Supporting information

Near-Infrared-Triggered Dynamic Surface Topography for Sequential Modulation Of Macrophage Phenotypes

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Synthesis of 2- and 4-branched polycaprolactone (PCL) macromonomers. Pentaerythritol (2.0 g, 14.7 mmol) was placed in a Schlenk bottle and dehydrated under reduced pressure for 12 h prior to polymerization. Distilled ε-caprolactone (48.8 ml, 439.8 mmol) and a catalytic amount of stannous octoate were added under flowing dry nitrogen. The mixture was stirred for 24 h at 120°C under a nitrogen atmosphere. After dilution with a little amount of tetrahydrofuran (THF), the solution was dropwise added into a large amount of cold methanol. The precipitated polymer was repeatedly washed with methanol to remove any unreacted monomer and catalyst. 4-branched PCL (4b-PCL) was obtained as a white powder by vacuum drying. Then, 20 g 4b-PCL, 12 mL acryloyl chloride (147.8 mmol) and 23.2 mL triethylamine (167 mmol) were reacted in dehydrated THF at room temperature for 24 h to obtain 4b-PCL macromonomer (4b-PCLm). The mixture was precipitated and washed with cold methanol. After purification, the macromonomer was dried under reduced pressure for 2 days. By the same procedure, the ends of 2-branched PCL (2b-PCL, Sigma) were also modified with double bonds using acryloyl chloride, termed 2b-PCLm (Figure S1A). The structures of 2b-PCLm and 4b-PCLm were confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR, Figure S1B) and Fourier-transform infrared spectroscopy (FTIR, Figure S2). The grafting degrees of double bonds calculated from the results of ^{1}H NMR were 89.0% and 97.3% for 2b-PCLm and 4b-PCLm, respectively. Characterizations of 2b-PCLm and 4b-PCLm by differential scanning calorimeter (DSC, TA Q200, USA) and gel permeation chromatography (GPC, Waters 1515 Isocratic HPLC, USA) were shown in Table S1. The

number-average molecular weights (M_n) of 2b-PCLm and 4b-PCLm were evaluated to be 18247 and 8612 g/mol, and the degree of crystallinity was calculated by the following Eq1:

$$\chi_c = \frac{\Delta H_m}{140} \times 100\% \quad (1)$$

Where ΔH_m (J/g) is the heat of fusion of 2b-PCLm or 4b-PCLm determined by DSC, and 140 J/g is the ΔH_m of a perfect PCL crystal[1].

Preparation of poly (ethylene glycol) (PEG)-modified gold nanorods (AuNRs). Gold nanoroads owned excellent photothermal effects. In order to enhance their dispersibility in toluene, gold nanoroads were modified by thiol-terminated PEG (SH-PEG) as the previous reports[2,3]. Briefly, cylindrical gold nanorods were prepared by a seed-mediated growth method and then modified by immersing into 1 mg/mL SH-PEG for 24 h. The obtained AuNRs were observed under transmission electron microscope (TEM, JEM-1230EX), and the maximum surface plasmon resonance (SPR) peak of AuNRs was measured by ultraviolet-visible spectrophotometer (UV-Vis) (Figure S3). The content of gold was determined using an ICP-MS (X series II, Thermo Elemental Corporation, USA).

Characterizations of AuNRs/PCL films. The melting temperatures (T_m) of the AuNRs/PCL films were measured by DSC (Figure S4). In this study, the AuNRs/PCL films with the weight ratios of 1:2, 1:1 and 2:1 of 2b-PCLm/4b-PCLm were prepared to determine a suitable T_m for further experiments. The dispersion of AuNRs in the films was observed by ultra-thin sections under TEM (Figure S5A). The mechanical properties of the AuNRs/PCL films (3 mm × 15 mm × 1 mm) were measured by

universal tensile testing machine (A5532, INSTRON, UK) with a speed of 40 mm/min (Figure S5B). The AuNRs/PCL films immersed in 1640 culture medium were exposed under an 808 near infrared (NIR) laser (LSR-PS-FA, Lasever Inc., China) at a power of 1.5 W/cm^2. The temperatures of the AuNRs/PCL films containing 1.2×10^{-3} wt%, 6×10^{-3} wt% or 12×10^{-3} wt% AuNRs were detected by infrared thermal imager (Fotric, USA) in real-time (Figure S6). The hydrophilicity of the AuNRs/PCL films were optimized by plasma treatment for a better affinity to cells, and the results of water contact angles before and after plasma treatment were carried on DSA100 (KRUSS, Germany, Figure S**7**).

Cell isolation and gene expression analysis. Femurs were harvested and bone marrow was flushed with 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, USA) to collect bone marrow cells. Cells were cultured with FBS contained 1640 medium supplemented with macrophage colony stimulating factor (M-CSF, PeproTech, USA) to induce differentiation to macrophages. After being cultured for 4 days, bone marrow-derived macrophages (BMDMs) were collected using cell scrapers and seeded onto experimental substrates. Flow cytometry was used to confirm the purity of isolated macrophages (Figure S8A). BMDMs were stained with allophycocyanin (APC)-conjugated F4/80 (1: 50, eBioscience, USA), corresponding isotype controls were used as recommended by the manufacturer. Labeled cells were analyzed using a flow cytometer (BD FACS Calibur with CellQuest software, BD Bioscience, San Jose, CA). Data were estimated using FlowJo software (Tree Star, Inc., USA).

The assay of quantitative real time polymerase chain reaction (qPCR) was employed to demonstrate the phenotypic shift ability of BMDMs. BMDMs were cultured with 1640 medium containing 100 ng/mL lipopolysaccharide and 10 ng/mL interferon-γ (LPS/IFN-γ) or 1640 medium containing 20 ng/mL IL-4 for 1 d. Thereafter, the expressions of M1-related genes (iNOS, TNF-α and CCR7) and M2-related genes (Arg-1, IL-10 and TGF-β1) were analyzed by qPCR. For reverse transcription polymerase chain reaction (RT-PCR), total RNA from samples was extracted by RNAeasy^{TM} animal RNA isolation kit (Beyotime, China), then used for first-strand complementary DNA synthesis with PrimeScript^{TM} RT reagent kit (Takara, China). Thereafter, qPCR was performed by using TB^{TM} Green PrimeScript^{TM} RT-PCR kit (Takara, China) on the CFX96 Real-Time System (Bio-Rad, USA). 18s ribosomal mRNA, which served as a housekeeping gene, was chosen as the internal references. The expression values were given as fold changes to the expression of genes in control group.

Biocompatibility of AuNRs/PCL films. The AuNRs/PCL films containing different ratios of AuNRs were prepared to optimize biocompatibility. The AuNRs/PCL films were put into a 96-well plate and seeded with BMDMs at a density of 2.5×10^4 cells/well. After being cultured for 1 day, the samples were irradiated by NIR for 60 s, and then further cultured for 2 days. The AuNRs/PCL films with adhered BMDMs were transferred to a new 96-well plate, which was filled with 200 μL 1640 culture medium and 20 μL CCK-8 per well. After incubating for 2 h, the absorbance at 450 nm of the supernatant was measured by a microplate reader (M200 pro, TECAN). The

cell viabilities were normalized to that on F before NIR (Figure S**9**). After the characterization of cell viability, the films with a 2b-PCLm/4b-PCLm ratio of 1: 1 and an AuNRs content of 6×10^{-3} wt% were selected for further experiments.

BMDMs transformation by the AuNRs/PCL films with fixed topography *in vitro*. Herein, the size of the microgrooves is equal to the spacing between the microgrooves, and the depth of all the microgrooves is 4 μm. To confirm the optimal parameters of microgrooved substrate for inducing macrophage orientation, the AuNRs/PCL films with 5, 10, 20 and 30 μm microgrooves were seeded with BMDMs at a density of 7.8×10^4 cells/cm^2 and incubated for 2 days. Then, BMDMs were stained with calcein-AM (Beyotime, China) and observed under a fluorescence microscope (IX81, Olympus, Japan). As shown in Figure S10A, BMDMs had the larger elongation on the films with 10 μm (P-10 μm) and 20 μm (P-20 μm) microgrooves than those on the films with 5 and 30 μm microgrooves. The morphologies of BMDMs were further observed by scanning electronic microscopy (SEM, Hitachi S-4800, Japan). The BMDMs on P-10 μm were fixed with 4% paraformaldehyde (PFA) for 15 min and washed 5 times with PBS. Subsequently, the samples were dehydrated in gradient aqueous ethanol with increasing concentrations of 30%, 50%, 70%, 80%, 90% and 100%, each for 10 min, then the ethanol was totally replaced with tertiary butanol. After freeze-drying, the samples were observed under SEM. As shown in Figure S10B, all the BMDMs on F remained round shapes, whereas most BMDMs on P-10 μm elongated. For demonstrating the phenotypic shift, BMDMs on P-10 μm and P-20 μm were fixed with 4% PFA for 10 min after being cultured for 2 d, and then blocked

with 3% bovine serum albumin (BSA, AMRESCO, USA)/PBS at 37°C for 60 min. These cells were stained with antibody of FITC-labeled inducible nitric oxide synthase (iNOS, 1: 50, BD Pharmingen, USA) and antibody of PE-labeled arginase-1 (Arg-1, 1: 50, Santa Cruz, USA) following with twice PBS washing, then counterstained with Hoechst 33342 (1: 200, Invitrogen, USA) and observed under a fluorescence microscope. As shown in Figure S11, BMDMs on P-10 μm showed a higher expression of Arg-1, indicating a pro-healing phenotype. Therefore, P-10 μm was abbreviated to P and used in the further experiments.

Adhesive structure of BMDMs observed under confocal laser scanning microscope (CLSM). After confirming transformation curves on different samples, we took several representative time points (6, 10, 18, 26, 42 h for F and P, and 30, 34, 42, 50, 66 h correspondingly for SMP), and stained for cytoskeleton and focus adhesion, then observed the BMDMs morphology under a CLSM (LSM-510, Zeiss, Germany). BMDMs were seeded on F, P and SMP for specified times, and then fixed with 4% PFA at room temperature for 10 min, then washed with PBS 3 times and permeabilized at 4°C using 0.5% Triton X-100 (Sigma-Aldrich, USA) for 10 min. After being rinsed with PBS 3 times, the samples were incubated into 3% BSA/PBS for 60 min at 37°C. Then BMDMs were washed 5 times and stained with mouse monoclonal vinculin primary antibody (Abcam, UK), and then washed thrice with PBS and incubated with FITC labeled goat-anti-mouse IgG (Boster, China) and rhodamine-labeled phalloidin (Thermo Fisher Scientific, USA) for 12 h at 4°C. Then BMDMs were stained with 4', 6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich,

USA) at room temperature for 10 min. After being rinsed thrice in PBS, BMDMs were observed under CLSM.

The morphology of the adhered host cells. After the AuNRs/PCL films were taken out from the animals, the morphology of the adhered cells on the films were observed by SEM. BMDMs were fixed with 4% PFA for 15 min and washed 5 times with PBS. Subsequently, the samples were dehydrated in gradient aqueous ethanol with increasing concentrations of 30%, 50%, 70%, 80%, 90% and 100%, each for 10 min, then the ethanol was totally replaced with tertiary butanol. After freeze-drying, the samples were observed by SEM (Hitachi S-4800, Japan, Figure S12).

The phenotypes polarization of the host macrophages in vivo. The phenotypes of macrophages at 2, 4 and 6 d were measured by immunostaining of iNOS/Arg-1 and CD86/CD163 (Figure S13, Figure S14). The samples were fixed with 4% PFA at room temperature for 15 min, then blocked with 5% goat serum (Absin, China)/PBS at room temperature for 10 min. Thereafter, the samples were incubated in primary antibodies of mouse monoclonal CD86 (Santa Cruz, USA) and rabbit monoclonal CD163 (Abcam, UK) with the dilution of 1: 60. After washing with PBS, the samples were then applied with corresponding secondary antibodies of goat-anti-mouse IgG (Alexa Fluor 594, ab150116, Abcam) and FITC conjugated goat-anti-rabbit IgG (BA1105, Boster, China) with the dilution of 1: 200 in PBS at 4 °C overnight. The adhered cells were counterstained with Hoechst 33342 and rinsed before observing under the fluorescence microscope. The cells were also stained for iNOS and Arg-1 by the similar method mentioned before with the dilution of 1: 50.

Western Blotting. The total protein was quantified using BCA Protein Quantification Kit (Beyotime, China). The equal amount of proteins of each sample were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transferred onto the polyvinylidene fluoride membrane. The membrane was incubated with TBST containing 5% nonfat dry milk for 1 h and then incubated with relevant primary antibodies (ROCK2 and Rac1, 1:1000) at 4 °C overnight. After that, the membranes were washed three times with TBST and hybridized with relevant secondary antibody (1:5000) at room temperature for 1 h. Finally, the membranes were rinsed and visualized by chemiluminescence using an enhanced chemiluminescence detection kit. The expressions of GAPDH were chosen as the internal references, respectively. The quantitative data of integral optical density were determined by Image J software. Protein expressions were normalized to that of F before NIR.

References

1. Ping, P., Wang, W., Chen, X., Jing, X., Poly (ε-caprolactone) polyurethane and its Shape-memory Property. *Biomacromolecules* **2005**, 6, 587-592.
2. Zhu, H., Chen, Y., Yan, F. J., Chen, J., Tao, X. F., Ling, J., Yang, B., He, Q. J., Mao, Z. W. Polysarcosine Brush Stabilized Gold Nanorods for In Vivo Near-infrared Photothermal Tumor Therapy. *Acta. Biomater*. **2017**, 50, 534-545.
3. Chen, J., Li, X., Zhao, X., Wu, Q., Zhu, H.H., Mao, Z.W., Gao, C.Y., Doxorubicin-conjugated pH-responsive Gold Nanorods for Combined

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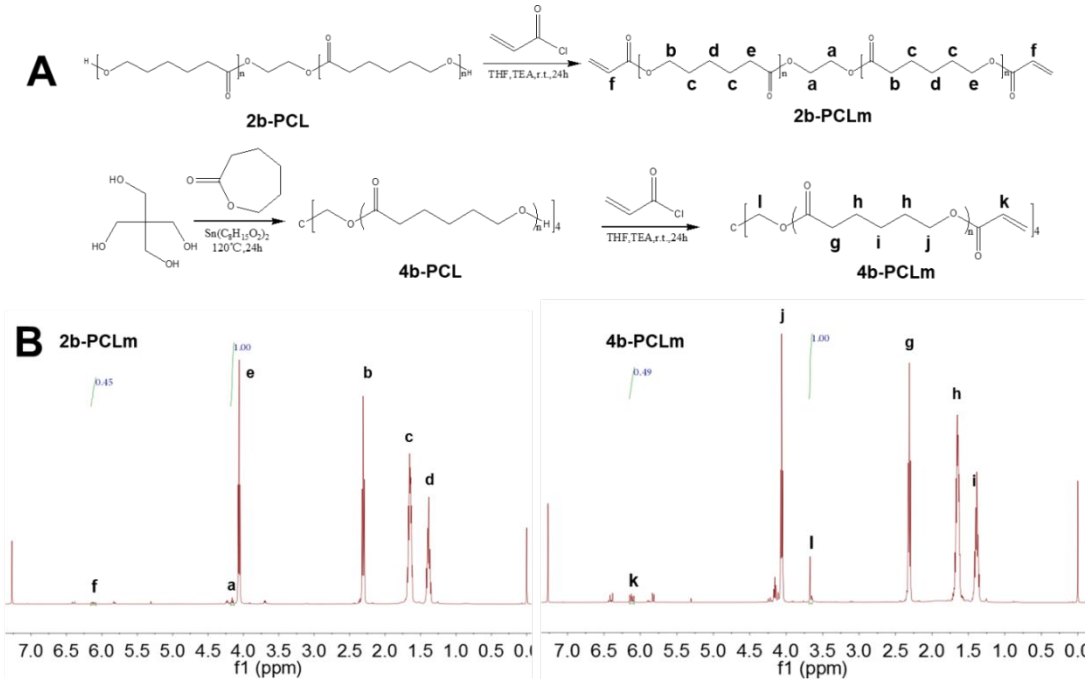

Figure S1. Syntheses and characterizations of 2b-PCLm and 4b-PCLm. (A) 2b-PCLm or 4b-PCLm were synthesized by the esterification of 2b-PCL or 4b-PCL with acryloyl chloride in THF for 24 h. (B) ^{1}H NMR spectra of 2b-PCLm and 4b-PCLm.

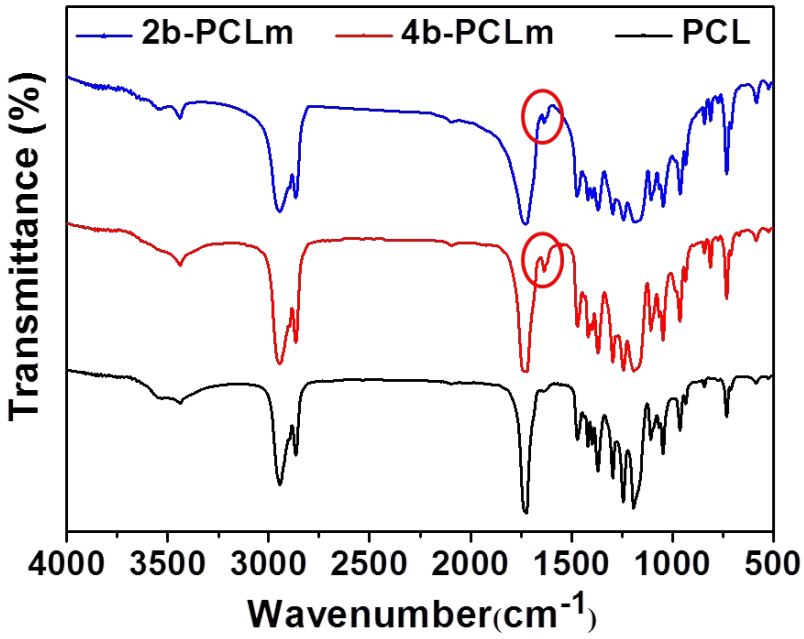

Figure S2. FTIR of PCL, 2b-PCLm and 4b-PCLm. The red circles are labeled as the vibration absorptions of carbon-carbon double bonds.

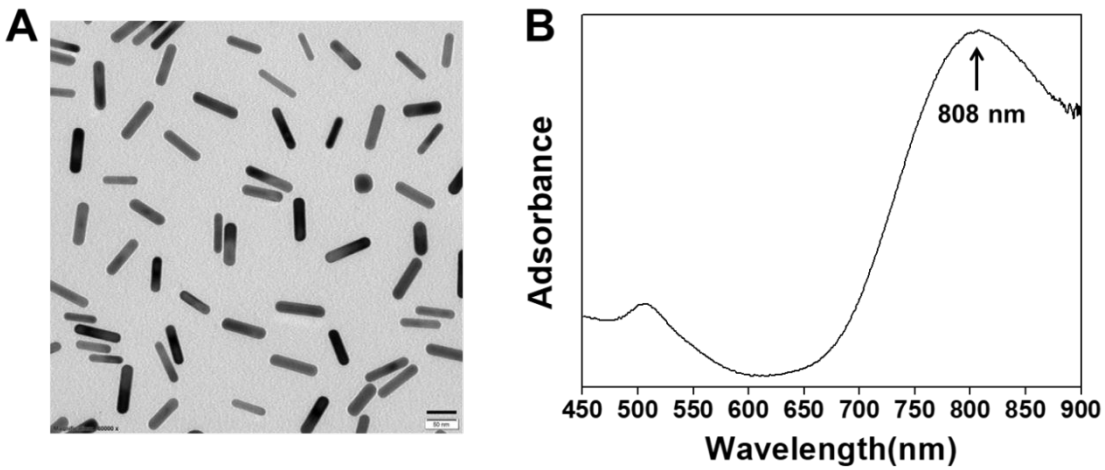

Figure S3. (A) The transmission electron microscopy (TEM) image for gold nanorods. Scale bar is 50 nm. (B) The maximum surface plasmon resonance (SPR) peak of AuNRs at 808 nm measured by ultraviolet-visible spectrophotometer (UV-Vis).

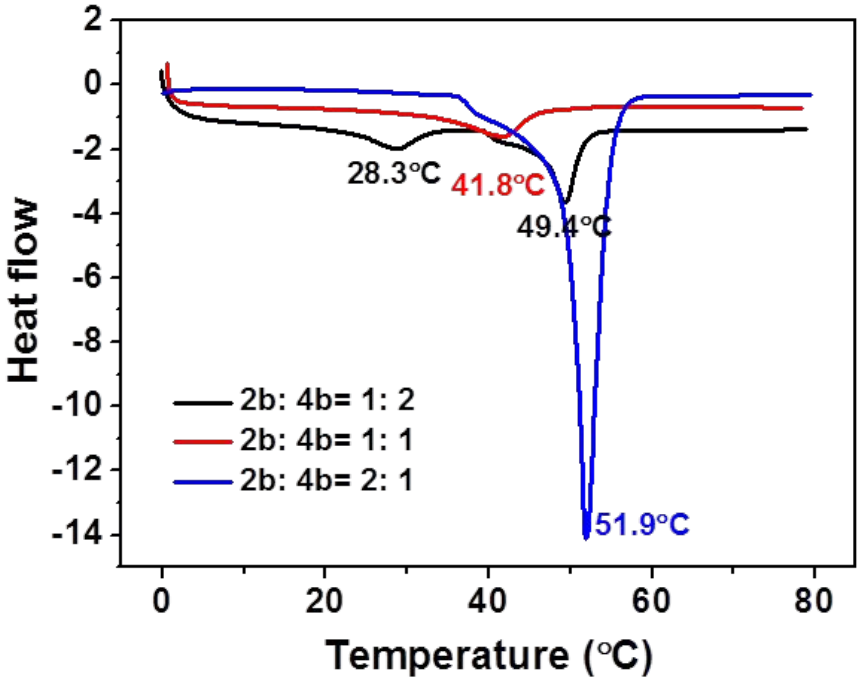

Figure S4. The melting temperatures of the AuNRs/PCL films polymerized from different ratios of 2b- and 4b-PCLm (1: 2, 1: 1 and 1: 2) measured by DSC. Heat history has been eliminated.

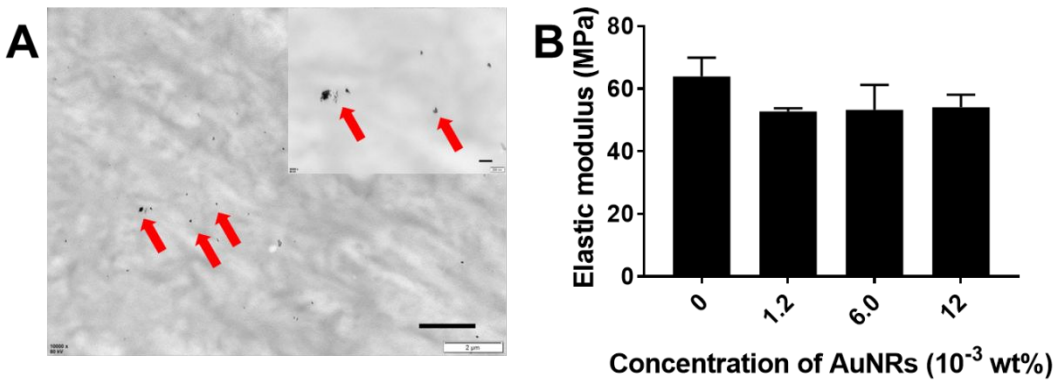

Figure S5. (A) TEM images show the dispersion of AuNRs (as indicated by red arrows) in the AuNRs/PCL films. Scale bar is 2 μm. The insert is the corresponding images with higher magnification and the scale bar is 200 nm. (B) Mechanical properties of the AuNRs/PCL films with different ratios of AuNRs.

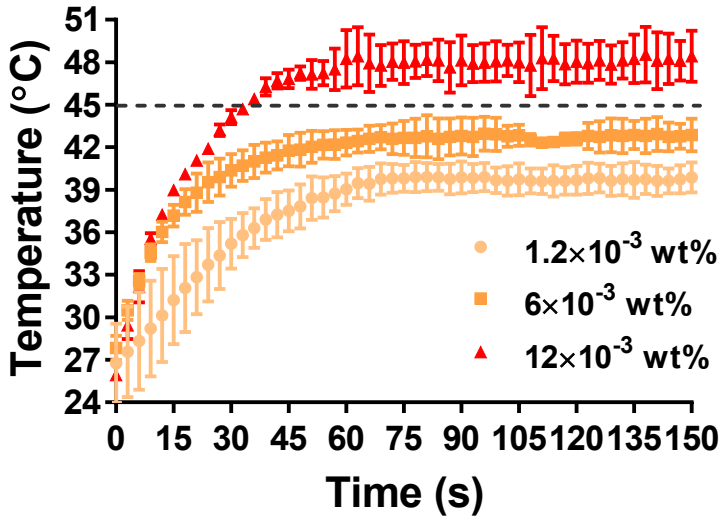

Figure S6. The surface temperature curves of the AuNRs/PCL films after being irradiated under near infrared (NIR) light for different time. Dash line represents 45°C, which indicates the temperature that starts to be harmful to cells.

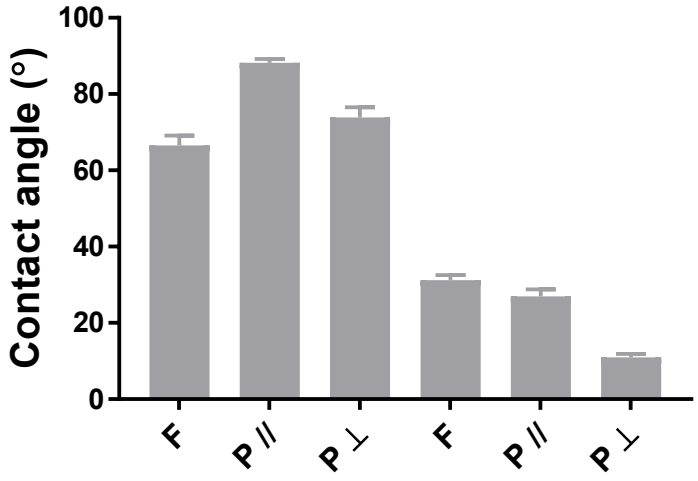

Figure S7. The water contact angles of the AuNRs/PCL films before and after plasma treatment. The contact angles of the AuNRs/PCL films with flat (F) and patterned (P) topographies are analyzed. P ⊥ means that the direction of microgrooves is perpendicular to the direction of light path. P // means that the direction of microgrooves is parallel to the direction of light path.

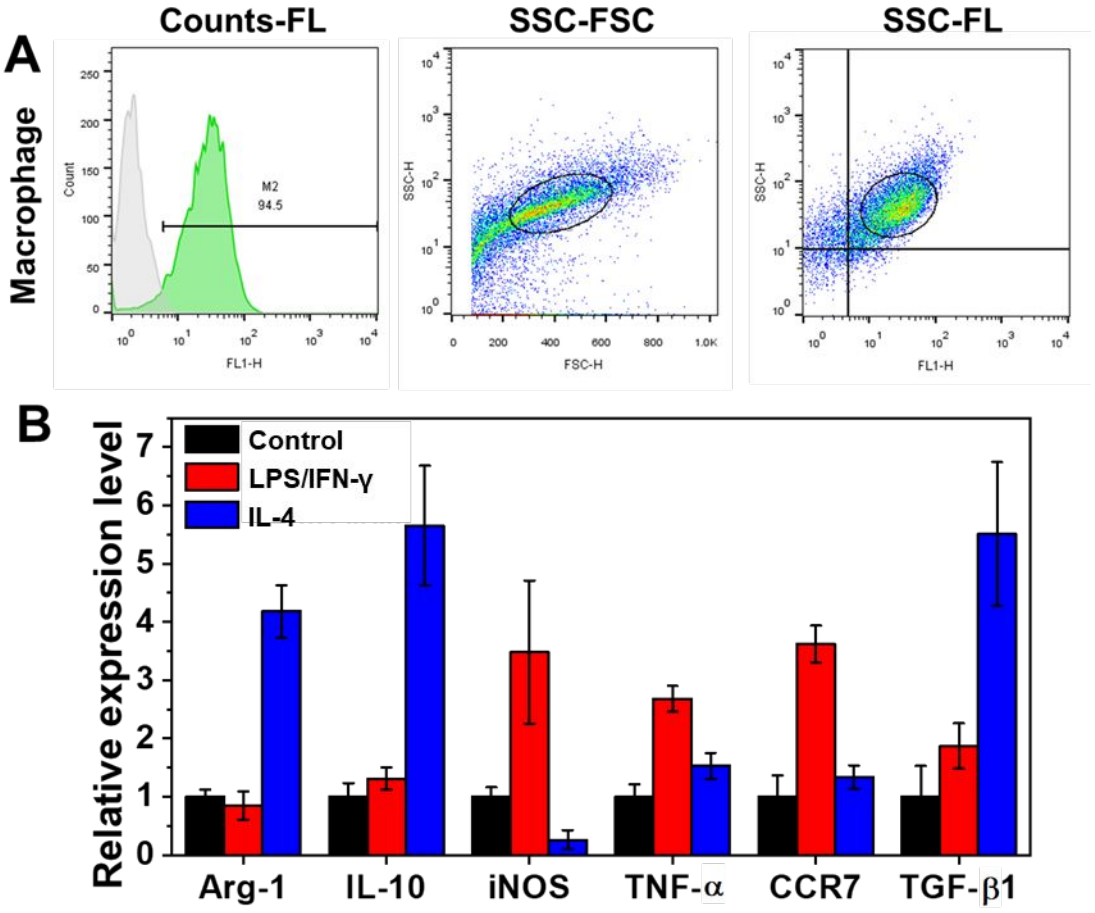

Figure S8. (A) Expression of the macrophage-related marker F4/80 by flow cytometry. (B) The expression level of Arg-1, IL-10, iNOS, TNF-α, CCR7 and TGF-β1 determined by qPCR in BMDMs after activated by 100 ng/mL lipopolysaccharide and 10 ng/mL interferon-γ or 20 ng/mL IL-4 for 1 d. BMDMs cultured in the normal medium without any cytokines are chosen as control.

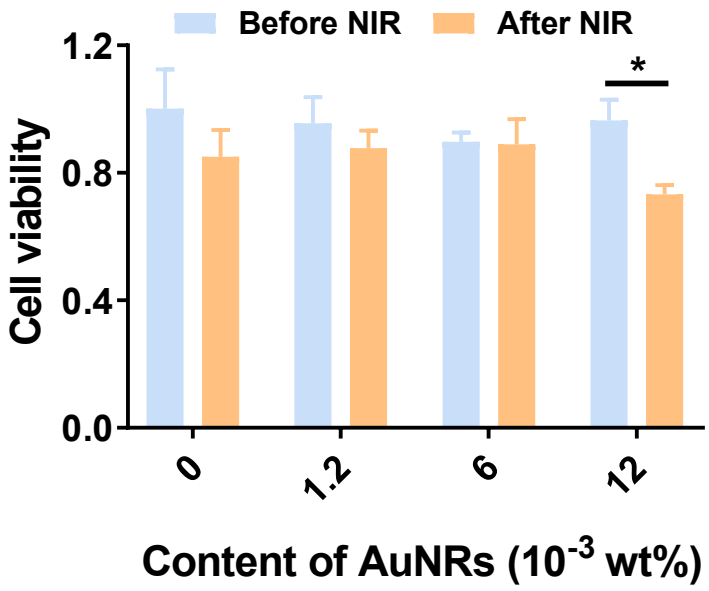

Figure S9. Cell viability of BMDMs cultured on the AuNRs/PCL films with different amounts of AuNRs before and after NIR irradiation.

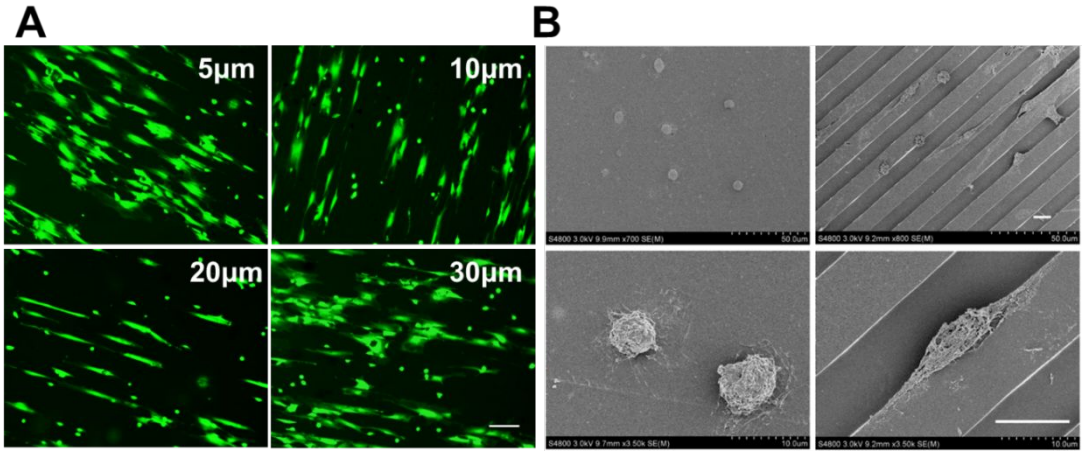

Figure S10. (A) The fluorescent images of BMDMs stained for calcein-AM in green to verify the most effective width of microgroove for BMDMs elongation. Scale bar is 100 μm. (B) SEM images of BMDMs being cultured on the flat films (left) and films with 10 μm-microgrooves (right). The bottom images correspond to a higher magnification of the top images. Scale bars are 10 μm.

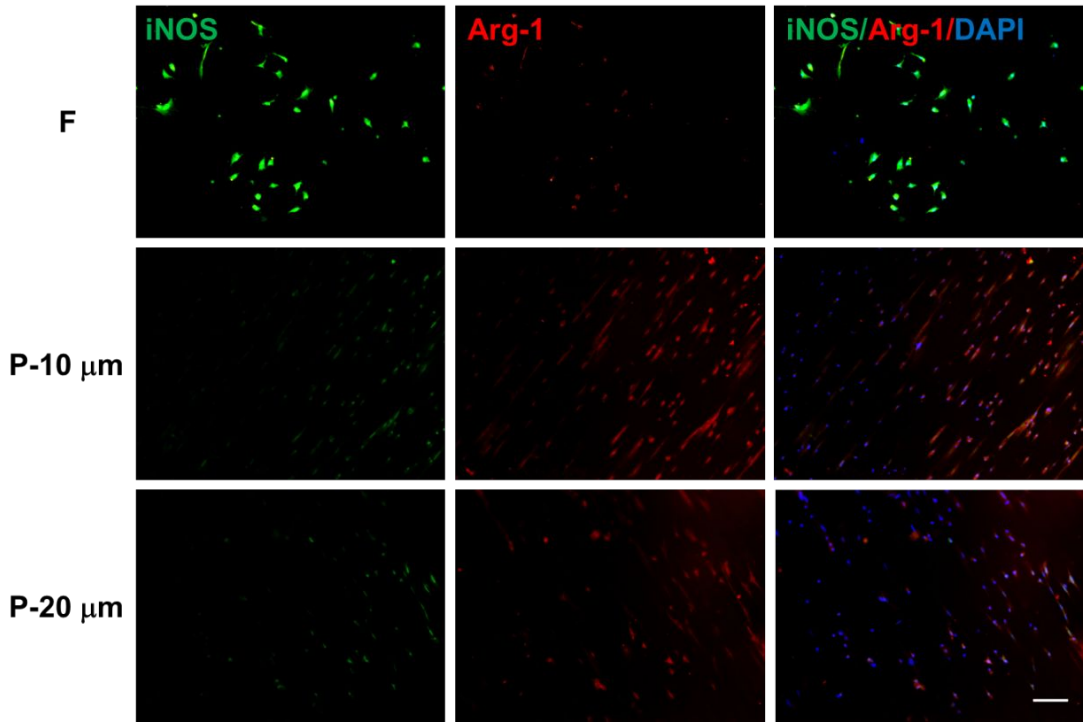

Figure S11. The fluorescent images of BMDMs on the films with 10 μm (P-10 μm) and 20 μm (P-20 μm) microgrooves immunostained for iNOS (M1 marker, green), arginase-1 (Arg-1, M2 marker, red), and nuclei (DAPI, blue) to verify the most effective width for BMDMs polarization. Scale bar is 100 μm.

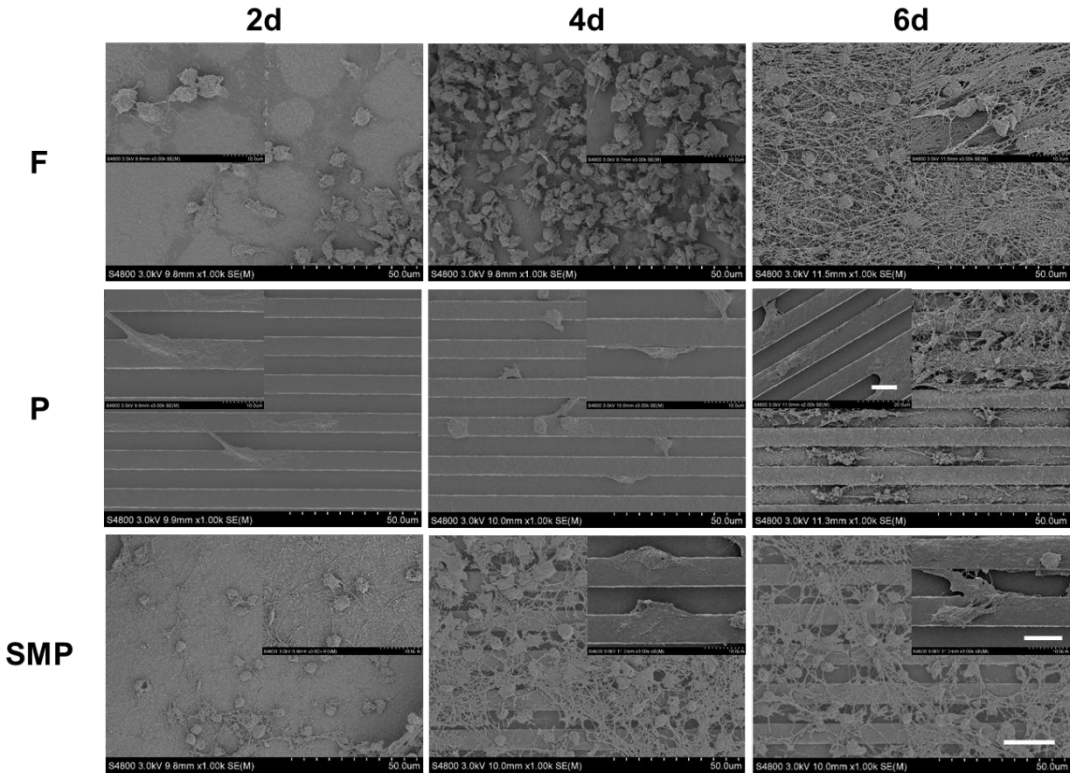

Figure S12. SEM images of the adhered host cells on F, P, and SMP after being subcutaneously implanted for 2, 4 and 6 d, respectively. Scale bar is 20 μm. Inserts are the corresponding images with higher magnification, and the insert scale bars are 10 μm.

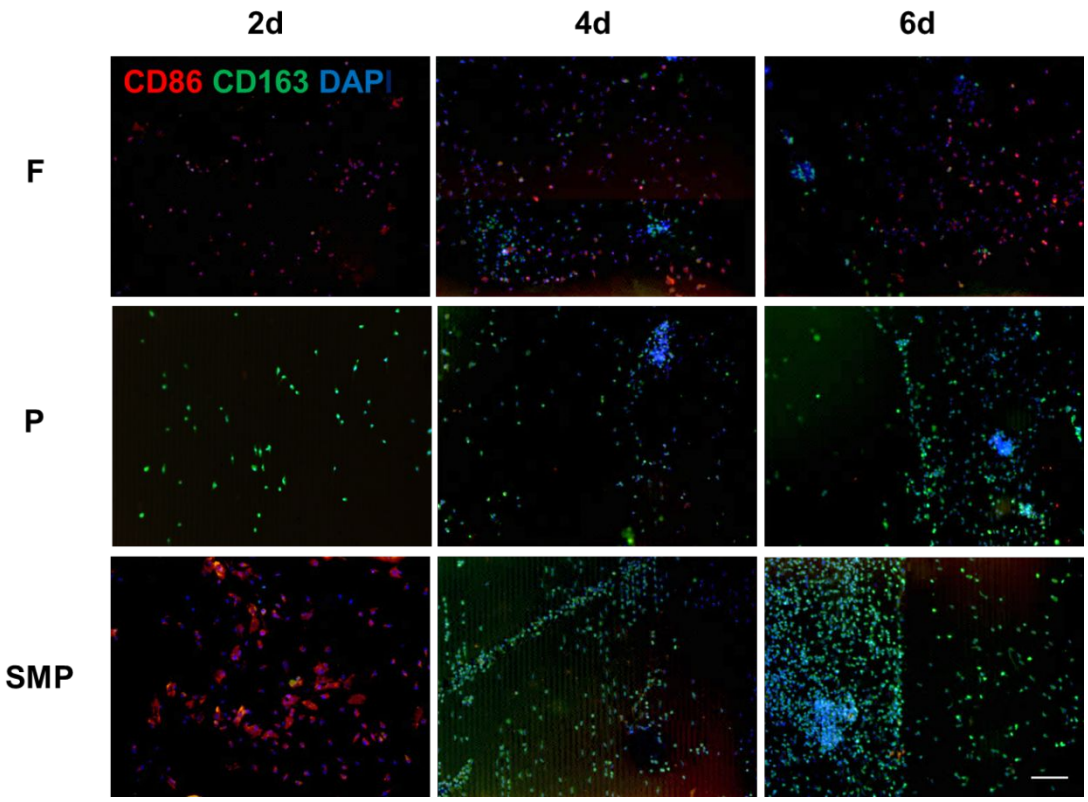

Figure S13. The fluorescent images of the adhered host cells immunostained for CD86 (M1 marker, red), CD163 (M2 marker, green), and nuclei (DAPI. blue) on F, P, and SMP after being subcutaneously implanted for 2, 4 and 6 d, respectively. Scale bar is 100 μm.

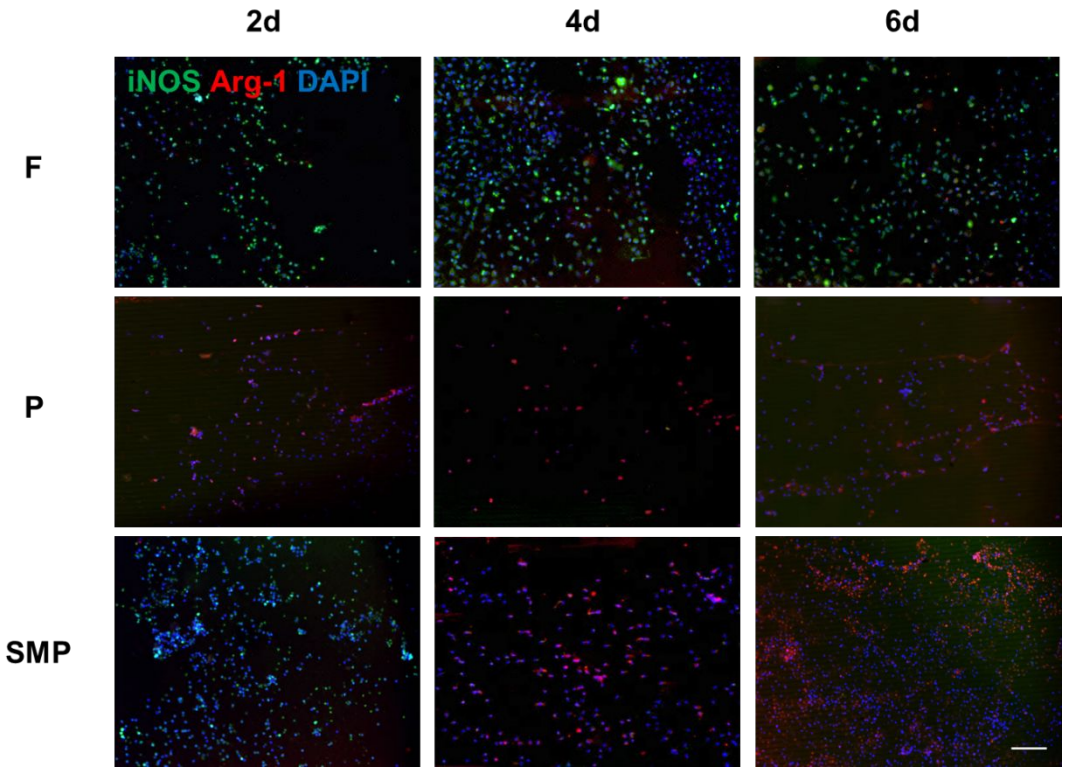

Figure S14. The fluorescent images of the adhered cells immunostained for iNOS (M1 marker, green), Arg-1(M2 marker, red), and nuclei (DAPI, blue) on F, P, and SMP after being subcutaneously implanted for 2, 4 and 6 d, respectively. Scale bar is 100 μm.

Table S1. Data of 2b-PCLm and 4b-PCLm measured by gel permeation chromatography (GPC) and differential scanning calorimeter (DSC).

Sample	Mn (g/mol)	PDI	Dg (%) [a)]	Tm (°C)	ΔH_m (J/g)	χ_c (%) [b)]
2b-PCLm	18247	1.2	90.0	52.2	61.7	44.1
4b-PCLm	8612	1.4	98.0	42.7	50.6	36.1

[a)] Dg(%)-grafting degree of double bonds; [b)] χ_c (%)- degree of crystallinity