SUPPORTING INFORMATION

Pentapeptide IKVAV-Engineered Hydrogels for Neural Stem Cell Attachment

Yixia Yin,[a] Wenwu Wang,[a] Qi Shao,[a] Binbin Li,[a] Dan Yu,[a] Xin Zhou,[a] Jayanti Parajuli,[a] Haixing Xu,[a] Tong Qiu,[a]* Ali Kemal Yetisen,[b] Nan Jiang[c,d]*

[a] State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, Wuhan 430070, China. Email: tongttqq@whut.edu.cn

[b] Department of Chemical Engineering, Imperial College London, London SW7 2AZ, UK

[c] West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, China. Email: jiangnansophia@scu.edu.cn

[d] School of Engineering and Applied Sciences, Harvard University, Cambridge 02138, USA

Content:

Materials

Preparation and characterization of IKVAV-PLEOF hydrogel

Isolation and Identification of NSC spheroids

Degradation of the IKVAV-PLEOF hydrogel

Cell proliferation in IKVAV-PLEOF hydrogels

Cells adhesion on hydrogels

The proliferation of NSC spheroids in the IKVAV-PLEOF hydrogel

Hemolysis Assay

IKVAV-PLEOF hydrogel implantation in vivo

Statistical Analysis

Figures

Materials:

N,*N*'-methylene bisacrylamide (BISAM) and *N*,*N*'-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co. Ltd (China). 4,4'-Azobis(4-cyanovaleric acid) (ACP) was purchased from Bailingwei Technology Co., Ltd (China). Antibodies, 4',6-diamidino-2-phenylindole (DAPI), collagenase (powder) were bought from Thermo Fisher Scientific (USA). Diethylene glycol (DEG), (triethyl)amines (TEA), tannous octoate (STANOCT), IKVAV (Purity = 95.16%), YIGSR (Purity = 97.01%) peptides, trypsin, N, N-dimethylaminopyridine (DMAP), B27, Fibroblast Growth Factor basic (bFGF), epidermal Growth Factor (EGF), penicillin-streptomycin, ANTI-NESTIN (S1409) antibody produced in rabbit (Nestin IgG), toctylphenoxyplyethoxyethanol (Triton-X,) phosphate buffer solution (PBS) were bought from Sigma-Aldrich (USA). Cell Counting Kit-8 was purchased from Beyotime (China). Calcein-AM/PI Double Stain Kit was purchased from Solibao (Beijing). Fetal bovine serum (FBS), alpha modified eagle minimum essential medium (αMEM), RIPM1640 culture medium, penicillin/streptomycin and DMEM/F12 were purchased from Gibco (USA). Anti-CD31 rabbit pAb and Cy3 conjugated goat anti-rabbit IgG were purchased from Servicebio (China). L929 (fibroblasts) were purchased from ATCC. Male Sprague Dawley (SD) rats and New England Rabbits were purchased from Hubei Center for Disease Control. The experimental procedures involving laboratory animals were approved by the Institutional Animal Care and Use Committee of Wuhan University of Technology and were performed in accordance with Guides for the Care and Use of Laboratory Animals from Wuhan University of Technology and the U.S. National Institutes of Health.

Preparation and characterization of IKVAV-PLEOF hydrogel:

Ultralow molecular weight poly(lactic acid) (ULMW-PLA, Mn = 2266) was synthesized by mixing DEG, STANOCT, and lactide. The molar ratio of DEG and STANOCT was 50:1, while the lactide and DEG were 12:1. The procedure was conducted at 140 °C for 24 h. Pure PLEOF was then fabricated by reacting ULMW-PLA, fumaryl chloride, PEG (Mw = 4000), and TEA at -5 °C with nitrogen gas for 6 hours.[1] The reactant was kept at room temperature for 12 h. ^{1}H-NMR spectrometer (Bruker, 600 MHz) was conducted to characterize the synthesized PLEOF.

Acrylated IKVAV(Ac-IKVAV) peptide and YIGSR (Ac-YIGSR) peptide were then synthesized. Briefly, both of the two peptides were reacted with an excess of acryloyl chloride

(dry methanol) with the catalyst of DMAP. This process was maintained for 2 h at room temperature, followed by rotary evaporation with methanol for three times after each step, to remove the methyl acrylate byproduct and unreacted reactant. The acrylated peptides were dissolved in deionized (DI) water and stored at 4 °C. Appropriate amounts of PLEOF monomer, Ac-YIGSR (as a cross-linking agent in the work), Ac-IKVAV, BISAM, ACP and DMF were placed in the flask, evacuated and sealed. The reactor was heated in an oil bath to 80 °C. The composition of the reactants can be seen in Table S1. The control group (PLEOF hydrogel) was obtained by mixing the PLEOF polymer with BISAM, ACP, and DMF. After 10 h of reaction, hydrogels with different IKVAV concentrations were obtained, followed by immersing in DI water. The DI water was replaced at intervals to remove unreacted monomers, cross-linkers, and linear polymers in the hydrogels. After 3 days, the hydrogel was placed under -20 °C for 24 h. Finally, the hydrogel was placed in a vacuum freeze-drying machine at -40 °C for 48 h, so that the water was sublimated from the solid state to a gaseous state to maintain the morphology and network structure of the gel. The functional groups of IKVAV-PLEOF hydrogel scaffolds were characterized by Fourier-transform infrared spectroscopy (FTIR). Infrared spectra were obtained in the range of 4000 to 500 cm^{-1}, with a spectral resolution of 4 cm^{-1}.

Isolation and Identification of NSC spheroids:

NSCs have the ability of self-renewal and differentiation, and can differentiate into other cells (such as neurons, oligodendrocytes, astrocytes) after asymmetric division. Pregnant SD rats within 14 days were anesthetized by 4 wt% chloral hydrate anesthetic at a dose of 0.1 mL/10 g and immersed in 75% alcohol for removing the fetus rat. The bilateral cerebral cortex of the fetal rat was removed quickly. After washing with PBS and transferring to DMEM/F12 medium, the blood vessels and meninges were peeled off and cut. 0.25 vol% trypsin-0.04 vol% EDTA was added and transferred to an incubator for digestion for 20 min followed by filtration using a nylon mesh to remove excess tissue fibers. The collected suspension was centrifuged (1000 rpm, 5 min), and the supernatant was removed. NSC culture medium (DMEM/F12, 2 vol% B27, 20 ng mL^{-1} bFGF, 20 ng mL^{-1} EGF, 1 vol% penicillin-streptomycin) was added and pipetted into a single cell suspension. After counting, the cell density was adjusted to 2×10^5 mL^{-1} medium, and seeded in a T25 cell culture flask. After adding the NSC culture medium, it was cultured in a 37 °C, 5 vol% CO_2 environments, and the NSCs were proliferated and split

into larger NSC spheroids and then passaged every 3 days. As cells are passaged, the NSCs were be purified.

After the purified NSC spheroids were cultured for 3 days. Subsequently, the NSCs were fixed with 4 wt% paraformaldehyde (Seville Biotechnology Co., Ltd., China) for 30 min at 4 °C. Incubation with 0.2% Triton-X for 5 min was preceded by two washes with PBS. The Nestin IgG as the primary antibody in PBS (at a volume ratio of 1:400) containing 5% goat serum was kept overnight at 4°C. Followed by washing with PBS, the NSCs were incubated with Cy3-conjugated secondary antibodies in PBS containing 5% goat serum at room temperature for 2h. Then all nuclei were stained with DAPI Stain (1:1000, Sigma) at room temperature for 10 min in order to visualize Nestin negative cells. Finally, the NSCs were imaged using a fluorescence microscope (IX70, Olympus, Japan).

Degradation of the IKVAV-PLEOF hydrogel:

The hydrogel was immersed in PBS and collagenase solutions (0.03 wt%) respectively to evaluate its degradation performance. Hydrogels (10 mm in diameter, 2 mm in depth) with different IKVAV concentrations were prepared and lyophilized at -40 °C. The structure of the hydrogel was imaged using a field-emission scanning electron microscope (SEM)(JEM-7500F, Japan). Each group of the freeze-dried hydrogel was weighed (m_0) and immersed in PBS. The hydrogel scaffold was recorded (m_1) every other week to measure its degradation rate.

Cell proliferation in IKVAV-PLEOF hydrogels:

To evaluate the biocompatibility of the hydrogels with different concentrations of IKVAV, different hydrogels were extracted with serum-free medium for 72 h at 37 °C with 150 rpm h^{-1}. L929 (fibroblasts) cells were seeded in a 96-well-plate (100 μL) at a density of 1×10^4 mL^{-1}, and cultured in a 37 °C, 5 vol% CO_2 for 24 h, the extracts of different hydrogel materials were added, the control group (without extract) and the blank group were set. On day 1, 3, 5 and 7, CCK8 solution was added then incubated for 1h. The absorbance of each group was measured at 450 nm using a microplate reader (Thermo-Scientific Multiskan GO1510, USA).

Cells adhesion on hydrogels:

The IKVAV-PLEOF hydrogels were prepared for cell adhesion and proliferation. NSCs were seeded in hydrogels at a density of 4×10^5 mL^{-1}. The control group was the pure PLEOF hydrogel. For NSC adhesion tests, the hydrogels were washed with PBS after incubated for 1 h in different groups, the unattached cells were washed away, the attached cells on hydrogel were stained with DAPI for 10 min, then imaged by a fluorescence microscope, images were randomly selected in each group for the calculation of adhesion rate. To evaluate the proliferation of NSC spheroids, we measured the size of the spheroids in the hydrogel using the software of ImageJ. 5 micrographs were captured for each group, which contains 50-150 spheroids. The spheroids with only 30-400 nm diameters were counted, and the AVONO statistical analyzation was further performed (n = 3, *: $p < 0.05$; **: $p < 0.01$).

After 3 days, NSCs were stained with Calcein-AM/PI Double Stain Kit for 20 min to evaluate the viability of NSCs. At 490 ± 10 nm excitation wavelength, the emitted green and red were observed simultaneously. In addition, 545 nm excitation wavelength was used to observe dead cells. On day 3, NSCs with hydrogels were fixed by 4 wt% paraformaldehyde, the growth and the morphology of NSCs with hydrogels are imaged by an Environment Scanning Electron Microscopy (ESEM, Quanta X50, USA).

NSCs on the IKVAV-PLEOF hydrogel-coated surfaces: 12-mm glass coverslips used for NSCs two-dimensional cultures experiments were soaked in ethanol to encourage hydrophilicity, then spin-coated with 50 μL of a 0.2 mmol L^{-1} IKVAV-PLEOF pre-gel solution. After coated with poly-lysine (encourage adsorption) and subsequently with IKVAV-PLEOF pre-gel solution, the coverslips were dried overnight, and then washed three times with distilled water to remove weakly adherent material before the addition of cell suspension. The NSCs spheres were seeded on different surfaces. The results of cultures were imaged after different time points. The neurons cultured on the hydrogel surfaces were fixed and stained with β-Tubulin III antibody and DAPI for 10 min and imaged by a fluorescence microscope.

The proliferation of NSC spheroids in the IKVAV-PLEOF hydrogel:

The 8×8×2 mm^3 freeze-dried hydrogel were sterilized under the UV exposure, and then transferred into 12-microwell plate. 200,000 of NSCs were then re-suspended in 0.2 mL of B27 culture medium containing 20 ng mL^{-1} of FGF-2 and 20 ng mL^{-1} of EGF, and loaded onto the hydrogel scaffolds. The NSCs were infiltrated into the hydrogel scaffold, and 0.8 mL of the complete medium were gently added along the side of microwells, avoiding to disturb the NSCs. The culture medium was replaced with fresh complete medium every day. After

culturing for 3 to 7 days, the NSC spheroids were imaged using the fluorescence microscope, and the diameters of the NSC spheroids were measured, and analyzed by ImageJ (software).

Hemolysis Assay:

Red blood cells (RBCs) from New Zealand Rabbit were used to evaluate the compatibility of the hydrogels. First, 2 mL of the blood sample was added to 4 mL of PBS, and RBCs were isolated from serum by centrifugation at 9700 rpm for 10 min. The RBCs were further washed 6 times with 20 mL of PBS. The purified blood was diluted to 80 mL of PBS. Prior to the extraction of the hydrogels, the absorbance spectrum of the positive control supernatant was checked and used only if it was in the range of 0.50-0.55 optical density units to reduce sample difference from different donors. Then, RBCs incubation with DI water and PBS were used as the positive and negative control groups, respectively. Then, 0.2 mL of diluted RBCs suspension was added to 0.8 mL extract of different groups of hydrogels and mixed by vortexing. All the sample tubes were kept in static conditions at room temperature for 3 h. Finally, the mixtures were centrifuged at 9700 rpm for 3 min, and 100 μL of the supernatant of all samples was transferred to a 96-well plate. The absorbance values of the supernatants at 570 nm were determined by using the microplate reader. The percent hemolysis of RBCs was calculated using the following formula:

$$Hemolysis = \frac{A_{sample} - A_{control}}{A_{positive\ control} - A_{negative\ control}} \times 100\% \qquad \text{(Eq.S1)}$$

IKVAV-PLEOF hydrogel implantation *in vivo*:

Eight-week-old, male SD rats were housed in plastic cages, under standard laboratory conditions (22-24 °C, relative humidity of 55-65% and a cycle of 12 h light/12 h dark), with free access to water and standard diet (granulated feed) except during the period of the experiments. After sterilization, the IKVAV-PLEOF hydrogels with 0.2 mmol L^{-1} (test groups) and PLEOF hydrogels (control groups) were implanted into the animal to test the histocompatibility of the material. 4 wt% chloral hydrate anesthetic was intraperitoneally injected at a dose of 0.1 mL/10 g, the cylindrical hydrogel (5 mm in diameter, 2 mm in depth) was completely buried under the skin mucosa and the skin was

closed with sutures. The PLEOF hydrogels were implanted as a control group. After the operation, the states of the rats were frequently monitored. The SD rats were sacrificed by carbon dioxide method at 4^{th} week post-surgery, and the surrounding tissues of the material were surgically excised, fixed in 4 wt% paraformaldehyde, and stored at 4 °C. Pathological observation of the local inflammatory reaction and local degradation of the hydrogel material were carried out by paraffin embedding, sectioning and H&E staining. The subcutaneous tissue sections were stained (red) with anti -CD31 rabbit pAb (1:1000), and Cy3 conjugated goat anti-rabbit IgG (1:400). The nucleus was stained in blue and observed under a fluorescence microscope

Statistical Analysis:

All the data were statistically analyzed to express the mean ± the standard deviation (SD) of the mean. Student's t-test was performed, and $p < 0.05$ was accepted to be statistically significant.

Figures

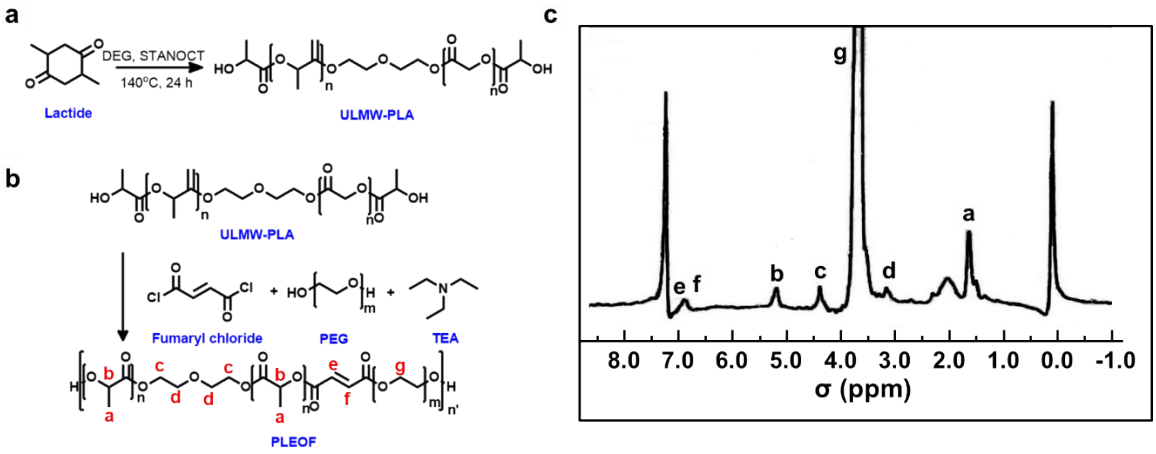

Figure S1. Synthesis of PLEOF hydrogel. (a) Step 1: Preparation of ultra-low molecular weight-poly(lactic acid) (ULMW-PLA). (b) Step 2: Preparation of PLEOF. (c) NMR spectrum of as-synthesized PLEOF.

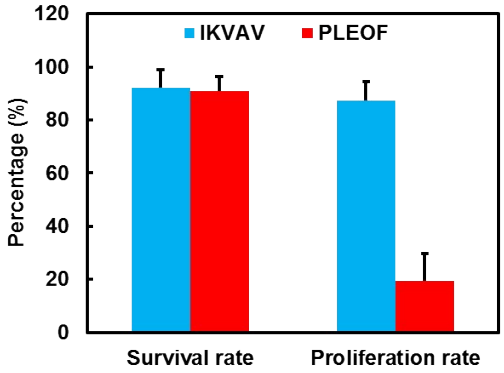

Figure S2. The statistics of Live/dead assay of adhered NSCs cultured on IKVAV-PLEOF hydrogel surface and pure PLEOF hydrogel surface using ImageJ software.

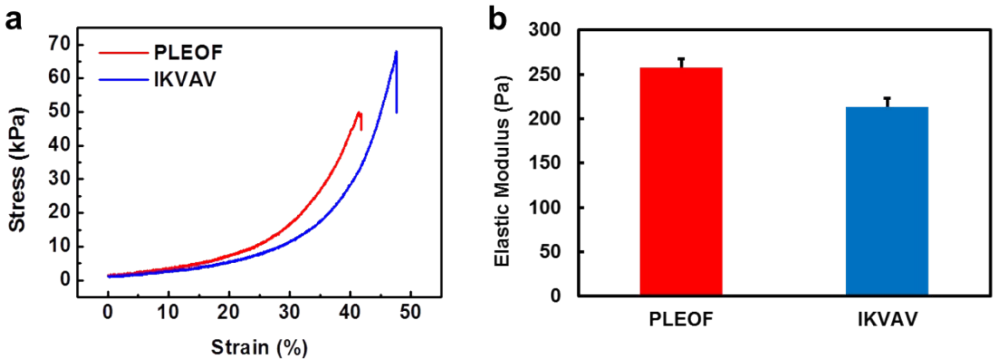

Figure S3. Mechanical tests of IKVAV-PLEOF hydrogels. (a) Stress-strain curve and (b) elastic modulus of PLEOF/IKVAV-PLEOF hydrogels, tested by mechanical test (MTS, Instron, America) under the condition of (1mm min^{-1}, diameter = 8 mm, height = 8 mm).

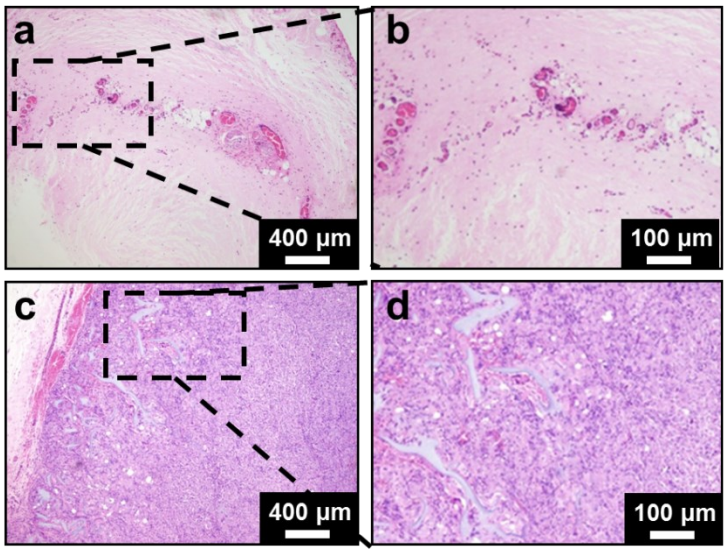

Figure S4. H&E staining of cross-section images of the tissue implanted with (a, b) the IKVAV-PLEOF hydrogel at a concentration of 0.2 mmol L^{-1} and with (c, d) PLEOF-only hydrogel.

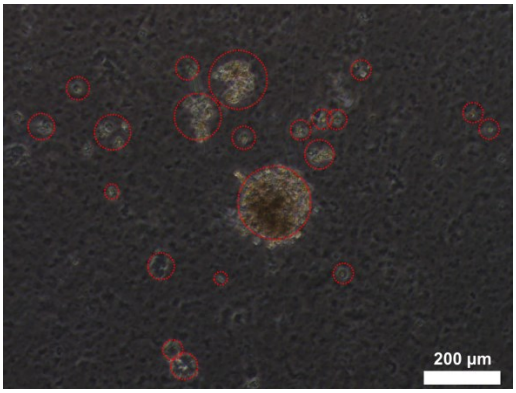

Figure S5. Micrograph of 3D-encapsulated NSC spheroids. Red circles indicate the NSC spheroids.

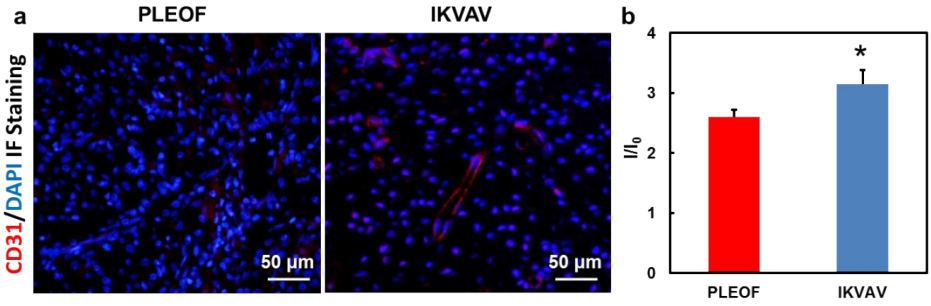

Figure S6. Immunofluorescent staining test. (a) Immunofluorescent staining with DAPI (blue, nucleus stained) and CD31(red, endothelial marker) of the subcutanuous tissue implanted with the pure PLEOF hydrogel and the IKVAV-PLEOF hydrogel. (b) Quantitative measurement of the CD31 staining by using Image J (n = 3, *: $p<0.05$).

Table S1. Raw material ratio of different IKVAV-PLEOF hydrogels

Sample	PLEOF (mg)	YIGSR (mg)	IKVAV (mg)	BISAM (mg)	ACP (mg)	DMF (mg)
M1	945	120	5	150	9.45	3
M2	945	120	25	150	9.45	3
M3	945	120	50	150	9.45	3
M4	945	120	100	150	9.45	3
M5	945	120	250	150	9.45	3

[1] B. Li, P. Zhang, Y. Yin, T. Qiu, Y. Tao, X. Wang, S. Li, *Journal of Wuhan University of Technology-Mater. Sci. Ed.* **2014**, *29*, 824.