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Chitosan-hyaluronic acid/VEGF loaded fibrin nanoparticles composite sponges for enhancing angiogenesis in wounds

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ABSTRACT

Reduced levels of endogenous growth factors and diminished angiogenesis are contributory factors for impaired wound healing in diabetic patients. Vascular endothelial growth factor (VEGF) is the most potent angiogenic growth factor which stimulates multiple phases of wound healing angiogenesis and thereby accelerates healing. The aim of this work was to develop chitosan-hyaluronic acid composite sponge incorporated with fibrin nanoparticles loaded with VEGF as a wound dressing for diabetic wounds. VEGF loaded fibrin nanoparticles (150-180nm) were prepared and characterized which was then incorporated to the composite sponge. The prepared sponges were characterized by SEM and FT-IR. Porosity, swelling, biodegradation, mechanical properties and haemostatic potential of the sponges were also studied. Release of VEGF from the composite sponges was evaluated using ELISA kit. More than 60% of the loaded VEGF was released in three days. Cell viability and attachment studies of the composite sponges were evaluated using human dermal fibroblast (HDF) cells and human umbilical vein endothelial cells (HUVECs). HUVECs seeded on VEGF containing sponges showed capillary like tube formation which was absent in control sponges. The results suggest that the prepared chitosan-hyaluronic acid/VEGF loaded nanofibrin composite sponges (CHVFS) have potential to induce angiogenesis in wound healing.

Keywords: Chitosan-hyaluronic acid, VEGF, Nanofibrin, Angiogenesis, Diabetic wound, composite sponge.
1. Introduction

Healing is a complex mechanism where different phases such as inflammation, proliferation and remodeling converge [1, 2]. It occurs as a cellular response to injury and involves various cell types like platelets, neutrophils, macrophages keratinocytes, fibroblasts and endothelial cells [3]. Many growth factors and cytokines are produced by these cell types which coordinates the whole process of wound healing. Multiple physiological factors contributes to impaired wound healing in diabetic patients, including decreased production of growth factors leading to micro and macro vascular insufficiency and decreased local angiogenesis [4, 5]. Angiogenesis is a major step in the proliferative phase of wound healing, which is the formation of new blood vessels from pre-existing capillaries that grow into the wound site and provides nourishment for healing [6, 7]. Wound healing angiogenesis involves multiple steps including vasodilatation, basement membrane degradation, endothelial cell migration and proliferation, capillary tube formation, loop formation, stabilization of the loop and establishes capillary network [8, 9].

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic growth factor which stimulates multiple phases of wound healing angiogenesis [9, 10]. Ischemic wounds show diminished production of VEGF and reduced angiogenesis which is a contributory factor to impaired wound healing [10, 11]. Some studies have shown that exogenously applied VEGF can improve the wound healing process by stimulating various phases of wound repair. Nevertheless there are many shortcomings for the local application of VEGF, once administered to a host environment VEGF has a half life of only 90 min, which requires high doses to achieve
therapeutic effect and may result in side effects such as development of malignant vascular tumors. This short half life necessitates the need for an adequate and constant supply of growth factor in order to attain desired results [12, 13]. Furthermore it is highly unstable and may to lead to rapid leakage and clearance of growth factor from the site of application [14]. Also, chronic diabetic wounds exhibits elevated levels of matrix metalloproteinases which may lead to degradation of growth factors [15].

A nanocarrier capable of constantly delivering the biologically active VEGF at the wound site can overcome the above mentioned shortcomings to an extent. Fibrin based biodegradable nanoparticles as delivery vehicle for VEGF was our choice of interest. Studies have shown the efficacy of fibrin nanoparticles as potential drug carriers [16, 17]. A study by Sudheesh et al has shown that fibrin nanoparticle incorporated chitosan bandages showed improved healing and complete re-epithelialization in *in vivo* wound models [18]. Nanocarriers loaded with growth factor if administered directly to the wound site may lead to a rapid leakage from the wound site, which requires frequent administration. Here we incorporated VEGF loaded nanoparticles to a chitosan-hyaluronic acid composite sponge, so that it can serve as a growth factor eluting wound dressing.

Chitosan being biocompatible and biodegradable is widely used in wound management area [18]. Chitosan based materials have gained attraction due to inherent ability of the material to combine with other polymers to show a combined effect. For example, a study by Mehta et al had shown that the blending chitosan with silk had improved the compressive modulus of the material [19]. Apart form the mechanical properties; the antibacterial activity of chitosan is another property which necessitates
the use of chitosan in wound dressing materials [20]. Hyaluronic acid (HA) is a major component of the skin extra cellular matrix with a high capacity to store water which provides a moist environment and prevents dryness of wounded tissue surface and promotes healing. Moreover, HA increases collagen secretion at the wound site by fibroblast proliferation [21, 22]. We have reported the preparation and characterization of chitosan-hyaluronic acid composite sponges loaded with nanoparticles exhibiting certain properties for wound dressing applications [23, 24].

2. Materials and methods

2.1. Materials

Chitosan (MW 100-150 kDa, degree of deacetylation-85%) was purchased from Koyo chemical, Co Ltd (Japan). HA was purchased from Qingdao Haitao Biochemical Co Ltd (China), Fibrinogen from Himedia (India), thrombin from Merck, recombinant human VEGF was purchased from PeproTech Inc. (Rocky Hill, USA) Prospec, human VEGF-ELISA kit from Ray Biotech (USA). Glutaraldehyde and hen lysozyme were obtained from Fluka, Human dermal fibroblast (HDF) and its media were purchased from Promo Cell (India). Gelatin and Iscove’s modified Dulbecco’s medium (IMDM) was obtained from Sigma Aldrich. 4, 6-Diamidino-2-phenylindole (DAPI), Alamar Blue, trypsin-EDTA, pen-strep antibiotic, fetal bovine serum (FBS), Geltrex and LIVE/DEAD® Viability/Cytotoxicity Assay Kit were obtained from Gibco, Invitrogen Corporation.
2.2. Methods

2.2.1. Preparation of CHVFS

2.2.1.1. Preparation of chitosan-HA blend

Chitosan-HA blend was prepared by a previously reported method with slight modification [21]. Here chitosan and HA were mixed in 5:1 ratio without the use of a cross-linking agent.

2.2.1.2. Preparation of VEGF loaded fibrin nanoparticles (VnF)

VnF were prepared by an already reported method by Praveen et al., which utilizes a surfactant-free water-in-oil emulsification-diffusion method [16]. Briefly VnF were prepared by the addition of 1 µg VEGF to 1 ml of Hank’s balanced salt solution and mixed with 4 ml aqueous suspension of fibrinogen. Thrombin was taken in a separate applicator. Fibrinogen to thrombin ratio was maintained to be 20-40 mg/ml: 5-10 U/ml. Aqueous suspension of VEGF containing fibrinogen and thrombin in separate applicators were simultaneously injected to 40ml pre heated purified vegetable oil which was kept under constant magnetic stirring at 400 rpm and a constant temperature of 45-50°C was maintained for 6-8 h. Special care should be taken to confine the said temperature, above which may lead to denaturation of VEGF. Cross-linking and uniform dispersion of fibrin clot occurs in oil. The emulsion containing nanoconstructs were centrifuged at 10,000 rpm for 10 min leading to formation of a density gradient layer of nano constructs at the oil-water interface which was scooped out from the emulsion. It was further redispersed in water and probe sonicated for 10 min to attain a uniform nano size distribution and lyophilized for 48 h.
2.2.2.3. Preparation of composite sponge

To the chitosan-HA blend, VnF (2% of chitosan-HA blend) was added and stirred for 4 h to obtain a homogenous mixture. The resulting mixture was then poured into a mould, freezed and lyophilized for 24 h.

2.3. Characterization

Size of VnF was analyzed using DLS (DLS-ZP/Particle Sizer NicompTM 380 ZLS particle sizing system). Measurements were carried out under a monochromatic, coherent laser source and the scattered intensity was measured. Morphology and size of VnF was analyzed using SEM (JEOL Ltd., JEOLJSM-6490LA) for which the nano suspension was diluted in distilled water and mounted on carbon-taped aluminum stubs, which was then gold sputtered (JEOL, JFC-1600) before imaging. Mallory’s phosphotungstic acid hematoxylin (PTAH) staining was used to stain the VnF. PTAH specifically stains fibrin component and gives blue colour. In brief, lyophilized nanoparticles were dehydrated in 80% ethanol and pre-stained with eosin for 5 min. It was then washed with double distilled water and stained with PTAH followed by 30 min incubation at 60ºC and imaged using the bright field mode of a fluorescent microscope (Olympus-BX-51).

The structural architecture of the CHVFS was analyzed using SEM. The sponges were cut into thin sections to expose its internal architecture. Samples were then mounted on carbon-taped aluminum stubs and gold sputtered before imaging by SEM. FT-IR measurements were performed using a FT-IR spectrometer (Perkin-Elmer RX1) to analyze the various functional groups present in the composite sponges. Each sample were finely ground, mixed with potassium
bromide and pelletized. IR spectra was obtained by scanning in the range of 400-4000 cm⁻¹

2.4. Porosity

Liquid displacement method was used to determine the porosity of the sponges using ethanol as the displacing liquid [18]. Dimensions of the samples were measured using a vernier caliper and volume (V) was calculated. Samples of known weight (Wi) was immersed in a graduated cylinder containing a known volume of ethanol and soaked for 24 h to allow ethanol to penetrate into the pores of the sponge. The final weight of the wet sponge was noted as Wf. Percentage porosity was calculated using the following equation.

\[
\text{% Porosity} = \left( \frac{(W_f - W_i)}{\rho_{\text{ethanol}} \times V} \right) \times 100
\]

\(\rho_{\text{ethanol}}\): density of ethanol

2.5. Swelling studies

Swelling ability of the composite sponges was studied in PBS (pH-7.4) [18]. Initial weight of the sponges was noted as Wi. Sponges were immersed in buffer for different time points and incubated at 37 °C. Samples were taken out and after removing the excess water wet weight was noted as Ww. Swelling ratio was determined using the formula

\[
\text{Swelling ratio} = \left( \frac{W_w - W_i}{W_i} \right)
\]

2.6. In vitro biodegradation studies

To study the in vitro biodegradation, preweighed (Wi) sponges were incubated in PBS (pH 7.4) with lysozyme (10⁴ units/ml) (Fluka) at 37 °C at different time points. Samples
were then taken out and freeze dried. Dry weight of the sponges was noted as \( W_d \). Degradation percentage was calculated using the equation.

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

2.7. Release Kinetics of VEGF

*In vitro* release of VEGF from CHVFS were analysed by ELISA technique using Ray Bio human VEGF-ELISA kit protocol [17,18]. For this study 10mg samples of CHVFS were used and Chitosan-HA sponges loaded with bare fibrin nanoparticles served as control. Samples were dipped in 2ml PBS at pH 7.4 and placed in a thermostatic bath at 37°C with gentle shaking. At scheduled time intervals, the release medium was withdrawn and replaced with the equal volume of fresh medium, with drawn medium was stored at -80°C. Released medium was analyzed for VEGF with ELISA kit. Briefly VEGF present in sample will bind to the wells precoated with immobilized antibody. The wells are washed and biotinylated antihuman VEGF antibody is added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin is added. The wells are again washed and a TMB substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. Results are expressed as cumulative release of VEGF ± SD (standard deviation) of three replicates.

2.8. Blood clotting studies

Blood was drawn from human ulnar vein and mixed with anticoagulant acid citrate dextrose (ACD) at 9:1 ratio [18]. The blood clotting efficiency of CHVFS was compared with chitosan-HA sponges and commercially available Calcium Sodium Alginate Dressing, Kaltostat (ConvaTec). Samples of equal dimensions and weight were used for
the study. Blood without samples was used as blank. Samples were placed in a 24 well plate and 200µl of blood mixed with ACD was added to each sample. To this 5µl of 1% CaCl$_2$ was added to initiate blood clotting and incubated at 37 °C for 15 min. After incubation 1ml distilled water was added slowly along the sides of the plate without disturbing the clot to hemolyze the red blood cells (RBC) that were not trapped in the clot [23]. 500µl from this was centrifuged in an eppendorf for 2min at 2000rpm. Supernatant was collected and optical density at 540nm was measured.

2.9. Mechanical properties

Mechanical properties of the composite sponges were evaluated by measuring the elongation at break and tensile strength. The percentage of elongation at break and tensile strength of the sponges were measured by a universal testing machine (Tinius Oisen H5KL) with a load cell of 10N and extension rate of 15mm/min.

2.10. Isolation and maintenance of human umbilical vein endothelial cells.

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cord as per the established protocol [16]. Umbilical cord was collected from ladies who underwent normal delivery at gynaecology Department of Amrita Institute of Medical Sciences and Research Centre (AIMS) with their prior written consent and ethical clearance of the institutional body of AIMS. Isolated cells were washed in serum-free Iscove's modified Dulbecco's medium (IMDM) and resuspended in complete IMDM (containing 20% fetal calf serum, 100U ml$^{-1}$ pen-strep antibiotic solutions and 150 mg ml$^{-1}$ endothelial cell growth factor [ECGF]). HUVECs were cultured on 2% gelatin coated flasks in complete IMDM and maintained in a humidified atmosphere of 5% CO2 at 37 °C.
2.11. Cell viability studies

Cell viability studies were done using alamar blue assay as per ISO specification 10993-5. EtO sterilized samples were used for the study. Studies were performed using HDF cells and HUVEC cells. Cells were seeded separately at a seeding density of 5x10^3 cells/sponge in 96 well plates and incubated at different time points (24, 48 h) at 37 °C and 5% CO₂. Alamar blue reagent diluted to 10 % in growth medium was added and after 6 h incubation, absorbance was measured at 570 and 600nm.

2.12. Cell attachment and proliferation

Cell attachment studies on the composite sponges were carried out using HDF cells and HUVEC for two different time points (12 and 48h). EtO sterilized samples were used for the study. Cells were seeded separately on the sponges with a seeding density of 2x10^4 cells/sponge in 24 well plates. After the incubation time the sponges were washed with PBS, fixed using 0.25% glutaraldehyde and dehydrated using alcohol gradient. The samples were then air dried, sputter-coated with gold and viewed under SEM.

For proliferation studies, after culturing HDF cells and HUVECs separately on sponges for 24 and 48 h, the samples were stained with Phalloidin red and DAPI. Briefly, samples were washed with PBS, fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton, blocked with 1% FBS and stained with Phalloidin red (actin stain), then incubated in dark for 2 h followed by staining with DAPI (nuclear stain). The stained samples were imaged using fluorescent microscope (Olympus-BX-51).

2.13. Live/Dead assay

In vitro qualitative analysis of cell viability was performed with live/dead assay (Invitrogen). HDF cells and HUVECs were seeded separately on etched cover slips at a
seeding density of $2 \times 10^4$ cells in 12 well plates and incubated for 12 h at 37°C. Samples were kept on top of the cell seeded cover slips and incubated for 24 and 48 h. After incubation period samples were taken and 200 µl of PBS solution containing 2 µl/ml ethidium bromide homodimer and 0.5 µl/ml calcein AM was added and incubated for 20 min at 37°C.


*In vitro* tube formation assay was performed using HUVEC cells of passage 4. The cells were trypsined and around $2 \times 10^4$ cells were added to geltrix coated wells. Meanwhile samples incubated conditioned media was added to the wells. The well plates were incubated and the tubes formed were observed under inverted phase contrast microscope (Leica) at fourth hour and was imaged at random different fields. The presence of tube like capillary structures indicates angiogenesis formation.

### 3. Results and discussion

3.1 Preparation and characterization of VnF

The prepared VnF was characterized using SEM and DLS. Morphological analysis by SEM showed spherical particles with size in the range of 150-180 nm (Fig. 1A). In DLS, number average weight distribution showed an average particle size of 180nm with a poly dispersity index of 0.4 (data not shown). The mean zeta potential of the nanoparticles were obtained as -28mV (data not shown) which shows the stability of nanoparticles. Histochemical staining using PTAH was adopted for visualizing the fibrin component in VnF. Fibrin particles stained blue can be seen in Fig. 1B.
3.2. Preparation and characterization of CHVFS

The CHVFS was prepared by homogenous mixing of chitosan hydrogel, HA solution and VnF suspension. The surface morphology of the composite sponges was shown by SEM image (Fig. 1 C & D). Both control and sample showed an interconnected porous structure.

HA and chitosan exhibited characteristic peaks at 3200-3600 cm\(^{-1}\) which corresponds to OH and NH stretching and 1640-1690 cm\(^{-1}\) for C=O stretching of amide I. Fibrin being a protein has shown characteristic peak at 1640-1690, 1550 and 1240 cm\(^{-1}\) corresponding to amide I, II and III [24, 22, 16]. In CHVFS, amide II peak at 1550 cm\(^{-1}\) in fibrin got shifted to 1590 cm\(^{-1}\) with an increased intensity. In the FT-IR spectrum of CHVFS, the characteristic peaks of chitosan, HA and fibrin were present (Fig. 2).

3.3. Porosity

Porosity of the composite sponges was evaluated by alcohol displacement method. All samples showed porosity in the range of 65-75%. No significant difference in porosity was noted in CHVFS.

3.4. Mechanical properties

Elongation at break and tensile strength of the composite sponges were measured. Elongation at break indicates the flexibility of composites which was in the range of 10-20\% (Fig. 3B). Tensile strength was in the range of 0.15 -0.02 MPa (Fig. 3C) for both control and sample. Ideal wound dressing bandages should be strong and flexible [25]. Studies have shown that presence of hyaluronic acid in composite scaffolds can reduce the tensile strength while increases the elongation at break point thereby improving the
flexibility. The flexibility of the sponge has been improved on addition of HA. Therefore, the sponges can be held on any surfaces without breakage.

3.5. Swelling studies

Swelling ability plays a significant role in wound dressings. Studies were carried out till 7 days. Swelling ratio was in the range of 8-12% for all the samples (Fig. 3D). Incorporation of VnF doesn't impart any significant difference in swelling. Composite sponges were saturated with PBS after 24 h which further does not improve the swelling ability. In a wound, exudate absorption is an important criteria which is satisfied largely by a sponge material. Dressings are normally replaced every alternate days clinically. Maximum swelling for the prepared sponges is attained within 24 hours which is advantageous even for a moderate to highly exudative wounds.

3.6. Degradation studies

Composite sponges were incubated at different time points in solution containing lysozyme to study the enzymatic degradation behavior. Lysozyme cleaves glycosidic linkage in chitosan and hyaluronic acid. Degradation percentage was in the range of 10-20% for all composite sponges in first week, whereas percentage degradation increased to 30-35% after second week (Fig. 3E). A wound environment is rich in proteases, therefore degradation of the sponges can occur at a faster rate [28]. Of all the enzymes, Lysozyme is responsible for the degradation of chitosan. Therefore, as the study indicated, only minimal degradation of chitosan sponge occurs by around week one. Hence the dressing can be applied on the wounds at least for seven days.
3.7. Blood clotting

Hemostatic potential of the composite sponges were shown in Fig. 4A. After contacting the samples with whole blood, RBCs that were not trapped in the clot were hemolyzed with water. Absorbance value of the resulting hemoglobin solution was noted at 540nm. Lower OD value indicates the high blood clotting. CHVFS showed higher blood clotting ability as compared to kaltostat and chitosan-HA composite sponges. Fibrin being natural coagulation protein, presence of nanofibrin in CHVFS enhances the blood clotting ability and platelet activation. Even though chronic wounds are not associated with heavy blood loss, there are certain wounds that are characterized by the presence of minor blood loss. The presence of fibrin can help prevent these.

3.8. Release kinetics of VEGF

In vitro release kinetics of VEGF from CHVFS was performed in PBS at 37°C for 7 days. An initial burst release of 29% was observed at the first time point (2 h), followed by a 64% release of bound VEGF at 72 h which was sustained till 168 h (7 days) (Fig. 4B). This particular kinetics observed with CHVFS make the system ideal as a wound dressing to restore impaired growth factor production, stimulate local angiogenesis and accelerates wound healing in diabetic wounds. An initial release of 29% is sufficient to enhance sprouting of blood vessels. The subsequent release can further accelerate angiogenesis. The release would be facilitated by the initial swelling followed by slower degradation of the chitosan HA matrix.

3.9. Cell viability studies

Cell viability of HDF cells and HUVECs in the presence of the composite sponges was analysed using alamar blue assay. Studies were done by contacting cells with samples
for 24 and 48 h. In HDF cells both samples and control showed more than 85% viability. No significant difference in viability was observed in cells contacted with CHVFS (Fig. 4C). Whereas there was a significant difference in viability in HUVECs contacted with CHVFS as compared to control sponges. At 24 h HUVECs in contact with CHVF showed above 90% viability and at 48 h it increased to more than 98%, whereas in presence of control sponge's viability was nearly 80% and 85% at 24 and 48 h respectively (Fig. 4D). VEGF released from CHFVS induced an increase in HUVEC viability. VEGF is the most potent growth factor that promotes angiogenesis which induces endothelial cell proliferation and migration [15]. Cell proliferation and migration is hampered in a diabetic wound [28]. Therefore, it is important to maintain the cells at the wound site viable and functional.

3.10. Cell attachment and proliferation

Morphology and spreading pattern of HDF cells and HUVECs on the composite sponges were analysed by SEM. Cytocompatible nature of CHVFS were revealed from the study. Images were taken after 24 and 48 h incubation of cells on composite sponges. HDF cells (Fig. 5A & C) and HUVECs were attached and began to spread on CHVFS and control sponges after 48 h incubation (Fig. 5B & D). HUVECs seeded on CHVFS showed higher proliferation due to the presence of VEGF (Fig. 5B & D). This was further confirmed by DAPI and Phalloidin staining, DAPI stains cell nucleus blue and phalloidin dye stains actin filaments red. HDF cells seeded on CHVFS did not show any significant difference as compared to cells seeded on control sponges (Fig. 6 A-D), Whereas, the HUVECs showed considerable difference in CHVFS with the presence of VEGF (Fig. 6D). HUVECs seeded on CHVFS showed capillary like tube structures which are clearly
visible in images at 48 h (Fig. 6 B & D). These tubular structures were absent in control sponges without the presence of VEGF. This evidently confirms CHVFS not only enhances viability and proliferation but also induces capillary like tube formation promoting functional angiogenesis.

3.11. Live/dead assay

Live/dead assay was performed with HDF cells and HUVECs in the presence of CHFVS. Ethidium-bromide homodimer stains dead cells as it binds fragmented deoxyribonucleic acid (DNA) in cells without an intact plasma membrane, and fluoresces red. Calcein AM is capable of permeating the plasma membrane of viable cells, where it is cleaved by intracellular esterases and gives green fluorescence. Fluorescent images of live/dead stained HDF and HUVECs in the presence of CHVFS and control sponges after 48 h is shown in Fig. 7A & C. These results were in correspondence with the DAPI- Phalloidin staining. Significant difference was seen in HUVECs seeded on CHVF composite sponges (Fig. 7 B&D).

3.12. In vitro tube formation assay

Tube like structures started forming by around 4th hour in the CHVFS sponges treated cells. HUVEC cells treated with control sponges didn't have tubular morphology. The results could be correlated with the initial 29% of VEGF released within 2 hours. The released VEGF was sufficient to initiate in vitro angiogenesis in endothelial cells. The capillary like structures formed is shown in Fig. 8 (A-B). After 4th hour the cells in both the system started dying, which was not considered. From the results it is clear that the presence of VEGF is enhancing tube formation in endothelial cells whereas the chitosan HA sponge control doesn't show the in vitro angiogenic property. The mechanism by
which VEGF induce angiogenesis involves activation of integrins that are essential for endothelial cell proliferation. In addition, the inherent vasodilatory property of endothelial cell is being enhanced via a nitric oxide mediated pathway [9].

4. Conclusions

A chitosan-hyaluronic acid composite sponge containing VEGF loaded nanofibrin was developed for the delivery of VEGF to diabetic wounds with poor angiogenesis. The prepared sponges were characterized and evaluated the porosity, mechanical strength, swelling, degradation and hemostatic potential. VEGF release studies from CHVFS showed an initial burst release followed by a sustained release till 7 days which is appropriate for a wound dressing material. Cell studies with HDF cells and HUVECs revealed the cytocompatible nature of CHVFS. Moreover endothelial cells seeded on CHVFS were well proliferated and showed capillary like tube formation which is a prominent process in wound healing angiogenesis. The proposed system provides a promising way for the treatment of diabetic wounds with poor granulation tissue, even though further studies' involving diabetic animal models are required to draw final conclusions regarding the potential of CHVFS to accelerate diabetic wound healing.

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References

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**Figure 1.** Characterization of VnF (A) SEM image (B) PTAH staining of VnF Characterization of composite sponges: (C) Images of Chitosan-HA (D) CHVFS inset shows magnified image.

**Figure 2.** (A) SEM images showing porous morphology of Chitosan-HA and (B) CHVFS. (C)FT-IR spectra (i) Chitosan (ii) HA (iii) VnF (iv) CHVFS.

**Figure 3.** In vitro evaluation of composite sponges: (A) Elongation at break (B) Tensile strength (C)swelling (D) In vitro biodegradation in presence of lysozyme. Student’s T test was performed for stastical analysis (n=3)

**Figure 4.** (A) Blood clotting studies of Chitosan-HA and CHVFS compared to kaltostat (B) *In vitro* cumulative release of VEGF from VnF in PBS at pH 7.4 and 37°C (C) Cell viability studies on HDF Cells and (D) on HUVECs at 24 and 48 h. Student’s T test was performed for stastical analysis (n=3)

**Figure 5.** Cell attachment studies: SEM images of HDF cell and HUVEC cell attachment on sponges after 48 h incubation on Chitosan-HA sponge (A & C) and CHVFS (B & D).

**Figure 6.** Fluorescent microscopic images of HDF cells and HUVEC cells (nucleus stained blue with DAPI and actin filaments stained red with phalloidin) after 48 h incubation on chitosan-HA sponge (A & C) and CHVFS (B & D). Scale bar denotes 20 μm.

**Figure 7.** Fluorescent microscopic images of live dead assay of HDF cells and HUVEC cells after 48 h incubation on chitosan-HA sponge (A & C) and CHVFS (B & D). Scale bar denotes 20 μm.
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Figure 1
Figure 2
Chitosan-HA/VEGF nanofibrin composite bandage

VEGF release

Promotes wound healing angiogenesis

blood vessel
basement membrane
collagen
endothelial cell
HIGHLIGHTS

- Chitosan-HA/VEGF loaded sponge was developed and characterized.
- Developed sponge showed controlled release of VEGF.
- Developed sponge showed better swelling, porosity and degradation.
- Developed sponge is cyto-compatible towards HDF cells.
- Enhanced proliferation & tube formation showed in HUVECs.