Supporting information

for

Facile synthesis of enzyme-inorganic hybrid nanoflowers and their application as an immobilized trypsin reactor for highly efficient protein digestion†

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2. Experimental

2.1 Reagents and Materials.

All other chemicals were of analytical grade or better. Copper sulfate pentahydrate ($CuSO_4.5H_2O$) was purchased from Sinopharm Chemical Reagent, Co., Ltd (Shanghai, China). Iodoacetamide (IAA), 1,4-dithiothreitol (DTT), and formic acid (FA), acetonitrile (ACN, HPLC grade), bovine serum albumin (BSA), horseradish peroxidase (HRP), immunoglobulin G (IgG), and lysozyme (Lyz) were obtained from Sigma (St. Louis, MO). TPCK-treated trypsin (Try) was purchased from Aladin (Shanghai, China). N_α-benzoyl-L-arginine ethyl ester (BAEE, Mw=342.82, Purity> 98%) was get from Yuanye Biotechnology Co. Ltd (Shanghai, China). Branford Protein Assay (P0006) was get from Beyotime Institute of Biotechnology. The deionized water used in all experiments was purified with a Milli-Q system from Millipore (Milford, MA). Healthy human serum sample was kindly gifted from Fujian Province Official Hospital (Fuzhou, China).

2.2 Preparation of enzyme-inorganic hybrid nanoflowers.

For the synthesis of enzyme-inorganic hybrid nanoflowers, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of phosphate buffered saline (PBS) (0.1 M, pH 7.4) containing different concentrations of enzyme. Followed by incubation at 25 ºC for 3 day, blue precipitates were obtained after centrifugation and then dried under vacuum at room temperature. Other protein-inorganic hybrid nanoflowers were aslo prepared by the same method as mentioned above.

2.3 Characterizations.

Scanning electron micrographs (SEM) of the prepared hybrid nanoflowers were carried out on a XL-30E scanning electron microscope (Philips, Netherlands). Fourier transform infrared (FT-IR) spectra of the nanoflowers were conducted with a FT-IR spectrophotometer (Nicolet 6700, Waltham, MA, USA). The crystal structures of the nanoflowers were determined by X-ray powder diffraction (XRD) (D/Max-2500 diffractometer, Shimadzu, Japan). Surface area and pore size analysis of the nanoflowers were performed by Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods using physisorption analyzer (Micromeritics ASAP2020 porosimeter, USA). The data of adsorption were obtained by using UV/Vis spectrophotometer (Shimadzu UV2550, Japan). The encapsulation yield of the trypsin was obtained by determining the supernatant at 280 nm using the UV/Vis spectrophotometer and weight percentage of trypsin in the nanoflowers was determined by the gravimetric method. Analysis of digested peptides was performed with LC-ESI-Q-TOF/MS (Agilent, Palo Alto, CA, U.S.A.).

2.4 Measurement of the trypsin activity.

The catalytic activity of the trypsin was determined using BAEE as the substrate. For this purpose, some modifications were made to determine the activity of immobilized trypsin following the method developed by Schwert and Takenaka.[1] 0.2 mg immobilized trypsin on the nanoflowers was dissolved in 20 μL, 50 mM NH_4HCO_3 buffer solution (pH 8.0). The blank solution, which contained 0.2 mg copper

phosphate and 280 μL, 0.5 mM BAEE in 50 mM NH_4HCO_3 buffer solution (pH 8.0), was used to calibrate the UV/Vis spectrophotometer at 253 nm; then a total of 280 μL, 0.5 mM BAEE was hydrolyzed with nanoflowers at 25 ºC, and the increase in absorbance at 253 nm was measured every 30 s for 10 min; finally, the kinetics curves with regards to the enzymatic reaction were drawn. The bioactivity was calculated in BAEE-units/mg trypsin, and 1 BAEE-unit is equal to an increase in absorbance of 0.001/min at 253 nm under specified conditions. The activity was calculated with following equations:

The activity (BAEE unit/mL) = (ΔA253 nm/min)/(0.001×V(trypsin)) (1)

The activity (BAEE unit/mg) = The activity (BAEE unit/mL)/(concentration of trypsin (mg/mL) ×V(trypsin)) (2)

In the case of free trypsin, the activity measurement was done following the procedures and the conditions similar to those for immobilized trypsin.

2.4 Digestion of proteins and human serum using hybrid nanoflowers.

1.0 mg protein (BSA or HRP) in 1.0 mL of 50 mM NH_4HCO_3 buffer solution (pH 8.0) (or 10 μL human serum in 590 μL of 50 mM NH_4HCO_3 buffer solution (pH 8.0), which corresponds to 600 μg total proteins by Brandford protein assay (Bio-Rad, Catalog. No. P0006) using BSA a standard) was denatured in a 95 ºC water bath for 5 min and then reduced in 15% (v/v) ACN and 5 mM DTT for 1h at 56 ºC. When cooled to room temperature, cysteines were alkylated in the dark in 10 mM IAA for 1h at room temperature. Subsequently, 0.10 mg dried nanoflowers was transferred to

300 μL of the above denatured protein solution (substrate-to-enzyme ratio of 20:1) in a 1.5 mL Eppendorf tube at 37 ºC for 1min. The supernatant was collected and directly analyzed by LC-ESI-Q-TOF/MS (Agilent, Palo Alto, CA, U.S.A.).

2.5 Digestion of proteins and human serum using free trypsin.

For comparison, the digestion of protein and human serum was performed according to the conventional procedures. Briefly, 1.0 mg protein (BSA or HRP) in 1.0 mL of 50 mM NH_4HCO_3 buffer solution (pH=8.0) (or 10 μL human serum in 590 μL of 50 mM NH_4HCO_3 buffer solution (pH 8.0) was denatured in a 95 ºC water bath for 5 min and then reduced in 15% (v/v) ACN and 5 mM DTT for 1h at 56 ºC. When cooled to room temperature, cysteines were alkylated in the dark in 10 mM IAA for 1h at room temperature. In solution, digestion was performed by adding trypsin into the pretreated protein sample with a substrate-to-enzyme ratio of 40:1(m/m). Then the solution was incubated at 37°C for 12h. After the digestion, 1% (v/v) formic acid was added to stop the reaction.

2.6 MS analysis and database searching.

The tryptic peptides were separated by reversed-phase HPLC using an Agilent 1260 HPLC. An Agilent C18 column (50 mm × 3 mm i.d.) packed with a 2.7 μm diameter particles, 120 Å pore size C18 resin was used for the separations. The solvents were as follow: (A) 0.1 % formic acid (FA) in water, and (B) 0.1 % FA in acetonitrile. The column was equilibrated at 3 % solvent B. Three minute after sample injection the concentration of buffer B was increased from 3 to 40 % B within 20 min followed by

a linear gradient of 40 % to 80% B over 3 min. A flush step was performed with 80 % B for 3 min and the column was equilibrated with 3 % B for 4 min. The column temperature was maintained at 40 °C.

MS detection was performed on an Agilent 6520 Q-TOF mass spectrometer with a dual ESI source and an Agilent G1607A coaxial sprayer (all from Agilent). All analyses were performed in a positive ion mode. Nitrogen was used as drying gas at a temperature of 350 °C and a flow-rate of 10 L min^{-1}. The voltage set for the MS capillary was 4 kV and the fragmentor was set to 175 V. Scanning mass range was from m/z 100 to 3000 at an acquisition rate of 3 spectra s^{-1} in the auto MS/MS mode. For MS^2 experiments, the collision energy was set to according to formula, in which the top three highest intensity peaks in each MS were chosen for collision-induced dissociation. Isolation width for MS^2 was ± 4 amu.

All the LC-MS/MS raw data were searched with Spectrum Mill versionA.03.03 against a database (target database of IPI bovine v3.80, IPI plants v3.80, and IPI human.v3.80). Trypsin restriction was set with two missed cleavages. Cyscarboxymethylation was set as the static modification; oxidizedmethionine and proglutamic acid were set as the variable modifications. The mass tolerances were 100 ppm for parent ions and 200 amu for fragment ions.

2.7 Stability and reusability.

The stability of the immobilized trypsin was assessed after storing at -20°C for 20 days. In order to avoid the loss of nanoflowers during washing process, a home-made digestion device was designed for cycle experiment. In brief, 0.1 mg nanoflowers was

packed into a pipette tip that was beforehand filled with a sieve, and then 300 μL of the above denatured protein solution was pipetted into the tip. After incubating at 37°C for 1min, the solution was pushed and collected for MS analysis. Then the tip was washed two times with 10 μL of 50 mM NH_4HCO_3 buffer solution (pH 8.0), and then resuspended in the same buffer to start a new run. The bioactivity to biomacromolecule were expressed in the way that the percentage of residual activity to the initial one.

References

1 G.W. Schwert and Y. A. Takenaka, *Biochim. Biophys. Acta.* 1955, **16**, 570-575.

Supporting Figures and Tables

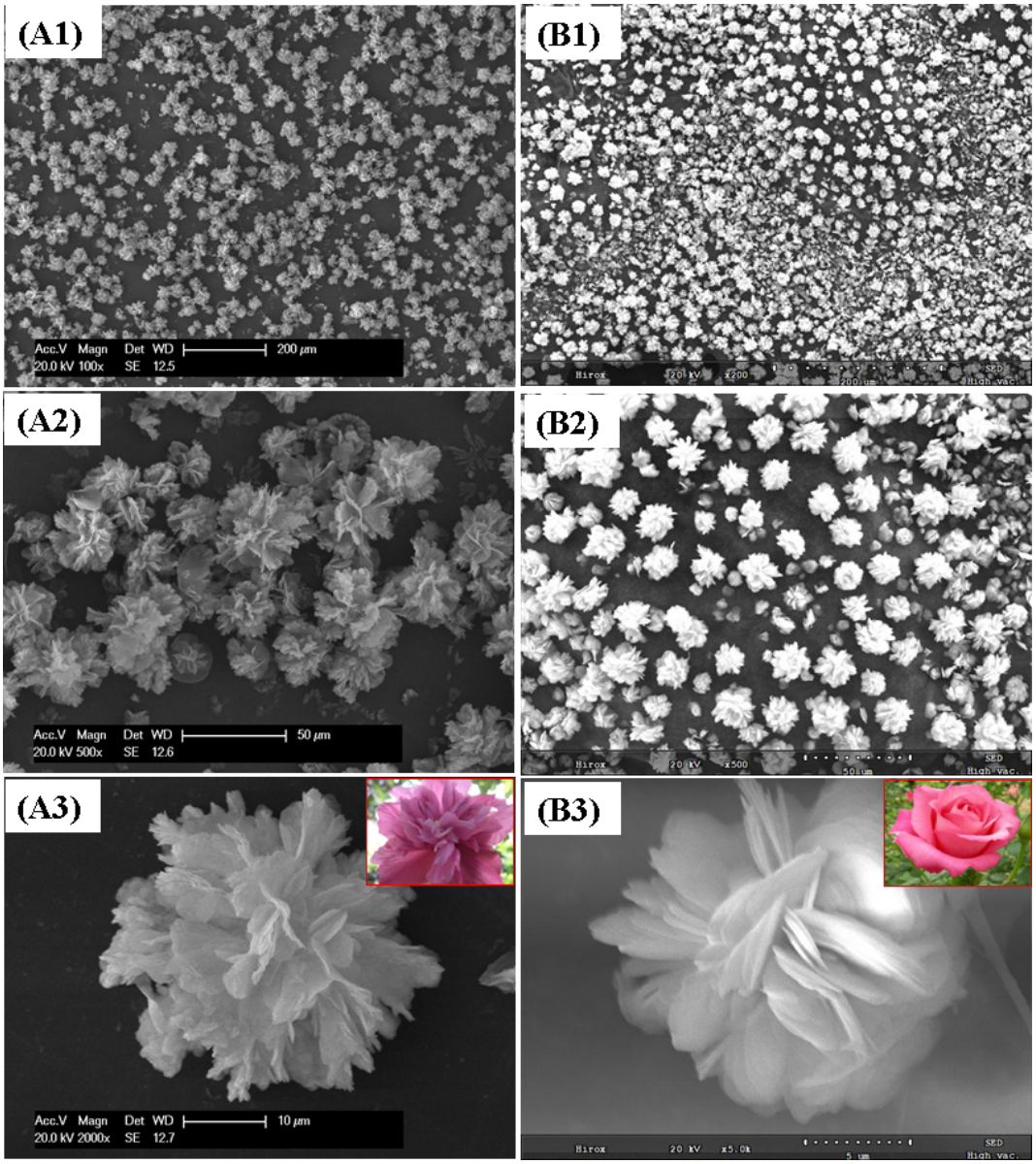

Figure S1. The morphologies of protein-inorganic hybrid nanoflowers. (A1-3) 2.0 mg • mL^{-1} Lyz; (B1-3) 0.1 mg • mL^{-1} IgG. Other conditions: 120 mM Cu^{2+}, 0.1 M PBS at pH 7.4, and reaction for 3 days at 25 ℃.

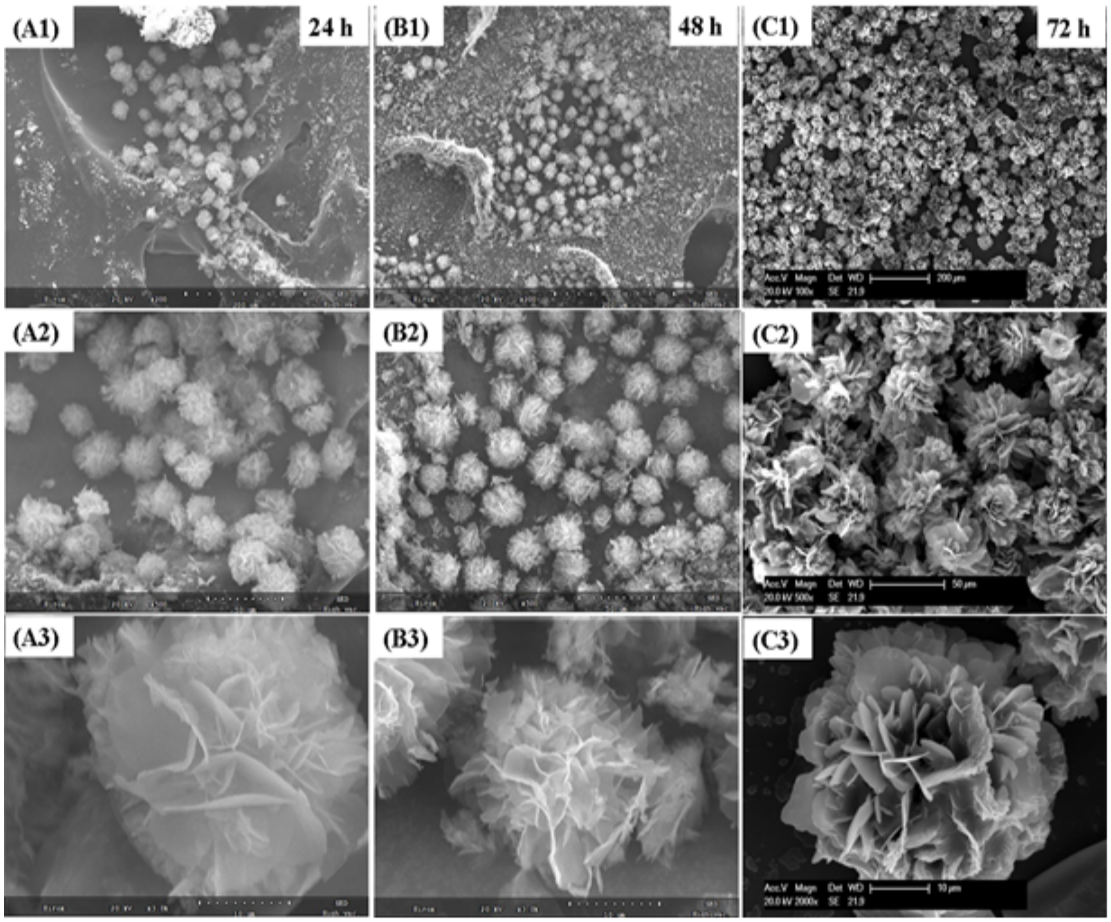

Figure S2 Effect of different incubation time on the morphologies of nanoflowers. (A1-3) 24 h; (B1-3) 48 h; (C1-3) 72 h. Other conditions: 0.5 mg mL^{-1} trypsin, 120 mM $Cu2^{+}$, 0.1 M PBS at pH 7.4, and reaction at 25 ℃.

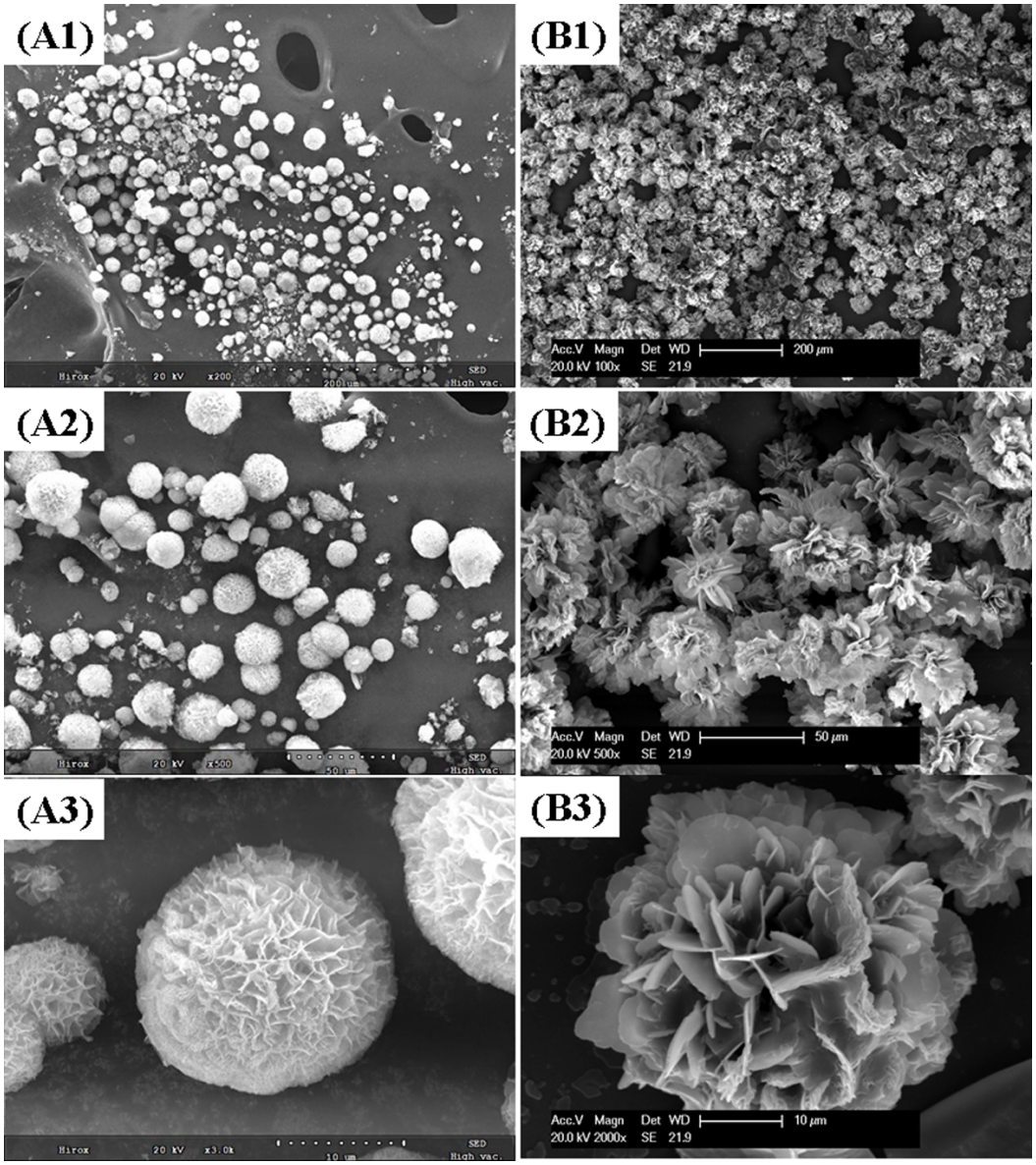

Figure S3. The effect of different incubation temperature on the morphology of the trypsin-inorganic hybrid nanoflowers. (A1-3) 0 ℃; (B1-3) 25℃. Other conditions: 0.5 mg • mL^{-1} trypsin, 120 mM Cu^{2+}, 0.1 M PBS at pH 7.4, and reaction for 3 days.

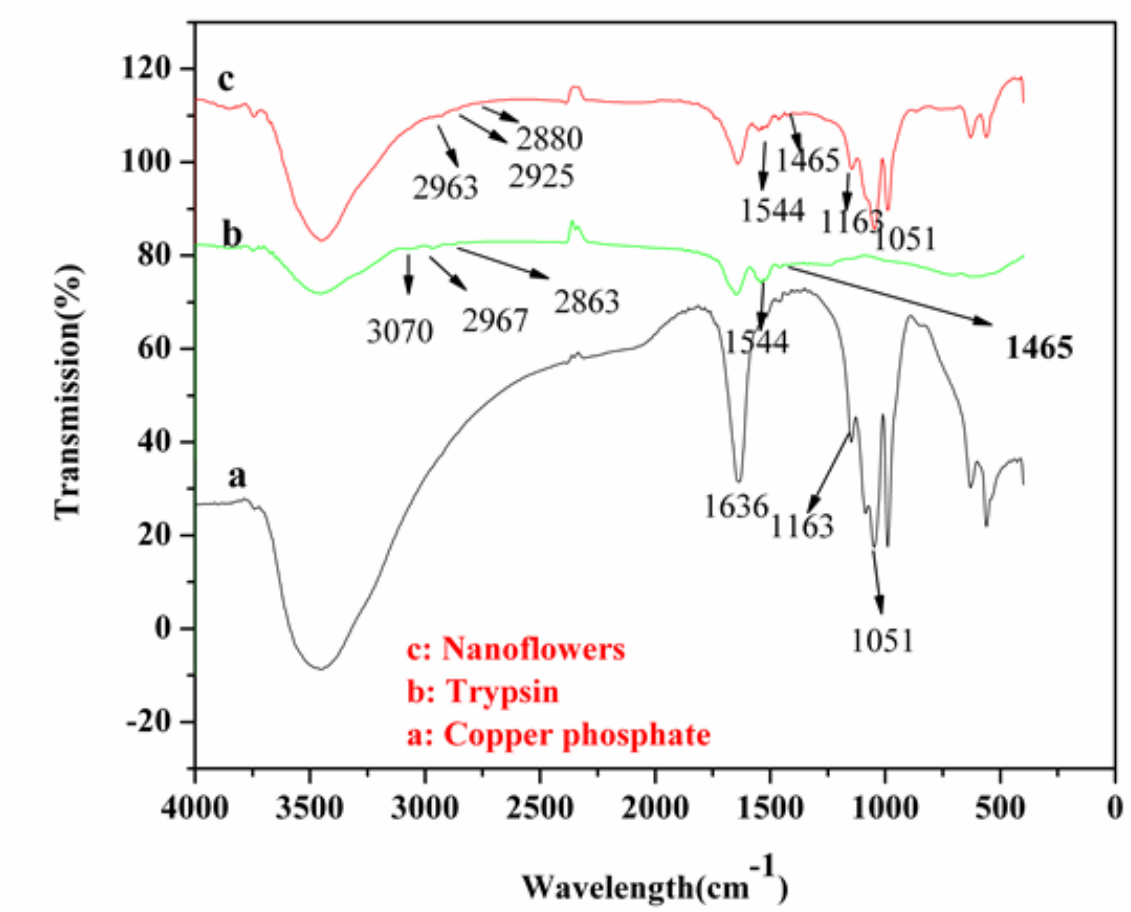

Figure S4 FT-IR spectra of (a) $Cu_3(PO_4)_2.3H_2O$; (b) trypsin; (c) the trypsin-inorganic hybrid nanoflowers.

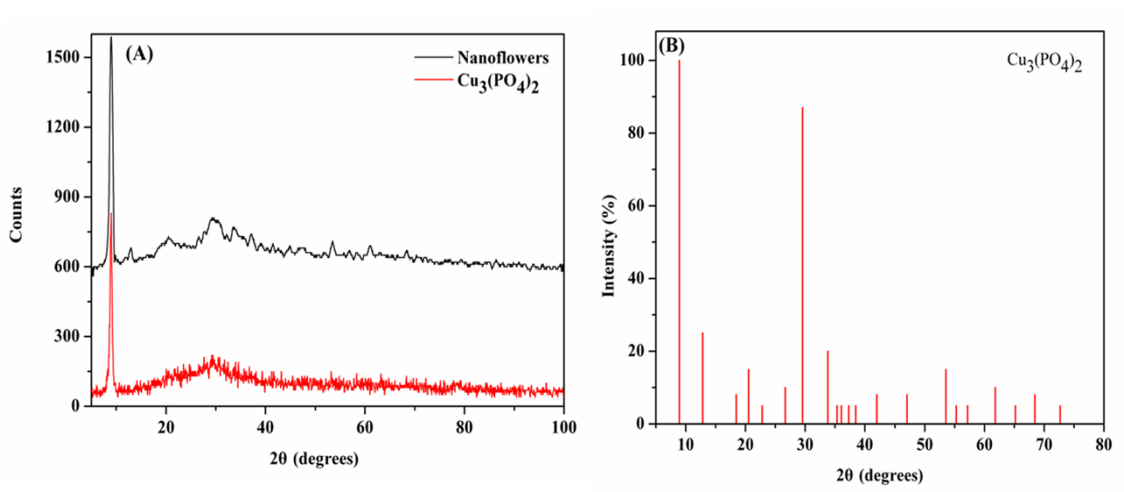

Figure S5. XRD patterns of (A) hybrid nanoflowers and $Cu_3(PO4)_2$; (B) peak of $Cu_3(PO4)_2{\cdot}3H_2O$ (JPSCD 00-022-0548).

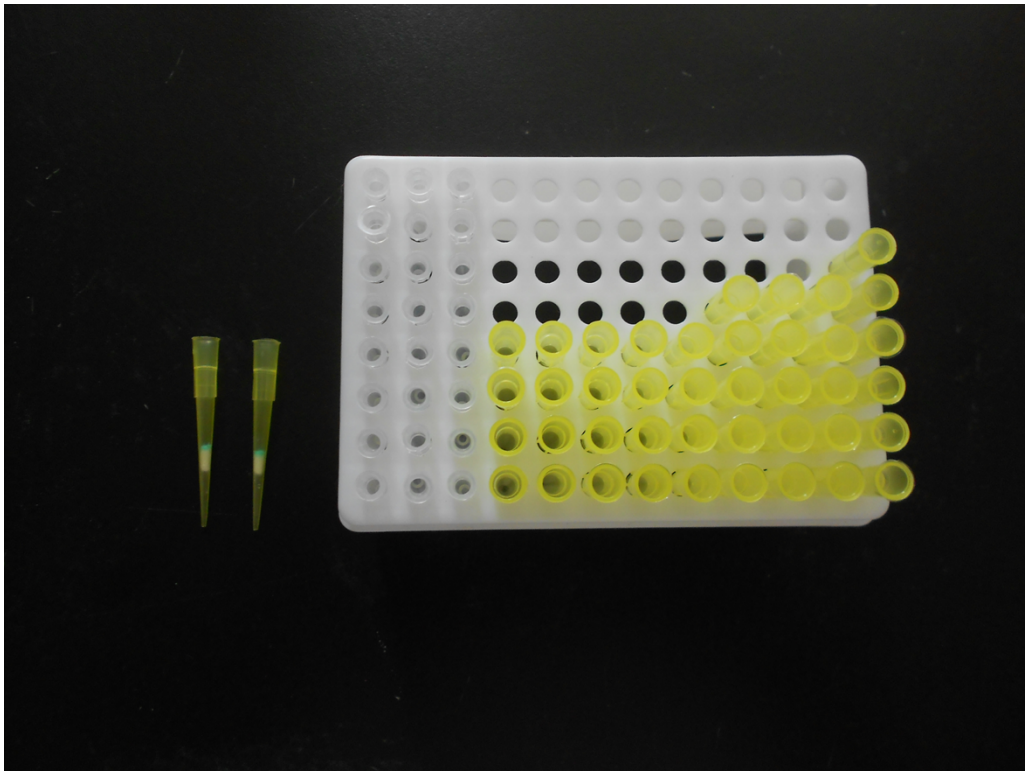

Figure S6. Photograph of a home-made IMER devices

Table S1. The actual encapsulation yield trypsin in the nanoflowers was further determined by UV/Vis methods[a)].

theoretical concentration (mg mL^{-1})	Actual concentration (mg mL^{-1})	Absorbance (253 nm)	The supernatant concentration (mg mL^{-1})	The supernatant absorbance (253 nm)	Encapsulation yield (%)
0.02	0.0227	0.05	0.0037	0.027	83.7%
0.1	0.1126	0.159	0.0235	0.051	79.1%
0.5	0.5103	0.641	0.4467	0.564	12.5%

(a) Regression equations: Y= 1.21209 X + 0.02252 (r=0.999) (X: trypsin concentration, Y: absorbance)

Table S2. Weight percentage of trypsin in the nanoflower were determined by gravimetric methods

theoretical concentration (mg mL^{-1})	$Cu_3(PO4)_2 \cdot 3H_2O$ (mg)	Trypsin-incorporated nanoflowers (mg)	Weight percentage (%)
0	3.13	0	0
0.02	3.38	0.25	7.4
0.1	3.44	0.31	9.0
0.5	3.58	0.45	12.6

Table S3. Comparison of the proteolytic efficiency of trypsin-immobilized nanolfower and free trypsin for BSA digestion (for 12h incubation, BSA: SwissProt Accession: P02769)

	Position	Mass	Peptide sequence	Free	Nanoflower[a]	Nanoflower[b]
	Incubation time			12h	1min	1min
	Matched peptides			72	70	68
	Sequence coverage, %			92	86	80
1	1-10	1193.602	(-)DTHKSEIAHR(F)	√	*	*
2	5-10	712.374	(K)SEIAHR(F)	√	√	*
3	11-20	1249.621	(R)FKDLGEEHFK(G)	√	√	*
4	11-41	3723.852	(R)FKDLGEEHFKGLVLIAFSQYLQQCPFDEHVK(L)	*	*	√
5	13-20	974.458	(K)DLGEEHFK(G)	√	√	*
6	21-41	2493.248	(K)GLVLIAFSQYLQQCPFDEHVK(L)	√	√	*
7	42-51	1163.631	(K)LVNELTEFAK(T)	√	√	*
8	42-64	2610.17	(K)LVNELTEFAKTCVADESHAGCEK(S)	*	*	√
9	52-64	1465.557	(K)TCVADESHAGCEK(S)	√	√	*
10	65-75	1420.678	(K)SLHTLFGDELCK(V)	*	√	*
11	76-81	545.341	(K)VASLR(E)	*	√	*
12	82-93	1480.491	(R)ETYGDMADCCEK(Q)	√	√	*
13	82-98	2119.789	(R)ETYGDMADCCEKQEPER(N)	√	√	*
14	94-114	2542.151	(K)QEPERNECFLSHKDDSPDLPK(L)	*	*	√
15	99-106	1035.456	(R)NECFLSHK(D)	√	√	*
16	99-114	1902.854	(R)NECFLSHKDDSPDLPK(L)	√	√	*
17	107-114	886.415	(K)DDSPDLPK(L)	√	√	*
18	115-127	1577.752	(K)LKPDPNTLCDEFK(A)	√	*	*
19	115-131	2020.953	(K)LKPDPNTLCDEFKADEK(K)	√	√	*
20	115-132	2149.048	(K)LKPDPNTLCDEFKADEKK(F)	√	√	*
21	132-136	665.377	(K)KFWGK(Y)	√	√	*
22	132-144	1573.853	(K)KFWGKYLYEIAR(R)	*	*	√
23	133-136	537.282	(K)FWGK(Y)	√	√	*
24	133-144	1445.758	(K)FWGKYLYEIAR(R)	*	*	√
25	137-143	927.493	(K)YLYEIAR(R)	√	√	*
26	137-144	1083.595	(K)YLYEIARR(H)	*	*	√
27	144-159	2045.028	(R)RHPYFYAPELLYYANK(Y)	*	√	*
28	145-159	1888.927	(R)HPYFYAPELLYYANK(Y)	√	√	*
29	160-170	2490.062	(K)YNGVFQECCQAEDKGACLLPK(I)	*	√	*
30	160-173	1749.673	(K)YNGVFQECCQAEDK(G)	√	*	*
31	174-180	759.407	(K)GACLLPK(I)	√	√	*
32	174-185	1389.723	(K)GACLLPKIETMR(E)	*	*	√
33	181-185	649.334	(K)IETMR(E)	√	√	*
34	197-204	976.524	(R)LRCASIQK(F)	√	√	*

35	197-208	1465.758	(R)LRCASIQKFGER(A)	*	*	√
36	199-204	707.339	(R)CASIQK(F)	√	√	*
37	199-208	1196.573	(R)CASIQKFGER(A)	*	*	√
38	205-208	508.251	(K)FGER(A)	√	√	*
39	209-217	1001.589	(R)ALKAWSVAR(L)	*	√	*
40	212-217	689.373	(K)AWSVAR(L)	*	√	*
41	218-224	847.504	(R)LSQKFPK(A)	√	*	*
42	218-232	1750.974	(R)LSQKFPKAEFVEVTK(L)	*	*	√
43	225-232	922.488	(K)AEFVEVTK(L)	√	√	*
44	225-239	1692.942	(K)AEFVEVTKLVTDLTK(V)	√	√	*
45	233-239	789.472	(K)LVTDLTK(V)	√	√	*
46	239-261	2615.117	(K)VHKECCHGDLLECADDRADLAK(Y)	√	√	*
47	243-256	1752.615	(K)ECCHGDLLECADDR(A)	√	√	*
48	243-261	2250.895	(K)ECCHGDLLECADDRADLAK(Y)	√	√	*
49	257-261	517.298	(R)ADLAK(Y)	*	√	*
50	262-273	1444.626	(K)YICDNQDTISSK(L)	√	*	*
51	262-275	1685.805	(K)YICDNQDTISSKLK(E)	*	*	√
52	262-285	2960.357	(K)YICDNQDTISSKLKECCDKPLLEK(S)	*	*	√
53	274-285	1534.749	(K)LKECCDKPLLEK(S)	√	*	*
54	276-285	1293.57	(K)ECCDKPLLEK(S)	*	√	*
55	286-294	1073.493	(K)SHCIAEVEK(D)	√	*	*
56	286-312	3010.435	(K)SHCIAEVEKDAIPENLPPLTADFAEDK(D)	*	√	*
57	286-316	3513.64	(K)SHCIAEVEKDAIPENLPPLTADFAEDKDVCK(N)	*	√	*
58	295-312	1955.96	(K)DAIPENLPPLTADFAEDK(D)	*	√	*
59	295-316	2459.165	(K)DAIPENLPPLTADFAEDKDVCK(N)	*	√	*
60	295-323	3192.504	(K)DAIPENLPPLTADFAEDKDVCKNYQEAK(D)	√	√	√
61	317-322	752.357	(K)NYQEAK(D)	√	√	√
62	317-335	2301.082	(K)NYQEAKDAFLGSFLYEYSR(R)	√	√	√
63	323-335	1567.743	(K)DAFLGSFLYEYSR(R)	√	√	√
64	323-336	1723.844	(K)DAFLGSFLYEYSRR(H)	√	√	√
65	336-347	1439.812	(R)RHPEYAVSVLLR(L)	√	√	√
66	337-347	1283.711	(R)HPEYAVSVLLR(L)	√	√	√
67	351-375	3041.201	(K)EYEATLEECCAKDDPHACYSTVFDK(L)	√	√	√
68	351-362	1504.582	(K)EYEATLEECCAK(D)	*	*	√
69	351-377	3282.38	(K)EYEATLEECCAKDDPHACYSTVFDKLK(H)	√	√	√
70	363-375	1555.637	(K)DDPHACYSTVFDK(L)	*	√	√
71	363-377	1796.816	(K)DDPHACYSTVFDKLK(H)	√	*	√
72	376-388	1546.895	(K)LKHLVDEPQNLIK(Q)	√	*	√
73	378-388	1305.716	(K)HLVDEPQNLIK(Q)	√	√	√
74	378-396	2356.124	(K)HLVDEPQNLIKQNCDQFEK(L)	*	*	√
75	378-409	3816.901	(K)HLVDEPQNLIKQNCDQFEKLGEYGFQNALIVR(Y)	*	*	√
76	388-412	2950.415	(K)QNCDQFEKLGEYGFQNALIVRYTR(K)	*	*	√

77	389-396	1069.426	(K)QNCDQFEK(L)	√	√	√
78	389-409	2530.203	(K)QNCDQFEKLGEYGFQNALIVR(Y)	√	√	√
79	397-409	1479.795	(K)LGEYGFQNALIVR(Y)	√	√	√
80	397-412	1900.008	(K)LGEYGFQNALIVRYTR(K)	*	*	√
81	410-427	2060.15	(R)YTRKVPQVSTPTLVEVSR(S)	*	*	√
82	413-427	1639.938	(R)KVPQVSTPTLVEVSR(S)	√	√	√
83	414-427	1511.843	(K)VPQVSTPTLVEVSR(S)	√	√	√
84	428-431	404.25	(R)SLGK(V)	√	*	√
85	428-435	817.489	(R)SLGKVGTR(C)	*	*	√

[a] 1 min of shake-assisted digestion at 37 ℃

[b] nanoflowers were stored at -20 ℃ for 20 days

Table S4. Comparison of the proteolytic efficiency of trypsin-immobilized nanolfower and free trypsin for HRP digestion (for 12h incubation, HRP: SwissProt Accession: P00433)

	Position	Mass	Peptide sequence	Free	Nanoflower[a]	Nanoflower[b]
	Incubation time			12h	1min	1min
	Matched peptides			14	19	16
	Sequence coverage, %			45	41	41
1	50-57	959.516	(R)DTIVNELR(S)	√	*	√
2	62-68	743.477	(R)IAASILR(L)	√	*	*
3	58-68	1198.69	(R)SDPRIAASILR(L)	*	*	√
4	93-105	1380.65	(R)TEKDAFGNANSAR(G)	√	√	√
5	93-112	2165.073	(R)TEKDAFGNANSARGFPVIDR(M)	*	√	*
6	96-105	1022.465	(K)DAFGNANSAR(G)	√	*	*
7	106-114	1062.576	(R)GFPVIDRMK(A)	*	√	√
8	106-123	2004.999	(R)GFPVIDRMKAAVESACPR(T)	*	√	√
9	113-123	1220.576	(R)MKAAVESACPR(T)	*	√	√
10	115-123	961.441	(K)AAVESACPR(T)	√	√	√
11	124-148	2603.313	(R)TVSCADLLTIAAQQSVTLAGGPSWR(V)	*	√	*
12	124-153	3125.641	(R)TVSCADLLTIAAQQSVTLAGGPSWRVPLGR(R)	√	√	√
13	124-154	3281.742	(R)TVSCADLLTIAAQQSVTLAGGPSWRVPLGRR(D)	*	√	√
14	154-179	2900.567	(R)RDSLQAFLDLANANLPAPFFTLPQLK(D)	*	√	*
15	154-183	3405.795	(R)RDSLQAFLDLANANLPAPFFTLPQLKDSFR(N)	*	√	√
16	155-179	2744.466	(R)DSLQAFLDLANANLPAPFFTLPQLK(D)	√	√	√
17	155-183	3249.694	(R)DSLQAFLDLANANLPAPFFTLPQLKDSFR(N)	*	√	√
18	190-204	1475.749	(R)SSDLVALSGGHTFGK(N)	√	√	√
19	190-208	2034.966	(R)SSDLVALSGGHTFGKNQCR(F)	√	√	√
20	237-254	1974.995	(R)GLCPLNGNLSALVDFDLR(T)	√	*	*
21	246-254	1035.547	(R)SALVDFDLR(T)	√	*	*
22	255-262	935.483	(R)TPTIFDNK(Y)	√	√	*
23	255-271	2102.044	(R)TPTIFDNKYYVNLEEQK(G)	*	√	*
24	263-271	1185.579	(K)YYVNLEEQK(G)	√	*	*
25	314-328	1586.832	(R)MGNITPLTGTQGQIR(L)	√	√	√

[a] 1 min of shake-assisted digestion at 37 ℃

[b] nanoflowers were stored at -20 ℃ for 20 days

Table S5. Identification of proteins from human serum digested by the nanoflower-based microreactor (n=2)

Group (#)	Distinct Peptides(#)	Distinct Summed MS/MS Search Score	% AA Coverage	Protein Name
1	79	1442.25	92	Serum albumin precursor
2	19	311	59	Apolipoprotein A-I precursor
3	18	300.96	30	Serotransferrin precursor
4	20	299.79	18	Alpha-2-macroglobulin precursor
4	1	17.79	0	Pregnancy zone protein precursor
5	17	294.63	43	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain]
5	8	139.28	22	Haptoglobin-related protein precursor
6	18	277.18	39	Alpha-1-antitrypsin precursor
7	10	189.16	96	Ig kappa chain C region
8	12	188.42	9	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complemen...
9	10	179.48	36	Ig alpha-1 chain C region
9	6	101.55	20	Ig alpha-2 chain C region
10	11	175.56	40	Ig gamma-1 chain C region
10	9	140.68	31	Ig gamma-2 chain C region
10	6	83.94	15	Ig gamma-3 chain C region
10	6	81.56	20	Ig gamma-4 chain C region
11	6	107.27	80	Ig lambda chain C regions
12	5	75.43	27	Alpha-1-acid glycoprotein 1 precursor
12	2	28.98	8	Alpha-1-acid glycoprotein 2 precursor
13	5	72.16	44	Apolipoprotein A-II precursor

14	5	67.77	12	Hemopexin precursor
15	3	47.1	7	Alpha-1-antichymotrypsin precursor
16	3	41.9	27	Ig kappa chain V-III region WOL
16	2	28.7	21	Ig kappa chain V-III region GOL
16	2	28.7	21	Ig kappa chain V-III region Ti
16	2	28.7	21	Ig kappa chain V-III region SIE
17	3	41.03	8	Alpha-1B-glycoprotein precursor
18	3	38.17	5	Complement factor B precursor
19	2	33.73	27	Serum amyloid A protein precursor
20	2	30.67	2	Complement C4-B precursor
20	2	30.67	2	Complement C4-A precursor
21	2	27.48	11	Ig kappa chain V-III region CLL precursor
21	2	27.48	13	Ig kappa chain V-III region POM
21	2	27.48	15	Ig kappa chain V-III region NG9 precursor
21	1	15.83	7	Ig kappa chain V-III region VH precursor
21	1	15.83	8	Ig kappa chain V-I region Lay
22	2	25.39	8	Beta-2-glycoprotein 1 precursor
23	1	21.32	14	Ig kappa chain V-IV region Len
24	1	21.31	15	Ig heavy chain V-III region BRO
24	1	21.31	15	Ig heavy chain V-III region TEI
25	2	21.02	13	Ig kappa chain V-III region VG precursor
26	1	20.93	15	Ig heavy chain V-III region WAS
26	1	20.93	15	Ig heavy chain V-III region POM
26	1	20.93	15	Ig heavy chain V-III region TUR
26	1	20.93	15	Ig heavy chain V-III region TIL
27	1	20.21	3	AMBP protein precursor [Contains: Alpha-1-microglobulin

Table S6. Identification of proteins from human serum digested by the nanoflower-based microreactor (stored at -20 ℃ for 20 days) (n=2)

Group (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Protein Name
1	79	1430.76	91	Serum albumin precursor
2	18	310.22	40	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain]
2	7	123.19	20	Haptoglobin-related protein precursor
3	15	247.24	21	Serotransferrin precursor
4	15	223.85	43	Apolipoprotein A-I precursor
5	11	200.88	41	Ig alpha-1 chain C region
5	7	123.28	25	Ig alpha-2 chain C region
6	11	186.75	94	Ig kappa chain C region
7	12	176.56	32	Alpha-1-antitrypsin precursor
8	11	168.9	9	Alpha-2-macroglobulin precursor
9	10	167.48	37	Ig gamma-1 chain C region
9	9	146.98	30	Ig gamma-2 chain C region
9	6	83.14	15	Ig gamma-3 chain C region
9	5	78.88	15	Ig gamma-4 chain C region
10	7	125.66	86	Ig lambda chain C regions
11	7	111.05	4	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complemen...
12	3	52.65	9	Hemopexin precursor
13	4	51	44	Apolipoprotein A-II precursor
14	2	42.08	11	Alpha-1-acid glycoprotein 1 precursor

14	1	16.09	4	Alpha-1-acid glycoprotein 2 precursor
15	2	34.77	27	Serum amyloid A protein precursor
16	2	31.67	1	Complement C4-B precursor
16	2	31.67	1	Complement C4-A precursor
17	2	30.89	21	Ig kappa chain V-III region GOL
17	2	30.89	21	Ig kappa chain V-III region Ti
17	2	30.89	21	Ig kappa chain V-III region SIE
17	2	30.89	21	Ig kappa chain V-III region WOL
18	2	29.39	3	Complement factor B precursor
19	2	26.61	7	Alpha-2-HS-glycoprotein precursor
20	2	24.9	4	Alpha-1-antichymotrypsin precursor
21	2	23.96	3	Alpha-1B-glycoprotein precursor
22	2	23.15	13	Ig kappa chain V-III region VG precursor
23	2	21.9	11	Ig kappa chain V-III region CLL precursor
23	2	21.9	13	Ig kappa chain V-III region POM
23	2	21.9	15	Ig kappa chain V-III region NG9 precursor
23	1	11.23	7	Ig kappa chain V-III region VH precursor
23	1	11.23	8	Ig kappa chain V-I region Lay
24	1	21.53	15	Ig heavy chain V-III region BRO
24	1	21.53	15	Ig heavy chain V-III region TEI
25	1	20.5	15	Ig heavy chain V-III region WAS
25	1	20.5	15	Ig heavy chain V-III region POM
25	1	20.5	15	Ig heavy chain V-III region TUR
25	1	20.5	15	Ig heavy chain V-III region TIL
26	1	20.24	14	Ig kappa chain V-IV region Len

Table S7. Identification of proteins from human serum digested by the free trypsin (n=2)

Group (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Protein Name
1	61	1132.43	84	Serum albumin precursor
2	17	280	40	Alpha-1-antitrypsin precursor
3	14	257.81	29	Serotransferrin precursor
4	16	256.33	13	Alpha-2-macroglobulin precursor
4	2	29.64	1	Pregnancy zone protein precursor
5	14	251.89	58	Ig gamma-1 chain C region
5	11	178.61	48	Ig gamma-2 chain C region
5	7	110.8	20	Ig gamma-4 chain C region
5	7	102.61	29	Ig gamma-3 chain C region
6	14	244.37	58	Apolipoprotein A-I precursor
7	13	225.88	43	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain]
7	6	106.59	20	Haptoglobin-related protein precursor
8	14	218.74	10	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha

				chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complemen...
9	11	182.6	41	Ig alpha-1 chain C region
9	8	131.9	32	Ig alpha-2 chain C region
10	8	161.37	87	Ig kappa chain C region
11	5	96.1	75	Ig lambda chain C regions
12	5	73.66	14	Alpha-1-antichymotrypsin precursor
13	4	71.72	16	Hemopexin precursor
14	4	66.79	23	Alpha-1-acid glycoprotein 1 precursor
14	1	14.7	4	Alpha-1-acid glycoprotein 2 precursor
15	4	49.1	68	Apolipoprotein A-II precursor
16	2	33.47	2	Complement C4-B precursor
16	2	33.47	2	Complement C4-A precursor
17	2	32.52	19	Ig heavy chain V-III region BRO
17	2	32.52	19	Ig heavy chain V-III region TEI
18	3	29.49	22	Ig kappa chain V-III region NG9 precursor
18	2	21.94	11	Ig kappa chain V-III region CLL precursor
18	2	21.94	13	Ig kappa chain V-III region POM
18	1	13.51	7	Ig kappa chain V-III region VH precursor
18	1	13.51	8	Ig kappa chain V-I region Lay
19	2	26.29	15	Ig heavy chain V-III region KOL
20	2	25.8	19	Ig heavy chain V-III region WAS
20	2	25.8	19	Ig heavy chain V-III region POM
20	2	25.8	19	Ig heavy chain V-III

				region TUR
20	2	25.8	20	Ig heavy chain V-III region TIL
21	2	21.04	7	Alpha-1B-glycoprotein precursor