Supporting Information

Hierarchically micro-patterned nanofibrous scaffolds with nanosized bio-glass surface for accelerating wound healing

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Experimental Section

Preparation of patterned electrospun scaffolds with nano-sized bioglass: The blend of PDLLA (Mn=45 kDa, D/L=50/50, Jinan Daigang Biomaterial Co, Ltd, China) and PCL (Mn=80 kDa, Sigma-Aldrich) (1/1, w/w) were dissolved in a mixture of DMF and THF (4:1, v/v), and then stirred for six hours to obtain a homogeneous and stable solution (4.8%, w/v). The solution was fed at a constant rate (0.2 mL h^{-1}) by a syringe pump (LSP01-1A, Baoding Longer Precision Pump, China) through a 21 gauge blunt tip needle. A high-voltage power supply (HV30, NanoNC) was used to apply a high voltage (8 kV) between the metal needle and the collector. The needle-to-collector distance was set to 12 cm. The electrospun nanofibrous scaffolds with different micro/nano patterns were fabricated using the same method introduced in our previous work.[1] In addition, electrospun scaffolds with non-woven structures were also prepared using a plane collector as controls. These patterned and non-woven electrospun scaffolds were named as PT and NW, respectively.

An akermanite (AKT; $Ca_2MgSi_2O_7$) ceramic disk (Ø20 × 3 mm) was prepared by sintering the AKT green compacts at 1350 °C according to a previous study.[2] The

AKT disk was used for the targets to coat samples. The prepared electrospun scaffolds were fixed on the sample stage in the chamber of the PLD instrument using double-sided adhesive. Then, the sintered AKT disk was ablated using focused laser fluence of 180 MJ with a pulsed repetition rate of 5 Hz at room temperature. The O_2 ambient was set with a pressure of 20 MPa. The treating time was set at 30 min to obtain AKT coatings with a uniform thickness. The prepared patterned and non-woven electrospun scaffolds with nano-sized bioglass coatings were named as PT-NBG and NW-NBG, respectively.

Microstructure, composition and surface wettability of the electrospun scaffolds: The morphology and microstructure of the prepared composite scaffolds were observed by scanning electron microscopy (SEM; FEI Magellan 400). The chemical composition and phase structure of the prepared films was characterized by energy dispersive spectrum (EDS) and X-ray diffraction (XRD, D8 ADVANCE). The hydrophilicity of the prepared films was investigated by testing the water contact angle (Kruss GmbH DSA 100 Mk 2).

ICP-AES analysis for ionic release: To measure the ionic release of Si, Ca and Mg from scaffolds, five scaffolds (25×25 mm) were soaked in 10mL of phosphate buffered saline (PBS) (pH 7.4) at 37°C for 1, 3 and 7 days. The solution was refreshed at each time point. The concentrations of released Si, Ca and Mg ions in the PBS were determined by inductive coupled plasma atomic emission spectrometry (ICP-AES) (Varian 715ES).

Cell culture: Human umbilical vein endothelial cells (HUVECs) were isolated from the human umbilical cord vein according to the descriptions of Bordenave et al. and Jaffe.[3,4] The procedure was approved by the Ethics Committee of Chinese Academy of Sciences. The obtained cell pellets were re-dispersed in endothelial cell medium (ECM) (Sciencell, USA) containing 5% (vol./vol.) FBS and 1% (vol./vol.) endothelial cell growth supplement/heparin kit (ECGS/H, Promocell). In this study, cells between passages 3 and 5 were employed.

HUVECs proliferation assay: Scaffolds were first sectioned into 10 × 10 mm^2 squares, and then and soaked in 75 vol.% medical alcohol solution for 2 h for sterilization prior to seeding cells on the samples. HUVECs were seeded on the surfaces of those scaffolds at a density of 1.5 × 10^4 cells per well in a 24-well culture plate, and then cultured in a humidified 37 ^oC/5%, CO_2 incubator for 6, 24 and 72 hours. Cells cultured on the culture plates were regarded as controls. The medium was replaced every 1 days. A CCK-8 assay (Cell counting kit-8, Dojindo, Kumamoto, Japan) was performed according to the manufacturer's instructions. The absorbance of the samples was measured at 450 nm using an enzyme-linked immunosorbent assay plate reader (Synergy 2, Bio-TEK).

Immunofluorescence staining: For further investigating the cytoskeletal and nuclear organizations of HUVECs cultured on different scaffolds, the actin filaments and nuclear of HUVECs were stained utilizing rodamine phalloidin R415 (Invitrogen) and 4-6-diamidino-2-phenylindole (DAPI) (Invitrogen) according to the supplier's

procedure, respectively. A confocal microscope (Leica TCS SP5) was used to image the morphologies of the seeded cells on different scaffolds.

Animal model of full thickness wound: All animals were obtained from the National Rodent Laboratory Animal Resources, Shanghai Branch of China. The mice were bred and maintained in the animal center in East China Normal University and all of the experimental protocols were approved by the Animal Investigation Committee of the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University.

A total of 55 Male, BALB/c mice weighing approximately 18 g and five mice for each group were applied to evaluate the wound healing efficiency. After mice were anesthetized with anesthetic ventilator and the dorsal hair was shaved. Then, we made a circular trace using a punch (diameter of 8 mm) and cut out a circular full-thickness wound along the trace. The created wounds were covered with four the prepared scaffolds. Four kinds of groups were (a) no treatment, (b) NW, (c) NW-NBG, (d) PT and (e) PT-NBG, respectively. All the scaffolds were firstly cut into round samples with diameter of 8 mm by using a punch, and then the samples were sterilized by 75% ethanol solution for 30 min and washed thrice with sterilized PBS before applied to cover the wound area. After that the wound area was treated with medical grade adhesive bandage for the conventional wound care. After surgery, each mouse was caged individually with free access to water and food under SPF environmental conditions so as to avoid antibacterial interference caused by external conditions.Once

wounded, digital pictures at a fixed distance and angle of the wound area were captured to measurement of wound size change. At 3 and 7days after the surgery, mice were sacrificed by inhalation of carbon dioxide. Moreover, the tissue surrounding the wounds (2 mm farther) was removed.

The method to calculate the wound sizes percent reduction in wound size:

Wound size reduction (%) =[A_0-A_t]/A_0 ×100%

Where A_0 and A_t are the initial wound area and wound area at a time interval "t", respectively.

Histomorphometric analysis and Immunohistofluorescence of wound tissue sections: On day 3 and 7, the wounds were excised. After fixation with 4% paraformaldehyde for at least 12 h, the samples were dehydrated in graded alcohols, dimethylbenzene and then the specimens were embedded in paraffin.

Finally the tissue sections were sectioned into samples with thickness of 5 μm. Histological evaluation and immunohistofluorescence assay was performed according to the method previously described.[5] Histological evaluation, the samples were deparaffinized, rehydrated and stained with haematoxylin and eosin for routine histology processing. Masson's trichrome stain was performed for observation of the collagen framework and collagen expression. All images were taken at ×20 magnification and Image Pro Plus version 6.0 (Media Cybernetics, Rockville, MD, USA) was used to quantify the re-epithelization and the collagen per area, the intensity RGB threshold wasset for the blue–green positive colour of collagen and the

number of positive pixelswas counted. For every counting, images with ×20 magnification were used and five random positions were chose for each image.[5] For immunohistofluorescence staining, the tissue sections (5μm thick) were deparaffinized and boiled for 20 min in sodium citrate buffer. After that the sections were cooled down to room temperature for at least 1 h and incubated with primary antibodies overnight in 4°C environment, including rabbit polyclonal to CD31 antibody (Abcam, product number ab28364). Then sections were washed in PBS solution, incubated with a secondary antibody for 2 hours at room temperature. Then, 4',6-diamidino-2-phenylindole (DAPI) was added to samples for 5min, avoiding light. Slides were evaluated using an optical microscope (Leica Confocal microscope).

Real-Time Polymerase Chain Reaction: The mRNA transcript level of VEGF was assessed by real-time PCR. The primer sequences were designed using the primer 5.0 software. The wound tissue total RNA was isolated with Trizol reagent (Invintrogen). 1 μg of total RNA was reverse transcribed into cDNA with reverse transcriptase and oligo dT primers (Promega, Madison, USA). In this study, the expression levels of genes were relative to expression of the housekeeping gene β-actin. The expression of genes for different biological materials treated wounds were compared with those without treatments. The following primer sequences were used:

VEGF sense: 5'-CTGCTCTCTTGGGTGCACTGT-3',

antisense: 5'-AGATGTCCACCAGGGTCTCA-3'.

PCR cycles were 5 minutes at 95°C, followed by 42 cycles with an annealing temperature of 58°C.

Western blotting: Protein were prepared from skin of the wound site and lysed with RIPA buffer containing protease/phosphotase inhibitors (Roche). Protein concentration was determined using a Bicinchoninic acid assay (Thermo Scientific) for western blotting. Then the simple boiled for 10 min for protein denaturation. Whole samples were run on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Gibco). Membranes were blocked in 5% milk for 1 h. The membranes were incubated at 4°C overnight using specific antibodies: COL3A1 (H-300) (product number sc-28888) and Pro-COL1A2 (Y-18) (product number sc-8787) (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma). The visual signals were visualized via the Odyssey Western blotting detection system. The β-actin levels were determined by Western blotting to correct the Col 1 and Col 3 protein band intensity.[6]

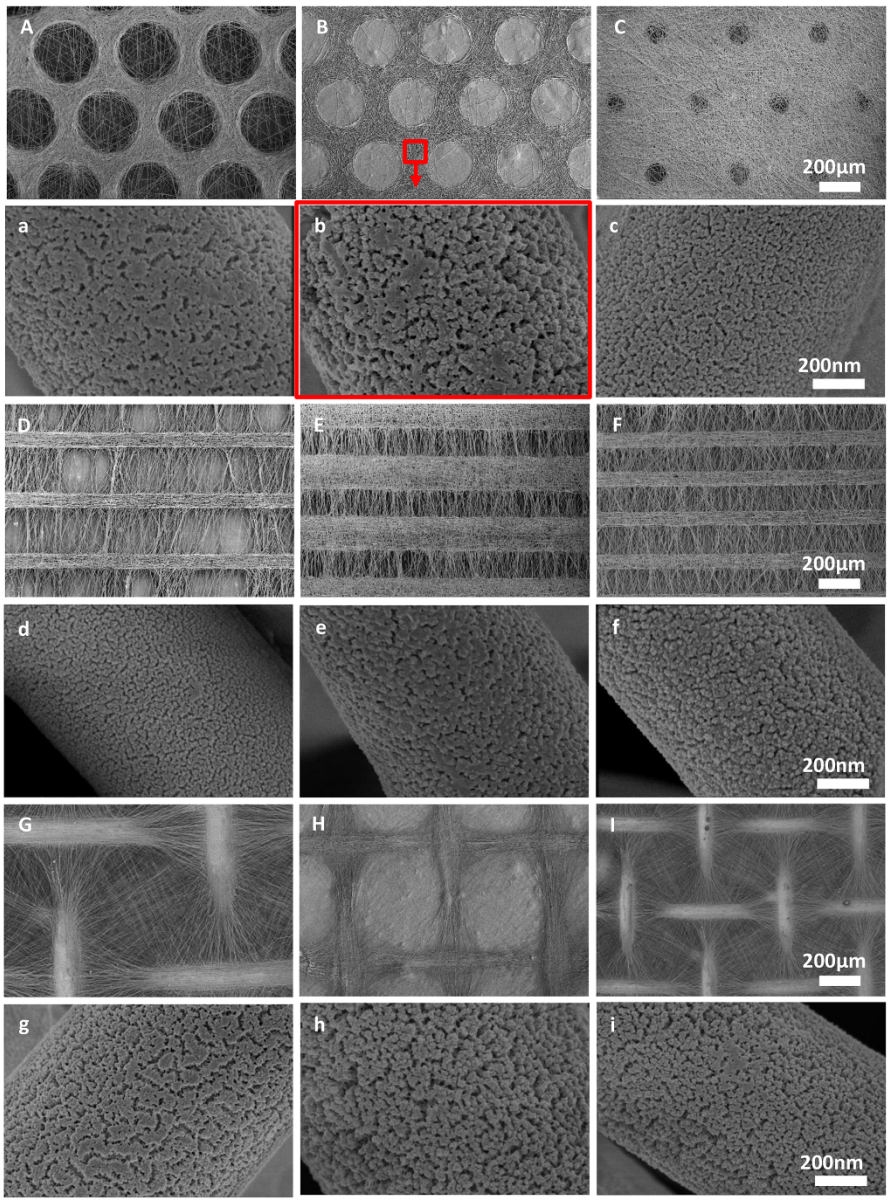
Statistical analysis: The results were expressed as the arithmetic mean ± standard deviation. Three independent experiments were carried out, and at least five samples per each test were taken for statistical analysis. Statistical significance between two groups was calculated using two-tailed analysis of variance (ANOVA) and performed with a Student's t-test program. Differences were considered significant when $p < 0.05$ (*)or $p < 0.01$ (**). In addition, a one-way ANOVA with Tukey's post hoc test

was used for statistical analysis of multiple comparisons. Significant difference was considered when $p < 0.05$ (*) or $p < 0.01$ (**).

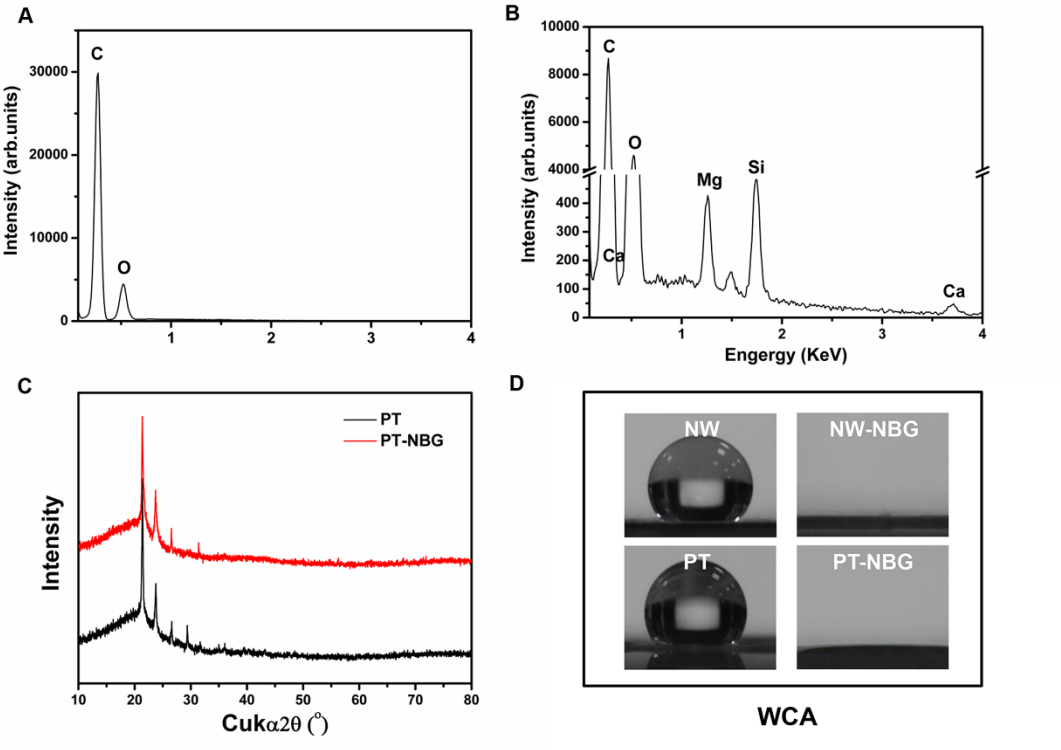
Supplementary Table S1. The ionic release from PT-NBG scaffolds after soaking them in PBS solution after 1, 3 and 7 days.

Ionic Concentrations (μg/mL)	Si	Ca	Mg
Day 1	9.12	12.95	4.36
Day 3	1.76	3.10	0.87
Day 7	1.05	1.56	0.47

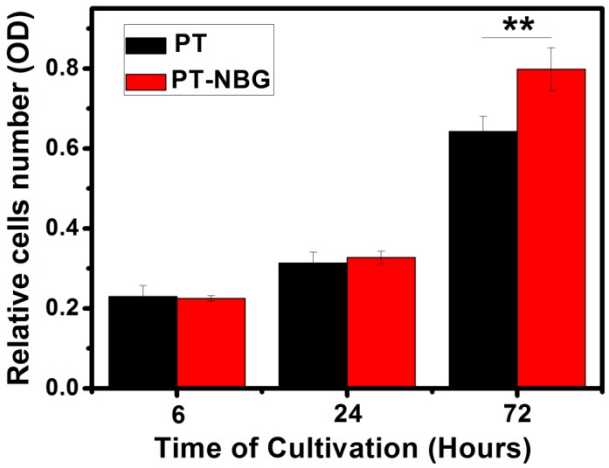
Supplementary Figures



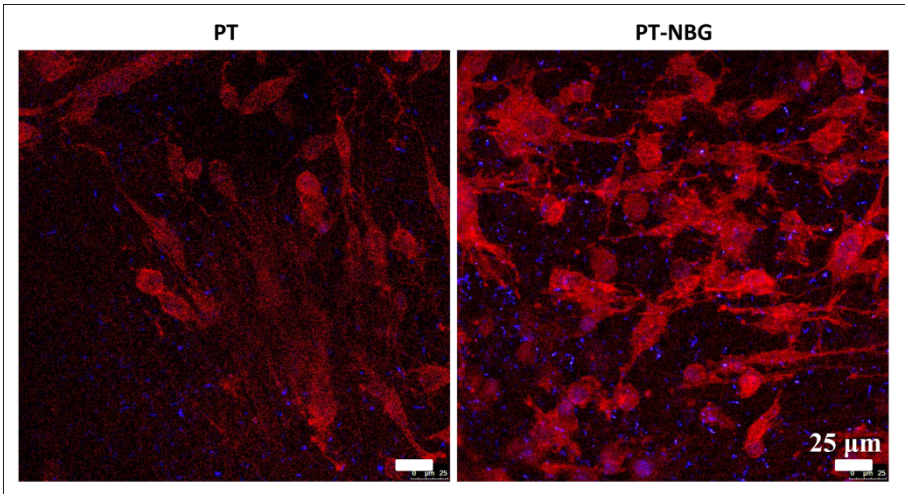
SupplementaryFigure S1. The SEM images of composite scaffolds with different size of holes: (A) Large; (B) Middle; (C) Small. The high magnification SEM images of the nano-sized particles on the surfaces of nanofibers in the corresponding scaffolds are shown in (a-c), respectively. The SEM images of the stripe-shaped electrospun scaffolds with altered distances between the stripes: (D) Large; (E) Middle; (F) Small. The high magnification SEM images of the nano-sized particles on the surfaces of nanofibers in the corresponding scaffolds are shown in (d-f), respectively. The SEM images of the square-shaped electrospun scaffolds with different side lengths: (G) Large; (H) Middle; (I) Small. The high magnification SEM images of the nano-sized particles on the surfaces of nanofibers in the corresponding scaffolds are shown in (g-i), respectively.



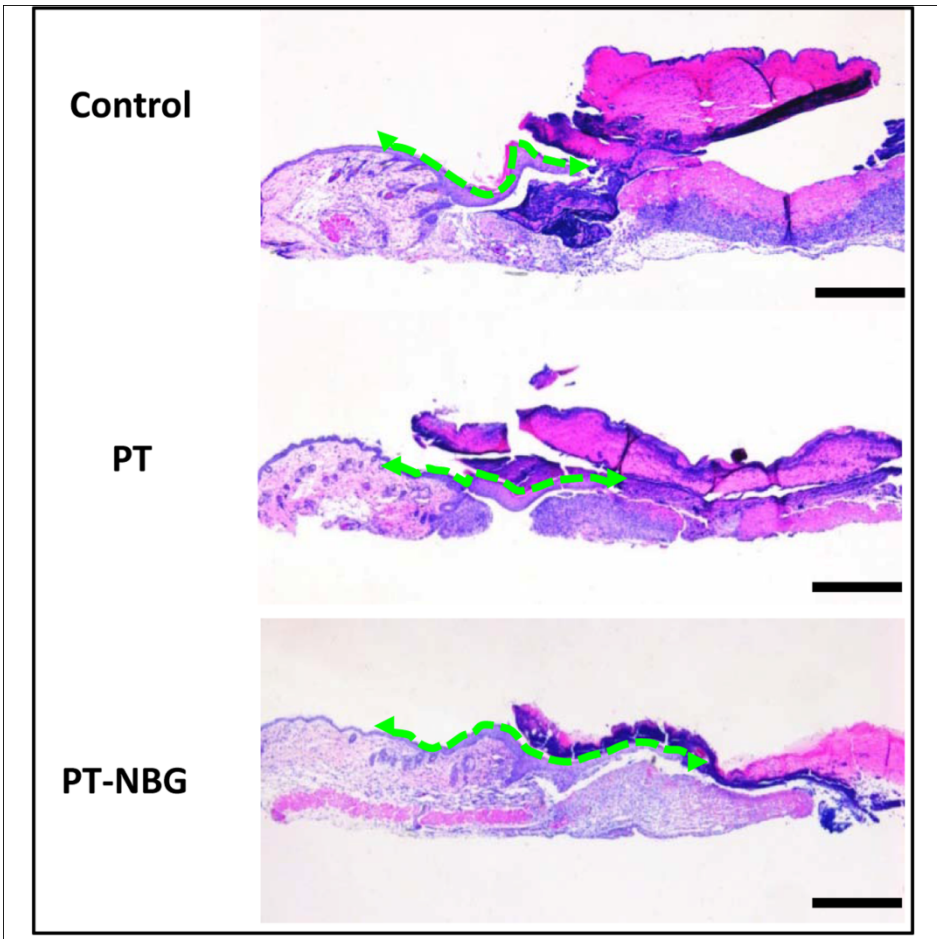
Supplementary Figure S2.(A) EDS of the PT scaffolds; (B) EDS of the PT-NBG scaffolds; (C) XRD patterns of the PT scaffolds and the PT-NBG scaffolds. (D)Different scaffoldswith various water contact angles: NW scaffolds(131.75°), NW-NBG scaffolds(0°), PT scaffolds(124.08°) and PT-NBG scaffolds(0°).



Supplementary Figure S3. Proliferation of HUVECs on PT and PT-NBG electrospun scaffolds. (** represent $p < 0.01$.)



Supplementary Figure S4. Cytoskeleton organizations of HUVECs after being cultured for 3 days on PT and PT-NBG scaffolds.



Supplementary Figure S5. Histological sections from the control, PT and PT-NBG groups after 7 days. The edge of the new epidermis is indicated with green dotted line. The left green arrows indicate the borders of the normal area and new epidermis, while the right green arrows indicate the borders of the new epidermis and wound area. Original magnification × 25; scale bar = 500μm.

References

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