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Drug loaded bi-layered sponge for wound management in hyperfibrinolytic conditions

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Excessive bleeding due to premature clot lysis and secondary bacterial wound infection are two significant problems that contribute to increased morbidity in patients with hyperfibrinolytic conditions. In this study, we have developed a bi-layered sponge that promotes fibrin clot stability and prevents secondary bacterial wound infections. Using the technique of freeze-drying, a bi-layer matrix consisting of hyaluronic acid (HA) containing aminocaproic acid (Amicar) and chitosan containing tetracycline loaded *O*-carboxymethyl chitosan nanoparticles (Tet-*O*-CMC NPs) were produced. We hypothesized that the top chitosan layer with Tet-*O*-CMC NPs will prevent wound infection and concomitantly act as a matrix for cellular migration and subsequent wound healing, while the amicar-containing layer would promote clot stability. Tet-*O*-CMC NPs and bi-layer sponges were characterized using Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and Fourier Transform Infra Red (FT-IR) spectroscopy. Physicochemical characterization such as porosity, swelling and mechanical testing were performed. The drug release study shows that the bi-layered sponge demonstrates a robust burst release of amicar and a sustained release of tetracycline. *Ex vivo* muscle permeation study indicated that Tet-*O*-CMC NPs have enhanced tissue permeation compared to free Tet. *In vitro* antibacterial activity of the bi-layer towards *Staphylococcus aureus* and *Escherichia coli* as well as against their clinical pathogens proved to be effective. The *ex vivo* bacterial sensitivity study using porcine muscle confirmed the antibacterial activity, while cell viability study using human dermal fibroblast (HDF) cells revealed its biocompatible nature. The *in vitro* antifibrinolytic study shows that the bi-layered sponge with amicar showed significant protection against streptokinase induced clot lysis. These studies suggest that the prepared amicar and tetracycline loaded chitosan/HA bi-layered sponge can be used effectively to promote better wound healing by simultaneously preventing bacterial infection, and enhancing clot stability.

Keywords: Aminocaproic acid, Hyaluronic acid, Chitosan, Tetracycline, Nanoparticles, Bi-layered sponge, Hyperfibrinolysis, Bacterial infections

INTRODUCTION

Hyperfibrinolysis is a devastating clinical condition that is characterized by uncontrolled and catastrophic bleeding.¹ Liver disorders, severe trauma, major surgical procedures, and certain congenital conditions are some of the known causes of hyperfibrinolysis.² Inability to sustain a stable clot and enhanced clot lysis leads to profuse bleeding from wounds, making routine wound management difficult. While management of excessive bleeding from wounds is challenging, an overlying secondary bacterial infection further complicates wound care. *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* and *Escherichia coli* (*E. coli*) are some common bacteria responsible for secondary bacterial infection of wounds.³ Hence, in clinical conditions where hyperfibrinolysis and secondary bacterial infection co-exist, a combinatorial therapeutic strategy would be more effective.

One of the fundamental mechanisms responsible for clot lysis in hyperfibrinolytic conditions is the enhanced conversion of the anti-clotting factor, plasminogen (PA) to

plasmin, by the enzyme tissue plasminogen activator (t PA).^{4,5} Degradation of fibrin and subsequent dissolution of the clot occurs as a direct activity of plasmin at the site of the wound. Currently, one of the drugs used to manage excessive bleeding due to hyperfibrinolysis is aminocaproic acid (amicar®), a lysine analogue that binds to plasminogen and prevents plasmin induced clot lysis.⁶ One possible therapeutic option to control bleeding from wounds in hyperfibrinolytic conditions could be the use of aminocaproic acid incorporated degradable polymeric matrices that could be clinically used as bandages. Additionally, inclusion of an antibiotic within the polymeric matrix would have the added advantage of preventing bacterial complication within the wound site. Among the commonly used antibiotics, tetracycline has a broad range of anti-bacterial activity and it has been previously demonstrated that tetracycline in a nanocarrier like *O*-CMC (*O*-carboxymethyl chitosan) exhibits improved activity, penetration, and has a sustained release profile.⁷

Natural polymers like chitin, chitosan, cellulose, alginate, hyaluronic acid are some of the more preferable and

robust materials for use as drug delivery matrices.⁸⁻⁹ Chitosan has antibacterial, anti-inflammatory and haemostatic property, which favors its application in wound healing.⁹⁻¹¹ Although chitosan is reported to have antibacterial activity but its activity is pH dependent and shows a minimal antibacterial activity in neutral pH. It can be enhanced by incorporation of antibacterial drugs/nanoparticles into chitosan.¹² Chitosan, the de-acetylation product of chitin degrades into its monomer *N*-acetyl- β -*D*-glucosamine that has several functions in wound healing. Chitosan has ability to enhance fibroblast proliferation, simulate type IV collagen synthesis, activate polymorphonuclear cells, modulate cytokine production, and stimulate giant cell migration in wound.⁹⁻¹¹ Additionally chitosan serves as an excellent drug loadable matrix that can be used to produce prolonged drug release.¹³

However, the slow degrading nature of chitosan in comparison to copolymers such as Hyaluronic acid (HA) demonstrates a reduced burst release of loaded drugs.¹⁴ HA, a copolymer of *N*-acetyl glucosamine and glucuronic acid is the chief component of extra cellular matrix (ECM).¹⁵ Due to its non-immunogenic and viscoelastic behavior HA is a suitable biocompatible material in wound management.¹⁶ Additionally, the by-products of HA degradation has been shown to facilitate fibroblast proliferation and angiogenesis.¹⁶ Further HA provides a moist environment and prevents dryness of wounded tissue surface and promotes healing by increasing collagen secretion at the wound site by fibroblast proliferation.¹⁶

In this study we fabricated a dual functional bi-layered sponge with anti-fibrinolysis and antibacterial properties. The anti-fibrinolytic, aminocaproic acid was loaded onto the fast releasing HA, while the antibacterial Tet-*O*-CMC NPs was loaded onto the sustained releasing chitosan matrix. The goal of the study was to develop a multifunctional sponge that could be used as a therapeutic bandage in the management of wounds in patients with hyperfibrinolytic conditions. Fig. 1 shows the schematic representation of the bi-layered sponge.

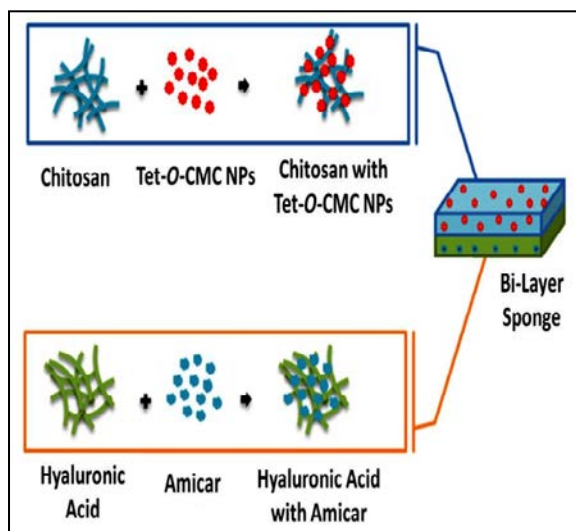


Fig. 1: Schematic representation of layers in bi-layer sponge.

Experimental

(a) Materials

Chitosan (MW 100-150 kDa, (degree of deacetylation-85%) and *O*-CMC sodium salt (degree of deacetylation-61.8% and degree of substitution- 54%) was purchased from Koyo Chemical Ltd (Japan). HA was purchased from Qingdao Haitao Biochemical Co Ltd (China). Tetracycline Hydrochloride purchased from HiMedia. Acetic acid and sodium hydroxide were purchased from Qualigens, India. 6-Amicar obtained from Sigma-Aldrich. Streptokinase purchased from Gland Pharma Limited. Luria Bertani broth (LB broth) and Agar-Agar were purchased from Himedia, India. Alamar Blue, Trypsin-EDTA, DAPI, antibiotic antimycotic solution, Minimum Essential Medium (MEM), and Fetal Bovine Serum (FBS) were obtained from Gibco and Himedia, India. All chemicals were used with no further purification.

(b) Bacterial strains and culture conditions

S. aureus strain SA113 (ATCC 35556)¹⁷ and *E. coli* strain (ATCC 25922) were used for this study.¹⁸ In liquid culture, all bacterial strains were cultured aerobically in LB broth at 37°C with shaking at 160 rpm.

(c) Cell culture

Normal human dermal fibroblast (HDF) cell line was purchased from Promocell. For the cell viability study HDF cells were cultured in MEM and supplemented with 10% FBS and 1% antibiotic antimycotic solution.

(d) Preparation of Chitosan Hydrogel

Chitosan hydrogel was prepared by neutralizing 2% Chitosan solution with 1% NaOH.¹⁹ The obtained hydrogel was washed 3 times with double deionized water before use.

(e) Preparation of Amicar-HA Hydrogel

2% HA solution was prepared by dissolving HA in distilled water by vigorous stirring under room temperature. 2% Amicar were measured and added into the HA solution and stirred for 1 h to dissolve the drug homogenously.

(f) Preparation of Tet-*O*-CMC NPs

Tet-*O*-CMC NPs were synthesized by ionic cross-linking of *O*-CMC using calcium chloride as a cross-linker as previously reported by our group.⁷

(g) Incorporation of Tet-*O*-CMC NPs into Chitosan Hydrogel

The prepared Tet-*O*-CMC NPs was then re-suspended in double distilled water which was then added drop wise into 2% chitosan hydrogel under constant vigorous stirring. Tet-*O*-CMC NPs was uniformly homogenised with chitosan hydrogel and was used for the preparation of bi-layer sponge after lyophilisation.

(h) Preparation of Bi-Layer Sponge

Initially, homogenized Amicar/HA hydrogel mixture was poured on to a Teflon mould and stored at -20°C overnight. After freezing, mould was taken and to the top of HA/Amicar hydrogel (bottom layer). Chitosan Hydrogel with Tet-*O*-CMC NPs were spread and kept for 20 min at room temperature. The mould with hydrogels was stored at -20°C overnight. The frozen samples were lyophilized (Martin Christ, Germany) for 24 h to obtain porous bi-layer sponge with top layer of Tet-*O*-CMC NPs incorporated in chitosan sponge layer and bottom layer of HA sponge containing Amicar (Fig. 2).

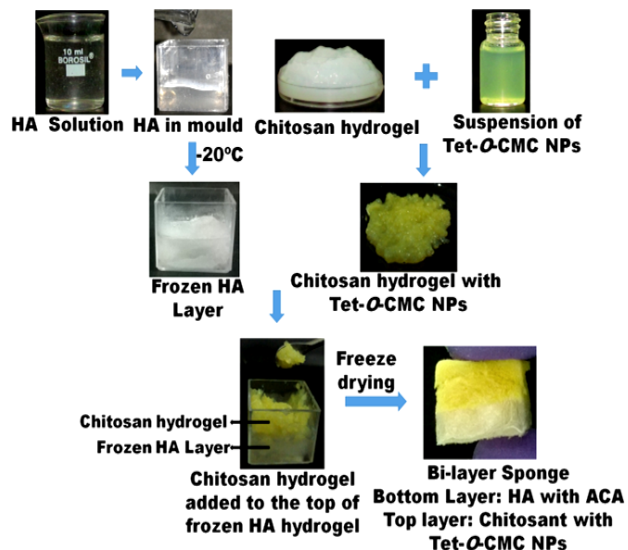


Fig. 2: Schematic representation of preparation of bi-layer sponge.

(i) Nanoparticle Size Analysis and Characterization

Particle size, particle size distribution, zeta potential etc. was analyzed using DLS (Malvern Zeta Sizer, Nano series). Surface charge and stability of the same was also analyzed using the same system. The average size and surface morphology studied using SEM (JEOL JSM-6490LA Analytical SEM). The Tet-*O*-CMC NPs suspension was diluted 5 fold with double distilled water and dropped on an aluminium stub and sputter coated with gold using an automatic fine gold coater (JEOL JFC-1600) at 10 mA for 120 s then scanned for SEM images. FT-IR analyzed potential chemical interaction between the constituents within the nanoparticle system. FT-IR spectra of *O*-CMC NPs were compared to Tet-*O*-CMC NPs.

(j) Encapsulation Efficiency (EE)

The supernatant obtained after particle separation from the reaction mixture was collected and quantified using a UV spectrophotometer (UV-1700 Pharma Spec, Shimadzu) at 275 nm. The encapsulation efficiency of the Tet-*O*-CMC NPs was calculated using the formula:

$$EE \% = \frac{(\text{Total amount of Tet added} - \text{Free Tet})}{(\text{Total amount of Tet added})} \times 100$$

(k) Characterisation of Bi-Layer Sponge

Lyophilized samples were cut into thin layers and placed on an aluminium stub using carbon tape and sputter coated with gold for SEM imaging. SEM (JEOL, JSM-6490LA, and Japan) was employed to observe the morphology of prepared sponges. The characteristic peaks of chitosan, HA, Amicar, tetracycline, *O*-CMC, Tet-*O*-CMC NPs were found out using Fourier transform infrared spectroscopy (FT-IR) (Perkin-Elmer Co. Model SPECTRUM RXI, FT-IR).

(l) Porosity Evaluation

Porosity of the sponges (average void volume) was studied using alcohol displacement method.²⁰ Chitosan bandage was taken as control and all the samples were in triplicate. Sponges of uniform size and shape were made using a scaffold punch. Dimensions of the sponges were measured using vernier calliper and volume was calculated. The displacing liquid was ethanol. Initial weight of sponges (dry weight) were measured and immersed in a known volume of absolute ethanol and allowed to penetrate into the porous sponge for 24 h. Final weights (wet weight) of the sponges were noted down and porosity was calculated using the equation below.

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

Where,

W_i = Initial Weight of the sponge (Dry Weight)

W_f = Final Weight of the sponge (Wet Weight)

ρ = Density of Absolute Ethanol (constant)

V = volume of sample before immersion in alcohol

(m) Swelling and Water Uptake Studies

The swelling study was carried out in PBS. Double distilled water was used for the water uptake studies. Water uptake ability of the sponges was also studied. Lyophilized samples were cut into uniform size and shape using a scaffold puncher and triplicates were used for the study. Initial weight of the samples were noted and immersed in PBS buffer / water and samples were placed at 37°C for incubation. At different time points samples were taken out and the wet weight was measured after removing the excess PBS/Water content by using filter paper. The buffer/water uptake was analysed by the formula.

$$\text{Swelling Ratio} = \left[\frac{(W_w - W_i)}{W_i} \right] \times 100$$

Where,

W_i = Initial Weight of the sponge (Dry Weight)

W_w = Final Weight of the sponge (Wet Weight)

(n) Tensile Strength and % Elongation Evaluation

Mechanical properties of the sponges were evaluated by measuring tensile strength and % elongation. The specimens were prepared by cutting it into a rectangular block of dimensions $50 \text{ mm} \times 10 \text{ mm} \times 2 \text{ mm}$. The specimens were clipped with a special pneumatic gripper on both ends. Samples were triplicated for the study. Tensile strength and percentage of elongation at break were measured by a universal testing machine (Tinius Olsen) with a load cell of 250 N, crosshead

speed of 25 mm/h and gauge length of 5cm at room temperature. Noted the values of both tensile and % elongation at break. Bare chitosan sponge was taken as control sample and the mechanical properties were compared with bi-layer sponge.

(o) Cell Viability

Cell viability of the prepared Bi-layer sponges on HDF cells were studied using Alamar Blue Assay.²¹ Sponges were cut into uniform size and shape using scaffold puncher and sterilized by ethylene oxide gas. 10,000 HDF cells were seeded per wells of a 96 well plate on sterile sponges and were incubated up to 48 h. After 24 h and 48 h the culture medium was replaced with 10% alamar blue in MEM and incubated for 4 h. After incubation period the optical density (O.D) was measured using micro plate spectrophotometer reader (BiotekPowerWave XS, USA) at 570 nm, with 620 nm set as reference wavelength. HDF alone used as positive control and Triton X100 treated cells used as negative control. The percentage of cell viability is calculated by using the formula:

$$\text{Cell viability (\%)} = \frac{\text{O.D of Test sample at 570nm} - \text{O.D of Test sample at 600nm}}{\text{O.D of Positive control at 570nm} - \text{O.D of Positive control at 600nm}} \times 100$$

(p) *In vitro* Drug Release Study and Its Kinetic Modelling

In vitro drug release study was carried out at pH 7.4. Chitosan sponge with Tet-*O*-CMC NPs and HA with Amicar were used for the drug release study. Samples were cut into uniform size and shape using a scaffold puncher in triplicates. Samples were soaked in 5 ml PBS and incubated at 37 °C under gentle shaking. At different time intervals a known volume of buffer was withdrawn and drug release was quantified spectrophotometrically. Drug release % was quantified using the formula:

$$\% \text{ Drug Release} = \left[\frac{\text{(drug released at definite time)}}{\text{total amount of drug in the sponge}} \right] \times 100$$

To interpret the release mechanism of the drugs, kinetic modeling was done.

(q) Antifibrinolytic Activity Study

Citrated blood was collected from blood bank of Amrita Institute of Medical sciences and Research, Kochi, India. The blood was transferred to a micro centrifuge tube containing different units of streptokinase (0, 10,100 and 1000 IU) and bi-layer sponge with/without amicar. Streptokinase is a plasminogen activator addition of which could mimic hyperfibrinolytic condition *in vitro*. The unit of streptokinase which caused complete lysis was optimised for further studies. The coagulation pathway was initiated with the addition of 50µl of 5% calcium chloride solution in to 500µl of citrated blood, followed by incubation at 37°C water bath for 15mins. After the incubation the serums is removed to measure the clot weight and were compared with controls. PBS and different units of streptokinase was used as negative control and tubes without streptokinase and amicar serves as positive control.

(r) *In vitro* Antibacterial Activity Assessment

McFarland standard 0.5 (~10⁸CFU /ml) was set and an aliquot of the *S. aureus* and *E. coli* suspension was added to the test tubes that contains 8 ml of LB broth containing pre-sterilized samples (10 X 5 mm²) to get a dilution of ~10⁶ CFU/ml. Test tubes were incubated at 37°C in a shaking incubator. After incubation, LB broth containing bacteria were serially diluted in sterile saline and plated onto LB agar plates. The number of bacterial colonies was counted and plotted for quantification.

(s) Validation of Antibacterial Activity against Clinical Isolates

As described above antibacterial activity against clinical isolates of *S. aureus* and *E. coli* were performed. The turbidity formed after incubation period of 12 h was measured by taking absorbance at 580 nm.

(t) *Ex-vivo* Antibacterial Activity Study

The antibacterial activity of bi-layer sponge was analyzed on infectious porcine muscle. The overnight cultures of *S. aureus* and *E. coli* were centrifuged at 5000 rpm for 10 min and washed two times with sterile PBS. The bacterial cells were finally re-suspended in 1 ml sterile PBS for the study.

Fresh hind limb muscle was cut in to 1 cm³ blocks and placed in 12 well plates. The muscles were infected by injecting 50 µl of the inoculums at the center of blocks by using a 1 ml syringe. 1 cm² chitosan-HA bi-layer sponge with and without Tet-*O*-CMC NPs were used for the study. Muscle tissue infected with bacteria was used as negative control. The hydrated samples were placed on the infected muscle. 1 ml of Sterile PBS was added to each well to avoid dehydration during the experiment. The entire system was incubated at 37°C for 7 h. Post incubation, the muscle tissue was isolated and minced into small pieces using a sterile surgical blade. The minced homogenate was transferred into a conical tube containing 10 ml sterile PBS, vortexed vigorously and plated on LB agar plates in serial dilutions. The reduction in bacterial colonies was quantified.²²

(u) *Ex- vivo* Muscle Permeation Studies

Nanoparticles released from the dressing material need to be penetrated through the muscles to avoid the deep tissue infections in open wounds. Franz diffusion cell apparatus was used to study the penetration of nanoparticles across tissue samples.²³ Human muscle and porcine hind limb muscles share physiological similarities. Porcine muscle was collected from local slaughterhouse immediately after butchering. The white fat layers was removed from the collected sample, washed thoroughly in double distilled water and was kept in normal saline to maintain the osmotic balance of the tissues.

Franz diffusion cell apparatus has two compartments-donor and receptor. The receptor chamber volume varied from 7.0 to 7.4 ml and area for diffusion was 2.54 cm². The compartment was filled with PBS (pH 7.4) and maintained at 37°C with circulating water jacket. The receptor media was kept under

gentle stirring to maintain mass and heat transfer. Porcine muscles were cut into appropriate size and thickness and kept on the top of the receptor compartment. The PBS in the receptor compartment should touch the muscle for a continuous penetration. Chitosan sponges containing Tet-*O*-CMC NPs were used for the study and compared with chitosan sponge containing same amount of tetracycline without any carriers. The dressing materials were clamped tightly to avoid any leakage. 2 ml of PBS was added to the donor compartment to hydrate the dressing material and tissue. 0.5 ml of the acceptor fluid was withdrawn at different time points (1, 2, 3, 6 and 24 h) and replaced with equal volume of PBS. The amount of drug in the withdrawn samples was quantified by taking the UV absorbance at 275 nm using double beam spectrophotometer. The penetration was analyzed by plotting cumulative percent of drug permeated per cm² for Vs time.

The muscles after the penetration study were fixed in buffered formalin and 5µm thick sections were taken using cryotome (Leica CM 1510 S). The tetracycline fluorescence in the tissue sections were observed under fluorescent microscope. The auto-fluorescence of the tissue sample has been corrected.

(v) Statistical Analysis

The data are expressed as mean ± standard deviation (SD). Student's t-test was conducted to analyze the results statistically. Probability level of p<0.05 was considered to be statistically significant.

RESULTS

Particle size distribution of Tet-*O*-CMC NPs in aqueous medium was determined using DLS technique, and was found to be in the size range of 200 ± 63 nm with mono-dispersion. The zeta potential of Tet-*O*-CMC NPs was -30 ± 5mV which confirms the stability in aqueous media. Particle size and morphology as analysed from the SEM image ensured the average particles to be in the range of 200 nm with spherical shape (Fig. 3A). Entrapment efficiency of the prepared Tet-*O*-CMC NPs was calculated using the formula, and was found to be about 64 ± 8 %.

Bi-layer sponge characterized using SEM showed well adhered layers of chitosan and HA with interconnected pores (Fig. 3D). HA and chitosan concentration was fixed at 2% w/v. Both the sponges showed pore size of 200-300 µm. Surface roughness is more in chitosan sponge when compared to the HA sponge. The SEM image of chitosan also shows the highly interconnected pores which favor the wound exudate absorption and moisture vapor transmission (Fig.3B).

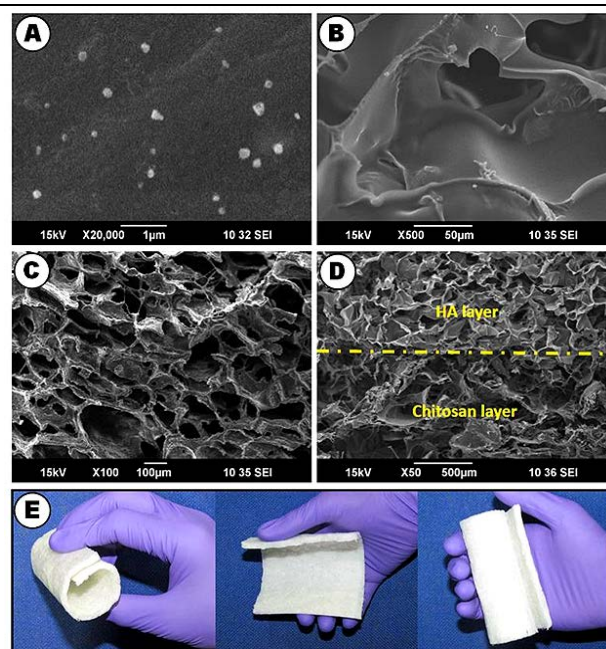


Fig. 3: (A) SEM images of Tet-*O*-CMC NPs, (B) Chitosan sponge, (C) HA sponge with Amicar, (D) HA and chitosan layers in bi-layer sponge and (E) Flexibility and foldability of prepared bi-layer sponge.

Fig. 4A-B shows the FT-IR peaks of chitosan sponge with Tet-*O*-CMC NPs layer and HA layer with amicar respectively. The FT-IR spectra of *O*-CMC showed a broad peak between 3500 and 3000 cm⁻¹ is due to the stretching vibrations of amine and hydroxyl group.²⁴ The stretching vibrations of protonated amino groups showed a peak of 1629 cm⁻¹.²⁵ *O*-CMC sodium salt did not show C-O stretching vibration peak at 1740 cm⁻¹.²⁵ The peak at 3365 cm⁻¹ is equivalent to hydroxyl group of tetracycline.²⁶ Stretching of N-H bonds and vibrations of C-N bonds of tetracycline were observed at 1240-1200 cm⁻¹.²⁶ In the FT-IR of Tet-*O*-CMC NPs depicts the slight broadening in intensity of the peak at 1639 cm⁻¹ compare to control *O*-CMC is due the interaction between *O*-CMC and tetracycline. Chitosan shows its characteristic peak for NH₂ stretching at 3400 cm⁻¹.²⁷ The peaks at 1640-1690 and 1035 cm⁻¹ corresponds to amide I band of chitosan and C-O-C stretching of HA, respectively.²⁸ Bands at 2850 and 2950 cm⁻¹ are due to C-H stretching vibrations of methylene groups of the amicar carbon chain.²⁹ Two bands at 1530 and 1545 cm⁻¹ are attributed to the symmetric and asymmetric stretching vibrations of uncoordinated -COO terminal groups of amicar.²⁹

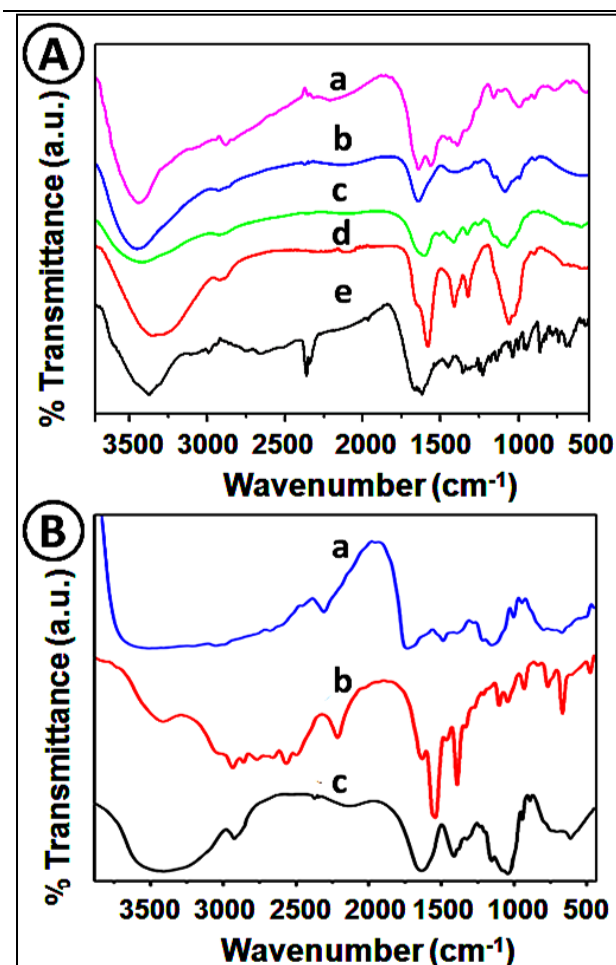


Fig. 4: (A) FT-IR spectra of chitosan layer in bi-layer sponge- (a) chitosan + Tet-*O*-CMC NPs, (b) Bare chitosan, (c) Tet-*O*-CMC NPs, (d) *O*-CMC, (e) Tetracycline, (B) FT-IR spectra of HA layer in bi-layer sponge- (a) Amicar in HA, (b) Amicar, (c) Hyaluronic Acid.

Porosity of the prepared sponges were analysed by previously reported method.¹⁹ Sponges were taken from the wells containing alcohol and weighed the wet weight, after removing the last drop of alcohol from the sponge. Porosity was found to be 75 to 85 % which will help to improve wound exudate absorption capacity.¹⁹ (Fig. 5A). Tet-*O*-CMC NPs incorporated chitosan sponge was found to be slightly more porous than the bare chitosan sponge which was not statistically significant.

Fig.5B shows the swelling studies of the prepared sponges. The study was done by immersing sponges in distilled water and PBS respectively. When compared to the control samples Tet-*O*-CMC chitosan sponge does not show any significant changes in swelling ability. There was a slight change in swelling ability of Tet-*O*-CMC chitosan sponge may be due to the swelling of *O*-CMC NPs. The remarkable point is that the sponges were saturated with liquid in the first day itself. There was not much significant difference in swelling of sponges after day 1 up to day 4.

Tensile and elongation properties of sponges were analyzed

using universal testing machine. Chitosan sponge used as control sample was compared to bi-layer sponge. Samples were cut into rectangular shape was hydrated to mimic wound conditions. Fig.5C shows the tensile strength evaluation data. Chitosan sponge showed tensile strength of 0.1 MPa whereas the tensile strength of bi-layer sponge was 0.08 MPa, which was adequate for a wound dressing material.^{13, 19}

Percentage of Elongation of samples was analyzed in order to evaluate the flexibility and softness of the sponges. Fig. 5D shows the % elongation data of chitosan and bi-layer sponge. Samples were triplicated and hydrated to mimic the wound conditions. Bi-layer sponge and chitosan sponge showed better elongation properties. Both sponges were elongated about 40±5 %.

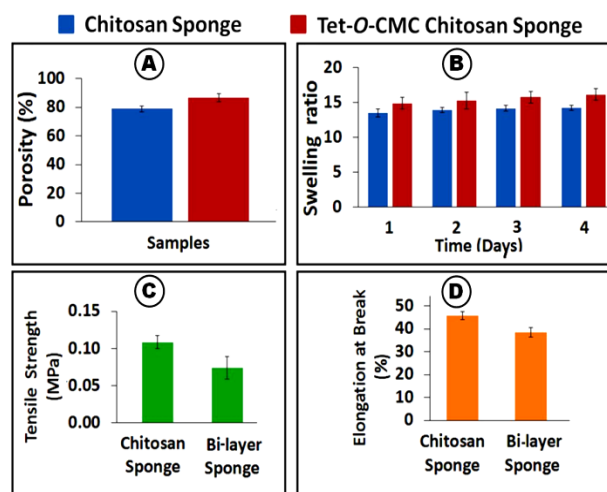


Fig. 5: (A) Porosity studies, (B) Swelling studies, (C) Tensile strength evaluation (D) % Elongation at break of bi-layer sponge.

Cell viability studies of bi-layer sponges and control samples were done using alamar blue Assay (Fig. 6). Bi-layer control showed 85-90% viability at 24 h which increased slightly after 48 h. Bi-layer sponge with drugs showed about 81 % of cell viability at 24 h and increased to 85% at 48 h. This proved that the bi-layer sponges are non-toxic to the HDF cells.

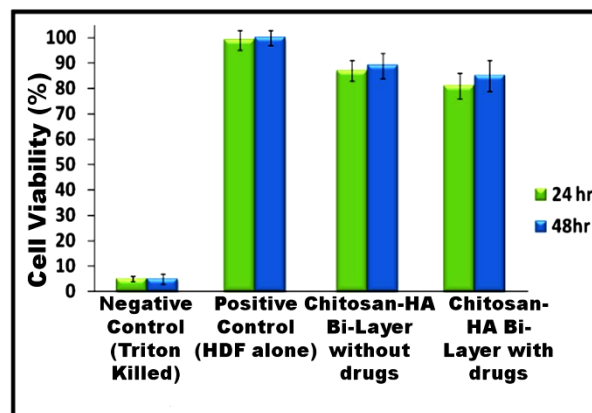


Fig. 6: Cell viability studies using HDF cell line at 24 and 48 h.

Fig.7 (A & B) shows the drug release data of amicar and

tetracycline from bi-layered sponge. From the drug release pattern, it was found that $48 \pm 5\%$ of drug released by 24 h. After that the release pattern is almost slow with a sustained pattern. The sustained dosage of drugs will be helpful in preventing the bacterial infection. Various kinetic models were plotted (Fig.7 C) and from the analysis it was found that Higuchi model shows closest value to the regression coefficient to unity ($R^2=0.9079$). Therefore, the best fit model that fits the drug release of tetracycline is Higuchi model.

Release study of amicar was done in PBS buffer. Fig.7B shows the percentage cumulative release data of amicar. 96% of amicar from the HA layer got burst release within 5 min.

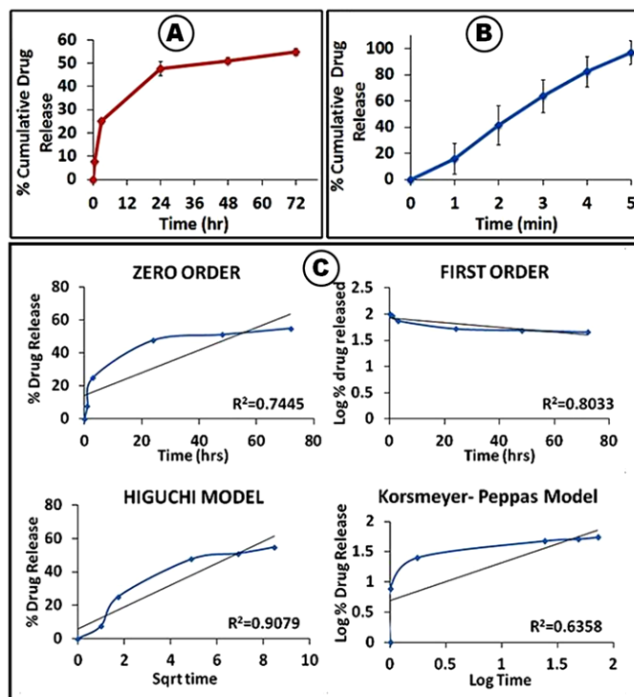


Fig. 7: (A) Drug release study of Tet-O-CMC NPs, (B) Drug release study of Amicar from bi-layer sponge and (C) *In vitro* Tet release data fitted using various kinetic models

Antifibrinolytic activity study shows that the developed amicar containing bi-layer sponge showed a significant activity against streptokinase induced hyperfibrinolysis (Fig. 8). 100 I.U. and 1000 I.U. of streptokinase treated blood showed a decrease in clot weight but lower concentration i.e. 10 I.U. of streptokinase did not. Bi-layer sponge without any amicar did not stabilize the clot. Whereas, the bi-layer sponge with amicar drug was showed excellent clot stability even at higher units of streptokinase treatment. We compared the activity of developed sponge with the same amount of amicar incorporated in the sponge and found that the sponge stabilized the clot similar to that of amicar control. This could be facilitated with the enhanced burst release of amicar from the HA layer that would disintegrate within minutes.

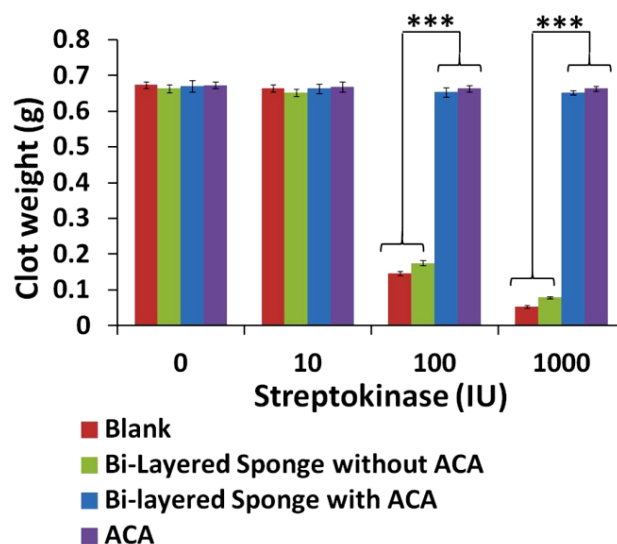


Fig. 8: *In vitro* antifibrinolytic activity of amicar and Bi-Layered Sponge with/without amicar.

Antibacterial activity of bi-layer sponge having Tet-O-CMC NPs was tested against typical gram positive bacteria *S.aureus* and a gram negative bacteria *E.coli*; which are the major infection causing bacteria. Fig. 9A shows the antibacterial activity evaluation by serial dilution method. In the figure-samples were indicated from 1 to 10 and top to bottom indicates the colonies of bacteria after serial dilution. The results represented graphically in Fig.9B. Bacteria alone were taken as negative control. Chitosan sponge, bi-layer sponge with amicar did not show any significant activity against both *S. aureus* and *E.coli*. Absence of bacterial colonies illustrates the effective activity of Tet-O-CMC NPs released from the chitosan sponge. The chitosan sponge with Tet-O-CMC NPs, tetracycline will be slowly released in a sustained manner which will inhibit the bacterial growth. The O.D of samples were plotted to note the activity of bi-layer sponge. Fig.10 B shows the antibacterial activity of bi-layer sponge and control samples. The O.D value of nutrient medium containing control samples and bi-layer sponge having Tet-O-CMC NPs were observed. Difference in turbidity was also analyzed visually. Fig. 10 A shows the photographs of the test tubes showing difference in turbidity which was clearly visible. More turbidity is due to the higher bacterial growth whereas the clear solution shows lower bacterial growth due to the activity of tetracycline released from the sponges. It was found that the bi-layer sponge with nano tetracycline showed significant reduction in bacterial growth against both *S.aureus* and *E.coli*.

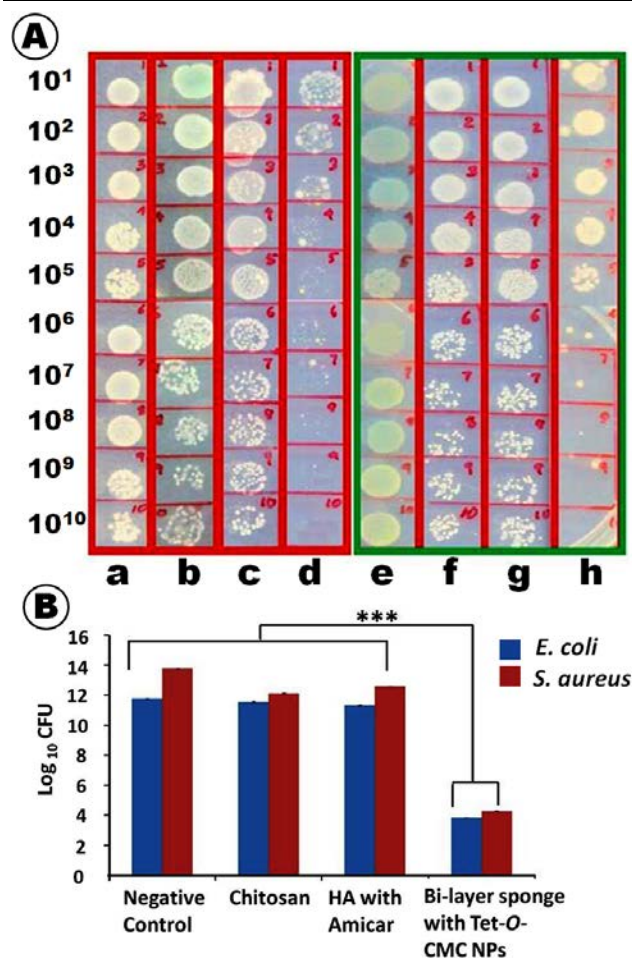


Fig. 9: (A) Antibacterial activity of bi layer sponge against *S.aureus* and *E.coli* by serial dilution method. (a) *E.coli* negative control, (b) Chitosan, (c) HA with amicar, (d) Bi-layer sponge with Tet-O-CMC NPs. (e) *S.aureus* negative control, (f) Chitosan, (g) HA with amicar, (h) Bi-layer sponge with Tet-O-CMC NPs, (B) Graphical representation of results obtained after serial dilution method.

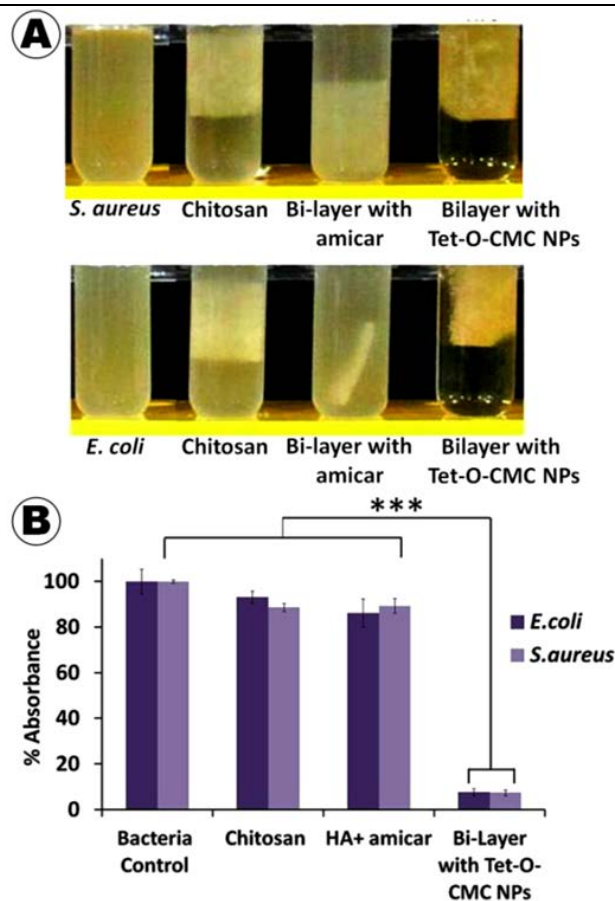


Fig. 10: Antibacterial activity of samples: photograph of tubes after incubation (A) and the percentage of absorbance of the LB broth from the tubes (B).

Optical density was measured by the same procedures used for clinical strains. The antibacterial activity of bi-layer sponge against clinical strains (Fig.11 A and B) was studied by measuring the reduction in absorbance at 580 nm. The bi-layer sponge significantly reduced the bacterial growth. Visual analysis also confirmed that the test tubes having bi-layer sponge with Tet-O-CMC NPs have lesser turbidity which implies its greater antibacterial activity.

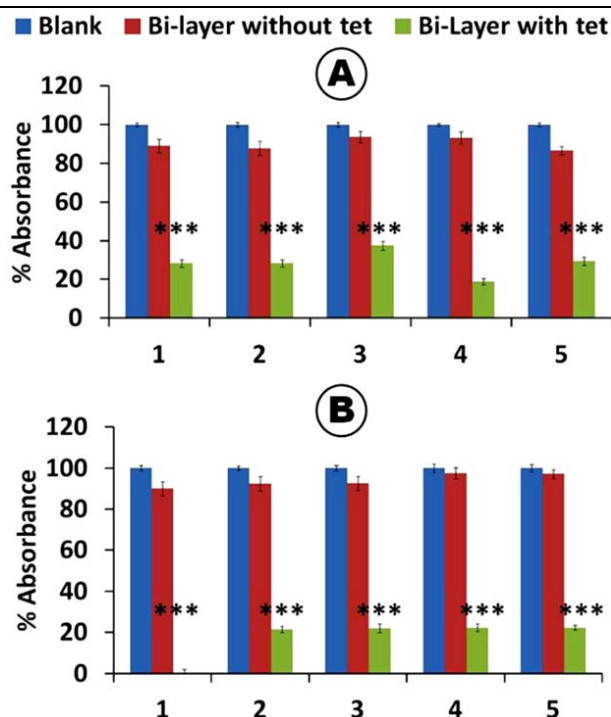


Fig. 11: Antibacterial activity of samples (A and B) against different clinical strains of *E. coli* and *S. aureus* respectively.

After *ex-vivo* antibacterial study bacterial CFU in muscle were analysed after incubation by plating on LB agar plates (Fig.12A-B). The results shows that the Tet-*O*-CMC NPs encapsulated scaffold had shown significant reduction in colony forming units towards both *E. coli* and *S. aureus* infected muscles. The infected muscle treated with bi-layer control had shown similar concentration as that of muscle without any treatment.

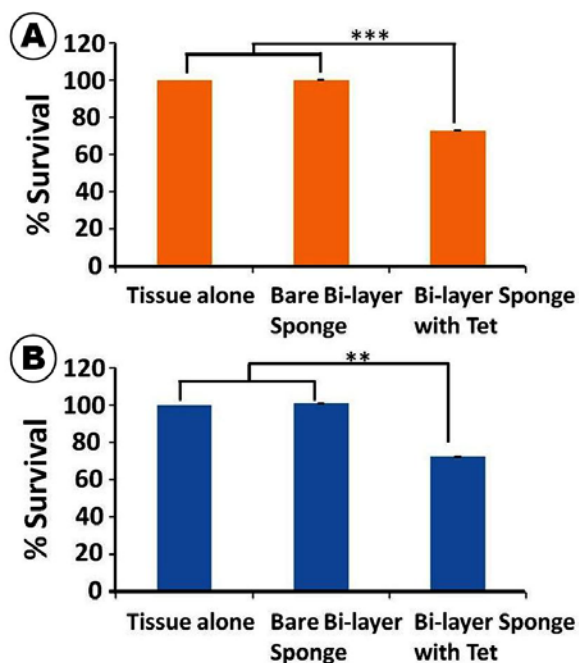


Fig. 12: *Ex vivo* antibacterial study of optimised bandages on porcine muscle infected with (A) *E. coli* and (B) *S. aureus*.

The cumulative percent of drug permeated per cm^2 were analyzed for both the systems. The kinetics shows that the nanoparticles improved the penetration of tetracycline across the porcine muscle. The penetrated drug reached the receptor compartment within second h. Within first h, the nanoparticles showed significant penetration. The cumulative percentage of drug permeated after 24 h was significantly higher in nanoparticles assisted penetration (Fig.13)

The fluorescent microscopic analysis of the muscle sections after penetration was analysed. The auto fluorescence of muscle was nullified by adjusting the intensity of light exposure and all the images were analysed in the same intensity. The image (Fig. 13 B-D) shows that the chitosan sponge with bare Tet showed more fluorescence and the boundaries of muscle fascicles were visible. But the nano Tet-*O*-CMC NPs penetrated more as evident in the fluorescent images. The nanoparticles accumulated in the fascicles, showed more fluorescence and can be easily identified.

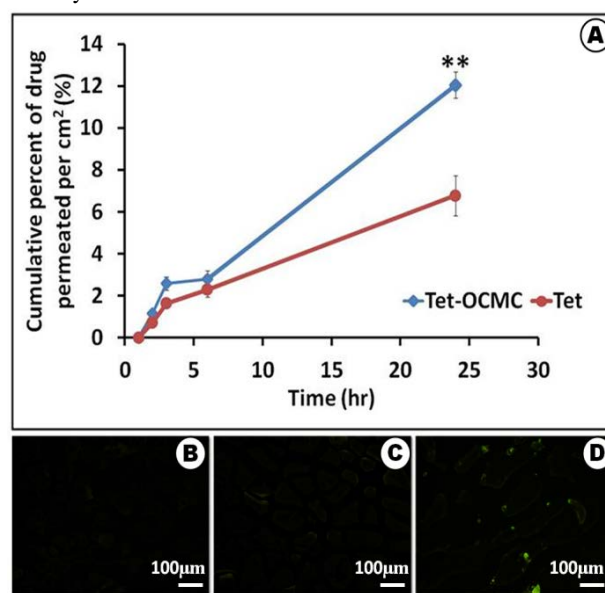


Fig. 13: (A) Drug penetrated across porcine muscle at different time points, *ex vivo* muscle penetration studies of (B) Bare porcine muscle section, (C) Porcine muscle with bare tetracycline, (D) Porcine muscle section with Tet-*O*-CMC NPs.

DISCUSSION

Tet-*O*-CMC NPs were prepared using ionic gelation method. Concentrations of tetracycline, *O*-CMC, CaCl_2 etc. were optimized by repeated experimental preparation methods. CaCl_2 was used as cross-linker. From the study it was found that the particle size can be tuned by changing stirring time, concentration of cross linker, rpm and temperature. Particle size, stability and drug entrapment also varies with the above mentioned parameters. Size of the Tet-*O*-CMC NPs changes with concentration of cross-linker added to Tet-*O*-CMC solution. CaCl_2 concentration was fixed to 0.1 % and was added using a syringe for the drop wise addition into the Tet-*O*-CMC solution and was stopped till an opalescent turbid solution is obtained. With increase in the polymer concentration, particle

size increases. Particles of smaller sizes were obtained when the polymer concentration was low. Tetracycline which was incorporated in the nano-matrix of *O*-CMC will release the drug in a sustained manner by the degradation of *O*-CMC which further improves the bioavailability of the drug.

From the SEM analysis showed the porous nature of sponges were well established. The highly porous nature of the sponge will be beneficial in faster healing of wound by providing controlled evaporation of wound exudates and its drainage. The porous nature of the bi-layer sponge showed that they can allow better gaseous and nutrients exchange. The chitosan and HA layers in sponges are well adhered. The chitosan layer will remain intact on the top of the wound site after the disintegration of HA layer. The cytocompatibility assessment of the control and bi-layer sponges using alamar blue assay confirmed non-toxic nature.

The porosity, swelling and water uptake ability of the bi-layer sponges did not change significantly when compared to the control. Porosity of the sponges has a major role in the swelling and water uptake ability. The data shows that the sponges have adequate swelling ability, which will help in absorbing wound exudates and maintaining a moist wound atmosphere at the wound site.¹⁹

Compared to the bare chitosan sponge, the tensile strength of bi-layer sponge was slightly reduced insignificantly. The reduced tensile value may be due to the soft HA layer, which is highly hygroscopic in nature. Samples were hydrated in order to mimic the wound condition. The disintegration of HA layer might have slightly reduced the tensile strength. When applying the sponges or bandages in the wounded area, such as in joints flexibility is more important. So from the % elongation data it is clear that the bi-layer sponge is suitable for the wound dressing application in any type of wound surfaces. This more flexible nature will also help to conform on any irregular wound surfaces.

One of the main characteristic of HA is its highly hygroscopic nature, which will easily absorb water and disintegrates quickly.¹⁴ In the bi-layer sponge, the base layer having HA with amicar is designed in such a way that when the sponge is in contact with blood or wound exudate will easily disintegrates and provides amicar to the wound site. The release pattern of amicar was found to be an immediate burst release which will be beneficial in a bleeding wound condition. This will help in controlling bleeding and thus minimizing the blood loss through the traumatic wound.

Tetracycline release study shows a sustained slow drug release. The surface bound drug got released at a faster rate which is attributed to initial burst release. The slower drug release of antibacterial drug at the wound site will help in prevention of bacterial growth. Reports suggested that Tet-*O*-CMC NPs are endocytosed by phagocytic cells and the colloidal carriers are degraded in endosomes by lysosomal esterases.⁷ The problems associated with over dosage can also be minimized by the slow release of tetracycline. Drug release kinetics of Tet-*O*-CMC NPs were also studied by plotting various kinetic models and checked for the best fit model by closeness of the

coefficient regression value to unity. From the analysis it was found that Tet-*O*-CMC NPs follows the Higuchi model. Here drug release is directly proportional to the square root of time and the release happens at a slower rate. This model is based on Fick's laws of diffusion, where the drug which is incorporated in porous matrix is dissolved and diffused by solvent. In an ideal case, controlled drug delivery systems are designed in such a way that, the drug delivery can be achieved in a controlled rate over a preferred duration. This will help in improving the efficacy of the drug and ensures the safety. Highly porous nature and enhanced swelling ability of the bi-layer sponges results in better drug release of Tet-*O*-CMC NPs as described in Higuchi model.

In vitro fibrinolytic study showed that, 100 and 1000 IU of streptokinase induced lysis of blood clot *in vitro*. The streptokinase enhances the conversion of plasminogen to plasmin which cleaves the fibrin networks. Sponge without amicar did not show any antifibrinolytic activity. But the amicar loaded sponges showed a significant increases in blood clotting similar to bare amicar. The peculiarity of the prepared bi-layer sponge lies in the immediate release of amicar from the HA layer that would favour inhibition of plasmin formation similar to amicar control. Amicar released from sponges binds reversibly to the kringle domain of plasminogen and blocks the binding of plasminogen to fibrin and its activation to plasmin.⁵

It was reported that tetracycline has greater activity against both gram positive and gram negative bacteria such as *S. aureus* and *E. coli*.³⁰ The serial dilution and turbidity assessment showed that bi-layer sponge with nano tetracycline effectively reduced the bacterial colony formation of ATCC and clinical strains of *S. aureus* and *E. coli*. Bacterial turbidity can be seen from the test tubes, but the tubes that contain bi-layer sponge with Tet-*O*-CMC NPs showed clear medium, which proves that they have very good activity against bacterial growth. Nano tetracycline from the bi-layer sponge would interact with the surface of the bacteria, and the encapsulated tetracycline will be released. Tetracycline is known to inhibit the protein synthesis in bacteria, and thus prevents the bacterial growth.³⁰ Tet-*O*-CMC NPs have greater activity against intracellular infection caused by *S. aureus*.⁷ From the study it was confirmed that the Tet-*O*-CMC NPs are effective in reducing the bacterial growth in *S. aureus*, *E. coli* and their clinical strains. So the bi-layer sponge with nano Tet having good anti-bacterial activity can be effectively utilized in wound sites to prevent infection and related problems. The chitosan sponge is not showing any considerable antibacterial activity when compared to bi-layer sponge with Tet-*O*-CMC NPs. It has been reported that antibacterial activity of chitosan is negligible at neutral pH due to its decreased solubility and protonation.³¹ Therefore the incorporation of antimicrobial drugs and nanoparticles like silver and ZnO into chitosan based wound dressing materials are necessary to increase its antimicrobial activity effectively towards infectious wounds.^{12, 19, 32-34}

The porcine muscle treated with Tet-*O*-CMC NPs incorporated to bi-layer sponge had shown significant reduction in bacteria compared to bare sponge treated muscles. Since

muscles are exposed to wound dressing material the delivery of drug in the muscles are more important. The nanoparticle and the released drug from the nanoparticle can penetrate through porcine muscle and can inhibit the growth of bacteria. *Ex vivo* studies provided similar results as that of *in vitro* studies. Even though the wound condition is different from experimental setup we can say that the sponge can effectively deliver antibiotic in to muscles and can decrease the bacterial load. One of the hurdles in chronic wound treatment is the control of bacterial load in the deep tissues. So our sponge can be used for the treatment of infectious chronic wounds.

The results shows that the drug loaded nanoparticles improved the penetration of the drugs. Even though drug can penetrate through the muscle fibres, these nanoparticles will improve its delivery. Major factors affecting better penetration are the nano size and cationic charges. The drug permeated in the muscle treated with nanoparticles containing sponge is contributed by the penetration of nanoparticles and drug released from the nanoparticles. The infections in the deep tissue can be easily controlled by incorporating drug loaded nanoparticles in the dressing material. Thus these sponges can be used for infectious wounds also.

Conclusions

The chitosan/HA bi-layer sponge was prepared and characterized using SEM and FT-IR. Top layer-chitosan sponge showed about 80-85 % porosity, which is an important property that is needed for an ideal wound dressing material. The highly interconnected pores will help in exudate absorption. The controlled swelling ability of chitosan sponge is also an added advantage in absorbing excess wound exudate and thus provides a moist wound atmosphere. Antifibrinolytic study showed that bi-layer sponge with amicar prevented thrombolysis as effective as amicar control and stabilized blood clot. This confirms that the amicar present in the HA layer would be released at a faster rate. The mechanical and elongation properties were confirmed the flexible and soft nature of the sponges. Tensile strength evaluation also showed that it has adequate tensile strength to be used as a dressing material. From the drug release study, it was clear that the chitosan layer having Tet-*O*-CMC NPs showed sustained/slow drug release pattern of Tet. From the *ex vivo* muscle penetration study using porcine muscle it was found that the Tet-*O*-CMC NPs have more penetration capacity when compared to the bare drug. Antibacterial evaluation by *ex vivo* study and using *S. aureus* and *E. coli* and their clinical strains proved that the bi-layer sponges have greater activity against both gram positive and gram negative bacteria. Cytocompatibility of bi-layer sponges were confirmed using HDF cell line. Hence the prepared bi-layer sponge could be a promising dressing material for post-surgical/traumatic wounds in order to reduce uncontrolled bleeding under hyperfibrinolytic conditions and to prevent bacterial infection.

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Notes and references

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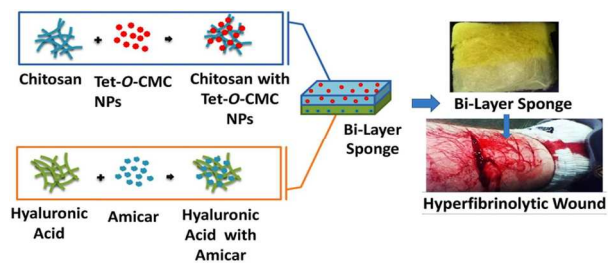
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Graphical abstract



Textual graphical abstract

Bi-layers consisting of hyaluronic acid containing amicar and chitosan containing tetracycline loaded *O*-carboxymethyl chitosan nanoparticles for hyperfibrinolytic wound management.