Online Supporting Information

Multiple Covalent Crosslink Soft Hydrogels for Bioseparation

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Experimental details

Materials. Acrylamide (AM), N,N'-methylene bis-acryclamide (MBA), sodium dodecyl sulfate (SDS), acrylic acid (AA) and ammonium persulfate (APS) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Prestained standard protein marker (molecular weights: 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa), Tris and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). HCl, Glycine, Coomassie brilliant blue, ethanol and acetic acid were obtained from Sinopharm (Shanghai, China). All materials were used without further purification. Ultrapure water with specific conductivity down to 18.2 $M\Omega \cdot cm^{-1}$ was obtained via a Milli-Q Ultrapure Water System.

Saliva sample. Saliva sample was collected from one healthy and age of 28 years volunteer. Written informed consent forms and questionnaire data sheets were obtained from the volunteer. The saliva sample preparation was performed as previously described.[6b,S1]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[S2] SDS-PAGE was used for evaluating the protein separation efficiency of P(AM-AA) and PAM gels through the following steps. **(a1) Preparation of PAM separation gel.** Gel stock solution of PAM (30%, m/v): 30 g AM and 0.8 g MBA were dissolved in 100 mL H_2O. The PAM gel solution was prepared by mixing 2.0 mL gel stock solution, 2.5 mL

Tris-HCl (1.5 mol/L, pH 8.80), 100 μL SDS (10% (w/v)), 100 μL $(NH_4)_2S_2O_8$ (10% (w/v)) and 8 μL TEMED, and then diluted to 10 mL with ultra pure water.[S2]

(a2) Synthesis of P(AM-AA) Separation gel. Gel stock solution of P(AM-AA) (30%, m/v): 30 g AM, 0.8 g MBA were dissolved in 100 mL H_2O. The P(AM-AA) gel solution was prepared by mixing 2.0 mL gel stock solution, 600 μL AA (10%, w/v), 2.5 mL Tris-HCl (1.5 mol/L, pH 8.80), 100 μL SDS (10%, w/v), 100 μL $(NH_4)_2S_2O_8$ (10%, w/v) and 8 μL TEMED, and then diluted to 10 mL with ultra pure water.

(b) Preparation of stacking gel. Stacking gel solution of PAM or P(AM-AA) was made by mixing 0.83 mL gel stock solution (AM and MBA contents were 6% (w/v) and 0.8% (w/v), respectively), with 0.63 mL Tris-HCl (1.0 mol/L, pH 6.8), 50 μL SDS (10%, m/v), 50 μL $(NH_4)_2S_2O_8$ (10%, m/v), 5 μL TEMED, and then diluted with water to 5 mL. A 1.0 mm thickness comb was used to create 10 wells on the top of each gel.

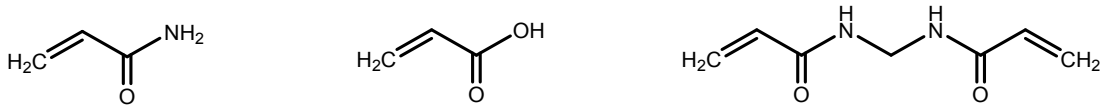
(c) Sample loading. Loading sample buffer consisted of 10% Tris–HCl stock solution (1 M, pH 6.8), 20% glycerol, 4% (m/v) SDS, 20% dithiothreitol (DTT, 1 M), and 0.2% (m/v) bromophenol blue. Standard protein marker was used directl. We loaded 7 μL prestained standard protein marker and 20 μL salive sample (17 μg total proteins).

(d) Run of SDS-PAGE. After the sample loading, the gels were run in Mini-Protean Tetra vertical electrophoresis cell (Bio-Rad Laboratories, Hercules, USA) and (DYCZ-2 4D electrophoresis power supply instrument (Liuyi Instrument Factory, Beijing, China)) using Tris-Glycine buffer (3.03 g Tris, 14.41 g glycine and 1 g SDS dissolved in 1 L water, pH 8.3). SDS-PAGE was run at 40 V for 15 min, 80 V for 15 min and 120 V for 1 h, the total running time was about 90 minutes. The upper and down electrodes were set as the cathode and anode, respectively.

(e) Chemical Staining and destaining.[S2] After a run, the gel was immediately immersed in fixing solution (50% ethanol and 10% acetic acid) for one hour. Then, the gel was set in 0.1% Coomassie Brilliant Blue R-250 staining solution in 40% ethanol and 8% acetic acid on an orbital shaker (LabLine Instruments Inc., Melrose Park, IL). After 15 min staining, the background stain of gel was removed by a destaining solution of 40% ethanol and 8% acetic acid, and then transferred back into water.

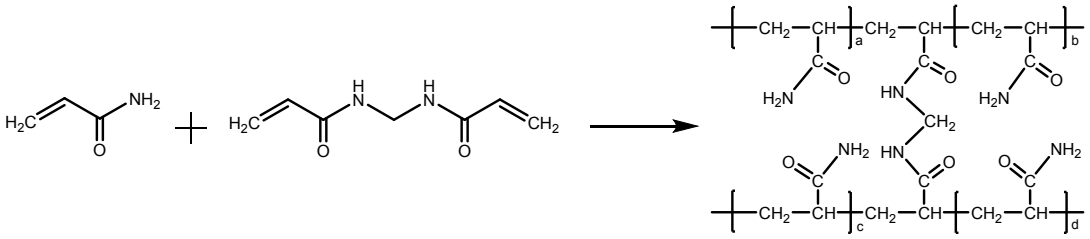
(f) Scanning of stained SDS-PAGE gel. All of the gels were scanned using an EPSON Scanner (Epson Perfection V700 Photo) and analyzed using Image J (v.1.47) software (USA).

Chemical Structures and Reactions

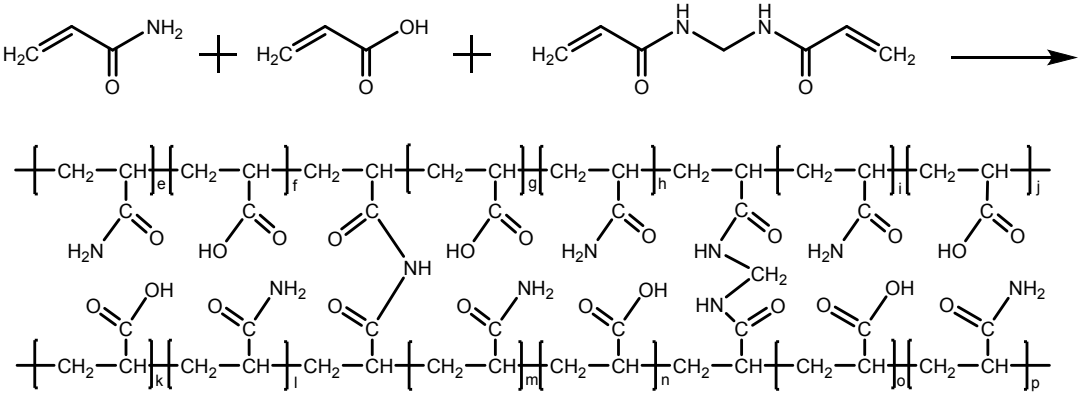


Acrylamide | Acrylic acid | N,N'-methylene bis-acrylamide

Figure S1. Chemical structures of acrylamide, acrylic acid and N,N'-methylene bis (acrylamide).



Scheme S1. Synthesis of polyacrylamide hydrogel by using acrylamide and N,N'-methylene bis-acrylamide.



Scheme S2. Synthesis of poly(acrylamide-acrylic acid) hydrogel by using acrylamide, acrylic acid and N,N'-methylene bis-acrylamide.

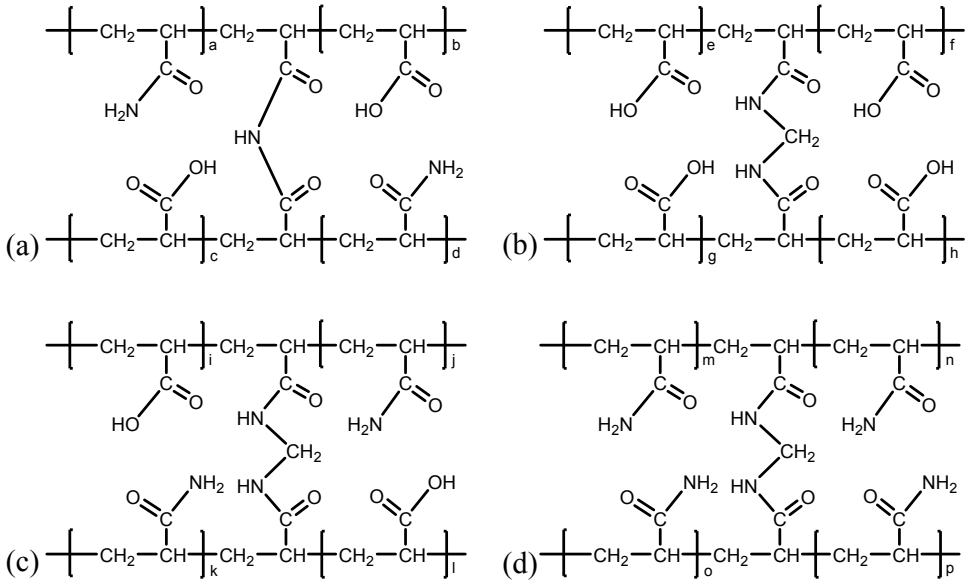


Figure S2. Four types of chemical bond structures of crosslinks in poly(acrylamide-acrylic acid) hydrogel.

Results of Protein Separation

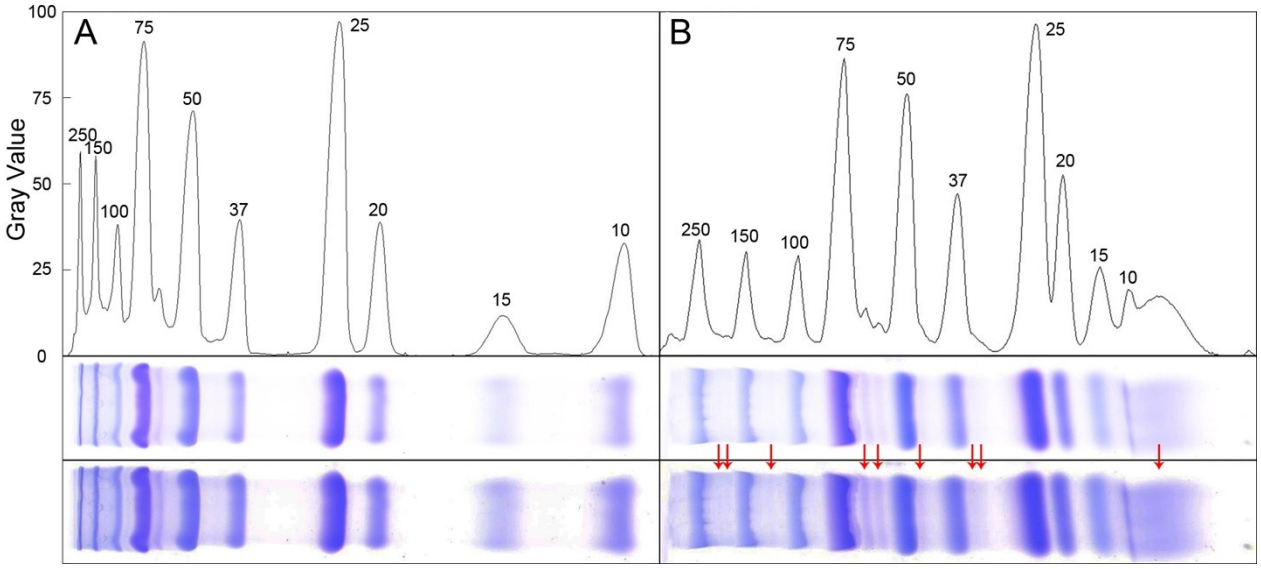


Figure S3. Comparative experiments of standard protein marker separation via P(AM-AA) gel under different running times of 90 minutes (A) and 100 minutes (B). The details of SDS-PAGE procedure was described in the supporting information given above. The red arrows indicate the minor protein bands separated via lengthened run of SDS-PAGE. The conditions were the same as those in Figure 2.

Table S1. Sensitivities of standard model protein determination in SDS-PAGE via PAM gel and P(AM-AA) gel.

Protein band	Gray value of PAM	Gray value of P(AM-AA)	Ratio [a)]
250 kDa	7.3	59.1	8.1
150 kDa	6.7	57.7	8.6
100 kDa	6.3	38.5	6.1
75 kDa	22.6	91.3	4.0
50 kDa	-	71.2	-
37 kDa	-	39.4	-
25 kDa	-	97.1	-
20 kDa	-	38.5	-
15 kDa	-	12.0	-
10 kDa	-	33.2	-

[a)] The ratio indicates the gray value of protein in P(AM-AA)-based PAGE run divided the one in PAM-based PAGE run.

Characteristics of Hydrogels

Scanning electron microscope (SEM). SEM images were taken on NOVA nanoSEM 230 (FEI Co., USA/ Oxford Instruments, UK) with an accelerating voltage of 5 kV. The samples were coated with Pt just before SEM observation. The hydrogels were briefly submerged in ethanol and frozen dried in vacuum for 72 h before examination of SEM.

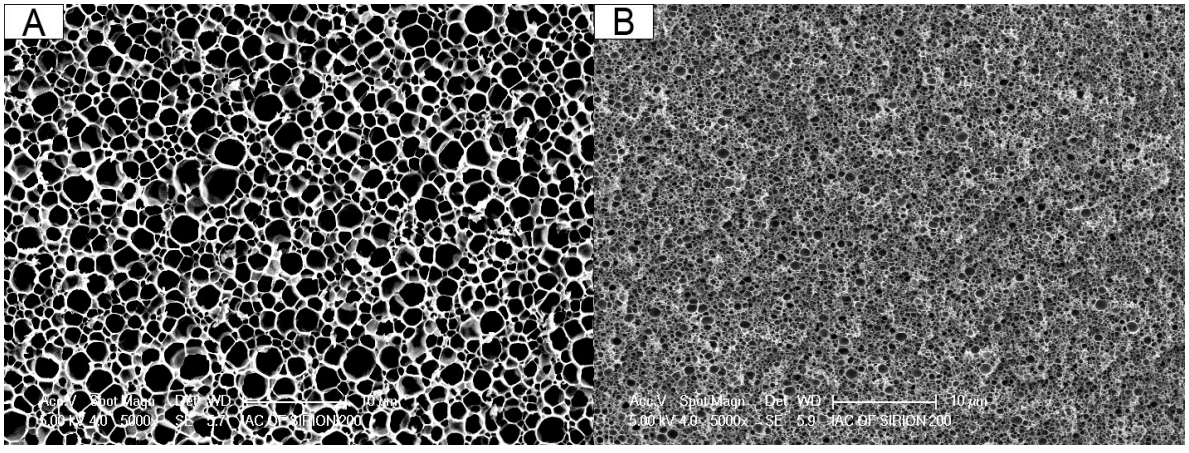
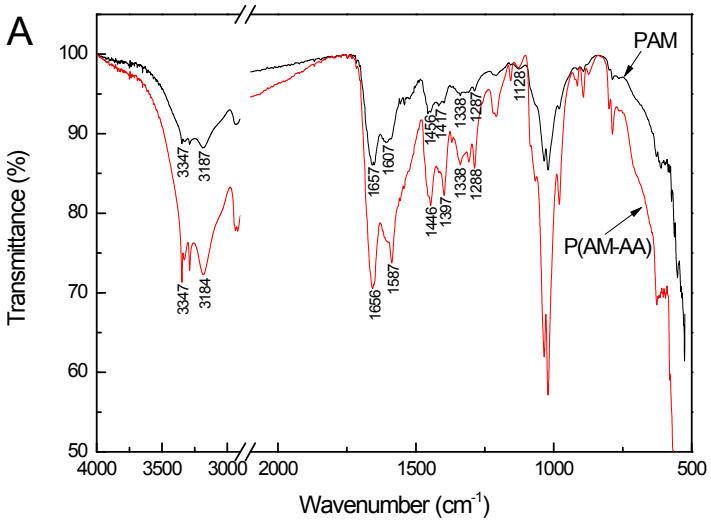


Figure S4. Whole SEM images of PAM gel (A) and P(AM-AA) gel (B).

SEM images (Figure S4) showed a porous three-dimensional network structures of PAM and P(AM-AA) hydrogels. The pores size of P(AM-AA) hydrogel was much smaller than that of the PAM hydrogel obviously. In the gel of P(AM-AA), the contents of AM, MBA and AA were respectively 6% (w/v), 0.8% (w/v) and 0.6% (w/v). When compared with the composition of PAM gel, only additional 0.6% (w/v) AA was added into the solution of P(AM-AA) gel.

Fourier transform infrared spectrometry (FTIR). FTIR spectra were taken into observation to investigate the possible crosslinks between acrylamide and acrylic acid. Samples of the same thickness (≈100 μm) were prepared from PAM gel and P(AM-AA) gel by applying a pressure of 300 kg/cm^2. FTIR was recorded between 4000 and 400 cm^{-1}on a Nicolet 6700 FTIR ESP spectrometer (Thermo Fisher Co., USA).



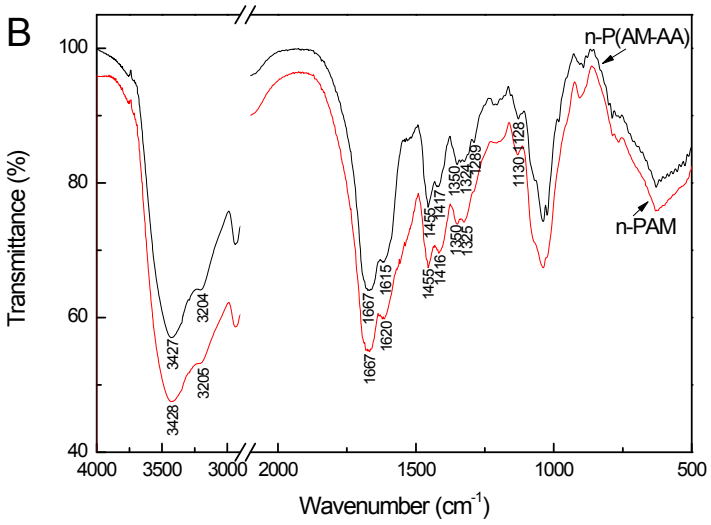


Figure S5. FTIR spectra of PAM and P(AM-AA) gels (A), n-PAM and n-P(AM-AA) gels (B).

FTIR spectra of P(AM-AA) and PAM gels are shown in Figure S5A. The polyacrylamide gel exhibited bands at 3347 cm^{-1} and 3187 cm^{-1}, corresponding to a stretching vibration of N-H, and at 1657 cm^{-1} for C=O stretching. The bands at 1607 cm^{-1} (N-H deformation for primary amine), 1456 cm^{-1} (CH_2 in-plane scissoring), 1417 cm^{-1} (C-N stretching for primary amide), 1338 cm^{-1} (C-H deformation), and 1128 cm^{-1} (NH_2 in-plane rocking) were also detected. In the spectra of the P(AM-AA) gel, the intensities of the N-H stretching peak (3184, 3347 cm^{-1}), C=O stretching peak (1656 cm^{-1}) and C-N stretching peak (1288 cm^{-1}) were increased. Furthermore, the intensity of NH_2 in-plane rocking peak (1128 cm^{-1}) was decreased. To avoid the interference of N,N'-methylene bis-acrylamide, we did not add the crosslinker in the n-PAM and n-P(AM-AA) gels. FTIR spectra of the n-P(AM-AA) and n-PAM gels are shown in Figure S5B. The bands at 3427 cm^{-1} and 3204 cm^{-1} (stretching vibration of N-H), 1667 cm^{-1} (C=O stretching), 1615 cm^{-1} (N-H deformation for primary amine), 1455 cm^{-1} (CH_2 in-plane scissoring), 1417 cm^{-1} (C-N stretching for primary amide), 1350 cm^{-1} (C-H deformation), and 1128 cm^{-1} (NH_2 in-plane rocking) were detected. In the spectra of the n-P(AM-AA) gel, a new peaks appeared at 1289 cm^{-1} for C-N stretching of secondary amide. Furthermore, the intensities of N-H stretching peak (3428, 3205 cm^{-1}), the intensity of the absorption bands (1620, 1416 cm^{-1}) which were related with primary amide, and the intensity of NH_2 in-plane rocking peak (1130 cm^{-1}) were decreased. These results indicated the new bonds formed between $-NH_2$ groups of acrylamide and carboxyl groups of acrylic acid.[21,S3,S4]

Rheological measurements. Rheological measurements were measured using an AR-G2 rheometer (TA Instruments, USA). The rheometer equipped with a parallel plate and a 40 mL aluminum cone was used for all experiments. Samples were cut with a rectangular punch (width = 4.5 mm) and carefully lifted out of the petri dish. They were then loaded onto the fixture between the drums (length = 13 mm). The thickness of each sample was 1.0 mm as measured by a caliper. Tests were conducted at 20°C, and the temperature was controlled to within 0.2 °C of the set point using an environmental temperature controller under a nitrogen atmosphere. The storage and loss moduli (G′ and G″, respectively) were measured as a fixed strain of 1.0%, and an angular frequency of 10 rad/s so as to assess the gel strength. A frequency sweep from 0.1 to 100 rad/s was conducted at 20 °C using a fixed strain of 1.0% and an equilibration time of 1 min between each measurement.

Thermogravimetric analysis (TGA). Thermal degradation of the samples were studied using TGA Q5000 (TA Instruments, USA) under a nitrogen atmosphere at a heating rate of 10 °C/min. Samples were scanned from 20 to 600 °C. P(AM-AA) hydrogel with the polymer ratio 1:10 of acrylic acid to acrylamide was used for this test. Samples with weight between 5 and 6 mg in weight were tested.

The integral results from TGA are shown in Figure S6, along with data for PAM and P(AM-AA) hydrogels. It was found that PAM had three pyrolysis stages, the first thermal degradation process occurred in the temperature range of 230-330 °C. During first stage, one ammonia molecule was liberated for every two amide groups, resulting in the formation of imide. The second stage occurred in the range of 330-480 °C, and was attributed to the depolymerization of polymer and formation of a carbonaceous residue, and finally yielded as char. Subsequently, thermal degradation of imides and breaking of the polymer backbone occurs as the second and third stages. The P(AM-AA) hydrogel clearly showed three pyrolysis stages, and its thermostability was not affected obviously when compared with the thermostability of PAM hydrogel.[16,S3,S4]

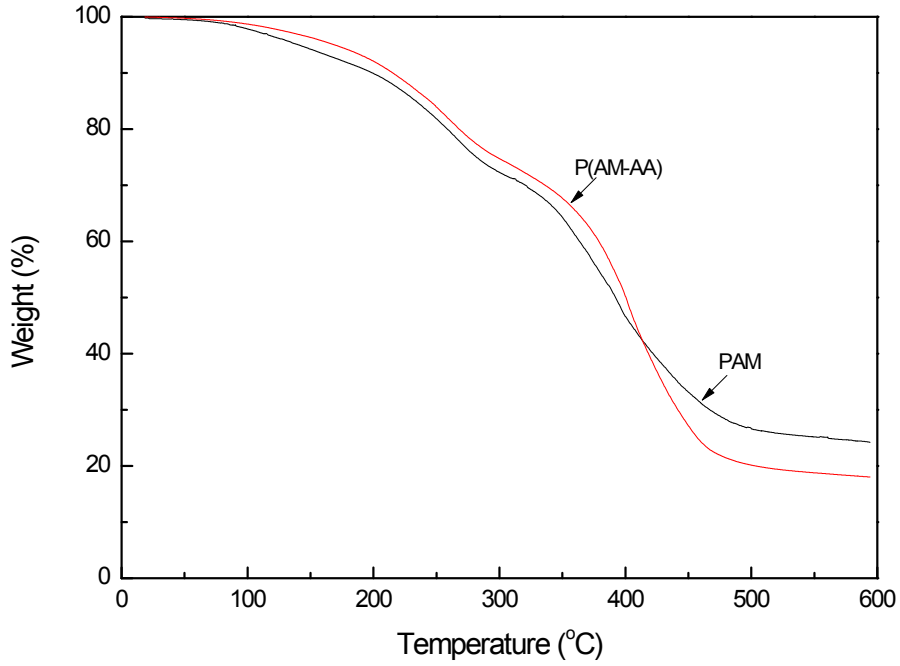


Figure S6. Thermogravimetric analyses of PAM and P(AM-AA) hydrogels.

Differential scanning calorimetry (DSC). A Netzsch DSC 204 F1 instrument (Netzsch, Germany) was used for differential scanning calorimetry analysis to determine the glass transition temperatures of the synthesized polymers. The midpoint of the specific heat increase in the transition region during the second heating was reported. Programmed heating cycles from 20 to 150 °C were used at a heating rate of 10 °C/min under nitrogen atmosphere. All gel samples were frozen dried in vacuum for a week to eliminate water molecules.

The glass transition temperatures, indicating the complex of the acrylamide and acrylic acid components in the symbol-networks, were determined by DSC. Representative DSC scans of these symbol-networks are shown in Figure S7. The glass-transition temperature increases from 50 to 58 °C. This could be explained by the formation of new covalent bonds between acrylamide and acrylic acid in the poly(acrylamide-acrylic acid) gel.[S3,S4]

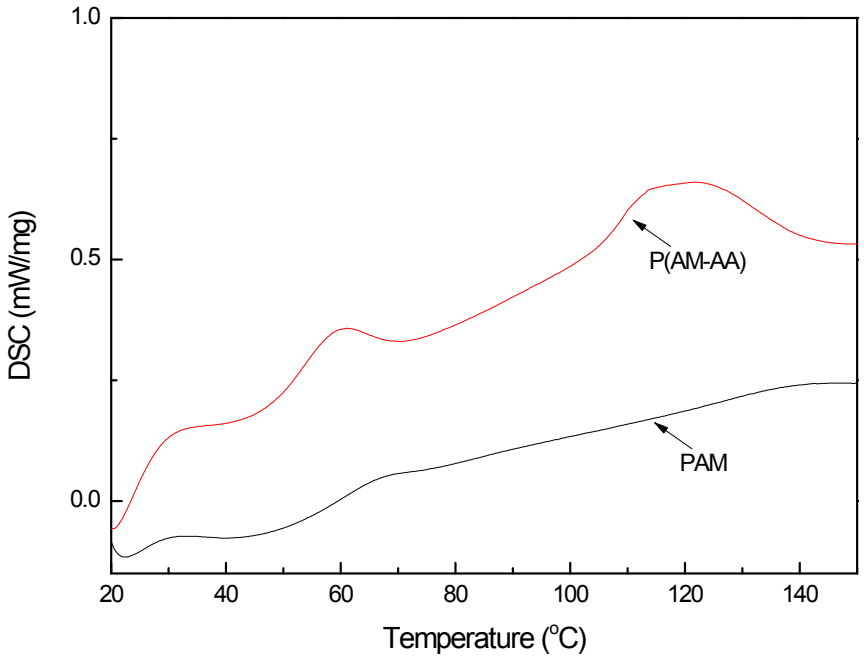


Figure S7. Thermogravimetric analyses (DSC) of PAM and P(AM-AA) hydrogels.

Protein Identification via MS

After excision of protein bands from electrophoresis gels of SDS-PAGE, the gel bands were washed with ultrapure water and dired in a centrifuge for 3 minutes, and treated with DTT (60°C, 1h), 50 mM NH_4HCO_3, CH_3CN, in turn. Trypsin digestion (with 10 μL of a 10 ng/μL trypsin solution, Promega) was performed overnight. The peptides were extracted with H_2O/CH_3CN (1:1) for 2 × 15 min, dried, and solubilized in loading buffer (CH_3CN/ HCOOH/water=2/0.1/97.9). The peptides were concentrated and separated by reversed-phase HPLC (Thermo scientific, USA) with a precolumn/analytical column nanoflow setup (Agela, Durashell-C18 Column, 2.1 mm × 250 mm, 5 μm, 100 Å). The peptides were analyzed using an LC system (Nano Pump, Ultimate 3000, Dionex,Thermofisher, USA) coupled with an ESI-Q-TOF mass spectrometer (maXis, Impact, Bruker Daltonik, Germany). The LC setup was coupled online to a Q-TOF using a nano-ESI source (Bruker Daltonik, Germany) in data dependent acquisition mode (m/z 350-1500). The source capillary was set at 1900 v, the flow and temperature of dry gas was 2.0 L/min and 120 °C respectively. The mass spectrometer was set as

one full MS scan followed by ten MS/MS scans on the ten most intense ions from the MS spectrum.

For protein identification, extracted MS/MS peak lists were compared to the MSDB database using Compass Data Analysis version 4.1 (Bruker Daltonics). All searches were performed with C+57 fixed modification and with M+16 variable modifications for carbamidomethylation of cysteines and for oxidation of methionines, with a maximum of one missed cleavage. MS2 spectra were searched with a mass tolerance of 20 ppm for precursor ions and 0.05 Da for fragment ions, respectively. The protein identification was validated if two peptides exhibited fragmentation profile scores higher than the average default value for significance using MASCOT (version 2.4.1, Matrix Science).

Table S2. Identification of proteins in minor bands 1 and 2 of Figure 2D via mass spectrometry.

No.	Gene	Protein name	MW (kDa)	Unique peptide	Score
1	DMBT1	Deleted in malignant brain tumors 1 protein OS=Homo sapiens GN=DMBT1 PE=1 SV=2	268	14	1835
2	DESP	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3	334	7	158
3	MUC5B	Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3	611.6	15	323

Table S3. Identification of proteins in minor bands 3 and 4 of Figure 2D via mass spectrometry.

No.	Gene	Protein name	MW (kDa)	Unique peptide	Score
1	CO6A1	Collagen alpha-1(VI) chain OS=Homo sapiens GN=COL6A1 PE=1 SV=3	109.6	2	65
2	MANBA	Beta-mannosidase OS=Homo sapiens GN=MANBA PE=1 SV=3	101.8	2	29
3	CO3	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	188.6	9	80
4	EGF	Pro-epidermal growth factor OS=Homo sapiens GN=EGF PE=1 SV=2	137.6	2	41

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