Flexible and Micro-Porous Chitinous Nanocomposite Bandages for Wound Dressing

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Chapter 1: Introduction

Diabetes is a group of metabolic diseases and it causes the formation of foot ulcers that is difficult to treat and occasionally require amputation. India is the leading country with more number of diabetic patients with foot ulcers. Impaired healing is the problem associated with diabetic wounds and it is increasing nowadays. Similarly, burn and chronic wounds also getting in to this category with scar formation and tissue loss. These wounds are associated with high volume of exudates and are susceptible to microbial infection. Chronic wounds are growing with incidence due to the growing age of the population and due to the lack of awareness and improper diagnosis. Wound infection is the major difficulty in the field of wound care management, because such infections can cause exudate formation, delay the wound healing, facilitate improper collagen deposition, etc. Microbes are the major reason for infection and the prevalence of the same is high in and around us. The major infection causing bacteria were Staphylococcus aureus and Escherichia coli. Once entered into the body, these microbes grow immediately and start to form colonies. The microbes can easily enter the body through the wounds and can reach into deeper portions of the tissue and, furthermore, can lead to internal infection. The remedy for the above-mentioned harms would be the use of wound dressing with antibacterial activity.

Kaltostat™ is the leading wound dressing bandage available in the market and it is made of alginate. Even though people are using this material since long it have drawbacks like adherence nature on to the wound, poor swelling ability and no antibacterial activity. Apart from Kaltostat, there are other dressings also available (Nu Derm™, Duoderm™, Tegaderm™ etc) and these dressings have de-merits like less flexibility, poor mechanical strength, lack of porosity, tendency of dressings to adhere onto the wound surface and majority of the dressings did not possess antibacterial activity. The adherence of wound dressing material on the wound surface cause the trauma to the healing wound when it peeled off. At times, even a wound dressing itself can cause infection, because of the wound dressing-exudate interface, non-sterility of the wound dressing, etc. Various types of wound dressing materials are available commercially, i.e., those made of synthetic polymers such as poly (vinyl alcohol)) or using natural polymers (such as alginate, chitin, chitosan. etc). Previous studies have been reported where chitosan films and membranes were used to treat patients with
deep burns, orthopedic injuries, etc. The reasons for choosing chitosan were the ease of availability, hemostatic potential, and biodegradability, and the biodegradation product was N-acetyl glucosamine, which was already present in the human body and will enhance the re-epithelization. The so-called membranes and films have poor mechanical strength, less flexibility, poor wound healing potential and has no antibacterial activity.

Hydrogel-based wound dressings would be helpful to provide a better healing effect on these types of wounds. Hydrogel based wound dressing materials provide cooling sensation and a moisture environment, as well as act as a barrier to microbes at the wound surface. These materials were capable of absorbing large volume of wound exudate from the wound surface and hence reduce the chance for infection. Further, the hydrogel based materials would be helpful to promote the cell adhesion and proliferation. Chitin and chitosan did not show antibacterial activity at neutral pH so, in order to impart antibacterial activity it is necessary to incorporate some antibacterial agents into it. ZnO nanoparticle is one of the most widely used engineered nano materials in commercial products due to its UV light absorption, antimicrobial, catalytic, semi-conducting, and magnetic properties. ZnO nanoparticle is, therefore, widely applied to personal care products, sunscreen, paints, electronic materials, rubber manufacture, food additives, and medicine. So, we selected ZnO nanoparticles for this purpose owing to its antibacterial activity as well as wound healing enhancing ability by migrating keratinocytes cells towards the wound site. In this work, we developed and evaluated the wound healing potential of nZnO incorporated chitosan and α and β-chitin hydrogel based nanocomposite bandages.

**Objectives of the study**

**The specific objectives of the study include:**

1. To synthesize and characterize ZnO nanoparticles, chitosan, α- chitin and β-chitin hydrogel
2. To develop and characterize nZnO incorporated chitosan, α-chitin and β-chitin hydrogel nanocomposite bandages
3. To evaluate the porosity, swelling capacity, biodegradation, hemostatic potential, antimicrobial activity and cytocompatibility of the composite bandages under *in vitro* conditions
4. To evaluate the cell attachment, proliferation and infiltration on the composite bandages
5. To evaluate the *in vivo* wound healing potential of the composite bandages in animal model (Sprague-Dawley rats)

**Review of literature**

An ideal wound dressing material should maintain a moist environment at the wound interface, allow gaseous exchange, act as barrier to microorganisms and it should have wound exudates absorbing ability.\(^1\)\(^,\)\(^2\) It should be non-toxic and non-allergenic. The advantages of using hydrogel bandages are retention of moist atmosphere, high swelling capacity and healing of wound without scar formation.\(^3\) Recent reports aiming on the acceleration of the wound repair by systematically designed dressing materials.\(^4\) Hydrogel based biodegradable scaffold materials gained interest in the field of wound dressing.\(^5\)\(^,\)\(^6\) These types of materials are capable of swelling when immersed in liquid medium. This would help the cells to get entrapped inside the scaffolds and also promotes the passage of oxygen and nutrients into the inner regions of the scaffolds. The scaffold provides necessary support for cells to proliferate and to maintain their differentiated function.\(^7\)\(^,\)\(^8\) In particular, efforts are focused on the use of biologically derived materials such as, chitin and its derivatives, which are capable of accelerating the healing processes at molecular, cellular, and systemic levels.\(^9\)\(^,\)\(^10\) These materials leads to the migration of keratinocytes towards the wound site to enhance wound healing.\(^11\)\(^,\)\(^12\) Chitin can be converted into different forms like hydrogel, scaffolds, fiber etc. The use of chitin would be beneficial in the wound dressing field.\(^13\)\(^-\)\(^15\)

Yen et al reported the wound healing effect of layered hydrogel dressing comprised of chitosan, alginate and polyglutamic acid.\(^16\) Takei et al reported the use of hydrogel dressing made of chitosan derivative for full thickness wound treatment in rats.\(^17\) Hydrogel sheets of honey, chitosan and gelatin was used to treat burn wounds and it was found that the wounds healed faster in comparison with the control.\(^17\)\(^,\)\(^18\) Chitosan has been reported to promote granulation and organization of wounds by the enhancement of the functions of inflammatory cells, such as polymorphonuclear leukocytes (PMN) and macrophages.\(^18\)\(^,\)\(^19\) These materials have poor mechanical strength and poor swelling ability. Previous studies had shown that in the size scale less
than 100 nm and at the appropriate concentration nZnO possess potent antibacterial activity but has no adverse effect on normal cells.\textsuperscript{20-22} Further, the zinc ions released from ZnO can enhance keratinocyte migration towards the wound site and promote healing.\textsuperscript{23, 24}

Chapter 2: Materials and Methods
Preparation of ZnO nanoparticles
Zinc oxide nanoparticles (nZnO) were prepared as follows; 0.1M sodium hydroxide solution was added drop wise to 0.1M zinc acetate dihydrate solution with continuous stirring. The solvent used for the preparation was methanol. The precipitated zinc oxide was centrifuged and washed several times with distilled water to remove the by-products and dried at 80 °C.

Preparation of chitosan hydrogel/nZnO composite bandage
Chitosan solution was prepared by dissolving 2 gram of chitosan in 1% acetic acid solution under room temperature. The solution was then filtered to remove undissolved particles. Chitosan hydrogel was prepared by raising the pH of chitosan solution to neutral pH by the addition of 1% NaOH solution followed by centrifugation of the hydrogel to remove unbound water. The prepared nZnO were then suspended in water followed by probe sonication for 10 minutes. The ZnO nanosuspension was added drop wise to the chitosan hydrogel and the whole mixture kept under vigorous stirring for 1 hr. The homogenized chitosan hydrogel/nZnO mixture was poured on to a Teflon mould and kept at -20°C overnight. The frozen samples were lyophilized for 24 hr to get porous chitosan hydrogel /nano ZnO bandage (CZB).

Characterization and biological evaluation
The prepared nZnO and CZBs were characterized using XRD, SEM and FT-IR. The morphology and size of nZnO were characterized using dynamic light scattering measurements and Atomic Force Microscope. The structural morphology of CZBs was characterized by scanning electron microscope.

Preparation of chitin hydrogel
Chitin powder was added to saturated CaCl\textsubscript{2}/methanol solvent and stirred vigorously for 48 hrs at room temperature. To the prepared chitin solution, excess water was added and stirred vigorously for 2 hrs to obtain chitin hydrogel. The precipitated hydrogel was
separated from the supernatant by centrifugation and purified by dialysis against distilled water for two days to get pure chitin hydrogel.

**Preparation of chitin hydrogel/nZnO composite bandage**

ZnO nanosuspension was prepared via probe sonication for 10 minutes. The ZnO nanosuspension was added drop wise to chitin hydrogel under vigorous stirring for 1 h. The homogenized chitin hydrogel/nZnO mixture was poured on to Teflon mould kept at -20 °C. The frozen samples were then freeze-dried to get porous chitin hydrogel/nZnO composite bandage.

**Material properties and biological evaluation**

The prepared nanocomposite bandages were characterized using FT-IR, XRD and SEM. In addition, blood clotting, antibacterial, swelling, cytocompatibility and cell attachment capability of the prepared nanocomposite bandages were evaluated.

**Preparation, characterization and evaluation of β-chitin hydrogel/nZnO composite bandages**

β-chitin hydrogel was prepared using β-chitin powder and saturated CaCl₂/methanol solvent. After getting a transparent β-chitin solution, the solution was filtered to remove the undissolved traces. To this solution, excess water was added and stirred for 2 hrs vigorously to obtain β-chitin hydrogel. The obtained hydrogel was dialyzed against distilled water for two days to get pure β-chitin hydrogel.

β-chitin hydrogel/nZnO composite bandages were prepared by adding 0.05 & 0.1% of nano ZnO to β-chitin hydrogel. The prepared composite bandages were characterized using FT-IR and XRD. Porosity, swelling ratio, biodegradation, blood clotting, antibacterial activity, cell viability, attachment, proliferation and in vivo evaluation studies were performed.

**Chapter 3: Results and Discussion**

**Chitosan hydrogel/nZnO composite bandages**

The prepared chitosan hydrogel/nZnO bandages (CZBs) that have interconnected pores showed ~80% porosity of the total bandage volume and were helpful with regard to absorbing large volumes of wound exudate (Fig.1). CZBs showed controlled swelling, degradation, enhanced blood clotting, and excellent platelet activation ability. In vitro cytocompatibility studies revealed that the bandages showed enhanced cell viability and infiltration. In vivo wound healing evaluation proved the enhanced healing ability of
CZBs without causing toxicity to cells (Fig. 2). *In vitro* and *in vivo* antibacterial activity studies proved that the antibacterial potential of the prepared CZBs.

**Fig. 1.** (A) Schematic representation of the chitosan hydrogel/nZnO composite bandage. (B, C, D) Photographs of the chitosan hydrogel, nZnO suspension, and chitosan hydrogel/nZnO mixture, respectively. (E, F, G, H) Photographs of chitosan hydrogel/nZnO composite bandage. (I, J, K) SEM images of chitosan control, chitosan + 0.01% nZnO, and chitosan + 0.005% nZnO composite bandages, respectively. (L) SEM image of the chitosan + 0.01% nZnO composite bandage; white arrows indicate the nZnO particles.

**Fig. 2.** Photographs of *in vivo* wound healing study.
α-Chitin hydrogel/nZnO composite bandages

The nanocomposite bandages (Fig. 3) showed enhanced swelling, blood clotting and antibacterial activity. The incorporation of nZnO helped to attain antibacterial activity. Cytocompatibility studies were carried out using human dermal fibroblast (HDF) cells and thus we proved the non-toxic nature of the composite bandages. HDF Cell attachment and infiltration analysis showed that the cells were attached and penetrated into the interior (250 µm) of the nanocomposite bandages.

β-chitin hydrogel/nZnO composite bandages

β-chitin hydrogel/nZnO composite bandages (Fig. 4) showed sufficient strength and we completed the in vivo evaluation in Sprague-Dawley rat model (Fig.5). The obtained in vivo data showed that these composite bandages showed enhanced healing in comparison with the control.
Chapter 4: Conclusion

Chitosan hydrogel/nZnO and α/β-chitin hydrogel/nZnO composite bandages were prepared and characterized by FT-IR, XRD and SEM. The FT-IR and XRD spectra were confirmed the intermolecular hydrogen bonding interaction between ZnO nanoparticles and chitosan & α/β-chitin. SEM images of the composite bandages showed that these bandages were highly porous and pores were well interconnected.
The results of blood clotting studies showed that chitosan hydrogel/nZnO composite bandages were capable of clotting blood quickly compared to the control. The nanocomposite showed inhibitory effects on the bacterial growth (S. aureus & E.coli); signifying its role as an antibacterial agent. Cell viability studies on human dermal fibroblast cells proved that the composite bandages were showing low viability at higher concentrations of nZnO and viability was increased at lower concentration of nano ZnO. The obtained results indicated that the β-chitin hydrogel/nZnO composite bandages showed sufficient strength and we completed the in vivo evaluation in Sprague-Dawley rat model. The obtained in vivo data showed that these composite bandages showed enhanced healing in comparison with the control.

REFERENCES
LIST OF PUBLICATIONS


PATENT