Supplementary Information

Bottom-up reconstitution design of a biomimetic atelocollagen microfibril for enhancing hemostatic, antibacterial, and biodegradable benefits

by

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S1 Supporting Methods

S 1.1 Visualization of biomimetic D-periodicity

Atomic force microscopy (AFM) was employed to investigate the *D*-periodicity of all tested samples. The purified samples were thoroughly dispersed in ultrapure water using high-speed stirring. A 10 μL aliquot of this dispersion was then deposited onto a freshly cleaved mica substrate and allowed to air dry at room temperature for 48 h. AFM measurements were conducted using an atomic force microscope (Eclipse Ti–U, Nikon, Japan) in tapping mode. The acquired images were subsequently analyzed with SPM Manager software.

S 1.2 Quantitation of atelocollagen and ε-PL incorporated into fibrils

Accurately weigh 2 g of atelocollagen microfibril suspensions, which were then centrifuged at 10,000 rpm for 15 minutes at 4 °C to collect the supernatants. Next, add 100 μL of the supernatants to 3 mL of 6 mol/L HCl and hydrolyze at 110 °C for 24 h. After hydrolysis, neutralize the solutions with 6 mol/L NaOH in an ice bath and dilute to a final volume of 25 mL using citric acid buffer (pH 6.0). Additionally, 100 μL of the initial mixed solution was used to replace the centrifuged supernatant, which was then hydrolyzed using the aforementioned method and subsequently diluted to volume. Finally, the hydroxyproline (Hyp) content in both the initial solution and the supernatant was determined according to the method described by Zhang et al. [1]

Another set of the supernatants and the initial mixed solution were analyzed to evaluate the ε-PL content utilizing a slightly modified Itzhaki colorimetric method. Specifically, A volume of 50 μL of the supernatants and the initial mixed solution were combined with 4 mL of the methyl orange solution and incubated at 30 °C for 30 minutes，respectively. Following incubation, the solutions were centrifuged at 6000 rpm for 15 min to isolate the testing supernatant. This supernatant was then diluted tenfold with

a 0.1 M phosphate buffer solution (pH 6.8), and the absorbance of the dilutions was measured against a blank using a UV-Vis spectrometer (PerkinElmer Ltd., Massachusetts).

S 1.3 In vitro hemostasis assay

The whole blood clotting array and index were evaluated as previously reported with slight modification. [2] A volume of 50 µL of rabbit whole blood, recalcified with 2.5 µL of 0.2 M $CaCl_2$, was added to the pre-warmed samples weighing 5 mg and incubated at 37 ℃ for 30, 60, 90, 120 and 150 s. Subsequently, 10 mL of distilled water was gently introduced to terminate the reaction and release unbound blood. Photographs were taken at various time points to document the speed of clot formation. The supernatant from each group was collected and the absorbance was measured at 540 nm (B_s) using a UV - Vis spectrometer (PerkinElmer Ltd., Massachusetts). The absorbance of 50 µL of recalcified whole blood in 10 mL of distilled water was used as the reference value (Br), while the absorbance of distilled water as the blank value (Bw). The blood clotting index (BCI) was calculated using the following equation:

$$BCI\ (\%) = \frac{B_s - B_w}{B_r - B_w} \times 100\%$$

Red blood cell (RBC) and platelet adhesion assays were conducted using a modified version of the reported method. [3] A 200 µL suspension of RBC was applied to 10 mg of the samples and incubated at 37℃ for 1 h. Non-adherent RBCs were removed by rinsing five times with PBS. The adherent RBCs were then transferred into 5 mL of distilled water to lyse the erythrocytes for 1 h. The supernatant from each group was collected and the absorbance at 540 nm (R_s) was measured using a UV - Vis spectrometer. A control consisting of a 200 µL RBC suspension mixed with 5 mL of distilled water was designated as the blank (R_b). The RBC adhesion rate was calculated using the the following formula:

$$RBC\ adhersion\ rate\ (\%) = \frac{R_s}{R_b} \times 100\%$$

Platelet-rich plasma (PRP) was obtained from the supernatant of rabbit whole blood after centrifugation at 200 × g for 15 min. PRP of 100 μL was added to 10 mg of samples and incubated at 37 °C for 1 h. Non-adherent platelets were removed by rinsing five times with PBS. The samples were then treated with 400 μL of Triton X-100 at a concentration of 1% to release lactate dehydrogenase (LDH). The platelet lysate from each group was mixed with LDH operating fluid at a 1:1 (V/V) ratio, following the LDH kit instructions, and the optical density (OD) value was determined at 490 nm (P_s). The absorbance of a solution containing of 100 μL of PRP and 400 μL of Triton X-100 was used as a blank (P_b). The platelet adhesion rate was calculated using the following formula:

$$Platelet\ adhersion\ rate\ (\%) = \frac{P_s}{P_b} \times 100\%$$

S 1.4 Antibacterial measurements

A antibacterial test was conducted using a broth dilution assay, following a modified method described by Wang. [4] Overnight bacterial suspensions were diluted to approximately 10^6 CFU/mL using 18% (w/v) Nutrient Broth (NB, Beijing Aoboxing Bio-tech Co, LTD.). Each sample of 10 mg was added to 5 mL of bacterial suspensions of *E. coli* and *S. aureus*, and the mixtures were incubated at 37 °C for 24 h. The sample-free bacterial suspensions served as the blank. Following incubation, the bacterial suspension was collected and subjected to serial dilution in physiological saline (10^2 - 10^5). A volume of 100 μL from the diluted bacterial suspension was inoculated onto agar plates. Colony-forming units (CFU/mL) were counted after a further incubation at 37 °C for 24 h. The relative inhibition rate of the bacteria was calculated as follows:

$$Bacteria\ relative\ inhibition\ rate\ (\%) = \frac{CFU_{blank} - CFU_{sample}}{CFU_{blank}} \times 100\%$$

Three independent experiments were performed and the mean measurements were assessed.

S 1.5 Hemocompatibility and biocompatibility evaluation in vitro

The hemocompatibility of the atelocollagen microfibrils was evaluated following a published protocol for observing and measuring hemoglobin release. [5] Red blood cells (RBCs) were isolated from sodium citrate anticoagulant rabbit blood through centrifugation at 200 × g for 15 min. The RBCs were then washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) and diluted to prepare an erythrocyte suspension at a concentration of 5% (V/V). The microfibrils were subsequently dispersed into erythrocyte suspension at concentrations of 1, 2, 3, 4, and 5 mg/mL. After incubating for 1 h at 37℃, the mixtures were centrifuged at 200 × g for 15 min. The resulting supernatants (200 μL) were then transferred into a 96-well plate to measure the absorbance at 540 nm (OD_s) using a microplate reader (Spectra Max 190). Gauze and Avitene hemostat served as control groups, while negative and positive controls were established using DPBS (OD_n) and 1% Triton X-100 (OD_p), respectively. The hemolysis rate was calculated as following:

$$Hemolysis\ rate\ (\%) = \frac{OD_S - OD_P}{OD_n - OD_P} \times 100\%$$

The biocompatibility of the microfibrils was evaluated using CCK-8 assay. In brief, 20 mg of the microfibrils was immersed in 10 mL of medium and incubated for 24 h. Subsequently, 100 μL of the extract was added to L929 fibroblasts cultured in 96-well plates, followed by incubation at 37 °C for 1, 3, and 5 days in a 5% CO_2 environment. At the conclusion of the incubation period, 20 μL of CCK-8 working solution was added to each well and incubated at 37 °C for an additional 3 h. The absorbance of the solutions was then measured at 450 nm using a microplate reader (Multiskan FC, USA). The

absorbance of the test solution without the sample and cells was used to zero the absorbance, while L929 cells without the sample served as the blank control (OD_0). The absorbance of the sample group was designated as OD_s.

The relative growth rate (RGR) of the cells was defined as follows:

$$RGR\ (\%) = \frac{OD_S}{OD_0} \times 100\%$$

The Live/Dead staining assay and morphology staining assay were conducted by incubating L929 fibroblasts with the aforementioned extracts at a 1:1 (V/V) ratio for 3 days. The AM/PI dye was employed for the Live/Dead staining assay in accordance with the instructions provided in the reagent kit. The morphology staining assay was performed as follows: the incubated cells were fixed with paraformaldehyde at 4 °C for 4 h, followed by staining with fluorescein isothiocyanate phalloidin (TRITC phalloidin) and a subsequent co-incubation period of 30 min at 37 °C in darkness. After washing the cells three times with PBS, the surface cells were re-stained with a 4,6-diamidino-2-phenylindole (DAPI) solution. The indicators of cytotoxicity and cell growth morphology were visualized using a fluorescence microscope (TI2-U, Nikon, Japan).

S 1.6 Scratch and transwell migration assay

The scratch migration assay was conducted according to the methods outlined by Yang et al. [6] Briefly, L929 fibroblasts were cultured to 80% confluence in a 6-well plate and then starved for 24 h in DMEM containing 0.1% fetal bovine serum (FBS) after washing away dead cells with PBS. The monolayer cells were subsequently scratched using a 100-μL sterile pipette tip and washed twice with PBS to remove cellular debris. Two milliliters of the microfibril extracts and two milliliters of fresh medium (10% FBS) were added to the 6-well plates. Avitene was used as the control group, with PBS

serving as the blank in place of the extracts. Images of the scratch area were captured immediately after scratching and at 6 and 12 h using an inverted phase-contrast microscope (IX2-SL Olympus, Japan). Results were analyzed using Image-J software, and the percentages of closed area were measured and compared with the PBS group.

The transwell migration assay was conducted according to the following procedure. L929 fibroblasts were seeded into 8 μm pore size transwell inserts in serum-free DMEM at a density of 1 × 10^5 cells per well. The microfibril extracts were added to the lower chamber of the insert, with Avitene and fresh medium serving as the control and blank, respectively. After 12 h, the cells on the upper side of the insert were removed by carefully scraping, and the migrated cells were fixed in paraformaldehyde. Finally, the cells were stained with 0.1% crystalline violet and quantified by counting the number of cells observed under an inverted phase-contrast microscope (IX2-SL Olympus, Japan).

S 1.7 In vitro biodegradation

The biodegradability of the microfibrils was investigated by incubating in PBS (pH=7.4) containing 1 U/mL type I collagenase (≥125 U/mg, C0130, Sigma-Aldrich, Munich, USA) at 37°C to simulate the degradation process under physiological conditions. [7] The microfibrils weighing 20 mg (W_0) were immersed in 10 mL of PBS and incubated at 37 °C. At different time points, incubated samples were placed in an ice bath immediately to terminate the enzymatic degradation and then were withdrawn from the solution, freeze-dried, and weighed (W_n). The weight loss of the microfibrils after degradation at each time point was calculated according to Eq.,

$$Weight\ loss\ (\%) = \frac{W_0 - W_n}{W_0} \times 100\%$$

S 1.8 Identification of collagen isoelectric point

The lyophilized atelocollagen was dissolved in 0.05 mol/L HCl at final concentration of 0.5 mg/mL and was continuously stirred at 4 ℃ for 24 h. The pH value of atelocollagen solution was adjusted to 2.5 – 8.5 using 1 mol/L NaOH. One milliliter atelocollagen solution was transferred to a capillary cell, and collagen Zeta (ζ) potential measured using a Zeta potential analyzer (NanoBrook Omni, Brookhaven Instr., UK). The isoelectric point (pl) was determined.

S 1.9 Preparation of pure atelocollagen fibrils and non-physiological rearrangement atelocollagen fibrils

Pure atelocollagen fibril, referred to as Col, free of ε-PL and TGase, were obtained following the method outlined for the preparation of BCF-10. Specifically, freeze-dried atelocollagen was dissolved in PBS solution at 4 ℃ for 24 h, after which it was placed at 25 ℃ and incubated with stirring for 24 h. Non-physiologically rearranged atelocollagen fibril, designated as ICol, were produced through isoelectric precipitation. In this process, freeze-dried atelocollagen was dissolved in 0.05 mol/L HCl to achieve a final concentration of 10 mg/mL and stirred continuously at 4 ℃ for 24 h. The pH of the atelocollagen solution was then adjusted to the isoelectric point while maintaining continuous stirring. Following centrifugation, the solutions were freeze-dried using 10% tert-butanol as the solvent.

S2 Supporting Figures

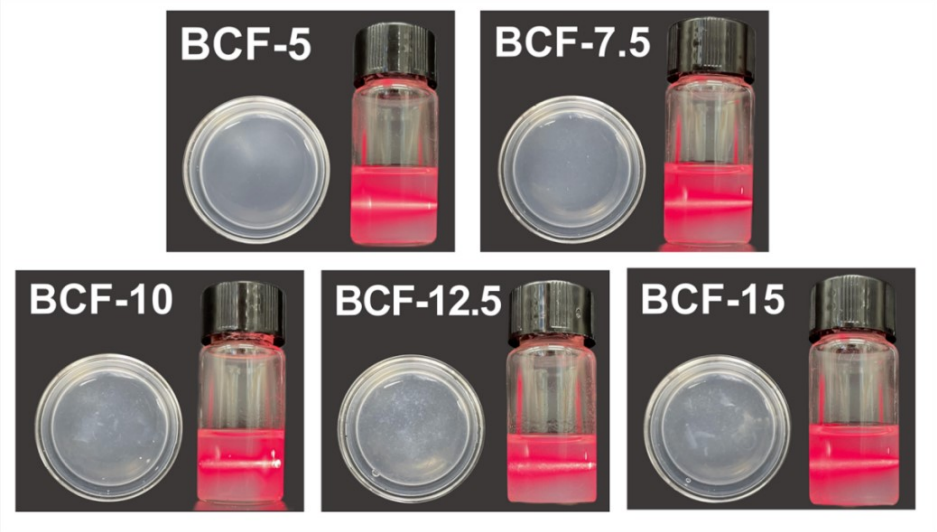

Fig. S1 Digital pictures of the atelocollagen fibrils (diluted to 2 mg/ml) reconstituted at various atelocollagen concentrations

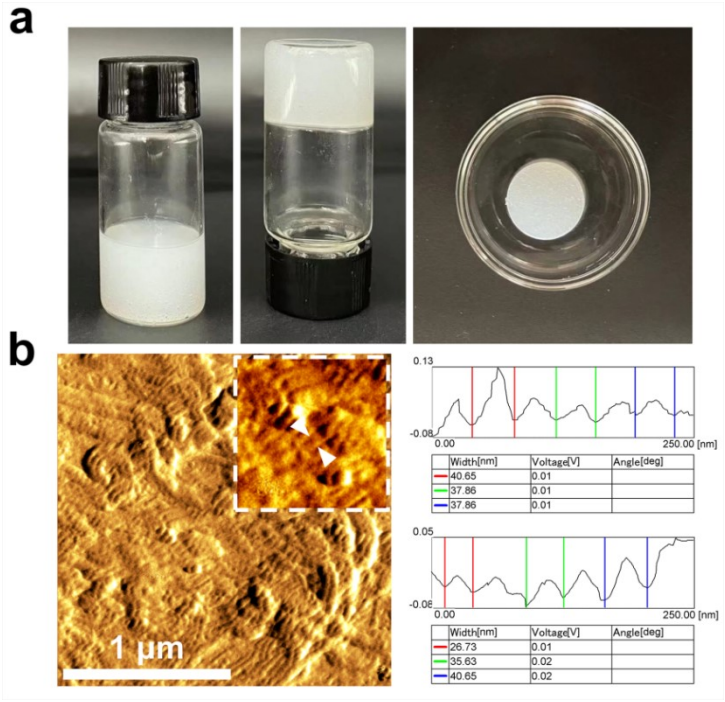

Fig. S2 Digital pictures and AFM images and the amplitude of fibrils surface along the white box (length = 250 nm) of the atelocollagen fibrils incubated under non-shear conditions at 37 °C at a concentration of 10 mg/mL

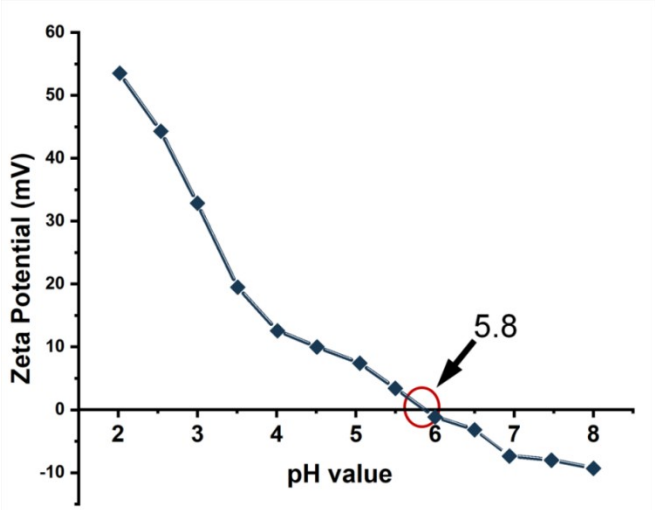

Fig. S3 Isoelectric point titration graph of atelocollagen

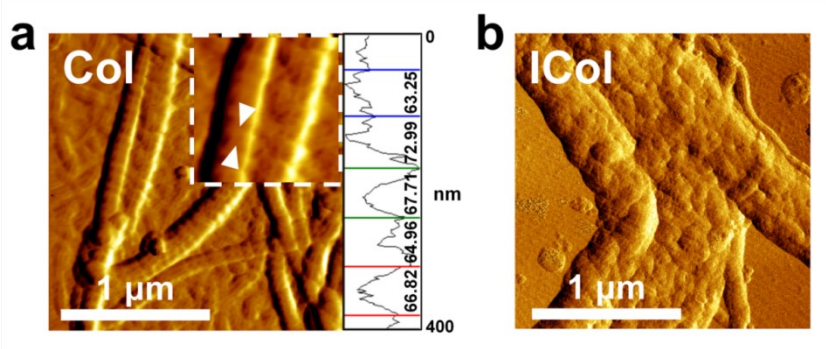

Fig. S4 AFM images of (a) Col and (b) ICol

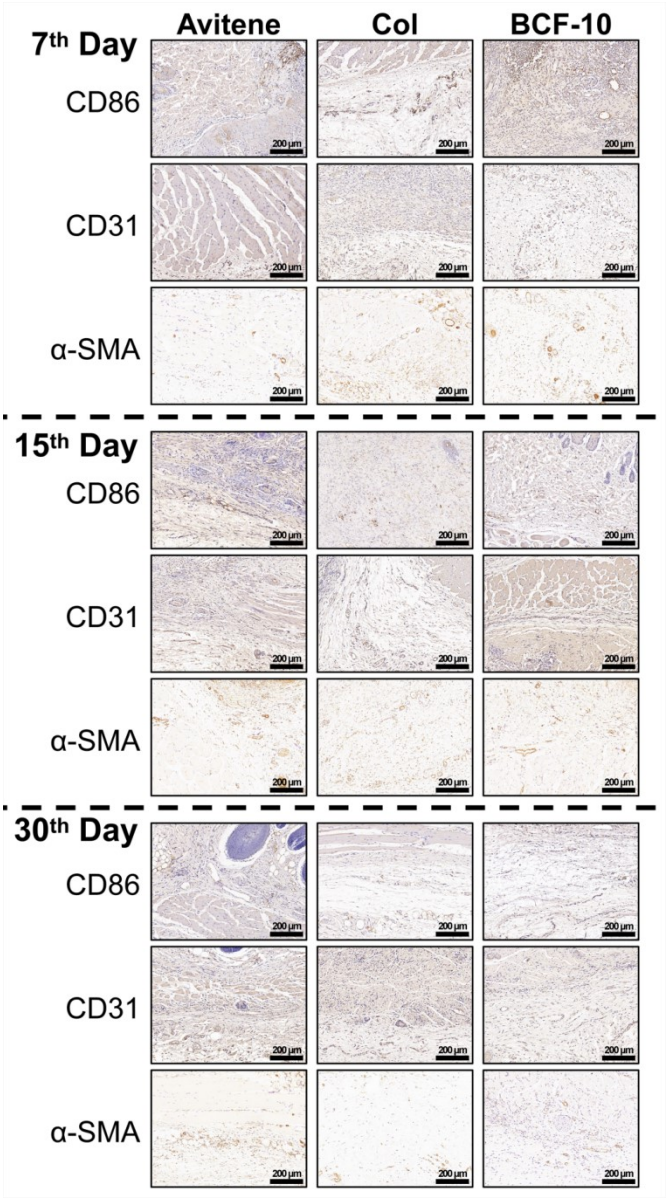

Fig. S5 The representative immune-identifications of CD86, CD31, and α-SMA of implantation areas after 7, 15, and 30 days.

References

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