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# Sol–Gel Assisted Fabrication of Collagen Hydrolysate Composite Scaffold: A Novel Therapeutic Alternative to the Traditional Collagen Scaffold

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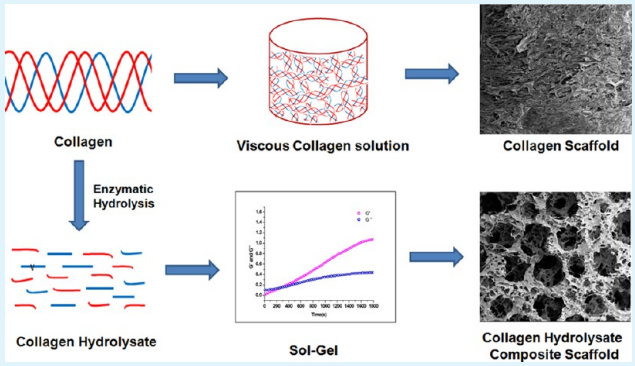
Supporting Information

**ABSTRACT:** Collagen is one of the most widely used biomaterial for various biomedical applications. In this Research Article, we present a novel approach of using collagen hydrolysate, smaller fragments of collagen, as an alternative to traditionally used collagen scaffold. Collagen hydrolysate composite scaffold (CHCS) was fabricated with sol–gel transition procedure using tetraethoxysilane as the silica precursor. CHCS exhibits porous morphology with pore sizes varying between 380 and 780 $\mu$m. Incorporation of silica conferred CHCS with controlled biodegradation and better water uptake capacity. Notably, 3T3 fibroblast proliferation was seen to be significantly better under CHCS treatment when compared to treatment with collagen scaffold. Additionally, CHCS showed excellent antimicrobial activity against the wound pathogens *Staphylococcus aureus, Bacillus subtilis*, and *Escherichia coli* due to the inherited antimicrobial activity of collagen hydrolysate. In vivo wound healing experiments with full thickness excision wounds in rat model demonstrated that wounds treated with CHCS showed accelerated healing when compared to wounds treated with collagen scaffold. These findings indicate that the CHCS scaffold from collagen fragments would be an effective and affordable alternative to the traditionally used collagen structural biomaterials.




**KEYWORDS:** *collagen, collagen hydrolysate, sol–gel, scaffold, tissue engineering, wound Healing*

## 1. INTRODUCTION

Collagen-based biomaterials have undergone numerous innovations in the field of tissue engineering and biomedical applications.[1−5] The valuable aspects of collagenous products including biocompatibility and biodegradability have made collagen an inevitable source for tissue engineering biomaterials. Three dimensional collagen scaffolds are invaluable for soft tissue engineering applications, especially for treating chronic wounds, such as burns, pressure sores, leg ulcers, and decubitus ulcers.[6,7] Although collagen is being widely used, it does have some drawbacks like a multistep extraction procedure, which increases the cost of collagen biomaterials,[8] low therapeutic loading because of limited solubility, faster collagen turnover because of ease of collagenase degradation, and low porosity of scaffolds.[9−11] These factors highlight the requirement of a better therapeutic and cost-effective alternative to traditional collagen biomaterial. In this study, we hypothesize that collagen hydrolysate, daughter fragments of collagen, could be a better therapeutic alternative to collagen because of its good bioavailability to the host tissue. Some recent findings have highlighted the possibility of using collagen hydrolysate as biomaterial for hard and soft tissue engineering applications.[12,13] Additionally, collagen hydrolysate would have the advantage of higher solubility than collagen and hence higher therapeutic loading is feasible. Also, the extraction of collagen hydrolysate is simple, cost-effective, and does not require a multistep extraction procedure. For the first time, we tested our hypothesis of using collagen hydrolysate as an alternative to traditional collagen for wound healing therapy. The present approach of using collagen hydrolysate as an alternative to collagen may offer novel insights in the field of biomaterial development.

There have been some previous attempts that support the use of collagen hydrolysate for skin health and wound healing

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applications.[14−16] The beneficial properties highlighted in these works include stimulation of collagen synthesis, hemostatic property, tissue adhesion, maintaining moisture balance, and antimicrobial property, which further emphasizes the need for the development of collagen hydrolysate based biomaterials. In addition, a previous clinical finding suggests that collagen hydrolysate can improve the rate of healing in patients with pressure ulcers by acting as a nutritional supplement.[17] It is also noted that collagen hydrolysate has chemotactic properties on fibroblasts and influences the growth of fibroblasts.[18] Therefore, the approach of using collagen hydrolysate as a main constituent in the scaffold was proposed.

In preparing 3-D sponge scaffolds, viscosity is the crucial property which determines the foam stability and sponge uniformity. Collagen sponge scaffold has been prepared through freeze-drying of the viscous collagen solution or gel. On the contrary, an aqueous solution of collagen hydrolysate is less viscous in nature. Therefore, it is challenging to fabricate a uniform sponge dressing using collagen hydrolysate unless a viscosity enhancing experimental design is adopted. Sol−gel methodology enables to manipulate the characteristics of the material required for a particular application.[19] In this work, sol−gel transition methodology was attempted using tetraethoxysilane (as silica precursor) to provide adequate viscosity to process collagen hydrolysate sponges. Sol−gel derived silica possesses many promising features, including low-temperature preparation procedure, porosity and chemical/physical stability.[20] In addition, silica incorporation would also enhance the exudate absorbing capacity and durability of the material.[21] Therefore, silica sol−gel transition methodology was adopted to develop a collagen hydrolysate composite scaffolds (CHCS), and the effectiveness of the CHCS was investigated against the traditionally used collagen scaffolds for wound healing therapy.

## 2. MATERIALS AND METHODS

**2.1. Materials.** Trypsin (4 BTEE units/mg solid), collagenase Type 1A (2.8 units/mg solid), chitosan (≥75% deacetylated) and MTT assay reagents were purchased from Sigma-Aldrich (Bangalore, India). Tetraethoxysilane (TEOS) was purchased from Alfa Aesar (Heysham, UK). All other chemicals used were of analytical grade, procured from SRL limited (Mumbai, India).

**2.2. Preparation of Type I Collagen Hydrolysate.** Type I collagen hydrolysate was prepared from bovine achilles tendon obtained from Regional slaughter house, Chennai, India. The preparation was carried out through successive steps: (i) The tendons were manually dissected out from the surrounding fascia, washed with saline followed by distilled water and frozen. (ii) The frozen tendons were minced and washed with three changes of 50 vol (vol/wt) of phosphate buffer saline distilled water, and (iii) the washed tendons were suspended in 20 vol of distilled water and heated at 90 °C for 1 h. (iv) The temperature is cooled down to room temperature, and trypsin (1:40, trypsin to initial wet weight of tendon) loaded on alginate beads was added and kept in orbital shaker maintained at 37 °C for 4 h to obtain collagen hydrolysate. (v) The hydrolyzed product was filtered and the filtrate was heated to 90 °C, and the collagen hydrolysate solution was annealed and stored at 4 °C.

**2.3. Preparation of Collagen and Collagen Hydrolysate Sponge.** Acid-solubilized collagen was isolated from bovine achilles tendon according to the procedure described elsewhere.[22] The concentration of the collagen solution was determined by hydroxyproline assay method.[23] Collagen sponge was prepared and its therapeutic potency was compared with that of collagen hydrolysate sponge. To the collagen solution (4 mg/mL), 400 μL of Triton X-100 was added and homogenized at 12000 rpm for 30 min using Ultra-Turrax IKA T25, and 10 g of dispersing element was added to produce uniform foam. Subsequently, the collagen foam was poured onto a petridish (90 mm × 15 mm) and frozen at −40 °C for 12 h, followed by freeze-drying for 12 h.

The collagen hydrolysate sponge was prepared using simple sol−gel transition methodology. Briefly, 8 mL of collagen hydrolysate solution containing 150 mg of collagen hydrolysate and 6 mL of 0.75% chitosan solution dissolved in 0.05 M acetic acid were mixed at 300 rpm for 15 min. Acid-catalyzed sol of tetraethoxysilane (TEOS) was prepared by mixing TEOS, 0.1 N hydrochloric acid, distilled water, and ethanol in the ratio of 66.66:16.00:12.88:4.44% v/v. The acid-catalyzed sol of TEOS was added to the collagen chitosan mixture solution while stirring; the pH was adjusted to 6.0 using 0.32 M ammonium hydroxide, and and the mixture was kept under mild stirring for 60 min. The subsequent steps were identical to that of the preparation of the collagen sponge. Similarly, chitosan−silica scaffold was prepared except the addition of collagen hydrolysate to appreciate the therapeutic benefit of collagen hydrolysate.

**2.4. Characterization of Collagen Hydrolysate Scaffold.**

*2.4.1. Rheology Study.* The rheological experiment was performed using Anton Paar Physica MCR 301 stress controlled rheometer with cone/plate geometry (1° cone angle and 40 mm cone diameter) for collagen hydrolysate−chitosan mixture with and without the addition of different concentrations of TEOS (0.5%, 1%, 1.5%, and 2%, w/v). Time sweep experiments were conducted at a constant strain of 5%, frequency of 1 Hz, with temperature maintained at 25 °C. The mechanical spectra, namely, storage ($G'$), loss modulus ($G''$), and complex viscosity ($\eta$) were recorded.

*2.4.2. Morphology, Porosity, and Water Uptake Capacity.* The morphology of the CHCS, chitosan−silica, and collagen scaffold were examined using scanning electron microscopy (SEM, JEOL-JFC 6360), operated at an accelerating voltage of 5 kV. The cross-section of the scaffold was placed on an adhesive stub, sputter-coated with gold using JEOL-JFC 1600 AUTO COATER, and examined using SEM. Pore size of the scaffold was obtained from the average pore diameter of 25 pores under the scope of the SEM. The pore size was measured using ImageJ, version 1.47, software according to the procedure described elsewhere.[24] The pores were measured and labeled.

Porosity of the sponges was determined by Archimedes' liquid displacement method using absolute hexane as the immersion medium.[25] Dimensions of the sponges were measured using vernier caliper and volume ($V$) was calculated. The preweighed scaffold ($W_i$) was immersed in a known volume of hexane in a graduated measuring cylinder for 30 min. The scaffold was removed, and the weight of the wet sponge was noted as $W_f$. Porosity of the scaffold was calculated by the following equation:

$$\%\text{porosity} = \frac{(W_f - W_i)}{\rho_{hexane} \times V} \times 100$$

where $\rho_{hexane}$ is the density of hexane.

To investigate water uptake capacity of sponges, CHCS and collagen scaffolds (triplicates) were separately immersed in distilled water at room temperature for 2 h. After they were removed from the water, they were hung over a table until no free water dripped from them and then weighed. The water uptake of the matrices was calculated by the following equation:

$$\text{water uptake } (\%) = \left[\frac{W_s - W_d}{W_d}\right] \times 100$$

where $W_d$ is the weight of the dry matrix and $W_s$ is the weight of the wet matrix.

*2.4.3. Collagenase Degradation.* A known weight of sample (triplicates) from CHCS and collagen scaffold were incubated with collagenase solution (100 μg/mL, prepared in PBS containing 0.05 M $CaCl_2$) at 37 °C. The ratio of collagen: collagenase was maintained at 50:1. The sampling was performed at desired time intervals (12, 24, 48, and 72 h). The samples after incubation of desired time intervals was immediately centrifuged at 2000 rpm at 4 °C for 10 min and an aliquot of supernatant was hydrolyzed with 6 M HCl at 120 °C for 12 h. Degree of collagen degradation of CHCS and collagen scaffold was

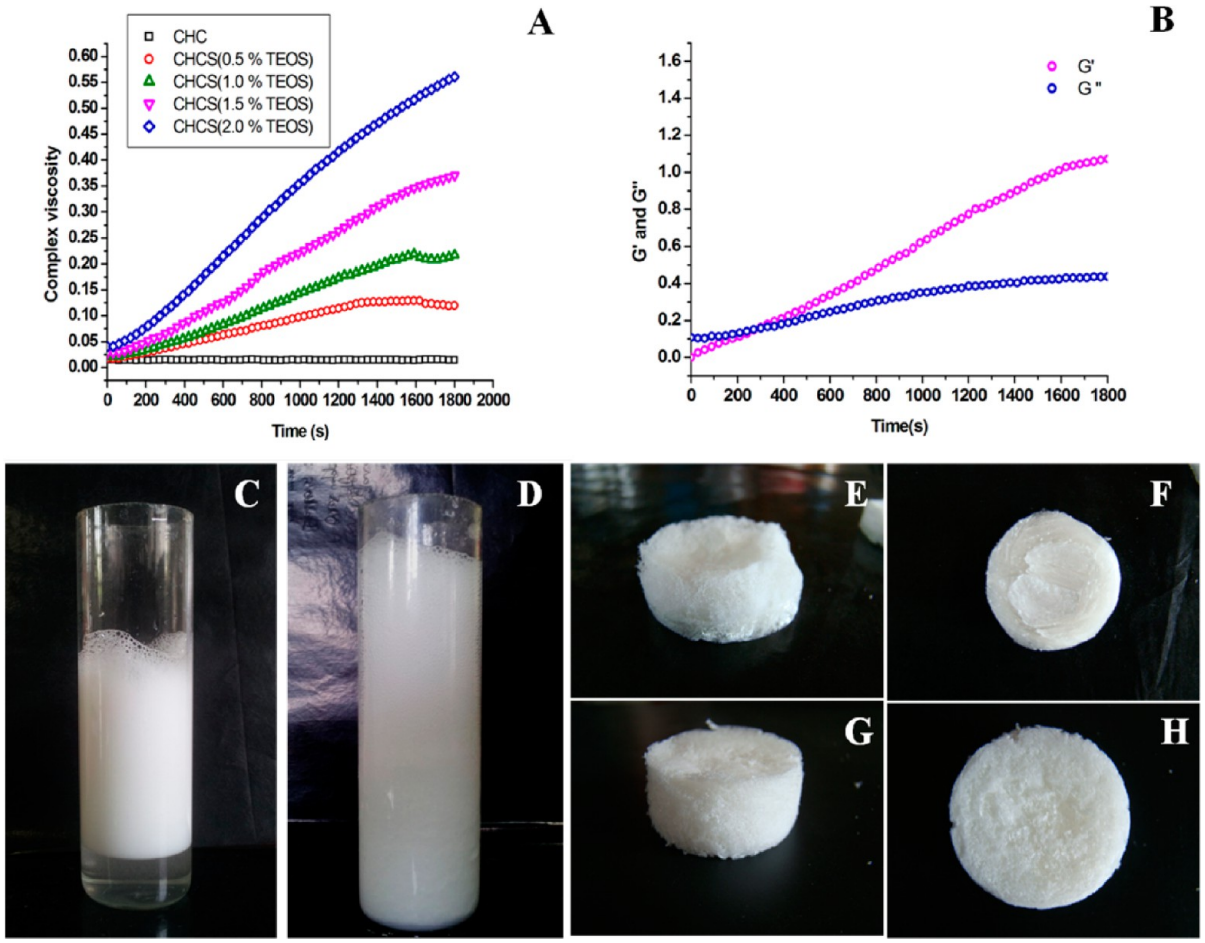


**Figure 1.** Rheological behavior of CHCS solution. (A) complex viscosity of CHCS at various concentrations of TEOS (0.5%, 1.0%, 1.5%, and 2.0%), (B) sol−gel transition of CHCS solution at 1.5% TEOS, (C) foam stability of CHCS without TEOS at 10 min, (D) foam stability of CHCS with TEOS at 10 min, (E, F) side and bottom view of collagen hydrolysate chitosan scaffold without TEOS, and (G, H) CHCS scaffold.

assessed through the measurement (spectrophotometrically) of hydroxyproline released in the supernatant.[23]

*2.4.4. In Vitro Release Study.* The release of the antibiotic model drug, mupirocin from CHCS and collagen scaffold samples (triplicates) was carried out using Franz diffusion model. A 1.5 $cm^2$ scaffold was placed in the upper chamber and 17.5 mL of physiological synthetic serum electrolyte solution (pH 7.4 maintained at 37 °C, with constant stirring) was poured into the lower chamber. These two chambers were separated by a wet dialysis membrane placed over the aperture of Franz diffusion apparatus. Aliquots of 1 mL were withdrawn at various time intervals and replaced by same volume of fresh medium. Mupirocin release was determined spectrophotometrically.

*2.4.5. Cell Proliferation Assay.* Briefly, 3T3 fibroblast cells at a density of $1 \times 10^4$ were seeded on CHCS, chitosan−silica, and collagen scaffolds in 24-well tissue culture plates and cultured for 1, 3, and 5 days. The cell-seeded scaffolds (duplicates) were cultured under 5% $CO_2$ atmosphere at 37 °C allowing attachment of cells to scaffolds. Then, 400 $\mu$L of media (DMEM containing 5% FBS) was added to each well. At desired intervals, 400 $\mu$L serum-free medium and 40 $\mu$L MTT solution (5 mg/mL in PBS) were added to each sample, followed by incubation at 37 °C for 4 h for MTT formazan formation. The supernatant was carefully discarded, and 400 $\mu$L DMSO was added to the sample to dissolve the formazan crystals. After solubilization, the absorbance at 490 nm was read on a microplate reader (Biotek, U.S.A.) versus untreated control.

*2.4.6. Antibacterial Activity.* The antibacterial activity of both CHCS and chitosan-silica scaffold were examined against four pathogenic strains commonly found in wounds, namely *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). All the cultures were subcultured periodically and maintained on nutrient agar. Mueller−Hinton agar was sterilized and poured on petriplates and allowed to solidify under laminar airflow. About 100 $\mu$L [$10^8$ CFU/ml (colony forming units)] of each bacterial culture was spread on the agar surface using sterile a glass spreader. The scaffolds with 0.6 mm diameter were placed and the plates were incubated for 24 h at 37 °C. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition against the test organisms.

**2.5. Full Thickness Excision Wound Healing Rat Model.** Male Wistar Rats (body weight range 200−220 g) were used for the study. All experimental protocols were approved by institutional animal ethical committee (Central Leather Research Institute, Chennai, India, IEAC No. 03/2012a) and are in agreement with the NIH guidelines for the proper use of animals for biomedical research. A total of 48 animals were divided into four groups, each consisting of 12 rats: group 1, CHCS group; group 2, collagen group (reference group); group 3, chitosan−silica group; group 4, untreated control group. Animals were anesthetized by intraperitonial injection of ketamine, at a dose of 60 mg/kg. The dorsal hair of the rats were removed by shaving and the skin was disinfected with 70% ethanol. Full thickness open excision wounds of 2 $cm^2$ area were created using scalpel blade by excising the dorsal skin. The wound was photographed and the initial wound area was traced using transparent sheet. The scaffolds were applied on excised wounds and covered with absorbent gauze to hold the material on wound area. On day 5, 10, and 15 of post wounding, four animals from each group were sacrificed by cervical dislocation, wound area was traced, and regenerated skin was exercised for

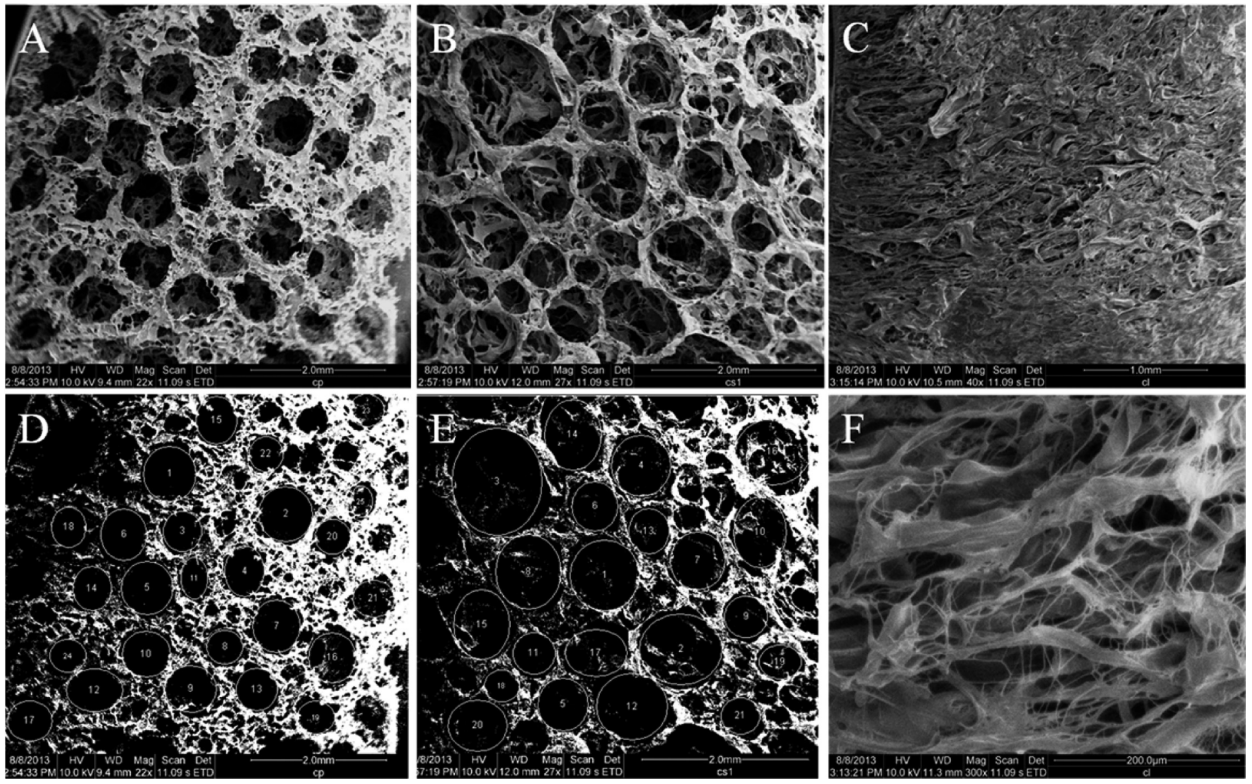


**Figure 2.** SEM morphology of the scaffolds: (A) CHCS and (B) chitosan−silica scaffold. Pore size measurement of scaffold using ImageJ software: (D) CHCS and (E) chitosan−silica scaffold. Collagen scaffolds at different magnifications: (E) 40× and (F) 300×.

histopathological investigation. The percentage wound closure was calculated according to the formula[26]

$$C_n = [(S_o - S_n)/S_o] \times 100$$

where $C_n$ is the percentage of wound size-reduction on days 5, 10, and 15 postwounding, $S_o$ is the original wound area, and $S_n$ is wound area on days 5, 10, and 15 postwounding.

The histology of exercised regenerated skin was performed by fixing in 10% neutral buffered formalin, stained with haematoxylin and eosin (H&E) and Masson's trichome stain. Histological interpretation and scoring was performed by the pathologist in a treatment blinded assessment manner. The histological scoring for inflammatory infiltrate, collagen deposition and neovascularisation was assessed based on the scoring system described elsewhere.[26]

**2.6. Statistical Analysis.** Data were expressed as mean ± SD. Statistical significance was determined by one way ANOVA using Dunnetts multiple comparison test. Differences were considered statistically significant for $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4.01 software.

## 3. RESULTS

**3.1. Preparation of CHCS Scaffold.** Viscosity is the prerequisite property for the preparation of a uniform collagen sponge scaffold. However, viscosity of the collagen hydrolysate solution is low even at high concentrations. Therefore, the objective is to increase the viscosity of the collagen hydrolysate solution by employing sol−gel methodology and by the addition of viscosity enhancing biopolymers as composites. Sol−gel-derived silica xerogels have found wide utility in the field of drug delivery and tissue engineering.[27−29] Collagen hydrolysate scaffold was prepared using the acid/base catalyzed sol−gel transition of a silica precursor, TEOS. The resultant material was fragile and did not have adequate physical integrity. In order to address this problem, chitosan was added to the collagen hydrolysate solution prior to the sol−gel process, which resulted in better physical integrity, thermal stability (see Figure S1 in Supporting Information), and spongy morphology. However, the concentration of chitosan in the scaffold was kept minimal to emphasize the therapeutic benefits of collagen hydrolysate.

Viscosity is a critical parameter in determining the foam stability of the composite. We studied the effect of varying concentrations of TEOS (0.5%, 1.0%, 1.5%, and 2.0%) on the viscosity of the composite solution and thus on foam stability. The viscosity versus foam stability correlation revealed that foam stability increased with increase in TEOS concentration, and that a TEOS concentration of ≥1.5% resulted in good foam stability. The scaffold with the final composition of collagen hydrolysate/chitosan/TEOS (1:0.3:1.5%, w/v) resulted in good foam stability and uniform spongy appearance.

**3.2. Rheological Behavior of CHCS.** Figure 1 shows the changes in complex viscosity of the CHCS composite at various TEOS concentrations. It is known that sol−gel transition of TEOS is pH dependent, and that the time for gelation varies with respect to the pH of the sol.[27] A gradual increase in complex viscosity was observed with increasing time and TEOS concentration (Figure 1A). In addition, collagen hydrolysate solution with TEOS (0.5 and 1%) exhibited shear thinning behavior, and this diminished with increase in TEOS concentration. Figure 1B shows the sol−gel transition behavior of CHCS (TEOS, 1.5% w/v), where the viscous modulus ($G'$) is equal to the elastic modulus ($G''$). In the beginning, the viscous modulus was dominant over the elastic modulus, which confirmed the fluid like behavior. Sol−gel transition occurred at the crossover region followed by a steady increase in the elastic modulus, which confirmed the gel system. Subsequently, the foam stability was tested after homogenization and there was a

significant increase in foam stability of sol–gel processed CHCS sample (Figure 1C and 1D). As a result, the CHCS solution with good foam stability exhibited scaffold uniformity and vice versa. Collagen hydrolysate sponges prepared with and without the addition of TEOS have been shown in Figure 1. The composite containing TEOS gave a scaffold with uniform spongy appearance (Figure 1G and 1H), whereas the composite devoid of TEOS gave a scaffold with a nonuniform spongy appearance, evident from the distinct layer at the bottom of the scaffold (Figure 1E and 1F).

**3.3. Morphology, Porosity, and Water Uptake Analysis of Scaffolds.** The morphology (Figure 2) of CHCS and chitosan–silica scaffolds revealed the presence of porous structure throughout the section, with larger pores being interconnected by a number of smaller pores, whereas the reference collagen scaffold was less porous with interconnected fibril appearance observed only at higher magnification (Figure 2C and 2F). The pore size distribution of the large pores was measured using imageJ software, version 1.17. The pore diameter of CHCS ranges from 380 to 760 $\mu$m with an average pore size of 596 $\mu$m, whereas the pore diameter for chitosan–silica scaffold, ranges from 350 to 980 $\mu$m with an average pore size of 588 $\mu$m. It is evident that the composite design, which includes chitosan and silica as chitosan–silica scaffold provides the porous morphology for CHCS. However, the pores of CHCS have a distinct serrated appearance, which is not present in chitosan-silica, which could be due to the presence of collagen hydrolysate. The presence of large pores in the scaffolds facilitates cellular infiltration and growth within the 3-D structure.[30] In addition, it could help better oxygen transfer to the host tissue. The smaller pores in the interconnected network could facilitate cell adhesion and infiltration within the scaffold.

Porosity is an important property in tissue engineering, as a highly porous structure provides greater surface area, thereby promoting better cell growth through the easier passage of nutrients to the growing cells.[25] For determination of porosity, hexane was used as a displacement liquid as it causes negligible swelling or shrinkage and weight loss of the test scaffolds during solvent treatment. The chitosan-silica scaffold showed porosity of 70.63 ± 3.32%, whereas collagen hydrolysate presence reduced the porosity of CHCS to 64.53 ± 2.54%. The % porosity was in accordance with the mean pore size of CHCS and chitosan-silica scaffolds. However, collagen sponge showed porosity of 62.99 ± 3.50%, despite less porous morphology and this could be due to the collagen swelling property.

The water uptake capacity of CHCS and collagen scaffolds is shown in Figure 3. CHCS showed good water uptake in comparison to collagen sponges. As indicated in SEM analysis, CHCS exhibited porous morphology which could facilitate the entrapment of water in the scaffold matrix. In addition, the incorporation of silica could contribute to increase in water uptake because of its water adsorbing property. Despite having lesser porosity, the swelling behavior of collagen scaffold is excellent which may be due to the presence of a large number of narrow pores which entrap and hold water through capillary action.

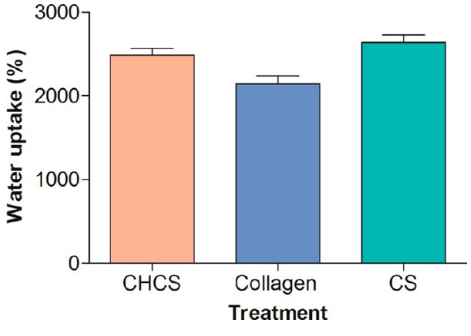


**Figure 3.** Water uptake capacity of CHCS, chitosan–silica, and collagen scaffold.

**3.4. Biostability of CHCS.** In vitro biodegradation study was performed to assess the biomaterial's potential to withstand collagenase action. The amount of collagen degradation was assessed through the estimation of hydroxyproline released in the supernatant after subjecting the scaffold to collagenolytic degradation. The CHCS scaffold showed slower degradation compared to the collagen scaffold (Figure 4). At 12 h sampling, collagen scaffold showed complete degradation, whereas CHCS showed only 56.88% degradation. At 72 h, CHCS demonstrates a degradation of 90.62%, which clearly indicates that the addition of chitosan and TEOS has made the collagen hydrolysate scaffold resistant to collagenolytic degradation. The faster biodegradation of the collagen scaffold limits its use for long-term application in wound-care therapy.

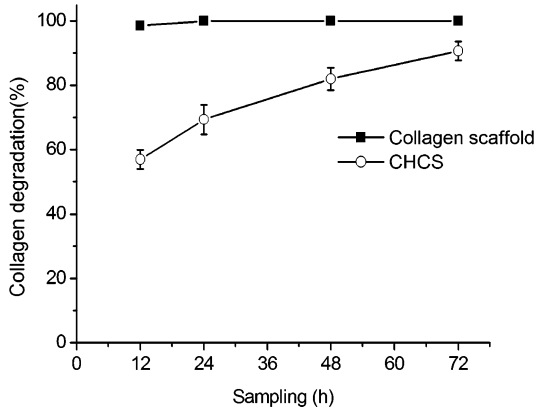


**Figure 4.** Collagenase degradation profile of CHCS and collagen scaffold.

**3.5. CHCS for Controlled Release of Drug.** The effect of CHCS composition on the release of a model drug was investigated. Franz diffusion model was used for carrying out in vitro drug release from the scaffold as it simulates the clinical application of a topical delivery system on wound surface.[21] Mupirocin-loaded collagen scaffold was used as a control to evaluate the effect of CHCS composition on the release of the drug. Figure 5 depicts the release profile of mupirocin from the CHCS and collagen scaffolds. CHCS exhibited prolonged drug release when compared to the collagen scaffold. In the first 12 h, 39.90 ± 4.46% of mupirocin was released from the CHCS, whereas 62.29 ± 3.06% of mupirocin was released from collagen scaffold. It is evident that the scaffold slowed down the initial drug release in CHCS which could be due to the presence of silica. Subsequent analysis showed that 75.52 ± 6.42% of mupirocin was released at 48 h from CHCS. On the contrary, mupirocin loaded collagen scaffold released 85.01 ± 2.87% within 24 h. The drug release of 88.14 ± 6.21% at 72 h from the CHCS scaffold showed that it is capable of delivering the drug for more than 3 days for clinical applications. Hereby, we clearly demonstrate that the CHCS can be used as a scaffold

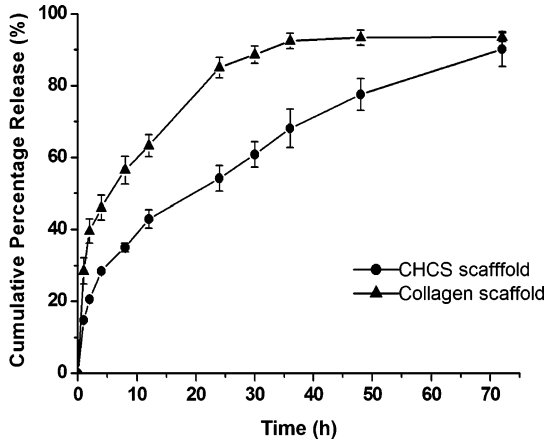


**Figure 5.** Cumulative release profile of mupirocin from CHCS and collagen scaffold.

for controlled drug release applications, which is one of the established characteristics of collagen sponges.

**3.6. Biocompatibility of CHCS.** Figure 6 shows the viability of fibroblasts cultured with the scaffolds at different

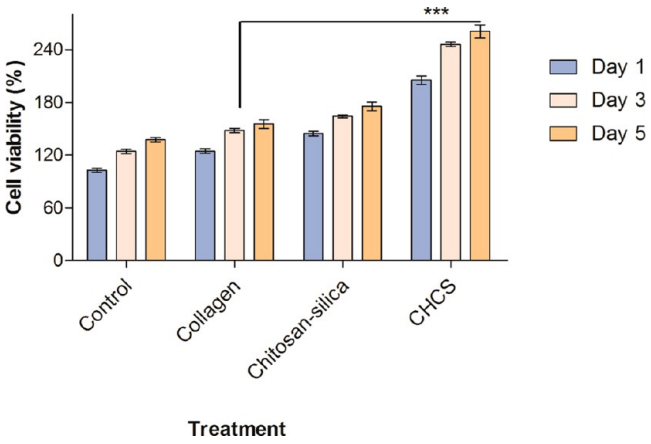


**Figure 6.** Cell proliferation of 3T3-L1 fibroblast cells seeded on culture plate well (control), collagen scaffold, chitosan-silica and CHCS scaffold. Significant difference (***$p < 0.001$) in cell proliferation was observed throughout the sampling interval between CHCS and the collagen group.

time period. The cell proliferation with CHCS treatment is significantly higher than that of collagen and chitosan−silica treatment. We have mentioned earlier that the increased solubility of collagen hydrolysate compared to that of collagen enabled us to load a considerably higher amount of material in the CHCS scaffold, that is, an equivalent amount of collagen cannot be loaded onto a collagen scaffold of equal volume. The higher therapeutic load of collagen hydrolysate present in the CHCS might be the reason behind the increased cell proliferation. The higher porosity of CHCS also provides greater surface area that could promote better cell growth through the easier passage of nutrients to the growing cells. The porous nature of the scaffolds not only helped the cells to penetrate into the interior but also enhanced the transfer of oxygen and nutrients to cells.[31] The presence of chitosan and silica in CHCS did not have any detrimental effect on scaffold biocompatibility.

**3.7. Antibacterial Activity.** Antibacterial activity was examined against four pathogenic strains, which are more prevalent in infectious wounds. Interestingly, CHCS exhibits moderate antimicrobial activity against two Gram-positive strains, *Staphylococcus aureus* and *Bacillus subtilis*, and one Gram-negative strain, *Escherichia coli* (Table 1). Collagen did

**Table 1. Antimicrobial Properties of CHCS, Chitosan−Silica, and Collagen against Wound Pathogens**

| | zone of inhibition in diameter (mm) | | |
|---|---|---|---|
| test organisms | CHCS | chitosan−silica | collagen |
| *S. aureus* | 10 | | |
| *E. coli* | 11.5 | | |
| *B. subtilis* | 14 | 10 | |
| *P. aeruginosa* | | | |

not exhibit activity against any bacterial strains. We further wanted to establish if the antimicrobial effect is due to the presence of chitosan and silica in CHCS and hence tested the antimicrobial effect of chitosan−silica scaffold. However, this control scaffold showed activity only against *B. subtilis*. Therefore, it is evident that the antimicrobial activity of CHCS against *S. aureus* and *E. coli* is due to presence of collagen hydrolysate and this further emphasizes the therapeutic benefit of collagen hydrolysate. The existence of peptides with antimicrobial activity is well established. It has been reported that proline-rich peptides, where the proline is associated with arginine, are able to exhibit activity against Gram-negative bacteria through a nonlytic mechanism, which might involve transport system and cytoplasmic targets.[32] It is also established that collagen peptides are abundant in proline and also that proline and arginine are neighboring amino acids in a small share of collagen peptides.[33] Therefore, we propose that the antimicrobial activity shown by collagen hydrolysate can be attributed to the proline-rich peptides, especially those where proline and arginine are closely associated.

The use of topical antimicrobial drugs is essential in combating infections. However, these drugs retard the cell proliferation because of their concentration dependent cytotoxic effects.[34,35] Consequently, the incorporation of these drugs into scaffolds may deter wound healing efficiency. But, the inherent antibacterial activity of collagen hydrolysate is a promising value addition to CHCS without compromising the cell proliferation. Therefore, collagen hydrolysate based biomaterials like CHCS may emerge as a potential therapy for combating even infectious chronic wounds.

**3.8. *In Vivo* Experiments using CHCS on Full Thickness Excision Wounds in Rat Model.** Collagen-based wound dressings are uniquely suited to address the issue of elevated levels of MMPs by acting as a "sacrificial substrate" in the wound.[36] It has also been demonstrated that collagen breakdown products are chemotactic for a variety of cell types required for the formation of granulation tissue.[37] Collagen hydrolysate, a prefragmented product of collagen, is hypothesized as a therapeutically better alternative to collagen in treating chronic wounds. The adherence of the CHCS scaffold to the wound surface was comparable to that shown by collagen scaffolds. However, collagen scaffold lose their counter shape during application due to the spongy nature and easy compressibility, whereas CHCS retain their counter shape, which could aid in retaining porous structure and help in better oxygen transfer. As presented in Figures 7 and 8, treatment

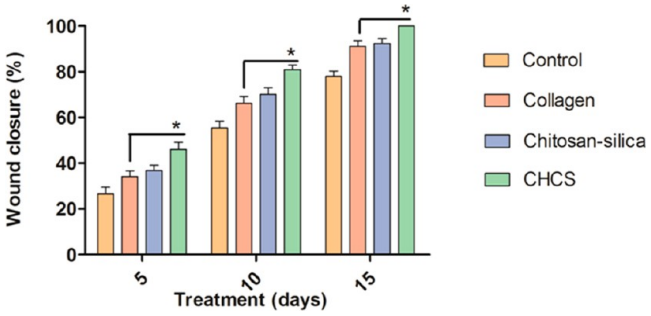


**Figure 7.** Percentage wound contraction for control (untreated wound), collagen, chitosan–silica, and CHCS. Significant difference (*$p$ < 0.05) in the efficacy was observed throughout the treatment duration between CHCS and the untreated group.

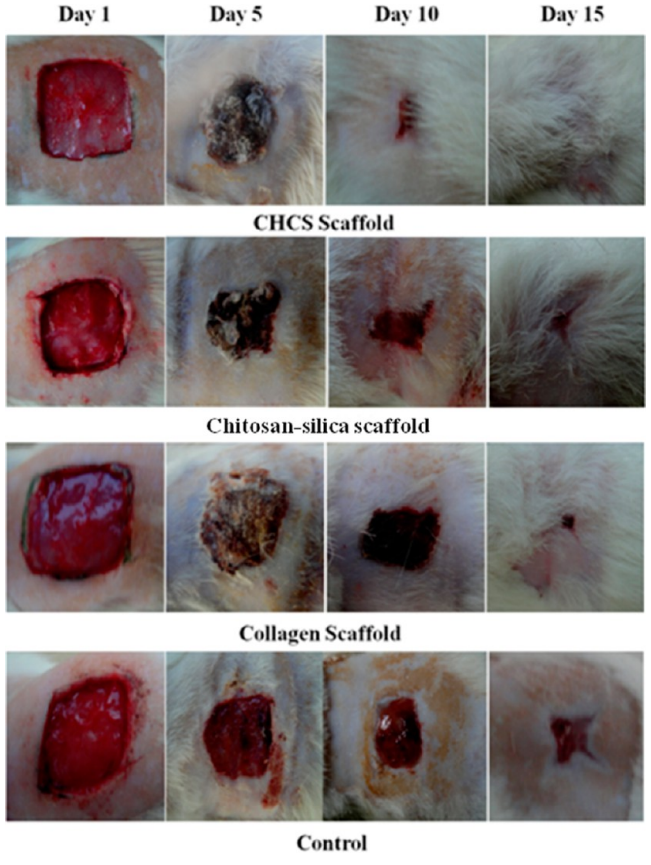


**Figure 8.** Photographs of the CHCS, collagen, and chitosan–silica treated and control (untreated) wounds. Each wound shown here is representative of four rats of that particular group on the given day.

with CHCS showed accelerated wound healing compared to the treatment with collagen scaffold and chitosan–silica scaffold. On day 5, area of the CHCS-treated wound reduced by 46.02 ± 5.58% compared to 34.09 ± 4.34% and 36.69 ± 4.00% for collagen and chitosan–silica treatment, respectively. On day 10, the rate of reepithelialisation increased to 80.99 ± 3.24% for the CHCS group and 70.10 ± 4.99% for chitosan–silica treatment, whereas this was 66.13 ± 5.35% and 55.37 ± 5.23% for collagen and control groups, respectively. Complete wound closure was observed on the 14th day of postwounding for CHCS treatment, whereas this was observed on the 16th and 17th day chitosan–silica and collagen treatment, respectively. There was a statistically significant difference in wound closure between the CHCS group and the collagen, chitosan–silica and control groups throughout the treatment period. These findings suggest that collagen hydrolysate is a better therapeutic choice for treating wounds.

Figures 9 and 10 depict the histopathological changes of wound tissue as a function of time (day 5 and day 15). The

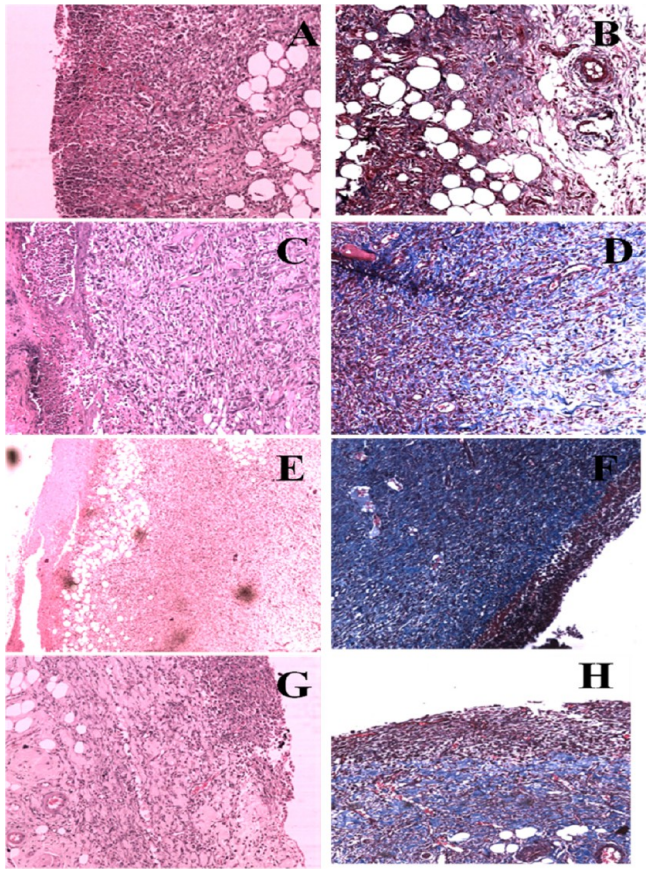


**Figure 9.** Haematoxylin/eosin-stained section of the regenerated skin tissues on day 5: (A) CHCS scaffold, (C) chitosan–silica scaffold, (E) collagen scaffold, and (G) control (no treatment). Masson's trichome-stained section at day 5: (B) CHCS scaffold, (D) chitosan–silica scaffold, (F) collagen scaffold, and (H) control (no treatment). Original magnification is 40× and the scale bar = 100 $\mu$m.

histology scoring for inflammatory cells, collagen deposition and neovascularisation (Figure 11) was performed as treatment blinded assessment on day 5, 10, and 15 postwounding. On day 5, dense inflammatory cells were observed in CHCS, as confirmed by the inflammatory score of 3.66 ± 0.33, whereas moderate inflammatory response was observed in the collagen and chitosan-silica group (3.00 ± 0.00). Inflammatory phase is a necessary prerequisite for normal wound healing,[38] however, factors like infection prolong the inflammatory phase, leads to wound chronicity. Neovascularisation is essential during early stages of healing, as the newly formed blood vessels aid in the oxygen transfer and essential nutrient supply for reepithelialisation. Interestingly, the wounds dressed with CHCS showed significant increase in neovascularisation score compared to the collagen, chitosan-silica and control groups. Therefore, it is evident that CHCS treatment offers a favorable environment during the early phase of healing. On day 10, Masson's trichome section revealed that CHCS resulted in greater

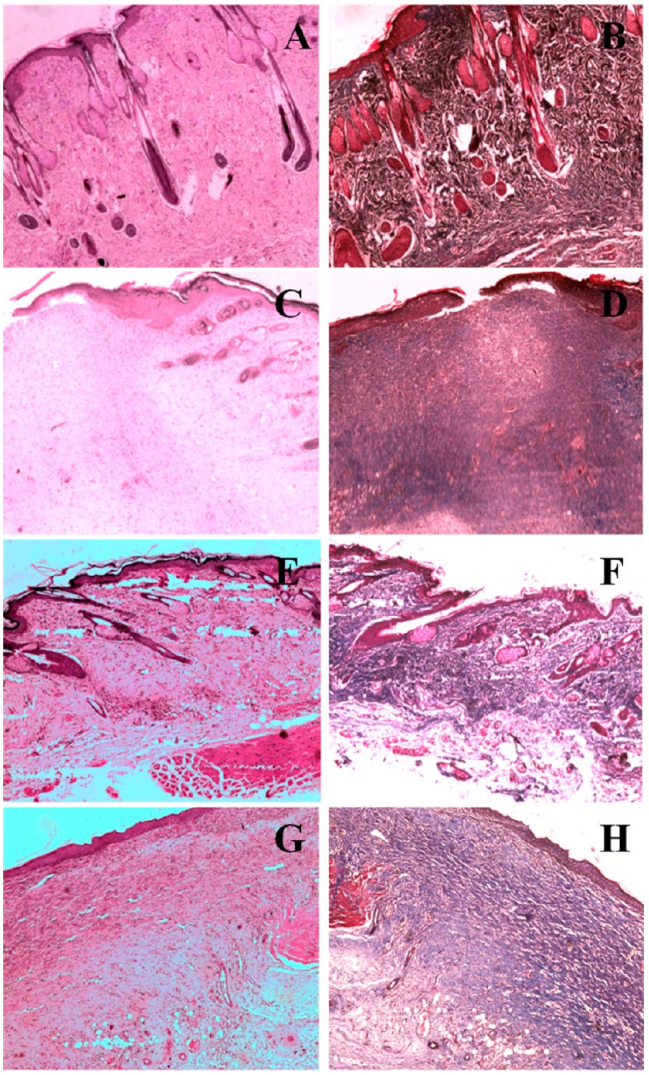


**Figure 10.** Haematoxylin/eosin-stained section of the regenerated skin tissues on day 15. (A) CHCS scaffold, (C) chitosan−silica scaffold, (E) collagen scaffold, and (G) control (no treatment). Masson's trichome-stained section at day 15, (B) CHCS scaffold, (D) chitosan−silica scaffold, (F) collagen scaffold, and (H) control (no treatment). Original magnification is 40× and the scale bar = 100 $\mu$m.

collagen content with compact alignment. Conversely, loose collagen alignment was seen in both collagen and control groups. CHCS-treated wounds showed almost complete absence of inflammatory cells, whereas moderate inflammatory response was observed for other treatment groups (see Figure S2 in Supporting Information). Although inflammation is a necessary part of the normal healing process, a prolonged inflammatory phase prevents the onset of the subsequent proliferative phase. The neovascularization score was higher for CHCS and chitosan−silica (3.66 ± 0.33) compared to collagen and the control groups (3.33 ± 0.33 and 3.00 ± 0.00). Conversely, the inflammatory score is lesser for CHCS (1.33 ± 0.33) compared to collagen, chitosan-silica and control groups (2.33 ± 0.33, 2.66 ± 0.33 and 2.66 ± 0.33). The prominent neovascularization and appropriately timed inflammatory response seen in the CHCS group facilitates faster regeneration of damaged tissue. The complete reepithelialisation with formation of epidermal layer along with focal acanthosis and adenexial structure was observed in the CHCS group on 14th day postwounding. In addition, new hair follicle formation was seen in the CHCS group. Therefore, all these findings lead us to conclude that the topical CHCS dressing could accelerate wound healing and may emerge as a better alternative to collagen dressings for wound therapy.

## 4. DISCUSSION

Collagen is a traditional structural biomaterial; there is an imminent need to engineer such traditional biomaterials for better performance in tissue engineering and regeneration.[39] In this paper, we demonstrate that the fragments from collagen engineered into scaffold results in an effective and affordable alternative to the traditionally used collagen scaffold. Collagen hydrolysate, enzymatic hydrolyzed fragments of collagen, is hypothesized to have better therapeutic value because of the possibility of increasing the therapeutic load and its ability to act as a nutritional supplement. In addition, collagen biomaterials have the issue of sterilization, as thermal stabilization is not possible because of the low thermal stability of collagen, whereas collagen hydrolysate was subjected to temperatures of about 90 °C. Currently, collagen-based sponge dressings are available under different brand names (PROMOGEN PRISMA, Medifil, ColActive) for wound healing therapy. The preparation of such collagen sponges is aided through a simple methodology of homogenization followed by freeze-drying of collagen. The sponge uniformity is mainly attributed to the foam stability attained because of the viscous nature of collagen solution. However, fabricating a spongy scaffold using collagen hydrolysate is challenging due to the low solution viscosity. In order to increase the viscosity of collagen hydrolysate solution, a TEOS-assisted sol−gel methodology was adopted. To provide physical integrity to the scaffold and to facilitate easy handling, chitosan was added to the composite. Both silica and chitosan, the components used in CHCS, have been used in a number of biomedical applications, such as wound dressings, drug delivery, and tissue engineering.[31,40−44]

In skin tissue engineering, material design should account for vital properties such as biocompatibility, biodegradation, porosity, and tissue regeneration capacity. To be effective for chronic wound therapy, a scaffold should neither show rapid degradation nor complete resistance against collagenase treatment. In addition, water uptake property of a scaffold is very essential for absorbing exudates and maintaining a moist environment for tissue debridement.[6,7] CHCS showed excellent adhesion on wound surface and complete absorption of the exudates within a short period of the dressing being applied, thus facilitating its application for wounds with heavy exudates. Porosity and structural stability of the CHCS could be the possible reasons for showing increase in the water uptake ability; as structurally stable porous sponges have the tendency to entrap a higher amount of water in between the porous network. Additionally the presence of silica in CHCS could enhance the ability to hold more water in the scaffold. As a result of good exudate absorption, rehydration of necrotic tissue is facilitated, and hence autolytic debridement of the wound is promoted.[45] In addition, it was noted that the biostability of CHCS scaffold is better than collagen scaffold (Figure 4) because of the presence of silica. This would minimize the frequency of dressing changes for CHCS when compared to a collagen sponge.

The scaffold porosity, pore size distribution, and shape have a prominent influence on cell adhesion, growth, and differentiation.[46] Doillon et al. reported that the mean pore size of

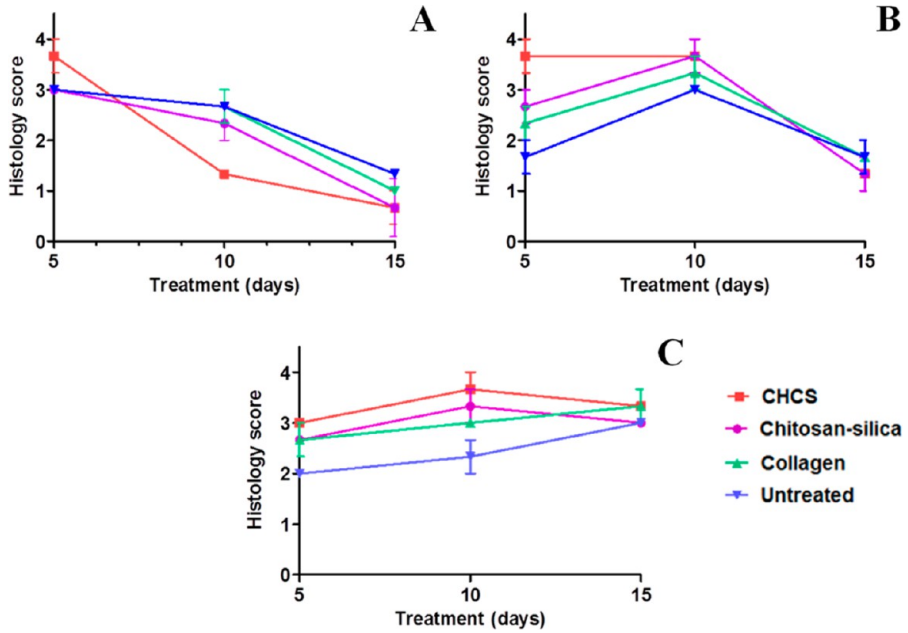


**Figure 11.** Histological evaluation of regenerated skin tissue during treatment at day 5, 10, and 15 using CHCS scaffold, chitosan−silica, collagen scaffold, and control (untreated): (A) inflammation, (B) neovascularization, and (C) collagen deposition.

the scaffold is affected by the viscosity of the material solution.[47] The viscosity of the CHCS material solution is sufficient to produce stable foam after homogenization, which results in porous morphology. In CHCS, the chitosan silica background frame provides a morphology consisting of small and large pores, which facilitate cell proliferation and good oxygen transport, respectively (Figure 2). Proliferation of fibroblasts was better in CHCS than in collagen scaffold (Figure 6), which could be due to the porous morphology of CHCS, the increased therapeutic load of collagen hydrolysate and the fact that collagen hydrolysate can also act as a nutritional supplement. All the above-mentioned properties of CHCS, that is, porous nature of the scaffold, increased water uptake, better biostability and cell proliferation are the outcome of the unique composite material design and the sol−gel methodology.

In vivo efficacy studies showed that the wounds dressed with CHCS healed faster than those dressed with collagen scaffold (Figure 7 and 8). In support of this observation, the neovascularization score during the early phase of healing (day 5) was significantly higher for the CHCS group compared to the collagen, chitosan−silica, and untreated groups. In addition, CHCS treated wounds elicited acute inflammatory response, as noted on day 5 (Figure 11), and it subsided in the subsequent sampling (day 10), which indicates the progress of healing, whereas collagen treated and untreated wounds elicited late inflammatory response and slow healing. Bacterial infection is the major underlying reason that prolongs the inflammatory phase. Consequently, therapeutic strategies to target infection and inflammatory phase are critical in the treatment of infected wounds. CHCS treated wounds had a relatively lower possibility for the onset of infection because of the antibacterial activity of CHCS against common wound pathogens.

## 5. CONCLUSION

A novel collagen hydrolysate composite scaffold was successfully developed using sol−gel transition methodology. The CHCS scaffold presented porous morphology, improved biostability, good water uptake capacity, and excellent biocompatibility. The antimicrobial activity of collagen hydrolysate is a great value addition for CHCS in wound care therapy. These findings suggest that the CHCS scaffold has a potential to be an effective alternative biomaterial for the treatment of chronic wounds. As a future perspective, collagen hydrolysate based biomaterial development may offer novel insights in the field of tissue engineering.

## ■ ASSOCIATED CONTENT

### Supporting Information

TGA curves of collagen, chitosan silica composite scaffold, and collagen hydrolysate composite scaffold and histopathological observation (H&E and Masson's trichome staining) of regenerated skin tissue at day 10. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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