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Der Pharmacia Lettre, 2018, 10 [6]: 37-57
[http://scholarsresearchlibrary.com/archive.html]

ISSN 0975-5071
USA CODEN: DPLEB4

Genipin Initiated Crosslinked Sericin/Chitosan Hydrogels: Accelerated Wound Healing in an Animal Model

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ABSTRACT

Genipin (Gn) initiated crosslinked hydrogel composed of sericin and chitosan was developed and evaluated for its wound healing potential in animal model. The prepared hydrogels were characterized for crosslinking degree, swelling studies, bulk and true density, pore volume, porosity, SEM, FTIR, MS/MS analysis, texture properties and rheological measurements. The hydrogels containing different concentration of Gn showed differences in morphology, swelling ability and physical properties. Crosslinking degree, FTIR studies and MS/MS analysis noticeably indicated the crosslinking of sericin and chitosan in the presence of Gn. The results of in-vivo studies indicated that 0.8G-S/C-H groups endorsed faster wound healing, enhanced reepithelialization and wound closure in albino rats when compared to the control and positive control groups. These results prove that Sericin/Chitosan hydrogels to be promising materials for wound healing.

Keywords: Sericin; Chitosan; Hydrogel; Genipin; Wound healing

INTRODUCTION

Intensive research in the field of biomaterials for wound healing application has led to the development of diverse range of wound dressings. Hydrogel type dressings portray special attention of biomaterial researchers for their inherent potential in wound healing phenomenon [1]. Hydrogels are three-dimensional polymeric networks that can swell significantly without dissolving or losing their structural integrity [2]. Hydrogel type dressings are the biomaterials (natural or synthetic) developed by physical or chemical interaction of polymer chains into network, which provides the ideal environment to accelerate the wound healing process [1,3]. Recent studies have shown that the hydrogel derived from synthetic polymers (polyvinyl alcohol, poly ethylene, poly acrylic acid) have advantage of lower inflammatory response and highly controlled gel properties [3,4]. However, the lack of biocompatibility in hydrogel derived from synthetic polymers leads to negative responses like toxicity and stimulation of chronic inflammatory reactions from the body and low biodegradability make them difficult to degrade in physiological conditions, restricts their use for wound healing application. In contrast, natural polymer based hydrogels possess innate biocompatibility, biodegradability, intrinsic cellular interaction and rich functional groups for easy modification.

Sericin has been successfully studied in wound healing application [4]. It is a cheap, naturally occurring, readily available, biocompatible and biodegradable product, obtained from cocoons of *Bombyx mori* [5]. Sericin comprises of hydrophilic $–NH_2$ group which enable easy crosslinking and blending with other polymers for development of modified hydrogel. Furthermore, several reports have proved that it increases the migration, adhesion, and growth of keratinocytes and fibroblasts and is also responsible for enhancement in collagen production and improved reepithelialization in wounded skin. Despite the excellent wound healing characteristics and crosslinking properties of sericin, its broad molecular weight range, high water solubility and amorphous structure limit its application in the biomedical field [6,7]. To overcome these problem chitosan can be used as material of choice because it is able to aid the development of modified biomaterial with improved mechanical properties for wound healing application, when combined with sericin. Chitosan is a polysaccharide, isolated from deacetylation of chitin [8,9]. The unique characteristics of chitosan such as non-toxicity, biodegradability, biocompatibility and stability make it a favorable material [10]. Additionally, the physico-chemical properties of prepared biomaterial can be easily modified by chitosan which readily blends with other biopolymers [8]. The presence of reactive $–NH_2$ and –OH groups in chitosan makes it most favorable for chemical crosslinking method [11].

Chemical crosslinkers such as aldehydes, carbodiimides and polyepoxy compounds have widely employed for preparation of hydrogels by chemical crosslinking method. However these substances possess the risk of toxicity that limits their uses for preparation of biomaterials [5,6,12].Therefore, many researchers have focused on finding naturally occurring and less toxic,

biocompatible crosslinker such as Genipin (Gn) [13]. It can be selected as a crosslinking agent over the other chemical crosslinkers. Gn is a chemical compound obtained from the fruits of the plant Gardenia jasminoides Ellis. It can be used as a tremendous natural crosslinker due to its extreme responsiveness towards the amino groups enabling efficient crosslinking of chitosan and proteins [14]. At the same time, it can produce crosslinked materials with mechanical and degradative properties as compare to the glutaraldehyde cross-linked materials [15].

The aim of present work was to develop a novel Gn crosslinked hydrogel based on chitosan and sericin for wound healing application. It was expected that bi-natural-polymer (chitosan and sericin) system can improve the physicochemical and biological properties of prepared hydrogel as well as also overcome the disadvantage associated with sericin. Furthermore, structural features and wound healing properties were conducted to investigate the therapeutic effect of chitosan/sericin hydrogel on wound model.

MATERIALS AND METHODS

Materials

Sericin was extracted in our lab from cocoons of *Bombyx mori* in consequence of the earlier described method by Pornanong et al. [16]. Chitosan (HiMedia, Laboratories Pvt. Ltd., Mumbai, India), genipin(Challenge Bioproducts, Taiwan), Trinitrobenzene sulfonic acid (TNBS) reagent (Sigma Aldrich) were used for preparation of the hydrogel. Isopropanol, methanol and deionized water were used in all the experimental procedures. All other chemicals were of analytical grade and were purchased from Himedia, Mumbai and used as obtained.

Preparation of non-crosslinked sericin/chitosan hydrogels

In brief, Sericin (1.5% w/v) and chitosan (1.5% w/v) were separately dispersed in water and acetic acid (1% v/v), respectively. Subsequently, three hydrogel batches were prepared by mixing different weight ratio of sericin and chitosan (2:1, 1:1 and 1:2 respectively) and stirred for 2 h at room temperature as shown in Table 1. The blends were allowed to develop into soft to hard gels and dehydrated with isopropanol for another 24 h. The dehydrated hydrogels were oven dried at 50°C for 2-4 h. All three dehydrated hydrogels were evaluated for swelling ratio in order to select the optimized hydrogel.

Preparation of genipincrosslinkedsericin/chitosan hydrogel

The hydrogel with the weight ratio of 2:1 revealed good swelling ability and high content of sericin. Therefore, this hydrogel was selected for the preparation of Gncrosslinked sericin/chitosan hydrogel (G-S/C-H) with different concentration of Gn (0.5%, 0.8%, 1.0 %w/w). G-S/C-H was prepared by above mentioned method (Table 1). The hydrogel samples containing sericin and chitosan in weight ratio of 2:1 were prepared and aqueous solution of Gn in different concentration (0.5%, 0.8% and 1%w/w) added to them with stirring. The homogenous blends were allowed to stand for 24 h at room temperature. The crosslinked mixture was poured in isopropanol (1:2 w/w) and the mixture was kept at room temperature for 24 h. The isolated hydrogel was washed thrice with methanol to terminate the crosslinking reaction by removing unreacted Gn. Finally the hydrogels were first air dried and then dried in an oven for 2 h at 50°C. Non-crosslinked hydrogel with the same weight ratio of sericin and chitosan (2:1) was also prepared without adding Gn (0G- S/C-H), as described in above section.

Crosslinking degree

The crosslinking degree of G-S/C-H determined the ratio of amino groups reacting with Gn to the unreacted amino groups in non-crosslinked hydrogel. The crosslinking degree was determined by Trinitrobenzene sulfonic acid (TNBS) assay using UV-Vis spectrophotometer [6,17]. 1 ml of TNBS (0.5%w/v) solution and 1 ml sodium hydrogen carbonate (4% w/v, pH 8.5) was added to the weight quantity of samples. The solution was heated at 40°C for 2 h using water bath and again heated at 70°C for another 2 h after the addition of 12.24 N HCl (2 ml). The optical absorbance of the solution was determined at 415 nm with suitable dilution using UV-Vis Spectrophotometer (Labtronics LT-2910). The crosslinking degree of the samples was calculated by using Eq. 1.

Degree of crosslinking (%) =1-Ach/Ach × 100---------Eq. 1

Where Ach is the absorbance of cross-linked hydrogel and Ach is the absorbance of non-cross-linked hydrogel. The experiment was performed in triplicate and values expressed as mean ± standard deviation.

Bulk density

Bulk density of the hydrogel was determined using dried sample of hydrogels. The dried samples were then filled upto mark (10 ml) in a volumetric flask and weighed. The bulk density of hydrogel was calculated using Eq. 2.

$B_d = W_t – W_f/10$……Eq. 2

Where W_f is the weight of flask and W_t is the total weight of hydrogel as well as flask.

True density

True density of the crosslinked and non-crosslinked hydrogels was determined by drying the sample up to constant weight (W_0). 0.35 gm of dried sample was placed into volumetric flasks (10 ml) of known weight at 20°C. Then, the flask was filled with 8 ml of cyclohexane and the mixture kept at 20°C for 24 h. Flask was then filled with cyclohexane up to the 10 ml mark and weighted (W). The true density (T_d) of crosslinked and non-crosslinked blends was calculated using Eq. 3.

$$T_d = W_0 [10 – (W - W_0)/d_c] \ldots\ldots \text{Eq. 3}$$

where W is the total weight of blends with the solvent, W_0 is the weight of the dry blends and d_c is the density of solvent (0.778 g/ml)

Pore volume and porosity

The weight gain in crosslinked and non-crosslinked hydrogels is taken as the measure of pore volume of the respective hydrogels. Both hydrogels (crosslinked and non-crosslinked) were dried and placed into porous glass bottom tubes. The tubes were placed inside a flask filled with cyclohexane and allowed to stand for 48 h at 20°C. Further, samples were centrifuges at 1500 rpm for 1 min to remove excess of cyclohexane. The porosity of the formulations was calculated from Eq. 4.

$$\text{Porosity} = V_p/V_t] \ldots\ldots \text{Eq. 4}$$

Where V_p is the pore volume of hydrogel and V_t is the true volume of hydrogel.

$$V_t = W_0/T_d] \ldots\ldots \text{Eq. 5}$$

where W_0 is the weight of dried hydrogel and T_d is the true densities of dried hydrogel, as true volume (V_t) determined according to the Eq. 5.

Syneresis index

Syneresis index is defined as the amount of water discharged from the hydrogel with time. Syneresis index was determined by a reported method [18,19]. Dried hydrogels were weighed (W_I) and kept into water for 2 h at 37°C ± 1°C. After 2h the

formulations were again weighed (W_f) and the difference between final and initial weight after 2 h, revealed the water holding capacity of each hydrogel.

Swelling ratio and moisture loss studies

The swelling ratio of the prepared hydrogel is an important parameter to investigate fluid absorbing property in order to maintain moist environment over wound bed. The dried samples (1 g of each, W_1) were soaked in 100 ml of PBS (pH 7.4) at 37°C ± 1°C. After the predefined time interval, the wet samples were blotted off with tissue paper to remove excess of liquid and then weighed (W_2). The swelling ratio was calculated using Eq. 6. All the experiments were performed in triplicate and obtained results reported as mean ± standard deviation.

$$\text{Swelling ratio (\%)} = W_2\text{-}W_1\ 100/W_1 \ldots\ldots \text{Eq. 6}$$

Fourier transform infrared spectroscopy (FTIR)

FTIR of the sericin, chitosan, 0G-S/C-H and G-S/C-H were determined by Shimadzu FTIR spectrophotometer. The KBr pellet technique was used for analysis, in the range of 4000-400$cm^{-1}$and resolution of 4 cm^{-1}.

Morphology

The morphology of the non crosslinked and G-S/C-H was observed through scanning electron microscopy (SEM, Jeol Model JSM 6400, Tokyo). The hydrogels were dried in a lyophilizer (Christ Alpha 1-4 lyophilizator, Osterodo, Germany). The coating of dried hydrogel samples was done via gold splutter coater under an argon atmosphere. The coated samples were mounted on stub and observed. Finally, images were recorded for morphological characterization.

MS/MS analysis

The Waters UPLCTQD mass spectrometer with negative mode ESIMS was used to determine whether Gn is involved physically or chemically in crosslinking reaction[16].The parameters used during analysis were capillary voltage 2700 V, sample cone voltage: 30.0 V, extraction cone voltage: 0.5 V, source temperature: 80°C, desolvation temperature 150°C, flow rate 0.5 µl/min, ion energy 2.0 V, collision energy 7.0 V.

Pure form of Gn was dissolved in 5 ml methanol (HPLC grade) and subjected to MS analysis. 0.8G-S/C-H was hydrolyzed in methanolic HCl (60°C for 1 h) and supernatant obtained after centrifugation was evaporated to dryness. The residue was further dissolved in methanol (5 ml, HPLC grade) and subjected to MS analyses,

The another samples were prepared by heating 0.8G-S/C-H in methanol (60°C for 1 h) and supernatant methanol obtained after centrifugation was evaporated to dryness. The residue was redissolved in methanol (5 ml, HPLC grade) and subjected to MS analyses.

Rheological measurements

The rheological behavior of optimized 0.8G-S/C-H and 0G-S/C-H was assessed using RHEOPLUS/32 V3.40 (Rheometer) through software as per reported method [20,21].

Texture Analysis

Texture properties such as hardness, adhesiveness, spreadability and extrudability of the 0G-S/C-H and 0.8G-S/C-H were investigated using advanced instrument CT3 Texture Analyzer (Brookfield Engineering Laboratories, USA) [22]. The hardness and adhesiveness is a force required to deform the hydrogel (hardness) and to break the attractive force between any surface and formulation (adhesiveness). Hardness and adhesiveness of the hydrogels were studied by TA 10 probe and fixture TA-BT-KI. Approximately, 30gm of hydrogel was filled in beaker with care in order to prevent bubble formation. The cylindrical probe (TA 10) was allowed to compress the sample at a predefined test speed (1mm/sec) to a depth of 30mm with trigger load of 4 g to complete one cycle of experiment. On the other hand, spreadibility and extrudability of hydrogels was measured using male and female cone type probe and TA-DEC probe. The experiment was performed to compare the textural properties of0G-S/C-H and 0.8G-S/C-H.

In-vivo wound healing activity

The wound healing activity of control group, positive control group and 0.8G-S/C-H group was performed on rat model. Animal study was conducted after obtaining the approval of Institutional Animal Ethical Committee of SDCOP&VS (Registration number SDCOP&VS/AH/CPCSEA/01/00260). Adult, healthy and pathogen free Wistar rats of either sex with body weight around 150-200 g were selected for the *in-vivo* wound healing activity. Plastic cages with clean bedding were used to keep the animals and they had free access to standard diet and water *ad libitum*. The acclimatization of rats was done for a week prior to

the study in controlled condition of temperature (25 °C) and humidity (50% ± 5%) with 12 hour light/dark cycle. The rats were anaesthetized individually with suitable dose of diethyl ether, the dorsal area was shaved and disinfected with 70% alcohol. The epidermal and dermal layer was removed using scalpel blade to make a full thickness excision wound of approximately 1 cm on the dorsal side. The rats were divided in three groups (n=6) with appropriate identification. Animals of group 1 (control) were left undressed to serve as control group, while the animals of group 2 (positive control) and group 3 (0.8G-S/C-H) were treated with Cipladine ointment, Cipla Ltd. India and optimized 0.8G-S/C-H respectively, for 14 days after cleaning with alcohol. The wound healing progress was determined by measuring contraction at the wound area at 1, 7 and 14 days. At the same time digital photographs of the wounds were taken. Wound closure (WC %) was calculated using the formula:

$$WC\ (\%) = WA_0 – WA_1/WA_0 \times 100$$

where WA_0 is the original wound area and WA_1 is the open area of wound at day 1, 7 and 14 during replacement of dressing [23].

Histology

The wound tissues of rats in group-1, group-2 and group-3 were excised and fixed in a 10% formaldehyde solution, processed and then embedded in paraffin. Sections were made perpendicular to the surface of wound. Afterwards, tissue sections were dewaxed, rehydrated and finally stained with hematoxylin and eosin (H&E) dye. The sections were analyzed using light microscope and photographed (Nikon Microhot FXA, Japan).

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analysis was carried out using (GraphPad Prism-5.01, Software, Inc).Significant statistical differences between the results were analyzed using Student's t-test. A difference of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and optimization of hydrogels

In this investigation firstly, non-crosslinked hydrogels with different weight ratio of sericin and chitosan (2:1, 1:1 and 1:2) were prepared and optimized on the basis of swelling ratio. The swelling ability of hydrogel with high chitosan content (1:2 w/w) was

found to be highest as compared to hydrogel ratio of 1:1 and 2:1 w/w (data not shown). However, we selected the weight ratio of sericin and chitosan (2:1) for the Gn induced crosslinking reaction due to its good swelling property and high sericin content which may accelerate wound healing process [15,23,24].The high swelling ability of this particular hydrogel may be due the hydrophilic character of sericin and chitosan.

Table 1: Compositions of non-crosslinked hydrogel and crosslinked hydrogel.

Non-crosslinked Hydrogel (0G-S/C-H)		**Gn Crosslinked Hydrogel (G-S/C-H)**		
S.No	**Sericin:Chitosan (%wt.)**	**Formulation Code**	**Sericin:Chitosan (%wt.)**	**Gn (% w/w)**
1	2:1	0G-S/C-H	2:1	0
2	1:1	0.5G-S/C-H	2:1	0.5
3	1:2	0.8G-S/C-H	2:1	0.8
Note: Sericin solution: 1.5 %w/v and chitosan solution: 1.5 %w/v				

Gn, a natural and non-toxic crosslinker were used to prepare crosslinked hydrogel with weight ratio of sericin and chitosan (1:2) followed by chemical crosslinking method. Chemical crosslinking is very simple and adaptable method to control physicochemical properties of hydrogel [25]. Figures 1a–1c illustrates apparent change of yellowish colored non-crosslinked hydrogel to dark bluish colored Gn crosslinked hydrogel. Bluish color of hydrogel attributed to the reaction of Gn with amino groups on sericin and chitosan. Bluish coloration is associated with the oxygen radical-induced polymerization of Gn and its reaction with amino groups of sericin and chitosan, as shown in Figure 1 (d). Therefore, it can be assumed that the Gn initiated crosslinking with chitosan and sericin chains produced intra- and intermolecular crosslinks to produce stable hydrogels.

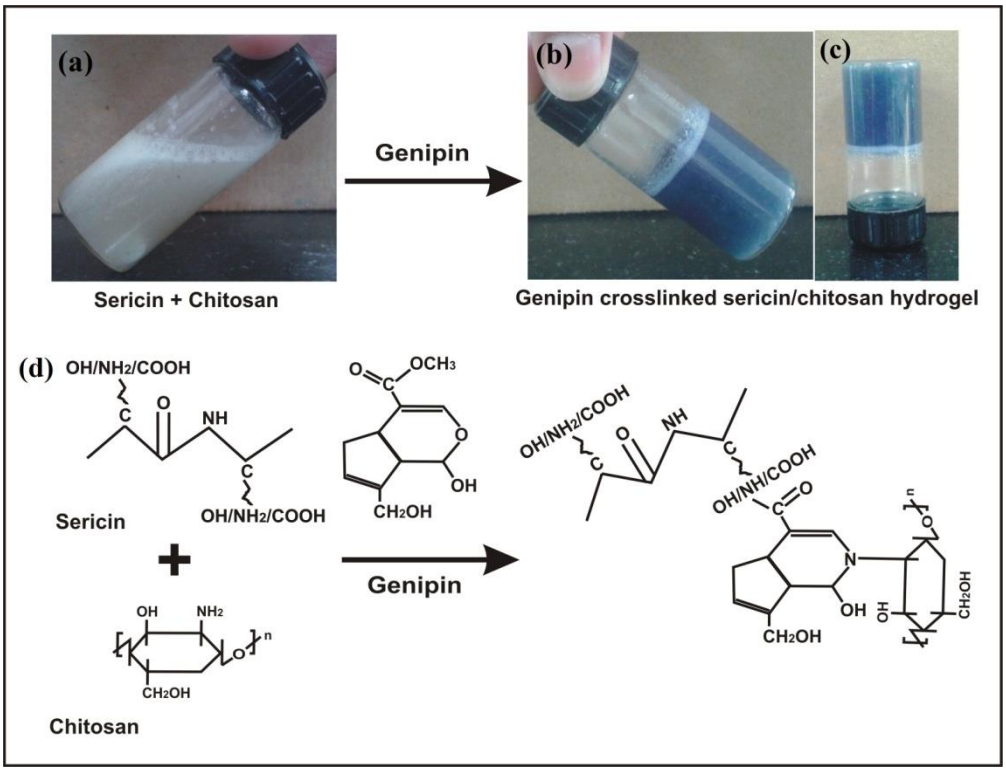

Figure 1: Preparation of sericin/chitosan hydrogel (a) before crosslinking, (b) (c) after crosslinking with Gn (d) schematic representation of synthesis of G-S/C-H

Crosslinking degree

The crosslinking degree of G-S/C-H is presented in Table 2. It is well known that the Gn is a nontoxic and preferred crosslinker for various polymers such as sericin, chitosan, gelatin, agar, kappa-carrageenan[6,8,18].The crosslinking degree of hydrogel crosslinked with Gn (0.5, 0.8, and 1%w/w) was found to be 21.1 ± 2.3%, 28 ± 1.9% and 50 ± 2.2%, respectively. The obtained data demonstrated that crosslinking degree increased with increasing concentration of Gn. Considerable difference in crosslinking degree at Gn concentration (0.8% and 1%w/w) was observed when compared with hydrogel crosslinked with 0.5%w/wGn. Moreover, as the concentration of Gn increased beyond 1%w/w, the higher crosslinking degree was observed but it compromised the physicochemical properties of hydrogel such as spreadibility. Hence, a concentration of Gn higher than 1%w/wwas not selected for crosslinking. Several research reports suggested that Gn can crosslink intra and inter-molecularly with proteins and polysaccharides. This may be attributed to number of steps involved in the nucleophillic attack of primary amine on the C_3 carbon of the Gn and dihydropyran ring opening resulting in crosslinking by radical reaction induced

dimerization process[12,26]. The appearance of blue colour as shown in Figure 1 again supports the crosslinking of Gn with the amine groups of sericin and chitosan.

Physical characterization

Table 2 demonstrates the effect of Gn concentration on physicochemical characteristic of the 0G-S/C-H and G-S/C-H. Bulk density and true density of 0G-S/C-H was found to be lower than that of crosslinked hydrogels. It has been found that as the concentration of Gn increased from 0.5% w/w to 1% w/w, bulk density increased from 0.393 ± 0.89 to 0.450 ± 1.0 g/ml and true density from 0.0220 ± 0.93 to 0.0341 ± 0.67 g/ml for the hydrogels. This may be attributed to the formation of more cross-linking bridges by Gn induced crosslinking reaction leading to denser hydrogel formation. Contradictory results were obtained for the pore volume, porosity and syneresis index when concentration of Gn increased. The higher concentration of Gn increases crosslinking degree of hydrogel leading to decrease in pore volume and porosity of hydrogel.

Table 2: Physicochemical properties of the non-crosslinked and G-S/C hydrogels.

Physicochemical Properties	**Formulations**			
	0G-S/C-H	0.5G-S/C-H	0.8G-S/C-H	1.0G-S/C-H
Crosslinking Degree (%)	-	21.1 ± 2.3	28 ± 1.9	50 ± 2.2
Bulk Density (g/ml)	0.355 ± 0.98	0.393 ± 0.89	0.395 ± 0.88	0.450 ± 1.0
True Density (g/ml)	0.0339 ± 0.11	0.0220 ± 0.93	0.0240 ± 0.99	0.0341 ± 0.67
Pore Volume (g/ml)	0.402 ± 0.76	0.325 ± 0.71	0.325 ± 0.62	0.300 ± 0.83
Porosity (Θ)	1.787 ± 0.87	1.444 ± 0.63	1.444 ± 0.77	1.333 ± 0.22
Syneresis index (%)	93.2 ± 0.82	69.6 ± 0.65	69.1 ± 0.69	51.9 ± 0.79

Swelling ratio studies

The swelling ratio of the hydrogels plays an important role in wound healing process. The swelling ratio of hydrogels with or without Gn is shown in Figure 2a. A higher swelling capability of the efficient wound dressing indicates that material will allow maximum absorption of fluids and exudates from the wound. Figure 2a demonstrates the effect of Gn concentration on the

swelling ratio in PBS pH 7.4 after soaking period of 24h. The swelling ratio decreased from 338 ± 11 to 180 ± 24 with increasing concentration of Gn from 0.5% to 1.0% w/w. With increase in concentration of Gn, higher cross-link density would take place, which furtherled to expansion of hydrogel network structure followed by reduced free spaces within the hydrogel resulting in the lowering of the swelling ratio[6,27, 28].The highest swelling ratio was attributed to the presence of hydrophilic ingredients i.e. sericin and chitosan in hydrogel with higher water absorption capabilities of polar groups –COOH, –OH, $–NH_2$ exists in sericin and chitosan. When these polar groups are crosslinked via Gn then hydrogel losses its water absorption properties further reducing its swelling ability [24]. The swelling ratio increased upto 770 ± 13.1% at 10 h of soaking period but beyond 10 h there was decrease in swelling ratio of non-crosslinked hydrogel. The swelling ratio of non crosslinked hydrogel displayed faster and maximum swelling properties but lacked the stable hydrogel structure.

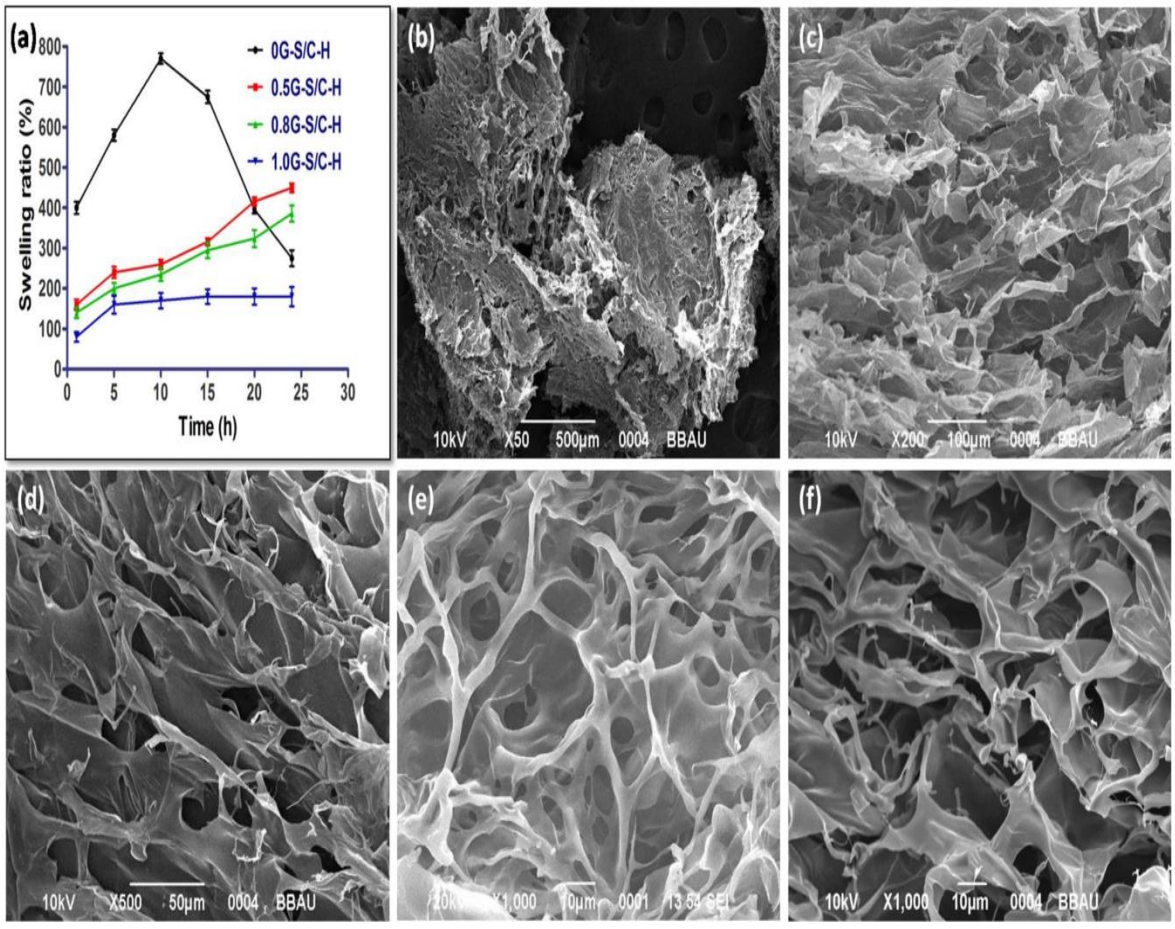

Figure 2: (a) Swelling ratio of S/C-H crosslinked via different Gn concentration over a period of 24 h. The cross-section SEM images of hydrogel (b) 0G-S/C-H at 50X (c) 0G-S/C-H at 200X (d) 0.5G-S/C-H (e) 0.8G-S/C-H, and (f) 1.0G-S/C-H

Morphology of hydrogels

Morphology is an essential parameter for wound dressing. It must have porous structure to absorb exudates from wounds. The SEM images of S/C-H before crosslinking (0G-S/C-H) and after crosslinking (0.5G-S/C-H – 1.0G-S/C-H) were studied to provide insight into the effect of Gn concentration on their internal microstructure of hydrogel as shown in Figure 2b-2f. Significant change was observed in SEM images of non-cross-linked and cross-linked hydrogel. Figure 2b and 2c shows the microstructural appearance and cross section image of 0G-S/C-H. The hydrogel before the crosslinking (Figure 2c) displayed poorly interconnected pores, heterogeneous pore distribution and the lack of networking attributed to absence of crosslinking among sericin and chitosan. On the other hand, SEM images of crosslinked hydrogel with different concentration of Gn (Figure 2d-2f) revealed characteristic structure consisting of interpenetrating mesh with clear visible pores and uniform pore distribution. As the concentration of Gn increased from 0.5 to 1.0%w/w, size of pores decreased. Based on physical properties, degree of crosslinking, swelling ratio and morphological character 0.8G-S/C-H was selected for mass analysis, rheology, texture analysis and *in-vivo* wound healing activity [3,4,6,12,25].

FTIR

The FTIR spectrum of 0G-S/C-H and 0.8G-S/C-H is depicted in Figure 3(a-d), along with the characteristic spectra of all the ingredients individually (sericin and chitosan) as reported in literature [26,27]. FTIR spectra of sericin (Figure 3a) showed typical adsorption band at 1650 cm^{-1}, 1524 cm^{-1} and 1240 cm^{-1} due to the amine I, II and III, respectively. The characteristic band at 3425 cm^{-1} corresponding to N–H stretching vibrations of the sericin was observed in Figure 3a. FT-IR spectra of chitosan was shown in Figure 3b, and chitosan exhibited characteristics band at 3445 cm^{-1} attributed to overlapping of amine and hydroxyl group and the amine and amide band at around 1643(cm^{-1})and 1458 cm^{-1}, respectively. The infrared spectrum of 0G-S/C-H (Figure 3c) represented all the characteristic bands of sericin and chitosan. Distinct difference was observed in FTIR spectra of0.8G-S/C-H when compared with spectra of sericin and chitosan. The shifting of amide I and amide II band to 1640 cm^{-1} and 1510 cm^{-1} indicated an interaction between sericin and chitosan via Gn [4,8,9,25].

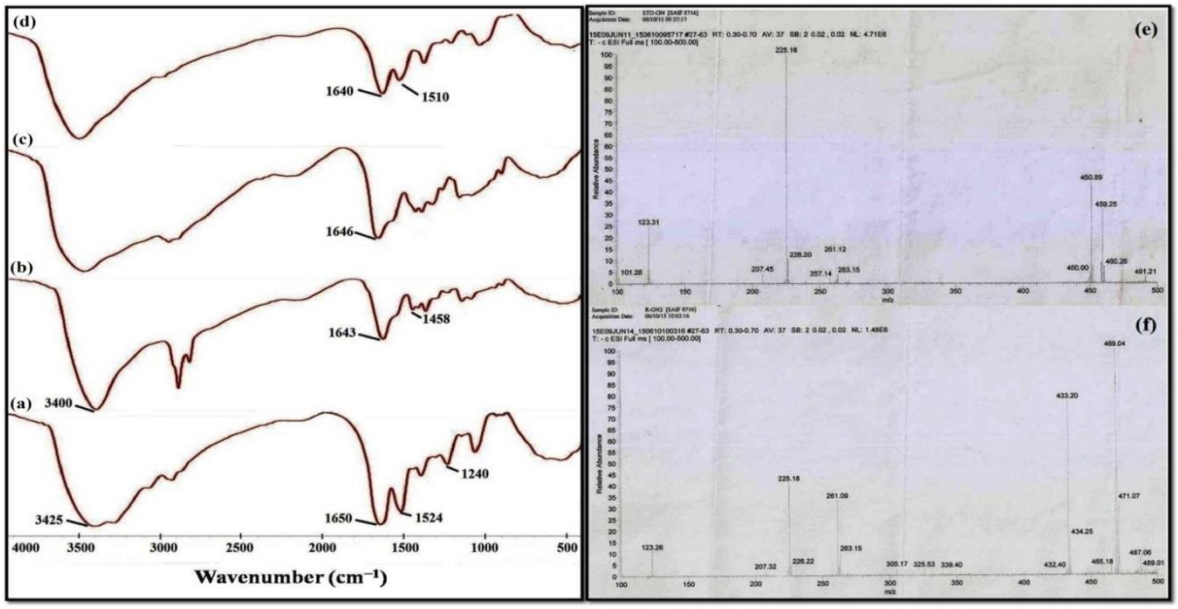

Figure 3: FTIR spectra of (a) sericin, (b) chitosan, (c) 0G-S/C-H, (d) 0.8G-S/C-H and MS/MS spectrum of (e) standard genipin and (f) recovered genipin after methanolic HCl degradation of 0.8G-S/C-H

MS/MS analysis

The mass spectra of standard Gn and methanolic HCl degraded hydrogel residue repectively, have been presented in Figure 3(e) and 3(f). The mass spectrum of standard Gn showed: *m/z* (% abundance) 225 (M^+ - 1, 100%), 207 (5%), 123 (25%), 101(3%). The mass spectrum of methanolic HCl degraded hydrogel residue obtained from crosslinked hydrogel produced the major ion fragmentation with *m/z* value of 225 (40%), 207 (4%), 123 (11%) with other contamination peaks. On the other hand, the mass spectrum of methanol extracted Gn did not show major peaks at *m/z* 207, 101 which was observed in mass spectrum of standard Gn. These results of MS analysis indicate that harsh treatment (methanolic HCl and 60 °C temperature) facilitate the breakdown of chemically crosslinked network through hydrolysis, which can be detected by mass analysis. Furthermore, Gn is soluble in methanol but on treatment with HPLC grade methanol and 60°C temperature, no major peak of Gn was found, which further supported that Gn is chemically involved in crosslinking reaction between amine groups of sericin and chitosan as supported previously reported results [19,29].

Rheological measurements

Rheology is an important parameter to investigate the structural property as it influences the texture, flow behavior, appearance and physical form of 0G-S/C-H and 0.8G-S/C-H. The viscosity curve Figure 4a, revealed increase in viscosity at low shear rate and remained constant at higher shear rates in both hydrogels. 0.8G-S/C-H showed increased viscosity as compared to 0G-S/C-H. The results indicated that the viscosity was dependent on crosslinked hydrogel density. Crosslinking of sericin and chitosan blends which resulted in hydrogel formation with gel like consistency (Figure 1b) increased the viscosity. Furthermore, the flow curves of G-S/C-H and 0.8G-S/C-H (Figure 4a) confirmed linear relationship between the shear stress and shear rate, which indicate the Newtonian flow behavior [21,29,30].

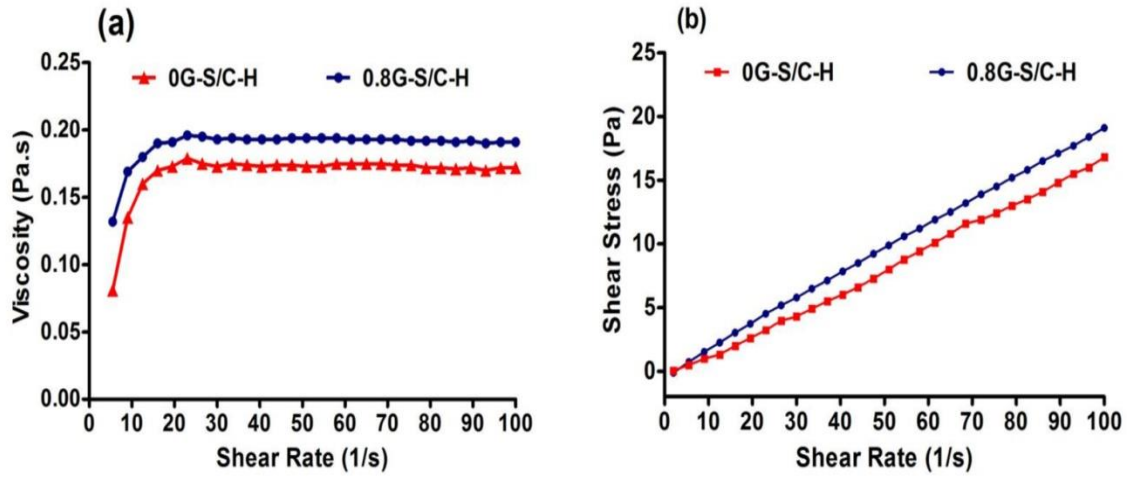

Figure 4: (a) Viscosity curve and (b) flow curve of 0G-S/C-H and 0.8G-S/C-H

Texture Analysis

Texture analysis is an advanced technique which is mainly concerned with measurement of the mechanical properties of semisolid products as they relate to its sensory properties. The texture properties of 0G-S/C-H were compared with 0.8G-S/C-H as represented in Table 3. The hardness of the 0.8G-S/C-H was found to be more (241 ± 1.65) than 0G-S/C-H (48 ± 2.19)attributed to the dense hydrogel network structure formed after crosslinking with Gn, which required high positive force to deform the hydrogel structure. Technically, spreadability and extrudability is the work done (mJ) required for spreading the formulation over a surface and to easily expel out formulation from its container. It has been observed that spreadability and extrudability of 0.8G-S/C-H (6.7 ± 3.84 mJ and 1220 ± 2.23 mJ) was found be higher than 0G-S/C-H (3.8 ± 1.99 mJ and 134 ±

1.50 mJ). The increase in spreadability and extrudability observed due to the Gn induced crosslinking by formation of bridges between sericin and chitosan resulted in strengthening the hydrogel system. Adhesiveness is the maximum force required to break the attractive force between any surface and formulation. It is a very important parameter to consider because a hydrogel with appropriate adhesive property is required for healing a wound, without the loss of tissue at wound site on removal of wound dressing. The adhesiveness of 0.8G-S/C-H was found to be 48 ± 1.34gm while of 0G-S/C-H was 43 ± 3.78gm, respectively. Furthermore, adhesive properties were not much affected by crosslinking as small difference in adhesiveness of non-crosslinked and cross-linked hydrogels was observed. The results of textural studies showed that addition of Gn in sericin and chitosan blend highly influenced their textural properties. Therefore, textural properties can be controlled by modulating the crosslinker amount and anaesthetic product can be prepared with improved patient compliance [20].

Table 3: Texture profile of S/C-SNPs hydrogels and marketed formulation

Formulations Code	**Texture Analysis Parameters**			
	Hardness (gm)	**Spreadability (mJ)**	**Extrudability (mJ)**	**Adhesiveness (gm)**
0G-S/C-H	48 ± 2.19	3.8 ± 1.99	134 ± 1.50	43 ± 3.78
0.8G-S/C-H	241 ± 1.65	6.7 ± 3.84	1220 ± 2.23	48 ± 1.34

In vivo wound healing activity

Figure 5 demonstrated the progress of wound healing induced by 0.8G-S/C-H (test treated), positive control treatment (Cipladine ointment, Cipla Ltd. India) and no treatment (control) in a rat wound model experiment. Macroscopic observations of wound treated with by 0.8G-S/C-H showed noticeable dryness without any discharge of pathological fluid from the wounds. The combined effect of wound contraction and re-epithelialization was observed in term of wound closure in each group as a percentage of the reduction in wounded area at day 1, 7, and 14 (Figure 5). On day 1, wounds showed inflammatory phase (redness) and this phase lasted for approximately 3 days. As shown in Table 4. 0.8G-S/C-H treated animals(33.3 ± 2.49% at day 7; 73.6 ± 3.22% at day 14) showed better wound closure than positive control group (25 ± 2.5% at day 7,$p<0.05$;43.7 ± 3.13% at day 14,$p<0.05$) and control group (21.4 ± 3.19% at day 7,$p<0.05$; 33.3 ± 3.17% at day 14, $p<0.05$) at day 7 and day 14,

respectively. At day 7 and day 14, wound contractions in 0.8G-S/C-H group were significantly higher than positive control and control group ($p<0.05$).Moreover, hydrogel dressings were easily removed from the surface of wound without any adherence.

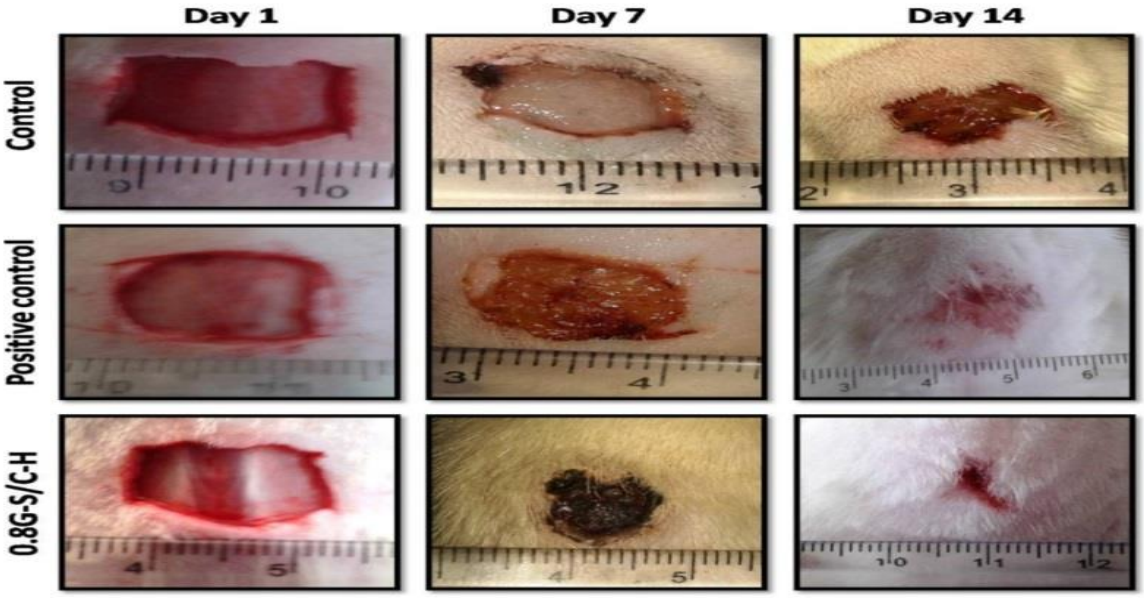

Figure 5: Photographs of the wound on days 1, 7 and 14 after treatment with 0.8G-S/C-H (test treated), positive control treatment and no treatment (control)

Table 4: Percentage wound contraction with 0.8G-S/C-H (test treated), positive control treatment and no treatment (control) on 1, 7 and 14 day

Days	Control	Positive control	0.8G-S/C-H
1	0 ± 0.0	0 ± 0.0	0 ± 0.0
7	21.4 ± 3.19	25.0 ± 2.5	33.30 ± 2.49*#
14	33.3 ± 3.17	43.7 ± 3.13	73.60 ± 3.22*#

Data shown as mean ± SD, * p value < 0.05 on a 2-tailed unpaired t test was considered statistically significant when compared with positive control, # p value < 0.05 on a 2-tailed unpaired t test was considered statistically significant when compared with control group

Histology

The healing phenomenon in wounds of control, positive control and test groups (Figure 6) were studied by histological examination at day 1, 7 and 14, respectively. At day 1, the macroscopic observation of all three groups showed that there was an ulceration area on epidermal layer which was covered with many inflammatory cells (neutrophils). These neutrophils and smaller number of other inflammatory cells (lymphocytes and macrophages) were observed in a thick layer of necrotic tissue to dispose the pathogens entrapped in the clot at the time of injury.At day 7, the epidermal layer of positive control and control treated group showed scab formation and dermal and subcutaneous region of skin revealed replacement of granulation tissues. On the other hand, in the 0.8G-S/C-H treated group, scabs reduced faster than other groups and high degree of epithelialization (thickening process of new epidermis) from the margins of the wound sites to the centers was observed. Dermal and subcutaneous region of skin revealed the existence of granulation tissue, fibroblasts, newly formed blood vessels, as evident in Figure 6.

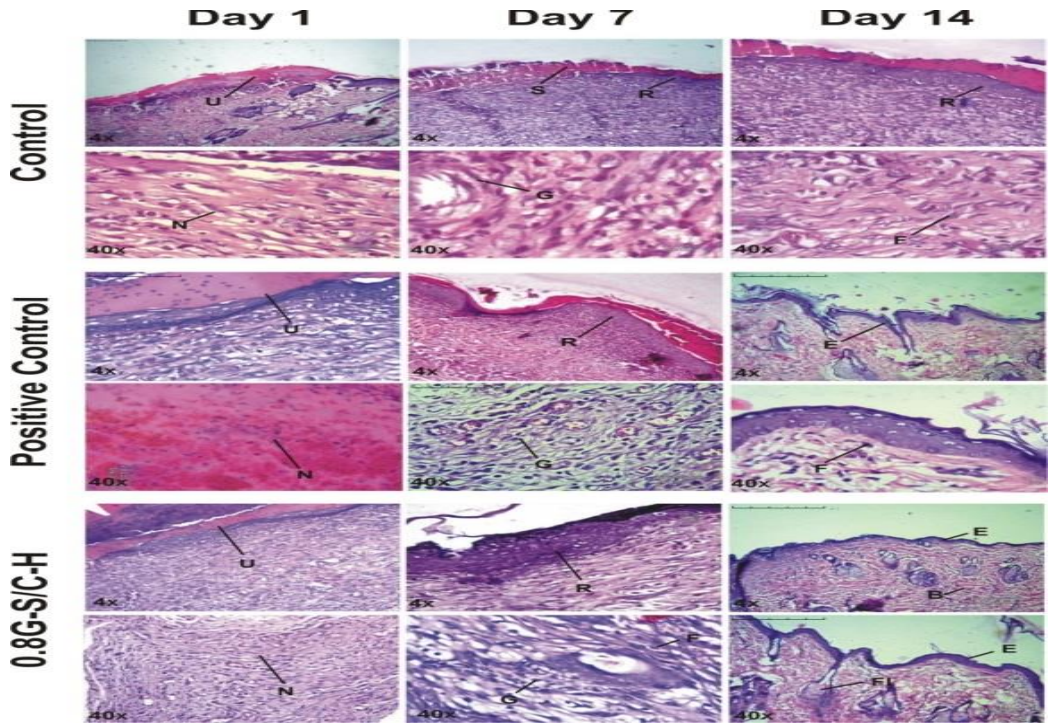

Figure 6: Histology of the wound healing process in various groups on days 1, 7 and 14 with H&E staining at magnification of 4X and 40X. Lines indicate wound healing events. B, blood vessels; E, epidermis; F, fibroblast cells; FI, hair follicle; G, granulation tissue; N, neutrophils; R, re-epithelialization; S, Scab; and U, ulceration

At day 14, highest degree of re-epithelialization, capillaries and vessels formation in the 0.8G-S/C-H group with well-developed granulation tissue and fibroblast, confirmed the rapid healing of wounds. No such changes were observed in control and positive control groups, indicating a slow healing process. Our results suggested that hydrogel composed of sericin and chitosan had synergistic effect on wound healing process. Sericin is a unique protein which promotes collagen formation in wound and thereby accelerates the process of re-epithelialization. The wound healing process and keratinocyte migration associated with the signaling events such as MEK1, PI3K, and JNK leads to c-Jun activation. The antibacterial activity, moisturizing action, and enhanced oxygen permeability are favorable features of sericin for rapid healing of wounds [30,31].

CONCLUSION

In the present study, bi-natural-polymer system comprising of sericin and chitosan, was successfully prepared by chemical crosslinking reaction. Our investigation focused on the use of natural biomaterials with natural and non-toxic crosslinker for the preparation of Gn crosslinked sericin/chitosan hydrogel wound dressings. Furthermore, addition of chitosan in the hydrogel system addresses the problem of wide molecular weight range, high water solubility and amorphous nature of sericin which limit its application in the biomedical field. The effect of Gn concentration on physical properties of prepared hydrogels was studied. 0.8G-S/C-H with high swelling ratio, homogenous porous structure, optimum viscosity and good textural properties, accelerated wound healing process at a faster rate than control and positive control groups. Thus, the current hydrogel dressing of sericin and chitosan crosslinked with Gn shows promising results and offers a potentially valuable, attractive and cost-effective approach for wound treatment with better patient compliance.

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