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Electronic Supplementary Information (ESI)

Poly[N-(2-acetamidoethyl) acrylamide] supramolecular hydrogels with multiple H-bond crosslinking enable mouse brain embedding and expansion microscopy

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Figures

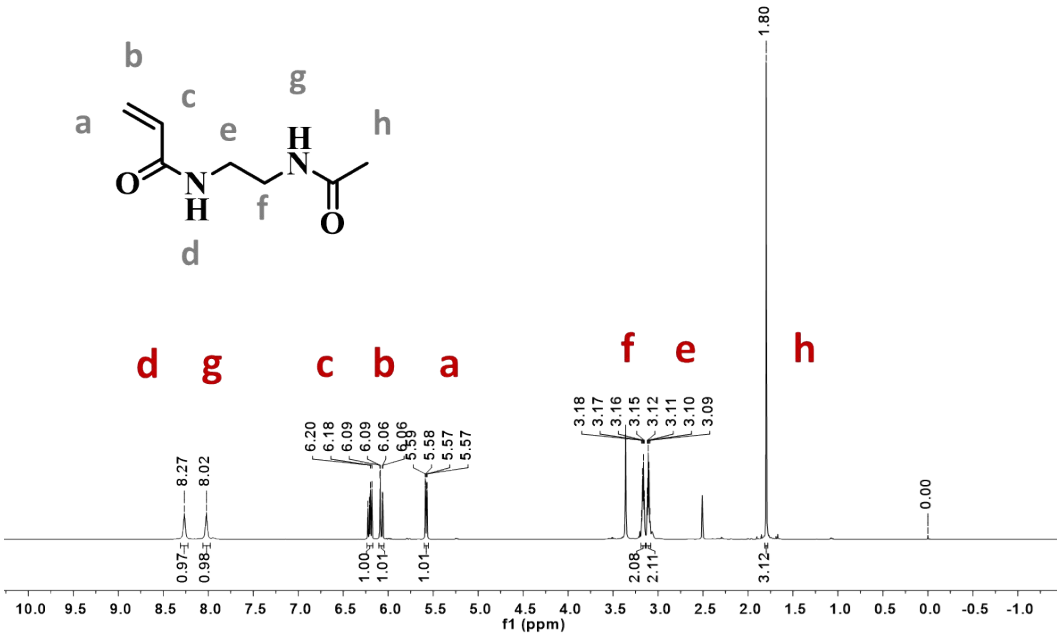

Figure S1 ^{1}H NMR spectrum of N-(2-acetamidoethyl)acrylamide in DMSO-d_6.

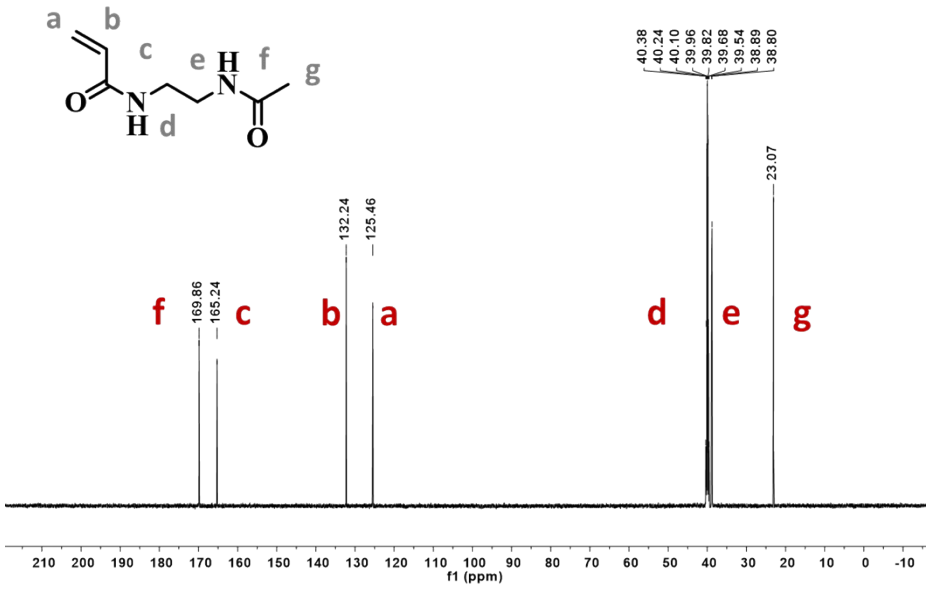

Figure S2 ^{13}C NMR spectrum of N-(2-acetamidoethyl)acrylamide in DMSO-d_6.

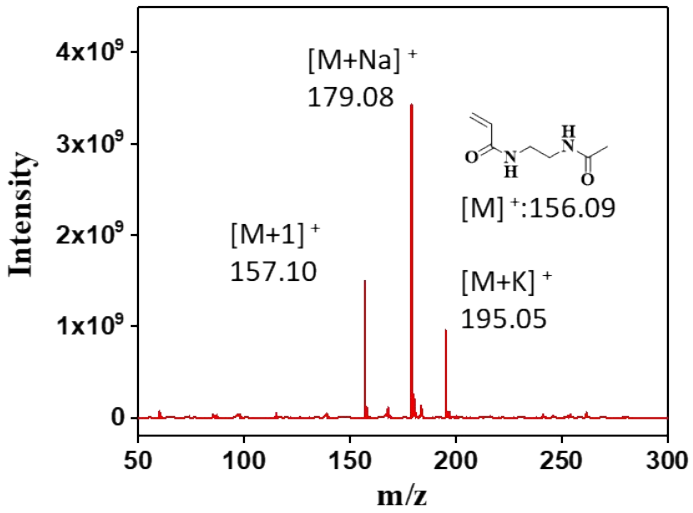

Figure S3 The Mass spectra of monomer AAE.

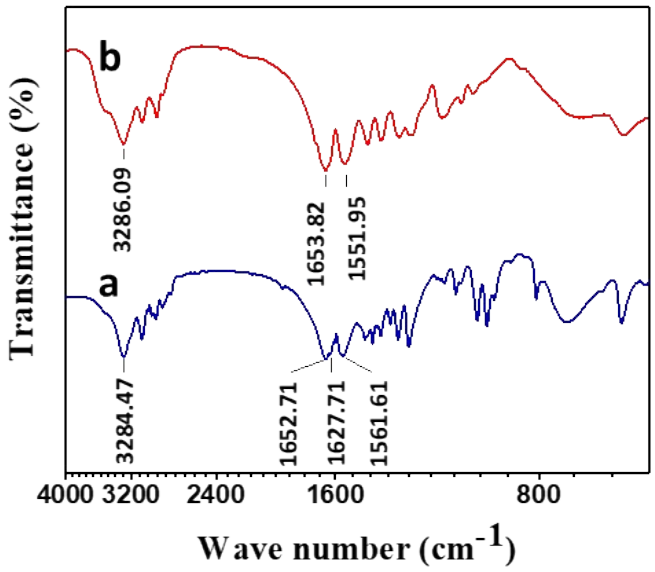

Figure S4 FT-IR spectra of (a) AAE and (b) PAAE.

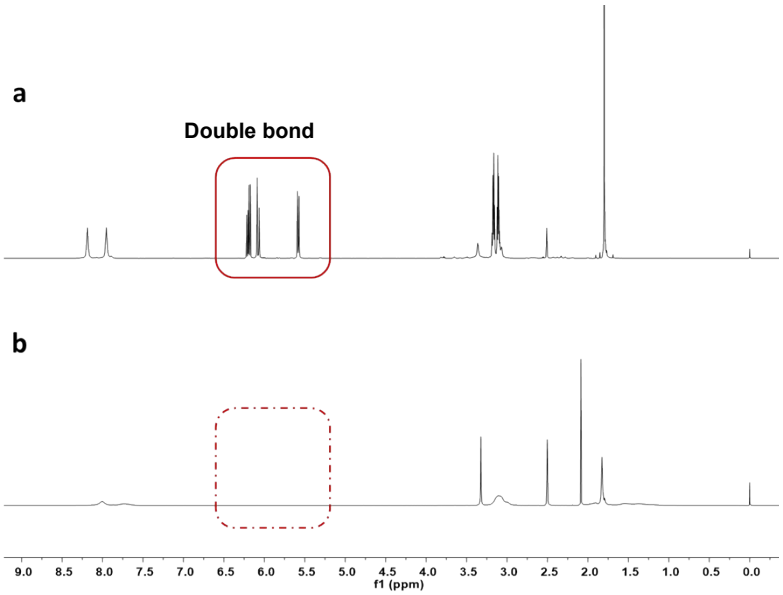

Figure S5 ^{1}HNMR spectra of (a) AAE and (b) PAAE in DMSO-d_6.

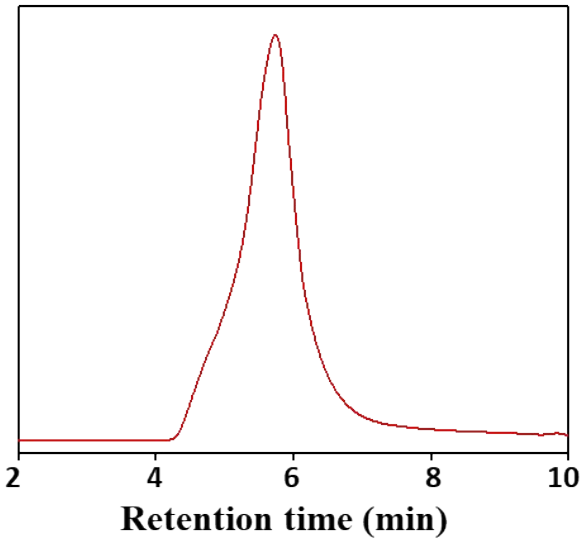

Figure S6 Gel permeation chromatography (GPC) elution trace of PAAE in water.

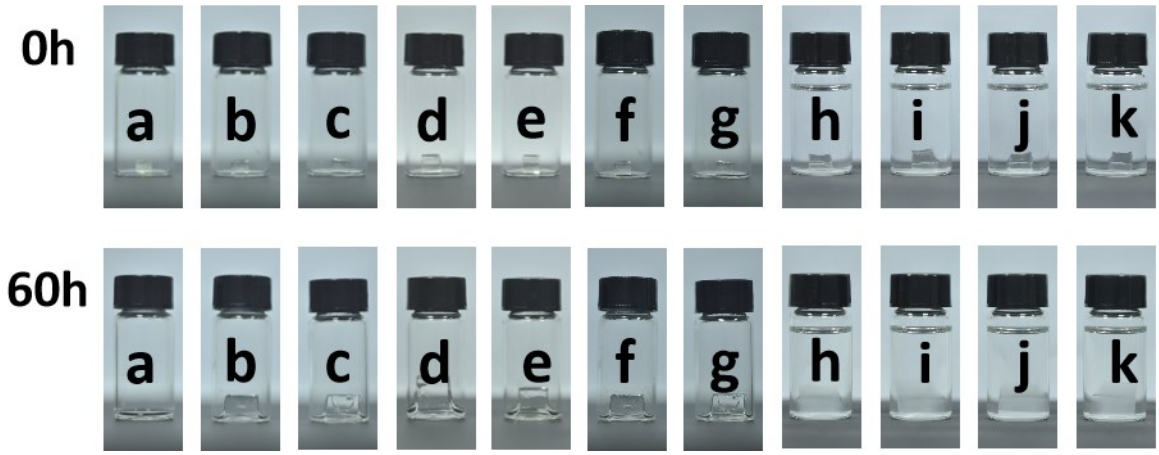

Figure S7 Swelling stability of supramolecular hydrogels. PAAm-30 hydrogel in deionized water (a). PAAE-30 hydrogels in varied solvents: (b) deionized water, (c) 5 mol L^{-1} urea solution, (d) pH=3 buffer, (e) pH=10 buffer, (f) 10 mol L^{-1} NaSCN solution, (g) 1 mol L^{-1} LiBr solution, (h) ethanol, (i) DMSO, (j) DMF, (k) acetonitrile.

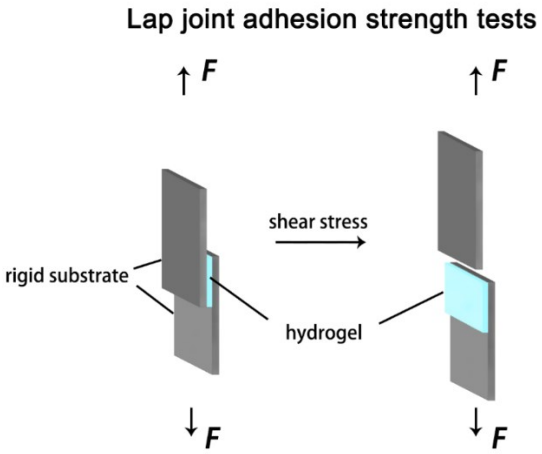

Figure S8 Lap joint adhesion strength tests.

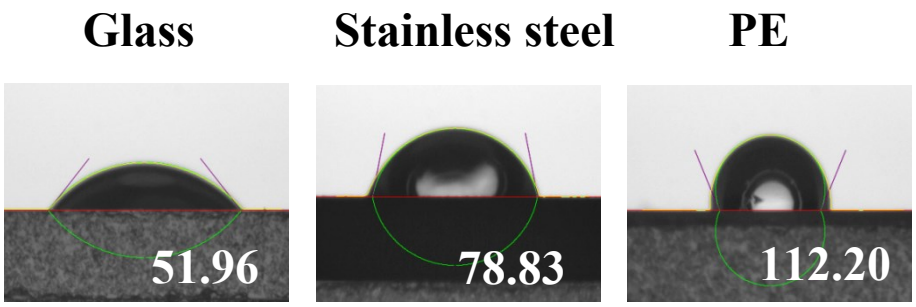

Figure S9 Contact angle of water on different material surfaces.

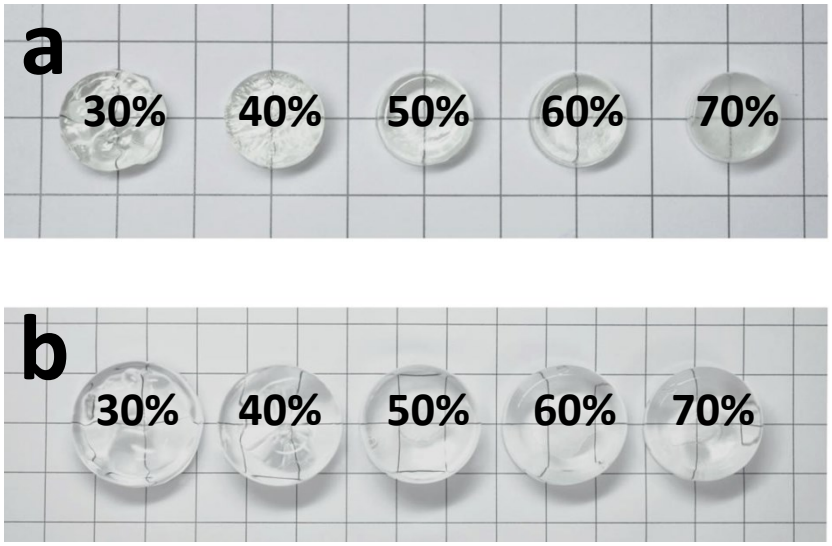

Figure S10 Comparison of swelling size for PAAE hydrogel. a) Original hydrogels; b) hydrogels swelling to equilibrium.

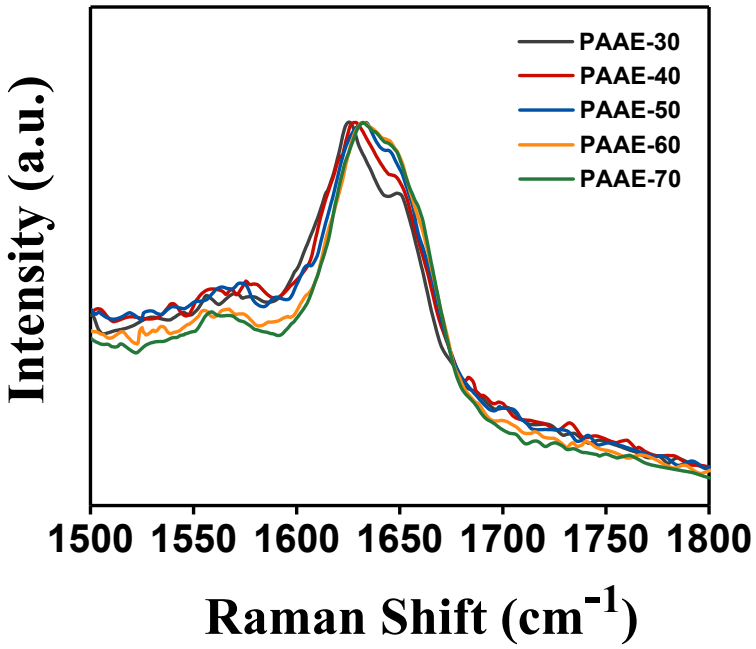

Figure S11 Raman spectra of hydrogel at different monomer contents.

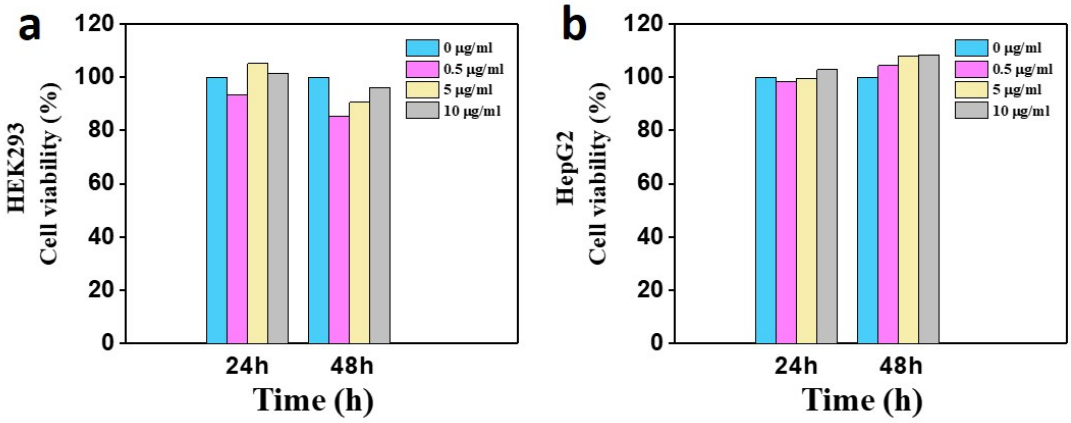

Figure S12 Cytotoxicity results (represented by percentage cell viability) of different concentrations of AAE monomer against a) HEK293 cells, and b) HepG2 cells from MTT assay of different culture time.

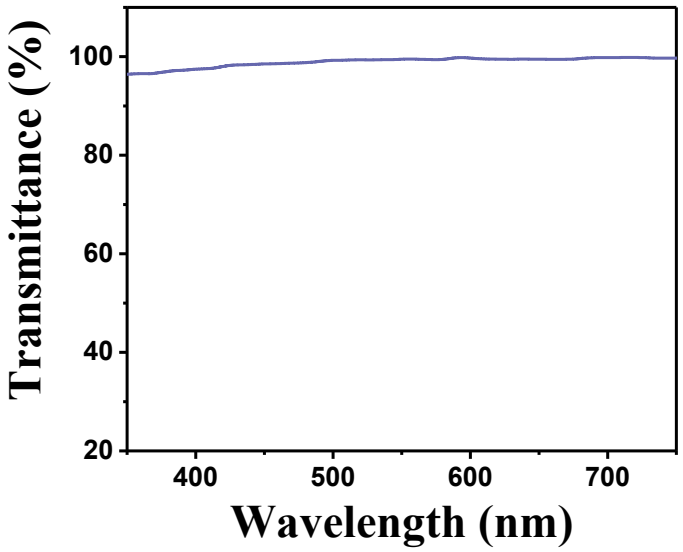

Figure S13 Transparency test of PAAE-40 hydrogel.

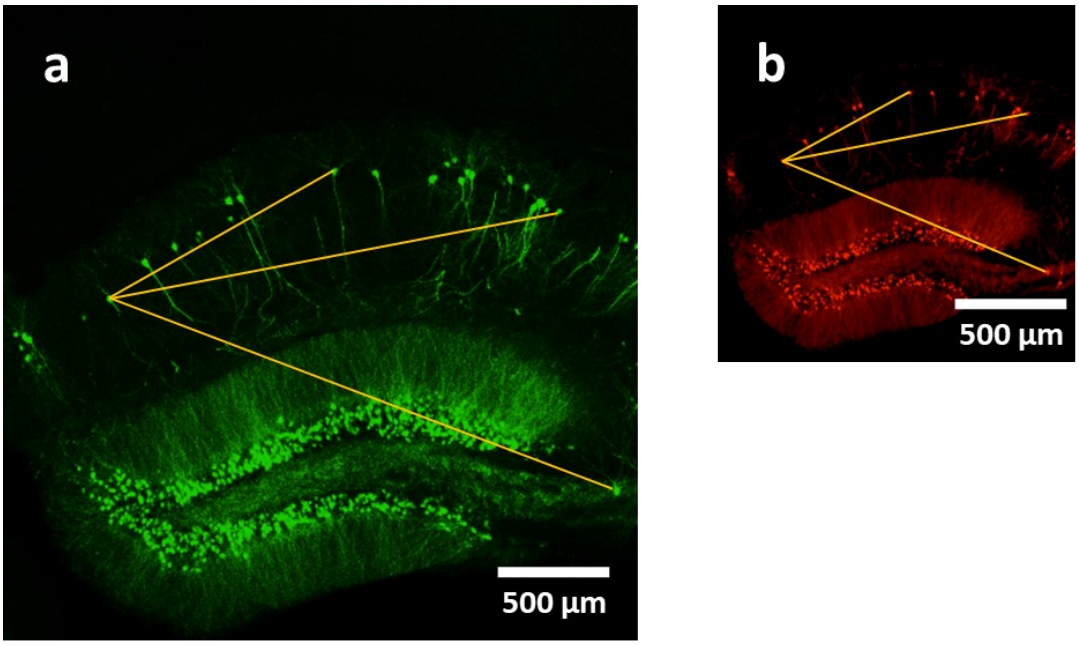

Figure S14 a) Fluorescence image with 3 measuring lines of 300 µm mouse brain slice after expanded; b) Original fluorescence image with 3 measuring lines of 300 µm mouse brain slice.

Tables

Table S1 Preparation of PAAE hydrogels with varied monomer concentrations.

Sample	AAE (g)	H_2O (μL)	Gel or solution
PAAE-1	0.010	990	sol
PAAE-5	0.050	950	sol
PAAE-10	0.100	900	sol
PAAE-15	0.150	850	sol
PAAE-20	0.200	800	gel
PAAE-25	0.250	750	gel
PAAE-30	0.300	700	gel
PAAE-40	0.400	600	gel
PAAE-50	0.500	500	gel
PAAE-60	0.600	400	gel
PAAE-70	0.700	300	gel

Table S2 Mechanical properties of PAAE hydrogels prepared with different monomer concentrations.

Sample	Young's modulus (MPa)	Elongation at break (%)	Tensile strength (MPa)	Compressive strength at 95% strain (MPa)	Compressive modulus (MPa)
PAAE-30	0.016	434	0.066	7.985	0.033
PAAE-40	0.048	415	0.126	12.328	0.058
PAAE-50	0.066	437	0.196	16.672	0.095
PAAE-60	0.082	425	0.285	22.568	0.130
PAAE-70	0.109	280	0.195	29.188	0.166

Table S3 Self-healing efficiency of PAAE hydrogels prepared with different monomer concentrations.

Sample	Original tensile strength (MPa)	Healed tensile strength (MPa)	Self-healing efficiency (%)
PAAE-30	0.036	0.032	88.9
PAAE-40	0.054	0.042	77.8
PAAE-50	0.135	0.117	86.7
PAAE-60	0.196	0.162	82.7
PAAE-70	0.175	0.156	89.1

Table S4. Fluorescence Intensity change during expanding procedures.

NO.	Fluorescence Intensity (a.u.)				Fluorescence preservation (%)		
	original	After permeation	After embedded	After expanded	After permeation	After embedded	After expanded
1	1042014	1455694	1391818	1067856	139.70	133.57	102.48
2	594601	770484	720300	558152	129.58	121.14	93.87
3	744838	1115916	1087463	827441	149.82	146.00	111.09
Mean± Std. Dev.	-	-	-	-	139.70±10.12	133.57±12.43	102.48±8.61

Table S5. Linear expansion factor calculated from Figure S14.

NO.	Length (μm)		Linear expansion factor
	original	expanded	
1	668.907	1140.192	1.705
2	1154.978	2027.471	1.755
3	1330.528	2993.923	1.799
Mean±Std. Dev.			1.753±0.047

Experiments

Materials and instrumentation

N-acetylethylenediamine (98%), Hubei Qifei pharmaceutical chemical industry Co. Ltd., Tianmen, China), acryloyl chloride (98%, Energy, China), potassium carbonate (99%, AR, Sinopharm Chemical Reagent Co. Ltd., China), acetonitrile (99%, AR, Sinopharm Chemical Reagent Co. Ltd., China), ammonium persulfate (99%, Sinopharm Chemical Reagent Co. Ltd., China), 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (99%, Energy, China). All commercially available starting materials, reagents and solvents were used as supplied, unless otherwise stated. All reactions were carried out under a dry nitrogen atmosphere and the temperatures were measured externally. The NMR spectra were recorded using a 600 M Bruker AscendTM 600 MHz in DMSO-d_6 and an internal standard of tetramethylsilane was used. The mass spectra were recorded using an Agilent 1100 LC/MSD Trap. MALDI TOF mass spectra were recorded with a MALDI-TOF-TOF (Bruker ultrafleXtreme). FT-IR spectra were recorded using a VERTEX 70 Fourier Transform Infrared Spectrometer (Bruker).

Synthesis and Characterization of N-(2-acetamidoethyl) acrylamide (AAE)

(a) Synthesis route

AAE was synthesized with some modification to the previously described method.[1, 2] Under a nitrogen atmosphere, K_2CO_3 (40.53 g, 293.7 mmol) was dissolved in a 1000 mL flask with three necks with 250 mL of deionized water. After cooling to room temperature, N-acetylethylenediamine (50 g, 489.5 mmol) and 70 mL acetonitrile was added under stirring. Subsequently, the mixture was maintained at 10 ^{o}C. Then a mixture of 40 mL acryloyl chloride in 160 mL acetonitrile was added dropwise under stirring. After stirring for another 12 hours at room temperature, all the solvents were evaporated under vacuum at 50 ^{o}C. After that, 40 0mL acetonitrile was added to extract the crude product and the salt was separated by filtration under reduced pressure. Then the filtrate was evaporated under vacuum again to give a white powder solid. Finally, the obtained solid were dried in vacuo at 50 ^{o}C to a constant weight (74.88 g, yield 98%). For qualitative purity tests, a single spot (visualized using UV-light at 254 nm and 365 nm) was obtained on thin film chromatography plate. The molecular structure of the AAE is depicted in Scheme 1A.

(b) Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.27 (s, 1H), 8.02 (s, 1H), 6.21 (dd, J = 17.1, 10.2 Hz, 1H), 6.08 (dd, J = 17.1, 2.2 Hz, 1H), 5.58 (dd, J = 10.2, 2.2 Hz, 1H), 3.17 (q, J = 6.0 Hz, 2H), 3.11 (q, J = 6.1 Hz, 2H), 1.80 (s, 3H). Figure S1.

^{13}C NMR (151 MHz, DMSO-d_6) δ 169.86, 165.24, 132.24, 125.46, 38.89, 38.80, 23.07. Figure S2.

(c) HR-MS. Figure S3, MALDI-TOF-MS calcd. for $C_7H_{12}N_2O_2$: 156.09; found: [M^+] 157.10.

(d) Fourier transform infrared spectrometry (FT-IR)

FT-IR spectrum of AAE in Figure S4 displays its feature bands: ν = 3284.47 cm^{-1} (vs, NH), 1652.71 cm^{-1} (vs, C=O), 1627.71 cm^{-1} (vs, C=C), 1561.87 cm-1 (vs, NH).

Synthesis of Poly[N-(2-acetamidoethyl) acrylamide] (PAAE) and PAAE hydrogels

The recipes for preparing hydrogels are listed in Table S1. According to different monomer mass concentration ratios, ammonium persulfate was used as initiator to conduct random radical polymerization of AAE monomer in aqueous solution under the heating condition of 30 ^{o}C, and finally hydrogel was formed. Specific preparation methods are as follows: The preparation of 60% initial monomer concentration PAAE hydrogel was taken as an example. First, 600 mg AAE monomer was dissolved completely with 400 μL of deionized water. Then 0.6 wt% APS (relative to the mass of AAE monomer) was added into the mixture and continue stirred vigorously for 10 minutes. Finally, the mixture was transferred to the corresponding mold and heated at 30 ^{o}C for 5 h to form hydrogels. A series of hydrogels were prepared by varying initial monomer concentration. The resulting hydrogels were named as PAAE-X (X is the monomer concentration in weight percentage). PAAE was prepared by the following method. First, the polymer was precipitated from a polymer solution with an initial monomer concentration of 5% by adding 5 volumes of acetone. Then, it was centrifuged at 5000 rpm for 30 minutes. The obtained crude product was thoroughly washed with acetone, then dried under vacuum at 50 °C for 40 hours, and finally ground to obtain a white polymer powder.
^{1}H NMR (600 MHz, DMSO-d_6) δ 8.00 (s, 1H), 7.73 (s, 1H), 3.10 (s, 4H), 2.09 (s, 1H), 1.82 (s, 3H), 1.46 (d, J = 102.2 Hz, 2H). FT-IR: ν = 3286.09 cm^{-1} (vs, NH), 1653.82 cm^{-1} (vs, C=O), 1551.95 cm^{-1} (vs, NH).
Figure S4 and Figure S5 shows that the purified PAAE presents identical characteristic features with those of AAE monomer, suggesting the successful polymerization of AAE.

Measurement of molecular weight of PAAE

The molecular weight and polydispersity index (PDI) were measured by GPC on a Waters Model TDA 302 instrument, using deionized water as an eluent at a flow rate of 900 μL min^{-1}. Polyethylene oxide standard was used for calibration. The number average molecular weight (M_n) was determined to be 1 680 000, and its polydispersity index (PDI) was 1.55 (Figure S6).

Determination of the swelling behavior

The swelling ratios (SRs) of the hydrogels were measured at room temperature. The as-prepared cylinder specimens were immersed in adequate deionized water or other solutions and exchange the media every 12 h. During this time, they were taken out every once in a while, gently wiped with filter paper, and immediately weighed on an electronic balance until a constant weight was obtained. The SR is defined as:

$$SR=W_t/W_0$$

Where W_t is the mass of sample at different time, and W_0 is the corresponding original weight of each sample. The average values and errors were calculated from at least four independent data for each specimen. The Equilibrium swelling ratios (ESRs) is defined as the swelling ratio when hydrogels swelling reaches equilibrium. PAAE-30 hydrogels in varied solvents are shown in Figure S7. The comparison of hydrogels size between the original hydrogels and the equilibrium hydrogels is shown in Figure S10.

Raman spectra of hydrogels at different monomer contents

For the Raman spectra of hydrogels at different monomer contents, PAAE hydrogels were prepared into square blocks of 10 mm in length and 1.5 mm in thickness. Then tested on LabRAM HR800 Raman spectra equipped with a laser light source of 785 nm wavelength. The results are show in Figure S11.

Measurement of mechanical properties

All mechanical properties of the hydrogels were tested on Electronic universal material testing machine (AGS-5kNX, SHIMADZU, Japan) at room temperature.[3, 4] At least four specimens were tested for each hydrogel sample.

(a) Tensile test of hydrogel

For tensile test, hydrogels were made into dumbbell-shaped, with a median uniform portion of 16 mm in length, 4 mm in width, and 2 mm in thickness. The extension speed was fixed at 16 mm min^{-1}, and a 100 N load cell was used.

(b) Compression test of hydrogels

For compression tests, hydrogels were made into cylinders 12 mm in diameter and 10 mm in height. The compression rate was set at 2 mm/min, and the maximum compression strain was set at 95% for the protection of the instrument, and a 5000 N load cell was used.

Strain is defined as:

$$W = (l-l_0)/ l_0 \times 100\%$$

Where, l is the distance between the upper and lower fixture of the instrument (l_0 is the initial distance).

And stress (engineering stress) is defined as:

$$\sigma = F/A_0$$

Where, F is the load applied to the sample, A_0 is the initial cross-sectional area of the tensile specimen.

Mechanical properties of PAAE hydrogels prepared with different monomer concentrations are listed in Table S2.

Self-healing of hydrogels

(a) Self-healing temperature and time

To evaluate heating temperature and time to the healing efficiency, the cross sections of separated PAAE-60 hydrogels were put together to heat at different temperature and time. The self-healing progression was monitored by recording the length of the sample when they were just pulled off. The photos were taken by a Nikon camera. In order to minimize the evaporation of water, we first wrapped the hydrogel samples in several layers of plastic wrap before heating, and then put them into aluminum foil bags for further sealing.

(b) Self-healing efficiency testing

The self-healing efficiency testing samples synthesized by the same method with tensile tests were partly cut in the middle. Then the separated ones were re-contacted just at the fracture interfaces. Afterwards, the original hydrogels and the re-contacted hydrogels were heated simultaneously at 90 ^{o}C in order to take into account of the evaporation of water. To evaluate the healing efficiency, the heat-treated and self-healed hydrogels were measured by tensile test. The healing efficiency is defined as:

$$HE = S_h / S_o \times 100\% \quad (4)$$

Where S_h is the tensile strength of self-healed hydrogel and S_o is the tensile strength of heated original hydrogel.[3] The average values and errors were calculated from at least four independent samples for each specimen. Self-healing efficiency of PAAE hydrogels prepared with different monomer concentrations are listed in Table S3.

Adhesion

(a) Adhesion strength

The hydrogels were fabricated to be membranes of 20 mm in length, 20 mm in width, and 2 mm in thickness. Then the quadrate samples were pressed between two same slices of rigid substrates. Paper and latex gloves were fixed on the surface of stainless steel with double-sided adhesive since they were flexible. The adhesion strength were tested on Electronic universal material testing machine (AGS-5kNX, SHIMADZU, Japan) at room temperature according to a previously reported procedure.[5] At least four specimens were tested for each hydrogel sample. The rate of crosshead speed was fixed at 4 mm min^{-1} for Lap joint shear strength tests (Figure S8). Where, adhesion strength is defined as the maximum stress upon the glued area. The cyclic adhesion of 60% PAAE hydrogels were tested on the stainless-steel substrate.

(b) Contact angle

Determination of surface hydrophilicity of different materials. The contact angle of water on stainless steel, glass and plastic were measured with a KINO' s Automatic Contact Angle Meter (Model: SL200K). The measurement results are shown in Figure S9**.**

Brain slice expansion and imaging

(a) Treatment of brain slice expansion

Firstly, the precursor solutions of 40% AAE monomer were prepared, which were consistent with the hydrogel preparation process except replacing APS with 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride as the initiator to prevent premature gelation. Under the condition of 5 ^{o}C, the 300 μm thickness mouse brain slices (mouse brain from Thy1-GFP-M mouse, fixed with Sigma 4% PFA solution, brain slices prepared by Vibration slicer, VT1000, Leica, German) were immersed in the precursor solutions for 2 hours to ensure the monomer fully penetrate into the tissue. Then the precursor solutions with brain slices were heated to form hydrogel-embedded brain slices at 30 ^{o}C. The brain slices embedded in hydrogel were soaked in abundant deionized

water under 5 ^{o}C and exchanged the water once every 12 h until expansion carried out 48 h above.

(b) Imaging parameters

All the images were achieved by a confocal fluorescence microscopy (LSM710, Zeiss, Germany), Ar laser 488 nm. The widefield fluorescent images of expanded and original slice were taken at on the LSM710 equipped with 5x/0.25NA dry objective (WD 12.5 mm), and 10X/0.50NA air objective (WD 2 mm) for details.

(c) Fluorescence intensity change during expansion

Determination of fluorescence retention in expansion process. Under the same imaging conditions, the fluorescence images of different states of the same brain slices were taken. Then the total fluorescence intensity of the same neuron cells was measured by ImageJ software. The fluorescent intensity retention rate is defined as:

$$Fl = F_t/F_0 \times 100\%$$

Where, F_t is the fluorescence intensity of neurons in different states, and F_0 is the corresponding fluorescence intensity of initial state neurons. Fluorescence Intensity change during expanding procedures were shown in Table S4.

(d) Linear expansion factor

The 5x objective of LSM710 were used to image the same brain slices. Then two corresponding marker sites in the brain slice before and after expansion were selected, and the distance between which were measured with ImageJ software. The Linear expansion factor is defined as: [6]

$$LEF = L_e/l_o$$

Where L_e is the distance between the two feature points on the expanded brain slice, and l_o is the distance between the corresponding two feature points on the original brain slice. In the same way, the other two groups of markers in different directions were selected for the same calculation, taking the average value as the linear expansion factor of brain slice, as shown in the Figure S14 and Table S5.

(e) Full width at half maximum

Intensity profiles perpendicular to axis cylinder was taken averaging over a line profile**.** Intensity profiles were fit to a Gaussian using the Origin 'fit' function and the FWHM calculated from the Gaussian fit.

(f) Transparency test of PAAE hydrogel

For the transparency test of PAAE-40 hydrogel at a swelling equilibrium on Shimadzu UV-vis-NIR spectrophotometer (UV-3600), the hydrogel sample was prepared into a 0.5 mm thick, 15 mm long and 15 mm wide film, which was attached to the cover glass. The blank cover glass was used as the substrate.

Cytotoxicity determination

For AAE monomer cytotoxicity determination,[7] normal human embryonic kidney cells(HEK293) and hepatocellular carcinoma(HepG2) were incubated with Dulbecco's modified Eagle's medium

(DMEM) in 96-well culture plate overnight(5×104 cell mL^{-1}, 200 μL each well). After the medium was removed and washed with PBS, cells were maintained in varied concentration solutions of AAE prepared with DMEM for 24 h or 48 h. Then 20 μL of MTT solution (5 mg mL^{-1}) were added and incubated for 4 hours. Afterwards, the MTT solution was removed and DMSO (150 μL) was added into each well. After incubated for another 15 minutes, the absorbance of MTT at 492 nm was studied by Enzyme standard instrument. The cells without any treatment were used as control. The cell viability was calculated following the equation below:

$$\text{Cell viability}=(OD_S-OD_O)/(OD_C-OD_O)\times 100\%$$

Where, OD_S is the absorbance of the wells with cells treated with the AAE monomer, OD_C is the absorbance of wells pretreated with only culture medium, and OD_O is the absorbance of wells with cells treated with no reagents. The cytotoxicity test results are shown in Figure S12.

REFERENCES.

1. Guo H, Mussault C, Marcellan A, Hourdet D, Sanson N. Hydrogels with Dual Thermoresponsive Mechanical Performance. *Macromolecular Rapid Communications* **38**, (2017).

2. Seuring J, Bayer FM, Huber K, Agarwal S. Upper Critical Solution Temperature of Poly(N-acryloyl glycinamide) in Water: A Concealed Property. *Macromolecules* **45**, 374-384 (2012).

3. Dai X*, et al.* A Mechanically Strong, Highly Stable, Thermoplastic, and Self-Healable Supramolecular Polymer Hydrogel. *Advanced Materials* **27**, 3566-3571 (2015).

4. Yang Y, Wang X, Yang F, Wang L, Wu D. Highly Elastic and Ultratough Hybrid Ionic-Covalent Hydrogels with Tunable Structures and Mechanics. *Advanced Materials* **30**, (2018).

5. Zhou Y*, et al.* Light-Switchable Polymer Adhesive Based on Photoinduced Reversible Solid-to-Liquid Transitions. *Acs Macro Letters* **8**, 968-972 (2019).

6. Chen F, Tillberg PW, Boyden ES. Expansion microscopy. *Science* **347**, 543-548 (2015).

7. Hu F, Cai X, Manghnani PN, Kenry, Wu W, Liu B. Multicolor monitoring of cellular organelles by single wavelength excitation to visualize the mitophagy process. *Chemical Science* **9**, 2756-2761 (2018).