CHAPTER 2
MATERIALS AND METHODS

2.0 Materials

Chitosan (molecular weight = 100-150 kDa, degree of deacetylation = 85%), α-chitin (Molecular weight 100 kDa, Degree of acetylation-85%) and β-chitin (Molecular Weight 150 kDa, Degree of acetylation-72.4%) were purchased from Koyo chemical Co Ltd, Japan. Sodium hydroxide, Acetic acid and hen egg lysozyme were purchased from Qualigens chemicals, India. Zinc acetate, Calcium chloride and Methanol were purchased from Merck, India. *S. aureus* (ATCC 25923) and *E.coli* (ATCC 25922) and *Candida albicans* (ATCC 10231) strains were provided by Microbiology lab of Amrita Institute of Medical Sciences, Kochi, India. Alamar blue reagent and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Invitrogen. Phalloidin dye was purchased from Sigma-Aldrich. Human dermal fibroblast cells (HDF) and growth medium were purchased from Promocell, Germany. Luria-Bertani broth, Sabouraud Chloramphenicol Agar and Agar-Agar were purchased from Himedia, India.

2.1 Preparation of Zinc Oxide nanoparticles (nZnO)

nZnO was prepared by the following method; 0.1 M sodium hydroxide solution was added drop wise to 0.1 M zinc acetate dihydrate solution with continuous stirring [149]. The solvent for the preparation was methanol. ZnO precipitate was formed and it was further centrifuged and washed several times with distilled water to remove the by-products and dried at 80°C.

2.2 Preparation of hydrogels

We have prepared hydrogels using α-chitin, β-chitin and chitosan under mild conditions. The details of preparation are discussed in the following sections.

2.2.1 Preparation of α-chitin hydrogel

Calcium solvent was used to dissolve α-chitin powder. The calcium solvent was prepared as follows [150]. Calcium chloride dihydrate was added and mixed with methanol till to get a saturated solution. This solvent is termed as calcium solvent. To the prepared calcium solvent, α
-chitin powder was added and stirred vigorously for 48 h at room temperature. After the specified duration, a viscous solution was obtained. To the prepared α-chitin solution, excess water was added and stirred vigorously for 2 h to obtain α-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 α-chitin hydrogel.

2.2.2 Preparation of β-chitin hydrogel

β-chitin powder was added to calcium solvent and stirred vigorously for 48 h at room temperature to get a solution with a concentration of 0.5% W/V. To the prepared β-chitin solution, excess amount of water was added and stirred vigorously for 2 h to obtain β-chitin hydrogel. The precipitated hydrogel was separated from system by centrifugation and purified by dialysis against distilled water for two days to get pure β-chitin hydrogel.

2.2.3 Preparation of chitosan hydrogel

Chitosan solution was prepared by dissolving 2 g 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 the unbound water.

2.3 Preparation of composite bandages

We have prepared nanocomposite bandages by using nZnO and hydrogels. Freeze-dry technique was used for the preparation of the composite bandages. The details of the preparation are given below.

2.3.1 Preparation of α-chitin hydrogel/nZnO composite bandage

ZnO nanosuspension was prepared by adding 100mg of nZnO in 10ml water followed by probe sonication (Sonics, Germany) for 10 mins. The ZnO nanosuspension was added drop wise to α-chitin hydrogel under vigorous stirring to get a concentration of 0.1, 0.05, 0.025 and 0.01% of the bandage weight (W/W) and kept 1 h with constant stirring. The homogenized mixtures were then poured on to a Teflon mould and kept at -20°C overnight. After that the frozen samples were freeze-dried (Martin Christ, Germany) to get porous α-chitin hydrogel/nZnO composite bandages.
2.3.2 Preparation of β-chitin hydrogel/nZnO composite bandage

ZnO nanosuspension was prepared and added to β-chitin hydrogel under vigorous stirring to get a concentration of 0.1, 0.05, 0.025 and 0.01% of the bandage weight (W/W) and the obtained slurry was kept for constant stirring (1 h). The homogenized mixtures were transferred to Teflon mould and kept at -20°C overnight. The frozen samples were then freeze-dried for 24 h to get porous β-chitin hydrogel/nZnO composite bandages.

2.3.3 Preparation of chitosan hydrogel/nZnO composite bandage

Chitosan hydrogel was kept for vigorous stirring and the prepared ZnO nanosuspension was added to this hydrogel to get a final concentration of 0.01, 0.005, 0.0025 and 0.001% of the bandage weight (W/W). The mixture was stirred for 1 h and transferred to a Teflon moulds for freezing. The frozen samples were lyophilized to get micro-porous and flexible composite bandages.

2.4 Quantification of nZnO in the composite bandages by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

The quantification of zinc contained in the composite bandages was done by ICP-OES (Perkin Elmer Optima 5300DV). The samples having area of 2 cm² were digested in conc. HNO₃- conc. HClO₄ mixture in a microwave digestion system (Multiwave 3000, Anton Paar). During the analysis, HNO₃-HClO₄ mixture was used as the reagent blank and a calibration plot was made by analyzing high purity standards. Win Lab 32 software was used to record and process the results.

2.5 Measurement of porosity of composite bandages

The porosity of the prepared composite bandages was found out using the alcohol displacement method [151]. Briefly, pre-weighed composite bandage pieces were immersed in absolute ethanol until it was saturated. After the saturation, the bandage pieces were taken out from the alcohol and weighed. The porosity was calculated using the formula,

\[ P = \frac{(W_2 - W_1)}{(\rho V_1)} \]

Where W₁ and W₂ indicates the weight of the composite bandages before and after immersing, respectively, and V₁ is the volume before immersing, \( \rho \) is a constant of the density of alcohol. All samples were triplicated in the experiment.
2.6 Evaluation of swelling ratio of composite bandages

The composite bandages were cut into small pieces having equal weights and immersed in phosphate buffered saline (PBS, pH 7.4, 37 °C) for swelling ratio evaluation. The composite bandages were taken out at predetermined time intervals and water adhered on the surface was removed by gently blotting with filter paper and immediately weighed (Wd). The swelling ratio and water uptake was calculated using the following formula

\[ DS = \left( \frac{W_w - W_d}{W_d} \right) \times 100 \]

where DS is the degree of swelling and Ww & Wd are the wet weight and dry weight of the composite bandages, respectively.

2.7 Evaluation of in vitro biodegradation of the composite bandages

The degradation profile of the composite bandages was evaluated in PBS (pH 7.4) medium containing lysozyme at 37°C [152]. The composite bandages were equally weighed and immersed in PBS containing lysozyme (10,000 U/ml) and incubated at 37°C for 21 days. Initial weight of the bandage was noted as Wi. After 7, 14 and 21 days; each set of bandages were taken out from the PBS containing lysozyme and washed with deionized water to remove ions adsorbed on surface and freeze-dried. The dry weight was noted as Wt. The degradation of bandages were calculated using the formula

\[ \text{Degradation} \% = \left( \frac{W_i - W_t}{W_i} \right) \times 100 \]

2.8 Mechanical properties evaluation

Tensile strength and elongation at break of the prepared composite bandages were analyzed. For tensile strength measurements, the bandage specimens were prepared with the dimension of 10 × 2 × 0.4 cm. Both ends of tensile specimens were clipped with a special gripper. The tensile strength and percentage elongation at break of the bandages were measured by a universal testing machine (Instron 3365) with a load cell of 5 kN and the crosshead speed was 25 mm min\(^{-1}\) at room temperature. Average value out of six measurements was reported for each sample.

2.9 Evaluation of hemostatic potential the composite bandages

The blood clotting ability of the composite bandages was assessed based on reported literature [153]. Blood clotting ability of composite bandages were analyzed and compared with
commercially available dressing (Kaltostat™, Convatec). Blood was drawn from human ulnar vein using BD discardit™ II sterile syringe and mixed with anticoagulant agent acid citrate dextrose at a ratio of 85% to 15%. Triplicate samples were used for this study and blank in a 25ml plastic petri dish without bandage was used as control. Blood was added to each bandage and placed in a 25 ml plastic petri dish, which was followed by the addition of 10µl of 0.2M CaCl₂ solution to initiate blood clotting. Then these α-chitin hydrogel/nZnO composite bandages were incubated at 37º C for 10 mins. 15ml of distilled water was then added drop wise without disturbing the clot. Subsequently, 10ml of solution was taken from the dishes and was centrifuged at 1000 rpm for 1 minute. The supernatant was collected for each sample and kept at 37°C for 1h. 200 µl of this solution was transferred to a 96-well plate. The optical density was measured at 540nm using a plate reader (BioTek PowerWave XS).

For platelet activation; platelet rich plasma (PRP) was isolated from the blood by centrifugation at 2500 rpm for 5 mins. 100 µl of PRP was poured onto the bandage piece (10 mg) and incubated for 20 mins. After that, the bandages were washed thrice with PBS solution and fixed by using 0.1% glutaraldehyde solution. The bandages were dried and SEM images were taken.

2.10 In vitro antimicrobial activity evaluation

We have evaluated the antimicrobial potential of the prepared bandages against bacteria as well as fungus. The culturing medium with the microbes were taken as control for the experiments.

2.10.1 In vitro antibacterial studies

*S. aureus* (gram positive) and *E. coli* (gram negative) were used for evaluating the antibacterial activity of the prepared composite bandages. LB broth and LB agar were used as culturing nutrient sources for bacteria. The bacteria were inoculated in sterilized LB broth and then incubated overnight at 37 ºC in a shaking incubator. The concentration of bacteria was 1 x \(10^6\) colony forming units per milliliter (CFU/mL). The concentration of bacteria was taken in comparison to the McFarland standard (McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range). The composite bandages having size 2cm x 2cm were then added to the medium and were incubated at 37 °C for 24 h. After the incubation, bacterial viability was measured by serial dilution of the culture in normal saline. The serially diluted bacteria culture were then plated on
LB agar plates and incubated overnight. After the incubation period, the bacteria colonies were counted and plotted for quantification [118].

Also, we have followed another method for finding antimicrobial potential of the prepared composite bandages [154, 155]. $10^6$ CFU bacteria suspension was added onto the surface of composite bandages having size 2cm x 2cm. and the bandages were incubated. The survival of bacteria was found out by culturing the bandage pieces in LB broth at different time points (6, 12 and 24 h). The optical density of the bacteria suspension was found out using colorimeter and plotted for quantitative determination.

2.10.2 In vitro antifungal studies

*Candida albicans* was used to assess the antifungal activity and SC agar was used as the culturing medium. *Candida albicans* were inoculated in sterilized LB broth and then incubated overnight at 37 ºC in a shaking incubator. The concentration of fungus was $1 \times 10^6$ colony forming units per milliliter (CFU/mL). The composite bandages were then added to the medium and were incubated at 37 ºC for 24 h. After the incubation, fungal viability was measured by serial dilution of the culture in normal saline, followed by plating on SC agar plates. The colonies were counted and plotted for quantification [118].

Also, we have followed another method for finding antifungal potential of the prepared composite bandages (154). $10^6$ CFU fungal suspension was added onto the surface of composite bandages and the bandages were incubated. The survival of bacteria was found out by culturing the bandage pieces in SD (saboured dextrose) broth at different time points (6, 12 and 24 h). The optical density of the bacteria suspension was found out using colorimeter and plotted for quantitative determination.

2.11 Evaluation of cell viability of the composite bandages

Cell viability of the prepared composite bandages was evaluated by Alamar blue assay [156]. The cell viability of composite bandages with different concentration of nZnO was evaluated on nHDF cells. Bandages were sterilized by ethylene oxide gas. nHDF cells were cultured in fibroblast growth medium provided by Promocell, Germany. Sterile bandage pieces were placed in 12 well plates containing $5 \times 10^4$ cells. The cells with the materials were then incubated up to 72 h and Alamar blue assay was performed. The optical density measured at 570 nm with 620 nm set as the reference wavelength using a microplate spectrophotometer (Biotek PowerWave XS, USA).
2.12 Cell attachment, DAPI staining and Phalloidin staining

The nHDF cells were seeded on composite bandages which were kept in 24 well plates and the concentration was $1 \times 10^5$ cells/well. After 24 h incubation, the bandages were washed with PBS and fixed with 2.5 % glutaraldehyde for 1 h. The samples were thoroughly washed with PBS and sequentially dehydrated in a graded ethanol series, air-dried, gold sputtered in vacuum and examined with SEM.

DAPI is a fluorescent stain commonly used as a nuclear stain. For DAPI staining the cell seeded bandages were fixed with 4% paraformaldehyde for 20 mins, permeabilised with 0.5% Triton X-100 (in PBS) for 5 mins. Then the bandages were treated with 1% FBS, washed with PBS and stained with 50μl of DAPI (1:30 dilution with PBS) and incubated in dark for 3 mins. The bandages were then washed with PBS and viewed under fluorescent microscope (Olympus-BX-51). For cell infiltration evaluation, the bandages with seeded cells (24 h) were treated with TRITC conjugated Phalloidin dye and images were taken using laser confocal microscope (Leica SP 5 II). The bandages with cells (48 h) were treated with TRITC conjugated Phalloidin dye and images were taken using laser confocal microscope (Leica SP 5 II).

2.13 Cell infiltration analysis

nHDF cells were seeded on the bandage pieces at a density of $4 \times 10^4$ cells and incubated for 48 h. Cell culture medium was changed when required. After incubation, the medium was decanted and washed with PBS. The cells were then fixed with 4% paraformaldehyde for 15 mins and washed twice with PBS. The cell permeabilization and blocking steps were carried out using 0.5% paraformaldehyde for 10 mins and 1% bovine serum albumin for 20 mins respectively. The samples were incubated in dark with TRITC conjugated phalloidin for 1 h. Confocal imaging was done and the obtained images were used for observing the cell infiltration.

2.14 Characterizations

The prepared nZnO and composite bandages were characterized using X-Ray diffraction (PANalytical X’Pert PRO, Cu Kα radiation, operating at a voltage of 40 kV), FTIR (PerkinElmer Co, SPECTRUM RX1, FT-IR). The morphology and size of nZnO were characterized using dynamic light scattering measurements (DLS-ZP /Particle Sizer NicompTM 380 ZLS, particle sizing system) and Atomic Force Microscope (Jeol JSPM-5200). The structural morphology of the composite bandages was characterized by scanning electron microscope (JEOL, JSM-6490LA, Japan).
2.15 *In vivo* excisional wound healing evaluation of β-chitin hydrogel/nZnO composite bandages in Sprague-Dawley rats

2.15.1 Detailed *in vivo* study plan

*In vivo* animal study was done after getting the approval from Institutional Animal Ethical Committee (IAEC), Amrita Institute of Medical Sciences and Research Center, Cochin, India. Sprague-Dawley (SD) rats weighing 200-250 g and 4-6 weeks age were used in this study. The rats were randomly divided into 5 groups and each group contains 6 rats (n= 6). Rats were allowed to take normal rat feed and water without restriction. On the day of surgery; the rats were anaesthetized by intramuscular injection of 35.0 mg/kg Ketamine and 5.0 mg/kg Xylazine. The dorsal areas of the rats were depilated and the operative area of the skin was cleaned with alcohol. Partial thickness skin wound of 1.5 cm$^2$ was prepared by excising the dorsum of the rat using surgical scissors and forceps [159, 160]. The β-chitin hydrogel/nZnO composite bandages, β-chitin hydrogel control bandage and Kaltostat were then applied on the wounds. Rats with bare wound were kept as negative control. After experimental procedure rats were individually housed under normal room temperature. Dressing materials were removed each week to observe and measure the wound closure. Wound area closure was measured by marking the area on a transparent sheet by placing the transparent sheet on the wound surface. The area on the sheet was transferred to a graph sheet to get the exact area. New dressings were applied after measuring the area and again housed in individual cages.

2.16 *In vivo* excisional wound healing evaluation of chitosan hydrogel/nZnO composite bandages in Sprague-Dawley rats

2.16.1 Detailed study protocol

*In vivo* animal study was approved by the Institutional Animal Ethical Committee (IAEC), at Amrita Institute of Medical Sciences and Research Center, Cochin, India. Sprague-Dawley (SD) rats, weighing 200-250 g and 4-6 weeks of age, were used in this study. The rats were divided into five groups and each group contains three rats (n= 3); rats were allowed to take normal rat feed and water without restriction. On the day of wounding, the rats were anaesthetized by intramuscular injection of 35.0 mg/kg Ketamine and 5.0 mg/kg xylazine. The dorsal area of the rats depilated and the operative area of skin cleaned with alcohol. A partial thickness skin wound of 1.5 cm$^2$ was prepared by excising the dorsum of the rat using surgical scissors and forceps. The prepared
wounds were then covered with the CZBs, chitosan control, and Kaltostat. Rats with bare wound were kept as negative control. After applying the dressing materials, the rats were housed individually in cages under normal room temperature. The dressing materials were changed at Weeks 1, 2, and 3. During the changing of dressings, photographs were taken and the wound area was measured using a transparent polyethylene sheet. The sheet was kept on top of the wound and area was marked using a marker pen. The marked area was then transferred to graph sheet for getting the exact value.

2.17 Hematoxylin-Eosin and Picro-Sirius Red staining

Skin wound tissue was excised after 1 and 3 weeks and fixed with 10% formalin solution. The tissues were processed in different grades of alcohol and xylene. Tissues were then embedded in paraffin wax and sections were taken with thickness of 5µm. Tissue sections were stained with Harris’s Hematoxylin-Eosin (H&E) reagent for histological observations under light microscopy.

Tissue sections were stained with Picro-Sirius red for collagen staining as well as quantification of collagen content on the healed wound area. Quantification of collagen content was done by histomorphometry using the Image Pro software, USA. Histomorphometry is a software assisted analysis of images of tissue sections taken by a microscope in which the quantitative study of the tissues and structure of a tissue can be evaluated.

2.18 In vivo antibacterial activity Evaluation

In vivo antibacterial efficacy of the bandage was assessed by collecting swabs from the rat wounds after 1 and 2 weeks of the experiments using sterile swabs. The individual swabs were dipped into 1 ml of sterile LB broth and serially diluted before plating on LB agar plates. The plates were incubated overnight at 37°C; the number of bacterial colonies counted and plotted for quantification. The bacterial colonies were identified using VITEK® 2 Compact, BioMérieux instrument.

2.19 In vivo inflammation studies

To understand whether the prepared composite bandages induced any inflammation in the animals, the serum cytokine level was analyzed using BD™ Cytometric Bead Array rat inflammation flex sets (CBA,BD Biosciences, USA) in FACS Aria (BD Biosciences, USA). 800µl of blood was collected in vacutainers by retro-orbital bleeding at week 0, 1, 2 and 3. The
tubes were centrifuged at 10,000 rpm for 3 min to separate the serum, which was stored at -20°C for analysis.

2.20 Statistics analysis

The values were expressed in a format of mean ±standard deviation (SD). The results obtained were analyzed statistically. A Student’s t-test was conducted to determine the significance. A probability level of p < 0.05 was considered to be statistically significant.