Supplementary Information

A self-assembling bioactive oligopeptide hydrogel for the treatment of edema following prepuce surgery

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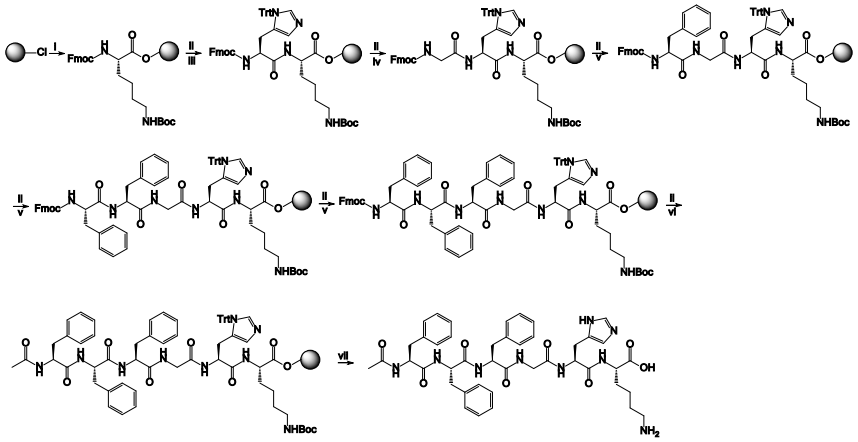
Experimental section

1) Chemicals and reagents

Fmoc-Lys(Boc)-OH, Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, and O-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) were obtained from Bide Pharmatech (Shanghai, China). Dichloromethane (DCM), methanol, *N,N*-dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIEA), acetic acid, triisopropylsilane (TIS), and trifluoroacetic acid (TFA) were purchased from Meryer (Shanghai, China). Piperidine and diethyl ether were purchased from Chengdu Kelong Chemical Co., Ltd., (Chengdu, China). 2-Chlorotriphenylmethyl chloride resin (1.2 mmol/g) was obtained from Tianjin Nankai Hecheng S&T Co., Ltd., (Tianjin, China). Ultrapure water (18.2 MΩ•cm) was prepared by an UPR-11-10L ultrapure water purification system (Chengdu, China). 0.9% Sodium chloride injection (saline) was purchased from Huiyinbi Group Jiangxi Dongya Pharmaceutical Co., Ltd. (Jiangxi, China). LPS (Lipopolysaccharides from E. coli O111:B4, S1732) was purchased from Beyotime (Shanghai, China). IL-6 and TNF-α ELISA kits were obtained from Invitrogen (Vienna, Austria). Antibodies against VEGF, β-actin, TNF-α, IL-6, and CD31 were obtained from Cell Signaling Technology (Beverly, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Solarbio Biotech (Wuhan, China). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (USA). Matrigel was purchased from Waltham (MA, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and 0.25% trypsin-EDTA solution were purchased from Gibco Laboratories (Grand Island, NY, USA). Other chemicals and reagents were purchased from Sigma-Aldrich.

2) Synthesis of Ac-FFFGHK-OH



I) Fmoc-Lys(Boc)-OH, DIEA; II) 20% piperidine; III) Fmoc-His(Trt)-OH, HATU, DIEA; IV) Fmoc-Gly-OH, HATU, DIEA; V) Fmoc-Phe-OH, HATU, DIEA; VI) Acetic acid, HATU, DIEA; VII) 95% TFA, 2.5% TIS, 2.5% H_2O

Scheme S1. Synthetic route of Ac-FFFGHK-OH.

The Ac-FFFGHK-OH was synthesized using the Fmoc-based solid-phase peptide synthesis approach. Briefly, Fmoc-protected amino acid (2 equiv.), HATU (2 equiv.), and DIEA (4 equiv.) in DMF were used for each amino acid coupling cycle. Each Fmoc-protected amino acid was activated for ~1 min before being incubated with the Fmoc-deprotected peptide resin for 1 h to complete each coupling cycle. The Fmoc-protecting group was cleaved by piperidine/DMF (1/4 by volume) for 30 min for each cycle. The N-terminal acetylated modification of the hexapeptide sequence was achieved by using acetic acid (2 equiv.), HATU (2 equiv.), and DIEA (4 equiv.) in DMF for 1 h before being detached from the resin with TFA/TIS/H_2O (19/0.5/0.5 by volume) for 1 h. The majority of the TFA was removed by rotary evaporation before the addition of an excess of cold diethyl ether, which afforded the hexapeptide precipitate. The crude product was purified by semi-preparative high-performance liquid chromatography.

3) Preparation of Ac-FFFGHK-OH hydrogel

The Ac-FFFGHK-OH hydrogel was prepared by fully dissolving the powders in saline at a concentration of 30 mg/mL (or 60 mg/mL) and incubating the solution for 24 h at room temperature. Subsequently, it was diluted to the requisite concentration for the specific experiment.

4) Determination of critical aggregation concentration (CAC) of Ac-FFFGHK-OH

The CAC value of Ac-FFFGHK-OH was measured by using the pyrene fluorescent probe method. Pyrene alcohol solutions (1 mL, 6.08 μg/mL) were dried in centrifuge tubes at 60 °C before incubation with different concentrations of self-assembling Ac-FFFGHK-OH solutions (4 mL, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625, 0.001953125 mg/mL) under continuous shaking for 24 h at room temperature in the dark. The emission spectra of the mixtures were acquired on a FluoroMax-4 spectrofluorophotometer (HORIBA Scientific, France) in the spectral range of 355-445 nm (λ_{ex} = 334 nm). The fluorescence intensity ratio of I_{394nm}/I_{374nm} was plotted against the log C of Ac-FFFGHK-OH, and the CAC value was calculated as the cross-point of the two fitted lines.

5) Rheology

Rheological studies of the Ac-FFFGHK-OH hydrogel (30 mg/mL) were conducted on a rheometer (DHR-2, TA Instruments, USA) at 25 °C using a 25 mm parallel plate with a gap distance of 500 μm. Amplitude sweeps were obtained in the strain range of 0.1-100% at a constant angular frequency (1 rad/s). Frequency sweeps were obtained from 0.1 to 100 rad/s at a constant strain of 10%.

6) Scanning electron microscopy (SEM)

The Ac-FFFGHK-OH hydrogel (30 mg/mL) was frozen in liquid nitrogen before lyophilization. The freeze-dried hydrogel was adhered to the conductive carbon tape and coated with ~4 nm of platinum using a sputtering system (EM ACE600, Leica, German) before visualization using a scanning electron microscope (SU8010, HITACHI, Japan).

7) Transmission electron microscopy (TEM)

The Ac-FFFGHK-OH assemblies in saline (3 μL, 5 mg/mL) were adsorbed onto a carbon film-coated copper grid for a brief period. The excess sample was then removed by filter paper and allowed to to dry in air. The sample-loaded carbon film was then stained with a 2 wt% uranyl acetate solution for a few seconds. The majority of the staining solution was subsequently removed by filter paper and the grid was again dried in air. The morphology of the Ac-FFFGHK-OH assemblies was imaged using a transmission electron microscope (FEI Talos F200S G2, Thermo Fisher, USA).

8) Atomic force microscopy (AFM)

The Ac-FFFGHK-OH assemblies in saline (10 μL, 5 mg/mL) were adsorbed onto a silica sheet (1 cm × 1 cm) for a brief period of time Most of the sample was taken away by filter paper prior to drying in air. The sample-loaded silica sheet was then washed with water for three times. Once more, the sample was allowed to dry in air. The morphology of the Ac-FFFGHK-OH assemblies was scanned using an atomic force microscope (Dimension Icon, Bruker, Germany).

9) Circular dichroism (CD) spectroscopy

The Ac-FFFGHK-OH hydrogel (3 μL, 30 mg/mL) was placed between two pieces of quartz sheets. The CD spectra were recorded on a CD spectrophotometer (Chirascan Plus, Applied Photophysics, England) in the spectral region of 200-275 nm at room temperature.

10) Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Prior to ATR-FTIR analysis, the Ac-FFFGHK-OH hydrogel (30 mg/mL) was dried in air. The FTIR spectrum of the air-dried hydrogel was recorded using a FT-IR spectrometer (Tensor II, Bruker, Germany) in ATR mode within the wavenumber range of 400-4000 cm^{-1}.

11) Cell culture

The human umbilical vein endothelial cells (HUVECs) and mouse macrophage cell line (Raw264.7) were cultured in DMEM complete medium containing 1% penicillin-streptomycin and 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

12) Cell Counting Kit-8 (CCK-8) assay

The HUVECs or Raw264.7 (5×10^3 cells/well) were cultured in a 96-well plate with DMEM complete medium for 12 h prior to the addition of the Ac-FFFGHK-OH hydrogel, which was then incubated at 37 °C for a further 24 h. Thereafter, the conditioned medium was replaced with fresh culture medium containing CCK-8 agent, which was incubated for a further 2 h at 37 °C. The absorbance at a wavelength of 450 nm was determined for each well using a microplate reader.

13) Cell migration assay

The HUVECs were cultured in a 6-well plate at a density of 2×10^5 cells/well for 36 h to form a confluent monolayer. Subsequently, the wells were scratched with a 200 μL sterile pipette tip to create a linear gap in the confluent cell monolayer. Following two washes to remove the scraped cells, the remaining cells were treated with fresh culture medium containing the Ac-FFFGHK-OH hydrogel for 24 h at 37 °C. Images of the migration areas were acquired using an inverted microscope.

14) Tube formation assay

To determine the effect of promoting vascular regeneration by the Ac-FFFGHK-OH hydrogel, we performed a tube formation experiment. The HUVECs (2×10^5 cells/well) were seeded into 24-well plates pre-coated with Matrigel. After HUVECs were seeded, the Ac-FFFGHK-OH hydrogel was added into the above 24-well plates containing HUVECs. After 5 h of incubation, the images were acquired on an inverted microscope.

15) Western blot analysis

To determine the protein expression level of VEGF after treatment with the Ac-FFFGHK-OH hydrogel, HUVECs (3×10^6 cells) were seeded in 100 mm dishes and allowed to incubate overnight. Thereafter, the cells were treated with culture medium containing the Ac-FFFGHK-OH hydrogel for a period of 48 h at 37 °C. The extraction of whole proteins and subsequent quantification of their concentrations were conducted using a BCA protein assay kit. Subsequently, the whole proteins were subjected to further separation by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes with a pore size of 0.22 μm. The PVDF was incubated with the primary antibodies for 12 h, followed by an additional incubation period of 2 h with the secondary antibodies. The protein bands were then detected using a chemiluminescence imaging system. The intensity of the protein bands was quantified using the ImageJ software (NIH, Bethesda, MD, USA).

16) Enzyme linked immunosorbent assay (ELISA)

The Raw264.7 (5×10^5 cells/well) were cultured in 6-well plates overnight. The cells were

stimulated with LPS (100 ng/mL) for 1 h and then co-treated with the Ac-FFFGHK-OH hydrogel for another 24 h. The supernatant was collected and determined by ELISA.

17) Surgeries

The animal experiment was conducted in accordance with the ethical standards set forth by the Ethics Committee on Laboratory Animal Management of Wenzhou Institute, University of Chinese Academy of Sciences (Approval Document No. WIUCAS23030115). The study utilized eight-week-old male Sprague-Dawley (SD) rats. The surgical procedure was analogous to that employed in human penile circumcision. In brief, the rats were anaesthetised via an intramuscular injection of ketamine and xylazine. To create the post-circumcision edema model, the outer prepuce was elevated, and then the outer prepuce tissue was excised to a depth of approximately 5 mm in a ring. The severed ends of the outer prepuce were then sutured to the inner skin with No. 5 sutures at the 12, 3, 6, and 9 o'clock positions. The edema of the prepuce was apparent on the first postoperative day. Subsequently, fifteen male SD rats were randomly allocated to three groups: a sham circumcision group (designated as the "sham" group), a circumcision group treated with saline (designated as the "saline" group), and a circumcision group treated with an Ac-FFFGHK-OH hydrogel (designated as the "Ac-FFFGHK-OH" group). All rats were administered the treatment thrice during the treatment period. The region of edema in the prepuce was photographed by optical microscopy on days 1, 3, 5, 7, 9, and 11 to observe the rate of edema resolution.

18) Histological and immunofluorescent analysis for tissues

All tissues were fixed in 4% paraformaldehyde for a period of 24 h prior to undergoing a series of dehydration, clearing, and embedding procedures, which were conducted in accordance with standard histological techniques. The resulting tissue blocks were then sectioned at a thickness of 4 μm. Subsequently, the tissue sections were subjected to histological analysis, which entailed staining in accordance with the protocol outlined in the Hematoxylin and Eosin (H&E) Staining Kit. For immunofluorescent analysis, the tissue sections were deparaffinized. Subsequently, 3% hydrogen peroxide was employed to quench the endogenous peroxidase activity in the tissue sections for a period of five minutes, after which they were washed with phosphate-buffered saline (PBS). Subsequently, the sections were incubated with 5% goat serum for 1 h at room temperature, after which they were incubated with the primary antibody at 4 °C overnight. Following this, the sections were washed and incubated with fluorochrome-conjugated secondary antibodies. Finally, the cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI).

19) Statistical analysis

Data in this study were statistically analyzed using GraphPad Prism 8 software. All data were expressed as mean ± standard deviation (SD). For data conforming to normal distribution, t-test was performed between two groups, one-way ANOVA was performed between multiple groups, and non-parametric test was used for data not conforming to normal distribution. The p-value less than 0.05 was considered as statistically different. And the significant differences in the figures are labeled as follows: * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$, ns = no significant difference.

Supplementary data

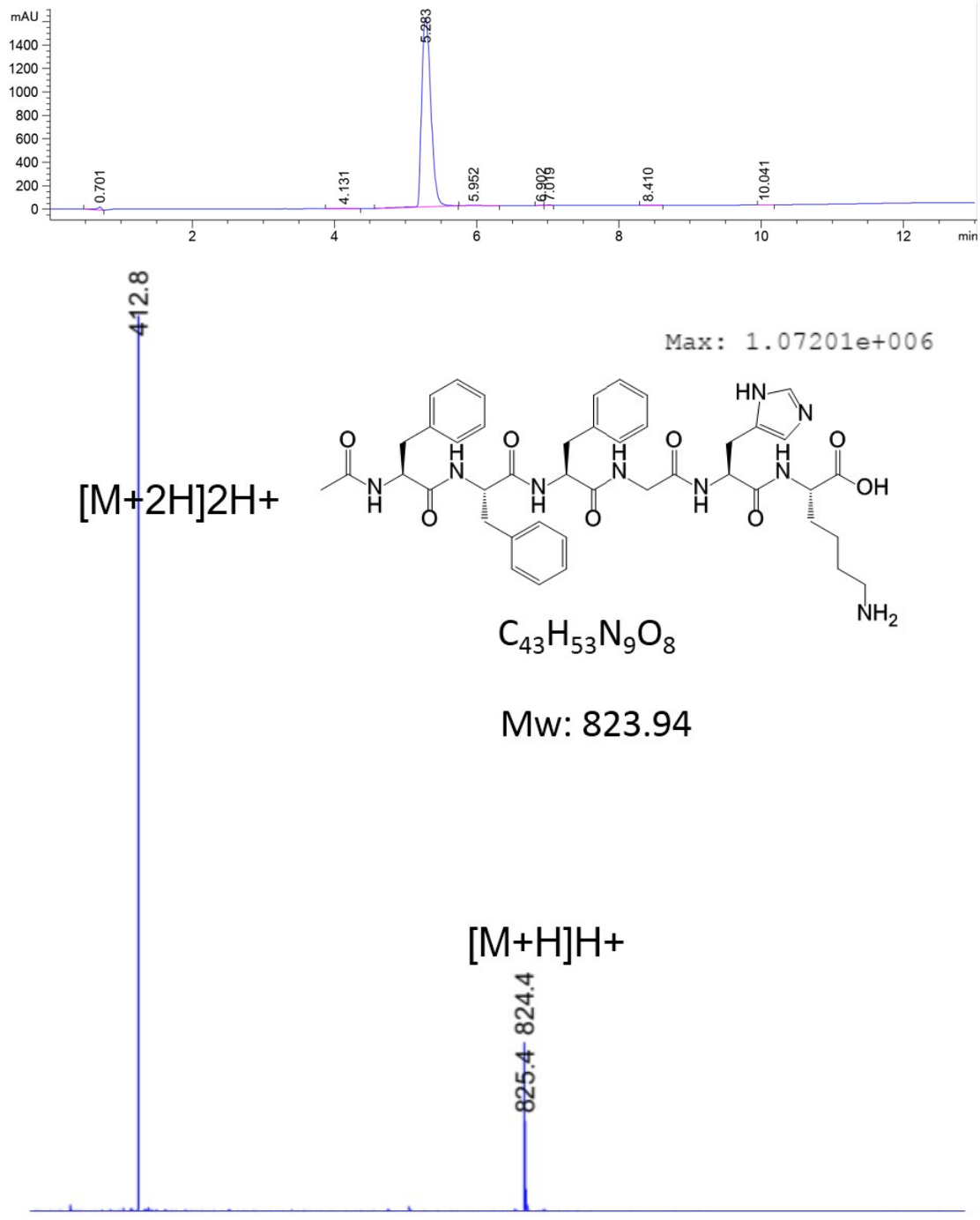


Fig. S1 HPLC-MS analysis of Ac-FFFGHK-OH.

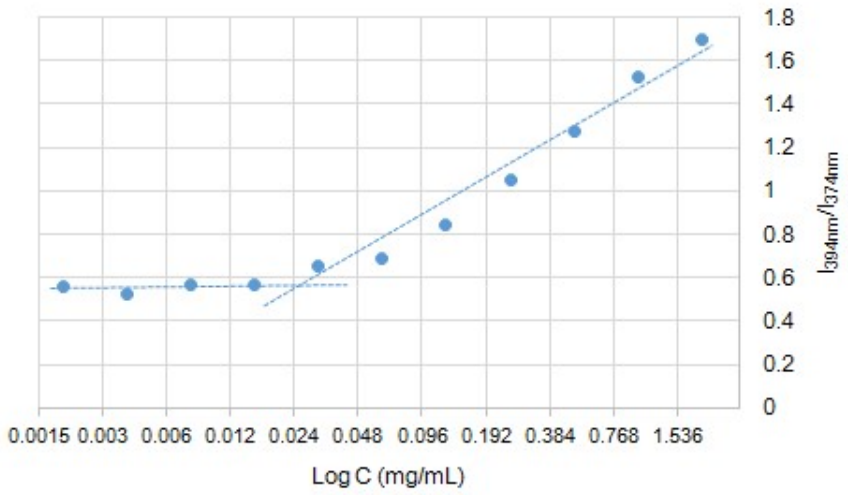
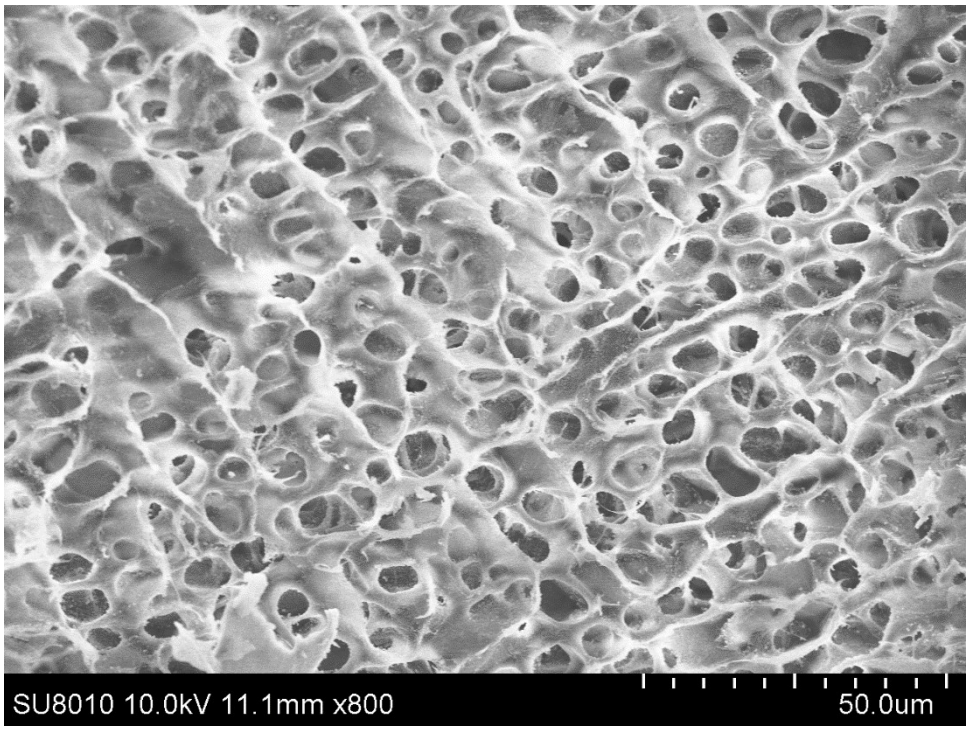


Fig. S2 Determination of the CAC value of Ac-FFFGHK-OH.



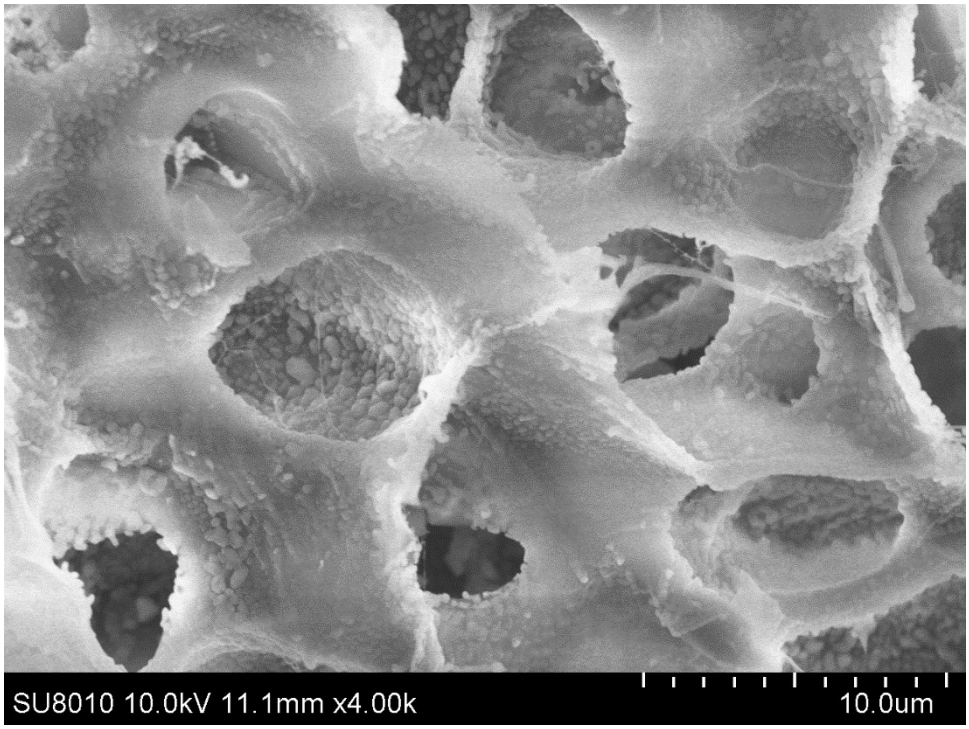
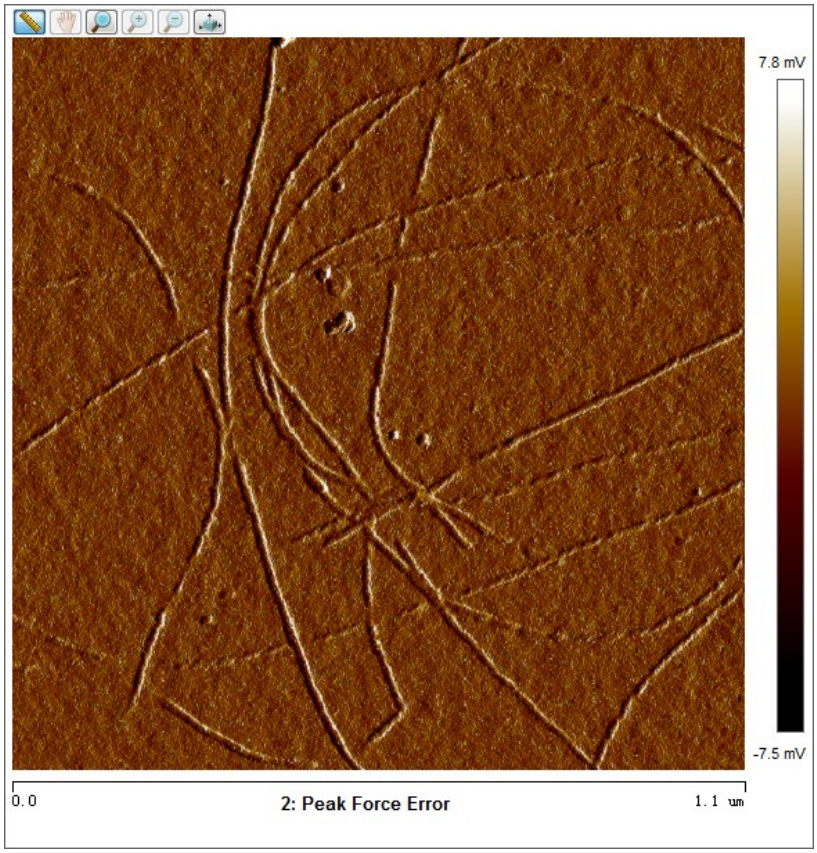


Fig. S3 SEM images of the Ac-FFFGHK-OH hydrogel.



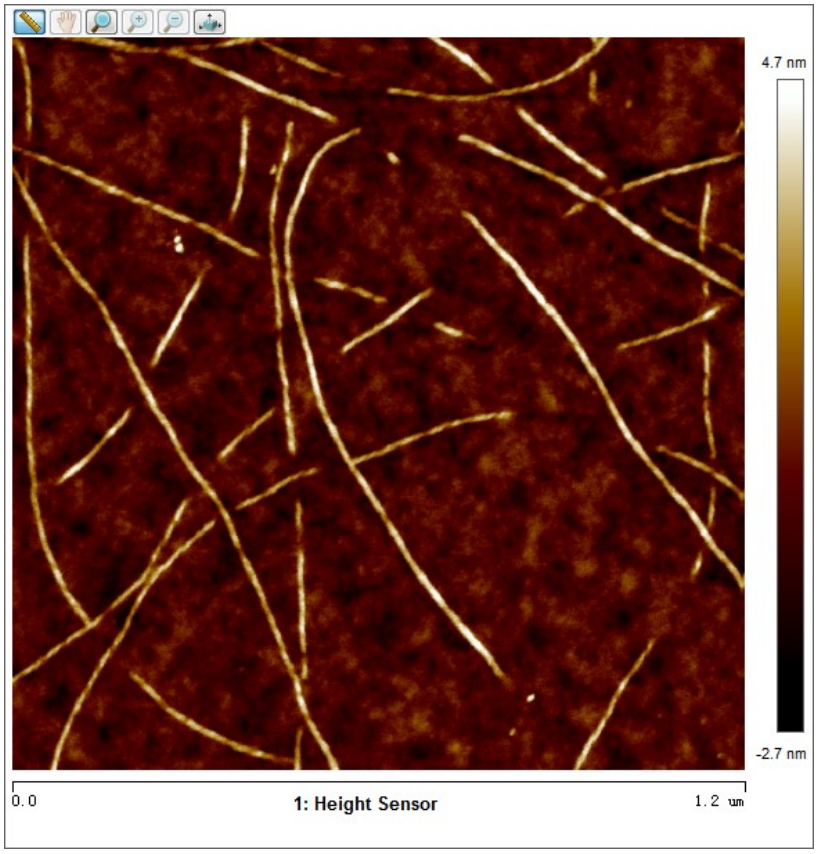


Fig. S4 AFM images of the Ac-FFFGHK-OH assemblies in saline.

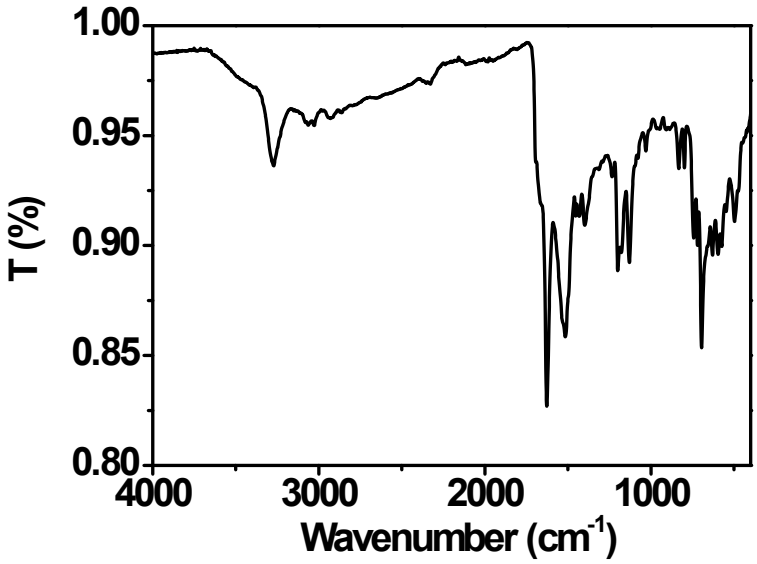


Fig. S5 ATR-FTIR spectrum of the Ac-FFFGHK-OH hydrogel.

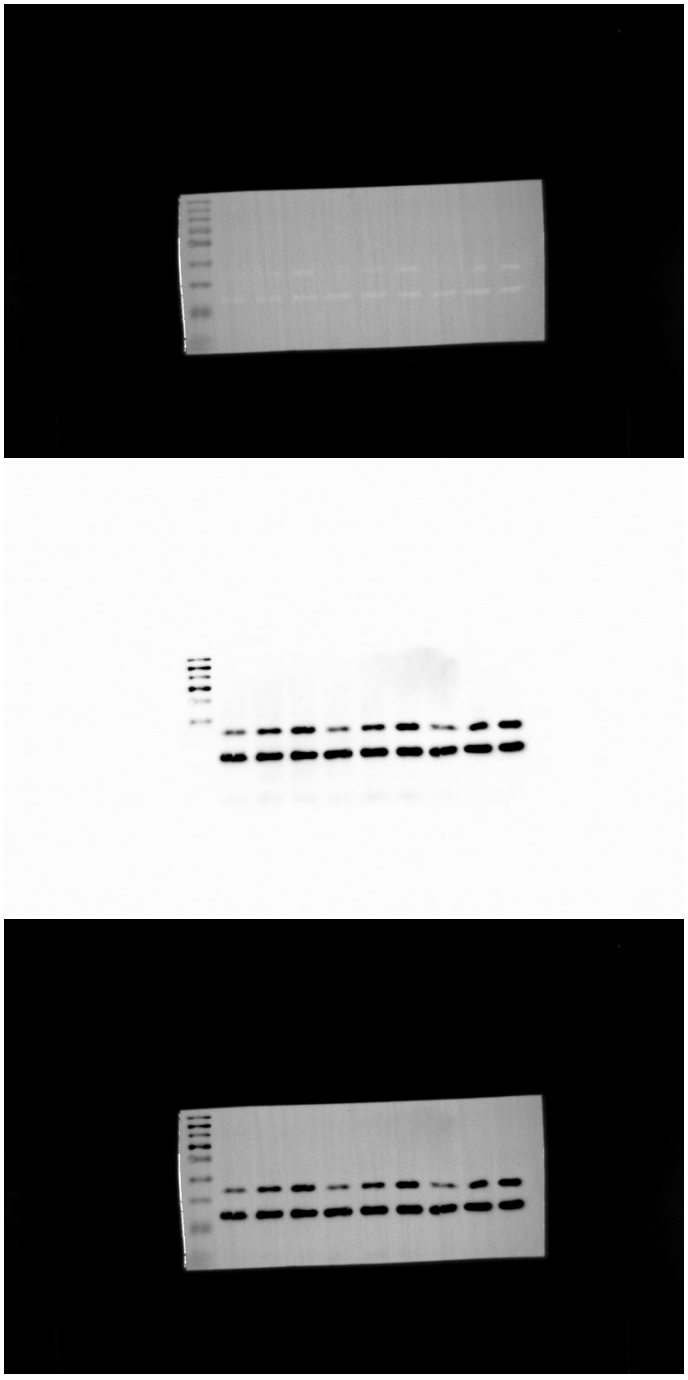


Fig. S6 Western blot analysis of the relative protein expression level of VEGF by HUVECs following different treatments for 48 h.