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Supporting Information

Nanofibrous artificial skin substitute composed of mPEG-PCL grafted gelatin / hyaluronan / chondroitin sulfate / sericin for 2nd degree burn care: *in vitro* and *in vivo* study

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Materials. Ethyl acetate and glacial acetic acid were obtained from Merck, Germany. Hyaluronan, Type A gelatin (Bloom Number: ~300), glutaraldehyde, mitomycin C, chondroitin-4-sulfate (CS), sericin and Bovine serum albumin (BSA) were bought from Sigma Aldrich, USA.

Synthesis of Bio-Syn polymer. Bio-Syn hybrid polymer was prepared in three steps syntheis as described in Figure 1. Firstly, Methoxypolyethyleneglycol-Polycaprolactone (mPEG-PCL di block copolymer) was synthesized via ring opening polymerization of ε-caprolactone (Sigma Aldrich, USA). In the next stage, mPEG-PCL-OH was modifed to, mPEG-PCL-O-CO-CH=CH_2 and in the third step, Michael addition reaction[1, 2] was performed to graft mPEG-PCL di block copolymer to gelatin polymer.

Synthesis of mPEG-PCL-OH. mPEG-PCL-OH was synthesized via ring opening polymerization of ε-caprolactone by using mPEG as initiator and Tin (II) 2-ethylhexanoate (Sigma Aldrich, USA) as a catalyst. Briefly, weighed amount of mPEG (Mn:-2000, 2 gm, 1 mM, Sigma Aldrich, USA) was heated in schlenk flask for azeotropic distillation to remove traces of moisture. Afterwards, 10 ml of toluene (Merck), 4.87 ml (40 mM) of ε-caprolactone (Sigma Aldrich, USA) and 40 µl (0.12 mM) of Tin (II) 2-ethylhexanoate (Sigma Aldrich, USA) was added into the mixture in a N_2 atmosphere . The mixture was continuously stirred for 24 h under N_2 atmosphere at 110 °C. Toluene was removed by rotatory evaporator (HB 10, IKA, Germany) and the crude product was dissolved in 5 ml of dichloromethane (DCM) (Merck, Germany) and precipitated in diethyl ether (Merck, Germany). The procedure was repeated twice. Finally product was dried and stored at 4 °C. The product was characterized by ^{1}H NMR (Brukers, USA), ATR−FTIR (PerkinElmer, USA) and gel permeation chromatography (GPC,

fitted with a refractive index detector (Waters 2414) and Styragel HR 3 and HR 4 columns) (Waters, USA). All values were measured using tetrahydrofuran as mobile phase (at a flow rate of 1.0 mL/min) at 30 °C. Polystyrene standards were used for molecular weight calibration. Yield= 90%.

Synthesis of mPEG-PCL-O-CO-CH=CH_2. Weight amount of mPEG-PCL di block copolymer (5 gm, 0.70 mM), 285 µl (3.5 mM) acryloyl chloride (Sigma Aldrich, USA) and 488 µl (3.5 mM) triethyl amine (Merck, Germany) were taken dissolved in 50 ml of DCM in a schlenk tube. The reaction was carried out at 37 °C for 12 h under inert atmosphere. The crude product was dissolved in DCM and purified by washing with concentrated sodium bicarbonate to remove excess amount of acryloyl chloride. The procedure was repeated twice to remove traces of impurities. The product containing DCM was dried over anhydrous magnesium sulfate and concentrated over rotary evaporator. For recovery, the product was precipitated in cold diethyl ether thrice and dried. The product was characterized by ^{1}H NMR and ATR−FTIR. Yield=85%.

Synthesis of mPEG-PCL-g-Gelatin. Michael addition reaction was performed for grafting mPEG-PCL-O-CO-CH=CH_2 to gelatin backbone. Weight amount (2 gm) of gelatin and 12.77 gm (1.82 mM) mPEG-PCL-O-CO-CH=CH_2 was dissolved into 20 ml dimethyl sulfoxide (DMSO)(Merck) by stirring at 40 °C for 3 h. The resulting solution was allowed to stir continuously for 96 h at 37 °C. The product was precipitate in cold chloroform (Merck, Germany) thrice and, finally the product was dried and characterized by ^{1}H NMR and ATR−FTIR. Yield=76%

The degree of grafting was calculated by evaluating the free amino groups of gelatin and Bio-Syn polymer via TNBS assay (2,4,6-trinitrobenzenesul-fonic acid), as per the method described by previously[3].

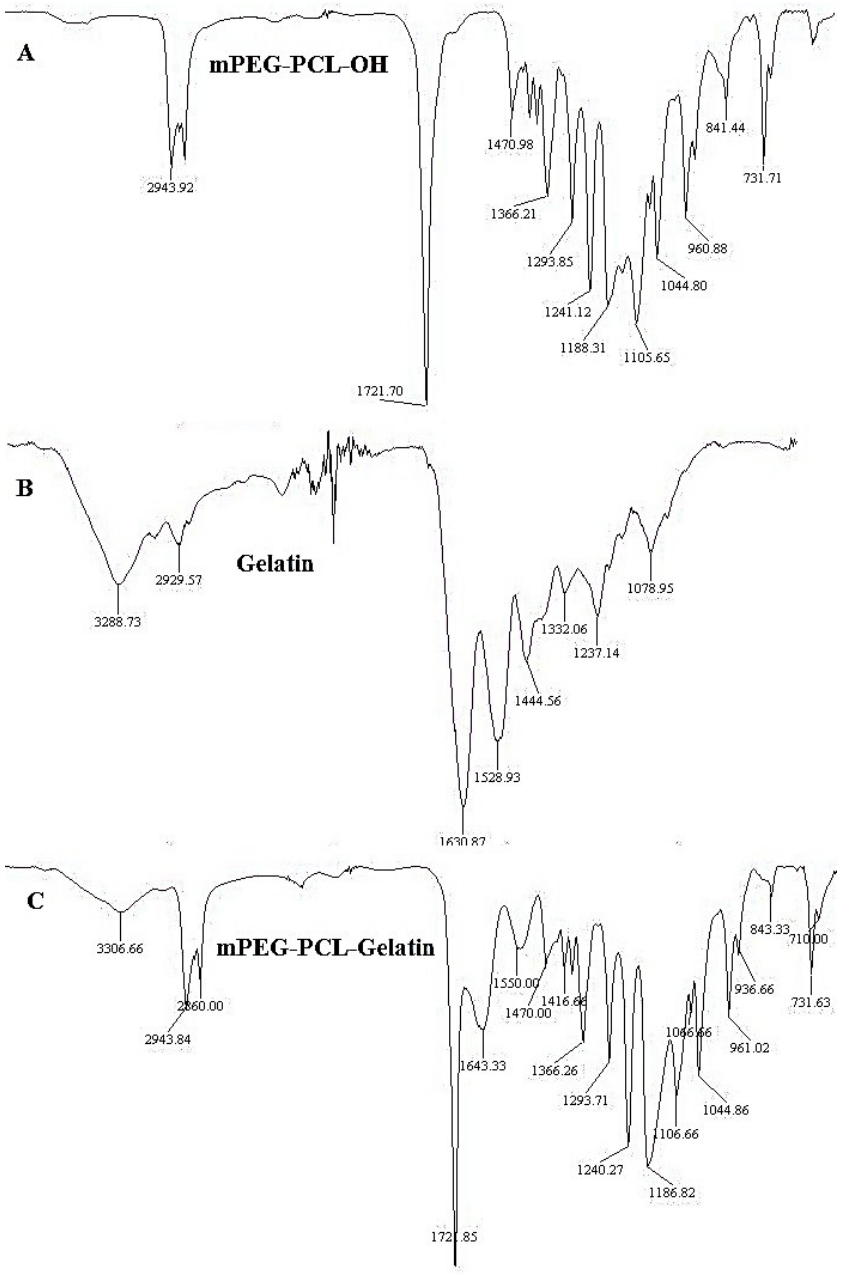

Figure S1. ATR-FTIR spectra of (A) mPEG-PCL-OH, (B) Gelatin and (C) mPEG-PCL-Gelatin

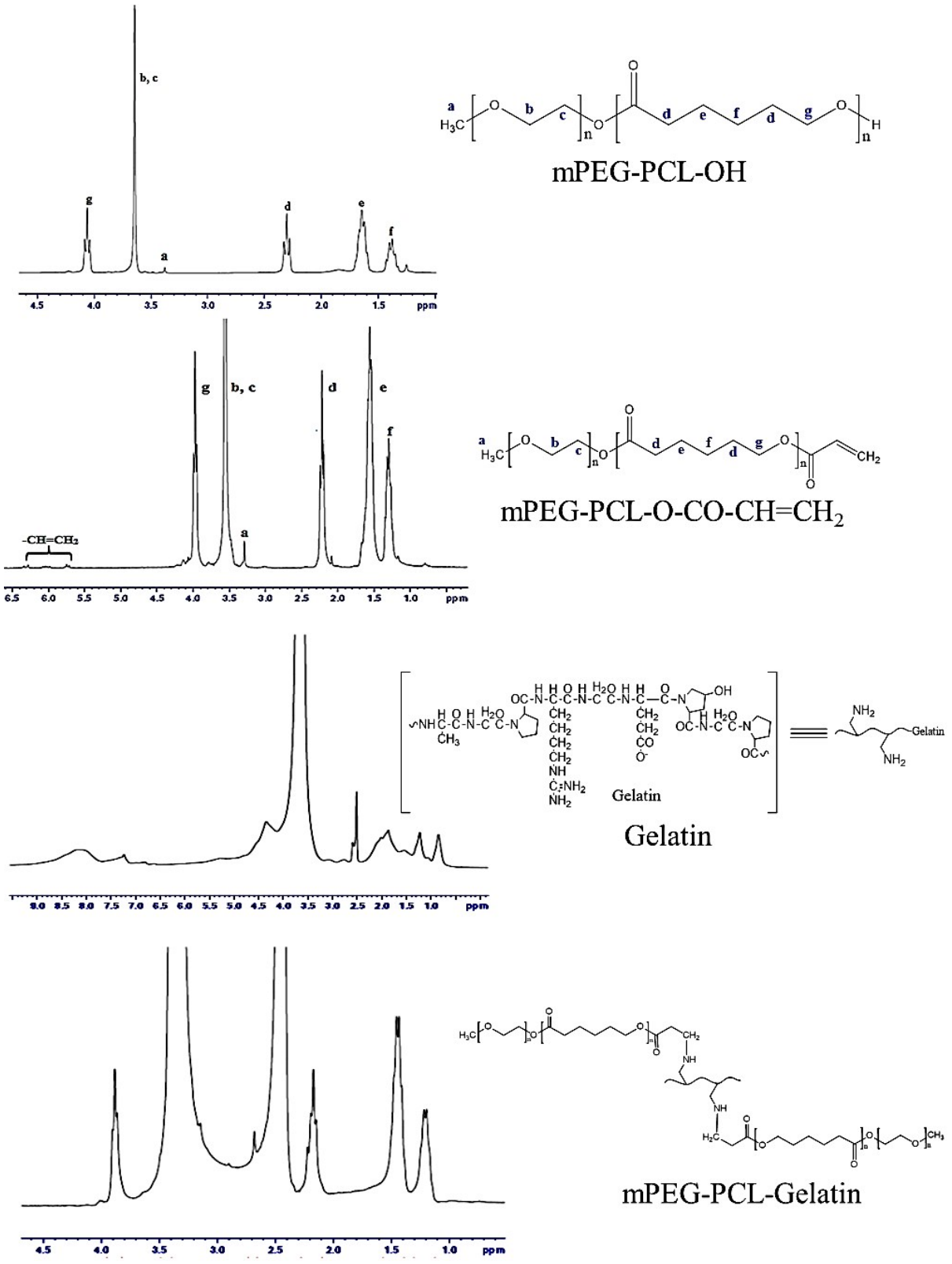

Figure S2. [1]H NMR spectra of mPEG-PCL-OH, Gelatin and mPEG-PCL-Gelatin

^{1}H NMR (300 MHz, $CDCl_3$, δ (ppm)- 1.35-1.43 (m, -$COCH_2CH_2$***CH_2***CH_2CH_2O-), 1.60-1.70 (m, -$COCH_2$***CH_2***CH_2***CH_2***CH_2O-), 2.28-2.32(t, -CO***CH_2***$CH_2CH_2CH_2CH_2O$-), 3.39(s, ***CH_3O***-,PEG unit), 3.64(s, -O***CH_2CH_2***-, PEG unit), 4.03-4.08(t, -$COCH_2CH_2CH_2CH_2$***CH_2***O-), 4.24 (t, -***CH_2***OH terminal OH group of polycaprolactone))

^{1}H NMR (300 MHz, $CDCl_3$, δ (ppm) - 1.16-1.34 (m, -$COCH_2CH_2$***CH_2***CH_2CH_2O-), 1.54-1.67 (m, -$COCH_2$***CH_2***CH_2***CH_2***CH_2O-), 2.19-2.24 (t, -CO***CH_2***$CH_2CH_2CH_2CH_2O$-), 3.29 (s, ***CH_3O***-), 3.5 (s, -O***CH_2CH_2***-, PEG unit), 3.95-4.07 (t, -$COCH_2CH_2CH_2CH_2$***CH_2***O-, 5.71-5.6.33 (m, -OCO***CH***=***CH_2***))

^{1}H NMR (300 MHz, DMSO, δ (ppm) - 0.850 (s, -CH_2***CH_2***$CH_2NHC^+NH_2$,-CH***CH_3***), 1.22 (b, -***CH_2***$CH_2CH_2CH_2NHC^+NH_2$), 1.54-2.01 (b, -CH_2CH_2***CH_2***NHC^+NH_2), 2.51-2.75(m, DMSO), 3.62 (b, H_2O), 4.34 (b, -Proline ring))

^{1}H NMR (300 MHz, DMSO, δ(ppm)- 1.43(m, -$COCH_2CH_2$***CH_2***CH_2CH_2O-), (-CH_2***CH_2***$CH_2NHC^+NH_2$), (-CH***CH_3***,-***CH_2***$CH_2CH_2CH_2NHC^+NH_2$), 1.45 (m, -$COCH_2CH_2$***CH_2***CH_2CH_2O-), 1.50 (m, -$COCH_2$***CH_2***CH_2***CH_2***CH_2O-), (-CH_2CH_2***CH_2***NHC^+NH_2), 2.17 (m, -***$COCH_2$***$CH_2CH_2CH_2CH_2O$-), 3.40 (s, -***OCH_2CH_2***-), 3.80 (m, -$COCH_2CH_2CH_2CH_2$***CH_2***O-), 2.15-2.75 (m, DMSO), 3.33 (H_2O))

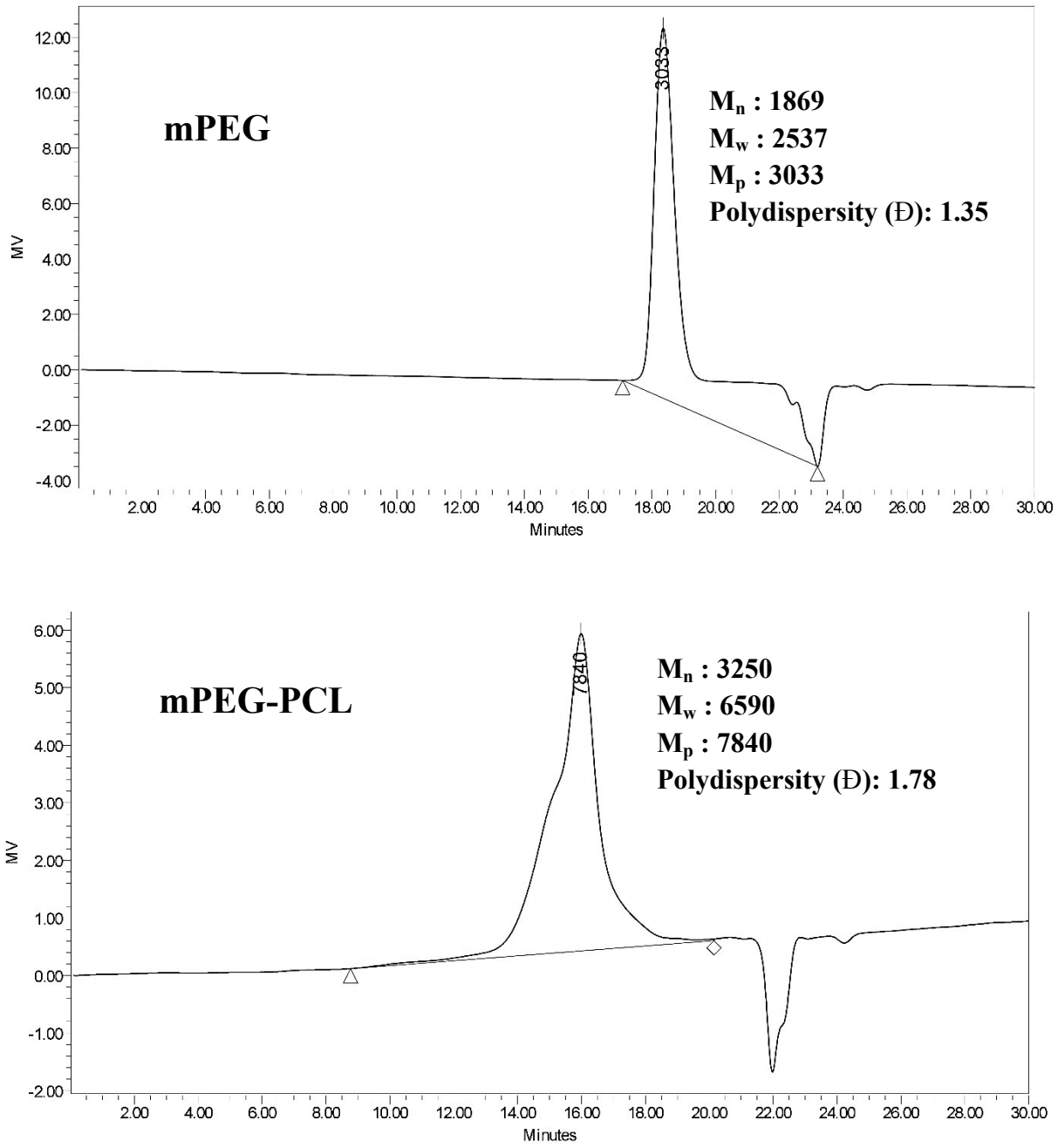

Figure S3. GPC chromatograms of mPEG and mPEG-PCL

Preparation of Bio-Syn composite electrospun scaffold. Aqueous solutions of sericin (0.5–1.0% w/v), hyaluronan (0.25% w/v) and chondroitin-4-sulfate (0.625% w/v) were mixed with 10% (w/v) Bio-Syn polymer solution in solvent system of glacial acetic acid: ethyl acetate

(GAA:EA=5:1), followed by ultra-sonic irradiation in an ice bath (UP200S, Hielscher, Germany). The final solution mixture was filled in six 5.0 ml luerlock plastic syringes, kept horizontally into the syringe pump of electrospinning machine (Physics Equipment, India) and the flow rate was adjusted to 0.5 ml/h. The voltage of electrospinning was set to 20-22 kV. The syringe spinneret's transition speed was fixed to 4.0 mm/sec. Drum collector (rotary) was fixed to drum rotation speed of 300 rpm. The distance of collector was set to 19 cm during electrospinning. The complete experiment was conducted under temperature (30±1°C) and controlled humidity (45±2% RH).

Crosslinking of the fibrous scaffold was performed using glutaraldehyde vapor (GTA) [4] for 30 min. After the crosslinking, the scaffolds were placed in laminar flow for 4 h to remove excess GTA vapor. Then the fibrous scaffolds were kept in a vacuum oven at room temperature for overnight and stored at 4°C in a desiccator until further use.

Evaluation of Bio-Syn electrospun composite fibrous scaffold. The surface morphology of electrospun nanofibrous mats were evaluated in Carl Zeiss Scanning Electron Microscope (SEM). Five SEM images were used to measure the average fiber diameter. At least 25 different fibers and 100 different sections were selected randomly to generate average fiber diameter, Photoshop 8.0 edition was used [3, 5, 6].

Pore size distribution in the electrospun nanofibrous mat was measured using porometer (Porous Materials Inc., USA; Model No: CFP 1100 AEX LH PC). Fibrous scaffold samples of 2.5 cm diameter were placed over metallic platform. Galvick (surface tension 15.9 dynes/cm^2, PMI, NY,USA) used as a solvent, was poured over the concerned area of whole circular fibrous specimen followed by covering with another metal plate. PMI CapWin software, USA was

programmed for the measurement of mean pore size and pore distribution, pore pressure at bubble point. 5 individual fibrous mats were used to plot the data as mean±SD. Galvick passes through fibrous mat by creating negative pressure using vacuum pump.

Mechanical property of scaffold was performed using an Instron micro-tensile tester, USA. The sample sheets were punched into suitable size[3] and placed between two soft clamps on the instrument. The thickness of the samples was calculated by digital micrometer (0.2μm precision). The test was conducted at a 10 mm/min crosshead speed and the testing parameters, i.e. maximum load, stress, elongation at break and, strain were documented using inbuilt software. Young's modulus was evaluated from maximum stress versus strain value [7].

The degradation behavior of the scaffolds were evaluated using method described earlier[3]. Concisely, gelatin/sericin's release was examined by analyzing the supernatant after scaffold incubation in solution. Scaffold of 10 mm diameter was kept in a microfuge tube containing phosphate buffer supplemented 1% (w/v) sodium azide and kept at 37 °C. Supernatant of 500 μl was collected in fresh microfuge tube after predetermined intervals and followed by refilling with fresh medium of same amount. Supernatant collected (100 μl) was mixed with solution S (500 μl) and incubated in the dark at room temperature for 15 minutes. Solution S is mixture of 1 ml solution of 2% (w/v) Na_2CO_3 in 0.1 (M) NaOH and 49 ml solution of 0.5% (w/v) $CuSO_4$ in 1% (w/v) sodium citrate. Afterwards, 50 μl of Folin reagent (Sigma Aldrich, USA) was mixed with every sample and incubated again for 80 min in the dark condition, followed by a brief mixing. The absorbance of the samples was measured at 700 nm. Standard curve was calibrated using gelatin's known concentrations.

Toluidin blue and sirius red staining was used to examine the distribution of GAGs and gelatin, respectively[3]. Incorporation of GAGs in the Bio-Syn composite scaffolds was further

established by 1% agarose gel electrophoresis according to procedure described by Rother et al [8]. Briefly, distilled water was used to dissolve the non-crosslinked scaffold and then the solution was mixed with 0.1 mg / ml of papain (Sigma Aldrich, USA) at 60 °C for 24 hours, followed by mixing with trichloroacetic acid for 2 hours. Afterwards, the solution was centrifuged at 15000×g for 30 min at 4° C. The supernatant was incubated overnight with absolute ethanol (1:4 = supernatant: ethanol) at -20° C. The samples were again centrifuged at 15000×g for 30 min at 4° C and supernatant was discarded. The pellets were dried in a laminar air flow for overnight at room temperature. Next day, the pellets were dissolved in double distilled water and loaded into 1% agarose gel for electrophoresis. After electrophoresis, overnight staining of the gel was performed using Stains All Dye (Sigma Aldrich, USA). Distaining of the gel was performed by exposing it to the daylight in double distilled water for 12-15 min. 1 mg/ml of CS and HA were used as markers.

Release profile of proteins (sericin/gelatin) and sulfated GAGs(CS). Sulfated GAGs release from the Bio-Syn composite scaffold was measured via dimethyl methylene blue assay (DMMB) [9]. Scaffolds were incubated in pH 7.5 phosphate buffer at 37°C. After fixed intervals (3, 6, 24, 96 and 192 h), samples (500 µl) were collected and replenished with same amount of fresh medium. Afterwards the DMMB solution was added to the samples and absorbance (595 nm) was examined using TECAN infinite UV-visible spectrophotometer (Model No. M200Pro, Switzerland). Standard calibration curve was calculated from known concentration of CS.

For protein release, scaffolds were incubated in phosphate buffer in a 37 °C incubator shaker (pH 7.5) (Orbitek, India). After schedules time intervals ,500 µl aliquot sample was isolated fresh microfuge tube. After each sample collection, tubes were replenished with equal amount of

fresh medium. Absorbance was measured (λ_{max} at 225 nm) by BioTek, PowerWave™ UV-visible spectrophotometer (Model No. XS/XS2, USA). The CS and proteins release kinetics from the scaffold was analyzed by theories of release kinetics: (a) Korsmeyer-Peppas equation model and (b) Diffusion model of Fick [3, 10]. Standard calibration curve was measured from known concentrations of sericin.

***In vitro* cell culture.** *In vitro* biocompatibility was performed on three different cells: (a) human foreskin fibroblasts (Hs27; ATCC-CRL-1634), (b) human keratinocytes (HaCaT; cell-line no. 300493, Cell Lines Service GmbH (CLS), Germany) and (c) human mesenchymal stem cell (hMSCs; Ethical No. EK 263122004). Details of cells and its culture medium has been described elsewhere [3].

Seeding of cells and Characterization of cellular activities on the Bio-Syn composite scaffold. Scaffold (~10mm diameter) was sterilized via UV irradiation for 72 hours. The scaffolds were immersed into complete medium at 37°C for overnight before cell seeding. 15,000 cells in 80 µl complete medium were seeded on each medium soaked scaffold and stored in CO_2 incubator for 2-3 hours before the addition of remaining medium to increase initial cellular adhesion efficiency. Medium was renewed after two days and samples were isolated after fixed interval (hMSC: 7 and 14 days; Hs27 & HaCaT: 1, 2 and 3 days) for evaluation. Collected samples were washed in PBS twice and stored at -80°C until further biochemical evaluation (DNA quantification and LDH assay). After PBS washing the scaffolds were stored at 4°C till immunocytochemical imaging.

Biochemical analysis

Cellular proliferation assessment using lactate dehydrogenase assay. Thawed samples were incubated with cell lysis buffer (1% Triton X-100 in PBS (Sigma Aldrich, USA)) in a shaker for 50 min at 75 rpm, followed by bath sonication at room temperature for 15 min. Cell proliferation was calculated by LDH activity assay (Takara Cytotoxicity Kit, France). Concisely, cell lysate was mixed with LDH substrate (1:1) for 5 min. Then, 50 µl (0.5 M) HCl was added and absorbance was evaluated at 492 nm. Standard curve of cell lysate of known cells was used to compare the LDH activity of the scaffold samples.

Cellular proliferation assessment using DNA quantification assay. Cellular proliferation was calculated by measuring the amount of DNA using Life Technologies Quant-iT™ PicoGreen® Kit, USA. Briefly, 190 µl of Pico Green dye in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) was mixed with 10 µl of cellular lysate at room temperature for 5 min. Fluorescence was evaluated at excitation of λ_{max}:485 nm and emission of λ_{max}:535 nm. Standard curve was used to determine the DNA content of the samples.

Immunocytochemistry-cellular behavior on Bio-Syn composite electrospun scaffold. Samples fixed using 3.7% formaldehyde solution, were incubated with 0.1% (v/v) TritonX100 for 5 min, followed by washing twice. Wash buffer is prepared from 0.05% Tween-20 solution in PBS. Afterwards the samples were treated with 1% BSA (blocking) for 25 min, followed by cytoskeleton staining with Phalloidin 546 (Invitrogen) for 50 min. Nucleus staining was performed by incubating for 2 min with DAPI (Invitrogen) and stored at 4°C till further experiment. Scaffolds were observed in an Carl Zeiss Axio Observer inverted fluorescence microscope (Model no. Z1m; Panfluor Objective: 40x/0.65 and 20x/0.45).

Contact co-cultivation of Keratinocytes-hMSCs on Bio-Syn composite electrospun scaffold. Keratinocytes were seeded into the scaffold and grown till they reached around 90% confluency. Afterwards keratinocytes were incubated for 30 min in mitomycin C (10 μg/ml) supplemented serum free DMEM medium and washed twice with complete medium. Around 10,000 hMSCs per scaffold were seeded onto the keratinocyte lawn and incubated for 2 h at 37 °C in a CO_2 incubator and then fresh medium was added to each sample and renewed every day during 5 days of contact co-culture study.

Immunocytochemical imaging of the Bio-Syn composite electrospun scaffold. Samples were fixed and blocked as per the procedure mention in earlier section (2.7.1.3) and stained with primary antibodies(Keratin 14, pan-cytokeratin and ΔNp63α), described elsewhere[3] and Phalloidin 488 (Invitrogen) for 60 min. After washing thrice using washing buffer, the scaffolds were treated again with Secondary antibody of goat anti-mouse Alexa Fluor 594 (Invitrogen) in 1% (v/v) goat serum solution for 90 min. DAPI (Invitrogen) was used to stain the nucleus for 2 min and washed thrice with washing buffer for 5 min and stored at 4°C till further experiment. Primary antibody was omitted for negative controls.

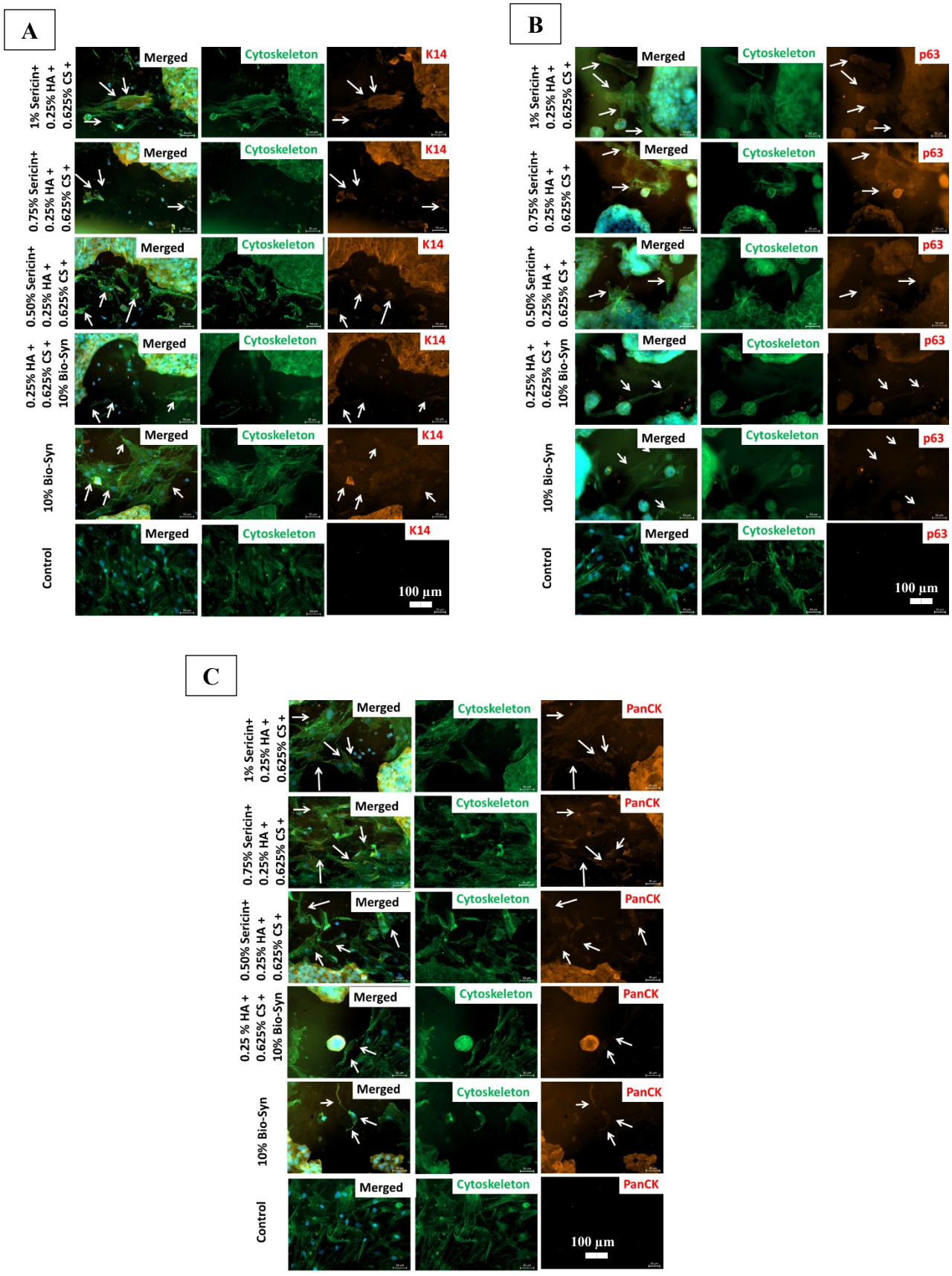
A
B
C
Merged
Cytoskeleton
K14
p63
PanCK
1% Sericin+ 0.25% HA + 0.625% CS +
0.75% Sericin+ 0.25% HA + 0.625% CS +
0.50% Sericin+ 0.25% HA + 0.625% CS +
0.25% HA + 0.625% CS + 10% Bio-Syn
0.25 % HA + 0.625% CS + 10% Bio-Syn
10% Bio-Syn
Control
100 µm

Figure S4. After 5 day of contact co-culture, the scaffolds were immunostained (color: red-orange) using (A) K14: Keratin 14, (B) p63: ΔNp63α, and (C) PanCK: Pan-cytokeratin monoclonal antibody, Phalloidin Alexa Fluor 488 (color: green) and DAPI (color: blue). White arrows point towards epithelial differentiated hMSCs in co-culture sub-population.

Isolation of non-fused hMSCs using fluorescence-activated cell sorting (FACS). Scaffolds confluent with keratinocyte were treated using Cell Tracker Blue® (CTB) [Invitrogen, USA] (20 μM) for 30 min in complete medium, followed by incubation with mitomycin C. hMSCs were stained using Cell Tracker Green® (CTG)[Invitrogen, USA] (0.1 μM) and then seeded on the scaffold confluent with CTB labeled HaCaT. Cells were lifted carefully using PBS containing 0.5 mM of magnesium chloride ($MgCl_2$), 10 mM of ethylenediaminetetraacetic acid (EDTA), 0.9 mM of calcium chloride ($CaCl_2$), and 1% (v/v) Fetal bovine serum (FBS) in Dulbecco's PBS (Ca^{2+}, Mg^{2+}). Sorting of cells was performed using BD FACS Aria™ II (BD Biosciences, USA). Around 50,000 cells were sorted from a mixed cell population of HaCaT cells (CTB), hMSCs (CTG), unlabeled HaCaT cells, and unlabeled hMSCs. Before the analyzing FACS data, cell clusters were removed from the examination data set. Afterwards, the unlabeled cells ($CTG^{(-)}CTB^{(-)}$) were also removed and then 20,000 $CTG^{(+)}CTB^{(-)}$ cells were collected in sterile microfuge tube for mRNA isolation and gene expression study (qRT-PCR). $CTG^{(+)}CTB^{(-)}$ represent the sub group of non-fused (mononucleated) hMSCs. Unlabeled cells (keratinocytes and hMSCs) were used as negative control and CTB labeled HaCaT and CTG labeled hMSCs were used as a positive control.

mRNA isolation and reverse transcription. RNA from non-fused $CTG^{(+)}CTB^{(-)}$ cells was isolated using Life Technologies Trizol (Gibco BRL, , USA) mRNA extraction method [11]. Briefly, 100 µl sample was incubated with 500 µl Trizol and then 200 µl chloroform was added. Afterwards, the solution was centrifuged and aqueous part of the supernatant was isolated in fresh microfuge tube and followed by mixing with 500 µl of isopropanol for 10-12 min. To precipitate the RNA, the solution was centrifuged at 12,000×g for 10 min at 4 °C. After gently washing with 75% (v/v) ethanol, the RNA pellet was centrifuged again at 7500×g for 5 min at 4 °C. The RNA pellet was dried under a laminar air flow and followed by re-dissolving in Ambion RNase free water (USA) and storing at -80 °C. RNA' purity and yield was measured spectrophotometrically using Thermo Scientific NanoDrop 1000 (USA) and found to be in the range of 100-250 ng/µl. cDNA was reversetranscribed from total RNA using Agilent Technologies AccuScript PfuUltra II RT-PCR kit (Waldbronn, Germany). Finally, RNase free water was used to dilute the cDNA (1:1) and stored at -20 °C.

Epithelial gene expression analysis using real-time polymerase chain reaction (qRT-PCR). Gene expression of the samples was evaluated by qRT-PCR (Model No. Mx3005P, Agilent Technologies) via Agilent Technologies Brilliant II QRT-PCR SYBR® Green Low ROX Master Mix kit (USA). Primers were acquired from Operon Eurofins MWG (Germany), described elsewhere[3]. Specificity of primers was confirmed by gel electrophoresis and melting curve analysis. Temperature cycle of PCR were optimized as per manufacture's instruction manual. Cycle of quantification (CT values) was determined using Mx3005P software (v 4.10). Relative expression of gene was evaluated as per δδCT method described by Livak et al. [12]. ΔNp63α and keratin 14 expression level of was standardized by considering the reference genes' average

mRNA expression (RPL13A -Ribosomal protein L13a, GAPDH- Glyceraldehyd-3-phosphate dehydrogenase, ACTB – β actin) ,as per the method defined by Vandesompele et al [13].

***In vivo* burn wound healing study on Wistar rats.** Burn wound healing efficacy of the Bio-Syn composite scaffold was examined on 2nd degree burn wound in female Wistar rat model. Ethical approval was obtained from Animal Ethical Committee of All India Institute of Medical Sciences, New Delhi (898/IAEC/15). Rats were divided into three groups: (a) control group with sterilized cotton gauze (Figure S5), (b) control group with commercial dressing (Neuskin™) and (c) experimental group treated with Bio-Syn composite scaffold. 24 Wistar rats were taken in each group and they were further divided into two sub-groups. Group A consists of 18 rats which were used for histological evaluation and wound contraction study on day 7, 14 and 21. Group B is made of 6 rats which were further used to evaluate the prohealing markers [total protein content, DNA, hexosamine (HAE) and hydroxyproline (HP)].

A

B

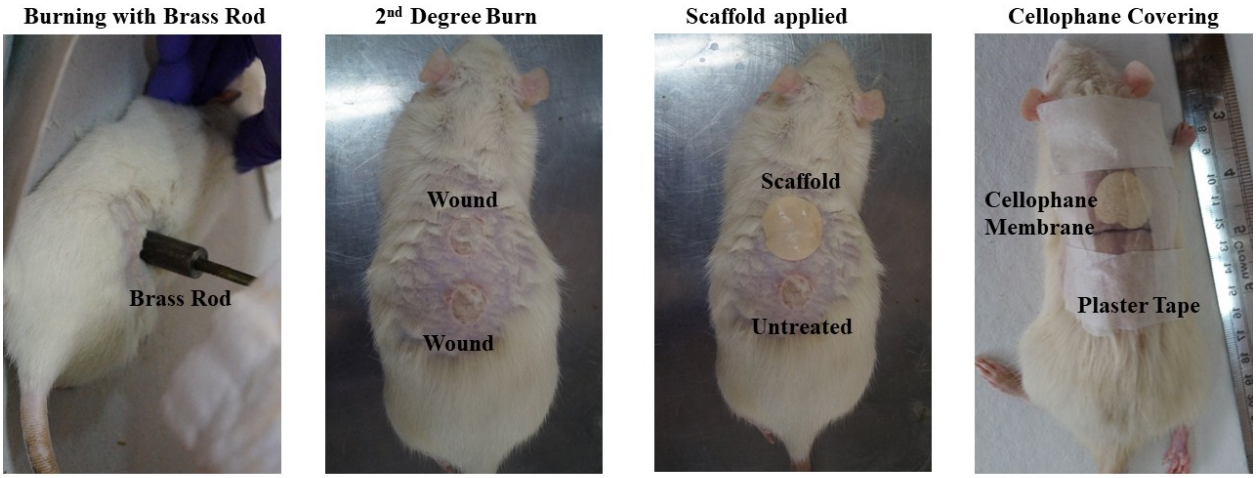

Figure S5. (A) Photograph of cotton gauze , (B) Second degree burn wound healing experiment on Wistar rats (Left to right).

Experimental design of burn wound healing study. Female Wistar rats (body weight 180±20 gm) were anaesthetized by intramuscular injection of 0.5% (w/v) Ketamine solution (100 µl) (AneKet®, Neo Laboratory limited, Mumbai, India). Before the experiment, the dorsal surface of rat skin was shaved with an electric hair shaver (Moeser, Unterkirnach, Germany) and cleaned with 70% v/v ethanol. 2nd degree burn wound was created using a metallic brass cylinder (diameter-1.1 cm, height-5.5 cm). Briefly, cylindrical brass templates were heated in a water bath with a constant temperature of 75±2 °C (measured with a digital thermometer) for 2 h. The metallic brass bar was held in direct contact with shaved portion of rat skin for 20 seconds to create a 2nd degree burn wound [14-18] (Figure S5 B). The wounded area was wiped with sterile saline water before applying dressing material. In control group, sterile cotton soaked in saline water was applied on the wound and covered with a cellophane membrane. In the experimental group, Bio-Syn composite scaffold was applied. The dressing was changed every week.

Assessment of healing parameters

Prohealing Parameters. The area of the wounded surface was measured on day 0, 7, 14 and 21 by making the healed boundary of the wound with a color marker on a transparent tracer paper [19]. In case of control group, the comparative rate of healing was evaluated by mean wound contraction rate. The percentage wound contraction rate was measured using equation no (1).

$$Wound\ contraction\ (\%) = \frac{(A_i - A_t)}{A_i} \times 100 \quad (1)$$

Where A_i represents the initial area of wound at day zero and A_t represents the wound area at time t

Evaluation of prohealing markers and histological factors. Regenerated skin tissue of sacrificed animal from both groups (control and experimental) was analyzed for evaluating prohealing markers and histological factors. Collected skin tissues were examined for assessment of total protein content, DNA, hexosamine and hydroxyproline [19].

For histological examination, skin tissue samples were fixed immediately after collection with formalin solution and was fixed in paraffin for section cutting by ultramicrotome. Micro-sections of skin tissues were stained with Van Gieson's (maturity of collagen fibril and its deposition pattern in ECM) and haematoxylin and eosin (inflammatory response) [19].

Morphological features of microsectioned tissue were evaluated by Olympus Microscope BX61 by using image analysis software (Image-ProPlus 6.2, Public domain, NIH, USA). Only epidermis and dermal regions were considered for this purpose. For grading purpose, around 50 high power fields (hpf) with 40× and 10× magnification were used examined. An average of acute inflammatory cells i.e. neutrophil per hpf were considered to evaluate acute inflammation

and they were categorized in the following manner; (a) for ± grade: less than 1/hpf , (b) for +grade: 1–2/hpf , (c) for ++ grade: 3–4/hpf ,(d) for +++ grade: 5–6/hpf , and (e) for ++++grade: 6/hpf [19]. Similarly, chronic inflammatory cells (plasma cells, histocytes and lymphocytes) present per hpf were measured to estimate chronic inflammation and degree of edema was evaluated via measuring the upper dermis regions' intercellular space. Out of 50 hpf, edema was represented by the following category: (a) grade ± if loose space was observed in <5, (b) grade+: if 5–10 hpf, (c) grade: if ++ in 11–20 hpf and (d) grade+++: if in >20 hpf. Proliferating fibroblasts, characterized by granulation tissue is graded as: (a) grade+: when respected cell number is <25 cells/hpf, (b) grade++: when 26–50 cells/hpf and (c) grade+++: when respected cell number is >50 cells/hpf. Mature fibroblasts categorized by eosinophilic abundant cytoplasm and spindle shaped nuclei, plays a vital role in collagenization. Population of mature fibroblasts per hpf during collagen deposition is directly related to the increase of eosinophilic hyaline material in intercellular spaces [19]. Presence of such material in histological slides was graded as: (a) + if number is <25 out of 50 hpf, (b) ++ if number is >25 out of 50 hpf. The re-epithelialization was assessed by measuring the regenerated epidermal portion and graded as: ± if number is <2 cell layer, (b) +if number is between 2–3 cell layer, (c) ++ if number is between 3–4 cell layer and (d) +++ if number is 5 or more cell layer[19].

Statistical analysis. Studies were performed in triplicate and examined statistically using following methods; (a) Twoway ANOVA and (b) Bonferroni Posthoc test among two groups where *p value < 0.05; **p < 0.01; ***p < 0.001 using GraphPad Prism® 5.

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