

Electronic Supplementary Information for

Layer-by-layer Coating and Chemical Cross-linking of

Multilayer Polysaccharides on Silica for Mixed-mode HPLC

Application

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Experimental Section

Reagents and Material

Spherical silica (5 μm , 120 \AA , 280 m^2/g) was purchased from DASIO Co., Ltd. (Osaka, Japan). Toluene (GR grade), NaCl (AR grade), acetic acid (HAc, GR grade), and NaHCO_3 (AR grade) were purchased from LingFeng Chemical Reagents Co., Ltd. (Shanghai, China). 3-Aminopropyltriethoxysilane (APTES, 98%) was purchased from Aladdin (Shanghai, China). Sodium hyaluronate (HA, Mw = 100 KDa) was purchased from Zhenjiang Dong Yuan Biotech Co., Ltd., (Zhenjiang, China). Chitosan (CS, low molecular weight), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride ($\text{EDC}\cdot\text{HCl}$, 98%), and N-hydroxysuccinimide (NHS, 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl alcohol (MeOH, AR grade) and ethyl alcohol (EtOH, AR grade) used in the synthesis procedure were purchased from Titan Scientific Co., Ltd. (Shanghai, China). MeOH (HPLC grade) and acetonitrile (ACN, HPLC grade) were purchased from ANPEL Laboratory Technologies (Shanghai, China). Click XIon column (5 μm , 100 \AA , 150 mm \times 4.6 mm) was obtained from Acchrom Technologies Co., Ltd. (Beijing, China). Ultrapure water (18.2 $\text{M}\Omega\cdot\text{cm}$) was obtained from a Sartorius Ultrapure Water System (Gottingen, Germany) and was used throughout the experiments.

Synthesis Procedure

The synthesis procedure is represented in scheme 1. First, 5 g of spherical silica was dispersed in a 50 mL water/ethanol (3:7, v/v) solution, and then 6 g of APTES was added to the suspension. After mixing, the mixture was refluxed at 110 $^\circ\text{C}$ for 24 h. The white solid product (named $\text{SiO}_2@(\text{NH}_2)$) was collected by filtration, washed with methanol and water successively and dried in an oven at 55 $^\circ\text{C}$. Next, the $\text{SiO}_2@(\text{HA-CS})_{12}$ stationary phase was synthesized via a step-by-step assembly strategy. $\text{SiO}_2@(\text{NH}_2)$ was dispersed into 60 mL of HA solution (5 mg/mL dissolved in 0.135 mol/L NaCl aqueous solution containing 2% HAc). The mixture was softly shaken for 30 min, and then the particles were centrifugally separated and washed several times

with water. Subsequently, the obtained product was dispersed into 60 mL of CS solution (5 mg/mL dissolved in 0.135 mol/L NaCl aqueous solution containing 2% HAc), and the mixture was treated in the same way as described above. After repeating for 6 cycles, the particles were washed thoroughly with water. Next, the cross-linking reaction of CS and HA, which had been assembled on the silica surface through electrostatic adsorption, was performed. The obtained product was dispersed into 150 mL of NH_3HCO_3 aqueous solution (10 mmol/L) that contained EDC·HCl (2 mg/mL) and NHS (2 mg/mL), and the reaction lasted overnight with gentle magnetic stirring. The product was successively washed with water and ethyl alcohol three times. Finally, the product (named $\text{SiO}_2@(\text{CS-HA})_{12}$) was dried in an oven at 50 °C. For the sake of description, the obtained products during the assembly procedure were named $\text{SiO}_2@(\text{HA-CS})_n$, where 'n' is the layer number of polysaccharides assembled on the silica surface.

Characterization

Scanning electron microscopy (SEM) images were obtained from a JSM-6360 LV Tungsten filament scanning electron microscope (JEOL Co., Ltd., Japan). Elemental analysis (EL) data were obtained from a Vario EL III CHNOS Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Fourier transform infrared (FT-IR) spectra were recorded on a Thermo Nicolet 380 spectrometer (Thermo Nicolet, Wisconsin, USA) by using the KBr pellet technique. Nitrogen adsorption/desorption isotherms were obtained from a TriStar II 3020 apparatus (Micromeritics Instrument Corporation, Norcross, GA, USA). Zeta potential (ζ) changes were acquired using a Delsa™ Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter Inc., Fullerton, CA, USA).

Chromatographic Experiments

The synthesized stationary phase was slurry-packed into stainless steel columns (150 mm × 4.6 mm I.D.) using MeOH/H₂O (40/60, v/v) as the slurry solvent under high pressure at 70 MPa. All chromatographic experiments were performed on a 5100 series

LC system (Dalian Elite Analytical Instruments Co., Ltd., Dalian, China), which consisted of a dual pump, an online degasser, an autosampler, a column thermostat and a UV-Vis detector. A Click XIon column (5 μm , 100 \AA , 150 mm \times 4.6 mm) obtained from Acchrom Technologies Co., Ltd. (Beijing, China), was utilized in the comparison experiment. The void volume of the $\text{SiO}_2@(\text{HA-CS})_{12}$ column was determined by injecting toluene dissolved in MeOH, with ACN/H₂O (99/1, v/v) as the mobile phase in isocratic mode and UV detection setting at 254 nm. The column temperature in all chromatographic experiments was set at 30 °C and the flow rate was set at 1 mL/min unless stated otherwise. All model analytes were dissolved in water or MeOH except nucleosides, which were dissolved in NaOH solution (0.1 mol/L), and the concentrations of all model analytes in solution were in the range of 0.1 - 0.5 mg/mL.

$$\frac{t_R - t_0}{t_0}$$

Retention factor k' = $\frac{t_R - t_0}{t_0}$, where t_R is the retention time of solutes, t_0 is the dead time of the column used.

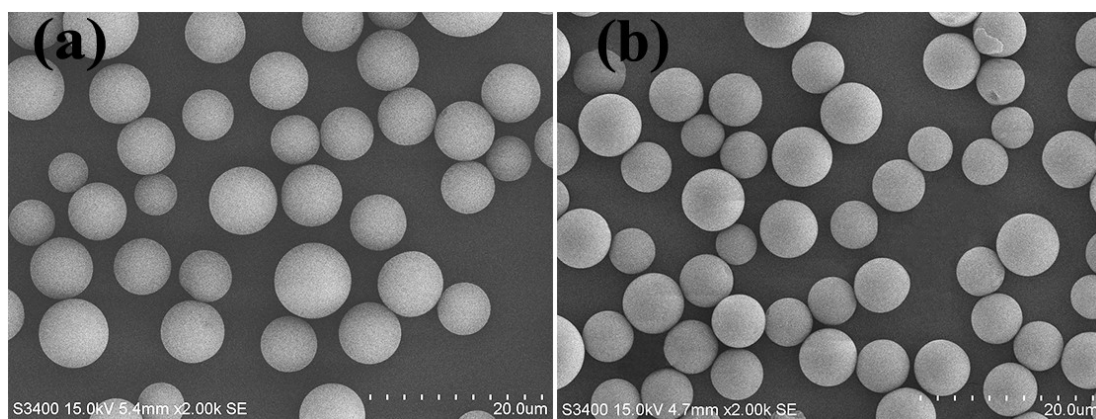


Figure S1. SEM images of (a) SiO₂@NH₂ and (b) SiO₂@(HA-CS)₁₂. The average diameter: $6.01 \pm 0.86 \mu\text{m}$ for SiO₂@NH₂ and $5.98 \pm 0.71 \mu\text{m}$ SiO₂@(HA-CS)₁₂.

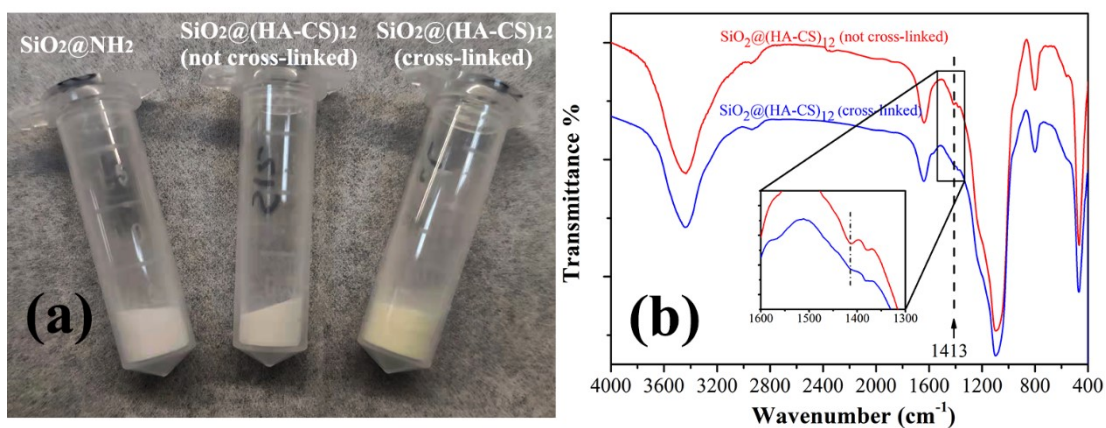


Figure S2. (a) digital photo of SiO₂@NH₂, SiO₂@(HA-CS)₁₂ (not cross-linked) and SiO₂@(HA-CS)₁₂ (cross-linked); (b) comparison of the FT-IR spectra of SiO₂@(HA-CS)₁₂ (not cross-linked) and SiO₂@(HA-CS)₁₂ (cross-linked).

Table S1. Results of elemental analysis and special surface area.

Sample	Elemental analysis (%)			Density of the disaccharide units ($\mu\text{mol/g}$) ^a	Special surface area (m^2/g) ^a	Pore size (nm) ^b
	C	N	H			
SiO ₂ @NH ₂	5.45	1.65	1.75	0	251.6	11.7
SiO ₂ @(HA-CS) ₄	6.96	1.70	1.93	72.7	/	/
SiO ₂ @(HA-CS) ₈	8.40	1.78	2.15	142.2	/	/
SiO ₂ @(HA-CS) ₁₂	9.82	1.89	2.39	210.6	186.3	7.8

^a: The immobilized density of the disaccharide unit on SiO₂ surface was calculated based on the change of the carbon content. According to the zeta potential (ζ) results, after 12-layer deposition of polysaccharides, the surface potential of SiO₂@(HA-CS)₁₂ showed little difference from SiO₂@NH₂. Thus, we suppose the immobilized density of HA and CS is equal. The molecular weight of the hypothetical disaccharide (formed from one HA monomer (C₆H₁₁NO₄) and one CS monomer (C₁₄H₂₁NO₁₁)) is 540.5, and the percentage of carbon mass to total mass is 43.89%. Then the calculation formula is:

$$\text{Density of the disaccharide unit } (\mu\text{mol/g}) = \frac{1/540.5}{\left(\frac{43.89\% - C_n}{C_n - C_0} + 1\right)} \times 10^6$$

where, C_n is the carbon content of SiO₂@(HA-CS)_n; C₀ is the carbon content of SiO₂NH₂.

^b: The results of specific surface area and average pore size were calculated from the adsorption isotherms by the BJH method;

/: untested.

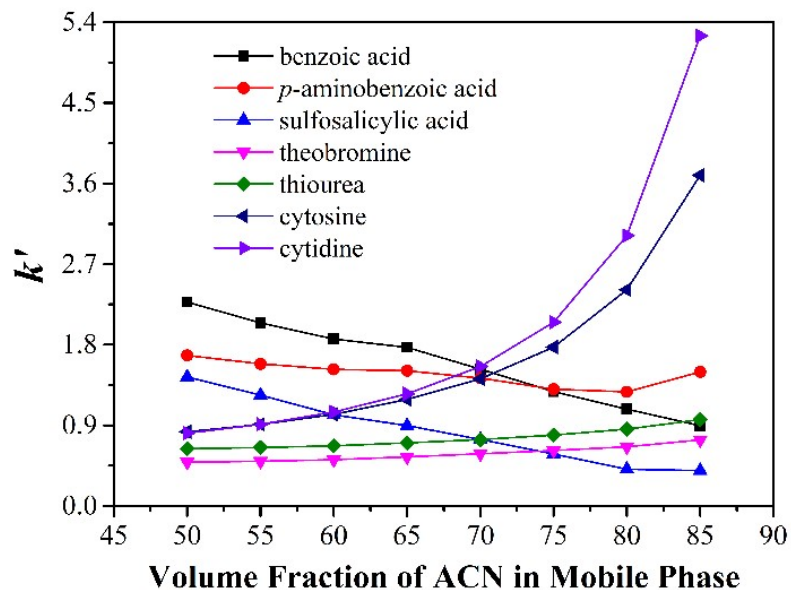


Figure S3. Effect of ACN volume fraction in the mobile phase on the chromatographic retention factors of basic, neutral and acidic probe molecules. Conditions: mobile phase: (A) H₂O, (B) ACN; flow rate: 1 mL/min; UV detection wavelength: 254 nm.

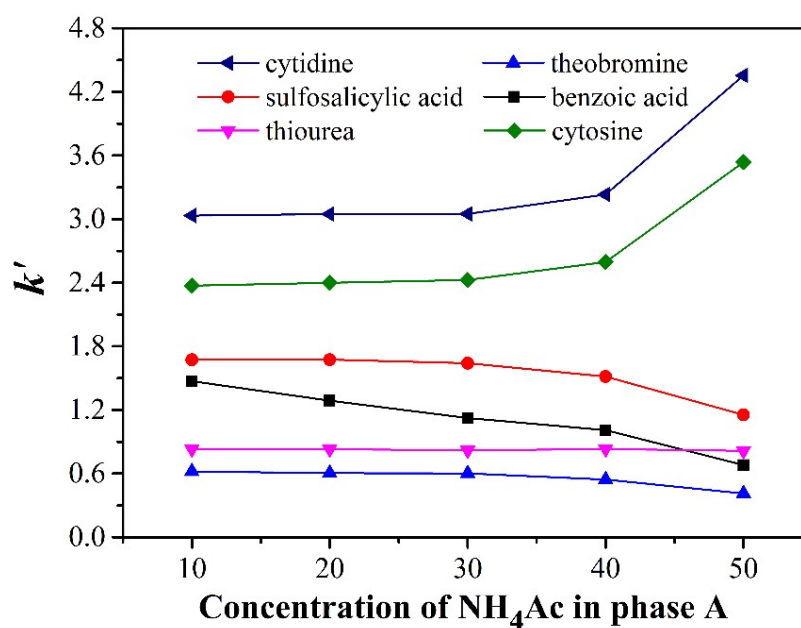


Figure S4. Effect of buffer concentration in the water phase on the chromatographic retention factors of basic, neutral and acidic probe analytes. Conditions: mobile phase: (A) NH₄Ac buffer solution (pH = 6.82), (B) ACN; flow rate: 1 mL/min; column temperature: 30 °C; UV detection wavelength: 254 nm.

The kinetic equation of the retention time versus the concentration of H⁺ in the mobile phase.

The equation was derived as follows:

First, taking an acidic solute (HA) as the sample, the equilibrium expression for the dissociation ($\text{AH} \xrightleftharpoons{K_a} \text{A}^- + \text{H}^+$) can be written as:

$$K_a = \frac{[\text{A}^-][\text{H}^+]}{[\text{AH}]} \quad (1)$$

$$[\text{AH}]_{\text{total}} = [\text{AH}] + [\text{A}^-] \quad (2)$$

where K_a is the dissociation equilibrium constant of HA. From Eq. (1, 2),

$$\alpha_{\text{AH}} = [\text{AH}]/[\text{AH}]_{\text{total}} = \frac{[\text{H}^+]}{K_a + [\text{H}^+]} \quad (3)$$

$$\alpha_{\text{A}^-} = [\text{A}^-]/[\text{AH}]_{\text{total}} = \frac{K_a}{K_a + [\text{H}^+]} \quad (4)$$

where α_{AH} and α_{A^-} are the molar fractions of the AH molecule and A⁻ anion, respectively. Because there is a transition equilibrium between the ionic form (A⁻) and molecular form (HA), we suppose that the linear velocity of the acidic solute in the column is proportional to the molar fraction of the two forms.

$$u_{\text{acid}} = \alpha_{\text{AH}} \cdot u_{\text{AH}} + \alpha_{\text{A}^-} \cdot u_{\text{A}^-} \quad (5)$$

where u is the linear velocity.

$$u_{\text{AH}} = L/t_{\text{AH}} \quad (6)$$

$$u_{\text{A}^-} = L/t_{\text{A}^-} \quad (7)$$

where L is the column length, and t is the retention time. From Eq. (3 - 7),

$$t_{\text{acid}} = L/u_{\text{acid}} = \frac{K_a + [\text{H}^+]}{[\text{H}^+]/t_{\text{AH}} + K_a/t_{\text{A}^-}} \quad (8)$$

In a similar way, for basic solutes ($\text{B} + \text{H}_2\text{O} \xrightleftharpoons{K_b} \text{BH}^+ + \text{OH}^-$), the retention time equation is:

$$t_{\text{base}} = L/u_{\text{base}} = \frac{K_b + [\text{OH}^-]}{[\text{OH}^-]/t_{\text{B}} + K_b/t_{\text{BH}^+}} \quad (9)$$

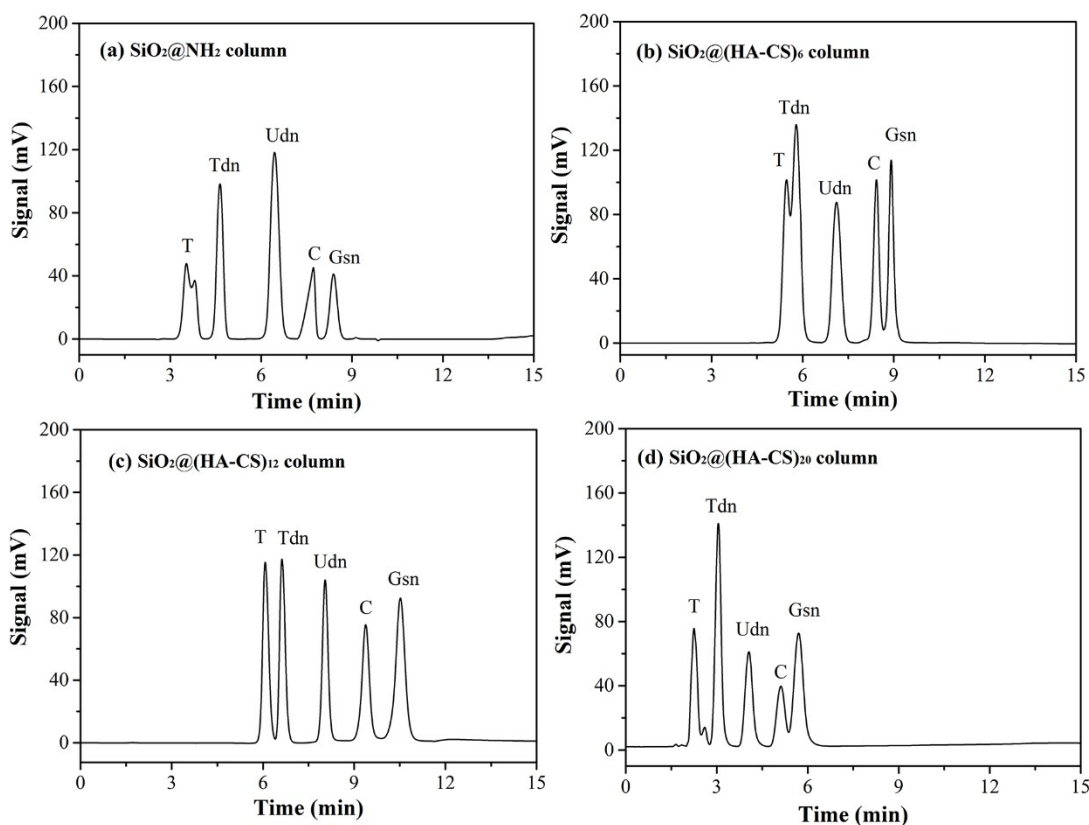


Figure S5. Comparison of chromatographic separation performance for nucleoside and bases on $\text{SiO}_2@\text{NH}_2$ column, $\text{SiO}_2@(\text{HA-CS})_6$ column, $\text{SiO}_2@(\text{HA-CS})_{12}$ column and $\text{SiO}_2@(\text{HA-CS})_{20}$ column. Conditions: (a): mobile phase (A): 50 mmol/L NH_4Ac in water, (B) ACN; isocratic: 0 - 10 min, 10 - 25% A; flow rate: 1 mL/min; UV detector wavelength: 254 nm; (b, c and d): mobile phase (A) 15 mmol/L NH_4Ac in water (pH 4.92), (B) ACN; gradient: 0 - 5 min, 5% A, 5 - 10 min, 5% - 30% A, 10 - 20 min, 30% A; UV detection wavelength: 254 nm.

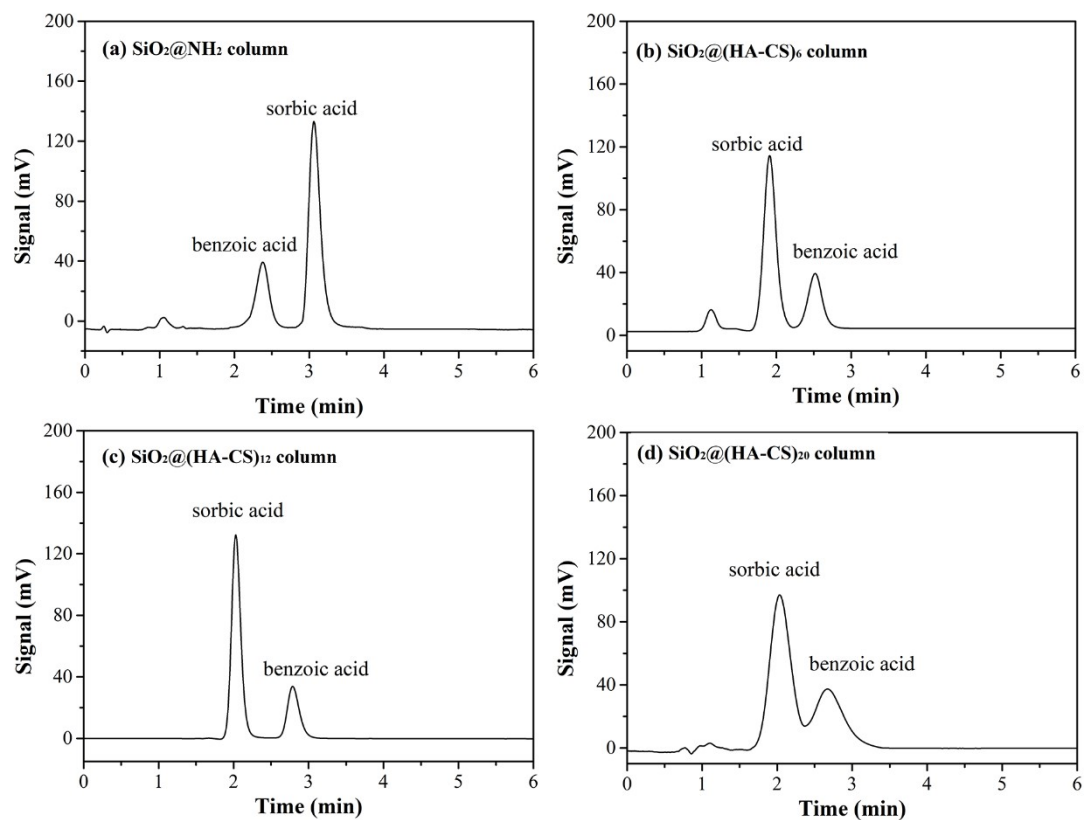


Figure S6. Comparison of separation performance for acidic solutes on $\text{SiO}_2@(\text{NH}_2)$ column, $\text{SiO}_2@(\text{HA-CS})_6$ column, $\text{SiO}_2@(\text{HA-CS})_{12}$ column and $\text{SiO}_2@(\text{HA-CS})_{20}$ column. Conditions: (a): mobile phase (A): 50 mmol/L KH_2PO_4 in water (pH 2.5, titrated with H_3PO_4), (B) ACN; isocratic: 35% A; flow rate: 1 mL/min; UV detector wavelength: 254 nm; (b, c and d): mobile phase (A) 1 mmol/L KH_2PO_4 in water, (B) ACN; isocratic 70%A; UV detection wavelength: 254 nm.

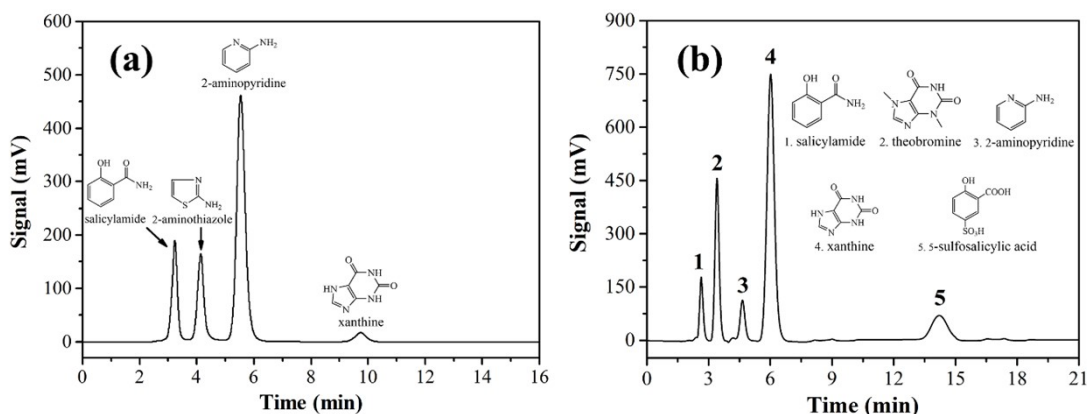


Figure S7. The chromatographic separation of several organic bases and organic acids on $\text{SiO}_2@(\text{HA-CS})_{12}$ column, conditions: (a): mobile phase (A): 5 mmol/L KH_2PO_4 in water, (B) ACN; isocratic: 35% A; flow rate: 1 mL/min; UV detector wavelength: 254 nm; (b): mobile phase (A): 25 mmol/L KH_2PO_4 in water (pH = 3.4), (B) ACN; isocratic: 30% A; flow rate: 1 mL/min; UV detector wavelength: 254 nm.

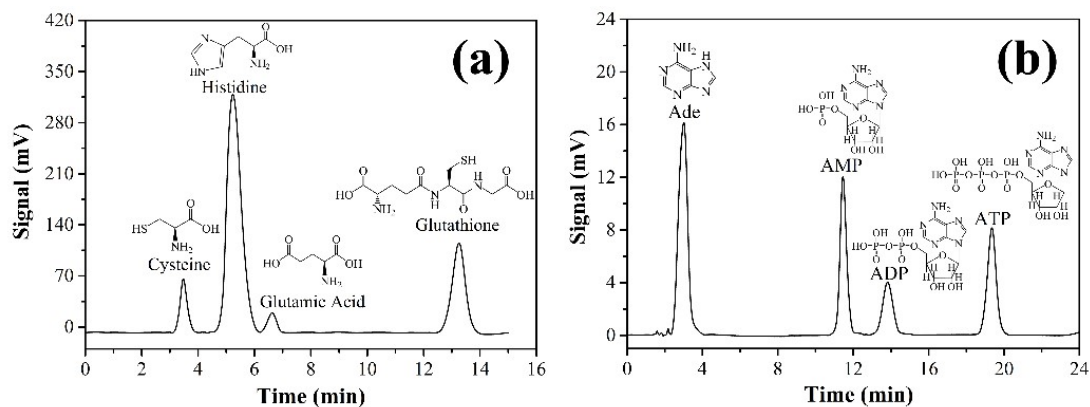


Figure S8. The chromatographic separation of (a) amino acids, small peptide and (b) adenosine phosphate derivatives on $\text{SiO}_2@(\text{HA-CS})_{12}$ column, conditions: (a) mobile phase (A) 0.3% H_3PO_4 in water, (B) ACN, isocratic: 5%A, detector wavelength: 210 nm; (b) mobile phase: (A) 0.2% H_3PO_4 in water, (B) ACN; gradient: 0 - 10 min, 35% - 70% A; detector wavelength: 254 nm.

Table S2. The pK_a values of organic acids solutes.

organic acids	pK_{a1}	pK_{a2}	pK_{a3}
sorbic acid	4.76	/	/
benzoic acid	4.17	/	/
<i>m</i> -phthalic acid	3.62	4.60	/
<i>p</i> -phthalic acid	3.54	4.46	/
trimesic acid	2.12	4.10	5.18
sulfosalicylic acid	-0.62	3.18	11.74
theobromine	11.0	/	/
cytosine	4.60	/	/
cytidine	4.22	/	/
thiourea	21.0	/	/
<i>p</i> -aminobenzoic acid	2.38	4.89	/

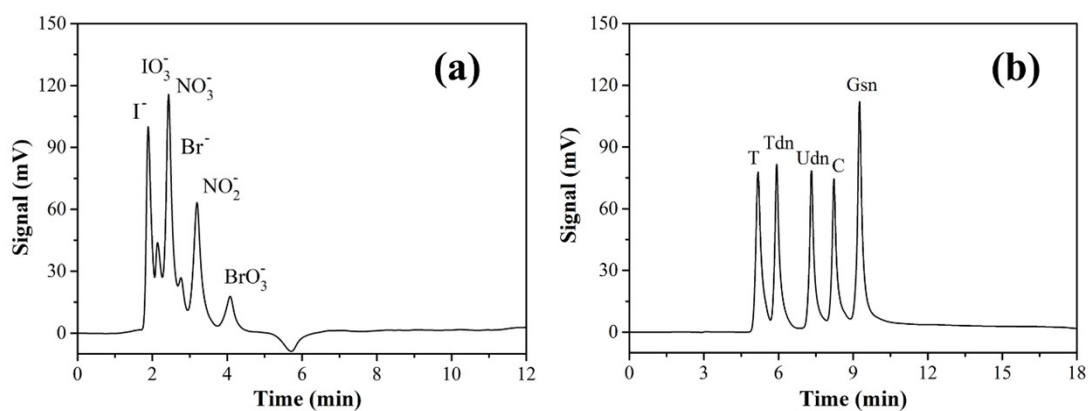


Figure S9. Comparison separation of (a) inorganic anions and (b) nucleoside and bases on Click XIon column, conditions: (a): mobile phase (A) 50 mmol/L NH_4Ac in water (pH 4.88), (B) ACN; isocratic 20% A; UV detection wavelength: 210 nm; (b): mobile phase (A) 15 mmol/L NH_4Ac in water (pH 4.92), (B) ACN; gradient: 0 - 5 min, 5% A, 5 - 10 min, 5% - 30% A, 10 - 20 min, 30% A; UV detection wavelength: 254 nm.

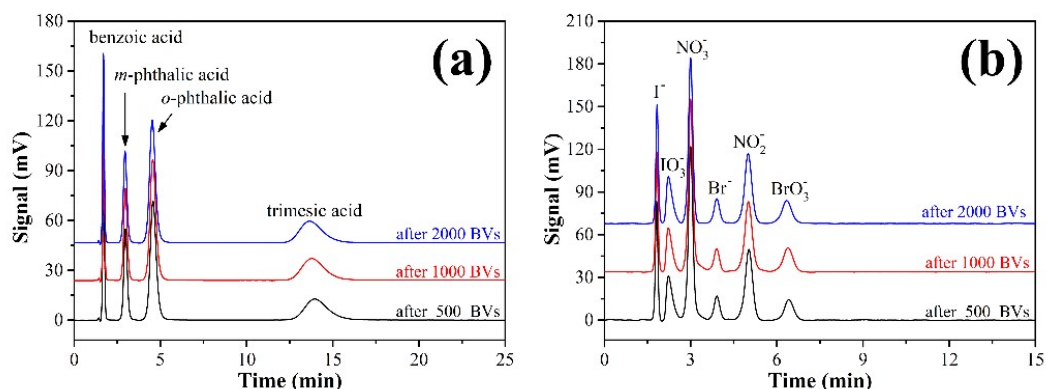


Figure S10. The stability test of the synthesized $\text{SiO}_2@(\text{HA-CS})_{12}$ (cross-linked) stationary phase, conditions: (a): mobile phase (A) 15 mmol/L KH_2PO_4 in water (pH 3.25), (B) ACN; isocratic 60% A; UV detection wavelength: 254 nm; (b): mobile phase (A) 50 mmol/L NH_4Ac in water (pH 4.88), (B) ACN; isocratic 20% A; UV detection wavelength: 210 nm. The Y axis was shifted for better display.

Table S3. The retention time of the solutes and RSDs on the $\text{SiO}_2@(\text{HA-CS})_{12}$ (cross-linked) column in the stability test.

Solute	Retention time			RSD (%)
	500 BVs	1000BVs	2000 BVs	
benzoic acid	1.69	1.71	1.69	0.545
<i>m</i> -phthalic acid	2.95	2.96	2.96	0.215
<i>o</i> -phthalic acid	4.53	4.55	4.56	0.352
trimesic acid	13.67	13.77	13.90	0.859
I^-	1.83	1.82	1.81	0.374
IO_3^-	2.22	2.23	2.23	0.158
NO_3^-	3.00	3.00	3.00	0.133
Br^-	3.91	3.90	3.91	0.174
NO_2^-	5.00	5.01	5.03	0.290
BrO_3^-	6.34	6.39	6.41	0.563

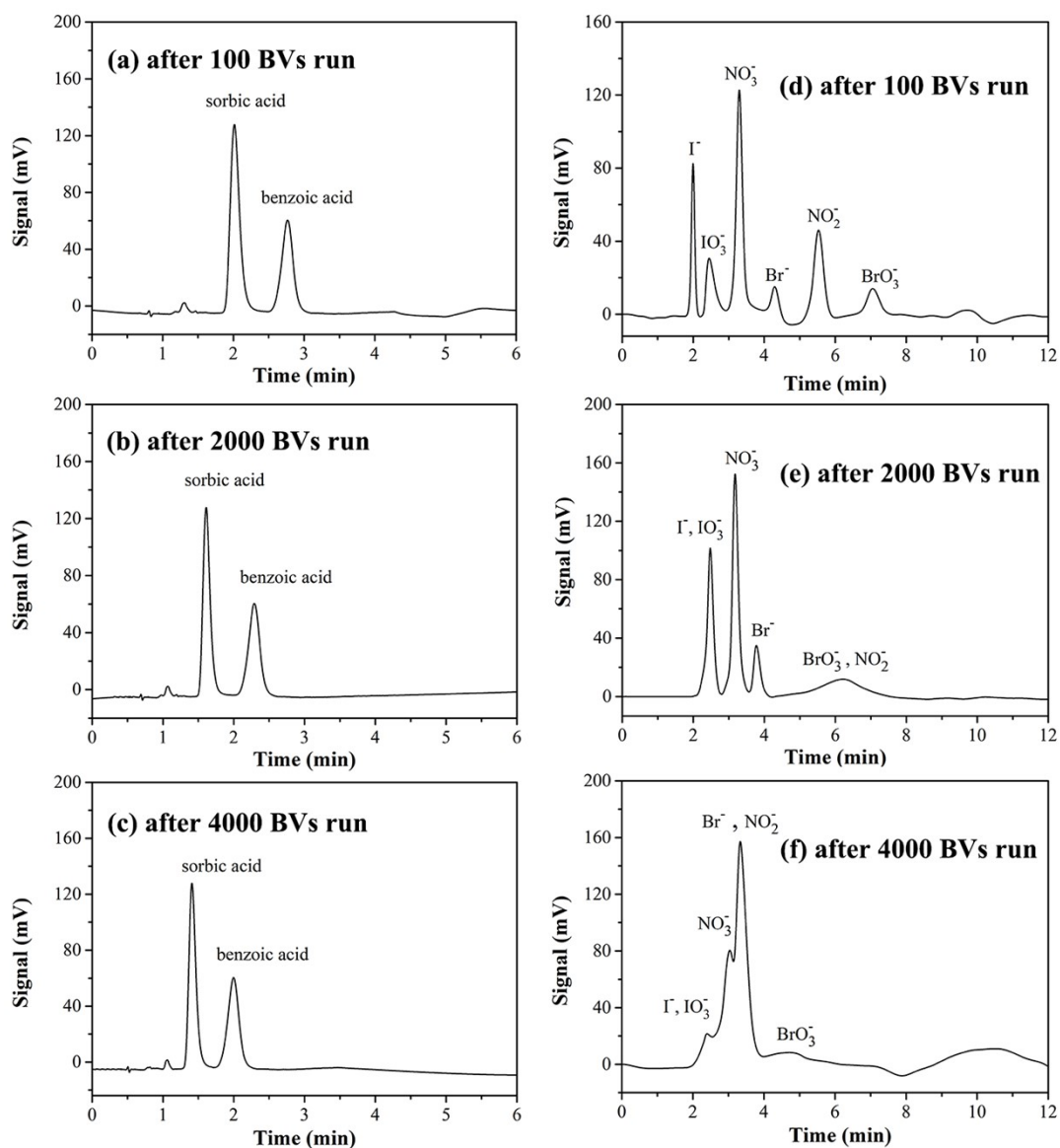


Figure S11. The stability test of the uncross-linked $\text{SiO}_2@(\text{HA-CS})_{12}$ stationary phase. Conditions: (a, b and c): mobile phase (A) 1 mmol/L KH_2PO_4 in water, (B) ACN; isocratic 70%A; UV detection wavelength: 254 nm; (d, e and f): mobile phase (A) 50 mmol/L NH_4Ac in water (pH 4.88, titrated with acetic acid), (B) ACN; isocratic 20% A; UV detection wavelength: 210 nm.

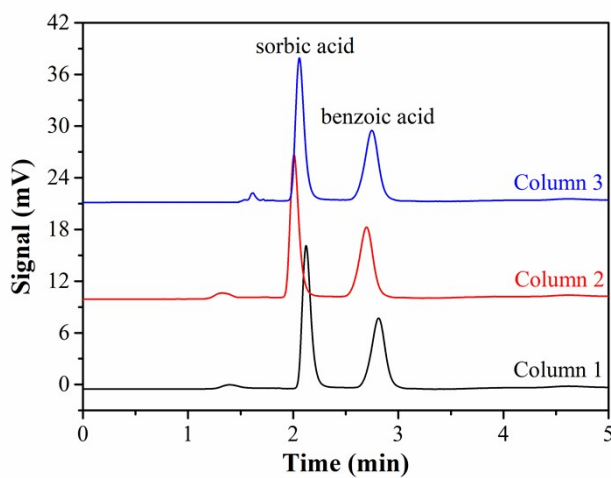


Figure S12. Lot-to-lot repeatability of the synthesis procedure. Chromatographic conditions: mobile phase (A) 1 mmol/L KH_2PO_4 in water, (B) ACN; isocratic 70%A; UV detection wavelength: 254 nm. The Y axis was shifted for better display.

Table S4. Results of lot-to-lot repeatability of the synthesis procedure.

	Retention time (min)		Resolution
	sorbic acid	benzoic acid	
Batch 1	2.06	2.75	2.37
Batch 2	2.02	2.70	2.40
Batch 3	2.12	2.81	2.28
RSD	2.44%	2.00%	2.64%