Supporting Information

3D Printing of High Strength Chitosan Hydrogel Scaffolds without any Organic Solvents

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SI. Materials and Methods

SI. 1. Preparation of Chitosan Ink

The chitosan ink was prepared by dissolving chitosan powders (Ruji Biotechnology Co., Ltd.) in an alkaline solvent. The alkaline solvent of chitosan was obtained by mixing LiOH, Urea, KOH and deionized water (Sigma-Aldrich) with a mass ratio of 5:8:7:80.5. Then, the chitosan powder with different mass ratio (3.5, 4 and 4.5 wt%) was added into the alkaline solution and was stirred for 5 minutes at room temperature until a homogeneous mixture was obtained. Then, the chitosan solution was stored in a refrigerator under -30°C for 6 h until completely frozen. After that, the frozen solid was thawed and stirred at room temperature. This freezing-thawing process was repeated for two times until all chitosan particles dissolved. Then, the air bubbles were eliminated by centrifuging (7000 rpm, 10 min under 5°C).

SI. 2. Printing Procedure

All printings were carried out using a customized 3D printer (EFL-BP-6800, Suzhou Intelligent Manufacturing Research Institute, Suzhou, China) equipped with a nozzle temperature controller and a plate temperature controller. During printing, the nozzle temperature was set at 5 °C and the plate temperature was set at the range of 40 to 70°C. Besides the syringes and digital pneumatic regulator, dispensing needles of 20 (nozzle diameter: 600μm), 22 (nozzle diameter: 500μm) and 25 gauge (nozzle diameter: 250μm) were used for extrusion. After printing, the printed samples were immersed in the coagulation bath for 30 minutes (T≥40 °C) to achieve a totally gelation. Then the samples were washed by distilled water for several times to remove the residual alkali. After that, the chitosan hydrogel sample was hold in deionized water at 5°C for further characterization.

SI. 3. Rheological Characterization

Rheological properties of the chitosan ink were measured by a rheometer (MCR102, Anton Paar, Austria) equipped with a plate- plate with a diameter of 25 mm. The measurements of viscosity and shear stress were performed by varying the shear rate from 0.01 to 1000 s−1 at 5 °C. The storage modulus (G') and loss modulus (G'') was measured as a function of temperature at a constant frequency of 1 Hz and a constant strain of 0.1%, while the hydrogel samples were equilibrated at 5 °C and then heated at a rate of 1 °C/min from 5 to 50 °C.

SI. 4. Mechanical Testing

The mechanical properties of chitosan hydrogels were measured using an electronic universal testing machine UTM2203 (Shenzhen Suns Technology CO., Ltd, China). The tensile test was carried out at 40 mm/min at a preforce of 0.005 N and the compressing test was carried out at 1 mm/min and a preforce 0.01 N. The Young's modulus was determined between 0.5% and 5% strain. Reported values for ultimate tensile strength and Young's modulus were averaged over at least five samples. The size of the hydrogel specimens for tension and compression tests were 60 mm × 10 mm × 1 mm and 10 mm × 10 mm × 10 mm, respectively.

SI. 5. Biocompatibility Characterization

SI. 5.1. Cell Culturing and Seeding

HUVEC lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The medium was changed every two days and the cell lines were passaged once they reached around 90% confluence.

SI. 5.2. Cell seeding

The printed scaffolds were immersed in the 70% alcohol and exposed under the UV light for 30min. After that, the scaffolds were washed with sterilized deionized water for three times. Then, the scaffolds were immersed in the DMEM and placed in a humidified atmosphere of 95% air and 5% CO2 at 37°C for 12 hours. At last, the HUVUCs were detached and resuspended in the DMEM and seeded on the treated scaffolds and cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

SI. 5.3. Cell Activity Analysis

Cell viabilities were measured after 1, 2 and 3 days culturing. The cell viabilities were tested with the cell LIVE/DEAD assay. The confocal fluorescence microscopy was used to image the cells. The live and dead cell quantifications were counted with Image software.

SI. 5.4. Cell Morphology Analysis

Cells were observed after 1, 2 and 3 days culturing. The morphologies of the HUVECs were visualized by cell cytoskeleton dyeing, including F-actin and nucleus dyeing with TRITC phalloidin and DAPI dyeing solution. The confocal fluorescence microscopy was used to image the cells.

SI. 6. *In vivo* wound healing model

SI. 6.1. Settings for rats wound healing models

The wound-healing efficacy of printed chitosan scaffolds was evaluated in male Sprague–Dawley rats weighing 250–300 g. 12 rats were used to finish these experiments. And for each rat, two full-thickness round skin wounds were created and one wound was treated with gauze and the other was treated with printed chitosan scaffold. The chitosan scaffolds were printed as size of diameter of 15 mm, height of 3 mm and porosity of 85%.

After anaesthetization with 3% pentobarbital sodium (45 ~ 60 mg / kg) by intraperitoneal injection, the dorsal region of the rats was shaved, and the hair around surgical areas was completely removed. Then the area was then antiseptically prepared with 10% w/v povidone-iodine. Two full-thickness round skin wounds (1.5 cm diameter) were created on the dorsum of each rat (Figure S3A). After the debridement treatment, the two wounds were affixed with radiation sterilization treated gauze and printed chitosan scaffold, respectively. Moreover, the gauze was fixed with a medical adhesive bandage (Figure S3B), and then the rats were returned to the corresponding cages for separate breeding. These experiments lasted 14 days.

SI. 6.2. Histopathological study

The infection and healing of the wound were observed at specific time points, and the wound area was recorded with a camera. The IPP 6.0 software was used to evaluate the size of wound area. The change in wound area over time is expressed as a percentage of the initial wound area. Rats were euthanized on days 3, 7 and 14 after fibrous membrane treatment, and wounds and surrounding healthy skin tissue were removed for subsequent experiments. After all the experiment, all rat carcasses were harmlessly treated.

Wound histology samples were collected on day 3, 7 and 14. Each specimen was trimmed into one or two parts based on the granulation tissue. All of the samples were fixed in 4% paraformaldehyde solution for at least 24 h. After dehydration, the samples were embedded in paraffin, and 4 μm-thick tissue sections were prepared. Representative sections were stained

with haematoxylin and eosin (H&E) and Masson trichrome by light microscopy.

SI. 6.3. Immunohistochemical staining of new blood vessel (CD31) and mature blood vessel (α-SMA)

After rehydration, the wound samples were placed in a repair box filled with EDTA antigen retrieval buffer (pH = 9.0) for antigen retrieval in a microwave oven. Then the samples were placed in a 3% hydrogen peroxide solution and incubated at room temperature for 25 minutes in the dark to block endogenous peroxidase. After blocking serum with 3% BSA, primary (TGF-β、TNF-α、IL-1β、IL-6) and secondary antibodies (IgG H&L (HRP)) were added to the samples sequentially. Then the wound samples were stained with DAB and hematoxylin chrome. The representative sections were collected by light microscopy, after dehydration.

SI. 6.4. Immunohistochemical staining of transforming growth factor-β (TGF-β), tumor necrosis factor (TNF-α), interleukin-1 (IL-1β), interleukin-6 (IL-6)

Then the samples were placed in a 3% hydrogen peroxide solution and incubated at room temperature for 25 minutes in the dark to block endogenous peroxidase. After blocking serum with 3% BSA, primary (TGF-β、TNF-α、IL-1β、IL-6) and secondary antibodies (IgG H&L (HRP)) were added to the samples sequentially. Then the wound samples were stained with DAB and Harris hematoxylin chrome. The representative sections were collected by light microscopy, after dehydration.

SI. 7. Statistical Analysis

Data were expressed as mean ±standard deviation. Statistical analysis is conducted using ANOVA, and single asterisk (*) indicates significant differences between groups ($p < 0.05$) and double asterisk (**) indicates very significant differences between groups ($p < 0.01$)).

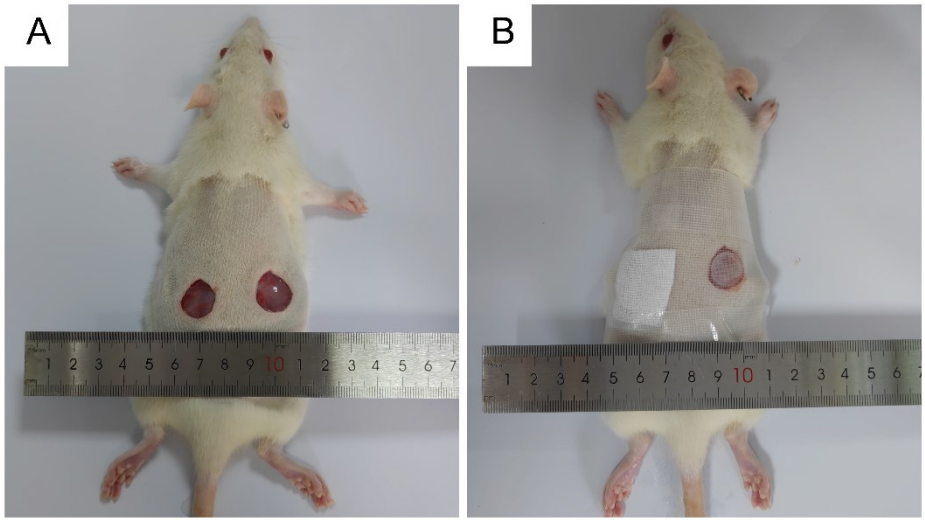

Figure S1. (A) Optical photographs of full-thickness skin lesions in SD rats. (B) Optical photographs of wounds treated with gauze (left) and printed chitosan scaffolds (right).

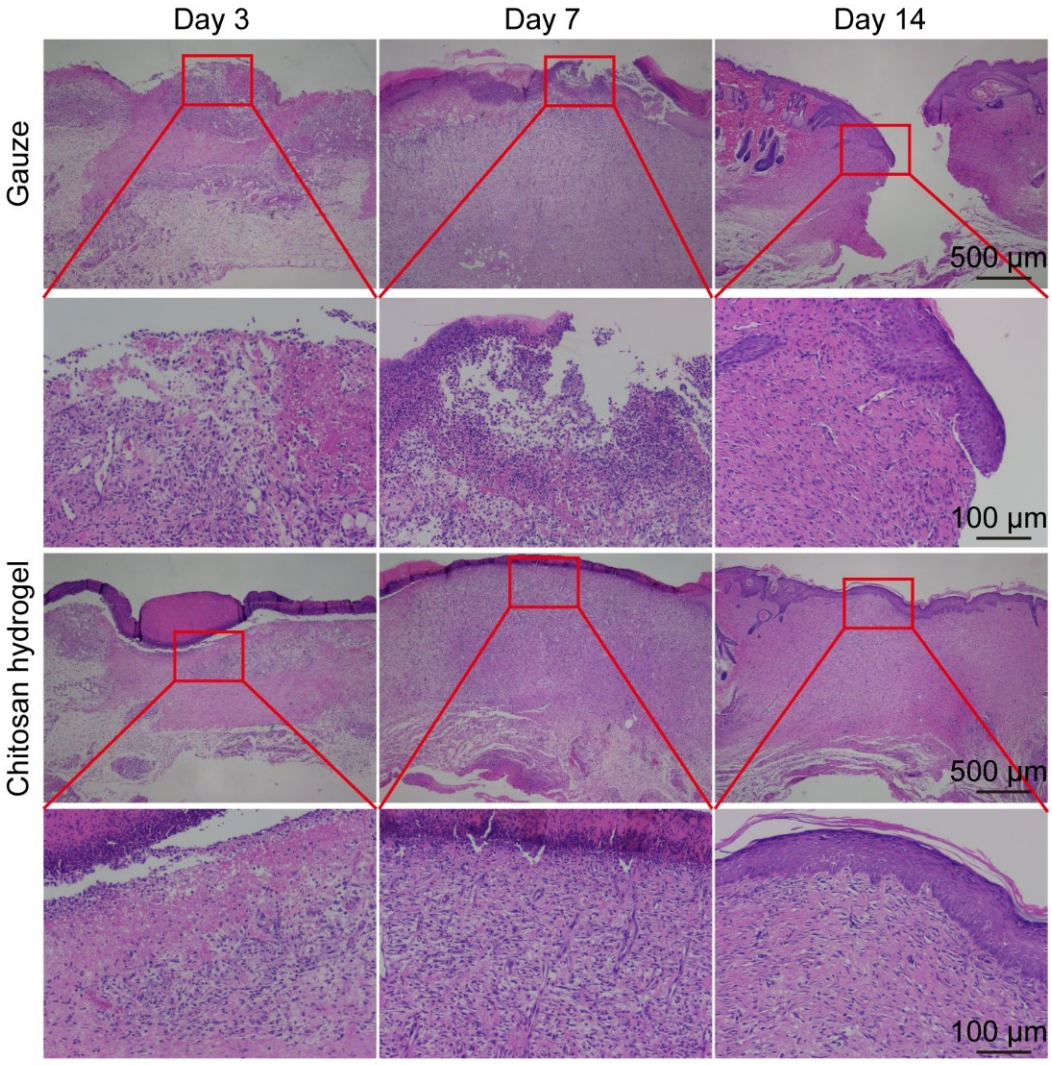

Figure S2. Representative photographs of tissue areas on Day 3, 7 and 14 by hemotoxylin and eosin (H&E) staining.

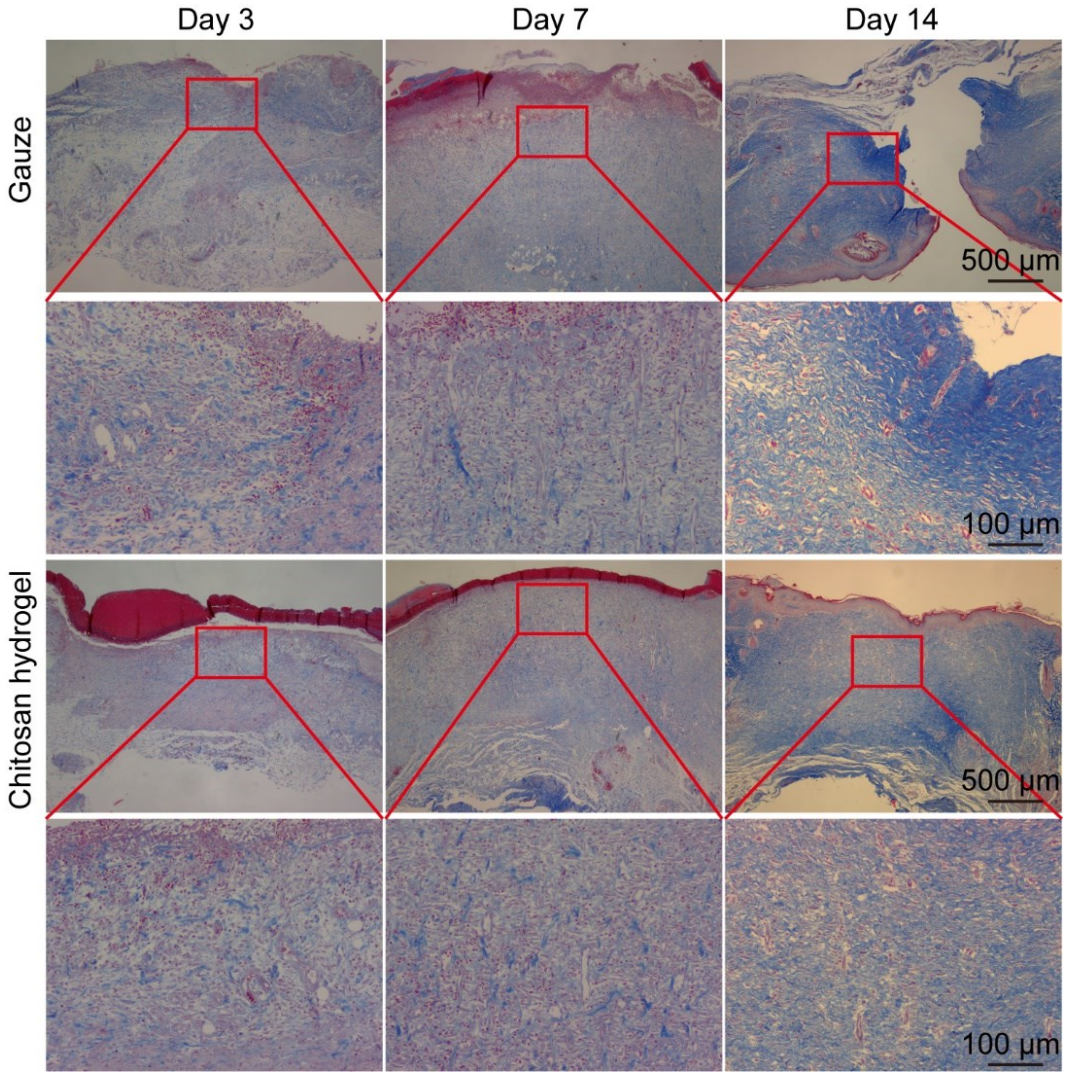

Figure S3. Representative photographs of tissue areas on Day 3, 7 and 14 by Masson's trichrome staining.

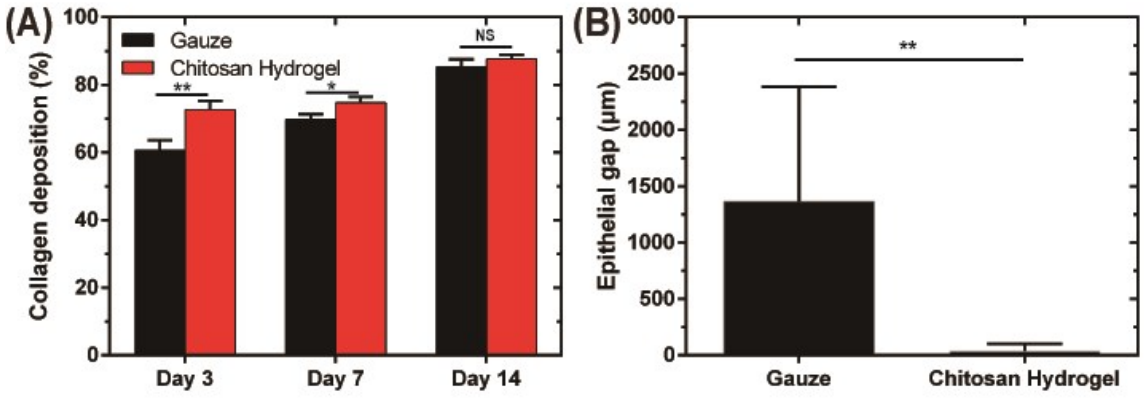

Figure S4. Quantitative analysis of (A) collagen content and (B) epithelial gap in wound tissue at day 14.

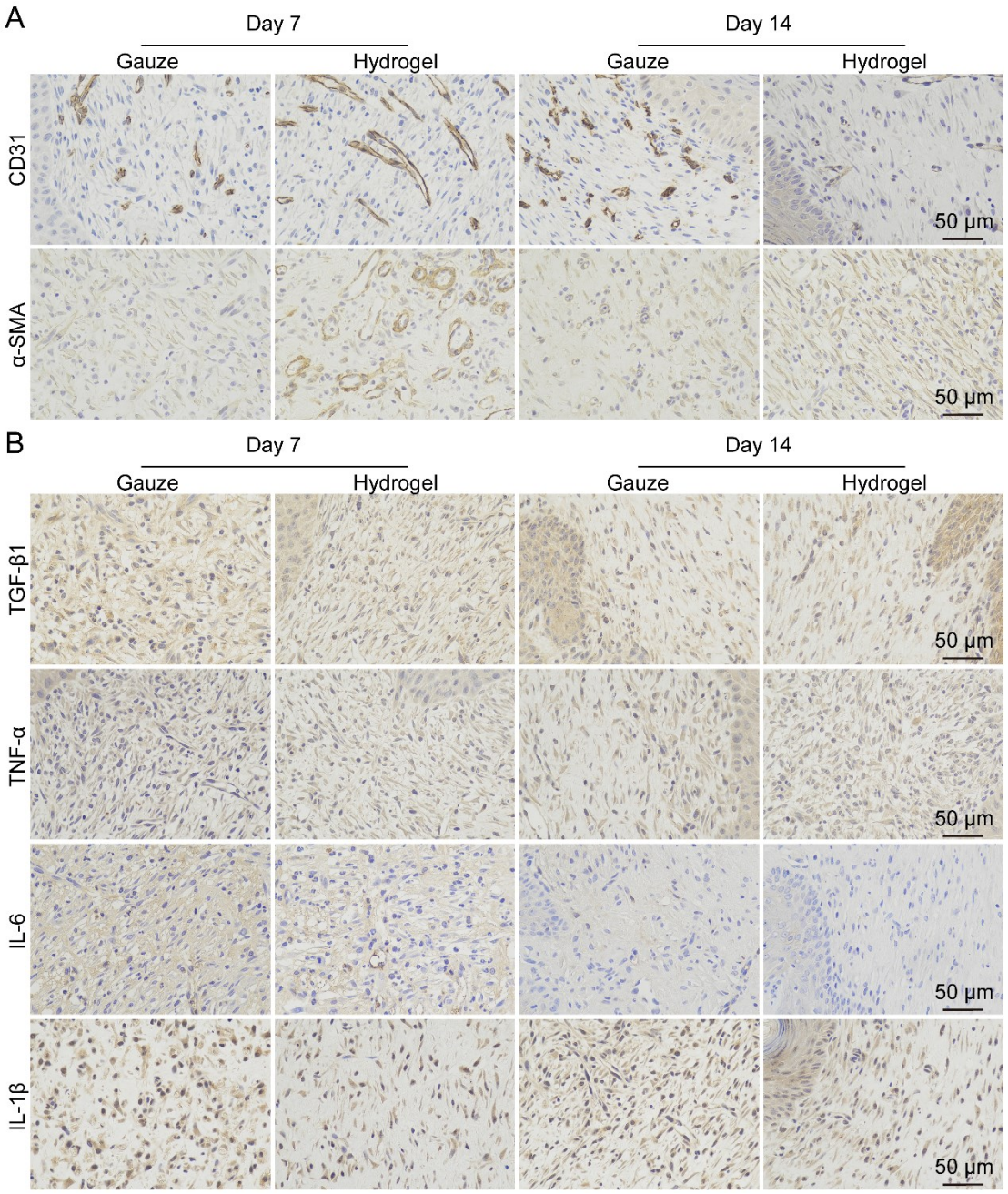

Figure S5. (A) Representative photographs of tissue areas on Day 7 and Day 14 by immunohistochemical CD31 and α-SMA staining. (B) Representative photographs of tissue areas on Day 7 and Day 14 by immunohistochemistry with anti-inflammatory factors (TGF-β1) and pro-inflammatory factors (TNF-a, IL-6 and IL-1β).