Supporting Information

Experimental Section

Preparation of Fe_3O_4 and SBA-15

Water-stable Fe_3O_4 was synthesized by a microwave-hydrothermal route in a microwave accelerated reaction system MARS-5 (CEM, USA). Briefly, 1g precursor $FeCl_3 \cdot 6H_2O$, 2g anhydrous sodium acetate and 6.5g 1,6-hexanediamine were mixed together into 30ml ethylene glycol to form transparent solution. Then, the mixture was transferred into the microwave system and treated at 200℃ for 30min. After washing with ethanol and water for three times, the prepared Fe_3O_4 was stored in water for use.

SBA-15 was prepared by dissolving 3g Pluronic P123 and 8.5g tetraethoxysilane (TEOS) in 120ml 1.5M HCl aqueous solution, with stirring at 35℃ for 20h. Subsequently, the solution was moved into autoclave and aged at 120℃ for 24h. The product was washed by water and ethanol, and dried at room temperature in air. The as-synthesized SBA-15 sample was calcined by slowly increasing temperature from ambient temperature to 500℃ for 8h and heating at 500℃ for 6h.

Synthesis of MMS hybrid Fe_3O_4-SBA-15and trypsin immobilization

3mg of obtained SBA-15 was dispersed in 1ml 50mM Tris-HCl buffer solution (pH=6.5) containing 0.3mg trypsin, and incubated in shaker at 25℃ for 6h. After that, the solids were separated from the mixture by centrifugation. To remove the enzyme leaching from the materials, the separated precipitate was resuspended in 10mM NH_4HCO_3 buffer solution (pH=8.0) at 37℃ for 2h, and the materials were collected by centrifugation. This procedure was repeated several times until no UV absorbance detected at λ=280nm in the supernatant. If trypsin was not introduced, the above procedure could be omitted.

400μl of Fe_3O_4 aqueous suspension (5mg/ml) was dispersed into 0.5% PDDA solution, and then shaken in a vortex for 30min. After magnetic separation, PDDA functionalized Fe_3O_4 was redispersed into the suspension which contained 3mg of SBA-15 (encapsulated with trypsin or not), and shaken in a vortex for another 30min. Finally, products were isolated by a magnet and stored in 10mM NH_4HCO_3 buffer solution.

Construction of the glass microfluidic devices

Photomask patterns used in these experiments were designed on the computer by Adobe Illustrator software. Standard photolithographic and wet chemical etching techniques were carried out as described in an established method[1]. In details, designed patterns were transferred onto a 1.7-mm thick 30×60-mm glass plate with chromium and photoresist coating (Shaoguang Microelectronics Corp., Changsha, China) by UV exposure. Afterward, S-shaped part was etched into the plate in $HF/NH_4F/HNO_3$ (2:1:1) bath at 37℃ in shaker (50r/min) for 1h (60μm depth, 200μm width) (Fig. S8a), and linear part was kept in the etching liquor for a longer time until the channel is deep enough (450μm depth, 1140μm width) for insertion of capillary tube (OD=360μm) (Fig. S8b). Ultimately, the cover and etched plates were irreversibly bonded by programmable heating to

620℃.

Two capillary tubes were inserted into both sides of the chip, and the interface between the glass chip and capillary tube was sealed by methacrylate resin. By measuring the volume of water filled in it, we obtained the dead volume (6μl) of this microfluidic device.

Immobilization of MMS in the microchip was achieved by placing a permanent magnet on the side of S-shaped channel. The hybrid material dispersion was injected into microchip by nitrogen pressure until the first two loops turned black.

On-chip digestion of model proteins

To evaluate the size-selective proteolysis effect, we utilized Cyt *c* as low-MW proteins, and BSA as a high-MW protein. Cyt *c* was coupled with BSA at the same molarity of 5pmol/μl in 10mM NH_4HCO_3 (pH=8.0) buffer solution to form a protein mixture. At first, the microchip is full of 10mM NH_4HCO_3 buffer solution in an incubater at 37℃. 20μl of the mixture was driven through the constructed microfluidic reactor by a KDS410 syringe pump (KD Scientific Inc., USA) at a flow rate of 1μl/min. Next, 10mM NH_4HCO_3 buffer solution was injected into the channel at 1μl/min for 6min to flush the protein solution out of the reactor. Until now, the solution pumped out at the outlet was denoted as early fraction. Afterward, 20μl buffer solution was used to elute the packed bed at 2μl/min, and thus the eluate was denoted as later fraction. The same procedure was performed for lysozyme and myoglobin.

Online digestion and analysis of human serum

Human serum employed as a real sample was collected from 100 healthy adults in Nanjing University Hospital according to their standard clinical procedures. After collection, the serum samples were stored in -80℃ refrigerator before usage. Without any denaturation procedures, the crude serum was diluted to 1ml by 10mM NH_4HCO_3 (pH=8.0) buffer solution, to reach the protein concentration of circa 0.1mg/ml.

Sample injection was performed by Finnigan Micro AS, with sample loop set as 20μl. As illustrated in Scheme S2, PEEK tube generated from Micro AS (Thermo Scientific, San Jose, CA, USA) was connected to the microreactor. The mobile phase of sample pump consisted of mobile phase A, 10mM NH_4HCO_3 (pH=8.0) buffer solution, and mobile phase B, H_2O. The mobile phase of MS pump consisted of mobile phase A, 0.1% formic acid in H_2O, and mobile phase B, 0.1% formic acid in ACN.

First, the 10-port valve was set as load position. Considering the dead volume of the PEEK tube and microchip, protein sample was pumped through the microfluidic reactor by 100% mobile phase A of sample pump at 1μl/min for 30min. High-MW proteins were driven out to waste, while low-MW ones entrapped onto the microreactor for proteolysis. Switching the valve, 100% mobile phase A was used to elute the peptides derived in the microreactor onto trap column at 2μl/min for 10min. After switching the valve back, peptides on trap column was eluted to separation column and then ion source for ionization by 90min linear gradient elution from 5% to 35% (mobile phase B of MS pump).

Mass spectrometry and data analysis

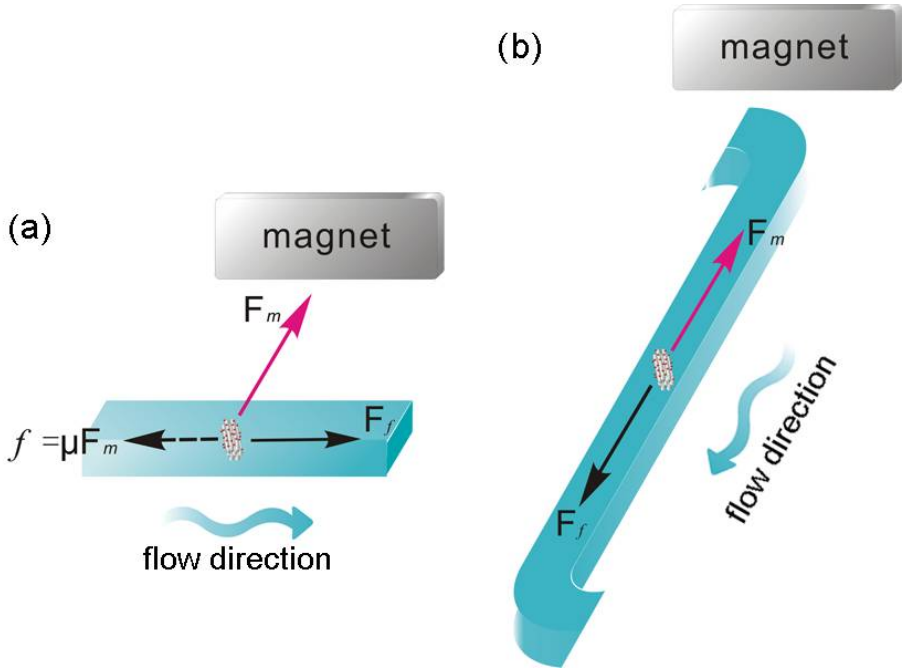
MALDI-TOF MS experiments were performed on a Bruker Autoflex II time-of-flight mass

spectrometer (Bruker, Bremen, Germany), equipped with a delayed ion-extraction device and a pulsed nitrogen laser operated at 337nm. The range of laser energy was adjusted slightly to obtain good resolution and S/N. The instrument was operated in the positive ion reflector mode for peptide analysis and linear mode for protein identification. The MALDI uses a ground-steel sample target with 384 spots. Each spectrum was summed with 100 laser shots.

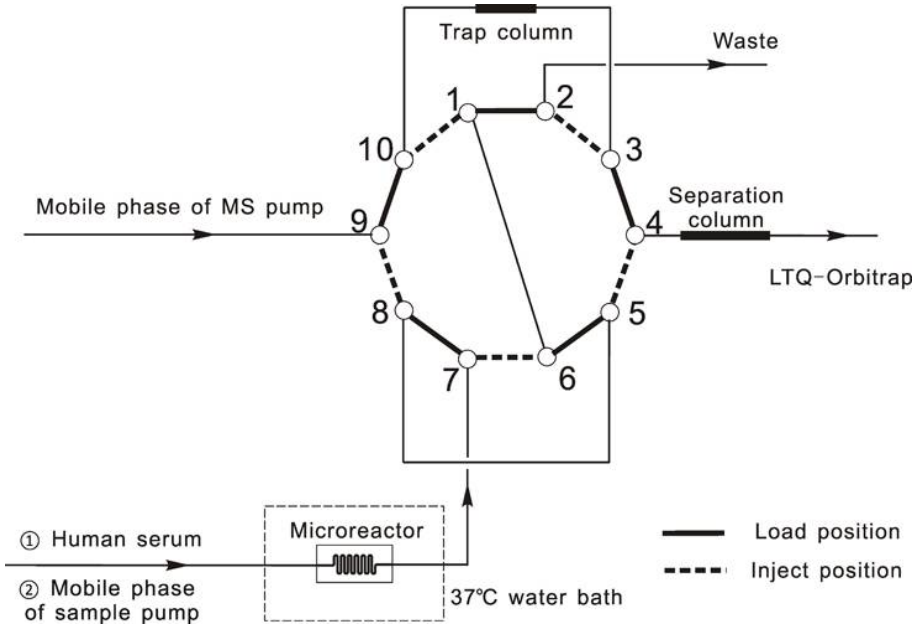
Nano-LC-MS/MS was performed on an LTQ-Orbitrap mass spectrometer equipped with a Finnigan surveyor MS pump (Thermo Scientific, San Jose, CA, USA). The MS pump flow rate was split by a PEEK Tee to achieve 200nl/min. The LTQ-Orbitrap instrument was operated in positive ion mode. A voltage of 1.9kV was applied to the Micro Tee before the C_{18} capillary column. The mass spectrometer was set as one full MS scan followed by six MS/MS scans on the six highest peaks.

The MS/MS spectra acquired from human serum were submitted to SEQUEST (Thermo Proteome Discoverer 1.0) for searching against a complex database containing original and reversed human protein database of International Protein Index (ipi.human.3.17.fasta). The database searching parameters were set as follows. Trypsin was selected as the enzyme, with KR/P as the cleavage site. Enzyme limits was set fully enzymatic, cleaving at both ends. The tolerance of missed cleavage was 2. Oxidation of methionine was set as dynamic modification. The database searching results of human serum samples were filtered with the target false detection rate (FDR) of 1%.

[1] Q. Fang, G. M. Xu, Z. L. Fang, *Anal. Chem.* **2002**, *74*, 1223-1231.



Scheme S1 Force analysis of MMS particle in (a) linear and (b) S-shaped channel. F_m, magnetic force; f, static friction; μ, static friction coefficient; F_f, force generated from the fluid.



Scheme S2 Scheme of integrated system for online digestion, separation and analysis of human serum sample.

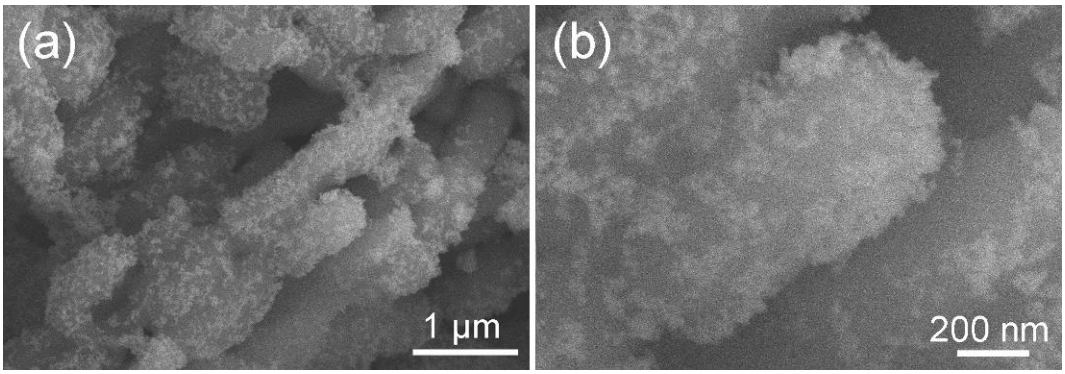


Fig. S1 SEM images of Fe_3O_4-SBA-15 hybrid.

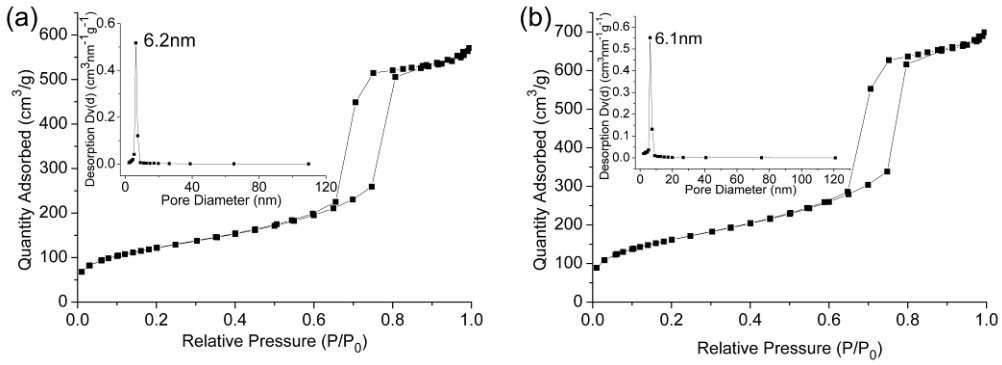


Fig. S2 Nitrogen adsorption-desorption isotherm and (inset) pore size distribution of (a) Fe_3O_4-SBA-15 and (b) SBA-15.

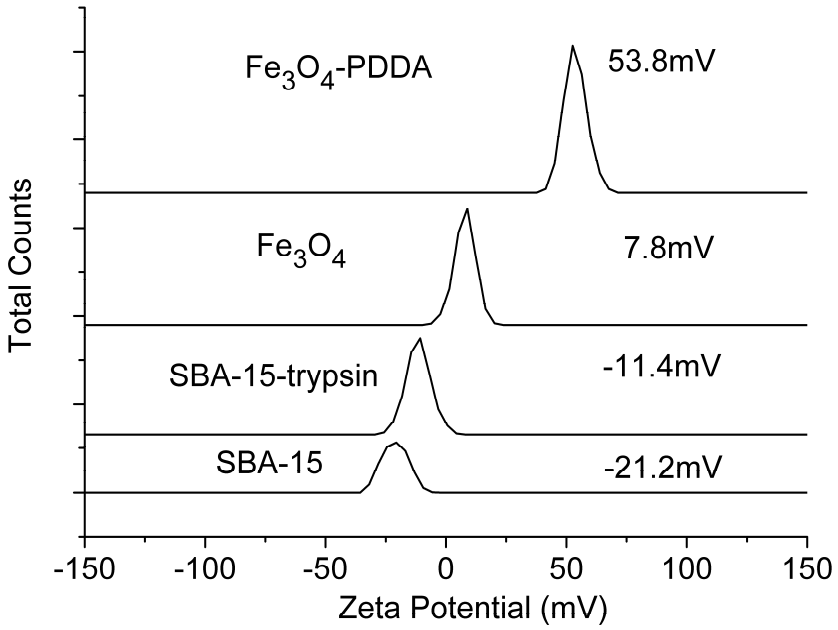


Fig. S3 Zeta potential distribution of Fe_3O4, Fe_3O_4-PDDA, SBA-15 and SBA-15-trypsin.

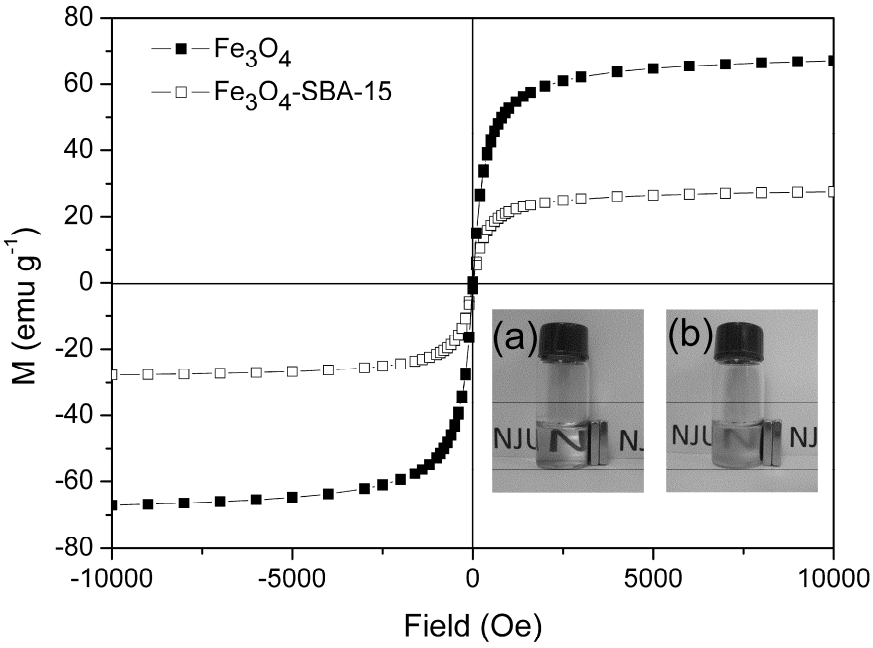


Fig. S4 Room-temperature magnetization curves of Fe_3O_4 and Fe_3O_4-SBA-15 hybrid. Insets show the magnetic separation behavior of Fe_3O_4-SBA-15 hybrid in the (a) presence and (b) absence of PDDA. The text "NJU" is used to demonstrate the transparency of liquid phase.

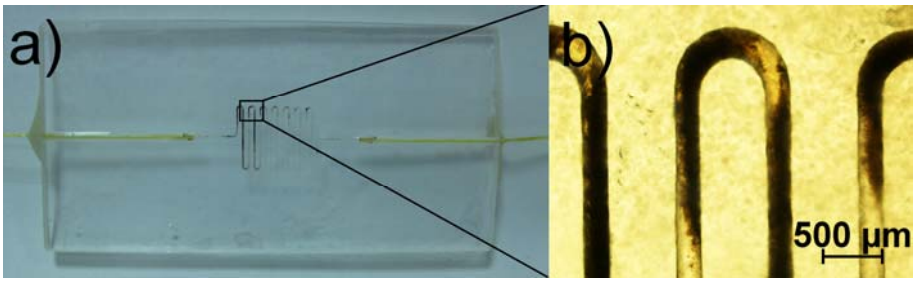


Fig. S5 (a) Photograph of S-shaped microfluidic device packed with trypsin-encapsulated MMS; (b) picture of the first three channel bends at high magnification

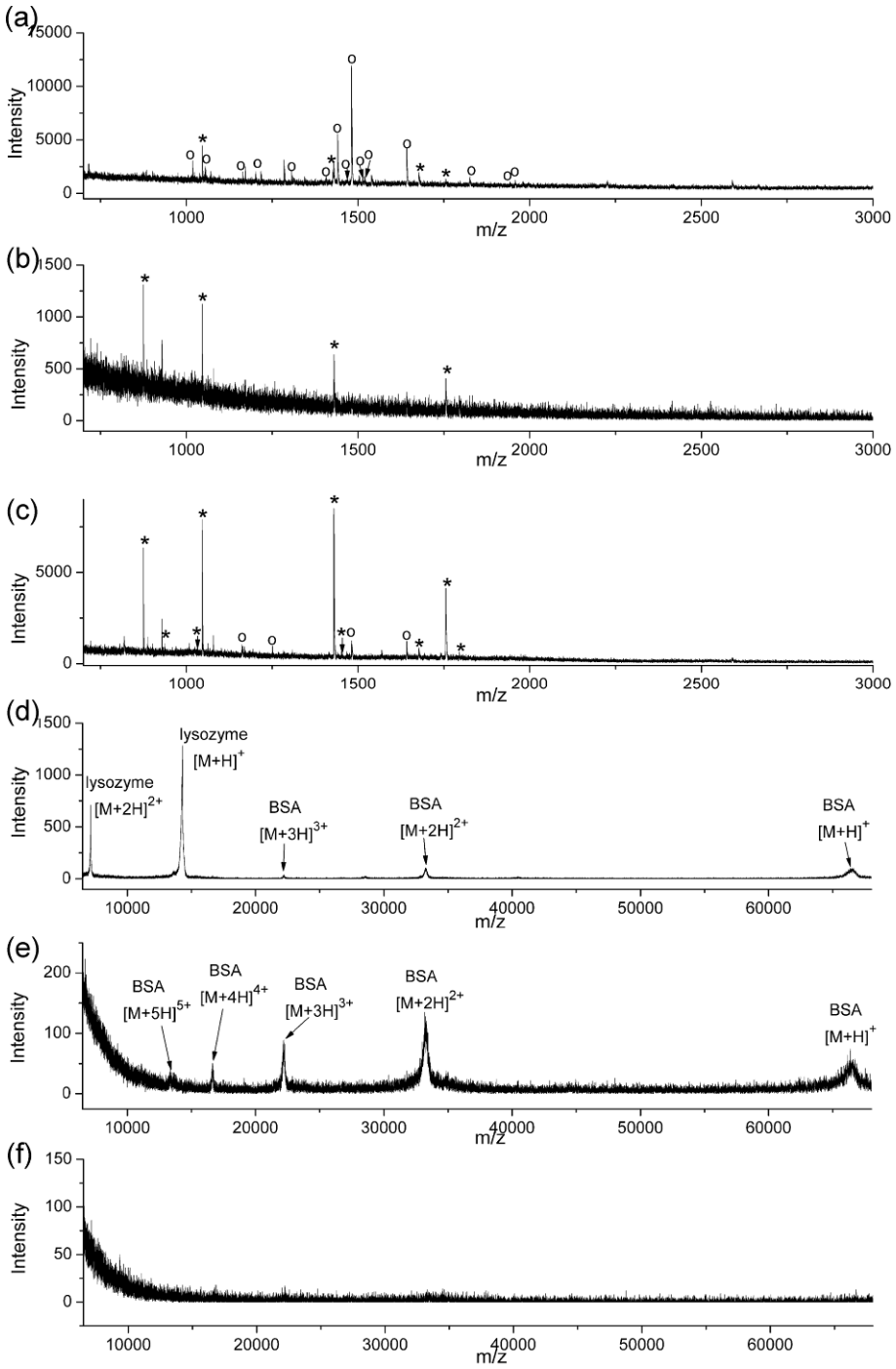


Fig. S6 MALDI-TOF spectra of peptides from mixture of lysozyme and BSA by (a) in-solution digestion and (b,c) on-chip digestion (b for the early fraction; c for the later fraction). MALDI-TOF spectra of proteins from mixture of lysozyme and BSA (d) before and (e,f) after on-chip digestion (e for the early fraction; f for the later fraction). Peptide peaks at relatively high S/N are labeled with asterisk and circle. Asterisk is assigned to peptides derived from lysozyme; circle is assigned to peptides from digest of BSA.

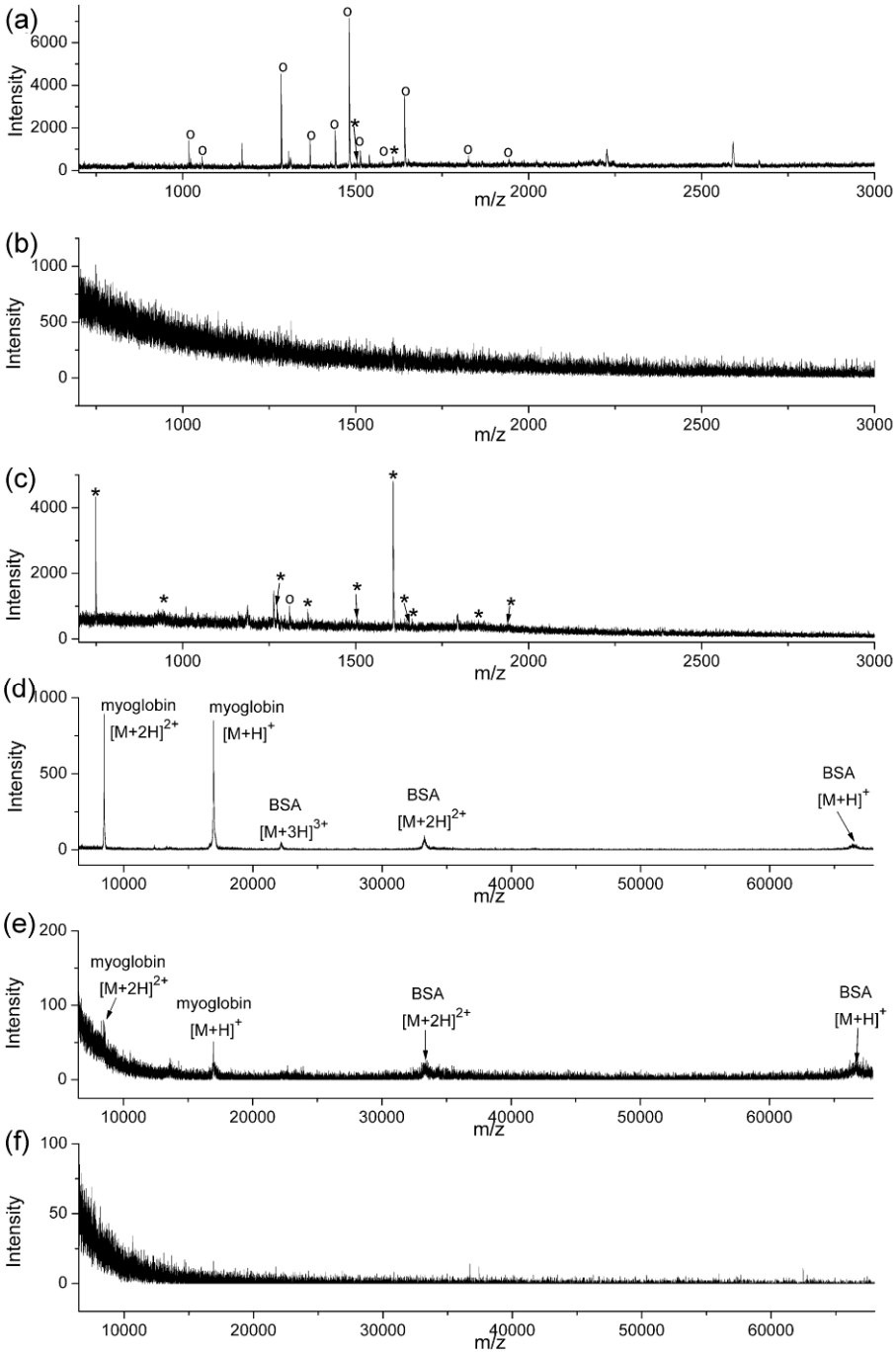


Fig. S7 MALDI-TOF spectra of peptides from mixture of myoglobin and BSA by (a) in-solution digestion and (b,c) on-chip digestion (b for the early fraction; c for the later fraction). MALDI-TOF spectra of proteins from mixture of myoglobin and BSA (d) before and (e,f) after on-chip digestion (e for the early fraction; f for the later fraction). Peptide peaks at relatively high S/N are labeled with asterisk and circle. Asterisk is assigned to peptides derived from myoglobin; circle is assigned to peptides from digest of BSA.

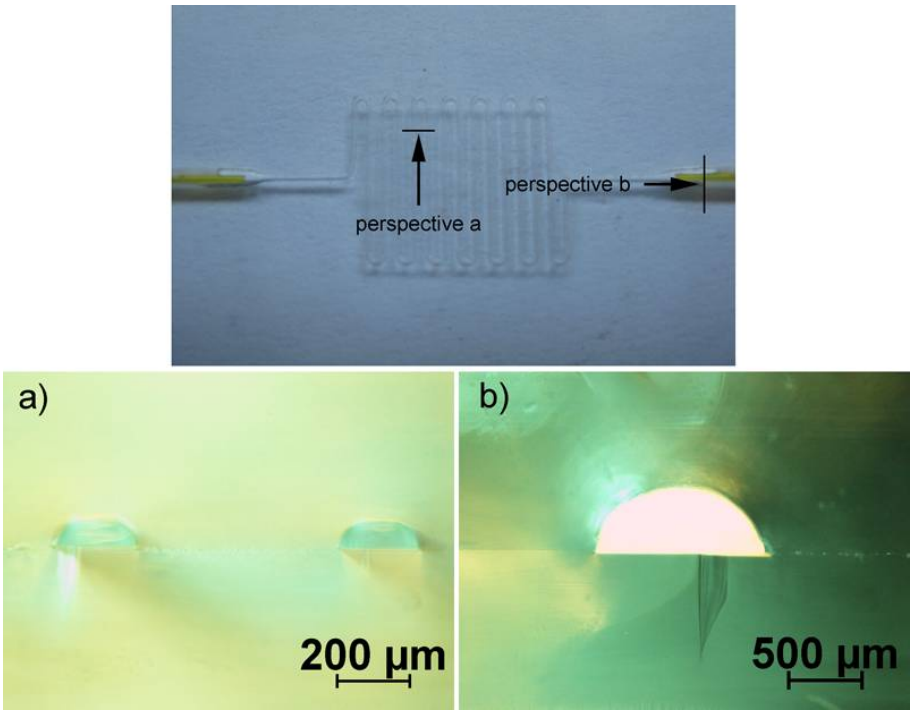


Fig. S8 Profile map of (a) S-shaped and (b) linear part of glass microfluidic chip.

Table S1 Peptide fragments observed in MALDI-TOF spectra in the protein mixture of Cyt *c* and BSA matched with the peptide mass in UniProtKB database.

Cyt *c*

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
2141.1106	10-28	3	IFVQKCAQCHTVEKGGKHK		√	
1675.9139	24-39	2	GGKHKTGPNLHGLFGR		√	√
1633.8189	10-23	1	IFVQKCAQCHTVEK		√	√
1606.9162	89-101	3	KTEREDLIAYLKK		√	
1598.7809	40-54	1	KTGQAPGFTYTDANK		√	
1495.6985	62-73	0	EETLMEYLENPK		√	
1478.8213	90-101	2	TEREDLIAYLKK		√	
1470.6859	41-54	0	TGQAPGFTYTDANK		√	
1433.7760	27-39	1	HKTGPNLHGLFGR	√	√	
1421.8297	81-92	3	MIFAGIKKKTER		√	
1168.6221	29-39	0	TGPNLHGLFGR	√	√	√
964.5349	93-100	0	EDLIAYLK		√	
762.4872	9-14	1	KIFVQK		√	

BSA

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
2060.1498	434-451	2	YTRKVPQVSTPTLVEVSR			√
2034.0575	588-607	1	EACFAVEGPKLVVSTQTALA			√
1994.0045	490-507	2	TPVSEKVTKCCTESLVNR			√
1823.8996	508-523	0	RPCFSALTPDETYVPK			√
1639.9377	437-451	1	KVPQVSTPTLVEVSR	√		√
1633.6621	184-197	0	YNGVFQECCQAEDK			√
1633.0159	548-561	2	KQTALVELLKHKPK			√
1578.5981	267-280	0	ECCHGDLLECADDR			√
1519.7461	139-151	0	LKPDPNTLCDEFK			√
1511.9519	545-557	2	QIKKQTALVELLK			√
1511.8427	438-451	0	VPQVSTPTLVEVSR			√
1490.8226	229-241	2	FGERALKAWSVAR			√
1479.7954	421-433	0	LGEYGFQNALIVR	√		√
1450.7835	223-235	2	CASIQKFGERALK			√
1439.8117	360-371	1	RHPEYAVSVLLR			√
1407.7525	221-232	2	LRCASIQKFGER			√
1386.6206	286-297	0	YICDNQDTISSK			√
1331.7174	198-209	1	GACLLPKIETMR			√
1305.7161	402-412	0	HLVDEPQNLIK	√	√	√
1283.7106	361-371	0	HPEYAVSVLLR			√
1202.6786	219-228	2	QRLRCASIQK			√
1193.6021	25-34	1	DTHKSEIAHR		√	
1177.5591	300-309	0	ECCDKPLLEK			√
1145.6425	236-245	1	AWSVARLSQK			√
1052.4499	460-468	0	CCTKPESER			√
1024.4550	499-507	0	CCTESLVNR			√
1015.4877	310-318	0	SHCIAEVEK			√
1002.5830	598-607	0	LVVSTQTALA			√
977.4509	123-130	0	NECFLSHK			√
906.4713	205-211	1	IETMREK			√

* Number of missed cleavage.

Table S2 Peptide fragments observed in MALDI-TOF spectra in the protein mixture of lysozyme and BSA matched with the peptide mass in UniProtKB database.

lysozyme

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
1803.8959	115-130	1	KIVSDGNGMNAWVAWR		√	
1753.8351	64-79	0	NTDGSTDYGILQINSR	√	√	√
1675.8009	116-130	0	IVSDGNGMNAWVAWR		√	√
1451.7609	20-32	2	VFGRCELAAAMKR		√	
1428.6502	52-63	0	FESNFNTQATNR	√	√	√
1045.5425	135-143	0	GTDVQAWIR	√	√	√
1030.5177	32-39	1	RHGLDNYR		√	
936.3781	80-86	0	WWCNDGR		√	
874.4166	33-39	0	HGLDNYR	√	√	√

BSA

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
1994.0045	490-507	2	TPVSEKVTKCCTESLVNR			√
1955.9596	319-336	0	DAIPENLPPLTADFAEDK			√
1823.8996	508-523	0	RPCFSALTPDETYVPK			√
1738.8105	387-401	1	DDPHACYSTVFDKLK			√
1700.7869	372-386	1	LAKEYEATLEECCAK			√
1639.9377	437-451	1	KVPQVSTPTLVEVSR	√	√	√
1517.8587	236-248	2	AWSVARLSQKFPK			√
1511.9519	545-557	2	QIKKQTALVELLK			√
1511.8427	438-451	0	VPQVSTPTLVEVSR			√
1479.7954	421-433	0	LGEYGFQNALIVR	√	√	√
1439.8117	360-371	1	RHPEYAVSVLLR			√
1362.6722	89-100	0	SLHTLFGDELCK			√
1305.7161	402-412	0	HLVDEPQNLIK			√
1283.7106	361-371	0	HPEYAVSVLLR			√
1249.6211	35-44	1	FKDLGEEHFK		√	
1202.6786	219-228	2	QRLRCASIQK			√
1163.6306	66-75	0	LVNELTEFAK		√	√
1052.4499	460-468	0	CCTKPESER			√
1015.4877	310-318	0	SHCIAEVEK			√
1002.5830	598-607	0	LVVSTQTALA			√
974.4577	37-44	0	DLGEEHFK			√
927.4934	161-167	0	YLYEIAR	√	√	√

* Number of missed cleavage.

Table S3 Peptide fragments observed in MALDI-TOF spectra in the protein mixture of myoglobin and BSA matched with the peptide mass in UniProtKB database.

myoglobin

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
1937.0167	33-48	2	LFTGHPETLEKFDKFK		√	
1853.9616	81-97	0	GHHEAELKPLAQSHATK		√	
1661.8533	33-46	1	LFTGHPETLEKFDK		√	
1651.9166	135-148	2	ALELFRNDIAAKYK		√	
1606.8547	18-32	0	VEADIAGHGQEVLIR		√	√
1502.6692	120-134	0	HPGDFGADAQGAMTK		√	√
1360.7583	135-146	1	ALELFRNDIAAK		√	
1271.6630	33-43	0	LFTGHPETLEK		√	
941.4727	147-154	1	YKELGFQG		√	
748.4352	135-140	0	ALELFR		√	

BSA

Mass	Position	MC*	Peptide sequence	On-chip digestion (early fraction)	On-chip digestion (later fraction)	In-solution digestion
2060.1498	434-451	2	YTRKVPQVSTPTLVEVSR			√
2045.0279	168-183	1	RHPYFYAPELLYYANK			√
1942.9769	29-44	2	SEIAHRFKDLGEEHFK			√
1942.8204	264-280	1	VHKECCHGDLLECADDR			√
1823.8996	508-523	0	RPCFSALTPDETYVPK			√
1639.9377	437-451	1	KVPQVSTPTLVEVSR		√	√
1578.5981	267-280	0	ECCHGDLLECADDR			√
1511.9519	545-557	2	QIKKQTALVELLK			√
1511.8427	438-451	0	VPQVSTPTLVEVSR			√
1504.9209	549-561	1	QTALVELLKHKPK			√
1490.8226	229-241	2	FGERALKAWSVAR			√
1479.7954	421-433	0	LGEYGFQNALIVR			√
1450.7835	223-235	2	CASIQKFGERALK			√
1439.8117	360-371	1	RHPEYAVSVLLR			√
1407.7525	221-232	2	LRCASIQKFGER			√
1364.4803	106-117	0	ETYGDMADCCEK			√
1308.7270	558-568	1	HKPKATEEQLK			√
1305.7161	402-412	0	HLVDEPQNLIK		√	√
1283.7106	361-371	0	HPEYAVSVLLR			√
1163.6306	66-75	0	LVNELTEFAK			√
1052.4499	460-468	0	CCTKPESER			√
1050.4924	588-597	0	EACFAVEGPK			√
1024.4550	499-507	0	CCTESLVNR			√
1015.4877	310-318	0	SHCIAEVEK			√
977.4509	123-130	0	NECFLSHK			√

* Number of missed cleavage.

Table S4 Comparison of spectral count generated from proteins identified in human serum by on-chip and in-solution digestion.

Protein	Molecular weight/Da	Spectral Count	
		On-chip digestion	In-solution digestion
Complement factor B	885532	0	14
Apolipoprotein B-100	515241	5	48
Zinc finger protein 292	304623	0	39
Histone acetyltransferase p300	263990	0	18
Fibronectin	221134	16	25
Sucrase-isomaltase, intestinal	209272	0	17
Complement component 4B preproprotein	192631	0	22
Complement C5	188186	0	20
Complement C3	187030	104	108
Alpha-2-macroglobulin	163175	15	71
Ceruloplasmin	122128	17	72
Inter-alpha-trypsin inhibitor heavy chain H2	106435	0	12
Inter-alpha inhibitor H4	99795	0	17
Zinc finger protein 347	95710	0	10
Serotransferrin	77000	0	97
Serum albumin	69321	48	1034
Complement component C9	63133	5	5
Histidine-rich glycoprotein	59541	0	67
Alpha 1B-glycoprotein	54239	0	7
Angiotensinogen	53121	0	4
Vitamin D-binding protein	52929	0	11
Antithrombin-III	52569	8	0
Hemopexin	51643	24	15
Alpha-1-antichymotrypsin	47621	12	0
Apolipoprotein A-IV	45371	68	8
Serum paraoxonase/arylesterase 1	39724	21	0
Alpha-2-HS-glycoprotein	39300	8	24
Apolipoprotein E	36132	25	8
Apolipoprotein F	35377	4	0
Alpha-1-antitrypsin	34734	28	9
Apolipoprotein A-I	30759	144	79
Cerberus	30064	6	4
Transmembrane gamma-carboxyglutamic acid protein	25859	12	0
Transthyretin	15877	72	0
Platelet basic protein	13885	22	0
Dermcidin	11277	13	0
Apolipoprotein C-II	11277	11	5

Apolipoprotein A-II	11168	5	11
Apolipoprotein C-III	10845	24	5
Apolipoprotein C-I	9326	4	0