Online Supporting Information

An Ionic Coordination Hybrid Hydrogel for Bioseparation

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Experimental And Methods

Materials. Calcium chloride ($CaCl_2$), acrylamide (AM), acrylic acid (AA), N,N'-methylene bis-acryclamide (MBA), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and Prestained standard protein marker were supplied by Sigma-Aldrich Ltd. Tris was obtained from Bio-Rad Ltd. Ethanol, acetic acid, Glycine, HCl and Coomassie brilliant blue were purchased from Sinopharm Ltd. Unless stated otherwise, all materials were used as received.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[16,S1] The details of the SDS-PAGE process as the following steps.

(a1) Synthesis of P(AM-co-AA) Separation gel. Milli-Q H_2O (1.98 mL, 18.2 $M\Omega \cdot cm^{-1}$), Gel stock solution (1.3 mL, AM and MBA contents were 30% (w/v) and 0.8% (w/v), respectively), AA (195 μL, 10%, w/v), $CaCl_2$ (25 μL, 10%, w/v), Tris-HCl (1.3 mL, 1.5 M, pH 8.80), SDS (50 μL, 10%, w/v), APS (50 μL, 10%, w/v) and TEMED (3 μL) were mixed.

(a2) Preparation of P(AM-AA) separation gel. Milli-Q H_2O (2.105 mL, 18.2 $M\Omega \cdot cm^{-1}$), Gel stock solution (1.3 mL, AM and MBA contents were 30% (w/v) and 0.8% (w/v), respectively), AA (195 μL, 10%, w/v), Tris-HCl (1.3 mL, 1.5 M, pH 8.80), SDS (50 μL, 10%, w/v), APS (50 μL, 10%, w/v) and TEMED (3 μL) were

mixed.

(a3) Preparation of PAM Separation gel. Milli-Q H_2O (2.3 mL, 18.2 MΩ·cm^{-1}), Gel stock solution (1.3 mL, AM and MBA contents were 30% (w/v) and 0.8% (w/v), respectively), Tris-HCl (1.3 mL, 1.5 M, pH 8.80), SDS (50 μL, 10%, w/v), APS (50 μL, 10%, w/v) and TEMED (3 μL) were mixed.

(b) Synthesis of stacking gel. Milli-Q H_2O (3.4 mL, 18.2 MΩ·cm^{-1}), Gel stock solution (0.83 mL, AM and MBA contents were 30% (w/v) and 0.8% (w/v), respectively), Tris-HCl (0.63 mL, 1.0 M, pH 6.8), SDS (50 μL, 10%, w/v), APS (50 μL, 10%, w/v) and TEMED (5 μL) were mixed. The top of each gel was created 10 wells by a comb (1.0 mm thickness).

(c) Preparation of Saliva sample. Saliva sample was collected from one 28 years old and healthy volunteer, who written informed consent forms and questionnaire data sheets. The saliva sample was prepared using previously published protocol. [16,17b,20c,S2]

(d) Sample loading. Loading sample buffer had 10% glycerol, 5% Tris–HCl (0.5 M, pH 6.8), 20% SDS, 0.2% bromophenol blue, and 20% dithiothreitol (DTT, 1 M). The salive sample (5 μL, 2.3 μg total proteins) and prestained standard protein marker (7 μL) were load in the wells.

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

(f) Chemical Staining and destaining. After electrophoresis, the gel was immediately immersed in fixing solution (10% acetic acid and 50% ethanol) for one hour. Then, the gel was stained by aqueous solution (40% ethanol, 8% acetic acid and 0.1% Coomassie Brilliant Blue R-250) 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 (8% acetic acid and 40% ethanol), and then transferred back into water.

(g) Scanning of stained SDS-PAGE gel. All of the gels were imaged with a Film Scanner (Epson Perfection V700 Photo) and analyzed via the analysis software (Image J, v.1.47, USA).

Chemical Structures and Reactions

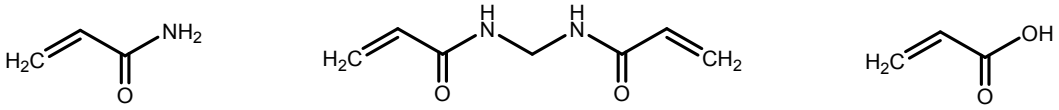

Figure S1. Chemical structures of acrylamide, N,N'-methylene bis-acrylamide and acrylic acid.

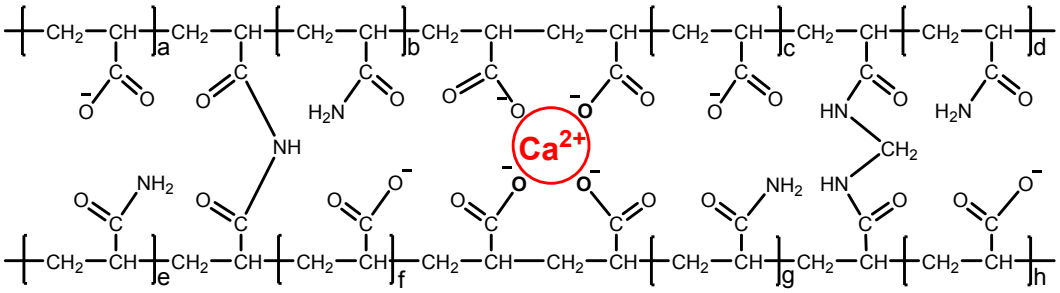

Figure S2. Chemical structures of crosslinks in poly(acrylamide-co-acrylic acid) hydrogel.

Results of Protein Separation

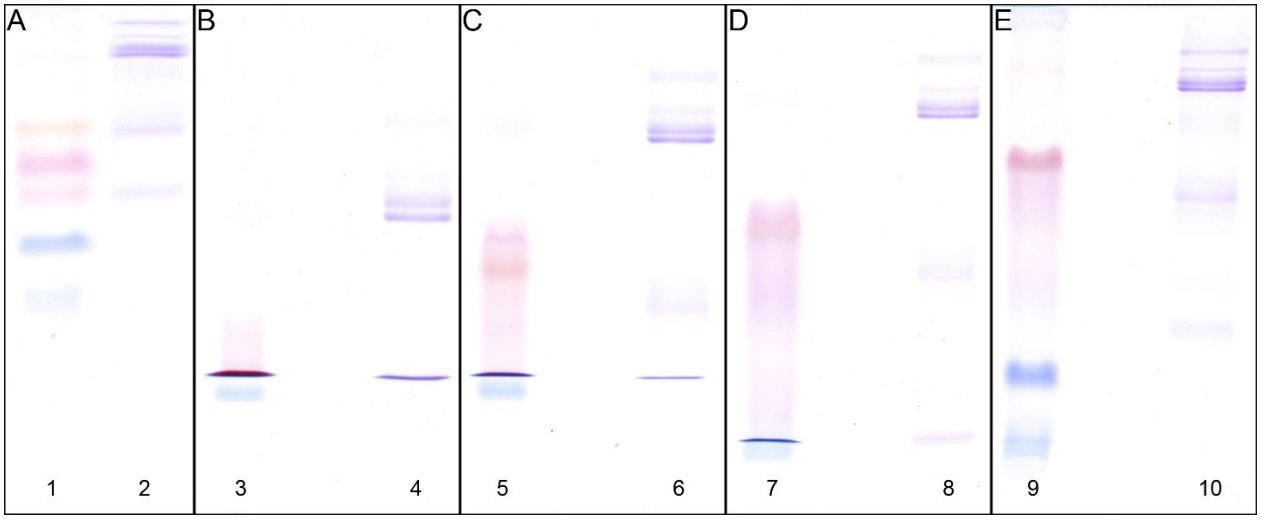

Figure S3. Comparative experiments of standard protein marker and saliva separation via P(AM-co-AA) gel (A), 8% PAM gel (B), 10% PAM gel (C), 12% PAM gel (D) and 15% PAM gel (E). The bands of 1, 3, 5,7 and 9 mean standard protein marker separation of P(AM-co-AA), 8% PAM, 10% PAM, 12% PAM and 15% PAM gels, respectively. The bands of 2, 4, 6, 8 and 10 mean saliva separation of P(AM-co-AA), 8% PAM, 10% PAM, 12% PAM and 15% PAM gels, respectively., respectively. The details of SDS-PAGE procedure was described in the supporting information given above. The conditions were the same as those in Figure 2.

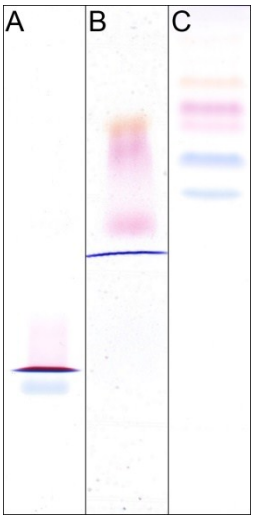

Figure S4. Comparative experiments of standard protein marker separation via PAM gel (A), P(AM-AA) gel (B) and P(AM-co-AA) gel (C). The details of SDS-PAGE procedure was described in the supporting information given above. The conditions were the same as those in Figure 2.

Table S1. Sensitivities of standard model peptide in SDS-PAGE via PAM and P(AM-co-AA) gels.

Protein band	Gray value of PAM	Gray value of P(AM-co-AA)	Ratio [a)]
26.6 kDa	-	47.4	-
17 kDa	-	98.2	-
14.2 kDa	-	37.5	-
6.5 kDa	-	94.7	-
3.5 kDa	-	70.3	-

[a)] The ratio was obtained via the gray value of peptide in P(AM-co-AA)-based PAGE run divided by the one in PAM-based PAGE run. Because poor separation, the gray value of PAM could not be detected as performed in our previous work,[16] resulting in no data of the ratio.

Characteristics of Hydrogels

Scanning electron microscope (SEM). The SEM images were obtained using a nanoSEM (NOVA 230, FEI Co., USA/ Oxford Instruments, UK). Before the scanning, the hydrogels were frozen and dried in vacuum for 3 days. After than the samples were coated with Pt just before SEM observation and the operating voltage at 5 kV.

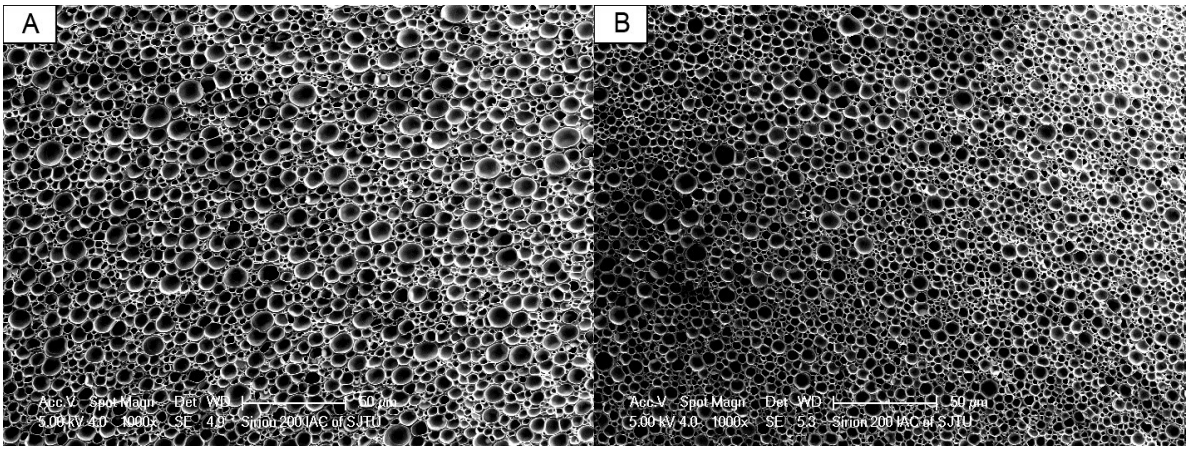

Figure S5. Whole SEM images of PAM gel (A) and P(AM-co-AA) gel (B).

Figure S5 displays the **S**EM images of the PAM and P(AM-co-AA) hydrogels. Both the PAM and the P(AM-co-AA) hydrogels exhibited a porous three-dimensional network structures. In contrast to the PAM

hydrogel, the pores diameter of P(AM-co-AA) gel is smaller and regular. In the gel of P(AM-co-AA), there were only additional 0.4% (w/v) AA and 0.05% (w/v) Ca(II).

Fourier transform infrared spectrometry (FTIR). FTIR spectroscopy can be used to study the possible crosslinks between the two types of gels by investigated the absorption bands. FTIR spectrometer (Nicolet 6700, Thermo Fisher Co., USA) was used for scanning the sample in a wavenumber range from 4000 and 400 cm^{-1}. Samples of the same thickness (≈100 μm) were prepared from PAM gel, P(AM-AA) gel and P(AM-co-AA) gel by applying a pressure of 300 kg/cm^2.

As shown in Figure S6, in the spectra of the polyacrylamide gel, the bands at 3445 cm^{-1} (N-H stretching vibration), 1658 cm^{-1} (C=O stretching), 1454 cm^{-1} (CH_2 in-plane scissoring), 1416 cm^{-1} (C-N stretching for primary amide), 1349 cm^{-1} (C-H deformation), and 1127 cm^{-1} (NH_2 in-plane rocking) were monitored. The poly(acrylamide-co-acrylic acid) gel showed a peak at 3428 cm^{-1} (stretching vibration of N-H), 1660 cm^{-1} (C=O stretching), 1455 cm^{-1} (CH_2 in-plane scissoring), 1417 cm^{-1} (C-N stretching for primary amide), 1338 cm^{-1} (C-H deformation), 1288 cm^{-1} (C-N stretching) and 1156 cm^{-1} (NH_2 in-plane rocking). In the spectra of the Poly(acryliamide-co-acrylic acid) gel, the intensities of N-H stretching peak (3431 cm^{-1}), C=O stretching peak (1659 cm^{-1}) and C-N stretching peak (1284 cm^{-1}) was decreased. Furthermore, the intensity of C-N stretching (1288 cm^{-1}) and NH_2 in-plane rocking peak (1128 cm^{-1}) were also reduced. These results indicated the new cross-linking bonds through imidization between amide groups of acrylamide and carboxylic acid groups of acrylic acid in the poly(acrylamide-co-acrylic acid) gel. [16,18,S3,S4]

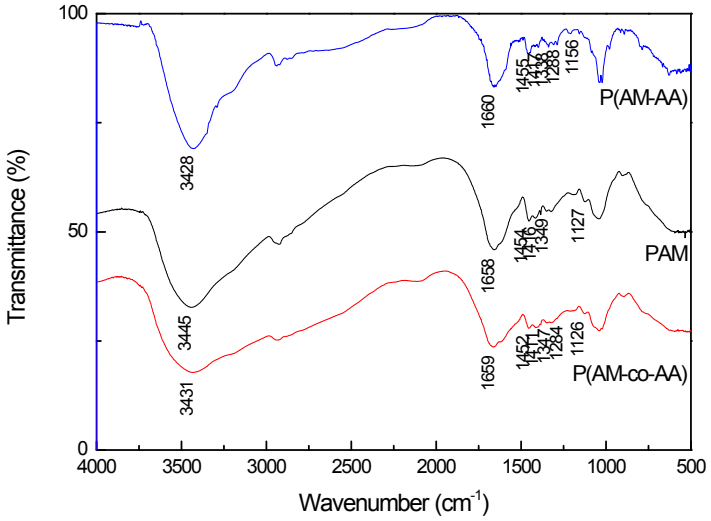

Figure S6. FTIR spectra of PAM, P(AM-AA) and P(AM-co-AA) gels.

Rheological characterization. Rheological characterization was performed on an instrument of rheometer (AR-G2, TA Instruments, USA) equipped with a parallel plate and a 40 mL aluminum cone. Samples were cut by using a rectangular punch (thickness = 1.0 mm, width = 4.5 mm) and taken out of the petri dish carefully. After then, the cut samples were then loaded onto the fixture between the drums (length = 13 mm). Tests were performed at a constant temperature of 20°C controlled by an environmental temperature controller under a nitrogen atmosphere. Frequency sweep was performed at 1.0% strain from 0.1 to 100 rad/s. The storage and loss moduli (viz., G′ and G″ values, 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.

Thermogravimetric analysis (TGA). Thermogravimetric analysis was conducted using a TGA Q5000 (TA Instruments, USA) equipped with a heating rate of 10 °C/min under a nitrogen atmosphere. Samples were scanned in the range of 20~600 °C. Gel samples of 5~6 mg were tested.

Figure S7 showed the integral results from TGA curves of PAM and P(AM-co-AA) hydrogels. From the TGA traces, it was apparent that the gel of PAM had three regions of major mass loss. The first mass loss occurred in the temperature range of 230-330 °C due to the liberate of ammonia molecule from every two amide groups, resulting in the formation of imide. The second one was observed from the range of 330~480 °C,

thanks to the depolymerization of polymer and formation of a carbonaceous residue. Furthermore, there existed thermal degradation of imides and breaking of the polymer backbone occurred as the second and third stages. The P(AM-co-AA) hydrogel clearly showed a three-stage thermal decomposition, and its thermostability was similar to the one of PAM gel.[16,S3,S4]

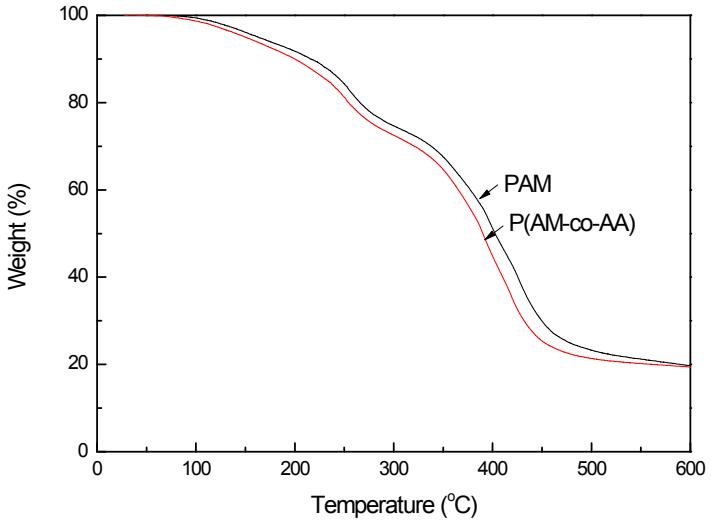

Figure S7. Thermogravimetric analyses of PAM and P(AM-co-AA) hydrogels.

Differential scanning calorimetry (DSC). Differential scanning calorimetry characterization were carried out using a Netzsch DSC 204 F1 instrument (Netzsch, Germany). Dry gel samples were placed in an aluminum pan and thermograms covered the temperature range of 20~150 °C at a heating rate of 10 °C/min under nitrogen atmosphere.

As shown in Figure S8, the glass-transition temperature increases from 50 °C to 60 °C. The DSC curve indicated the complex of the acrylamide and acrylic acid in the hybrid network, attributing to the formation of new bonds formed between carboxyl groups of acrylic acid and $-NH_2$ groups of acrylamide and acrylic acid in the P(AM-co-AA) gel.[16,S3,S4]

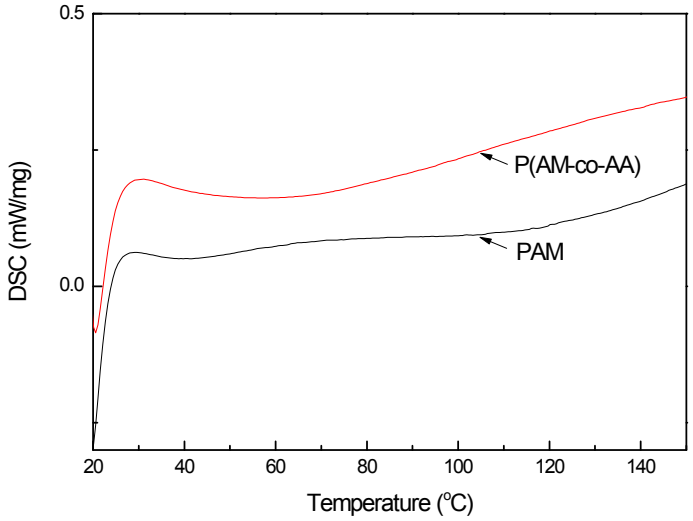

Figure S8. Differential scanning calorimetry (DSC) of PAM and P(AM-co-AA) hydrogels.

Peptide Identification via MS

The peptide bands were excised from electrophoresis gels of SDS-PAGE. The excised peptide band gels were deposited into centrifuge tube, washed with ultrapure water and dried in a centrifuge for 3 minutes. The samples were treated with DTT (60°C, 1h), 50 mM NH_4HCO_3, CH_3CN, in turn. The samples were digested overnight with trypsin solution (10 μL, 10 ng/μL, Promega). The digested samples were extracted for 2 × 15 min with extracting solution (H_2O/CH_3CN=1/1), dried and solubilized in loading buffer ($CH_3CN/HCOOH/H_2O$ = 2/0.1/97.9). The extracted samples were concentrated and separated by reversed-phase HPLC (Thermo scientific, USA) with a 5 μm 100 Å Durashell-C18 (Agela) 2.1 mm × 250 mm Column. Peptides identification was carried out using an LC system (Nano Pump, Ultimate 3000, Dionex, Thermofisher, USA) coupled with an ESI-Q-TOF mass spectrometer (maXis, Impact, Bruker Daltonik, Germany) in data dependent acquisition mode (m/z 350-1500). The flow and temperature of dry gas was 2.0 L/min and 120 °C, respectively, and the source capillary was set at 1900 V. The mass spectrometer was set as one full MS scan, and then ten MS/MS scans on the ten most intense ions from the MS spectrum.[S5]

The extracted MS/MS peak lists were compared with the MSDB database via the software of Compass Data Analysis version 4.1 (Bruker Daltonics) for peptide identification. The relevant searches were all

conducted for carbamidomethylation of cysteines and for oxidation of methionines, with a maximum of one missed cleavage. The peptide identification had validity if two peptides showed fragmentation profile scores more than the average default value for significance using MASCOT (version 2.4.1, Matrix Science), with mass tolerance of 20 ppm for precursor ions and 0.05 Da for fragment ions in MS/MS data.

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

No.	Gene	Protein name	MW (kDa)	Unique peptide	Score
1	IGLL5	Immunoglobulin lambda-like poly-peptide 5 OS=Homo sapiens GN= IGLL5 PE=2 SV=2	23.4	14	555
2	ZG16B	Zymogen granule protein 16 homolog B OS=Homo sapiens GN=ZG16B PE=1 SV=3	22.7	17	949

Table S3. Identification of proteins in minor bands b of Figure 2D via mass spectrometry.

No.	Gene	Protein name	MW (kDa)	Unique peptide	Score
1	LCN1	Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1	19.4	9	409
2	LC1L1	Putative lipocalin 1-like protein 1 OS=Homo sapiens GN=LCN1P1 PE=5 SV=1	18	7	347
3	PIP	Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1	16.8	19	287

Table S4. Identification of proteins in minor bands c of Figure 2D via mass spectrometry.

No.	Gene	Protein name	MW (kDa)	Unique peptide	Score
1	IGKC	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	11.8	12	446
2	LAC2	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	11.5	3	127

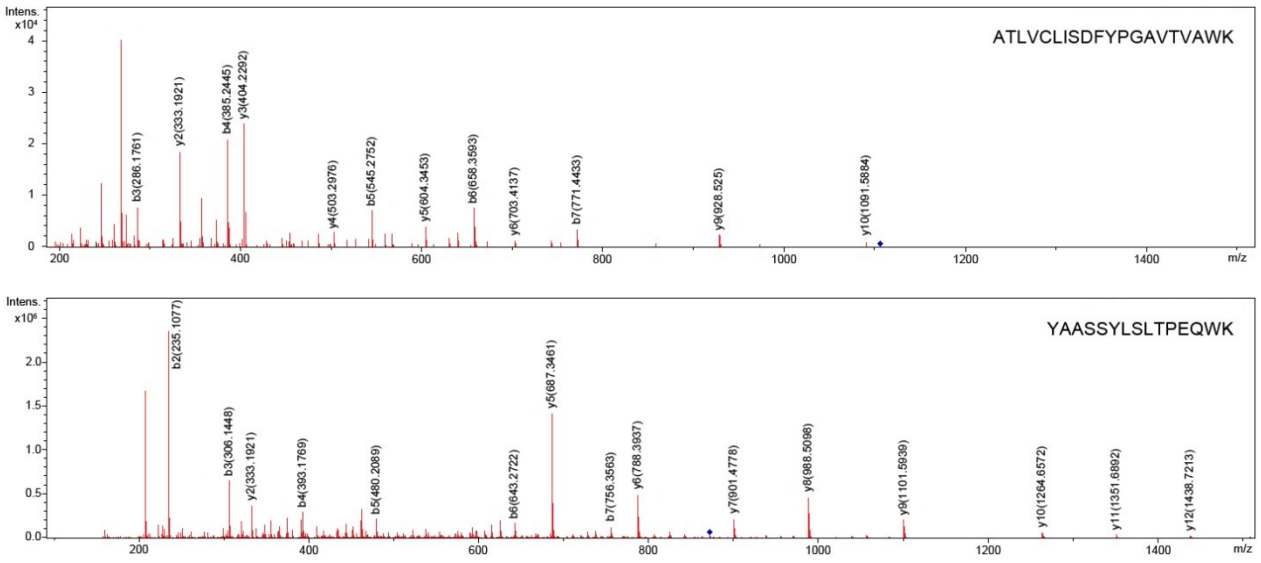

Figure S9. MS/MS spectrum of IGLL5

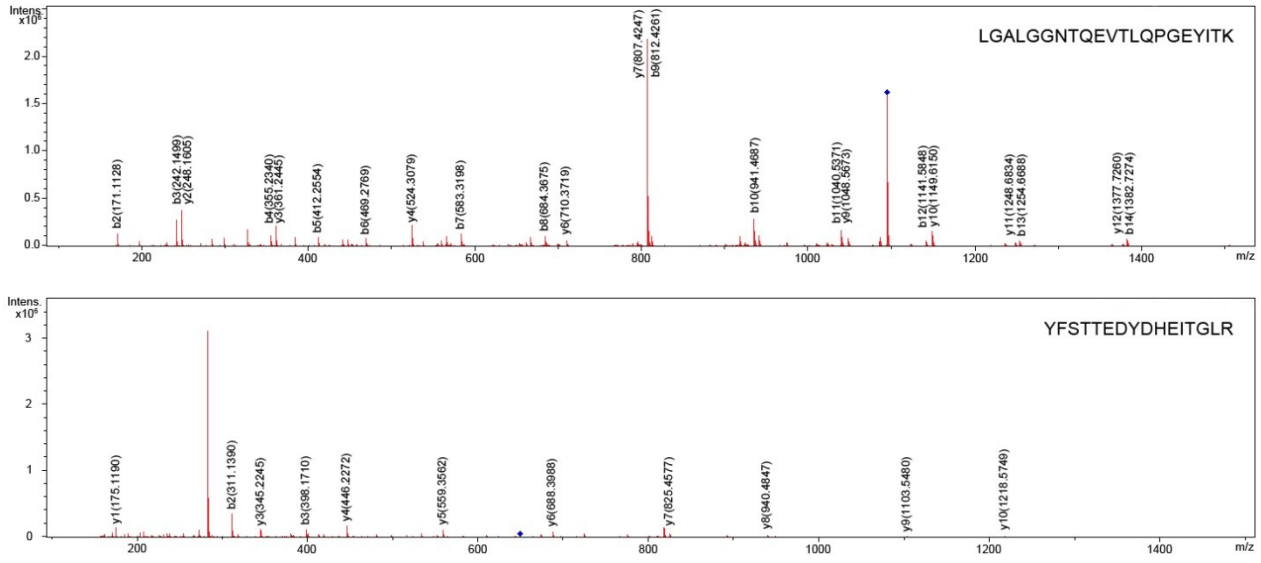

Figure S10. MS/MS spectrum of ZG16B

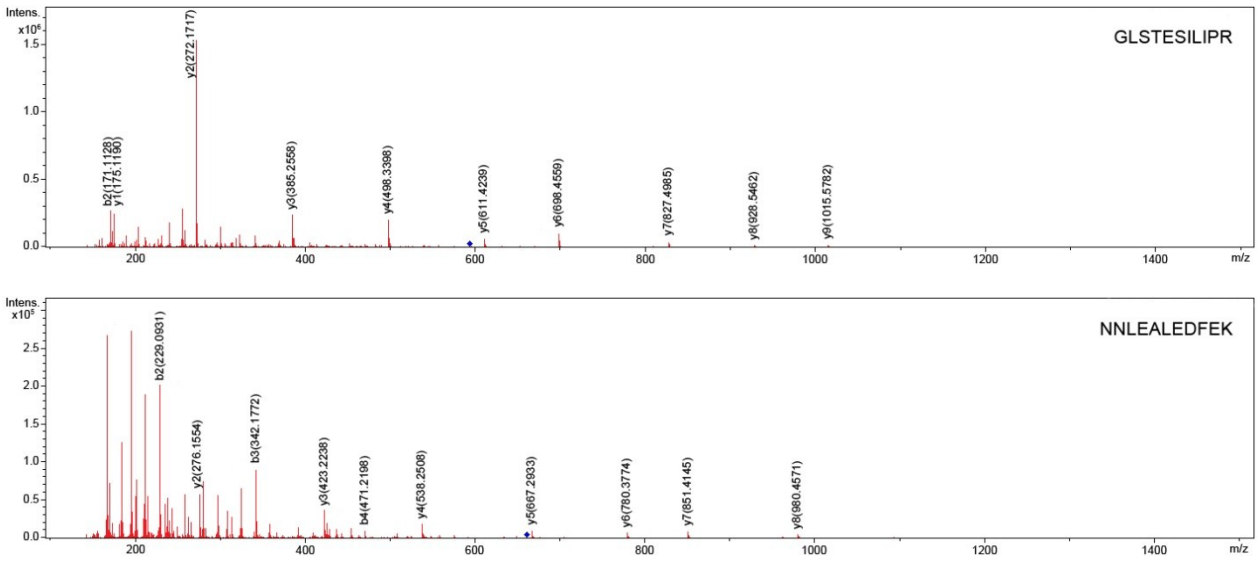

Figure S11. MS/MS spectrum of LCN1

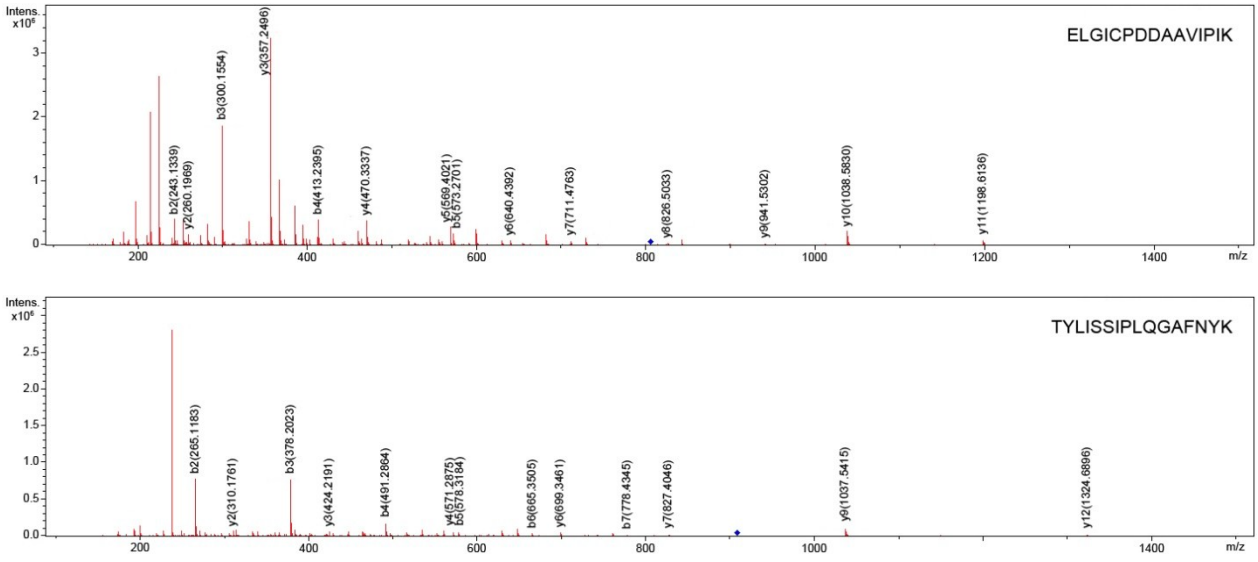

Figure S12. MS/MS spectrum of PIP

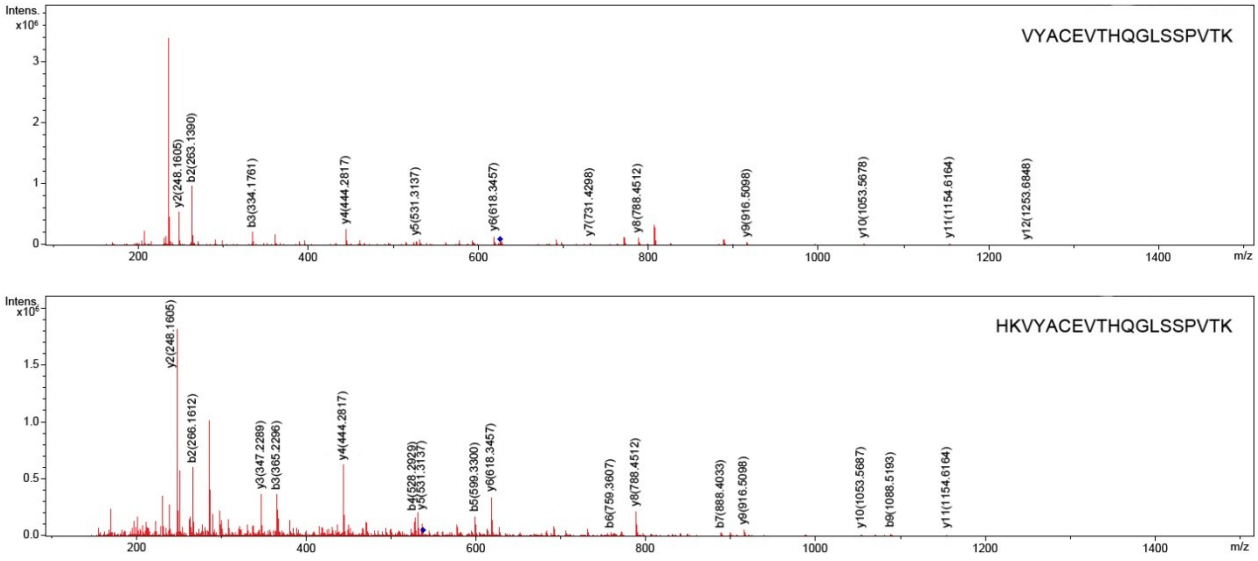

Figure S13. MS/MS spectrum of IGKC

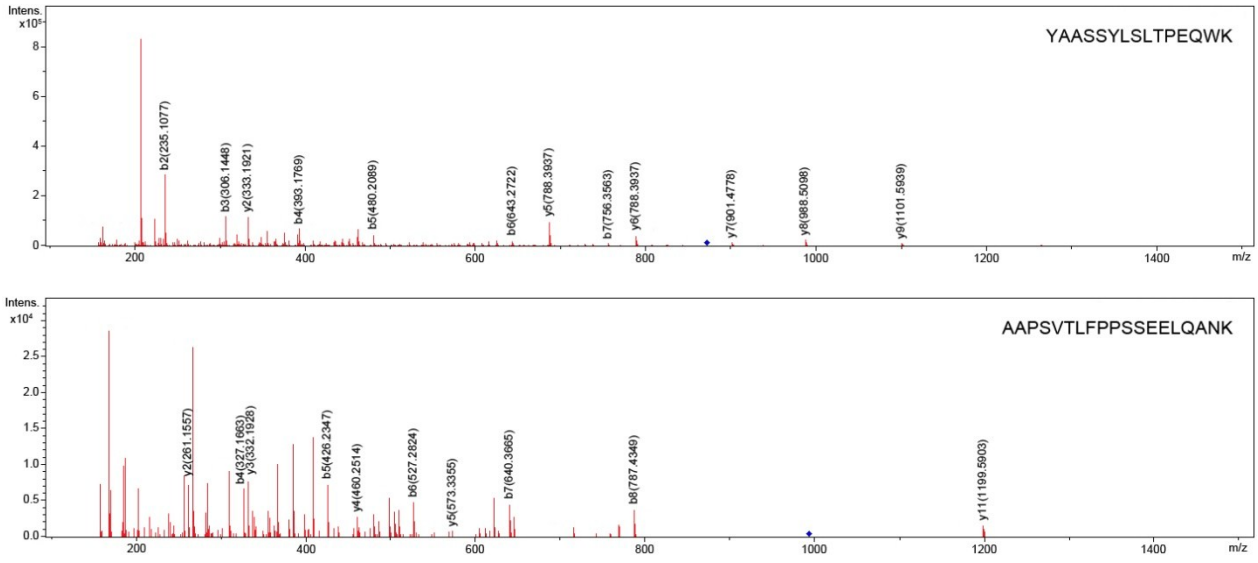

Figure S14. MS/MS spectrum of LAC2

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