Supporting Information for

Peptide and Protein Assays Using Bio-Affinity Arrays Combined with Ambient Ionization Mass Spectrometry

Xuemeng Zhang,[a]‡ Wei Wang,[a]‡ Richard N. Zare[b] and Qianhao Min*[a]*

a. State Key Laboratory of Analytical Chemistry for Life Science, Chemistry and Biomedicine Innovation Center, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, P. R. China

b. Department of Chemistry, Stanford University, Stanford, California 94305, United States

‡ These authors contributed equally to this work.

***Corresponding authors**

E-mail: rnz@stanford.edu (Richard N. Zare)

E-mail: minqianhao@nju.edu.cn (Qianhao Min)

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Experimental Section

1. Chemicals and materials

Cytidine (C), uridine (U), adenosine (A), inosine (I), guanosine (G), xanthosine (X), 5-methyluridine (5-U), deoxyadenosine (DA), thymidine (T), deoxyuridine (DU), isoprenaline hydrochloride (IE), dopamine hydrochloride (D), (3-glycidoxypropyl) trimethoxysilane (GPTS), polyethyleneimine (PEI, MW ~10,000 Da), 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 3-aminophenylboronic acid (APBA) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Trifluoroacetic acid (TFA), formic acid (FA), trypsin (from bovine pancreas, TPCK treated), α-casein (from bovine milk), β-casein (from bovine milk), albumin (from bovine serum), peroxidase (from horseradish), cytochrome *c* (from bovine heart), L-tyrosine (Y), L-arginine (R), L-valine (V), L-tryptophan (W), epinephrine hydrochloride (E), sodium cyanoborohydride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Titanium(IV) oxide (TiO_2), niobium(V) oxide (Nb_2O_5), potassium carbonate (K_2CO_3), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), glutaraldehyde 25% aqueous solution (GDA), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), and 10 % tetrabutyl ammonium hydroxide (TBAH) were obtained from Shanghai Reagent Co. (Shanghai, China). Fetal bovine serum (FBS) and Human α-thrombin were purchased from Thermo-Fisher Scientific (Waltham, MA, USA). The human α-thrombin binding aptamer (TBA) was synthesized by Sangon Biotechnology Company (Shanghai, China). The sequence of TBA is 5′-HS-$(CH_2)_6$-TT TTT TTT TTG GTT GGT GTG GTT GG-3′. Silica microspheres (SMSs) with particle size of 3 μm and pore size of 120 Å were provided by Alfa Aesar China (Tianjin, China). Acetonitrile (ACN) and methanol (MeOH) were chromatographic grade from Merck (Darmstadt, Germany). Ultrapure water was obtained from MilliQ gradient ultrapure water system (Millipore Inc., MA, USA). All other chemicals were of analytical grade without further treatment.

2. Instrumentation and characterization

Scanning electron microscopy (SEM) was done on a Hitachi S-4800 field emission electron microscope at an accelerating voltage of 5 kV. UV-vis spectra were recorded on a UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan). Zeta potential was tested on a Nano-Z zeta potential analyzer (Malvern Instruments, USA). Fourier transform infrared spectroscopy (FT-IR) spectra were acquired from a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific, USA).

3. Experimental procedures

3.1 Tryptic digestion of standard proteins

Standard protein (1 mg α-casein, β-casein, ovalbumin, HRP, Cyt c or BSA) was dissolved in 1 mL 25 mM ammonium bicarbonate buffer solution (pH = 8.1) and denatured at 100 °C for 5 min. Then the protein was digested with trypsin at the ratio of enzyme-to-substrate of 1:20 (w/w) in a water bath at 37 °C for 18 h. The tryptic digestion was terminated by addition of 10 % TFA. The obtained digests were kept at -80 °C for further use.

3.2 Preparation of bio-affinity nanomaterials

3.2.1 Preparation of STiNbNSs. The STiNbNSs were prepared according to the previous literature.[1] Layered $KTiNbO_5$ was prepared by grinding and heating a stoichiometric mixture of K_2CO_3, Nb_2O_5, and TiO_2 at 1100 °C for 24 h. Afterwards, 1 g prepared $KTiNbO_5$ in 5 M HNO_3 aqueous solution was stirred at room temperature for 3 days. After being rinsed three times by water, layered oxides $HTiNbO_5$ was collected and heated in 60 °C until dryness. Then 1 g layered $HTiNbO_5$ was suspended in 100 mL water and stirred with the dropwise addition of tetrabutyl ammonium hydroxide (TBAH) until the pH value increased to 10. After continuous stirring for 7 days, the suspension was centrifuged at 9000 rpm for 10 min, with the supernatant collected for further use. In the next step, 100 mL 1 M HNO_3 aqueous solution was dropwise added into 100 mL supernatant containing STiNbNSs while stirring. The introduction of H^+ induced restacking of STiNbNSs and generated white aggregates of restacked STiNbNSs. The precipitates were recovered by centrifugation, washed three times by ethanol and water to remove TBAH and HNO_3, and finally dried in an oven at

70 °C.

3.2.2 Preparation of APBA-SMSs. As shown in Figure S10, the APBA-SMSs were prepared according to the previous literature.[2, 3] In detail, 2.0 g SMSs were dispersed into 18% HCl solution and stirred at room temperature for 24 h to activate the hydroxyl groups on the surface of the SMSs. The activated SMSs were rinsed thoroughly with deionized water until the pH value increased to 7 and dried under vacuum at 40°C overnight. Then the activated SMSs (2.0 g) were reacted with 3.0 mL GPTS in 40 mL anhydrous toluene under nitrogen with continuous stirring at 110°C for 24 h. After being rinsed three times by anhydrous ethanol, the product (denoted as SMSs-epoxy) was collected and dried under vacuum at 40°C overnight. Afterwards, 0.4 g of SMSs-epoxy were dispersed in 30 mL 1:1 ethanol/water containing 0.5 g $PEI_{10,000}$. After continuous stirring at 65°C for 24 h, the resulting SMSs (denoted as SMSs-PEI) were collected by centrifugation and rinsed successively with anhydrous ethanol to remove the excess PEI. Subsequently, 100 mg SMSs-PEI and 100 mg sodium cyanoborohydride were dispersed in 40 mL anhydrous methanol containing 5% glutaraldehyde with continuous stirring for 12 h at room temperature. After washing with anhydrous methanol, the glutaraldehyde-activated SMSs-PEI were dispersed in 30 mL anhydrous methanol containing 50 mg APBA for another 24 h reaction. Meanwhile, 100 mg sodium cyanoborohydride was added every 4 h during the reaction. The obtained products (denoted as SMSs-PEI-APBA) were isolated from the supernatant, rinsed three times with anhydrous methanol, and dried under vacuum at 40°C overnight.

3.2.3 Synthesis of gold nanoparticles. Gold nanoparticles (AuNPs) were synthesized according to the classic protocols reported in the literature.[4] Briefly, 90 mL of 1 mM $HAuCl_4$ solution was added into a beaker with continuous stirring at 120 ℃. Until the solution violently boils, 9 mL of 38.8 mM trisodium citrate was added quickly to the beaker. Afterwards, the solution was stirred rapidly for 15 min. After cooling to room temperature, the solution was filtered through 0.22 μm microporous membrane, and the color of obtained solution is deep red.

3.3 Fabrication of BAAs

Figure S1b shows the parameters of BAAs. The BAAs were fabricated by recruiting a polydimethylsiloxane (PDMS) mold covered glass slide as substructure, and the slurry of bio-affinity nanomaterials were deposited and dried in the arrayed wells (3 mm in diameter, 3 mm in depth). To fabricate the PDMS mold, the PDMS prepolymer mixture (mass ratio of monomer and curing agent = 10:1) was poured into a plastic dish to reach a height of ~3 mm and cured horizontally at 70 °C for 2 h. After the PDMS mold was peeled off and cut into a rectangle, a series of holes with a 4 mm gap were drilled by a biopsy punch (i.d. 3 mm). The perforated PDMS membrane was then bonded to the glass slide after exposing both of them to oxygen plasma for 12 s.

For STiNbNSs-BAAs and APBA-SMSs-BAAs, the arrayed wells on the PDMS-glass sheet were respectively filled with 10 μL of 10 mg/mL suspension of STiNbNSs or APBA-SMSs; then the PDMS-glass sheet was subjected to evaporation under 70 °C until dryness of the nanomaterials at the bottom of each well. They were stored at room temperature for further sample loading.

For TBA-AuNPs-BAAs, 10 μL of 80 nM gold colloid solution was added to the arrayed wells on the PDMS-glass sheet. Then the PDMS mold was peeled off, leaving the AuNPs-covered spots on the glass slide for further heating at 200 ℃ for 2 h. The resulting glass slide was washed twice with deionized water to remove weakly bounded AuNPs and dried for TBA modification. Prior to modification, TBA was denatured at 95℃ for 10 min and cooled to room temperature. Then TBA was activated with 10 mM freshly prepared TCEP (pH 5.2) for 1 h to break the disulfide bond formed between two DNA strands. Afterward, 10 μL of 10 μM TCEP-activated TBA was deposited onto the AuNPs-covered spots and incubated in a dark humidity chamber at 4 ℃ overnight. Afterwards, the slide was rinsed with deionized water and binding buffer (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in deionized water, pH 7.4) twice, respectively. Then 10 μL of 10 mM MCH in binding buffer was dropped onto the arrayed spots to block active sites and nonspecific adsorption. Finally, the TBA-AuNPs-BAAs were washed with binding buffer twice and kept at 4 ℃ for sample loading.

3.4 Configuration of BAAs-nano-DESI-MS

Figure S1a shows schematically the configuration of the BAAs-nano-DESI-MS setup. The ambient ionization MS system contains the following six main components: (1) the BAAs preloaded with target biomolecules; (2) the primary capillary (o.d. 360 μm, i.d. 250 μm, Polymicro Technologies, USA) connected with a syringe (500 μL, Hamilton Co.) for continuously infusing the solvent to the sample spots; (3) the secondary capillary (length 3.5~4.7 cm, o.d. 360 μm, i.d. 250 μm) for directing the solvent into MS inlet via self-aspiration caused by the negative pressure; (4) liquid microjunction forming between primary and secondary capillary tips (Figure S1c, o.d. 140 μm, i.d. 70 μm) to extract the surface species on the sample spots; (5) an automated x−y−z translational platform for placing and moving the BAAs; and (6) an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) for MS interrogation. The syringe pump was used to deliver a solvent stream with flow rate at 2-5 μL/min. The distance between the emitter terminal of secondary capillary and the MS inlet was optimized to be 1-2 mm for stable ion signal and flow rate. The electrospray voltage was set at 4.0-4.5 kV and applied onto the needle of the syringe. A camera was oriented to the liquid microjunction for finely tuning the relative position of capillaries and monitoring the sampling process.

3.5 Sample loading and processing on BAAs

Figure 1a illustrates sample loading process. For ambient ionization MS detection of phosphopeptides and glycopeptides, the samples were first diluted with loading solution (2 % TFA for phosphopeptides, 15 mM NH_4HCO_3 with pH 9.0 for glycopeptides). Then, 10 μL of sample was dropped into the wells of the STiNbNSs-BAAs or APBA-SMSs-BAAs and incubated at room temperature for 5 min. Afterwards, the remaining sample was pipetted out and the wells were refilled with 10 μL wash solution (0.1 % TFA in acetonitrile/water (50:50, v/v) for phosphopeptides, 7.5 mM NH_4HCO_3 in acetonitrile/water (50:50, v/v) for glycopeptides) to wash the affinity material for 2 times (3 min for each time). After drying under vacuum, the PDMS mold was peeled off, leaving arrayed spots preloaded with target biomolecules for further nano-DESI-

MS analysis.

For ambient MS detection of human α-thrombin, 10 μL of thrombin in binding buffer (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in deionized water, pH 7.4) was dropped onto the TBA-AuNPs-BAAs and then incubated in dark humidity chamber at room temperature for 1 h. Afterwards, the remaining sample was pipetted out and the BAAs were washed by binding buffer, deionized water, and 2 mM NH_4HCO_3 in turn (3 min for each time). Trypsin (0.05 μg) in 2 mM NH_4HCO_3 solution (5 μL) was deposited onto the thrombin-preloaded spots, allowing for on-site digestion of thrombin in the humidity chamber for 1 h at 37 ℃. Then the resulting tryptic digest on the BAAs is subjected to nano-DESI- MS analysis.

3.6 Serum sample preparation and processing on BAAs

The whole blood samples were drawn from pancreatic cancer patients in Affiliated Drum Tower Hospital of Medical Department of Nanjing University using BD Vacutainer (No Additive, Catalog No. 366703) at their admission to the hospital. After centrifugation at 1500 g for 10 min, the supernatant serum was pipetted out, divided into 200 μL Eppendorf tubes (65 μL per tube), and stored at -80 °C. Control serum samples were collect from the healthy volunteers in Nanjing University Hospital and stored at -80 °C for further use. The experiments were approved by Institutional Ethics Board of Affiliated Drum Tower Hospital of Medical Department of Nanjing University. Written informed consent was obtained from the patients or next of kin prior to the study.

The standard phosphopeptide (FQ[pS]EEQQQTEDELQDK, MW 2062.0) stock solution was diluted by 2% TFA for 5 times (final concentration 0.02 mg/mL) and used as the internal standard (IS). 5 μL of raw serum, 4.5 μL of IS solution and 5.5 μL loading solution (2 % TFA) were added into the sample wells on the STiNbNSs-BAAs in turn. After incubation at room temperature for 5 min, the remaining sample solution was pipetted out, and the BAAs was further washed three times by 10 μL 0.1 % TFA in acetonitrile/water (50/50) for 2 min each time. After peeling off the PDMS mold, the sample spots were further incubated with a droplet (3 μL) of 1% NH_3H_2O in

methanol/water (50/50) for 30 s, followed by immediate nano-DESI-MS analysis in drop-and-desorb mode.

3.7 BAAs-nano-DESI-MS detection of target biomolecules

In touch-and-desorb mode, the translational platform carrying BAAs was raised up until the liquid microjunction droplet, the so-called nano-DESI probe, contacted with the surface of biomolecule-preloaded BAAs. Continuous solvent infusion delivered from primary capillary extracted compounds on the localized surface of BAAs, and transported them to secondary capillary and finally into the MS inlet. In detail, 1% NH_3H_2O, 1% formic acid and 0.1% formic acid in methanol/water (50:50, v/v) were respectively used as the desorption and ionization solvent of phosphopeptides, cis-diol compounds, and the tryptic digest of thrombin. Line scan mode is similar to the touch-and-desorb mode, except that nano-DESI probe was dragged horizontally at a constant rate to cross spot by spot on BAAs while sampling and MS recording. Drop-and-desorb mode was invented to increase the desorption efficiency and detection sensitivity of target proteins/peptides by using an aliquot of desorption solvent for pre-elution prior to MS analysis. Typically, after sample loading and processing on BAAs, 3 μL desorption solvent was dropped onto each spot and kept for 0.5-min pre-elution. Nano-DESI-MS analysis were performed by elevating the liquid droplet of desorption solvent to contact with nano-DESI probe, rather than extracting target molecules from the dry surface of BAAs.

3.8 Quantification and spiking-and-recovery experiments of phosphopeptides

The tryptic digests of β-casein were first diluted with loading solution (2 % TFA) at different loading amount (2-200 pmol). A standard phosphopeptide (EAIpYAAPFAKKK, MW 1335.8) was added as an internal standard (IS, m/z 706.82) with concentration of 0.01 mg/mL. The quantification experiment was performed in drop-and-desorb mode. In the spiking-recovery experiment, the tryptic digests of β-casein (20 pmol) mixed with 10-fold tryptic digests of Cyt c and BSA were loaded on STiNbNSs-BAAs. After sample processing on STiNbNSs-BAAs, the spiking-recovery experiments were performed in line scan and drop-and-desorb mode, respectively.

3.9 Nano-DESI MS imaging of phosphopeptides on an individual sample spot of BAAs

The translational platform carrying BAAs was raised up until the nano-DESI probe contacted with the surface of spot (diameter, 4 mm) capturing phosphopeptides from tryptic digests of β-casein (500 pmol). Then the platform moved to the spot on the BAAs for imaging. Solvent (1% NH_3H_2O in methanol/water (50:50, v/v)) was supplied by a syringe pump at a flow rate of 1 μL/min through fused silica capillaries. The motorized stage was set to move at a rate of 30 μm/s laterally, and to step 200 μm between line scans. The one spot shown here required approximately 1.5 h to acquire.

3.10 In-solution pretreatment for the analysis of phosphopeptides

Digests of model proteins were diluted to certain concentrations with a loading buffer containing 2% TFA. 0.1 mg of the STiNbNSs were dispersed into a 100 μL sample solution, and vibrated in a vortex for 30 min. After the supernatant was removed by centrifugation at 12000 g for 3 min, the STiNbNSs combined with the phosphopeptides were rinsed three times with 100 μL of 0.1 % TFA in acetonitrile/water (50:50, v/v). Afterwards, 10 μL of 1% $NH_3 \cdot H_2O$ in methanol/water (50:50, v/v) was introduced to elute the captured phosphopeptides from the STiNbNSs under sonication for 15 min.

3.11 Mass spectrometry settings and data processing

LTQ-Orbitrap XL mass spectrometer was operated at the following main parameters: 1) positive ion mode, spray voltage 4-4.5 kV, capillary voltage 35 V, tube lens voltage 110 V and capillary temperature 300 ℃; 2) negative ion mode, spray voltage 4 kV, capillary voltage -25 V, tube lens voltage -110 V and capillary temperature 300 ℃. A representative nano-DESI mass spectrum was obtained by averaging all the mass spectra in the range of appearance time of corresponding ion signals in the TIC.

Parameters of the BAAs-nano-DESI-MS setup

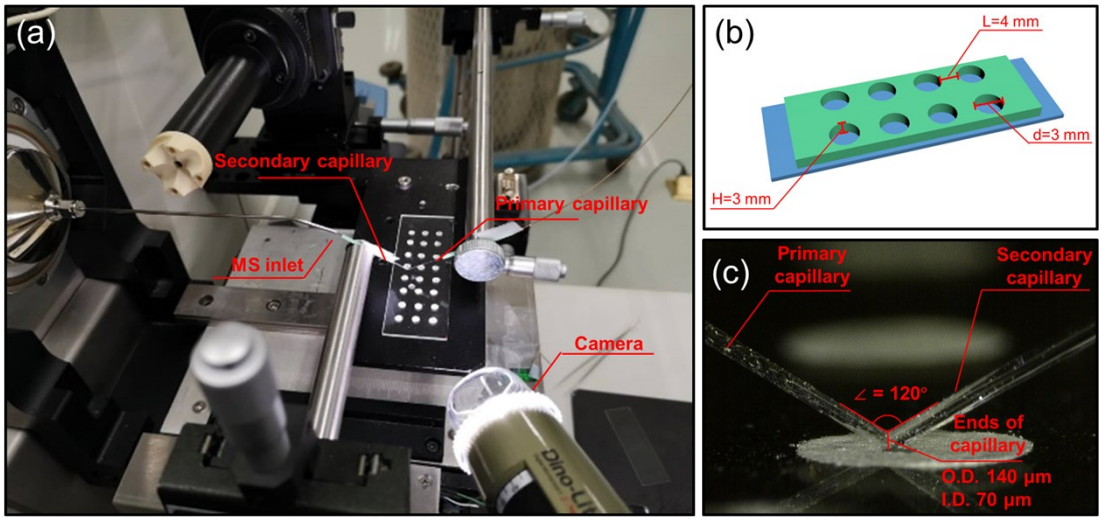


Figure S1. (a) Photograph of the BAAs-nano-DESI-MS setup. (b) Parameters of the arrayed sample wells in the fabrication of BAAs. (c) Photograph of the nano-DESI probe composed of primary capillary and secondary capillary, and the liquid microjunction that mobilizes the analytes on the sample spot.

Nano-DESI-MS analysis of tryptic digests of β-casein, α-casein, and ovalbumin

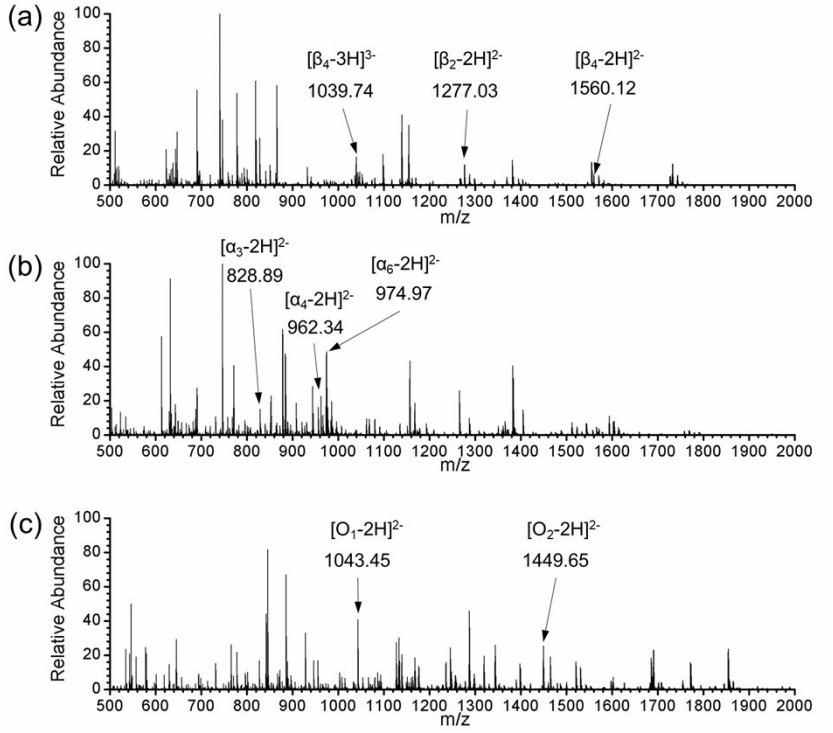


Figure S2. Negative ion mode nano-DESI mass spectra of tryptic digests of (a) β-casein, (b) α-casein, and (c) ovalbumin.

Optimization of solvent for detachment of phosphopeptides from the STiNbNSs-BAAs

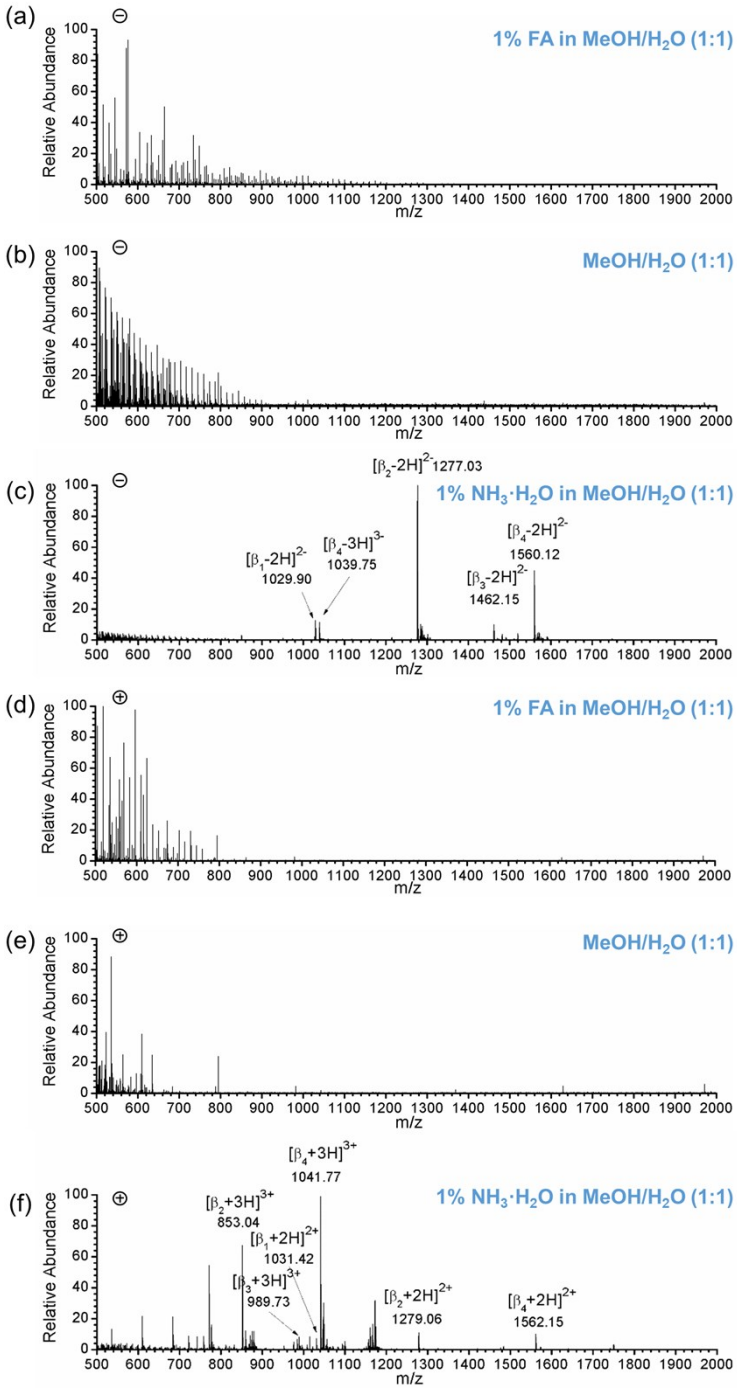


Figure S3. (a-c) Negative and (d-f) positive ion mode mass spectra of phosphopeptides from tryptic digests of β-casein obtained by STiNbNSs-BAAs-nano-DESI-MS respectively using (a, d) 1% formic acid (FA) in MeOH : H_2O (1:1), (b, e) MeOH : H_2O (1:1) and 1% (c, d) NH_3□H_2O in MeOH : H_2O (1:1) as the solvent.

Comparison between nano-DESI-MS, DESI-MS and LDI-MS for analysis of phosphopeptides preloaded on STiNbNSs-BAAs

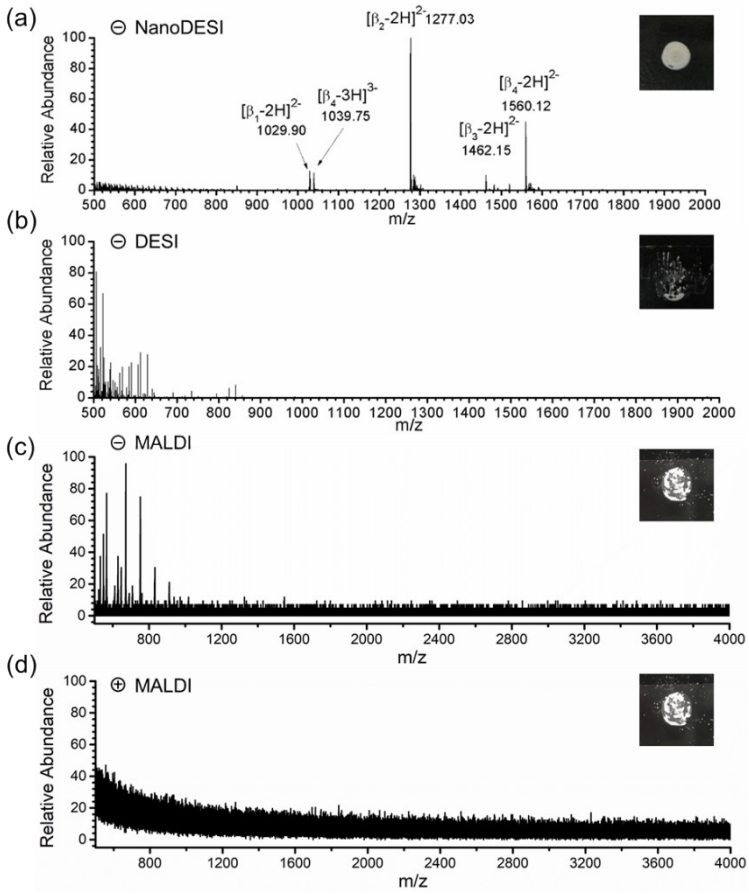


Figure S4. (a-c) Negative ion mode and (d) positive mode mass spectra of phosphopeptides from tryptic digests of β-casein (200 pmol) preloaded on STiNbNSs-BAAs obtained by (a) nano-DESI-MS, (b) DESI-MS and (c and d) LDI-MS. Each inset shows the sample spot after MS analysis.

Sensitivity of STiNbNSs-BAAs-nano-DESI-MS for detection of phosphopeptides

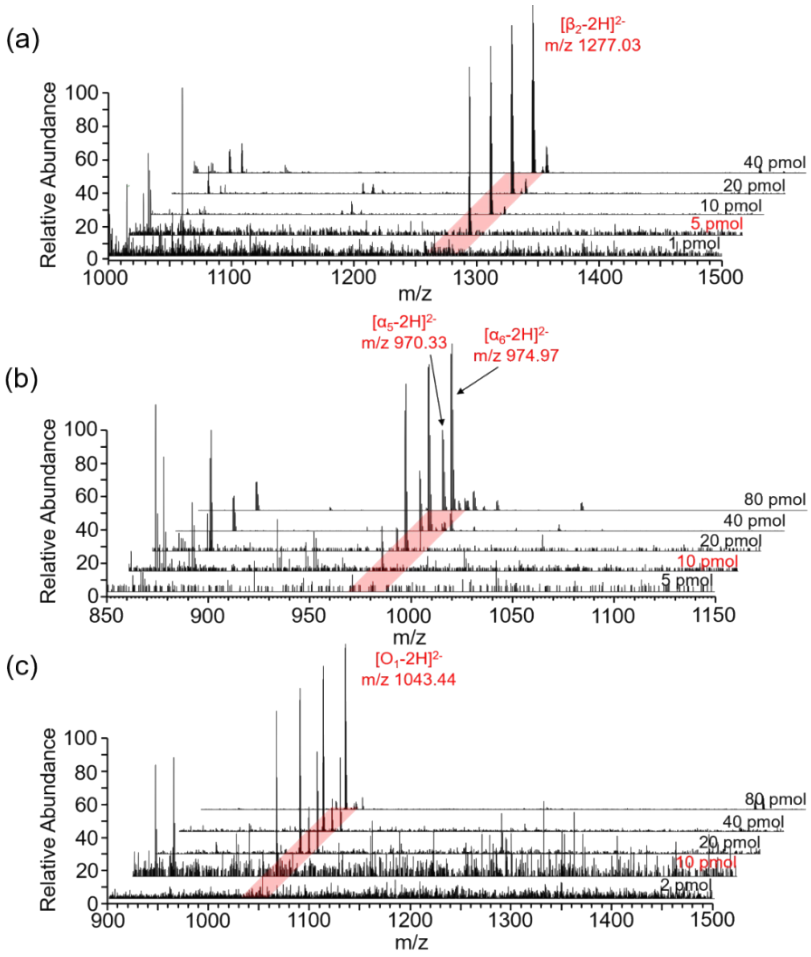


Figure S5. Negative ion mode mass spectra of phosphopeptides from tryptic digests of (a) β-casein, (b) α-casein, and (c) ovalbumin with different loading amount obtained by STiNbNSs-BAAs-nano-DESI-MS.

Co-sampling of peptides from tryptic digests of β-casein and Cyt c on SMSs sample spots by nano-DESI-MS

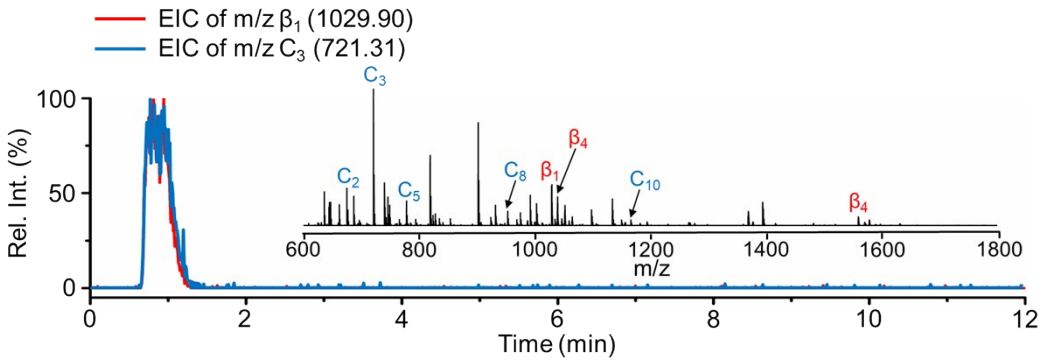


Figure S6. EIC of the ions respectively corresponding to phosphopeptide β_1 (*m/z* 1029.90, red) from tryptic digests of β-casein and peptide C_3 (*m/z* 721.31, blue) from tryptic digests of Cyt c.

Specificity of STiNbNSs-BAAs-nano-DESI-MS to phosphopeptides

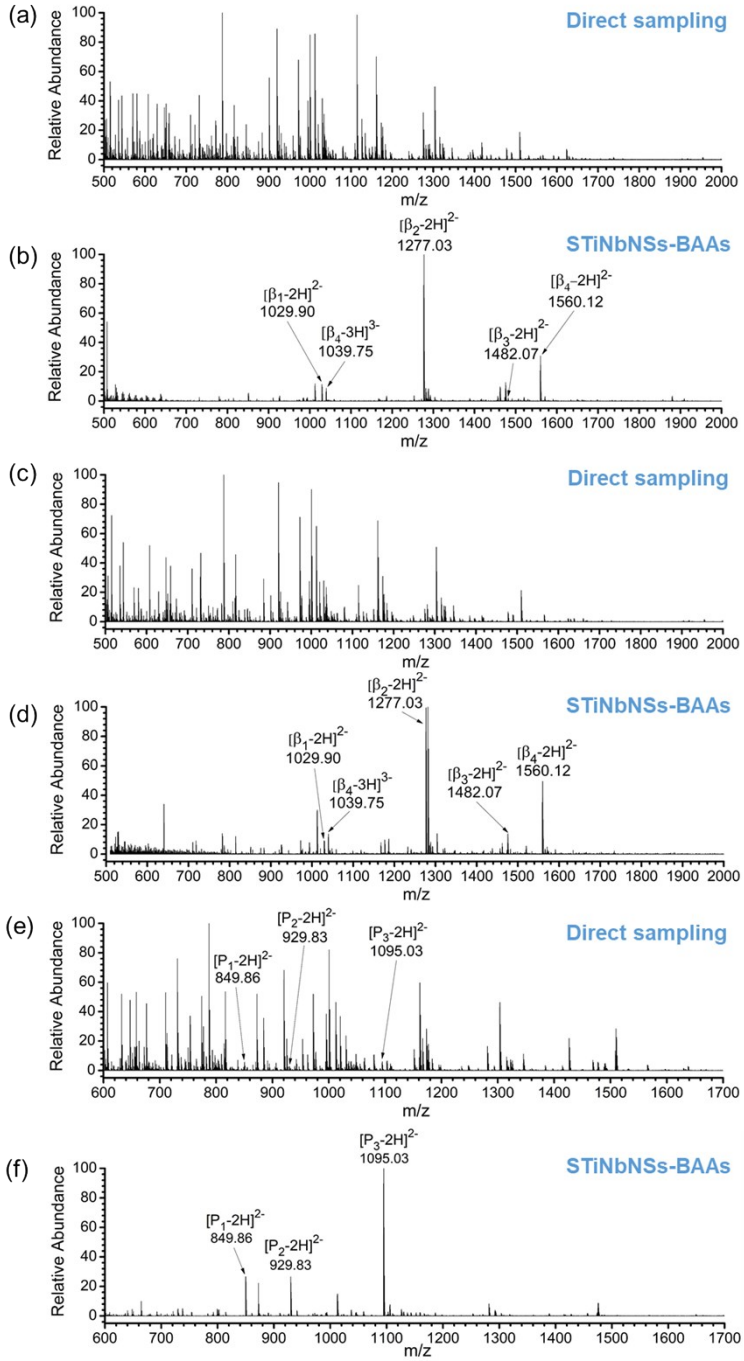


Figure S7. Negative ion mode mass spectra of the mixtures of tryptic digests of β-casein (40 pmol) and BSA at mass ratio of (a, b) 1:10 and (c, d) 1:100 obtained by (a, c) direct nano-DESI-MS and (b, d) STiNbNSs-BAAs-nano-DESI-MS. (e, f) Negative ion mode mass spectra of the 3 standard phosphopeptides (40 pmol) mixed with 10-fold tryptic digests of Cyt c, lysozyme and BSA obtained by (e) direct nano-DESI-MS and (f) STiNbNSs-BAAs-nano-DESI-MS.

Heterogeneity assessment of single sample spot of STiNbNSs-BAAs for MS detection of phosphopeptides

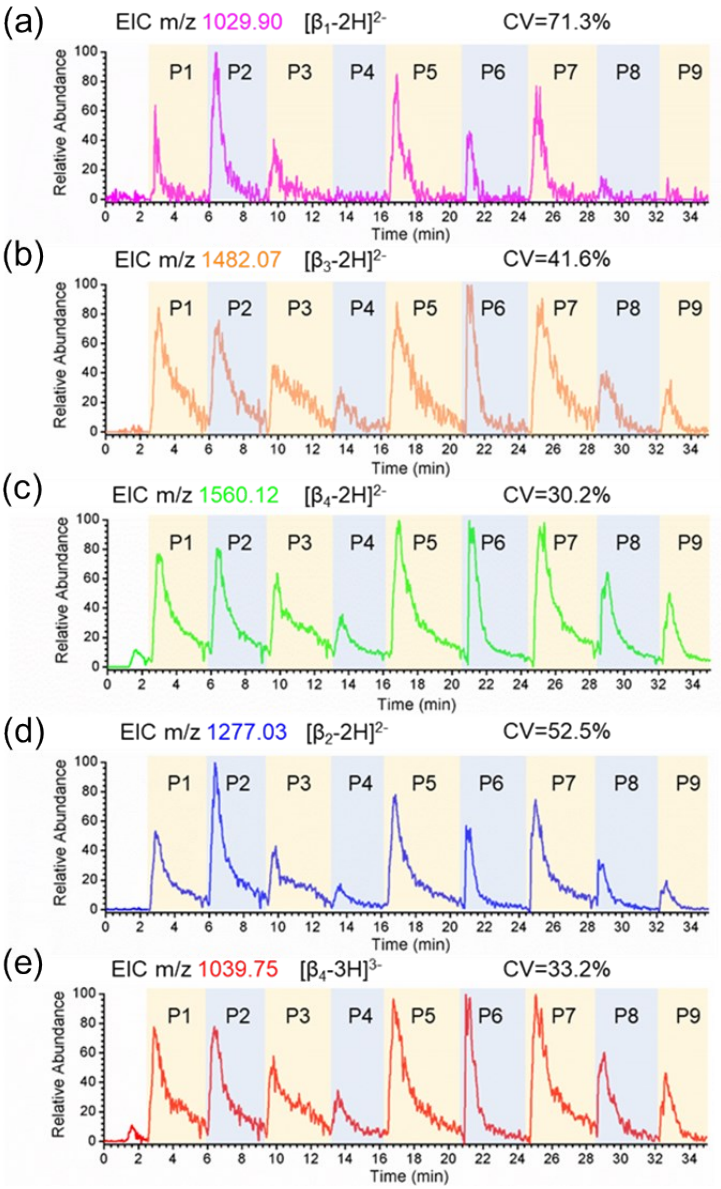


Figure S8. EIC of individual ion signal of phosphopeptides including (a) *m/z* 1029.90, (b) *m/z* 1482.07, (c) *m/z* 1560.12, (d) *m/z* 1277.03 and (e) *m/z* 1039.75 and from tryptic digests of β-casein at 9 distributed micro-areas (~ 200 μm × 200 μm) of a single spot obtained by BAAs-nano-DESI-MS in touch-and-desorb mode.

Nano-DESI-MS imaging of phosphopeptides on a single sample spot of STiNbNSs-BAAs

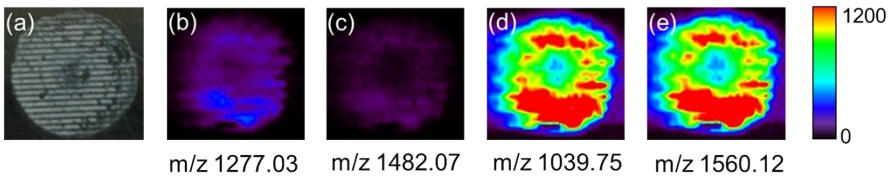


Figure S9. (a) Photographs of individual sample spot after nano-DESI mass spectrometry imaging. Nano-DESI MS imaging of phosphopeptides including (b) *m/z* 1277.03, (c) *m/z* 1482.07, (d) *m/z* 1039.75 and (e) *m/z* 1560.12 from tryptic digests of β-casein (200 pmol) trapped by STiNbNSs-BAAs.

Comparison between STiNbNSs-BAAs-nano-DESI-MS in drop-and-desorb mode and in-solution pretreatment for analysis of phosphopeptides

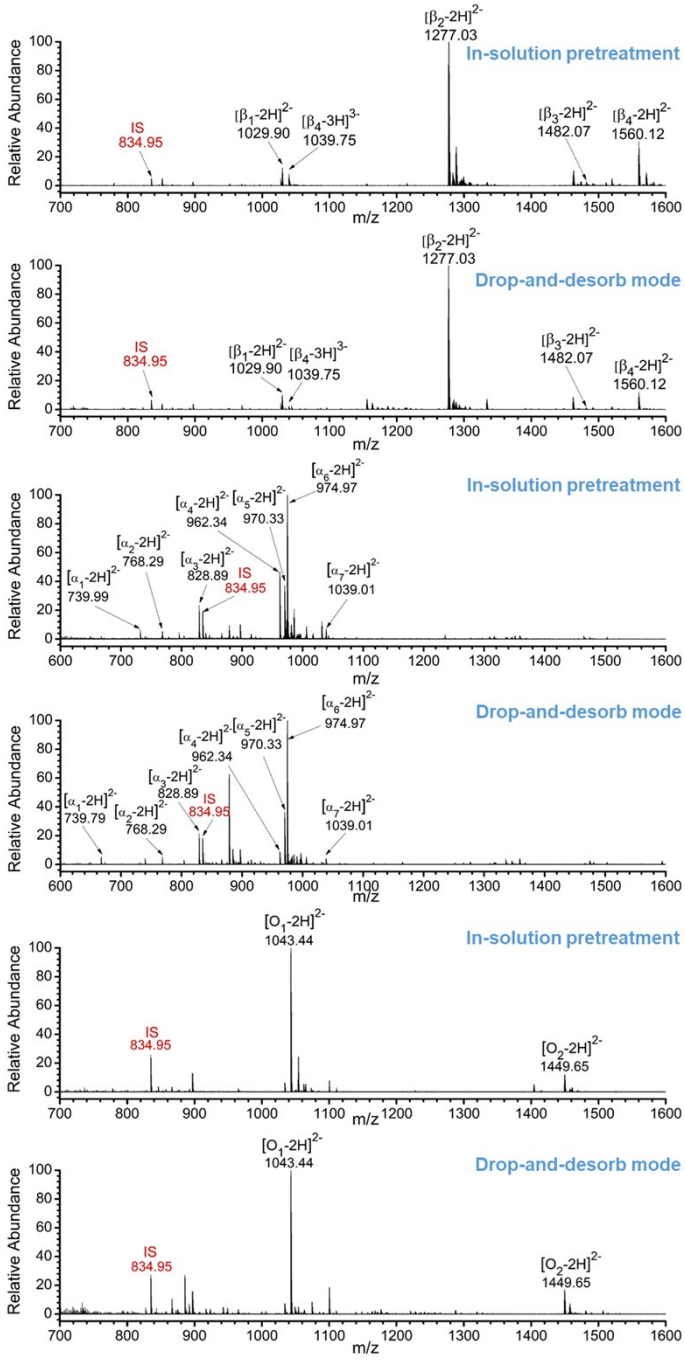


Figure S10. Negative ion mode nano-DESI mass spectra of phosphopeptides from (a, b) β-casein (200 pmol), (c, d) α-casein (200 pmol), and (e, f) ovalbumin (200 pmol) obtained by (a, c, e) in-solution pretreatment with STiNbNSs and (b, d, f) STiNbNSs-BAAs-nano-DESI-MS in drop-and-desorb mode.

The quantification and spiking-and-recovery experiments of phosphopeptides

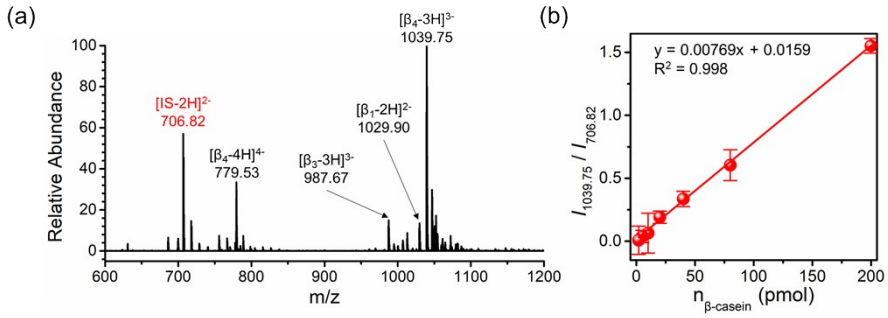


Figure S11. (a) Negative ion mode nano-DESI mass spectra of phosphopeptides β_4 (m/z 1039.75) from β-casein (80 pmol) obtained by STiNbNSs-BAAs-nano-DESI-MS in drop-and-desorb mode. A standard phosphopeptide (EAIpYAAPFAKKK) was added as an internal standard (IS, m/z 706.82). (b) Ion signal intensity ratio of phosphopeptide β4 (m/z 1039.75) to internal standard phosphopeptide (m/z 706.82) as a function of the loading amount of phosphopeptide from β-casein. The data were obtained from three independent experiments.

Synthetic route and characterization of APBA-SMSs

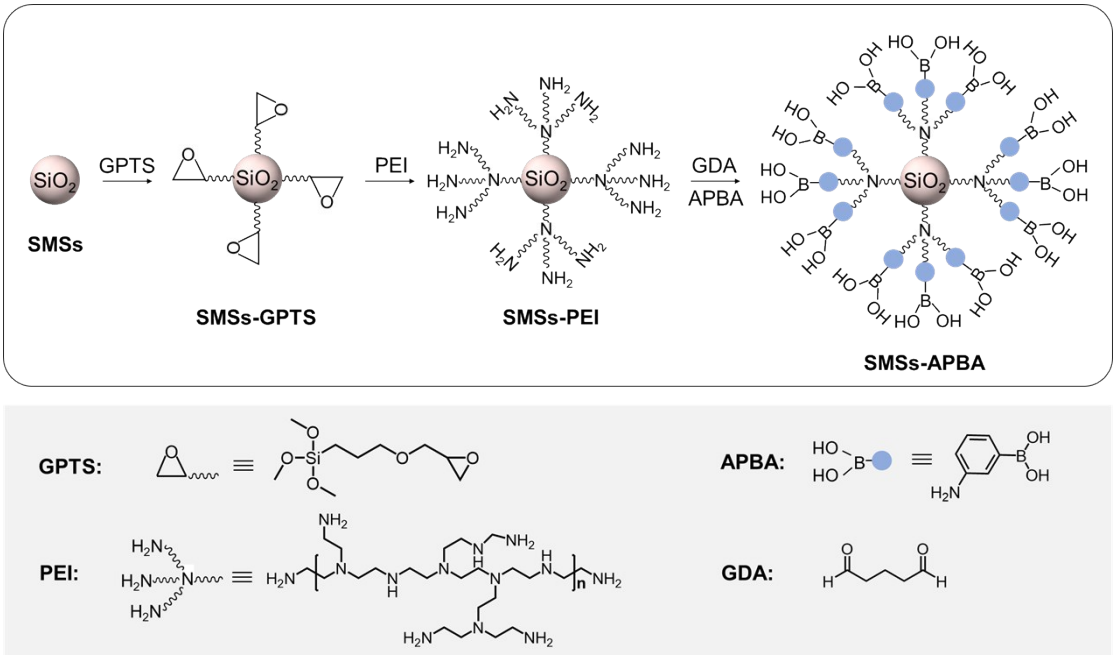


Figure S12. The synthetic route of the APBA-SMSs.

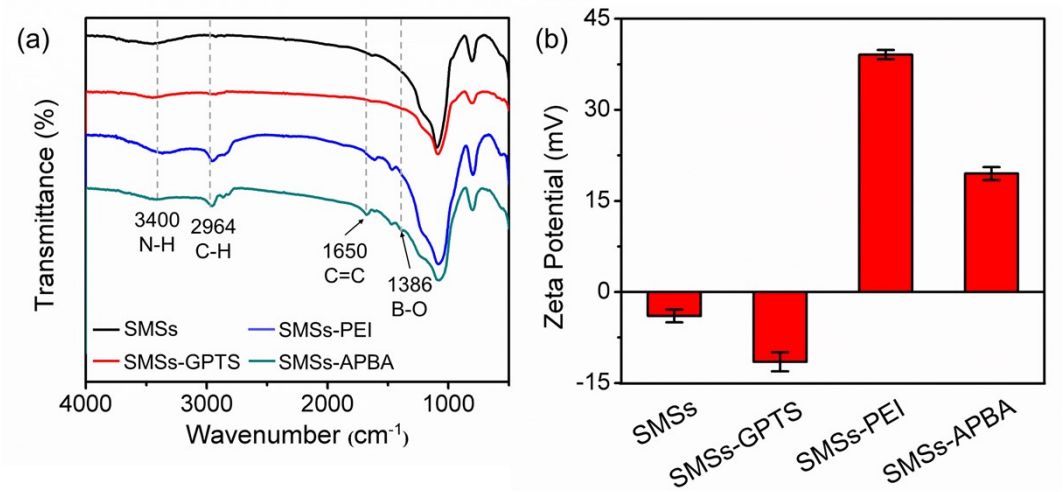


Figure S13. (a) FT-IR spectra of SMSs, SMSs-GPTS, SMSs-PEI and SMSs-APBA. The peak around 2964 cm^{-1} of SMSs-GPTA was attributed to the stretch vibration of C-H. The peak at 3400 cm^{-1} was originated from vibration of N-H, which demonstrates the modification of PEI through ring-opening reaction between epoxy group and amino group. Due to further functionalization with APBA, the peaks at 1386 cm^{-1} and 1650 cm^{-1} appear in the FT-IR spectra of SMSs-APBA, which were derived from vibration of B-O and benzene skeleton vibration, respectively. (b) Zeta potential of SMSs, SMSs-GPTS, SMSs-PEI, and SMSs-APBA. The zeta potential increased from -11.5 to 39.1

mV due to the higher surface density of amino groups caused by the coverage of PEI. Then, this value was decreased to 19.5 mV after further derivatization with APBA, indicating the conversion of initial amino groups to negatively charged phenylboronic acids.

Specificity of APBA-SMSs-BAAs-nano-DESI-MS to neurotransmitters and nucleosides

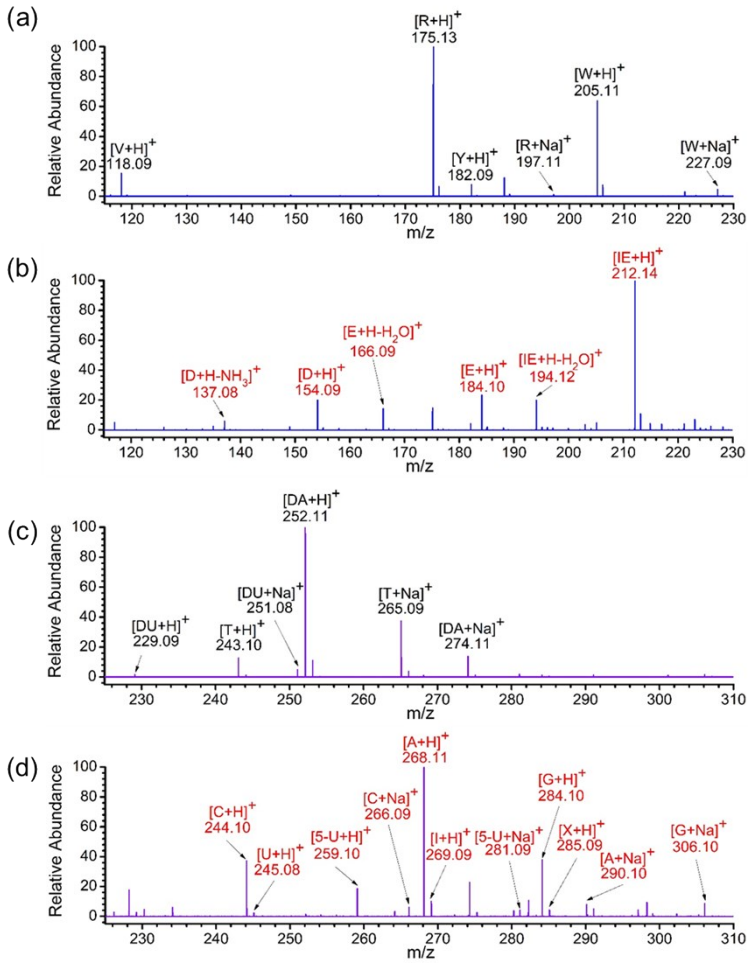


Figure S14. Positive ion mode nano-DESI mass spectra of (a, b) the mixtures of 3 neurotransmitters (10 μg/mL) and 4 amino acid at mass ratio of 1:100, and (c, d) the mixtures of 7 nucleosides (10 μg/mL) and 3 deoxynucleosides at mass ratio of 1:100 obtained by (a, c) direct nano-DESI-MS and (b, d) APBA-SMSs-BAAs-nano-DESI-MS. Table S6 lists detailed information of the neurotransmitters, amino acids, nucleosides, and deoxynucleosides. .

Specificity of APBA-SMSs-BAAs -nano-DESI-MS to glycopeptides

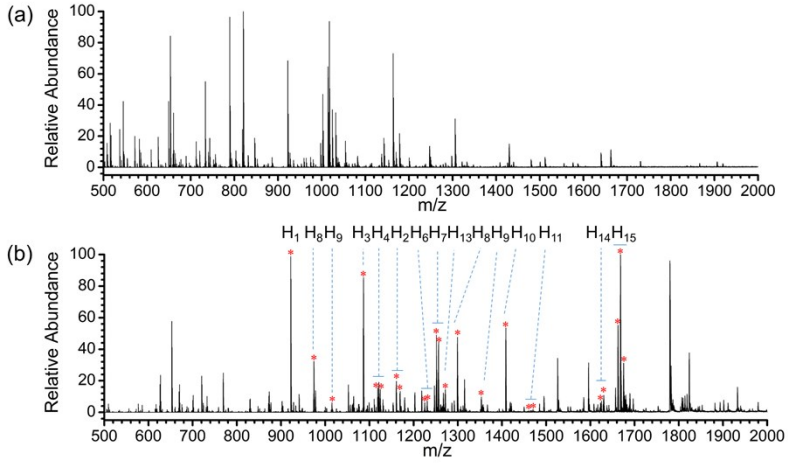


Figure S15. Positive ion mode mass spectra of mixed tryptic digests of HRP (40 pmol) and BSA at mass ratio of 1:100 obtained by (a) direct nano-DESI-MS and (b) SMSs-APBA-BAAs-nano-DESI-MS.

Verification of TBA modification on AuNPs-BAAs

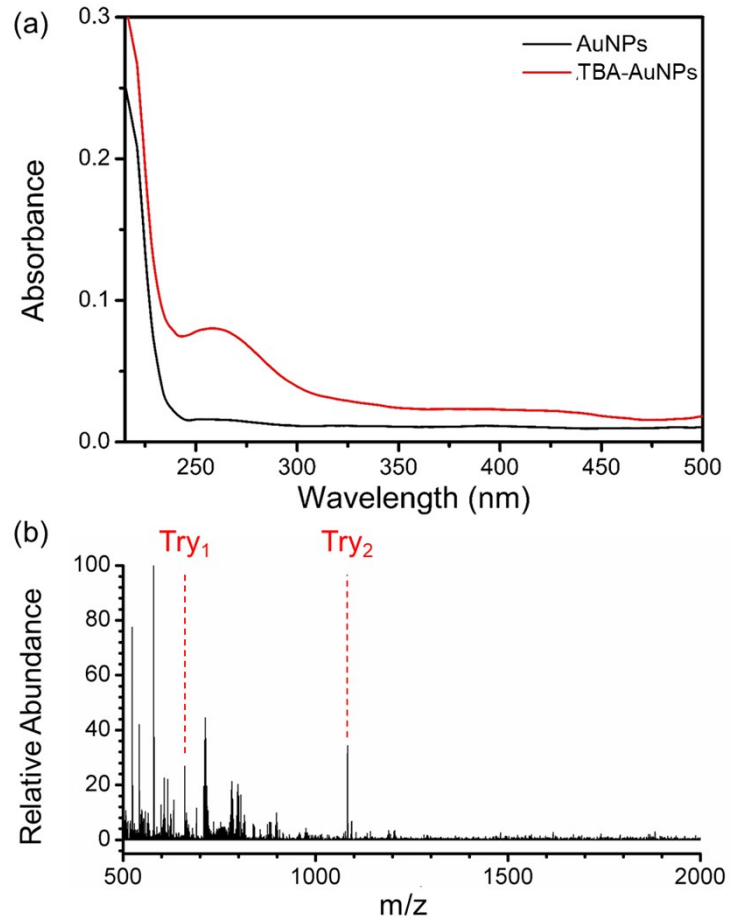


Figure S16. (a) UV absorption spectra of AuNPs and TBA-modified AuNPs respectively scraped off from AuNPs-BAAs and TBA-AuNPs-BAAs. (b) Positive ion mode nano-DESI mass spectrum of thrombin (80 pmol) processed on AuNPs-BAAs as a control.

Specificity of TBA-AuNPs-BAAs to human α-thrombin

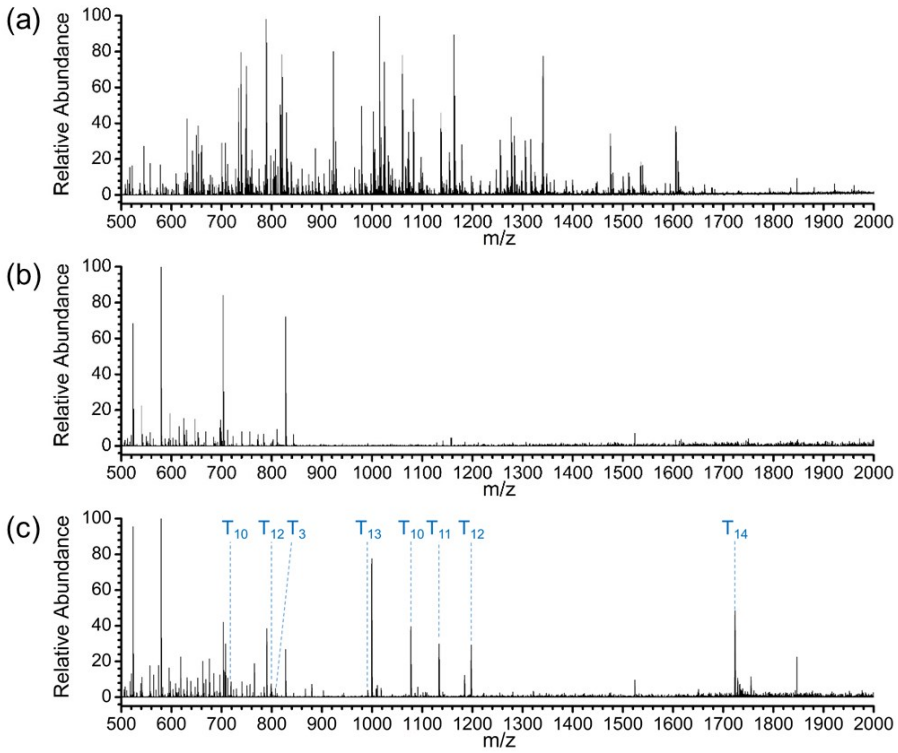


Figure S17. (a) Positive ion mode nano-DESI mass spectrum of tryptic digests of 10 times diluted fetal bovine serum (FBS). (b, c) Nano-DESI mass spectra of (b) 10 times diluted FBS and (c) thrombin (13.6 pmol) spiked 10 times diluted FBS after processing on TBA-AuNPs-BAAs.

Specificity of TBA-AuNPs-BAAs to human α-thrombin

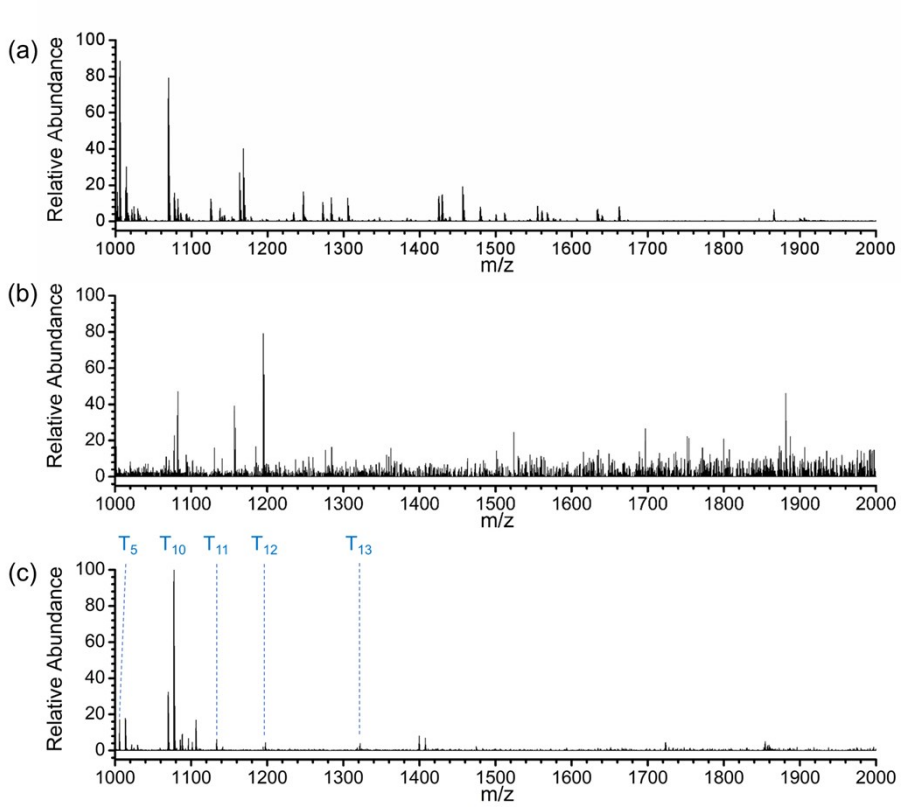


Figure S18. (a) Positive ion mode nano-DESI mass spectrum of mixed tryptic digests of BSA and Cyt c (136 pmol). (b, c) Nano-DESI mass spectra of (b) mixed digests of BSA and Cyt c (136 pmol) and (c) thrombin (13.6 pmol) spiked mixed digests of BSA and Cyt c (136 pmol) after processing on TBA-AuNPs-BAAs.

The quantification experiments of human α-thrombin

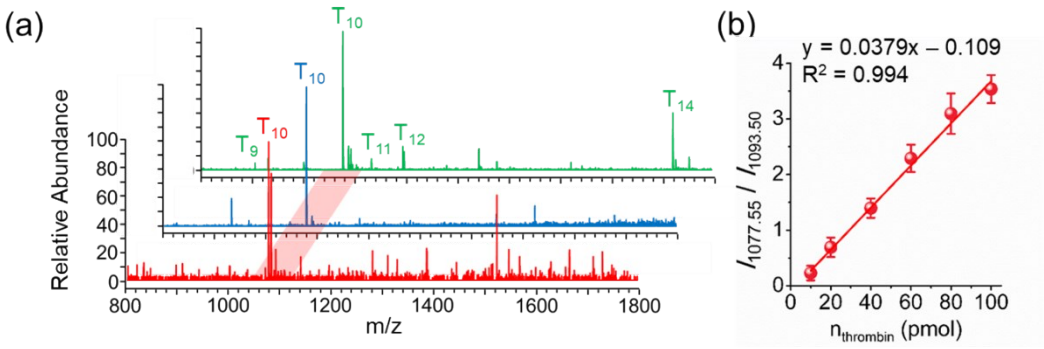


Figure S19. Positive ion mode mass spectra of tryptic digests of thrombin with loading amount of (c) 40 pmol (green), (c) 20 pmol (blue) and (c) 5.4 pmol (red) obtained by AuNPs-TBA-BAAs-nano-DESI-MS. (d) Ion signal intensity ratio of peptide T_{10} to internal standard peptide as a function of the loading amount of thrombin. The data were obtained from three independent experiments.

Identification of T_{10} ion signal from human α-thrombin by MS/MS

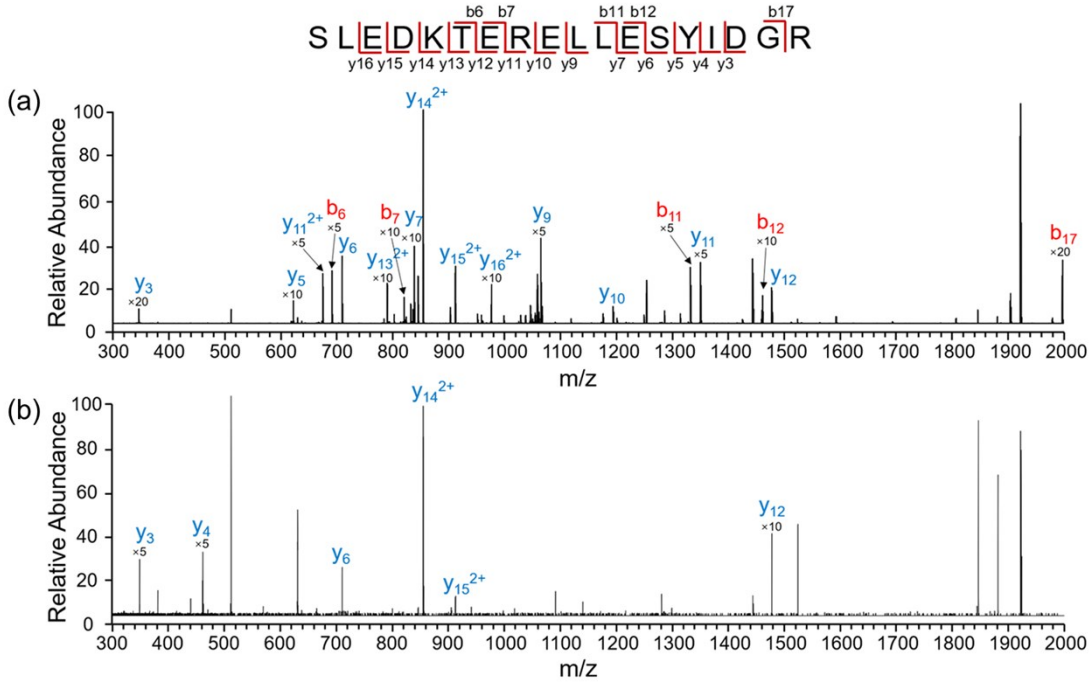


Figure S20. MS/MS spectra of peptide $[T_{10}+2H]^{2+}$ from (a) standard human α-thrombin and (b) tryptic digests of 10-fold diluted FBS spiked with human α-thrombin.

Table S1. Sequence information of phosphopeptides detected from tryptic digests of β-casein.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
β_1	1029.90	2	2061.82	FQ[pS]EEQQQTEDELQDK
β_2	1277.03	2	2556.09	FQ[pS]EEQQQTEDELQDKIHPF
β_3	1482.07	2	2966.16	ELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
β_3	987.67	3	2966.16	ELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
β_4	779.53	4	3122.26	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
β_4	1039.75	3	3122.26	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
β_4	1560.12	2	3122.26	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR

[pS]: phosphorylated serine.

Table S2. Sequence information of peptides from the tryptic digests of Cyt c.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
C_1	632.98	1	633.38	IFVQK
C_2	676.37	1	677.37	YIPGTK
C_3	721.31	1	722.35	EDLIAY
C_4	737.90	2	1477.81	KTEREDLIAYLK
C_5	777.45	1	778.44	MIFAGIK
C_6	797.88	2	1597.77	KTGQAPGFTYTDANK
C_7	801.95	2	1605.91	KKTEREDLIAYLK
C_8	953.49	1	954.49	TGPNLHGLF
C_9	1103.55	2	2208.11	GITWKEETLMEYLENPKK
C_{10}	1166.61	1	1167.62	TGPNLHGLFGR
C_{11}	1520.14	2	3042.58	EETLMEYLENPKKYIPGTKM IFAGIK

Table S3. Sequence information of detected phosphopeptides from tryptic digests of α-casein.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
α_1	739.79	2	1481.61	TVD[Mo]E[pS]TEVFTK
α_2	768.29	2	1538.59	EQL[pS]T[pS]EENSKK
α_3	828.89	2	1659.79	VPQLEIVPN[pS]AEER
α_4	962.34	2	1926.69	DIG[pS]E[pS]TEDQAMEDIK
α_5	970.33	2	1942.68	DIG[pS]E[pS]TEDQA[Mo]EDIK
α_6	649.31	3	1950.95	YKVPQLEIVPN[pS]AEER
α_6	974.97	2	1950.95	YKVPQLEIVPN[pS]AEER
α_7	1039.01	2	2080.04	KYKVPQLEIVPN[pS]AEER
α_8	1316.44	2	2634.33	NT[Mo]EHV[pS][pS][pS]EESII[pS]QETYK
α_9	1346.00	2	2694.01	VNEL[pS]KDIG[pS]E[pS]TEDQA[Mo]EDIK
α_{10}	1358.93	2	2719.91	QMEAE[pS]I[pS][pS][pS]EEIVPNPN[pS]VEQK
α_{11}	1474.57	2	2950.94	KEKVNEL[pS]KDIG[pS]E[pS]TEDQA[Mo]EDIKQ
α_{12}	1503.00	2	3008.02	NANEEEYSIG[pS][pS][pS]EE[pS]AEVATEEVK

[pS]: phosphorylated serine;

[Mo]: oxidation on methionine.

Table S4. Sequence information of detected phosphopeptides from tryptic digests of ovalbumin.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
O_1	1043.44	2	2088.91	EVVGS[pS]AEAGVDAASVSEEFR
O_2	1449.65	2	2903.46	FDKLPGFGD[pS]IEAQCGTSVNVHSSLR

[pS]: phosphorylated serine.

Table S5. Sequence information of 3 standard phosphopeptides detected by STiNbNSs-BAAs-nano-DESI-MS.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
P_1	849.86	2	1701.80	TRDIYETD[pS]YYRK
P_2	929.83	2	1861.66	TRDI[pS]YETD[pS]Y[pS]YRK
P_3	1095.03	2	2192.40	DLDVPIPGRFDRRV[pS]SVAAE

[pS]: phosphorylated serine.

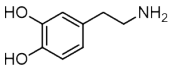
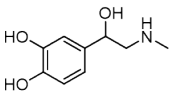
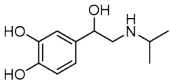
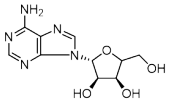
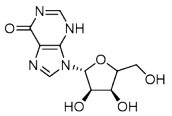
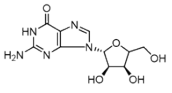
Table S6. Recovery of phosphopeptides from tryptic digests of β-casein (20 pmol) mixed with 10-fold tryptic digests of Cyt c and BSA obtained by STiNbNSs-BAAs-nano-DESI-MS in line scan mode. The data were obtained from three independent experiments.

Added (pmol)	Found (pmol)	Recovery (%)
20.0	21.9	109.3
	19.1	95.6
	21.4	107.1

Table S7. Recovery of phosphopeptides from tryptic digests of β-casein (20 pmol) mixed with 10-fold tryptic digests of Cyt c and BSA obtained by STiNbNSs-BAAs-nano-DESI-MS in drop-and-desorb mode. The data were obtained from three independent experiments.

Added (pmol)	Found (pmol)	Recovery (%)
20.0	21.7	108.4
	20.5	102.4
	21.3	106.5

Table S8. Detailed information of neurotransmitters, amino acids, nucleosides and deoxyribonucleosides used in this work.

Analyte	Structure	Formula	Theoretical MW (Da)	Observed *m/z*	Adduct type
Dopamine (D)		$C_8H_{11}NO_2$	153.08	154.09 137.08	$[M+H]^+$ $[M+H-NH_3]^+$
Epinephrine (E)		$C_9H_{13}NO_3$	183.09	184.10 166.09	$[M+H]^+$ $[M+H-H_2O]^+$
Isoprenaline (IE)		$C_{11}H_{17}NO_3$	211.12	212.14 194.12	$[M+H]^+$ $[M+H-H_2O]^+$
Adenosine (A)		$C_{10}H_{13}N_5O_4$	267.10	268.11 290.10	$[M+H]^+$ $[M+Na]^+$
Inosine (I)		$C_{10}H_{12}N_4O_5$	268.08	269.09	$[M+H]^+$
Guanosine (G)		$C_{10}H_{13}N_5O_5$	283.09	284.10 306.10	$[M+H]^+$ $[M+Na]^+$

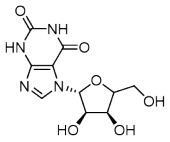
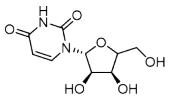
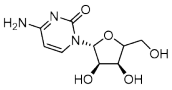
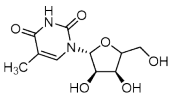
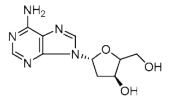
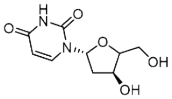
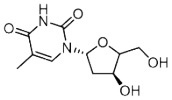
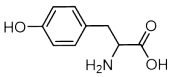
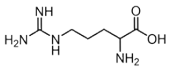
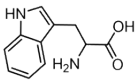
Xanthosine (X)		$C_{10}H_{12}N_4O_6$	284.08	285.09	$[M+H]^+$
Uridine (U)		$C_9H_{12}N_2O_6$	244.07	245.08	$[M+H]^+$
Cytidine (C)		$C_9H_{13}N_5O_5$	243.08	244.10 266.09	$[M+H]^+$ $[M+Na]^+$
5-Methylunidine (5-U)		$C_{10}H_{14}N_2O_6$	258.08	259.10 281.09	$[M+H]^+$ $[M+Na]^+$
2'-Deoxyadenosine (DA)		$C_{10}H_{13}N_5O_3$	251.10	252.11 274.11	$[M+H]^+$ $[M+Na]^+$
2'-Deoxyuridine (DU)		$C_9H_{12}N_2O_5$	228.07	229.09 251.08	$[M+H]^+$ $[M+Na]^+$
Thymidine (T)		$C_{10}H_{14}N_2O_5$	242.09	243.10 265.09	$[M+H]^+$ $[M+Na]^+$
L-tyrosine (Y)		$C_9H_{11}NO_3$	181.07	182.09	$[M+H]^+$
L-arginine (R)		$C_6H_{14}N_4O_2$	174.11	175.13 197.11	$[M+H]^+$ $[M+Na]^+$
L-valine (V)		$C_5H_{11}NO_2$	117.08	118.09	$[M+H]^+$
L-tryptophan (W)		$C_{11}H_{12}N_2O_2$	204.09	205.11 227.09	$[M+H]^+$ $[M+Na]^+$

Table S9. Detailed information of the glycopeptides processed by APBA-SMSs-BAAs-nano-DESI-MS. N# denotes the N-linked glycosylation site.

No.	Observed m/z	Charge	Adduct type	Theoretical MW (Da)	Glycan composition	Amino acid sequence
H_1	921.95	2	$[M+2H]^{2+}$	1841.79	XylMan3FucGlcNAc2	NVGLN#R
H_2	1161.04	2	$[M+2H]^{2+}$	2316.09	Man2GlcNAc2	MGN#ITPLTGTQGQIR
H_2	1169.55	2	$[M+H+NH_4]^{2+}$			
H_3	1086.57	3	$[M+3H]^{3+}$	3254.56	Man2GlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR
H_4	1118.86	3	$[M+3H]^{2+}$	3352.40	XylMan3FucGlcNAc2	SFAN#STQTFFNAFVEAMDR
H_4	1122.96	3	$[M+2H+NH_4]^{3+}$			
H_5	1198.23	3	$[M+3H]^{3+}$	3587.61	XylMan3FucGlcNAc2	NQCRGLCPLNGN#LSALVDFDLR
H_6	1224.96	3	$[M+3H]^{3+}$	3670.70	XylMan3FucGlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR
H_6	1230.63	3	$[M+2H+NH_4]^{3+}$			
H_7	1251.60	3	$[M+3H]^{3+}$	3748.60	XylMan3GlcNAc2	LHFHDCFVNGCDASILLDN#TTSFR
H_8	1299.27	3	$[M+3H]^{3+}$			
H_8	1306.62	3	$[M+2H+Na]^{3+}$	3894.66	XylMan3FucGlcNAc2	LHFHDCFVNGCDASILLDN#TTSFR
H_8	974.47	4	$[M+4H]^{4+}$			
H_9	1353.29	3	$[M+3H]^{3+}$	4057.80	XylMan3GlcNAc2	QLTPTFYDNSC(AAVESACPR)PN#VSNI

						VR-H_2O
H_{10}	1408.68	3	$[M+3H]^{3+}$			
H_{10}	1414.35	3	$[M+2H+NH_4]^{3+}$	4221.87	XylMan3FucGlcNAc2	QLTPTFYDNSC(AAVESACPR)PN#VSNI VR
H_{10}	1416.02	3	$[M+2H+Na]^{3+}$			
H_{10}	1421.35	3	$[M+2H+K]^{3+}$			
H_{11}	1458.69	3	$[M+3H]^{3+}$			
H_{11}	1464.36	3	$[M+2H+NH_4]^{3+}$	4379.97	XylMan3FucGlcNAc2 XylMan3GlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR
H_{11}	1470.04	3	$[M+H+2NH_4]^{3+}$			
H_{12}	1573.75	3	$[M+3H]^{3+}$			
H_{12}	1578.08	3	$[M+2H+NH_4]^{3+}$	4718.09	Man3FucGlcNAc2 Man3FucGlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR
H_{12}	1583.75	3	$[M+H+2NH_4]^{3+}$			
H_{13}	1271.64	2	$[M+2H]^{2+}$	2541.23	XylMan3FucGlcNAc2	SSPN#ATDTIPLVR
H_{14}	1624.79	3	$[M+2H+NH_4]^{3+}$	4854.33	XylMan3FucGlcNAc2 Man3FucGlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR
H_{14}	1630.47	3	$[M+H+2NH_4]^{3+}$			
H_{15}	1662.47	3	$[M+3H]^{3+}$			
H_{15}	1668.14	3	$[M+2H+NH_4]^{3+}$	4982.17	XylMan3FucGlcNAc2 XylMan3FucGlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR
H_{15}	1669.82	3	$[M+2H+Na]^{3+}$			
H_{15}	1675.14	3	$[M+2H+K]^{3+}$			

Table S10. Sequence information of peptides from the tryptic digests of human α-thrombin.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
T_1	690.39	1	689.38	DIALMK
T_2	734.37	1	733.35	SPFNNR
T_3	807.40	1	806.37	VIDQFGE
T_4	874.49	1	873.48	VTGWGNLK
T_5	1005.51	1	1004.49	ETWTANVGK
T_6	597.81	2	1193.59	ELLESYIDGR
T_6	1194.61	1		
T_7	626.34	2	1250.65	ETAASLLQAGYK
T_7	1251.67	1		
T_8	681.86	2	1361.71	WIQKVIDQFGE
T_9	930.99	2	1859.96	VTGWGNLKETWTANVGK
T_{10}	1077.55	2	2152.06	SLEDKTERELLESYIDGR
T_{11}	756.05	3	2264.09	IVEGSDAEIGMSPWQVMLFR
T_{11}	1134.07	2		
T_{12}	799.08	3	2392.20	IVEGSDAEIGMSPWQVMLFR K
T_{12}	1197.62	2		
T_{13}	991.52	4	3961.97	IVEGSDAEIGMSPWQVMLFR KSPQELLCGASLISDR
T_{13}	1321.69	3		
T_{14}	1379.05	5	6892.33	QECSIPVCGQDQVTVAMTPRSEGS SVNLSPPLEQCVPDRGQYQGRLAV TTHGLPCLAWASAQAK
T_{14}	1723.56	4		

Table S11. Sequence information of peptides from the trypsin autolysis.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
Try$_1$	659.39	1	658.38	SGIQVR
Try$_2$	1082.05	2	2162.05	LGEDNINVVEGNEQFISASK

Table S12. Sequence information of phosphopeptides obtained by STiNbNSs-BAAs-nano-DESI-MS.

No.	Observed m/z	Charge	Theoretical MW (Da)	Peptide sequence
HS1	693.25	2	1388.588	D[pS]GEGDFLAEGGGV
HS2	728.77	2	1459.677	AD[pS]GEGDFLAEGGGV
HS3	771.30	2	1544.750	D[pS]GEGDFLAEGGGVR
HS4	806.82	2	1615.738	AD[pS]GEGDFLAEGGGVR

[pS]: phosphorylated serine

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