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Bi-layered nanocomposite bandages for controlling microbial infections and overproduction of matrix metalloproteinase activity

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ABSTRACT

Chronic diabetic wounds is characterised by increased microbial contamination and overproduction of matrix metalloproteases that would degrade the extracellular matrix. A bi-layer bandage was developed, that promotes the inhibition of microbial infections and matrix metalloprotease (MMPs) activity. Bi-layer bandage containing benzalkonium chloride loaded gelatin nanoparticles (BZK GNPs) in chitosan-Hyaluronic acid (HA) as a bottom layer and sodium alendronate containing chitosan as top layer was developed. We hypothesized that the chitosan-gelatin top layer with sodium alendronate could inhibit the MMPs activity, whereas the chitosan-HA bottom layer with BZK GNPs (240 ± 66 nm) would enable the elimination of microbes. The porosity, swelling and degradation nature of the prepared Bi-layered bandage was studied. The bottom layer could degrade within 4 days whereas the top layer remained upto 7 days. The antimicrobial activity of the BZK NPs loaded bandage was determined using normal and clinical strains. Gelatin zymography shows that the proteolytic activity of MMP was inhibited by the bandage.

1. Introduction

Wound healing is a cascade of sequential overlapping phases involving a number of cell types, extracellular matrix (ECM) proteins and growth factors which will help in wound closure and ultimately help restore functional integrity [1]. Matrix metalloproteinases are group of enzymes secreted by inflammatory cells such as lymphocytes, monocytes, macrophages and non-inflammatory cells such as fibroblast cells, endothelial cells and keratinocytes, that degrade collagen and gelatin of the ECM for the process of wound remodelling. Fibroblast cells and other granulation forming cells invade through the matrix to the wound site. Therefore, moderate productions of MMPs are essential for the process of healing [2]. MMPs are of different types which include collagenases, gelatinases, stromelysins, matrilysins, metalloelastases, membrane-type MMPs and other MMPs depending on the type of substrates. These are secreted as basal levels [2]. However Nwomeh et al. has found a difference in MMP expression levels in chronic and acute wounds [3]. The MMPs are found to be highly over expressed in chronic wounds which may contribute to their delayed closure. A moist wound environment is an ideal ground for proliferation of pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella etc* which may further delay wound closure and if left untreated can also lead to septicaemia and be fatal [4]. An approach to regulate the activity of the overexpressed MMP activity along with the suppression of infection is an interesting concept to achieve accelerated wound closure in case of chronic wounds.

An ideal bandage system must be biocompatible at the same time highly porous to enable exudate absorption, gaseous exchange and also should act as a barrier against microbial colonization [5]. Chitosan, hyaluronic acid and alginate are some of the natural polymers that act as a suitable matrix for drug delivery [6–11]. Chitosan is an ideal material for developing a bandage system due to its biocompatibility, haemostatic potential, non-toxic nature and biodegradability [12]. Chitosan hydrogel based bandage system was found to be highly flexible and porous, making it suitable for moderate to heavy exudate generating wounds and also acts as a good drug delivery system [13]. Chitosan has been reported to

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show antibacterial property depending on its pH [14]. However chitosan at neutral pH has no much antibacterial activity, therefore an external agent can be introduced to impart antibacterial property. The degradation of chitosan relates to its degree of deacetylation and slow degradation rate may prolong the drug release [15]. Thus incorporation of hyaluronic acid (HA), which is highly degradable, will help in faster degradation rate of the bandage system. Hyaluronic acid was selected because it is a major component of extracellular matrix and upon degradation releases products that can facilitate angiogenesis and proliferation of fibroblasts [16]. Previously a bilayered bandage was prepared from Chitosan-HA incorporated with Amicar which was effective in inhibiting blood clot by faster release of Amicar from HA layer and eliminating secondary microbial infections by sustained release of antibiotic from chitosan layer *in vitro* [15].

Various synthetic molecules like bisphosphonates [17], doxycycline [18], 2, 3-dihydroxybenzoic acid [19], *N*-hydroxyurea [20] have shown to inhibit the activity of MMPs. Alendronate (4-amino-1-hydroxybutane-1, 1-bisphosphonic acid), a bisphosphonate molecule that has a potential to chelate ions like Zn^{2+} and Ca^{2+} which are in fact the co-factors of MMPs [21]. The chelations of the co-factor ions by alendronate inhibit the proteolytic activity of the highly over-expressed MMPs and thereby accelerate the healing of chronic wounds. Since different MMPs have Zn^{2+} and Ca^{2} as co-factor, alendronate non-specifically inhibits all MMPs at a concentration dependend manner [21]. Several antiseptic and antibiotic agents are being used currently to overcome the issue of infection in wounds. Benzalkonium chloride (BZK) is an antiseptic agent that has been reported to show good antimicrobial properties especially against *Staphylococcus aureus* [22,23]. Gelatin based nanoparticle system prepared by coacervation method was found to be a suitable system for a controlled release of drugs [24]. Gelatin contains RGD sequence making it a suitable component for biological applications [25]. Thus the incorporation of BZK in gelatin nanoparticle will help to ensure a sustained release of the drug.

The study aims at development and characterisation of a bilayered bandage system with antimicrobial and MMP inhibition property. In the prepared bi-layered bandage, the bottom layer could completely degrade within 4 days and release the antimicrobial drug that could eliminate infection within this 4 day period and top layer that could release alendronate in a sustained manner and ensure the inhibition of proteolytic activity of the over expressed MMP and thereby facilitate accelerated wound closure.

2. Experimental

2.1. Materials

Chitosan (MW-100–150 kDa, DDA-85%, Koyo Chemical Ltd, Japan). Gelatin (type B-Nitta Gelatin, Japan),Hyaluronic Acid (Qingdao Haitao Biochemical Co Ltd,China), Benzalkonium chloride (Alfa Aesar), Sodium alendronate (Apex Healthcare Limited), Luria Bertani broth, Agar–Agar etc (Himedia, India), Alamar Blue (Invitrogen, India) were used for the study. All chemicals were used as such.

2.2. Preparation and characterisation of BZK GNPs

Gelatin (type B) (200 g) was dissolved in double deionised water (10 mL) under constant heating at 40 ± 1 °C, pH-3(by adding 0.1 M HCl) [23]. BZK drug (20 mg) was added followed by drop wise addition of acetone (30 mL) to form GNPs. At the end of the process, cross-linking agent, glutaraldehyde (100 μL) was added and the solution was stirred for 30 min. Dispersion was centrifuged at 10,000 rpm for 30 min. The particles were purified by centrifugation and re-dispersed in water. After the last re-dispersion, fabricated particles were incorporated into the bandage system. The nanoparticles were characterized by SEM, FT-IR, size and zeta potential measurement through DLS.

2.3. Drug entrapment efficiency

The nanoparticles were prepared as discussed above. The encapsulation efficiency of BZK was determined in water at an absorbance of 275 nm in UV (UV-1700 Pharma Spec, Shimadzu, spectrophotometer). The efficiency of drug encapsulation was calculated using the equation

$$\text{Encapsulation efficiency\%} = \frac{(\text{Total amount of drug added} - \text{free drug})}{\text{Total amount of drug added}} \times 100 \quad (1)$$

2.4. Preparation of chitosan-HA (hyaluronic acid) hydrogel for the bottom layer

Chitosan hydrogel was prepared by previously reported method [14]. 4% (w/v) HA gel in double deionised water was prepared. Equal weight of obtained chitosan gel and HA gel (1:1) were mixed uniformly to obtain chitosan-HA hydrogel.

2.5. Preparation of chitosan hydrogel for the top layer

2% (w/v) chitosan solution was prepared to that 10% glycerol was added. Using 1% NaOH, the solution was neutralized. The centrifuged hydrogel pellet was washed several times with distilled water before use. The chitosan hydrogel thus obtained is further mixed with HA hydrogel to obtain Chitosan-HA hydrogel. The chitosan-HA hydrogel was freezed at −80 °C and lyophilised to obtain bandages.

2.6. Preparation of GNPs incorporated chitosan-HA hydrogel

The prepared BZK GNPs was added into the chitosan-HA hydrogel (bottom layer) during the mixing of chitosan-HA hydrogel. The hydrogel was allowed to undergo magnetic stirring at 500 rpm for uniform distribution of nanoparticles. The NPs were added in different concentrations such that the final hydrogel systems of different drug concentrations such as 4, 6, 8 and 10 mM respectively are formed. To obtain sponges, these hydrogels were freezed in moulds and subjected to lyophilisation.

2.7. Preparation of alendronate incorporated chitosan hydrogel

Sodium alendronate was added into the Chitosan hydrogel (top layer) to obtain a final drug concentration of 150 mM during the mixing of Chitosan hydrogel. The hydrogel is mixed at an uniform speed and stirring followed by freezing and lyophilisation.

2.8. Preparation and characterisation of bi-layer bandage system

To prepare bandage, chitosan-HA hydrogel mixture was transfered on to a rectangular Teflon mould of thickness of 2.5 mm, freezed at −20 °C for 3–4 h, followed by lyophilisation at −40 °C for 24 h. To prepare bilayer, 2% chitosan gel was added on top of lyophilised chitosan-HA bandage. The frozen samples were further subjected to lyophilized for 24 h to obtain bi-layer bandage. The characterization of the bi-layer was done by SEM and FT-IR.

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