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Supporting Information

Micropatterned shape-memory polymer substrate containing hydrogen bonds creates a long-term dynamic microenvironment for regulating nerve-cell fate

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1. Experimental Section: Preparations and Characterizations
2. Additional Results: Fig. S1 to S14, Table S1 and S2

1. Experimental Section

Materials

Six-armed polyethylene glycol (^{6}PEG) was purchased from Liming Chemical Research and Design Institute Co., Ltd. (China). Anhydrous sodium sulfate and calcium hydride were obtained from General Reagent (China). ε-Caprolactone (ε-CL) was purchased from Alfa Aesar Chemical Co., Ltd. Isocyanyl ethyl methacrylate, anhydrous dimethyl sulfoxide (DMSO), benzoyl peroxide (BPO), stannous octoate ($Sn(Oct)_2$), and acryloyl chloride (AC) were purchased from Adamas Reagent Co., Ltd. (China). 2-Amino-4-hydroxy-6-methylpyrimidine and potassium carbonate were obtained from TCI (Shanghai) Development Co., Ltd. (China). Other organic reagents were obtained from Chengdu Kelong Reagent Corp. (China).

Synthesis of PHU

First, several of the raw materials had to be purified before use. ε-CL was purified by reduced pressure distillation after dehydration with calcium hydride, and then ^{6}PEG-PCL was synthesized by ring-opening polymerization of ε-CL (40 mL) using $Sn(Oct)_2$ as the catalyst and ^{6}PEG (20 g) as the initiator (140 °C, 8 h). ^{6}PEG-PCL-AC was obtained by esterification of ^{6}PEG-PCL (30 g) with excess AC (3 mL) in anhydrous dichloromethane (150 mL), where potassium carbonate ($1.5 \times n_{AC}$) was used as the acid-binding agent (25 °C, 48 h). UPyMA was synthesized from isocyanyl ethyl methacrylate (2.4 mL) and 2-amino-4-hydroxy-6-methylpyrimidine (2 g) in anhydrous DMSO (40 mL) at 170 °C (10 min). Finally, PHU was obtained by free-radical polymerization of ^{6}PEG-PCL-AC, UPyMA, and HEMA using BPO as the initiator. The ratios of the prepolymer are displayed in Table S1. After polymerization, PHU was placed in ethyl alcohol for 3 days, replacing ethanol every 12 h. Then, PHU was placed in RO water until swelling equilibrium was reached, before conducting the follow-up experiments.

The aligned micropatterns were constructed on the surface of the dehydrated PHU by hot-pressing at 120 ℃ for 5 min and then transferring the patterned PHU to an ice bath to stabilize the permanent shape.

Characterizations of PHU

^{1}H-NMR spectra were obtained using an AVANCE NEO 400 MHz spectrometer (Bruker, Germany) and $CDCl_3$ as the solvent. FT-IR spectra were obtained using a NICOLET5700 spectrometer (Thermo Scientific, USA), and variable-temperature IR spectra were obtained by testing every 10 ℃ from 10 ℃ to 160 ℃.

Swelling behavior was analyzed by measuring the weight increase of the dried specimens after immersion in water (swelling ratio, formula (1)) and the weight loss after immersion in trichloromethane (gel content, formula (2)).

$$Swelling\ Ratio = \frac{m_s}{m_0} \times 100\% \quad (1)$$

where m_0 was the original mass of the sample, and m_s was the mass of the sample after swelling equilibrium in water.

$$Gel\ Content = \frac{m_d}{m_0} \times 100\% \quad (2)$$

where m_0 was the original mass of the sample, and m_d was mass of the sample after swelling in trichloromethane for 48 h and completely drying.

The crystallinity was measured using an Empyrean X-ray diffractometer (Malvern Panalytical, Netherlands). WCAs were measured using a DSA 100 contact-angle meter (Kruss, Germany). Static tensile tests were performed using an INSTRON 5965 electronic universal testing machine (Instron, USA), and the Young's modulus was calculated using the slope of the first 5% strain.

Thermal properties were evaluated using a DSC-2500 differential scanning calorimeter

(Waters, USA) with a temperature-change rate of 5 °C/min from –30 °C to 160 °C. The dissociation temperature of UPyMA was measured by temperature-modulation DSC with a heating rate of 2 °C/min from –30 °C to 160 °C, a period of 110 s, and an amplitude of 0.3 °C.

The R_f and R_r that reflect shape-memory properties were calculated by measuring the length changes during the shape-memory process using Equations (3) and (4). The shape-memory process was divided into three stages: deforming at 80 °C (or other temperatures) under external stress in water, fast cooling to -30 °C and removing the external stress and then unloading at 4 °C, and finally recovering at 37 °C (in water).

$$R_f = \frac{(l_2 - l_0)}{(l_1 - l_0)} \times 100\% \quad (3)$$

$$R_r = \frac{(l_2 - l_t)}{(l_2 - l_0)} \times 100\% \quad (4)$$

where l_0 was the original length of the sample, l_1 was the length of the sample after deforming under external stress, l_2 was the length of the deformed sample after fast cooling and unloading in 4 °C refrigerator for 12 h, and l_t was the residual length of the sample after recovering for different times.

The micropatterns on the PHU were directly observed using a SZX16 type microscope (OLYMPUS, Japan), and the gap width was calculated using Image J after placing the PHU in different environments. To construct different dynamic environments, temporary shapes of patterned PHU were obtained by deformation along different directions (vertical or horizontal), using the direction of the pattern as the reference direction. Changes in the pattern during the shape-memory process were also observed, and width changes were calculated using a Contour GT white-light interferometer (Bruker, Germany).

Cell Culture, Proliferation, and Plating

Poorly and highly differentiated PC12 cells were purchased from Wuhan SAIOS Biotechnology

Co. Ltd. (China) and were cultured with 1640 RPMI Medium (Gibco) containing 10% fetal bovine serum (ExCell Bio) and 1% penicillin/streptomycin solution (Cytiva) at 37 ℃ with 5% CO_2. The cell viability of PHU was measured in a CCK-8 assessment (Beyotime) by removing materials and adding medium with 10% (v/v) CCK-8 after co-culturing the cells with material for 1, 3, and 5 days and then measuring absorbance at 450 nm after 2 h. Live and dead cells were stained by removing materials and replacing the medium with 0.1 M PBS containing calcein (1:2000, Yeasen) and propidium iodide (1:1000, Yeasen) for 15 min at 37 ℃ after co-culturing cells with materials for 1, 3, and 5 days. Cells were observed using a fluorescence microscope after washing with PBS once.

PC12 cells were stimulated with 50 ng/mL NGF (Novoprotein) for at least 3 days before plating. The cells were divided into five groups: static and unpatterned (SU), static but patterned (SP), programmed (dynamic) but unpatterned (DU), vertically programmed and patterned (VP), and horizontally progranmmed and patterned (HP). The dynamic groups (DU, VP, HP) before plating were preprogrammed, and after plating cells on the surface at a density of 5×10^4 cells mL^{-1}, the materials were placed in a cell-culturing environment (37 ℃, 5% CO_2) to slowly recover.

Investigation of cell alignment and distribution

First, the Image J software was used to calculate the distribution intensities of PC12 cells in different groups at different directions (relative to the horizontal line), and thus to study the orientation and distribution of PC12 cells. Then, the oriented angels (θ) of PC12 cells in each group were calculated from the average values of the distribution intensities, and the degree of orientation (F) of PC12 cells was calculated using formula S1.

$$F = 100 \times 0.5 \times [3 \times \cos^2(\theta - \theta_0) - 1] \qquad \text{(S1)}$$

In formula S1, θ_0 was the angel for reference. In detail, for groups with patterns, θ_0 was the angel between the pattern direction (indicated by the yellow arrows in Fig.5a and 6a) and the horizontal line. For groups without patterns but with a dynamic recovery, the reference direction was assumed to be perpendicular to the recovery direction (indicated by the white arrows), and then θ_0 was determined as the angel between the reference direction and horizonal line. For groups with neither pattern nor dynamic recovery, θ_0 was 0°. The differences in the *F* between these groups were taken both the results in 1 day and 3 days into account.

Immunofluorescent Staining of cell F-actin, nucleus, and YAP

To observe the changes in cell morphology, TRITC Phalloidin (1:200, Yeasen) and DAPI (1:200, Biofroxx) were used to mark F-actin and the nucleus, respectively, following the manufacturer's instructions. Images captured by fluorescence microscopy were analyzed using ImageJ to assess the orientational arrangement of the cells, where at least three duplicate samples were obtained for each group.

The distribution of YAP was observed using a primary rabbit anti-YAP antibody (1:200, Cell Signaling Technology) and goat anti-rabbit Alexa Fluor 488 (1:500, Beyotime) to mark protein following the manufacturer's instructions. After staining the F-actin and nucleus, the distribution of YAP in the cells could be observed by comparing the fluorescence intensity of YAP in the nucleus and cells.

Immunofluorescent Staining of MAP2 and Tuj1

The common neurospecific proteins (MAP2 and Tuj1) were labeled with primary antibodies of mouse anti-Tuj1 (1:500, Beyotime) and rabbit anti-MAP2 (1:500, Proteintech). They fluoresced via secondary antibodies of goat anti-mouse Alexa Fluor 594 (1:500, Solarbio) and goat anti-rabbit Alexa Fluor 488 (1:500); besides, the nuclei were marked with DAPI following the

instruction manual.

qPCR test of MAP2 and Tuj1

The steps for mRNA extraction were as follows: First, 600 μL of cell lysis buffer (Tsingke) were used to lyse cells in each group, and then trichloromethane (120 μL) was used for extraction. After centrifuging (14000 g, 4 ℃, 15 min), the upper clear-water phase was collected carefully, and then isopropanol (equal amount to water phase) was added to separate the milky floccule (mRNA). After centrifuging (14000 g, 4 ℃, 10 min) and removing the solution, the floccule was washed with 75% ethyl alcohol (enzyme-free water preparation) and then centrifuged (14 000 g, 4 ℃, 5 min) to collect the floccule for re-washing. Finally, the floccule was dried at 4 ℃, and then enzyme-free water was added to dissolve it for subsequent experiments.

The gene expression of MAP2 and Tuj1 was tested by qPCR using the HiScript III Strand cDNA Synthesis Kit (Novizan) to obtain cDNA and the Taq Pro Universal SYBR qPCR Master Mix (Novizan) to conduct qPCR tests. All operations were conducted following the manufacturer's instructions. The primer sequences are listed in Table S2.

2. Additional Results

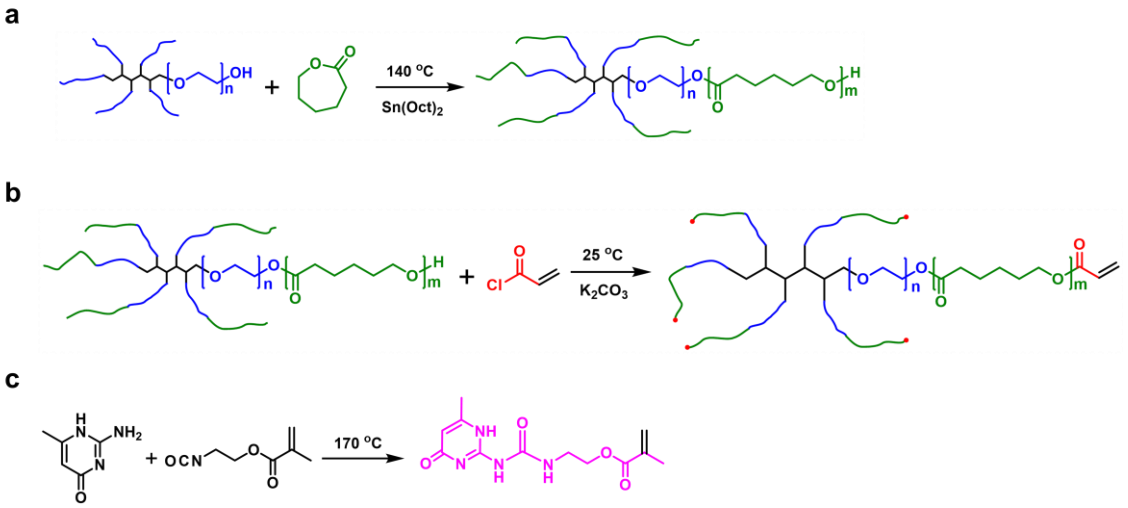

Fig. S1. Preparation of prepolymers and monomer: (a) ^{6}PEG-PCL, (b) ^{6}PEG-PCL-AC, and (c) UPyMA.

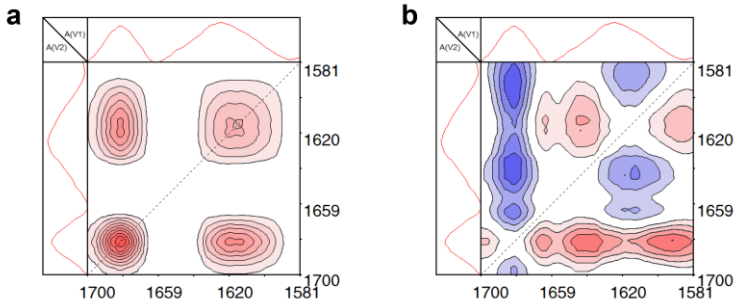

Fig. S2. 2D FT-IR of Amide I and II. (**a-b**) Synchronous spectrum (a) and asynchronous spectrum (b) of 2D FT-IR in the range of 1700-1581 cm^{-1}.

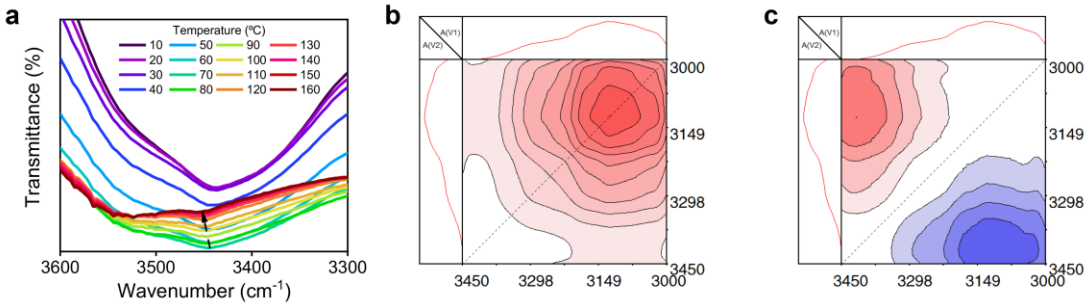

Fig. S3. 2D FT-IR spectra. (**a**) Variable temperature infrared spectrum in the range from 3600 cm^{-1} to 3300 cm^{-1}; (**b-c**) Synchronous spectrum (b) and asynchronous spectrum (c) of 2D FT-IR in the range of 3450-3000 cm^{-1}.

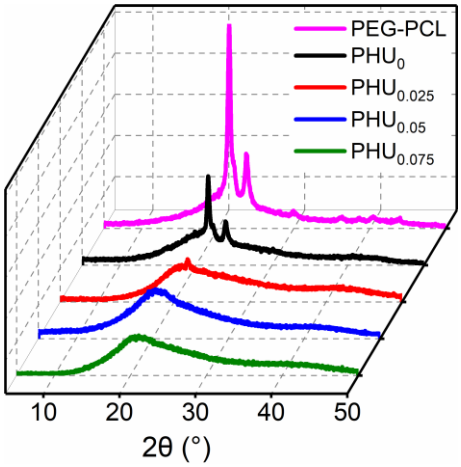

Fig. S4. The results of XRD for PHU with different UPyMA concentrations.

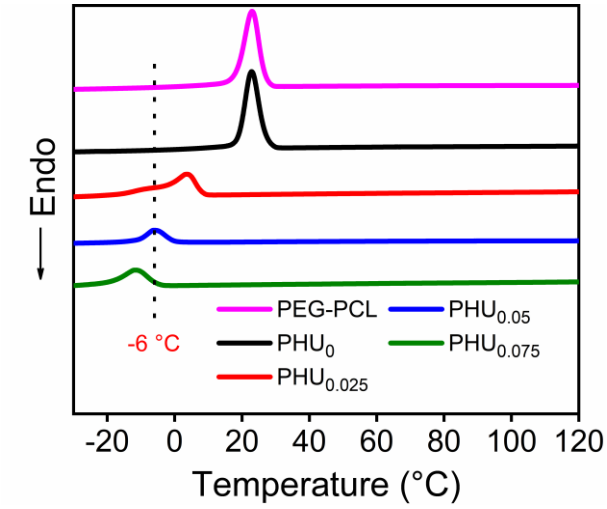

Fig. S5. The DSC results of PHU with different UPyMA concentrations (cooling).

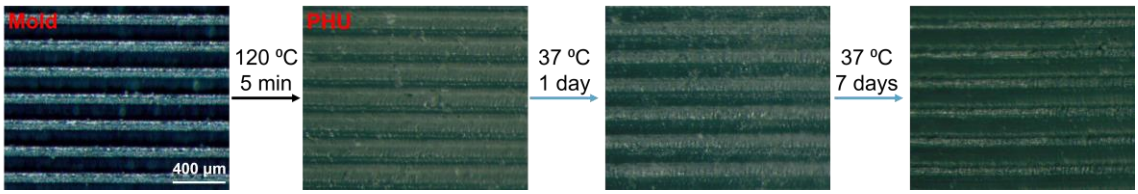

Fig. S6. Photos showing the stability of the micropatterns of PHU after placing at 120 °C for 5 min or 37 °C for 7 days.

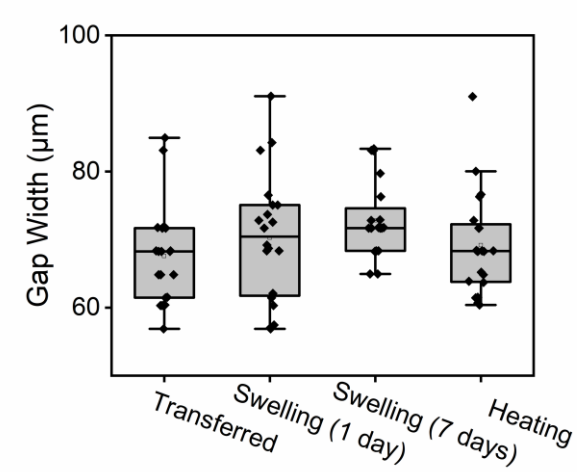

Fig. S7. Gap width of micropatterned PHU in different environments, swelling for 7 days or heating at 80 °C for 2 h.

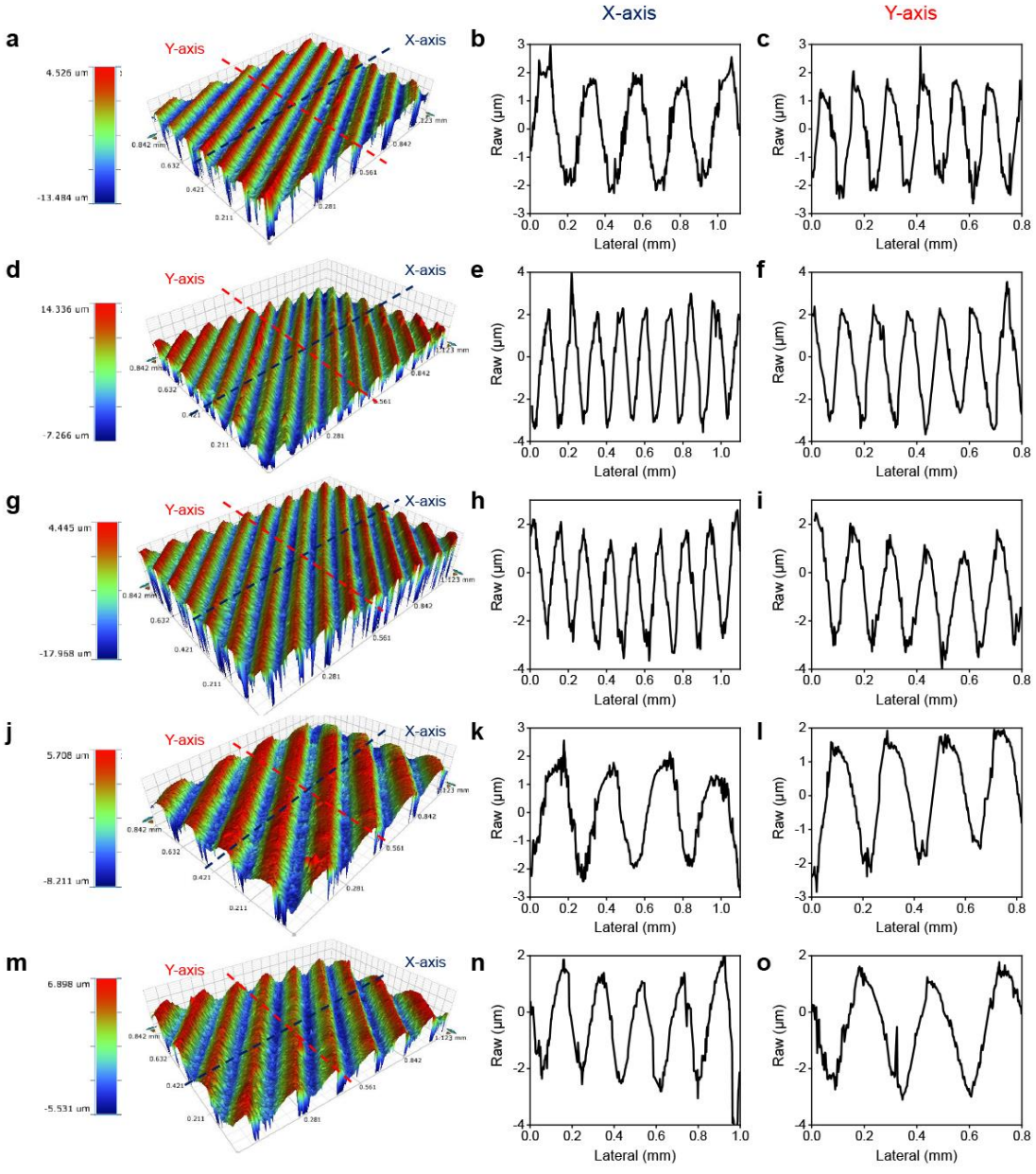

Fig. S8. The results of white light interference tests. (**a-c**) Aligned microstructures of PHU (a) and the

depth along X axis (b) and Y axis (c); (**d-f**) Microstructures of PHU after stretching horizontally (d) and the depth along X axis (e) and Y axis (f); (**g-i**) Microstructures of PHU after stretching vertically (g) and the depth along X axis (h) and Y axis (i); (**j-l**) Recovering of microstructures after stretching horizontally (j) and the depth along X axis (k) and Y axis (l); (**m-o**) Recovering of microstructures after stretching vertically (m) and the depth along X axis (n) and Y axis (o).

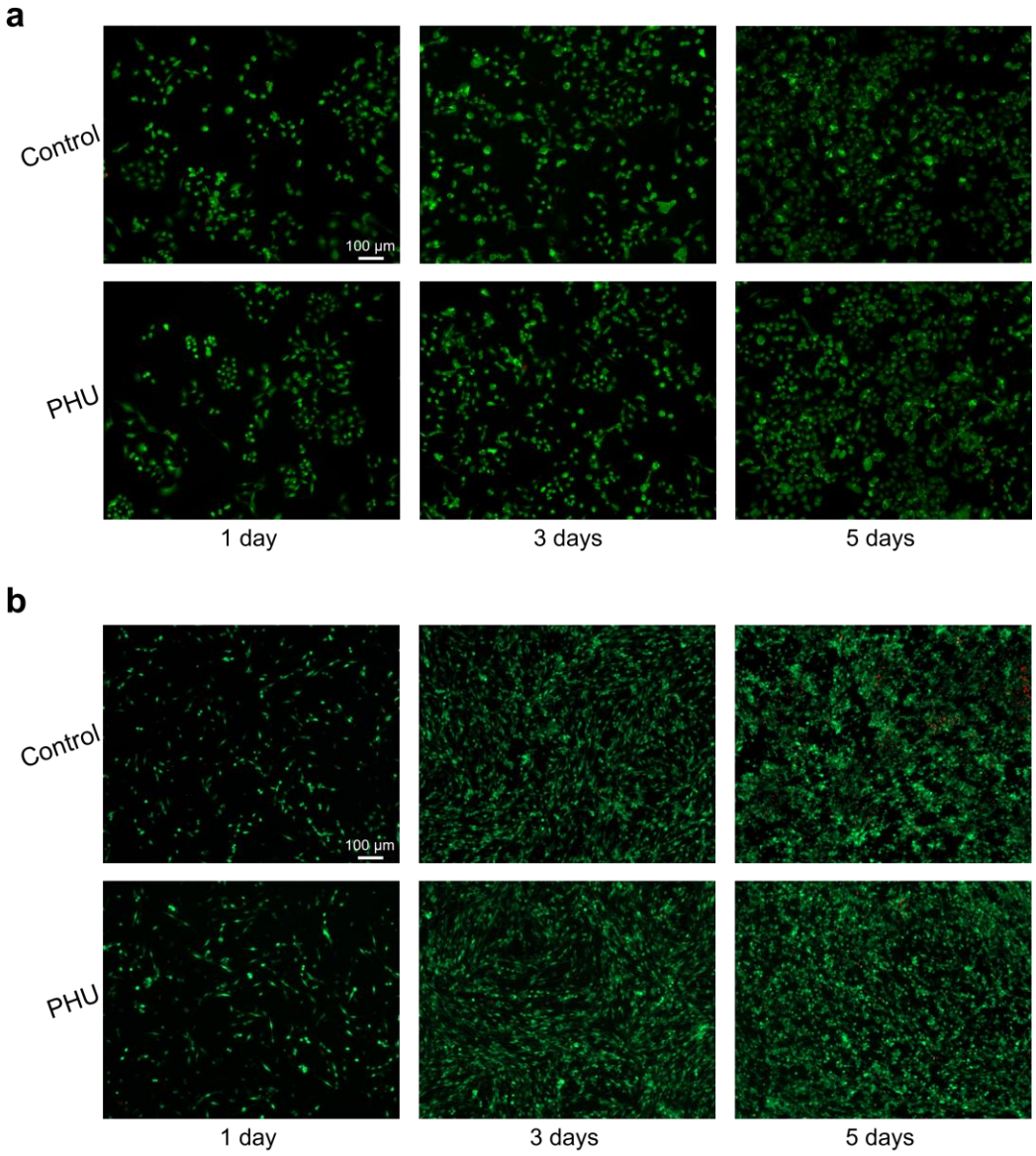

Fig. S9. The results of Live/Dead cell staining. (**a**) Poorly differentiated PC12 cells; (**b**) Highly differentiated PC12 cells.

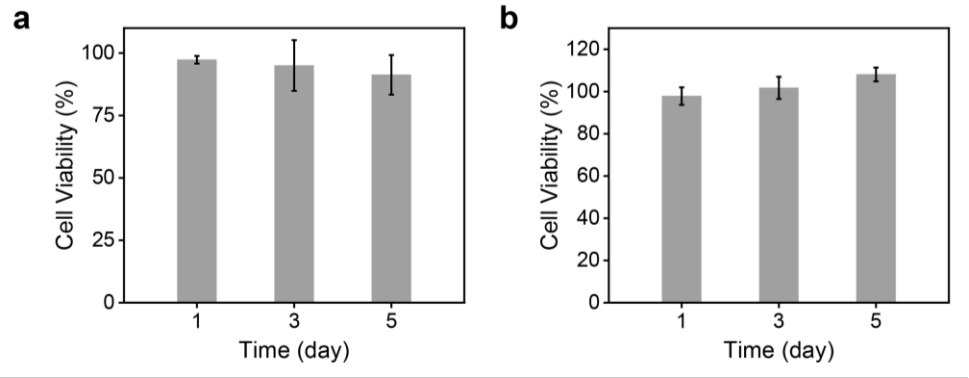

Fig. S10. The results of CCK-8. (**a**) Poorly differentiated PC12 cells; (**b**) Highly differentiated PC12 cells.

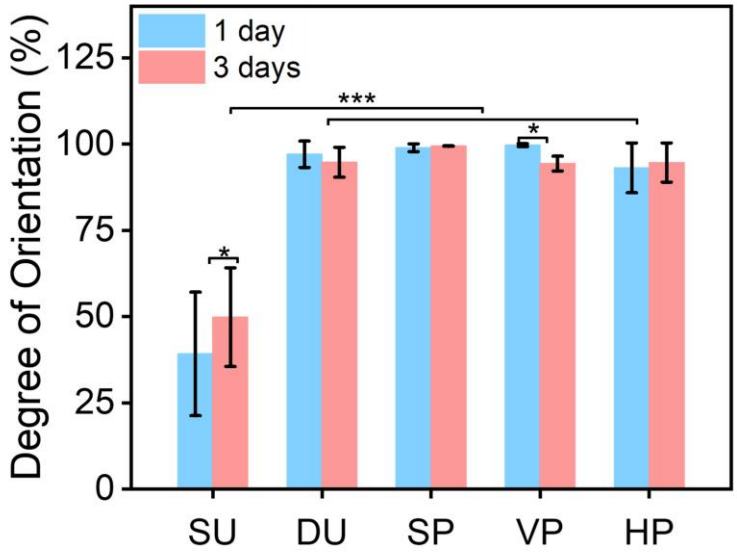

Fig. S11. Degrees of orientation of poorly differentiated PC12 cells. The reference direction of SP, VP, and HP groups is the direction of aligned pattern and it changes to the direction perpendicular to shape recovering in DU group. Besides, it is 0 ° for SU group. Statistical differences: *$p < 0.05$, ***$p<0.001$.

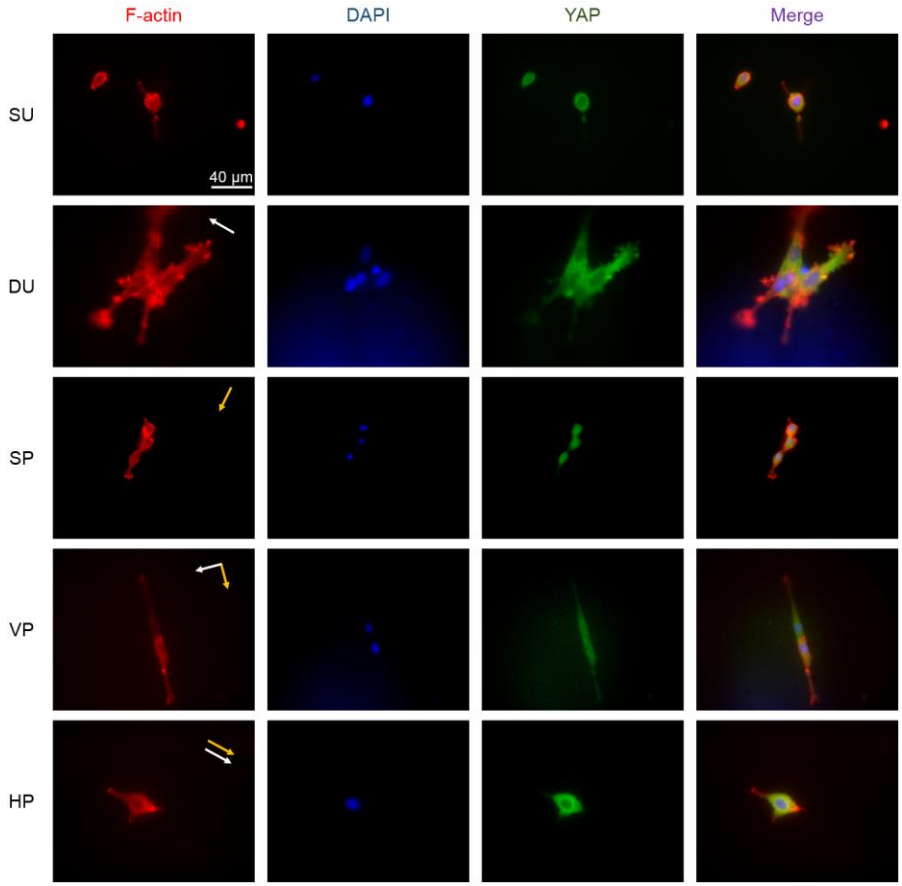

Fig. S12. Immunofluorescence of YAP (1 day) of poorly differentiated PC12 cells.

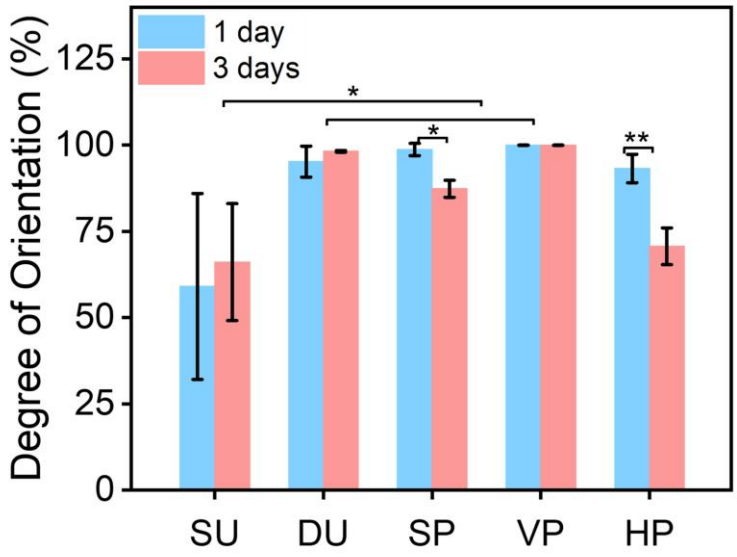

Fig. S13. Degrees of orientation of the highly differentiated PC12 cells. The reference directions of each group are the same as Fig. S11. Statistical differences: $*p < 0.05$, $**p < 0.01$.

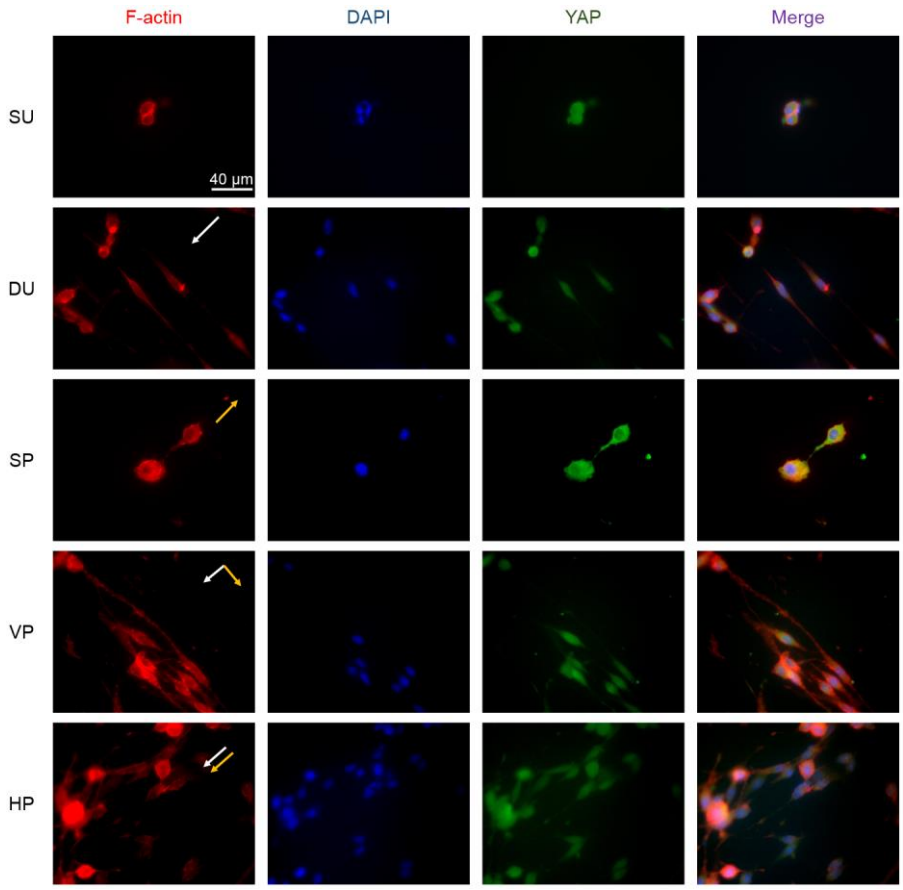

Fig. S14. Immunofluorescence of YAP (1 day) of highly differentiated PC12 cells.

Table S1 The Preparation of PHUx with different compositions.

Abbreviation	^{6}PEG-PCL-AC (g)	HEMA (mL)	UPyMA (g)	BPO (mg)	DMF (mL)
PEG-PCL	0.4	0	0	7	2
PHU_0	0.4	0.1	0	7	2
$PHU_{0.025}$	0.4	0.1	0.05	7	2
$PHU_{0.05}$	0.4	0.1	0.1	7	2
$PHU_{0.075}$	0.4	0.1	0.15	7	2

Table S2 The primer sequences of qPCR.

Abbreviation	Forward primers	Reverse primers
β-Tubulin	5′-ggaacgcatcagtgtctactac-3′	5′-ggcctgaataggtgtccaaa-3′
MAP2	5′-cagctgcactggaagaagc-3′	5′-taaaggctcagcgaatgagg-3′
GAPDH	5′-agttcaacggcacagtcaag-3′	5′-tactcagcaccagcatcacc-3′