Supporting Information

Asymmetric fabrication and in-vivo evaluation of wound healing potency of electrospun biomimetic nanofibrous scaffold based on collagen crosslinked modified-chitosan and graphene oxide quantum dot nanocomposites.

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1. EXPERIMENTAL SECTION

1.1. Materials

Potassium permanganate ($KMnO_4$, ACS grade), sulphuric acid (98% H_2SO_4, GR grade), sodium nitrate ($NaNO_2$, extra pure), hydrogen peroxide (30% H_2O_2), acetic acid (CH_3COOH, glacial 100%), formic acid (HCOOH, 98-100% pure) were purchased from Merck Specialties Private Ltd., India. Chloroform ($CHCl_3$, anhydrous), phosphate buffered saline (PBS), methanol (CH_3OH, anhydrous), 2,2,2-trifluoroethanol (TFE), trichloroacetic acid (TCA), thiobarbituric acid (TBA), nitro blue tetrazolium chloride (NBT) and riboflavin were procured from Sisco Research Laboratories Pvt. Ltd. (India). Glutaraldehyde (25% in water) and N, N-dimethylformamide (DMF, anhydrous) were purchased from Spectrochem Pvt. Ltd. (Mumbai, India). Phthalic anhydride and decanoyl chloride were purchased from TCI Chemical Pvt. Ltd., Japan. L929 fibroblast cell was purchased from the National Centre for Cell Science (NCCS, Pune, India). Chitosan, polycaprolactone (Mn 80000, PCL), nylon syringe filter (0.2 μm pore size), dialysis tubing (molecular weight cut-off 2000 Da), collagen (type I), 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), fluorescein diacetate (FDA) and 2,2-diphenyl-1-picrylhydrazyl were obtained from Sigma-Aldrich, Germany. Dulbecco's modified eagle medium (DMEM), fetal bovine serum, MTT reagent, tetramethylethylenediamine (TEMED), penicillin, and streptomycin were procured from HiMedia, Mumbai, India.

1.2. Characterization

Fourier transform infrared (FTIR) spectroscopy was performed using Perkin-Elmer spectrum Two FT-IR spectrometer over wave number 400-4000 cm^{-1}. The surface morphology and the fiber dimension were elucidated by the scanning electron microscope (ZEISS EVO-MA10, Germany) and transmission electron microscopy (HRTEM, JEOLJEM 2100) operated at

200 kV acceleration voltage. The particle and fiber size distribution were displayed by Image J (1.51K) software. The X-ray diffraction (XRD) analysis was carried out between $2\theta = 3$ and 40° by employing an X-PERT-PRO Panalytical diffractometer with CuKα (40 kV/30 mA) as the X-ray source comprising a wavelength of approximately 1.4506 nm. UV-visible spectroscopy was recorded by a Perkin-Elmer UV-vis Spectrophotometer Lambda 25. X-ray photoelectron spectroscopy (XPS) study was characterized by a Thermo-VG Scientific ESCA Lab250 microprobe equipped with AlKα monochromatic source comprising 1486.6 eV photons operating at 14 kV and 20 mA. ^{13}C solid state nuclear magnetic resonance (NMR) spectroscopy was carried out using AV 500S Bruker 500 MHz instrument. ^{1}H solution NMR spectroscopy was done with the employment of a 400 MHz Bruker instrument. NMR grade trifluoroacetic acid (5% v/v) incorporated D_2O was taken as a solvent for all the compounds. The mechanical properties were estimated according to the ASTM method D882-95a with the assistance of the Zwick Roell (ZO10) instrument. The water contact angle measurements of all the scaffolds were evaluated using an Apex ACAM-D2 contact angle system.

1.3. Porosity measurement

The liquid displacing method was utilized to estimate the porosity of the scaffolds. Briefly, the scaffold (weighing W_i) was immersed in a tube containing absolute ethanol. The total volume (V_1) of the ethanol with the scaffold was recorded. The scaffold was kept immersed in ethanol until saturation. The ethanol-saturated scaffold was taken out and the remaining volume (V_2) of the ethanol left was noted. The weight (W_f) of the ethanol-soaked scaffold was also recorded. The recorded data were employed in equation 1 to calculate the porosity of the scaffold. The data sets were calculated thrice experimentally and the average value was taken.

$$\text{Porosity} = \frac{W_f/\rho - W_i/\rho}{V_1 - V_2} \quad \ldots\ldots\ldots\ldots\ldots (1)$$ $\rho =$ density of ethanol

1.4. Water vapor transmission rate (WVTR)

The WVTR of the scaffolds was determined by pouring 20 mL of phosphate buffer saline (PBS) into a 50 mL centrifuge tube with an open area of S cm^2. The centrifuge tube was covered with a piece of a scaffold, and the total weight was recorded (W_1). The scaffold covered tube was incubated at 37 ^{O}C for about 24 hours and subsequently, the total weight was again recorded (W_2). Equation 2 was employed to evaluate the WVTR of the scaffolds in 24 hours. After three times of experiments, the average values were taken.

$$\text{WVTR} = \frac{W_1 - W_2}{S} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

1.5. Catalase-Like activity

The catalase-like activity assay was estimated by the addition of 10 (M) H_2O_2 in 100 µg mL^{-1} scaffolds. After 10 min, the evolution of gas bubbles was seen. The catalase-like activity of the scaffolds was also detected by analysing the reduction rate of H_2O_2 levels. The standard initial concentration of H_2O_2 was 25 mmol L^{-1}, and the final concentration of H_2O_2 after the incubation with the scaffold was evaluated spectrophotometrically with regard to the absorbance peak of H_2O_2 at 230 nm.

1.6. Antimicrobial activities assay

The antimicrobial activity of the scaffolds was assessed with the help of the plate counting method. Both *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* bacteria

were cultured in Luria-Bertani (LB) medium over a period of 16 hours at 37 °C with continuous shaking, followed by washing with fresh sterile 0.9% saline thrice. The cultures were then diluted in sterile 0.9% saline solution to ~10^7 colony-forming units per milliliter (CFU mL^{-1}). To expose the active surface of the bacteria, all the scaffolds with an area of 2 cm^2 were placed in a plastic holder. Bacterial suspensions (0.5 mL per cm^2) were subjected to contact with the scaffold surface over a period of 60 min at 37 °C temperature. With the discarding of excess bacterial suspension, the scaffolds were washed thoroughly with sterile 0.9% saline. After thorough washing, scaffolds were put into a 50 mL tube containing 10 mL of sterile 0.9% saline and subjected to vortex for 8 min to remove the bacterial cells from the scaffold's surface. Finally, the collected bacteria cells were put on LB agar plates and incubated for a period of 16 hours at 37 °C.

To get the SEM image of bacteria, the bacterial suspensions *(S. aureus)* were exposed to the surface of the scaffold by the method mentioned above, followed by a thorough washing of the scaffold with sterile 0.9% saline solution and fixed with Karnovsly's fixative (2.5 wt% glutaraldehyde in 0.2 M PBS buffer) for 3 hours. The dehydration step of the samples was carried out by sequential immersion in water/ethanol (30, 50, 60, 70, 80, 90, 100%), and kept in a desiccator (below 30 °C) for 12 hours to dry. Finally, the samples were sputter-coated with platinum and were imaged by SEM.

1.7. *In vitro* blood coagulation test

According to reported literature[1], the nanofiber scaffolds and medical gauze (served as a control) were cut into a circular pattern (approximately 20 mm in diameter). The experimental part was initiated with the casting of 100 μL of blood (comprising 10% sodium citrate by volume) onto the upper surface of individual samples and subjected to incubation at 37 °C for 5 min. The

samples were mildly rinsed in 50 mL of distilled water to separate uncoagulated red blood cells. Drabkin's reagents (D5941 Sigma-Aldrich) were employed for quantitative estimation of blood coagulations on each scaffold in compliance with the manufacturer's guidelines of the same. The absorbance values of individual samples were measured at a wavelength of 540 nm in a UV spectrophotometer. Other than that, the assessment of blood clotting time was done in accordance with the previously reported study[1]. The medical gauze and the scaffolds with coagulated blood was air dried for 4 hours at 37 ºC. Then the samples were fixed in 0.25% glutaraldehyde for 45 minutes. After fixation the samples were subjected to alcohol gradation (50%, 70%, 90% and 100%) for 10 minutes at each step. Finally, the samples were air dried and studied by SEM imaging.

1.8. Cell culture

Under an atmospheric condition of 5% CO2 and a temperature of 37 °C, a mouse fibroblast cell line L929 was cultured in Dulbeccos modified eagle medium (DMEM) with the subsequent addition of 10 wt% fetal bovine serum and 1wt% antibiotic. Coverslips were coated by scaffold and stationed onto 24 well cell culture plates. The cells in the culture flask were disengaged by trypsin-EDTA and subsequently seeded on the scaffold at a density of 1.5×10^4 cells per well.

1.9. *In vitro* biocompatibility, cell adhesion, and proliferation studies

An MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was engaged to estimate the biocompatibility of the scaffold at different culture duration up to 72 hours for three times. MTT solution was subsequently added to each well and was subjected to incubation for 3 hours. The optical density of the formazan complex dissolved in dimethyl sulfoxide was evaluated with a microplate reader at the wavelength of 570 nm. The percentage of cell viability was determined by investigating the optical density of the control and samples.

The adhesion and proliferation of L929 fibroblast cells were assessed at regular time intervals (day 1, 3, and 5) through fluorescein diacetate staining. First, the scaffolds with cells were removed from the culture medium and washed with PBS. The cells, after thorough washing, were incubated with FDA solution (10 µg/mL) for 30 min and again washed with PBS. Finally, the obtained cells were viewed with a Leica fluorescence microscope.

For SEM analysis, the scaffolds with cells were removed from the culture medium and washed with PBS, followed by incubation with 4% paraformaldehyde for 30 min. The scaffolds were dehydrated in a graded series of ethanol solutions and dried overnight. The dehydrated scaffolds were examined under SEM at 5 kV accelerating voltage.

1.10. *In vivo* wound healing experiments

For excisional wound healing investigation, healthy male Wistar rats of weight 200−230 g were chosen. All the experimental procedures were done before the approval of the Institutional Animal Ethical Committee (Department of Zoology, University of Calcutta, India, 885/GO/RE/S/05/CPCSEA). An intraperitoneal administration of ketamine (35 mg/kg) and xylazine (5 mg/kg) was done to anesthetize individual animals. A complete thickness excision wound was made by excising the dorsal skin of rats after shaving the dorsal surface and disinfecting it with 70% ethanol. 18 Wister rats were subdivided into three groups (six rats per group). Group 1 (control) rats were tended with gauze, while group 2 and 3 were tended with scaffold 1 and 3 respectively. In order to keep the healing material on the incised area, scaffolds were additionally enveloped with gauze and fixed with a bandage. Photographs of wounds were taken on days 3,7 and 10 after the wound incision. Wound size was traced with the equipment of a transparent sheet, followed by a new dressing. The wound closure percentage was determined by the following formula.

$$C_n = \frac{(S_0 - S_n)}{S_0} \times 100 \quad \ldots\ldots\ldots\ldots (3)$$

Where C_n is the percentage of wound size reduction on days 3, 7, and 10 post-wounding, S_0 is the initial wound area, and S_n is the wound area on days 3, 7, and 10 post-wounding.

1.11. Histological analysis

On day 3, 7, and 12 after surgery, a full-thickness skin biopsy was taken from the center of wounds with surrounding tissues. Tissue samples were fixed in 10% formalin for histological analysis of tissue samples, and these were embedded in paraffin wax after dehydration using a series of graded alcohol. The tissues were sectioned at 5 µm for hematoxylin and eosin (H&E) and Masson–Goldner trichrome (MGT) staining.

1.12. Biochemical Assays

Malondialdehyde (MDA) levels in the tissue were determined by mixing 0.5 mL of homogenate with 2.5 mL of trichloroacetic acid (20%) and 1.0 mL of thiobarbituric acid (0.6%). The mixture was heated for 30 min in a boiling water bath, cooled, and centrifuged at 3000 g for 10 min. The absorbance was measured at 535 nm[2].

Superoxide dismutase activity was determined by method according to literature[3]. The reaction mixture composed of 80 mM Tris (pH 8.9), 0.12 mM EDTA, 10.8 mM TEMED, 0.003% BSA, 30 µM riboflavin in 5 mM KOH, 300 µM NBT (SRL, 11207), 50 µL supernatant was added. It was kept under light (150 W) for 2 min, and the absorbance was taken at 560 nm. A blank without enzyme and light showed NBT present in the reaction mixture. Unit SOD was calculated as U/mg protein.

The rate of decomposition of hydrogen peroxide (H_2O_2) at 240 nm absorbance over 1 min was used to assess catalase activity in the wound tissue biopsy samples according to the literature[4]. Catalase activity was expressed as U⁄g protein.

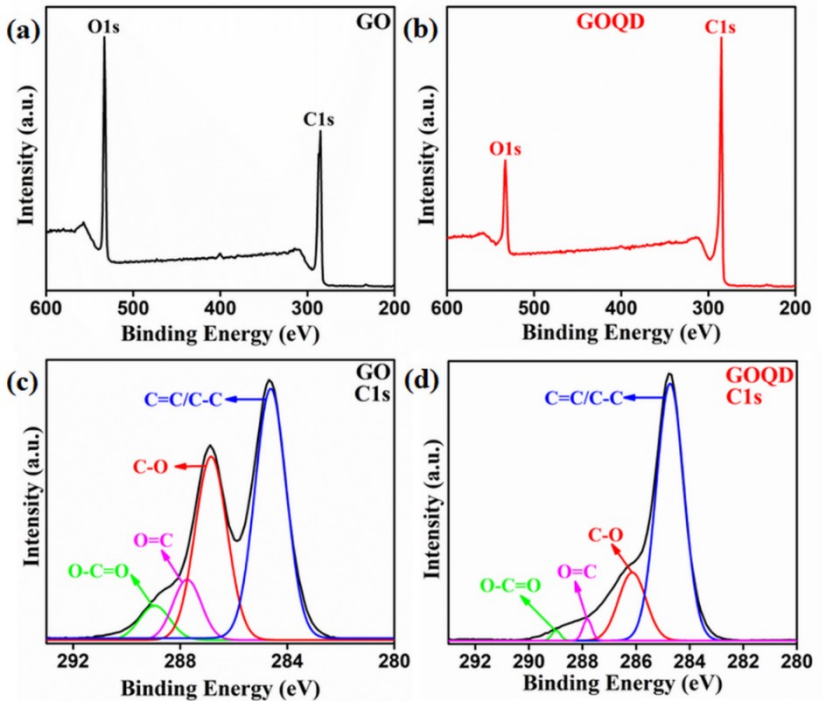
1.13. ELISA

Proteins were isolated from wound tissue biopsy samples in RIPA buffer. Then levels of inflammatory cytokines IL-6, TNF-α, and IL-1β were measured by using ELISA using a commercial kit from Sigma.

2.20. Statistical Analysis

Each and every experimental sets were conducted thrice with data written in the form of average ± standard deviation. Statistical analysis was executed by one-way ANOVA with Tukey's post hoc test by employing Origin Pro 8.0. P values < 0.05 were assessed to be statistically noteworthy.

Supplementary Figures



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194 **Figure S1.** XPS survey spectra of GO (a) and GOQD (b). High-resolution C1s peaks of GO (c)

195 and GOQD (d).

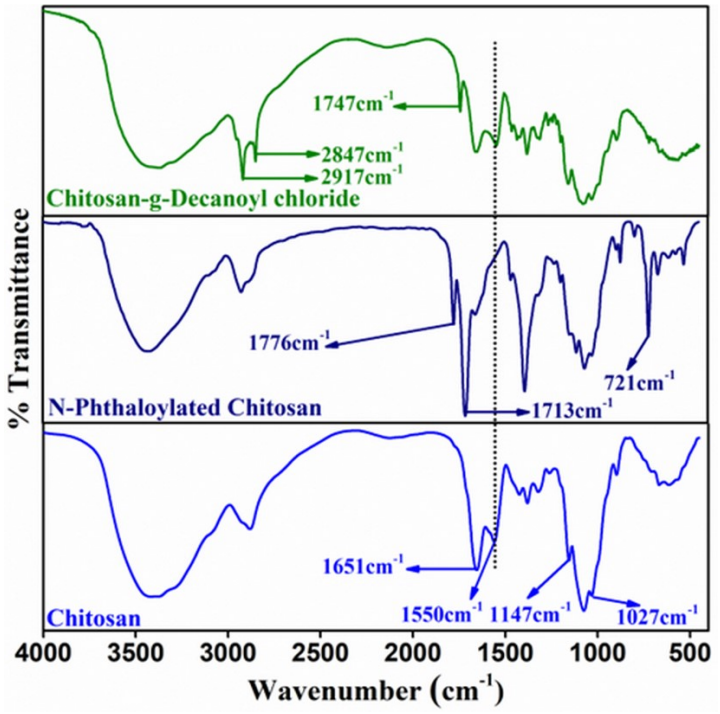

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Figure S2. FTIR spectroscopy of chitosan, N-phthaloylated chitosan, and CS-g-Dc.

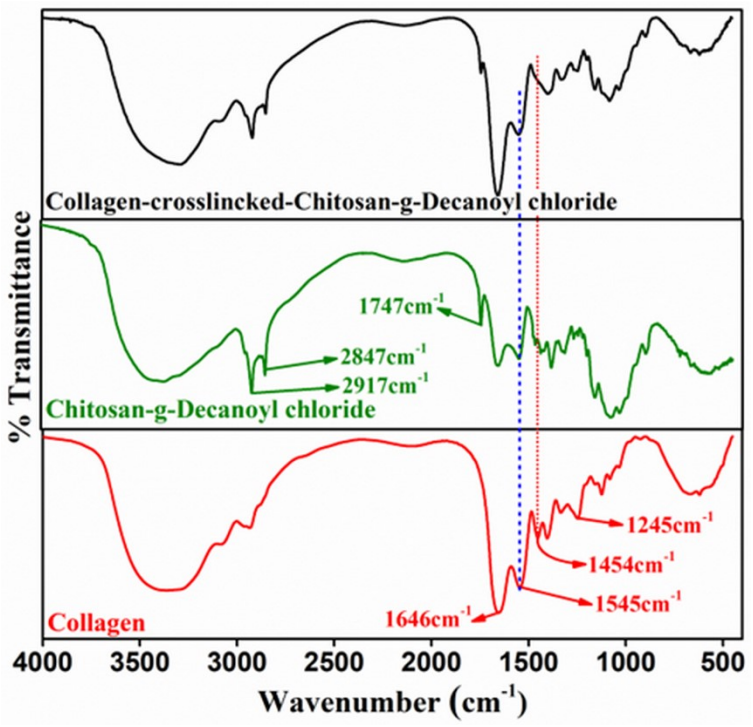

Figure S3. FTIR spectroscopy of collagen, CS-g-Dc, and CG-cross-CS-g-Dc.

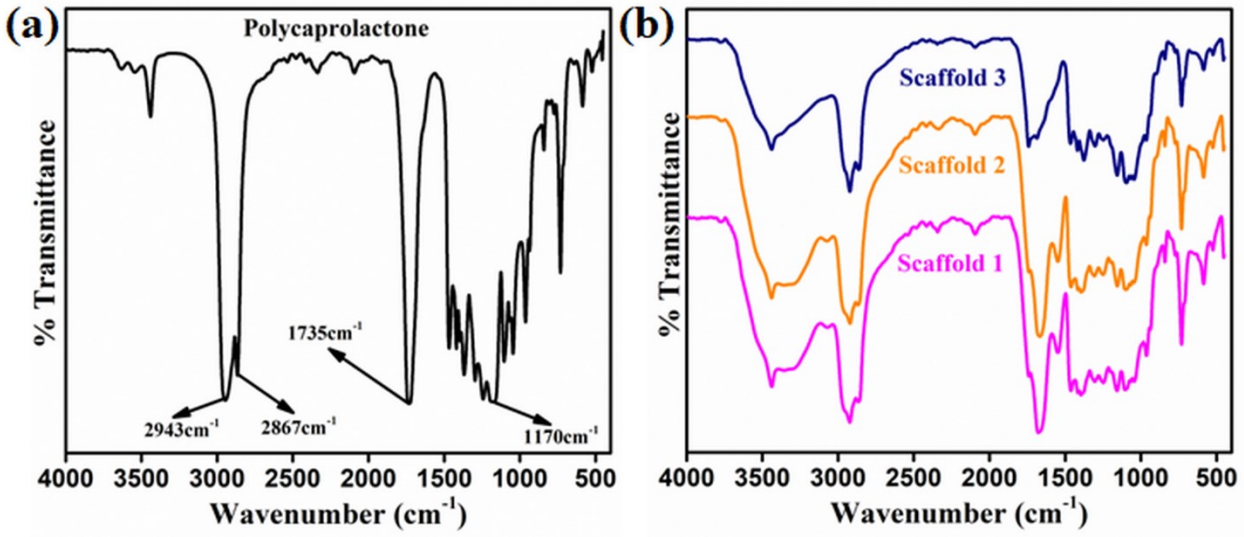

Figure S4. FTIR spectroscopy of polycaprolactone (a) and scaffold 1, 2, and 3 (b).

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