S1 Physico-chemical characterization of scaffold

S1.1 Microscopic Imaging

The morphologies of NFC, BL, and NFM were examined using digital microscope (Celestron Handheld) and scanning electron microscopy (SEM, EVO 60/ Zeiss, Germany). For SEM observation, the scaffolds were initially gold coated at an accelerating voltage of 10-20 kV using a plasma gold sputter. ImageJ software was used to measure fiber diameters of the samples with 20 random fibers under consideration.

S1.2 FTIR

Fourier transform infrared (FTIR)/ Attenuated total reflectance (ATR) spectra of NFC and NFM layer were recorded using Thermo Nicolet spectrophotometer (Model NEXUS-870; Thermo Nicolet Corporation, Madison, WI) with ZnSe crystal in reflectance mode between 4000 - 500 cm^{-1}.

S1.3 Contact angle

Hydrophilicity of NFC and NFM were evaluated by sessile drop technique. Water droplet of 2 µl was placed onto the scaffold surface and the equilibrium angle between scaffold surface and water droplet was determined using Protractor software (n = 3).

S1.4 Mechanical properties

Universal testing machine (Model H25KS; Hounsfield, UK) was used to measure the tensile properties of BL (n=5) scaffold. The samples were sectioned into thin strips (5 mm width, gauge length 10 mm and thickness approx. 0.05 mm) and 25-N load cell with 3 mm min^{-1} crosshead speed was used.

S1.5 Swelling behaviour

BL samples (n=5) of approx. equal weight (W_i) were incubated in PBS at 37 °C for 48 h. After specific time period, surface liquid was gently wiped and the weights were noted (W_f) using microbalance (Mettler Toledo, 162 US). The percentage swelling of samples was calculated as -

$$\% \text{ Swelling} = [(W_f - W_i) / W_i] \times 100$$

S1.6 Degradation kinetics

BL samples (n=5) of approx. equal weight (W_i) were incubated for 60 days in PBS supplemented with 10µg/ml lysozyme at 37 ºC. After every 5th day, the samples were washed with distilled water, air-dried, and weight (W_f) was recorded. Rate of degradation was calculated as:

$$\% \text{ Weight remaining} = (W_f / W_i) \times 100$$

S1.7 Antimicrobial property

Antimicrobial property of chitosan film, PCL and bilayer scaffold was tested against Staphylococcus aureus by zone of inhibition method (n=3). Briefly, after culturing bacteria for 24 hours in nutrient broth, bacterial suspensions were diluted and spread onto nutrient broth agar plate. After 15 minutes, the samples were placed onto the bacteria spread agar plates and incubated for 5 days. The zone of inhibition formed around scaffolds were examined and measured.

S2 Fibroblast and keratinocyte culture

Foreskin samples were initially diced into small pieces and incubated overnight in Dispase II (Sigma). The epidermal and dermal layer was then separated, epidermis was incubated in trypsin-EDTA (Gibco) and dermis in collagenase I (Gibco). After neutralization and

centrifugation, keratinocyte cells were cultured in keratinocyte specific media (KSFM) with media supplements (Gibco) and fibroblast cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml amphotericin B. Passage three to six of fibroblast cells and two to four of keratinocyte cells were used in these studies.

S3 Cell cytotoxicity and proliferation assay

Cell cytotoxicity on scaffold was done through MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. 5×10^4 fibroblast cells were seeded on scaffold (n=3) and also in tissue culture plate (control), and incubated at 37 °C. After 1, 3, and 7 days, the scaffolds and TCP were washed with PBS and kept for 4 h in 0.5 mg/ml MTT solution at 37 °C. DMSO was added to dissolve the formazan crystals and absorbance was recorded at 595 nm on a microplate reader.

To evaluate cell proliferation on scaffold, DNA quantification assay was performed according to manufacturer's protocol (DNA Quantitation Kit (DNAQF), Sigma). Briefly, after specific time period of cell growth on scaffold, DNA was isolated and incubated with fluorescent dye (bisBenzimide H 33258) and the developed complex was measured fluorometrically at excitation wavelength 350 nm and emission wavelength 460 nm.

Cell morphology and growth on scaffold

Scaffolds (NFC and NFM) (~ 1 cm^2) were initially sterilized by 70% ethanol for 3 h followed by repeated washing with PBS and incubation of NFC overnight in DMEM and NFM in KSFM. Fibroblast cells at count of 5×10^4 cells/NFC scaffold were seeded and grown in complete DMEM media while keratinocyte cells were seeded at density of 1×10^5 cells/NFM scaffold and

incubated in EGF and BPE supplemented KSFM with regular replacement of the culture medium. After 1, 3, and 7 days of incubation, the scaffolds were fixed in 4% paraformaldehyde, dehydrated by ascending series of ethanol (30–100%) and examined under SEM as described above. . The cell seeded samples after dehydration were embedded in paraffin also. Sections of 5 μm thickness were cut and after deparaffinization, the sections were stained with hematoxylin – eosin (H&E).

S4 Protein adsorption assay

Bicinchoninic acid (BCA) protein estimation assay was used to quantify the protein adsorbed on scaffolds. Initially, samples were incubated in 5% FBS/PBS solution at 37 ^{o}C and after 1, 3, 6, and 24 h they were rinsed in PBS, incubated for 45 min in BCA working solution (50 parts BCA reagent with 1 part 4% copper sulphate pentahydrate) at 37 ^{o}C and measured at 562 nm using UV absorbance spectrophotometer (Model-UV-1601, Shimadzu, Japan).

S5 Mechanical property of cell seeded scaffold

BL samples (~ 2×4 cm) were cultured with fibroblast cells at density of 2×10^5 cells/scaffold, and after 7 and 14 days of incubation, the constructs were fixed in 4% paraformaldehyde and air-dried. The tensile strength of the samples (n=3) were measured with the conditions detailed above.

S6 ECM protein expression

Fibroblast cells were grown in samples (~ 2 cm^2) at density of 1×10^6/scaffold and total RNA was extracted from cell/scaffold construct after 7 and 14 days using RNA extraction kit (Himedia, India) following manufacturer's instruction. Using RevertAid First Strand cDNA

Synthesis Kit (ThermoFisher Scientific), cDNA was synthesized from RNA pool and eventually amplified by polymerase chain reaction (PCR) with primers enlisted in Table S1. PCR amplification was carried out for 30 cycles using conditions: denaturation 94 °C for 30 s, annealing for 30 s, extension 72 °C for 30 s and final extension for 10 min at 72 °C. Resulted PCR products were examined by 1% agarose gel electrophoresis and analyzed using ImageJ program (NIH Bethesda, MD) to compare gene expression. Collagen I, collagen III, fibronectin and matrix metalloproteinases (MMP) protein expression was subjected in this study.

Table S1

List of primers for PCR of targeted genes

Name of Gene	Sequence	Fragment Size (Bp)	Tm (°C)	Gene Bank ID
Laminin	F-TGACCTTTTCTGGCTCGTCT R-GTTCAGCACAAAGGGCTCTC	201	57	NM_005560
Keratin 10	F-CATGAGTGTCCCCCGGTATC R-CAGTATCAGCCGCTTTCAGA	79	59	NM_000421
E-Cadherin	F-CATGAGTGTCCCCCGGTATC R-CAGTATCAGCCGCTTTCAGA	88	59	NM_004360
Collagen IV	F-GGATCGGCTACTCTTTTGTGATG R-AAGCGTTTGCGTAGTAATTGCA	152	58	NM_001845
Collagen I	F-GCGCCAGAAGAACTGGTACATCAGCAA R-GCGCGCCATACTCGAACTGGAATC	100	60	NM_000088.3
Keratin 14	F-TTCTCACAGCCACAGTGGAC R-CATTGACATCTCCACCCACC	281	59	NM_000526.4
Involucrin	F-TGCCTCAGCCTTACTGTGAG R-GCAGTCCCTTTACAGCAGTC	251	59	NM_005547.2
Fibronectin	F-AAGATTGGAGAGAAGTGGGACC R-GAGCAAATGGCACCGAGATA	179	58.8	NM_002026.2
MMP	F-TTGAGAAAGCCTTCCAACTCTG R-CCGCAACACGATGTAAGTTGTA	250	58.4	NM_002421.2
Collagen III	F-CTGAAATTCTGCCATCCTGAAC R-GGATTGCCGTAGCTAAACTGAA	236	58.4	NM_000090.3
GAPDH	F-CCATGGAGAAGGCTGGGG R-CAAAGTTGTCATGGATGACC	195	54	NM_002046.3

S7 Fibroblast and keratinocyte co-culture

Initially, fibroblast cells (1×10^6) were grown in samples (~ 2 cm^2) and cultured for two days in DMEM media supplemented with FBS and antibiotic, keratinocyte cells (5×10^6) were then seeded on scaffold and grown in KSFM media with supplements for further two days. Thereafter, the scaffold was kept on a stainless-steel grid with keratinocyte cell seeded surface on top and raised to air–liquid interface for 14 days. The media during this period was a 1:1 mixture of KSFM and DMEM with 3% FBS and replaced every alternate day.

S8 TGF-β1 quantification assay

TGF-β1 cytokine was quantified by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (Thermo Fisher Scientific). Briefly, fibroblast and keratinocyte cells were seeded individually on scaffold and culture media was collected after 3 and 7 days of growth period. Scaffolds were also incubated with fibroblast and keratinocyte co-culture growing at air-liquid interface following method detailed above and after 3 and 7 days media was collected. TGF-β1 protein secreted in the media was calculated in ng corresponding to standard curve.

S9 Third-degree burn wound healing experiment

Adult Wistar rats (*Rattus norvegicus*) (n=24) of weight 200 ± 50 g were used with approval from Institutional Animal Ethics Committee (IAEC) which follows guidelines of Committee for the purpose of control and supervision of experimentation on animal (CPCSE, Govt. of India). Throughout the experimentation period, the animals were kept in humidity and temperature controlled room maintaining 12 h photoperiod where the general health condition of the animals was monitored and they received balanced food and water. The animals were segregated into two

groups (n = 3 for each group). For experimentation, the animals were at first anesthetized with intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (90 mg/kg), followed by shaving the dorsal region and outlining an area of 2.5 x 2.5 cm (6.25 cm^2) on the dorsal lateral plane. Stainless steel plates were heated to ~ 300 °C and pressed on the outlined area of skin for 3 s to create a third-degree burn wound. Burnt skin flap was removed by incision to the subcutaneous panniculus carnosus layer. Prior to application, the scaffolds were sterilized using 70% ethanol for 3 h, washed with PBS repeatedly and incubated in PBS. Bilayer scaffold covering on the third-degree burn wound represented the sample group while control group had Tegaderm™ dressing. As no bandage was applied to surgical site, hence to secure the sample in wound bed, wound of both the groups was covered with sterile, transparent Tegaderm™ dressing. The Tegaderm™ dressing was removed after two days from both wounds and replaced with fresh dressing in control group and repeated after every fifth day. In sample group however no fresh scaffold or Tegaderm™ dressing was applied. Digital photographs were taken on day 3, 10, 20 and 30 to evaluate wound closure rate. Rate of wound closure was determined by measuring the size of wounds using image J software.

Percentage wound closure = (initial area of wound – nth day area of wound) / initial area of wound ×100

After 3, 10, 20 and 30 days, specific animals of each group were euthanized, and the repaired skin tissue was excised, and processed for H&E and Masson's trichrome (MT) staining. Immunostaining was performed using Biogenex Super Sensitive ™ Polymer-HRP Kit (Biogenex, US) according to the manufacturer's protocol. Primary antibody for p63 (Abcam, UK) with dilution 1: 200; keratin 10 (Abcam, UK) with dilution 1: 200; collagen I (Abcam, UK) with dilution 1: 500; and collagen III (Abcam, UK) with dilution 1: 500 were used in this study.

Results

Antimicrobial activity

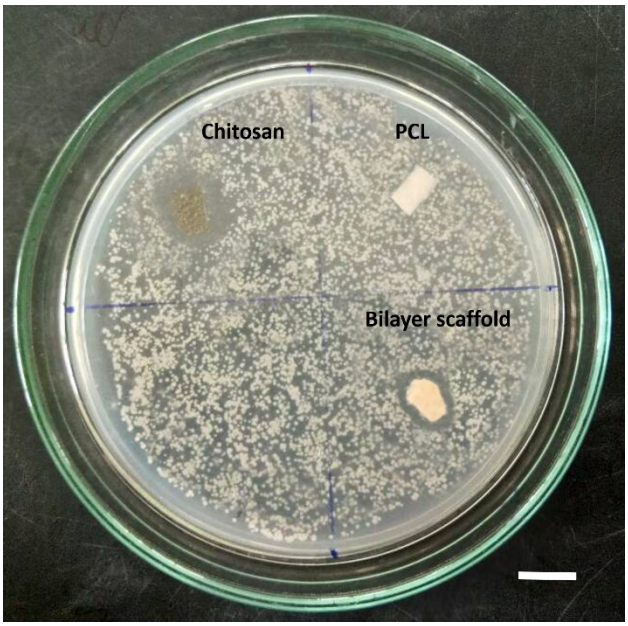

Figure S1: Antimicrobial activity of chitosan film, PCL and bilayer scaffold. Scale bar is 1 cm.