Electronic Supplementary Information for

Nanoconfining affinity materials for pH-mediated protein capture/release

Qianjin Li, Xueying Tu, Jin Ye, Zijun Bie, Xiaodong Bi, and Zhen Liu*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P.R. China

Table of Contents

1. Experimental section

1.1. Reagents and materials

Ribonuclease A (RNase A), Cytochrome c (Cyt C), Myoglobin (Myo), Bovine serum albumin (BSA), human serum albumin (HSA), Apo-transferrin (Trans), β-lactoglobulin A (β-Lac A), hemoglobin (Hemo), albumin from chicken egg white (OVA), lysozyme (Lyz), β-casein (β-Cas), avidin (Avi), trypsin (Try), glycidyl methacrylate (GMA), γ-methacryloxypropyltrimethoxysilane (γ-MAPS) and HPLC grade acetonitrile (ACN) were purchased from Sigma (St. Louis, MO, USA). α-Fetoprotein (AFP) from the normal fetal blood, monoclonal antibodies of α-Fetoprotein (anti-AFP), prostate specific antigen (anti-PSA) and carcinoembryonic antigen (anti-CEA) from mouse were purchased from Shuangliu Zhenglong Biology and Chemistry Research Institute (Chengdu, China); Recombinant human erythropoietin (EPO) was purchased from the European Pharmacopoeia. *N*,*N*′-methylenebisacrylamide (MBAA), dodecanol, D-tryptophan (D-Trp) and L-tryptophan (L-Trp) were purchased from Alfa Aesar (Ward Hill, MA, USA). Tetramethoxysilane (TMOS), L-histidine (His), L-cysteine (Cys), L-lysine (Lys), DL-serine (Ser) and DL-proline (Pro) were purchased from Aladdin Industrial Corporation (Shanghai, China). Horseradish peroxidase (HRP) was purchased from Sinopharm Chemical Reagent (Shanghai, China). L-aspartic acid (Asp), L-glutamic acid (Glu), L-asparagine (Asn), L-glutamine (Gln), L-arginine (Arg), L-phenylalanine (Phe) and L-tyrosine (Tyr) were purchased from J&K Scientific (Shanghai, China). Dimethyl sulfoxide (DMSO) and 2,2-azobisisobutyronitrile (AIBN, recrystallized in methanol before use) were purchased from Shanghai Fourth Reagent & H.V. Chemicals (Shanghai, China). Poly (ethylene glycol) (PEG, M_n = 10 000) and acetic acid (HAc) were purchased from Nanjing Chemical Reagent (Nanjing, China). *N*-(β-Hydroxypropyl)ethylenediamine (HPEDA), vinyltrimethoxysilane (VTMS) and ethanolamine were purchased from Tokyo Chemical Industry (Shanghai, China). Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford, MA, USA). Other chemical reagents were of analytical grade. Fused-silica capillaries with 50, 150 and 250 μm I.D. and 375 μm O.D. were purchased from Yongnian Optic Fiber Plant (Hebei, China). Profinity™ epoxide resin microbead was produced by Bio-Rad Laboratories (Hercules, CA, USA). The mean particle size of the microbead is about 60 μm.

1.2. Instrumentation

Scanning electron microscopy (SEM) analyses were performed on a Hitachi FE-SEM S-4800 (Tokyo, Japan). Nitrogen adsorption-desorption measurements were conducted at 77 K on an ASAP2020 instrument (Micromeritics, Norcross, GA, USA). The FT-IR spectrum was acquired on a Thermo Nicolet iS10 FT-IR spectrometer (Waltham, MA, USA). UV absorbance data were acquired from a Shimadzu UV-3600 spectrophotometer (Kyoto, Japan). Elemental analysis was measured on Elementar Vario Micro (Hanau, German).

All capillary liquid chromatography (CLC) were performed on an UltiMate 3000 nano-liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an LPG-3x00 micropump, an FLM-3100 microflow manager (1:100 split ratio) which guarantees a constant flow rate, an VWD-3400 variable-wavelength UV-vis absorbance detector with a 3 nL flow cell for on-column detection and an WPS-3000 automatic sampler. Chromeleon software from Dionex was used for system operation and data acquisition and processing. The flow rate was set at 1 μL/min and the column temperature was set at 25 ºC in all experiments. The effective lengths of monolithic columns used in all CLC experiments were 25 cm unless other specifications.

Capillary electrophoresis (CE) experiments were conducted on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a diode array detector (DAD). A fused-silica capillary of 50 μm I.D. × 60 cm (50 cm to detector) was used in separation. All CE experiments were performed at 30 ºC under optimum voltage settings (typically 18 kV). Prior to each run, the capillary was rinsed with 0.1 M NaOH (2 min) and running buffer (2 min) in sequence at 20 psi. Samples were injected under pressure at 0.5 psi for 5 s.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) experiments were implemented on an Autoflex mass spectrometer (Brucker Daltonics, Germany). Positive ion spectra were recorded using stainless steel target plate with standard parameter: a nitrogen laser (λ = 337 nm), 250 ns pulse duration and 20 kV for accelerating voltage. CHCA (10 mg/mL) and SA (10 mg/mL) dissolved in the mixture of 0.1% trifluoroacetic acid solution: ACN (70: 30, v/v) were used as the matrix for peptides (M_w < 6000 Da) and proteins (M_w > 8000 Da) detection, respectively. First, 0.5 μL sample was spotted on the plate and allowed to dry. Then, 0.5 μL matrix solution was added on the sample spot and allowed to dry. Each spectrum was typically summed with 100 laser shots.

1.3. Preparation of no-ligand-modified monolithic material

No-ligand-modified organic monolithic material (poly(MBAA)) was synthesized by thermo-initiated free radical copolymerization. Prior to the preparation of the monolithic material, the capillary was pretreated with acid, alkali and γ-MAPS according to the procedure reported previously.[1] Briefly, a mixture of MBAA (43 mg), DMSO (140 mg), dodecanol (125 mg) and AIBN (1 mg) was vortexed for 5 min and sonicated for 30 min to obtain a homogeneous solution. The vinylized capillary was filled with the polymerization mixture, sealed with rubber at both ends and then submerged into a water bath at 75 ºC for 12 h. After the polymerization reaction, the resulting monolithic column was washed with methanol and acetonitrile successively to remove non-reacted residues. This poly(MBAA) monolithic material is designated as NL-NCAM.

1.4. Preparation of HPEDA-modified materials

1.4.1. Preparation of epoxy-functionalized materials

The preparation of epoxy-functionalized organic-silica hybrid monolithic material is illustrated in Fig. S1 (reactions i). Prior to the preparation of the hybrid monolithic material, the capillary was pretreated with acid and alkali according to the procedure reported previously.[2] The epoxy-functionalized hybrid monolithic material was prepared by one-pot process. Briefly, a prepolymerization mixture was first prepared by mixing HAc (0.01 M, 5 mL), PEG (540 mg), TMOS (1.8 mL) and VTMS (0.6 mL) for 1 h at ice bath to form a homogeneous solution. Then, GMA (38.4 mg) and AIBN (1 mg) were added into 0.5 mL prepolymerization mixture. After 15 min sonication, the mixture was injected into the pretreated capillary of 70 cm length with positive pressure. Both ends of the capillary were sealed with silicone rubber. The capillary was immersed into a water bath at 45 ºC for 12 h and then at 75 ºC for another 12 h. After the polymerization reaction, the resulting monolithic column was washed with water and acetonitrile successively to remove non-reacted residues. This epoxy-functionalized organic-silica hybrid monolithic material is designated as Epoxy-NNCM.

The synthesis route of epoxy-functionalized organic monolithic material (poly(MBAA-co-GMA)) has been previously reported and illustrated in Fig. S1 (reactions i).[1] This epoxy-functionalized organic monolithic material is designated as Epoxy-NCAM.

1.4.2. Preparation of HPEDA-modified monolithic materials

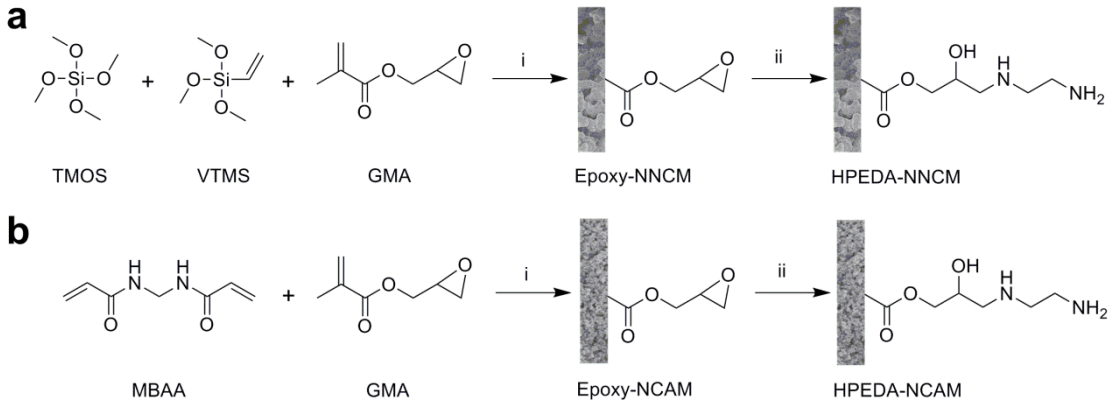

Fig. S1. a) Schematic of preparation of HPEDA-modified organic-silica hybrid monolithic material; b) Schematic of preparation of HPEDA-modified organic monolithic material.

The obtained Epoxy-NNCM and Epoxy-NCAM and the commercial Profinity™ epoxide resin microbead (designated as Epoxy-NCAMB) were then reacted with a solution of ethylenediamine/ACN (1:1, v/v) at 60 ºC for 12 h, respectively. After the epoxy opening reaction, the resulting materials were washed with acetonitrile thoroughly. Finally, the HPEDA-modified organic-silica monolithic material,

organic monolithic material and microbead were obtained and designated as HPEDA-NNCM, HPEDA-NCAM and HPEDA-NCAMB, respectively.

1.5. Preparation and post-modification of bulk materials

Bulk epoxy-functionalized monolithic materials were prepared in centrifuge tubes under the same preparation conditions as described in Section 1.4.1. A piece of bulk Epoxy-NNCM or Epoxy-NCAM (300 mg) was mixed with ethylenediamine/ACN (1:1, v/v), and the mixture was stirred at 60 ºC for 12 h. Then the mixture was filtrated and the solid was washed with methanol through a Soxhlet extractor for 12 h, followed by drying under vacuum at 60 ºC for 12 h. The resulting bulk HPEDA-modified organic-silica hybrid monolithic material and organic monolithic material were used for nitrogen adsorption-desorption measurements, FT-IR determination and element analysis.

1.6. Preparation of protein-functionalized NCAM monolithic columns

HSA-functionalized NCAM monolithic column was prepared as following: an HPEDA-NCAM column (150 μm I.D. × 60 cm) was first conditioned with the loading buffer (67 mM phosphate buffer, pH 7.4) for 60 min. Then 150 μL of HSA solution (1 mg/mL) was passed through the column under the nitrogen pressure. Thereafter, the column was washed with the loading buffer for 30 min in order to completely flush out the non-adsorbed proteins. The resulting HSA-functionalized NCAM monolithic column was used for the separation of chiral compounds.

Trypsin-functionalized NCAM monolithic column was prepared as following: an HPEDA-NCAM column (150 μm I.D. × 25 cm) was first conditioned with the loading buffer (50 mM ammonium bicarbonate, pH 8.5) for 60 min. Then 30 μL of trypsin solution (1 mg/mL) was passed through the column under the nitrogen pressure. Thereafter, the column was washed with the loading buffer for 30 min in order to completely flush out the non-adsorbed proteins. The trypsin-functionalized NCAM monolithic column was used as enzyme reactor for protein hydrolysis.

1.7. Determination of dissociation constant

1.7.1. Determination of dissociation constant (K_d) between HPEDA-NNCM and amino acids

The retention times of 13 representative amino acids (containing all types of natural amino acids) on HPEDA-NNCM column (150 μm I.D. × 50 cm) were determined. The flowing rate was set at 1.5 μL/min. All of the retention times reported here were the averages of three measurements. The K_d values were calculated according to the equation (**1**) reported in the literature:[3]

$$K_d = \frac{B_{max}}{V_t - V_0} \qquad (1)$$

where B_{max} stands for the total amount of ligands in the column, V_t is the elution volume of analyte and V_0 is the dead volume.

The total amount of ligands (B_{max}) in the column was estimated by element analysis method. The amounts of elements C, N and H in the material of Epoxy-NNCM were measured to be 30.71%, 0.04% and 5.06%, respectively; while those in the material of HPEDA-NNCM were measured to be 30.25%, 2.61% and 5.42%, respectively. From the contrast of the relative amounts of element N between Epoxy-NNCM and HPEDA-NNCM, the amount of the modified ethylenediamine in HPEDA-NNCM was calculated to be 5.52%; the HPEDA ligand density was thereby calculated to be 0.9 mmol/g. The monolith densities of HPEDA-NNCM was determined to be about 0.5 g/mL. The total amount of ligand HPEDA (B_{max}) in the monolithic column can be calculated by the multiplication of ligand density, monolith density and monolith volume.

Because the B_{max} value for poly(MBAA) monolithic column was not measurable, the binding strengths of the poly(MBAA) monolithic column toward amino acids were also not measureable.

1.7.2. Determination of K_d between HPEDA and proteins

Dissociation constants between HPEDA and proteins were determined according to the affinity capillary electrophoresis (ACE) method reported previously[4] with minor modifications. Briefly, the running buffers were prepared with 100 mM phosphate buffer (pH 7.0) and proteins of varied concentrations, and the samples contained 0.1% (v/v) DMSO (electroosmotic flow marker) and the HPEDA under investigation (10^{-5} M each). The electropherograms were recorded under a wavelength of 214 nm. Apparent mobility was calculated by the 32Karat software provided by Beckman Coulter. Thus, K_d can be calculated according to equation (**2**) on OriginPro8 software:

$$(v\mu_i - \mu_f) = (\mu_b - \mu_f)\frac{[L_f]^n}{K_d + [L_f]^n} \qquad (\mathbf{2})$$

where v is the viscosity correction factor to enhance the measurement accuracy, μ_i is the electrophoretic mobility of the target with the presence of ligand, μ_f is the electrophoretic mobility of free target, μ_b is the electrophoretic mobility of bound target, L_f is the concentration of the free ligand, K_d is the apparent dissociation constant, the binding stoichiometry between target and ligand is 1 : n. Because the solubility of MBAA in water is very poor, the binding constant between MBAA and proteins in free solution cannot be measured by the ACE method.

1.7.3. Determination of K_d between NCAMs and proteins

The monolithic column (250 μm I.D. × 7.5 or 20.0 cm) was first conditioned with 50 μL loading buffer (20 mM phosphate buffer, pH 7.0) under the nitrogen pressure. Then 110 μL of protein solution of

a certain concentration was passed through the column, which was collected and designated as solution **S**. Thereafter, the column was washed with 10 μL loading buffer in order to flush out the non-retentive components. Finally, the components bound on the column were eluted by 110 μL of 100 mM HAc (pH 2.7) solution and the collected solution is designated as solution **E**. After repeating above experiment with different protein concentrations, a series of solution **S** and solution **E** were obtained. Standard protein solutions of a series of concentrations were prepared using the loading buffer. The standard protein solutions, solutions **S** and **E** were subsequently subjected to UV absorbance measurement at the wavelength of 280 nm.

Dissociation constant and apparent maximum binding capacity (Q_{max}) were calculated according to the following Scatchard equation (**3**):[5]

$$\frac{Q_e}{C_s} = \frac{Q_{max}}{K_d} - \frac{Q_e}{K_d} \qquad (3)$$

where Q_{max} is the saturated adsorption capacity, Q_e is the amount of protein bound to the monolithic column at equilibrium, C_s is the free protein concentration at equilibrium. By plotting Q_e/C_s versus Q_e, the dissociation constant, K_d, and the apparent maximum binding capacity, Q_{max}, can be calculated from the slope and the intercept, respectively.

1.7.4. Determination of K_d between HPEDA-NCAMB and proteins

HPEDA-NCAMB (0.5-2.0 mg) were mixed with 1 mL protein solution (20 mM phosphate buffer, pH 7.0) in a 1.5 mL centrifuge tube, then the mixture was shocked to make the adsorption sufficient at room temperature. After centrifugation, the upper solution was collected and designated as solution **C**. Thereafter, the microbeads were washed three times using the phosphate buffer, once 1 mL. Finally, 100 μL of 100 mM HAc solution was used to elute out the protein adsorbed by the microbeads. After centrifugation, the elution solution was collected and designated as solution **D**. After repeating above experiment with different protein concentrations, a series of solutions **C** and solutions **D** were obtained. Standard protein solutions with a series of concentrations were prepared using the loading buffer. The standard protein solutions, solutions **C** and solutions **D** were subsequently subjected to UV absorbance measurement at the wavelength of 280 nm. Dissociation constant and apparent maximum binding capacity were calculated according to the Scatchard equation.

1.8. Binding proteins on HPEDA-NCAMB

HPEDA-NCAMB (5.0 mg) was mixed with 0.5 mg/mL protein solution (0.5 mg protein was dissolved in 1 mL 20 mM phosphate buffer, pH 7.0) in a 1.5 mL centrifuge tube, then the mixture was shocked for 1 h at room temperature. After centrifugation, the microbeads deposited. Then, the upper solution was depleted. Thereafter, the deposited microbeads were washed three times using the phosphate

buffer, once 1 mL. Finally, 120 μL of 100 mM HAc solution was added to elute out the protein adsorbed by the microbeads. The elution solution was collected for UV absorbance measurement at wavelength of 280 nm.

1.9. Protein hydrolysis on trypsin-functionalized monolithic column

The trypsin-functionalized monolithic column (150 μm I.D. × 10.0 cm) was first conditioned with 30 μL of 50 mM ammonium bicarbonate buffer with pH 8.5 under the nitrogen pressure. Then 2 μL HRP solution (1 mg/mL) was filled into the column. Thereafter, both ends of the column was sealed with silicone rubber and immersed into a water bath (37 ºC) for 2 h. Finally, the eluted components by 4 μL loading buffer from the column were collected, diluted by 3-fold trifluoroacetic acid solution (0.1%) and subjected to MALDI-TOF MS for peptide analysis.

2. pH-dependent interactions of MBAA and HPEDA with amino acids

Table S1. Retention times of amino acids on monoliths at different pH values

Amino acid	Retention time / min			
	HPEDA-modified monolith		poly(MBAA) monolith	
	pH 7.0	pH 2.7	pH 7.0	pH 2.7
Asp	4.87	-[a]	9.52	9.42
Glu	4.78	-	9.48	9.47
Ser	4.71	4.54	9.68	9.44
Cys	4.79	4.65	10.03	9.97
Asn	4.64	4.49	9.89	9.48
Gln	4.63	4.52	9.74	9.49
Arg	4.79	4.11	10.23	9.26
Lys	4.57	4.25	9.74	9.45
Phe	5.05	4.78	10.90	10.40
Tyr	5.33	4.95	11.39	11.37
Trp	6.46	5.71	14.12	11.54
His	4.83	4.06	10.27	8.65
Pro	4.68	4.58	10.02	9.37

[a] "-" means the signals of the amino acids were unobservable under the conditions used.

Table S2. Dissociation constants of amino acids on HPEDA-modified monolith

Amino acid	K_d (M)
Asp	3.26±0.13
Glu	3.67±0.48
Ser	4.06±0.24
Cys	3.63±0.59
Asn	4.53±0.34
Gln	4.66±0.48
Arg	3.61±0.27
Lys	5.16±0.63
Phe	2.68±0.19
Tyr	2.07±0.10
Trp	1.10±0.03
His	3.44±0.37
Pro	4.24±0.18

Because the interactions of HPEDA with amino acids were too low to be measurable by the ACE method, the expected properties of MBAA and HPEDA were evaluated in terms of the chromatographic retention of amino acids on a poly(MBAA) monolithic column and a HPEDA-modified organic-silica hybrid monolithic column. Since the molecular size of amino acids are too limited as compared with the pore sizes of the monolith, nanoconfinement effect can be ignored here. All amino acids exhibited stronger retention on these columns at high pH (7.0) than at low pH (2.7) (Table S1). Besides, the binding strengths of the HPEDA-modified monolith toward amino acids were rather weak, with dissociation constant (K_d) of 1-5 M (Table S2). These results verify the expected pH-dependent binding properties.

3. Interactions between proteins and HPEDA in free solution

Table S3. Dissociation constants between proteins and HPEDA in free solution

Protein	K_d (M)	Correlation coefficient	n[a]
β-Lac A	$(4.0\pm4.0)\times10^{-2}$	0.991	2.1±1.9
β-Cas	$(2.2\pm0.3)\times10^{-2}$	0.994	2.4±1.3
BSA	$(1.7\pm0.3)\times10^{-2}$	0.977	4.6±4.3

[a] Binding stoichiometry between protein and HPEDA is 1 : n

We evaluated the binding properties of HPEDA toward proteins in free solution by the ACE method. HPEDA exhibited weak binding strength with three representative proteins (K_d, ~10^{-2} M) (Table S3). As compared with the weaker affinity of HPEDA toward amino acids, the higher binding strength toward proteins is attributed to the cooperativity of multiple binding. Thus, if the density of HPEDA on a NCAM is adequate, multiple binding between HPEDA moieties and proteins may also occur on the NCAM, which can enhance the total affinity. Because the solubility of MBAA in water is very poor, the dissociation constant between MBAA and proteins in free solution cannot be measured by the ACE method.

4. FT-IR spectra of materials

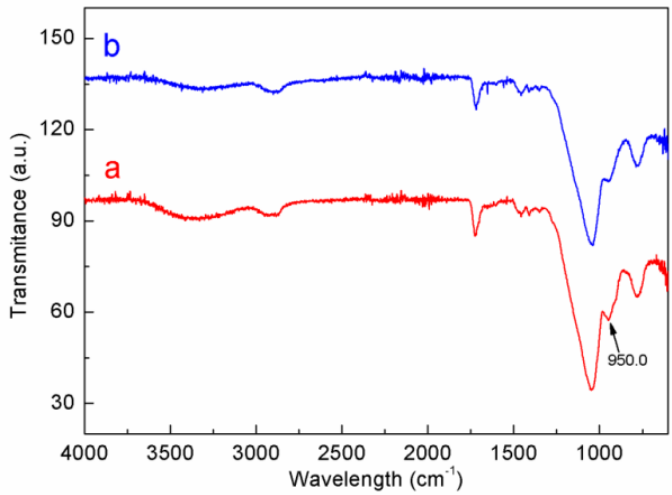

Fig. S2. a) FT-IR spectrum of Epoxy-NNCM; b) FT-IR spectrum of HPEDA-NNCM. In comparison with the peaks in trace **a**, the intensity of peak 950.0 cm^{-1} decreased in trace **b**, which can be an indicator for the successful reaction between ethylenediamine and epoxy groups on the surface of Epoxy-NNCM.

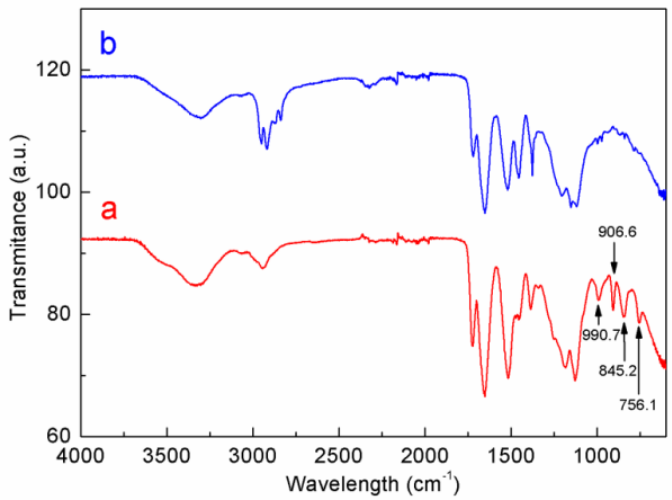

Fig. S3. a) FT-IR spectrum of Epoxy-NCAM; b) FT-IR spectrum of HPEDA-NCAM. In comparison with the peaks in trace **a**, the disappearance of peaks between 990.7 and 756.1 cm^{-1} in trace **b** can be an indicator for the successful reaction between ethylenediamine and epoxy groups on the surface of Epoxy-NCAM.

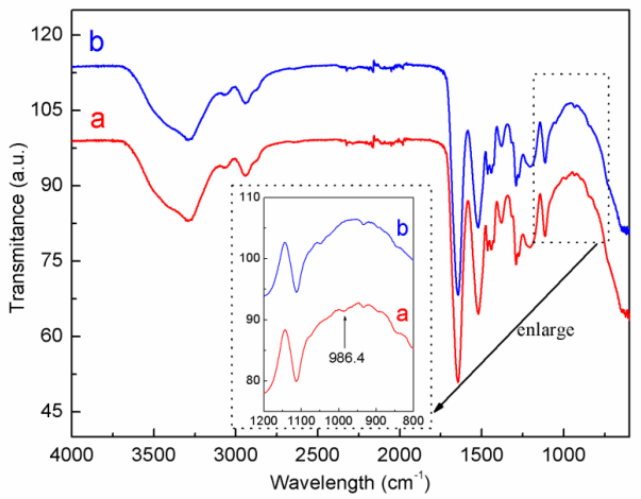

Fig. S4. a) FT-IR spectrum of Epoxy-NCAMB; b) FT-IR spectrum of HPEDA-NCAMB. In comparison with the peaks in trace **a**, the disappearance of the peak 986.4 cm^{-1} in trace **b** can be an indicator for the successful reaction between ethylenediamine and epoxy groups on the surface of Epoxy-NCAMB.

5. Material characteristics measured by nitrogen adsorption method

Table S4 Material characteristics measured by nitrogen adsorption method

Material	BET surface area (m^2/g)	Adsorption average pore width (nm)	BJH adsorption pore volume (cm^3/g)
NL-NCAM	28.7	3.3	0.029
HPEDA-NCAM	21.9	6.7	0.050
HPEDA-NNCM	55.8	2.6	0.029
HPEDA-NCAMB	116.5	9.6	0.450

6. Protein characteristics

Table S5. The properties of the test proteins

Name	M_w (kDa)	Dimension (nm × nm × nm)	pI
Cyt C	12.4	4.2×5.8×5.8[6]	10.0-10.5
RNase A	13.7	4.5×4.6×5.3[7]	9.6
Lyz	14.3	2.7×3.2×3.4[8]	11.4
Myo	17.6	3.1×3.5×6.5[9]	7.3, 6.8
β-Lac A	18.4	6.8×6.8×13.1[10]	5.1
Try	24.0	5.4×5.5×10.7[11]	10.1-10.5
β-Cas	24.0	unknown	4.6-5.1
EPO	34.0	7.3×8.0×13.5[12]	4.4-5.1
OVA	44.3	6.3×7.2×8.5[13]	4.5, 4.9
Hemo	64.5	5.3×5.3×19.2[14]	6.8
Avi	66.0	8.0×8.0×8.5[15]	10.0
BSA	66.4	4.5×14.3×21.2[16]	5.3
HSA	66.4	8.1×18.7×18.7[17]	4.6
AFP	67.3	unknown	4.7, 5.3
Trans	80.0	8.8×10.3×20.0[18]	5.2-6.2
IgG[a]	150	17.5×27.1×27.1[19]	7.5-7.9

[a] The properties of IgG can represent those for the antibodies anti-AFP, anti-PSA and anti-CEA.

7. Protein binding on HPEDA-NCAM

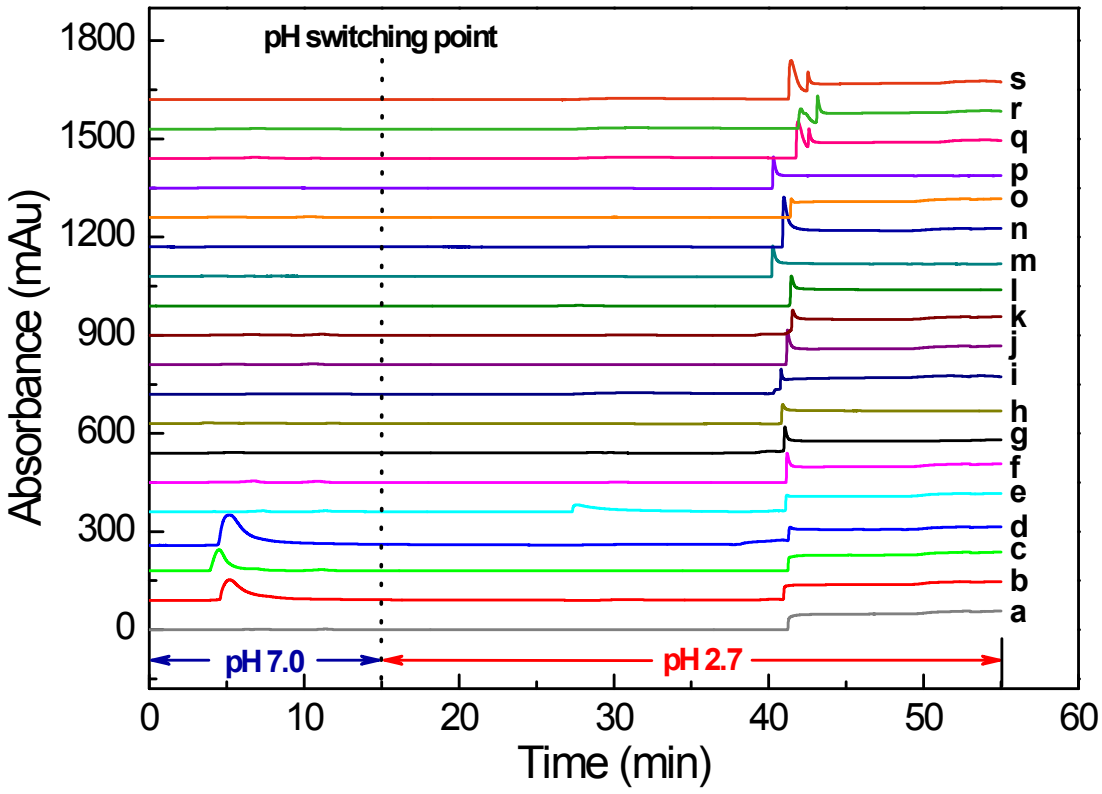

Fig. S5. Chromatographic retention of proteins on HPEDA-NCAM monolithic column. Mobile phase: 20 mM sodium phosphate buffer at pH 7.0, switched to 100 mM HAc (pH 2.7) at 15 min; Sample: 1.0 mg/mL protein dissolved in the loading buffer, blank (**a**), Cyt C (**b**), RNase A (**c**), Myo (**d**), Lyz (**e**), β-Lac A (**f**), Try (**g**), β-Cas (**h**), EPO (**i**), OVA (**j**), Hemo (**k**), Avi (**l**), BSA (**m**), HSA (**n**), AFP (**o**), Trans (**p**), anti-AFP (**q**), anti-CEA (**r**), anti-PSA (**s**); Sample injection volume: 600 nL. Proteins Cyt C (12.4 kDa, trace **b**), RNase A (13.7 kDa, trace **c**) and Myo (17.6 kDa, trace **d**) were not captured by the monolith HPEDA-NCAM. Protein Lyz (14.3 kDa, trace **e**) was captured loosely by HPEDA-NCAM through electrostatic interaction (Fig. S6). While proteins with molecular weight more than 18 kDa were all captured by HPEDA-NCAM.

8. Effect of NaCl on protein binding on HPEDA-NCAM

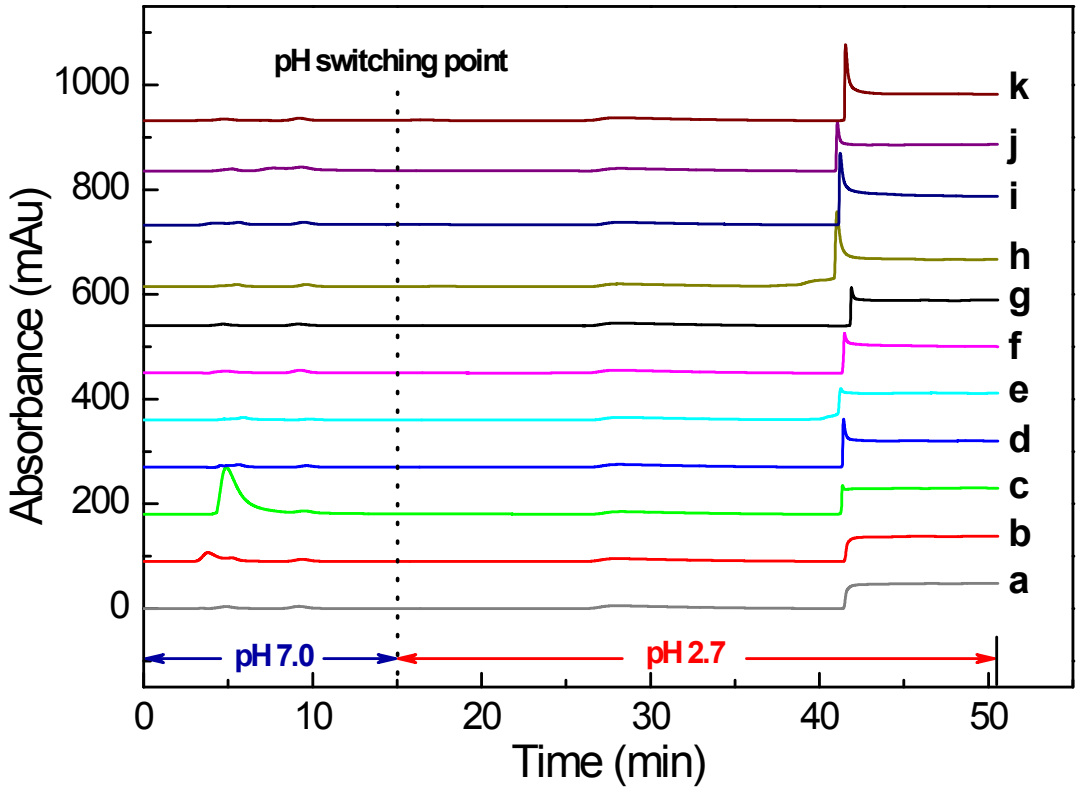

Fig. S6 Chromatographic retention of the effect of NaCl on the retention of proteins on HPEDA-NCAM monolithic column under salt-containing mobile phase. Mobile phase: 20 mM sodium phosphate with 150 mM NaCl buffer at pH 7.0, switched to 100 mM HAc (pH 2.7) at 15 min; Sample: 1.0 mg/mL protein dissolved in the loading buffer, blank (**a**), RNase A (**b**), Lyz (**c**), β-Lac A (**d**), β-Cas (**e**), OVA (**f**), Hemo (**g**), BSA (**h**), AFP (**i**), Trans (**j**), anti-AFP (**k**); Sample injection volume: 600 nL. The NaCl exhibited no influence on binding proteins by HPEDA-NCAM except for Lyz. In compare with the binding behavior in phosphate buffer without NaCl (Fig. S5), it can be confirmed that Lyz was captured mainly through electrostatic interaction on HPEDA-NCAM.

9. Effect of pH on protein binding on HPEDA-NCAM

Table S6. Effect of pH on protein binding on HPEDA-NCAM

Protein	pH 10.0	pH 8.5	pH 7.0	pH 6.0
RNase A	N[a]	N	N	N
β-Lac A	Y[b]	Y	Y	Y
OVA	Y	Y	Y	Y
Hemo	Y	Y	Y	Y
BSA	Y	Y	Y	Y
AFP	Y	Y	Y	Y
Trans	Y	Y	Y	Y
anti-AFP	Y	Y	Y	Y

[a] "N" means protein can not be captured by HPEDA-NCAM; [b] "Y" means protein can be captured by HPEDA-NCAM.

10. Retention of peptides on HPEDA-NCAM

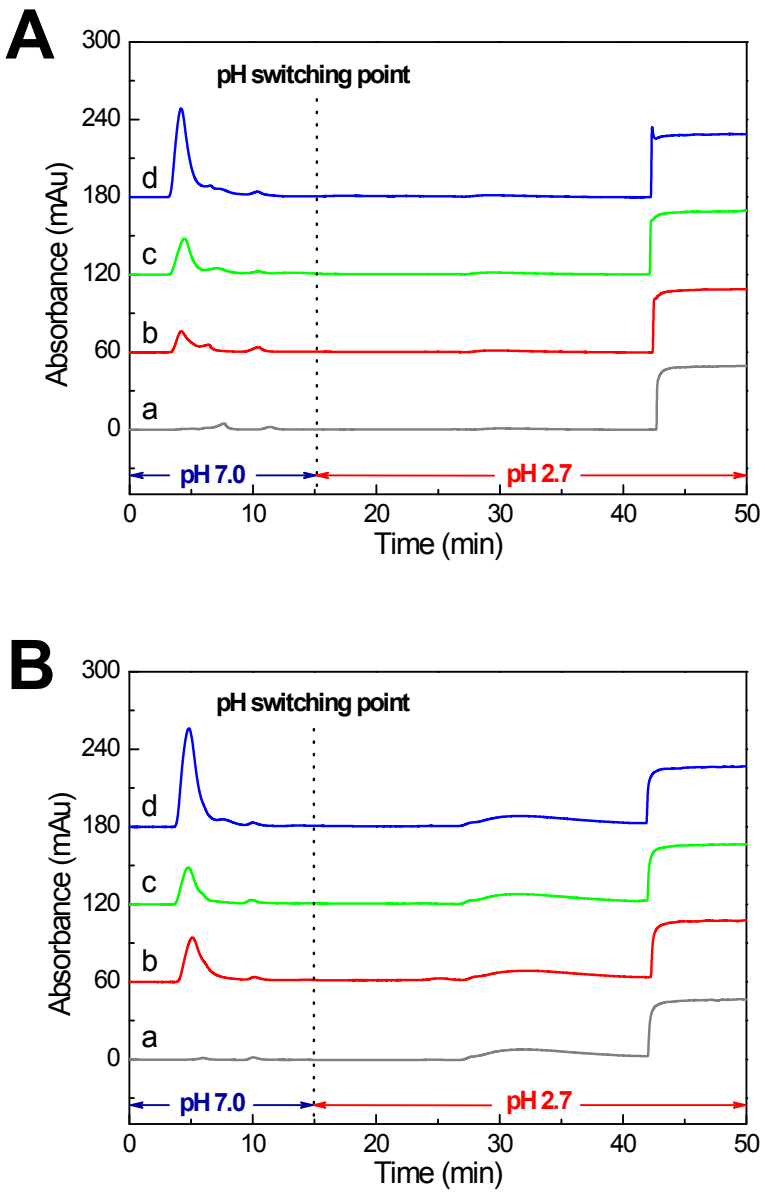

Fig. S7 Chromatographic retention of peptides on HPEDA-NCAM monolithic column. Mobile phase: 20 mM sodium phosphate buffer at pH 7.0 (**A**) and 20 mM sodium phosphate buffer with 150 mM NaCl at pH 7.0 (**B**), switched to 100 mM HAc (pH 2.7) at 15 min; Sample: blank (**a**), peptides from OVA (**b**), BSA (**c**) and Trans (**d**), hydrolyzed by trypsin; Sample injection volume: 600 nL. Peptides from OVA and BSA exhibited no retention on HPEDA-NCAM (**A**, traces **b** and **c**), only a few peptides from Trans were captured through electrostatic interaction (**A**, trace **d**). All of the peptides from the three proteins exhibited no retention on HPEDA-NCAM under neutral phosphate buffer with 150 mM NaCl (**B**).

11. Pore size distribution of HPEDA-NCAMB

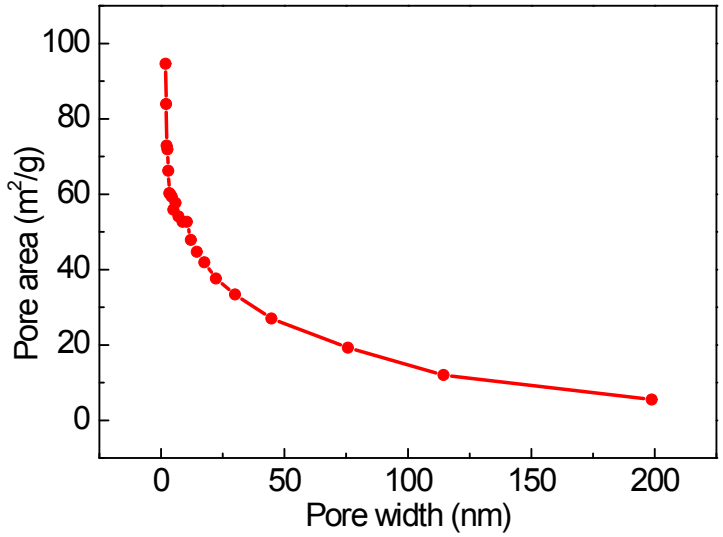

Fig. S8 Pore size distribution of HPEDA-NCAMB. There are nanopores (5-100 nm) distributed in the material.

12. The affinity of HPEDA-NCAMB toward different proteins

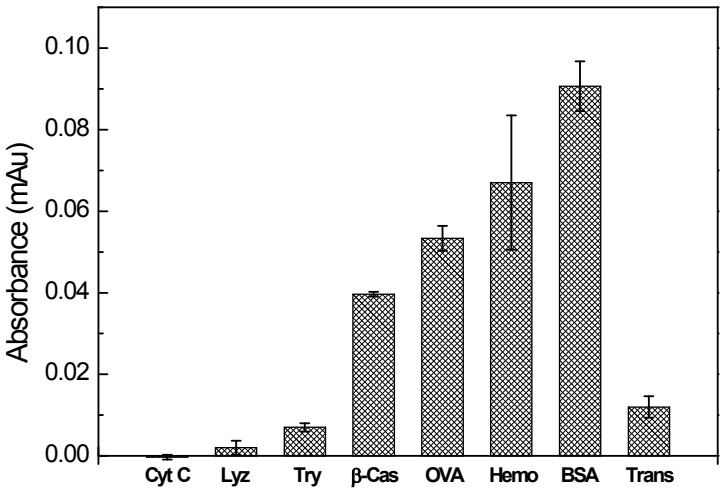

Fig. S9 Comparison of the amount of different proteins captured by HPEDA-NCAMB. Binding buffer: 20 mM sodium phosphate buffer at pH 7.0; elution solution: 100 mM HAc (pH 2.7); Sample: 0.5 mg/mL protein dissolved in the binding buffer. Proteins with molecular weight more than 18 kDa were all captured by HPEDA-NCAMB, which was very compatible with that for monolithic material HPEDA-NCAM (Fig. S5).

13. Enantiomer resolution on HSA-functionalized NCAM monolithic column

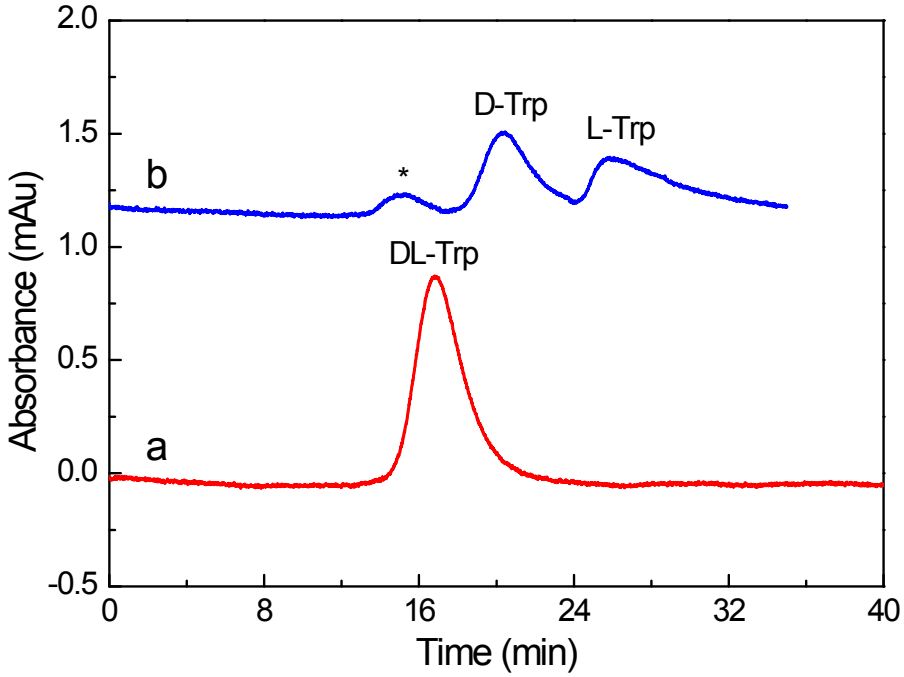

Fig. S10 Chromatograms for the chiral separation of racemic Trp on HSA-functionalized NCAM monolithic column. Trace **a**, HPEDA-NCAM column exhibited no chiral separation ability. Trace **b**, DL-Trp was separated into two peaks (D-Trp and L-Trp) by HSA-functionalized NCAM monolithic column, which indicated that the HSA adsorbed on the monolith HPEDA-NCAM still retained its biological activity. Mobile phase: 67 mM sodium phosphate buffer at pH 7.4; Sample: 1 mg/mL DL-Trp dissolved in the loading buffer; Sample injection volume: 50 nL; Flowing rate: 0.6 μL/min; Detection wavelength: 280 nm. "*" means system peak.

14. Capture of serum proteins by HPEDA-NCAM

Table S7. Proteins mass observed and identified from human serum

Peak	m/z	Protein name[a]	Captured protein[b]
1	14033	Transthyretin, glutathionylated	N
2	17377	Apolipoprotein A2 ($[2M+H]^+$)	Y
3	22225	β_2-Glycoprotein I ($[M+2H]^{2+}$)	Y
4	28046	Apolipoprotein A1	Y
5	33243	HSA ($[M+2H]^{2+}$)	Y
6	39441	α_1-Acid glycoprotein	Y
7	44272	β_2-Glycoprotein I	Y
8	50818	α_1-Proteinase inhibitor	Y
9	56174	Hemopexin or Antithrombin III	Y
10	66398	HSA	Y
11	73724	IgG ($[M+2H]^{2+}$)	Y
12	79296	Transferrin	Y
13	83324	unknown	Y
14	88652	Plasminogen	Y
15	94653	unknown	Y
16	99922	Haptoglobin	Y
17	111038	unknown	Y
18	117562	unknown	N
19	132804	HSA ($[2M+H]^+$)	Y
20	146126	Ig G	Y
21	161016	Ig A or Inter-α-trypsin inhibitor	Y
22	199162	C3 complement	Y

[a] Proteins were identified by comparing m/z values with the references reported[20]; [b] "Y" means the protein was captured, while "N" means the protein was not captured by HPEDA-NCAM.

15. Protein hydrolysis by trypsin-functionalized NCAM monolithic column

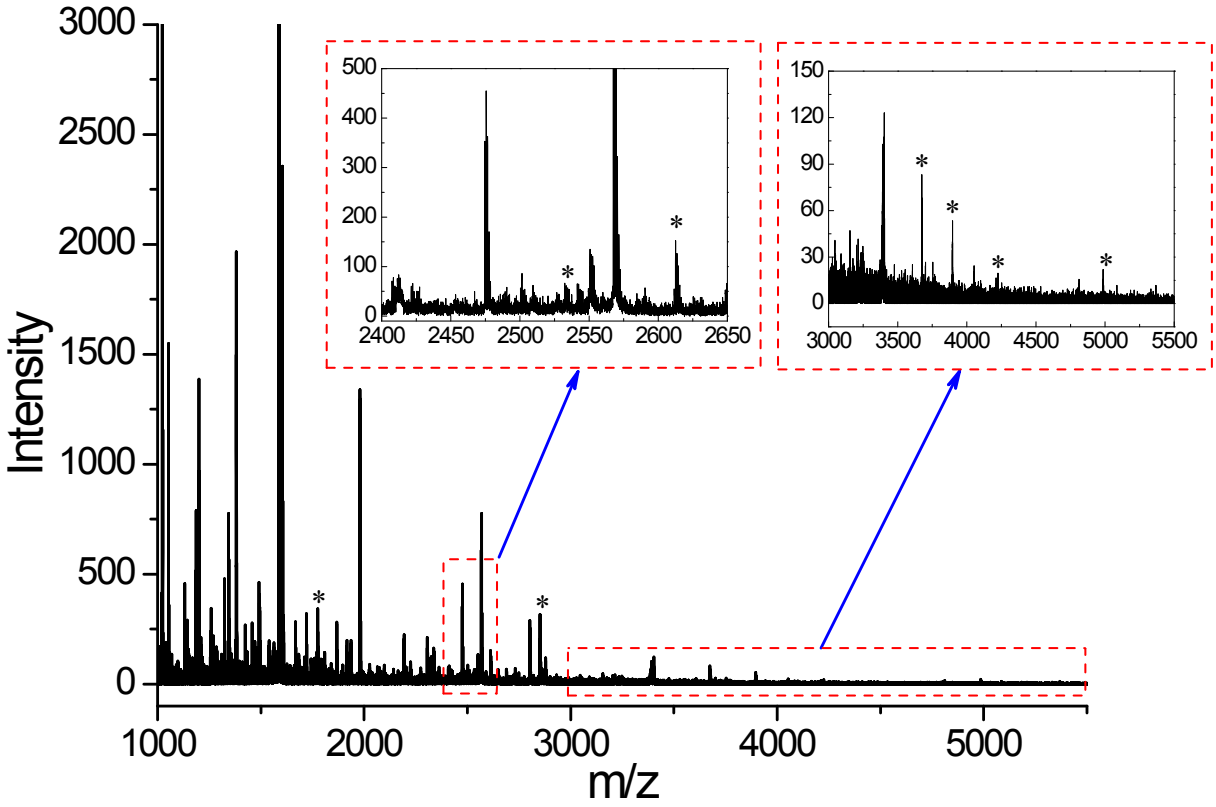

Fig. S11 MALDI-TOF MS spectrum of HRP digest by trypsin-functionalized NCAM monolithic column. The glycopeptides were marked with the symbol "*". HRP was hydrolyzed in the trypsin-functionalized NCAM monolithic column, indicating that the trypsin retained its enzyme activity in column.

Table S8. Identified peptides from HRP digest by trypsin-functionalized NCAM monolithic column

NO.[a]	m/z	Peptide sequence[b]
1	1022.6	DAFGNANSAR
2	1162.7	MKAAVESACPR
3	1185.7	YYVNLEEQK
4	1199.7	SDPRIAASILR
5	1380.8	TEKDAFGNANSAR
6	1475.9	SSDLVALSGGHTFGK
7	1587.0	MGNITPLTGTQGQIR
8	1808.1	DAFGNANSARGFPVIDR
9*	1843.0	NVGLNR
10	1915.1	GLCPLNGNLSALVDFDLR
11	1977.2	SSDLVALSGGHTFGKNQCR
12	2074.3	MGNITPLTGTQGQIRLNCR
13	2183.3	SFANSTQTFFNAFVEAMDR
14	2501.5	GLIQSDQELFSSPNATDTIP LVR
15*	2532.4	SFANSTQTFFNAFVEAMDR
16*	2611.6	MGNITPLTGTQGQIR
17*	2850.8	GLIQSDQELFSSPNATDTIP LVR
18*	3672.2	GLIQSDQELFSSPNATDTIP LVR
19*	3895.2	LYNFSNTGLPDPTLNTTYLQ TLRGLCPLNGNLSALVDFDL R
20*	4224.4	QLTPTFYDNSCPNVSNIVR
21*	4985.6	LYNFSNTGLPDPTLNTTYLQ TLRGLCPLNGNLSALVDFDL R

[a] The glycopeptides were marked with the symbol "*"; [b] Peptide sequence was identified according to the way reported by the reference[21].

16. Improvement of MS detection capability toward serum peptides

Table S9. Peptides mass observed and identified from human serum[a]

NO.	m/z (**b**)	Peptide[b]
1	1017.6	Fibrinopeptide A, fragment
2	1033.5	unknown
3	2086.1	unknown
4	3165.6	unknown

NO.	m/z (**c**)	Peptide
1	1078.0	Fibrinopeptide A, fragment
2	1113.4	unknown
3	1786.8	ITIH4, fragment
4	1793.8	unknown
5	1808.8	Apolipoprotein A1, fragment
6	1824.9	unknown
7	1943.8	Kininogen, fragment
8	2010.9	unknown
9	2032.9	ITIH4, fragment
10	2267.1	ITIH4, fragment
11	2390.2	unknown
12	2754.3	Apolipoprotein A4, fragment
13	3362.6	unknown

NO.	m/z (**d**)	Peptide
1	1018.6	Fibrinopeptide A, fragment
2	1032.6	unknown
3	1076.6	Fibrinopeptide A, fragment
4	1121.5	unknown
5	1129.6	unknown
6	1143.7	unknown
7	1171.6	unknown
8	1187.6	unknown
9	1240.7	unknown
10	1254.7	unknown
11	1276.7	Clusterin beta, fragment
12	1298.7	unknown
13	1324.6	unknown
14	1337.5	unknown
15	1351.8	Fibrinopeptide A, fragment
16	1365.8	unknown
17	1387.8	unknown
18	1395.6	unknown
19	1409.8	unknown
20	1432.8	unknown
21	1436.7	unknown
22	1462.8	Fibrinopeptide A, fragment
23	1476.9	unknown
24	1498.8	Complement C4a, fragment
25	1519.6	unknown
26	1573.9	unknown
27	1587.9	unknown
28	1609.9	unknown
29	1615.9	unknown
30	1632.9	unknown
31	1685.0	unknown
32	1699.0	unknown
33	1728.0	unknown
34	1740.9	Complement C4a,fragment
35	1762.9	Complement C4a,fragment
36	1786.8	ITIH4, fragment
37	1796.0	unknown
38	1802.8	unknown
39	1808.8	Apolipoprotein A1, fragment
40	1810.1	unknown
41	1839.1	unknown
42	1855.8	unknown
43	1891.0	unknown
44	1921.1	unknown
45	1943.9	Kininogen, fragment
46	1949.1	unknown
47	1965.1	unknown
48	2010.9	unknown
49	2026.9	ITIH4, fragment
50	2032.9	unknown
51	2076.2	unknown
52	2109.0	unknown
53	2143.3	unknown
54	2238.1	unknown
55	2254.3	unknown
56	2271.1	unknown
57	2283.1	ITIH4, fragment
58	2299.1	unknown
59	2305.1	Complement C4a, fragment
60	2358.1	ITIH4, fragment
61	2425.2	unknown
62	2602.3	Factor XIIIa,fragment
63	2627.3	ITIH4, fragment
64	2724.3	ITIH4, fragment
65	2753.0	Apolipoprotein A4, fragment
66	2816.3	Fibrinogen A, fragment
67	2829.4	unknown

[a] The color blue, red and black represents the peaks observed for b, c and d in Fig. S12. [b] Peptides were identified by comparing m/z values with the references reported[20].

Supplementary references

[1] Q. J. Li, C. C. Lu, Z. Liu, *J. Chromatogr. A* **2013**, *1305*, 123-130.

[2] Q. J. Li, C. C. Lu, H. Y. Li, Y. C. Liu, H. Y. Wang, X. Wang, Z. Liu, *J. Chromatogr. A* **2012**, *1256*, 114-120.

[3] H. A. Engstrom, R. Johansson, P. Koch-Schmidt, K. Gregorius, S. Ohlson, M. Bergstrom, *Biomed. Chromatogr.* **2008**, *22*, 272-277.

[4] C. C. Lu, H. Y. Li, H. Y. Wang, Z. Liu, *Anal. Chem.* **2013**, *85*, 2361-2369.

[5] L. Li, Y. Lu, Z. J. Bie, H. Y. Chen, Z. Liu, *Angew. Chem. Int. Ed.* **2013**, *52*, 7451-7454.

[6] G. W. Bushnell, G. V. Louie, G. D. Brayer, *J. Mol. Biol.* **1990**, *214*, 585-595.

[7] M. H. Dung, J. A. Bell, *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **1997**, *53*, 419-425.

[8] M. A. Walsh, T. R. Schneider, L. C. Sieker, Z. Dauter, V. S. Lamzin, K. S. Wilson, *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **1998**, *54*, 522-546.

[9] H. C. Watson, *Prog.Stereochem.* **1969**, *4*, 299.

[10] J. J. Adams, B. F. Anderson, G. E. Norris, L. K. Creamer, G. B. Jameson, *J. Struct. Biol.* **2006**, *154*, 246-254.

[11] H. K. S. Leiros, B. O. Brandsdal, O. A. Andersen, V. Os, I. Leiros, R. Helland, J. Otlewski, N. P. Willassen, A. O. Smalas, *Protein Sci.* **2004**, *13*, 1056-1070.

[12] R. S. Syed, S. W. Reid, C. W. Li, J. C. Cheetham, K. H. Aoki, B. S. Liu, H. J. Zhan, T. D. Osslund, A. J. Chirino, J. D. Zhang, J. Finer-Moore, S. Elliott, K. Sitney, B. A. Katz, D. J. Matthews, J. J. Wendoloski, J. Egrie, R. M. Stroud, *Nature* **1998**, *395*, 511-516.

[13] P. E. Stein, A. G. W. Leslie, J. T. Finch, R. W. Carrell, *J. Mol. Biol.* **1991**, *221*, 941-959.

[14] S. Y. Park, T. Yokoyama, N. Shibayama, Y. Shiro, J. R. H. Tame, *J. Mol. Biol.* **2006**, *360*, 690-701.

[15] L. Pugliese, A. Coda, M. Malcovati, M. Bolognesi, *J. Mol. Biol.* **1993**, *231*, 698-710.

[16] A. Bujacz, *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **2012**, *68*, 1278-1289.

[17] D. C. Carter, X. M. He, S. H. Munson, P. D. Twigg, K. M. Gernert, M. B. Broom, T. Y. Miller, *Science* **1989**, *244*, 1195-1198.

[18] J. Wally, P. J. Halbrooks, C. Vonrhein, M. A. Rould, S. J. Everse, A. B. Mason, S. K. Buchanan, *J. Biol. Chem.* **2006**, *281*, 24934-24944.

[19] E. O. Saphire, P. Parren, R. Pantophlet, M. B. Zwick, G. M. Morris, P. M. Rudd, R. A. Dwek, R. L. Stanfield, D. R. Burton, I. A. Wilson, *Science* **2001**, *293*, 1155-1159.

[20] G. L. Hortin, *Clin. Chem.* **2006**, *52*, 1223-1237.

[21] J. Liu, F. Wang, H. Lin, J. Zhu, Y. Bian, K. Cheng, H. Zou, *Anal. Chem.* **2013**, *85*, 2847-2852.