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Fibrin nanoparticles were incorporated to chitosan-gelatin hydrogel matrix to develop composite bandage having wound healing potential.
Flexible, Micro-Porous Chitosan-Gelatin Hydrogel/Nano Fibrin Composite Bandages for Treating Burn Wounds

P. T. Sudheesh Kumar #, G. Praveen #, Mincy Raj #, K. P. Chennazhi, R. Jayakumar

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In this study, we have developed chitosan-gelatin hydrogel/nano fibrin ternary composite bandages (CGFB) for the treatment of burn wound. The characterization of the material was done by SEM. The spherical nano fibrin moieties of 229 ± 3 nm size scale was prepared through emulsification method and allowed to distribute within the chitosan-gelatin matrix. The presence of fibrin component within the matrix was confirmed by SEM analysis and phosphotungstic acid-hematoxylin (PTAH) staining. In addition, the swelling, biodegradation, porosity, whole-blood clotting, platelet activation, cell viability, cell attachment and cell infiltration of the prepared composite bandages were evaluated. It was found that the nanocomposite bandages were more flexible, degradable and showed enhanced blood clotting and platelet activity compared to the control. The prepared nanocomposite bandages showed adequate swelling ability when immersed in water and PBS. Cell viability studies on normal human dermal fibroblast (HDF) and human umbilical cord vein endothelial cells (HUVEC) cells proved the non-toxic nature of the composite bandages. Cell attachment and infiltration studies showed that the HDF and HUVEC cells were found attached on the bandage. The enhanced collagen deposition and re-epithelialization with intact matured epidermis formation was noted in CFGBs treated animal groups compared to the experimental controls. The above results proved the use of this ternary nanocomposite bandages as an ideal candidate for burn wound dressing.

Introduction

Burn injury has become a serious health issue due to the lack of proper medication, long term disability, prolonged hospitalization, loss of body extremities and even death. Burn wound is usually caused by the thermal exposure of the body surface which can damage the skin 1. Inappropriate caring of the wound can delay the healing process which involves complex mechanisms such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, re-epithelialization, contraction and remodelling 2-4. Burn wound is usually characterized by the membrane destabilization, protein coagulation, associated energy depletion and hypoxia at the cellular level which leads to extensive tissue necrosis 1. Burn wounds have to be treated based on the severity of the injury. Various formulations such as ointments and wound dressings have been developed for the treatment of severe burn wounds. However, when ointments or creams are used, their frequent reapplication and washing of the wound region often lead to pain or burden on the patient 5-7. In order to avoid this discomfort, we are introducing a novel hydrogel based nanofibrin incorporated chitosan-gelatin bandages for burn wounds. This can be dealt with more simply and are more durable during application. An ideal dressing should maintain a moist environment at the wound interface, remove excess exudates from the wound surface, and allow proper gaseous exchange 8,9.

Chitosan is one of the typical marine polysaccharides obtained from the exoskeleton of invertebrates 10. Chitosan is the primary derivative of chitin and it has got tremendous properties which makes it an ideal material for biomedical applications. The distinctive properties of chitosan comprised of biocompatibility, biodegradability, antimicrobial activity, blood clotting potential, etc 9-11. Chitosan provides a non-protein matrix for 3D tissue growth and activates macrophages for tumoricidal activity and for the production of interleukin-1 which in turn stimulates cell adhesion and proliferation. Chitosan is a haemostat, which helps in natural blood clotting and blocks nerve endings reducing pain 12-15. Gelatin is a biocompatible polymer and used for many biomedical applications including wound dressing with hemostatic potential 15. Haemostatic action is based on platelet activation at the contact of blood with gelatin, which activates the coagulation cascade 16,17. Because of the gelation property it can act as a binding agent and causes the stoppage of blood flow into the blood vessel by a constriction of the vessel 17,18. Based on clinical and in vivo studies, it has already been proved that gelatin is effective in the treatment of wound healing 15-18. Topical skin gelatin treatment has been proven to be effective for accelerating wound healing as part of photographic film 19,20. Hydrogels can minimize the hypoxic condition which is a major problem for burns, by providing a moist environment on the wound surface 21,22. The haemostatic potential of both chitosan and gelatin can provide a better healing by initiating the blood clotting cascade.
Fibrin is an insoluble protein involved in blood clotting cascade. Fibrin is developed in the blood from a soluble protein called fibrinogen. Fibrin is formed after thrombin-mediated cleavage of fibrinopeptide A from the alpha chains and fibrinopeptide B from the beta chains. This results in the subsequent conformational changes and exposure of polymerization sites which generates the fibrin monomer that has a great tendency to self-associate and form insoluble fibrin. Nanofibrin have high surface to volume ratio which can enhance cell attachment, cell migration and cell proliferation. Other benefits of nano fibrin for the wound healing process have already been reported.

In light of the above, we aimed to develop a chitosan-gelatin hydrogel formulation of nanofibrin bandages for the treatment of burn wound. The CFGB is expected to protect the wound from further infections and provide a better healing environment. In vivo studies were also carried out and evaluated histopathologically.

Experimental Details
(a) Materials

Chitosan (MW; 150 kDa, degree of deacetylation-85%) was purchased from Koyo Chemical Ltd. Minimum essential medium (MEM) and thrombin and gelatin were purchased from Sigma-Aldrich Company. Fibrinogen was purchased from Himedia, India. Acetic acid, sodium hydroxide and hen lysozyme were purchased from Qualigens, India. DAPI (4’, 6-diamidino-2-phenylindole), Alamar Blue, Trypsin-EDTA, Fetal Bovine Serum (FBS) and TRITC (tetramethyl rhodamine iso-thiocyanate) were obtained from Gibco, Invitrogen Corporation. The chemicals were used without any further purification.

(b) Fabrication of CFGBs

As per the reported method from our laboratory, the chitosan hydrogel was prepared. In briefly, 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. The unbound water was removed by centrifugation to obtain chitosan hydrogel. Gelatin solution was prepared by dissolving 1gm of gelatin in 100 ml of water followed by mild heating at 50°C for 1 min. The nano fibrin was synthesized by a method that has already been reported previously and the prepared nanoparticles were suspended in distilled water and probe sonicated for 20 min. Nanofibrin suspensions were mixed with 1:1 concentration of chitosan hydrogel and gelatin solution. Vigorous stirring was carried out for 1 hr to get 2% of nano fibrin incorporated chitosan hydrogel/gelatin composite bandages. Stirring enable the homogeneous distribution of nano fibrin and gelatin on chitosan hydrogel. This suspension was poured on to a Teflon mould and kept at -20 °C overnight. The frozen samples were then lyophilized for 24 h (Martin Christ, Germany) to obtain CFGBs.

(c) Characterization of CFGBs

The lyophilized samples of chitosan bandages (CBs), Chitosan fibrin bandages (CFBs), chitosan-gelatin bandages (CGBs) and CFGBs were examined using SEM to know the structural morphology of the bandages.

(d) Swelling studies

The swelling study was carried out in Posphate Buffer Saline (PBS) and distilled water. The pH of the PBS solution was maintained at 7.4. Bandages of bare chitosan, chitosan-gelatin, CFBs and CFGBs were cut into small pieces and immersed in 5 ml of the water and PBS solution. The samples were placed at 37°C for incubation and at definite time intervals the samples were taken out of the falcon tube, gently blotted with filter paper and the wet weight was noticed. The swelling study in PBS was carried out up to 12 days and that in water was done for 48 hrs. The swelling ability was evaluated by the following formula.

\[ DS = \frac{W_w - W_d}{W_d} \times 100 \]

Where DS is the degree of swelling, \( W_w \) and \( W_d \) represents the wet weight and dry weight of the bandages respectively.

(e) Biodegradation studies

Each lyophilized samples having a dimension of 0.5 cm × 0.5 cm were taken and the dry weight was found out. For each samples triplicates were taken. Each sample were immersed in 5 ml of the PBS-lysozyme solution and were incubated at 37 °C. At definite time intervals, the samples were taken out and washed with PBS. The study was carried up to 2 weeks. The samples were freezedried and dry weight was noted. The degradation was calculated by the formula.

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

Where \( W_i \) is the initial weight and \( W_f \) is the dry weight of the bandage after lyophilization.

(f) Porosity evaluation

Cylindrical shaped CBs, CGBs, CFBs and CFGBs were prepared and the height and diameter of the bandages were found out using a vernier caliper to calculate the volume. All the samples were triplicatied in the experiment. The bandages were immersed in absolute ethanol until it is saturated. The weights of the bandages before and after immersion in alcohol were noted. The porosity was calculated using the following formula.

\[ \text{Porosity} = \frac{W_i - W_f}{W_i} \times 100 \]
\[ P = \frac{W_2 - W_1}{\rho V_1} \times 100 \]

5 (g) Whole-blood clotting

Blood clotting study was done by drawing blood from human ulnar vein and anticoagulated with acid citric dextrose ACD (20 Mm Citric acid, 110 Mm Sodium citrate, 5mM Dextrose) at a \( V/V \) ratio of 8:2. Bare chitosan and Kaltostat were used as the control and all samples were triplicated. Citrated whole blood was dispensed on to the bandages and 10µl of 0.2 M CaCl₂ solution was added to initiate the clotting process. Incubation was done at 37°C for 15 min. By adding 2 ml of water the red blood cells that were not trapped on the bandages were haemolysed and washed out. The absorbance of the supernatant at 540nm using a plate reader (BioTek PowerWave XS) was observed to analyze the blood clotting ability.

20 (h) Platelet activation studies

Platelet-rich plasma (PRP) was isolated from the blood by centrifugation at 2500 rpm for 5 minutes. One hundred microliters (100 µL) of PRP was added on to the bandage pieces weighing 10 mg and incubated at 37°C for 20 minutes. Here also bare chitosan and Kaltostat were used as the control along with CFGBs. The bandages were then washed with PBS solution and fixed using 0.1% glutaraldehyde solution. The bandages were dried, fixed on aluminum stubs and sputter coated for the SEM images to be taken.

30 (i) Cell viability using alamar blue assay

Alamar Blue assay was performed to evaluate the cell viability of the prepared CFGBs on HUVEC and HDF cells. The bandages were cut into small pieces and were sterilized by ethylene oxide gas. The cell seeding density was 5 × 10⁶ cells, each for HDF and HUVECs and they were incubated up to 48h. Alamar Blue Assay was performed by adding Alamar blue into the plates containing the sterilized bandage materials along with the cells. The optical density was measured at 570 nm, with 620 nm set as the reference wavelength; using a micro plate spectrophotometer (Biotek PowerWave XS, USA) was taken.

(j) Cell adhesion and proliferation studies

The cell morphologies of HUVECs within the bandages were observed using SEM. The cells were seeded on the CFGBs at a concentration of 1×10⁴ cells /well. After 24 h and 48 h of incubation, the bandages were rinsed by PBS and fixed with 2.5% glutaraldehyde for 1 h. The samples were thoroughly washed with PBS and dehydrated through a series of graded-ethanol solutions and air-dried. After gold sputtering in vacuum the samples were examined by SEM.

For DAPI staining, the HUVECs were seeded on CFGBs and the bandages were fixed with 4% para formaldehyde for 20 min. DAPI (4′, 6-diamidino-2-phenylindole) is a fluorescent stain which can particularly stain the nucleus of cells. After adding 0.5% Triton X-100 (in PBS) and incubated for 5 min, the bandages were then treated with 1% FBS and washed with PBS. The cell-seeded bandages were stained with 50 µL of DAPI (1:30 dilution with PBS). The bandages were then incubated in darkness for 5 min and viewed under fluorescent microscope (Olympus-BX-51).

65 (k) In vivo wound healing evaluation

The wound healing evaluation in animals was approved by the Institutional Animal Ethical Committee (IAEC), Amrita Institute of Medical Sciences and Research Center, Cochin, India. Sprague-Dawley rats were used in this study and they were kept under standard laboratory conditions. The rats were divided into 5 groups randomly (n= 6). On the day of burn creation, 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 were depilated and cleaned with Wokadine™. The burn was created by using a copper bar having an area of 1.5cm². The copper bar was heated to 150°C using electric hot plate and the temperature was monitored using a thermometer. The heated copper bar was placed on the depilated area for 15 seconds and the rats were individually housed. On day three after the burn creation, rat skin from the burned area was removed surgically. The wounds were then covered with the composite bandage materials and covered with a secondary dressing. It was further sutured to ensure the dressing materials were intact. The rats were then individually housed. Wound closure was noted each week by taking the photographs and marking the wound area using a graph sheet. Wounds treated with Gelpson™ were taken as positive control and bare wounds taken as negative controls. After 2 and 4 weeks, wound tissue was excised for histological and collagen deposition analysis. The histological and collagen deposition analysis was done according to reported protocols.

(l) Statistical analysis

All the experiments were done in triplicates and a student T-test have been performed to find out the statistical significance. Data with \( p<0.05 \) considered as statistically significant.

Results and discussion

Fig. 1a represents the average hydrodynamic particle size distribution and SEM representation of the Nano fibrin component showing an average particle size of 229 ± 7 nm with an average zeta potential value of -34 mV. Fig. 1b is the representative photographs of the prepared composite bandage. The flexibility of the bandage is clearly evident from the photographs of CFGBs. SEM images of the CGBs, and CFGBs are shown in Fig. 1c. SEM images showed that all the bandages were porous enough to absorb the excess exudates and the pore size was in the range of 200-300 µm. The porous structure of CFGBs is retained even after the incorporation of nano fibrin and gelatin.
The swelling study was carried up to the day 12. Swelling ratio analysis revealed that after the incorporation of gelatin, the swelling ability of the bandages increased when compared to the bare chitosan. Since the gelatin incorporation can enhance the porosity by disrupting the porous structure, the swelling ability of the bandages has also got increased. There was no significant difference in the swelling ratio of CFGBs when compared to CGBs and CFBs. In water, the swelling study was carried up to 2 weeks. All the bandages showed a swelling ratio from 10-15 and there was no difference in the swelling ratio after the incorporation of gelatin and fibrin when compared to bare chitosan bandages. The presence of nanofibrin or gelatin had no impact on the swelling ratio of the composite bandages. This might be due to the fact that the concentration of nanofibrin used was very less (2% of the total bandage weight). The gelatin might have well incorporated within the chitosan polymers and formed a well-integrated matrix all together. Due to these facts, the composite bandages retained the swelling ratio even after the addition of nanofibrin and gelatin.

In the in vitro biodegradation studies, the degradation rate of the bandages in the week 2 was greater than week 1. The CGBs showed a maximum degradation rate of around 65% than compared to all other bandages. The presence of gelatin might have reduced the strength of chitosan matrix and hence it became more susceptible to biodegradation compared to chitosan control and nanofibrin incorporated bandages. Also, the gelling property of gelatin had an impact in the degradation profile. The gelatin might have easily undergone degradation after swelling. But the fibrin incorporation reduced the degradation rate of CFGBs. Here the pores of the bandages will be occupied with fibrin nanoparticles which can provide strength to the bandages. This may be the reason for reduced degradation percentage after the incorporation of fibrin.

Alcohol displacement method was done to evaluate the porosity of CFGBs. After the incorporation of gelatin, the bandages showed porosity in the range of 60-70%. This showed a significant difference when compared to bare chitosan and fibrin incorporated chitosan bandages. The incorporation of gelatin might have altered the porous structure of the bandages and this may be the reason for a high porosity of gelatin incorporated bandages. There was no significant change in the porosity after the incorporation of nano fibrin when compared to the gelatin bandages. Thus it was proved that the bandages are capable enough to absorb large volume of wound exudates from the wound surface and can enhance the distribution of nutrients, provides gaseous exchange and thereby promote the wound healing process.

The haemostatic potential of CFGBs was assessed and the Fig. 2a show representative photographs of the blood clotting caused by the CFGBs. The lower O.D value indicates the high blood clotting. CFGBs showed enhanced blood clotting ability in comparison with Kaltostat and blank (Fig. 2b). Chitosan control, CGBs and CFGBs were showed same sort of blood clotting potential compared to the controls. The blood clotting data of chitosan control, chitosan-nanofibrin has been reported previously from our laboratory. The presence of fibrin and gelatin did not alter the hemostatic ability of chitosan. This might be due to the availability of some positive charges on the chitosan matrix which were not diminished due to the presence of nanofibrin or gelatin. The presence of fibrin and positive charges of chitosan triggers the blood clotting cascade. The blood clotting was also initiated by the activation of platelets by the composite bandages. The activated platelets form a mesh together with fibrinogen and other factors which eventually converted as a blood clot.
Fig. 3. Cell viability of bandages using (a) HDF cells and (b) HUVECs.

Cell attachment studies on the bandages were performed using SEM to determine the cytotocompatible nature of the bandages. The SEM images revealed that HUVECs were attached onto the CFGBs and began to spread on the bandages after 24 h of incubation. After 48 h of incubation, the attached cells started proliferating and further confirmation was done by the DAPI staining. The images showed that more cells were attached on CFGBs containing 2% of nanofibrin. Cell attachment and proliferation studies on HUVECs after 24 h and 48 h were shown in Fig. 4.

Fig. 4. Comparison on cellular attachment on CFGBs visualized through SEM (represented as upper panels) and subsequent cell proliferation visualized through DAPI nuclear staining (represented as lower panels) using HUVECs.

Fig. 5 represents the wound area closure after 1, 2, 3 and 4 week of the treatment. The wounds treated with chitosan-fibrin-gelatin composite bandage showed faster healing after 2 weeks compared to the other group wounds. The rate of healing was further enhanced after 3 weeks for the chitosan-fibrin-gelatin group compared to others. The rate of healing was 80% after 2 weeks and the healing were completed at week 3 of the wounds treated with chitosan-fibrin-gelatin composite bandages (Fig. 6). The presence of gelatin improved the healing rate and it might be due to the migration and proliferation of keratinocytes and fibroblasts on the wound site. It can also be hypothesize that the matrix assisted cell migration and enhanced deposition of collagen is a related phenomena that is promoted by the cumulative action of inherent growth factors in the system as well as due to the effect of fibrin degraded products from the matrices.

Fig. 5. Photographic representations of the burn wound area closure.

Fig. 6. Wound area closure measurement data. Group (1) bare wound, (2) chitosan control, (3) chitosan-fibrin, (4) chitosan-fibrin-gelatin and (5) Gelspon (positive control).

Figure 7 depicts the H and E stained images of the wound tissue excised after 2 and 4 weeks. The wounds treated with chitosan-fibrin-gelatin composite bandages showed intact re-epithelialization and enhanced epidermis formation. After 4 weeks the re-epithelialization is complete in the groups treated with the composite bandages compared to other groups. Chitosan-fibrin group also showed improved re-epithelialization. Chitosan assisted the migration of fibroblast and keratinocytes to the wound site and hence that helped the improved re-epithelialization and better collagen deposition. Figure 8 show the Picro-Sirius red stained images of the wound tissue excised after 2 and 4 weeks. The composite bandages showed enhanced collagen deposition on the wound sites compared to controls. The presence of gelatin, chitosan and fibrin enhanced the keratinocyte cell migration to the wound site and that eventually assisted the deposition of collagen on the wound site. The collagen deposition would help the intactness and elasticity of skin tissue. Since, fibrin is a bio-polymer naturally synthesized during the wound healing cascade and under in situ systems will serve as a reservoir of growth factors for proliferating cells, the degradation of fibrin from the wound site is in synergy with the native tissue formation. So in the present study also it was evident...
that the wound healing process is triggered by the addition of nano fibrin component to the matrices.

Conclusions

Several research approaches are employed to the time for improving the functionality of chitosan based wound dressing materials that enhanced the proper regeneration of skin tissue at chronic burn wound sites. This study is such an attempt to fabricated composite bandages of chitosan, gelatin and fibrin, which were found to be superior to bare chitosan as well as commercially used Gelspon and Kaltostat bandages. The prepared CFGBs were macro porous, biocompatible and biodegradable that can able to absorb the excess exudates from the wound interface and was found to have adequate flexibility as well as tensile strength. The enhanced blood clotting and platelet activation ability of the CFGBs proved their plausible usage as an effective wound dressing material for the patients with blood clotting disabilities. In vivo evaluation of the CFGBs in healing burn wounds created in SD rats revealed its efficacy compared to the control wound dressing materials. The enhanced collagen deposition and re-epithelialization with intact matured epidermis formation was noted in CFGBs treated animal groups compared to the experimental controls. Altogether, these research findings suggest that the composite CFGBs prepared through this study is better in supporting the healing process and skin tissue regeneration in chronic burn wound sites.

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Authors contributed equally.

Amrita Centre for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences and Research Centre, Amrita Vishwa Vidyapeetham University, Kochi-682041, India.

*Corresponding author. E-mail ID: rjyakumar@aims.amrita.edu & jayakumar77@yahoo.com (Prof. R. Jayakumar)

Tel.: +91 484 2801234 Fax: +91 484 2802020.

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