance of blood which had been in contact with sample}}{\text{Absorbance of solution of distilled water and ACD blood}} \text{ X } 100$$

Antibacterial activity Studies

Antibacterial property of the nano dressing material (impregnated with 100 ppm of H-ZnO NPs) was tested qualitatively (EN ISO 20645) and quantitatively (AATCC-100). For qualitative analysis, the wound dressings were placed on the agar medium swabbed with bacterial cultures (*Escherichia coli* MTCC 443, *Staphylococcus epidermidis* MTCC 2639, *Pseudomonas aeruginosa* MTCC 424, *Bacillus cereus* MTCC 430 and *Enterobacter aerogenes* MTCC 2822). Zone of inhibition was measured after 24 h of incubation at 37ºC. In quantitative method, the

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dressing material was immersed in a nutrient broth (10mL) inoculated with 10µl (7x10CFU/mL) of mid log phase bacterial culture and incubated in a shaker (200 rpm) at 37ºC for 12 h. The samples were serially diluted and transferred to nutrient agar plates. Percent reduction in number of colonies after 24 h of incubation at 37ºC was calculated using the formula,

$$\% \text{ reduction} = \frac{A - B}{A} \text{ X } 100$$

A represents the number of bacterial colonies (CFU/mL) in the control and B is the number of bacterial colonies obtained on exposure to nano wound dressing material.

Results & Discussion

The physicochemical parameters of honey collected from the bee hives is depicted in Table 1. The sample was found to be acidic (4.5). Acidity of the honey may be due to the fermentation of sugar into organic acid. Acidity is reported to be responsible for honey's flavor and stability against microbial spoilage (CDEU, 2001). Further the pH value was within the permissible limit indicating the freshness of the honey as described by Khalil *et al.* (2012). The moisture content of the honey was found to be 16.6 which might be due to the different floral source. The low moisture content prevents the microbial attack during storage (Moniruzzaman *et al.*, 2013). The moisture content of the honey collected was consistent with the previously reported values (Khalil *et al.*, 2010).

Electrical conductivity (EC) is a key physicochemical parameter for the authentication of honey quality (Mateo *et al.*, 1998). The EC value depends on the ash and acid content of the honey (Bogdanov *et al.*, 2002). This parameter was recently included in the International standards, replacing the determination of ash content (Alimentarius, 2001). In the present studies EC value of honey sample was found to be within the recommended range (0.12 mS/cm). HMF value indicates the purity and freshness of honey. The HMF concentration (5.65) of the honey collected was found to be within the limit set by the Codex Alimentarius Commission and the European Union. The result obtained illustrates that the honey harvested was found to be of good quality in respect to HMF content, reducing sugar concentration, pH, acidity etc.

Synthesis and characterization of Zinc oxide nanoparticles

Zinc oxide nanoparticles have attracted great attention because of its superior optical properties and wide application in biomedical sciences. In the present study, ZnO NPs was synthesized using honey as a reducing agent. The addition of honey to 0.05mM solution of zinc nitrate led to the appearance of white precipitate resulting in the formation of H-ZnO NPs. The nanoparticles exhibit a strong UV absorption spectrum with the absorption peak ranging from 350-370 nm due to its surface plasmon resonance and attains a plateau above 3.3eV (360 nm). The absorption peak centered around 350-370 nm wavelength confirms the presence of ZnO NPs (Fig. 1)

SEM with EDAX analysis

SEM image illustrates the surface morphology, size and shape of the nanoparticles. Scanning electron microscopic image of H-ZnO NPs along with EDAX was presented in Fig. 2. SEM imaging revealed the crystalline nature of the synthesized nanoparticles with the particle size approximately 52 nm. EDAX spectrum confirms the purity and elemental composition of the nanoparticle with the presence of zinc and oxygen.

Hemolysis assay

Hemolysis is considered as a reliable measure of determining the blood compatibility of biomaterials. Generally, smaller the hemolysis ratio value, better the blood compatibility of the biomaterial. In the present study, H-ZnO NPs impregnated dressing material induced 1.8% hemolysis (Fig.3). Whereas hemolysis was found to be 3.2% and 4.7% with negative and positive control. Autian *et al.* (1975) reported that a value of up to 5% hemolysis is permissible for biomaterials. So the dressing material impregnated with H-ZnO NPs was considered highly hemocompatible.

Whole blood clotting

In this study, the antithrombogenic activity of the biofabricated dressing material is qualitatively expressed by a relative parameter blood clotting index (BCI). Blood clotting index of the nano dressing material possessed shorter clotting time than honey impregnated and untreated materials. The quicker the absorbency and shorter the clotting time better the hemostatic effect of the material used. In the present study, after 10 minutes of incubation, BCI of H-ZnO NPs loaded

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wound dressing and honey impregrated wound dressing was found to be 77 and 53 respectively. Untreated wound dressing material possessed a BCI of 22 after 10 minutes of incubation.

Antibacterial

The antibacterial activity of H-ZnO NPs loaded dressing materials was evaluated qualitatively and quantitatively by standard methods. It is evident from the results (Fig.4) that the gram negative bacteria was found to be highly susceptible to nano dressings than gram positive bacteria (Table 2). The Gram-negative bacteria possess a negatively charged outer membrane and a thin peptidoglycan layer (~7-8 nm), which facilitates the anchoring and penetrating of the ZnO NPs. In contrast, the gram positive bacteria have a thick three dimensional rigid structured peptidoglycan layer (~20–80 nm), which limits the penetration of the positively charged ZnO NPs (Hebeish *et al.,* 2010). In this study, bacterial inhibition was considerably lesser with honey impregnated dressing materials. Furthermore, untreated dressing materials failed to inhibit the growth of bacteria.

Conclusion

To conclude we have reported green one-step cost effective biofabricated nano dressings impregnated with H-ZnO NPs. This study is the first to report the synthesis of ZnO NPs using honey as a reducing agent. Moreover, combination or synergistic effect of zinc oxide with honey against pathogenic bacteria is a new finding. Thus the biofabricated nano dressing materials can compete with commercial antibiotics used in the treatment of microbial wound infections and are even better. However more research work on animal models needs to be done before commercial application.

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Table 1 Physicochemical constituents of natural honey

Parameters	**Units**	**NH**
pH		4.5
Ash content	%	0.46±0.14
Moisture content	%	16.60±1.69
Free acidity	Meq/kg	13.76±0.17
Lactic acidity	Meq/kg	2.31±0.12
Total acidity	Meq/kg	27.3±0.32
Color		Light amber
Viscosity	Centipoise	3.4±0.12
Electrical conductivity	ms/cm	0.12±0.23
Hydroxy methyl furfural	Mg/kg	5.65±0.45
Carbohydrate composition		
Total sugar	g/100 g	72.35±1.43
Reducing sugar	g/100 g	69.24±0.24
Sucrose	g/100 g	5.32±1.50

Data are given as mean±standard error of three replicates

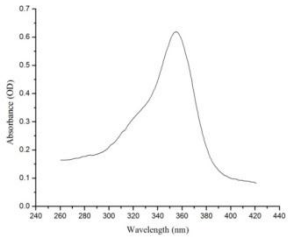
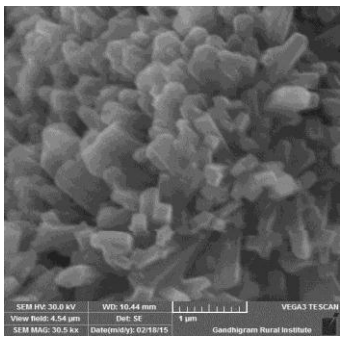


Fig.1 UV-vis absorption spectrum of H-ZnONPs



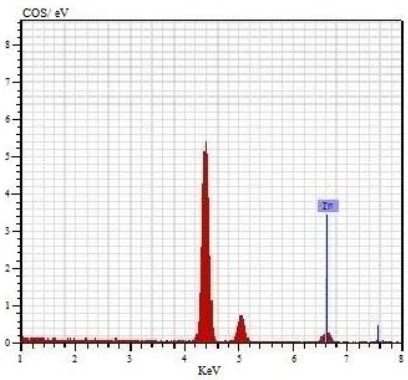


Fig 2. SEM – EDAX analysis of H-ZnO NPs

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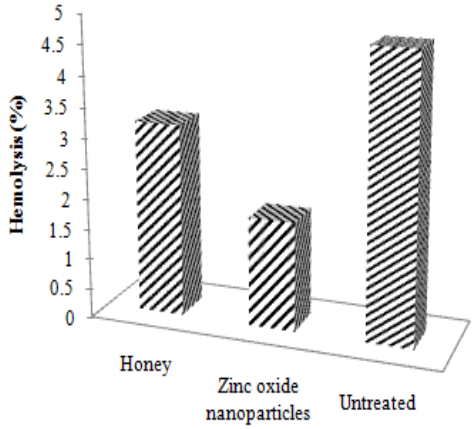

Fig.3 Hemolytic activity of biofabricated wound dressing materials

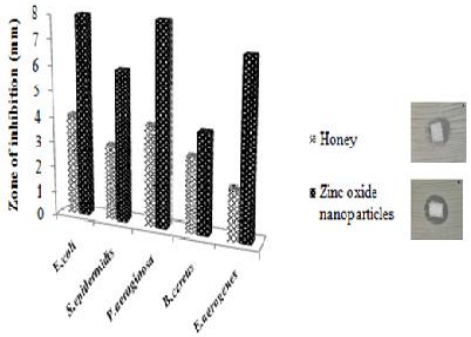

Fig. 4 Antibacterial activity of biofabricated wound dressing materials against different pathogens

Table 2 Quantitative assessment of antibacterial activity of wound dressing materials by percentage reduction method

Samples	Percentage reduction				
	E. coli	***S.epidermidis***	***P. aeruginosa***	***B. cereus***	***E. aerogenes***
Honey	50	60	52	33	44
Zinc oxide nanoparticles	87	84	81	76	79