Electronic Supplementary Information (ESI)

Effective Enrichment of Glycated Proteome Using Ultrasmall Gold Nanoclusters Functionalized with Boronic Acid

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Experimental Section

Materials

Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, reagent grade), reduced L-glutathione (≥ 98%), benzyl chloroformate (CBz-Cl, ≥98%), sodium bicarbonate ($NaHCO_3$, 99.7%), 3-aminophenylboronic acid (PBA, MQ 100), trifluoroacetic acid (TFA, ≥ 99.0%), N-hydroxysuccinimide (NHS, ≥ 99.0%), sodium phosphate dibasic dihydrate (BioUltra, ≥ 98%), sodium phosphate monobasic dihydrate (BioUltra, ≥ 99%), tetraoctylammonium bromide (TOAB, 98%), dichloromethane (DCM, ≥ 99.9%), deuterium oxide (99.9 atm% D), 2,5-dihydroxybenzoic acid (DHB, HPLC, ≥ 99.0%), Teicoplanin (Teicoplanin A2, HPLC, ≥ 80%), Ramoplanin (Ramoplanin A2, ≥ 75%), Bleomycin (pharmaceutical primary standard), Vancomycin (Vancomycin B, ≥85%), iodoacetamide (IAA, ≥ 99.0%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, MQ 300), and human serum (MQ 300) were purchased from Sigma-Aldrich. Ultrahigh-pure grade tetrahydrofuran (THF) and acetonitrile were purchased from Thermo Scientific. N,N'-dicyclohexylcarbodiimide (DCC, 99%) and 5-amino-2-(hydroxymethyl) benzeneboronic acid hemiester hydrochloride (BX, 95%) were purchased from Alfa Aesar. Sodium hydroxide (NaOH, 98%), hydrochloric acid (HCl, 35-37%), isopropyl alcohol (IPA, 99%), and acetone were purchased from Burdick & Jackson. For polyacrylamide gel electrophoresis (PAGE), triza base (≥ 99%), glycine (≥9 9%), acrylamide/bisacrylamide (bioreagent, 40%), N,N,N′,N′-tetramethylethylenediamine (TEMED, 99%), and ammonium persulfate (≥98%) were purchased from Sigma-Aldrich. Amicon ultra-4 centrifugal filter units and Amicon ultra-0.5 centrifugal filter units (desalting column) were purchased from Merck Millipore. Water was purified using a Millipore Milli-Q system (18.2 MΩ cm). All the chemicals were used as received without further purification.

Methods

The optical absorption and photoluminescent (PL) spectra of product solutions were obtained using a Shimadzu ultraviolet-visible-near-infrared (UV–Vis–NIR) spectrophotometer (UV-3600) and Edinburgh Spectrofluorometer (FS5), respectively. The Fourier transform nuclear magnetic resonance (NMR) measurements were conducted on a Bruker Avance III HD 400 MHz spectrometer at 25 °C. NMR samples were prepared by dissolving 5 mg nanocluster (NC) sample in 500 μL D_2O. PAGE was conducted using a Mini-PROTEAN Tetra Cell (Biorad). The homemade stacking and resolving gel were added at 4 and 30 wt%, respectively, and PAGE was employed at 150 V for 1.5 h until the bands are clearly separated. Electrospray ionization (ESI) mass spectroscopy was carried out using a 6230B Accurate mass time-of-flight (TOF) liquid chromatography–mass spectrometry (LC/MS) system (Agilent Technologies). The ESI mass spectra were obtained in negative-ion mode (flow rate: 3.0 μL min^{-1}; capillary voltage: 4.0 kV; capillary temperature: 200 °C). The NC samples were dissolved in 0.1 m triethylammonium acetate dissolved in 1:1 (v/v) water–methanol mixture at a concentration of 2 mg mL^{-1} and directly injected into the mass spectrometer. The triethylammonium acetate was used as the volatile ion-pairing reagent to suppress the sodium adducts. Transmission electron microscopy (TEM) images were collected on a JEOL transmission electron microscope (JEM-ARM 200F, NEOARM). TEM samples were prepared by drop casting a DCM solution of the NCs with TOAB on a 400 mesh Formvar/C-coated Cu grid (01814-F, Ted Pella), followed by overnight drying under vacuum conditions prior to imaging.

Preparation of Au_{22} NCs Functionalized with Boronic Acid (BA)

$Au_{22}SG_{18}$ NCs and CBz-protected Au_{22} NCs (Au_{22}–CBz) were synthesized according to previously reported methods.[1, 2] Into an aqueous solution of 1 mL Au_{22}–CBz (3 mM) and 1 mL NHS (0.3 M), 3 mL DCC (88 mM) dissolved in THF was added. Water and THF were further added to achieve a clear solution, if needed. After vigorous stirring for 1 h, 5 mL BA (60 mM) dissolved in an 80:20 THF/water mixture was added to the solution, along with minimal amounts of water and THF to achieve a clear solution. The optimum THF/water ratio was found to be 3. After stirring for 24 h, the solution was dried via rotary evaporation. The dried product was dissolved in 20 mL water and centrifuged, and the supernatant containing unreacted BA and byproducts was removed. The resulting precipitate was dissolved in water, and the pH was adjusted to 8.5 using 1 M NaOH, which completely dissolved the precipitate. The product was finally purified by PAGE separation. Subsequently, the BA-functionalized Au_{22} NCs (Au_{22}–BA) band was extracted in water after 1 h and further purified with 10 kDa Amicon Ultra-4 Centrifugal Filter Unit.

In Vitro Glycation of Human Serum

Human serum was incubated with a glycation buffer composed of 1 M D-(+)-glucose and 50 mM Tris-HCl (pH 7.5) for 48 h at 37 °C. Free glucose in the glycated human serum was removed using 3 kDa Amicon Ultra Centrifugal Filters (Millipore, USA).

Protein Digestion

Glycated human serum samples (2 mg) were reconstituted in a 400 μL solution of 50 mM tris-HCl buffer (pH 8.5) and 8 M urea, resulting in an initial concentration of 1 μg μL^{-1}. The denatured protein sample was then reduced with TCEP (500 mM, 4 μL) at 37 °C for 30 min and alkylated with IAA (500 mM, 12 μL) at 25 °C for 30 min in the dark. The samples were subsequently diluted with 1 M tris buffer to reduce the urea concentration to 2 M. Trypsin (0.1 μg μL^{-1}; trypsin/protein = 1/50, w/w) dissolved in 80 μL ammonium bicarbonate (50 mM) was added. Subsequently, the solution was incubated at 37 °C overnight. The reaction was quenched by adding 60 μL formic acid (100%) to the digestion and passing the mixture through C18 Sep-Pak cartridges (Waters) for desalting. The final tryptic digestion was kept at −20 °C for further use.

Enrichment of Glycated Peptides by Au_{22}–BA

For the glycated peptide enrichment, 0.05 μmol synthetic Au_{22}–BA dissolved in 100 μL binding buffer (0.1 M pH 9.0 phosphate buffer with 50 mM NaF) were mixed with 50 μg tryptic digests (or 0.05 μmol standard glycopeptides). Subsequently, the system was incubated on a thermomixer at 13 °C for 30 min. The composite was washed eight times with the washing buffer (0.1 M pH 7.4 phosphate buffer with 10 mM NaF and 0.2 M NaCl) by 10 kDa Amicon Ultra-0.5 Centrifugal Filter Unit. Finally, glycated peptide elution was performed at 37 °C for 30 min in an eluting buffer (ACN/H_2O/TFA = 50/49/1, v/v/v). The released glycated peptides were separated from Au_{22}–BA by adding excess 0.1% formic acid to induce the precipitation of the NCs. After centrifugation, the glycated peptides were collected from the supernatant. The separation of NCs from the eluted sample was monitored based on their PL. The NIR emission characteristics of Au_{22}–BA were used as an indicator for verifying the purification of the eluted peptide samples. We monitored the sample's emission during the elution process, and as shown in Fig. S7, we halted the separation only after the NCs' PL had completely disappeared from the sample. Finally, the eluted glycated peptides were desalted by C18 spin columns (Harvard Apparatus) for the MS analysis.

Further Optimization of Incubation Conditions for Glycated Peptide Enrichment

Incubation time. Considering the speed of the boronated affinity binding reaction, extending the incubation time would not yield noticeable positive effects on the enrichment efficiency. Similar amounts of standard glycopeptides were captured when the incubation time was varied from 30 min to 3 h (Fig. S13b). Therefore, we selected an incubation time of 30 min, considering the stability issue of the biological samples.

Incubation temperature. We explored the temperature dependence of the reaction system and opted to maintain the incubation process at 13 °C based on the results depicted in Fig. S13c. This choice is consistent with the theory that higher temperatures can accelerate the hydrolysis rate. As such, a higher temperature of 37 °C was set during the elution process.[3]

Further Optimization of Washing Conditions to Suppress Nonspecific Bindings

Effect of salt addition on the washing condition. Unexpected adsorption may occur by positively charged nonglycated peptides via electrostatic interaction with the negatively charged species on the NC surface. We attempted to alleviate this interaction by adding NaCl salts into the washing buffer, enhancing the ionic strength of the medium.[4] Another factor contributing to the nonspecific binding is the charge transfer interaction involving certain Lewis-base-type peptides. We introduced NaF salts to the washing buffer and analyzed the MS intensity of five selected nonglycated peptides from the human serum samples. This approach was adopted as robust Lewis base buffers can mitigate such interactions.[4] A concentration of 10 mM NaF was determined to be suitable (Fig. S14).

MS Analysis of Peptides

Matrix-assisted laser desorption ionization (MALDI) MS on standard glycopeptides were conducted using an Autoflex Max MALDI–TOF/TOF spectrometer (Bruker Daltonics, Germany). The dried droplet sampling method was used. In detail, 1 μL sample was first deposited on a MALDI plate, followed by 1 μL matrix solution prepared by dissolving 20 mg mL^{-1} DHB in TA-30 solution (30% ACN in 0.1% TFA). MS (5000 laser shots for every spectrum) was carried out in reflector-positive mode at the m/z scan range of 700–3500 and analyzed using FlexAnalysis version 3.4 software (Bruker Daltonics).

Nano-LC-tandem MS (nano-LC–MS/MS) was conducted using Q-ExactiveTM Hybrid Quadrupole-Orbitrap MS coupled with a Dionex Ultimate 3000 HPLC instrument (Thermo Scientific, USA). We used an AcclaimTM PepMapTM 100 C18 nanotrap column (75 μm × 2 cm, particle size: 3 μm, pore size: 100 Å) and a PepMapTM RSLC C18 nano column (75 μm × 5 cm, particle size: 2 μm, pore size: 100 Å) depending on the hydrophobicity of the peptide. The phase solvents consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in 80% acetonitrile, with the flow rate fixed at 300 nL min^{-1}. The gradient of mobile phase was as follows: 4% solvent B in 14 min, 4%–15% solvent B in 61 min, 15%–28% solvent B in 50 min, 28%–40% solvent B in 20 min, 40%–96% solvent B in 2 min, held at 96% solvent B in 13 min, 96%–4% solvent B in 1 min, and 4% solvent B for 24 min. A data-dependent acquisition method was adopted, and the top 10 precursor peaks were selected and isolated for fragmentation. The ions were scanned in high resolution (70,000 in MS1, 17,500 in MS2 at *m/z* 400) with the MS scanning range of 400–2,000 *m/z* at both MS1 and MS2 levels. The precursor ions were fragmented with normalized 27% collisional energy. Dynamic exclusion was set to 30 s. The peptide concentration was measured using a NanoDropTM 2000 Spectrophotometer (Thermo Scientific) with an injection amount of 1 μg.

MS Database Search

MS/MS raw files for the analysis were converted to mzXML[5] format using MSConverter and then analyzed with COMET and the Homo sapiens database downloaded from Uniprot. The search parameters were set as follows: 10 ppm tolerance of precursor ion masses, 0.02 Da tolerance of fragment ion masses, and a maximum of two missed cleavages with trypsin enzyme. The modification on peptide sequence was as follows: fixed carbamidomethylation of cysteine, +57.012 Da; variable modifications of methionine oxidation, +15.995 Da; acetylation of N-term, +42.011 Da; carbamylation of N-term, +43.006 Da; and glycation of lysine, +162.053 Da. The search results in pepXML[6] format can be imported into the Trans-Proteomics Pipeline (TPP)[7] and processed using PeptideProphet.[7] The cut-off probability score of 0.99 was used for TPP. A peptide false discovery rate of ≤0.01 was obtained based on a PeptideProphet[8] probability cut-off score of 0.99. Modified peptides were identified with high and medium confidence, considering the charge-state-specific Xcorr scores (≥2 for z = 1.5, ≥1.9 for z = 3, ≥2.8, and for z = 4).[9]

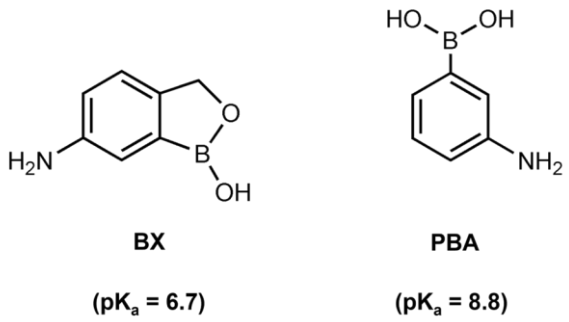

Fig. S1 Structures and pK_a values of BA derivatives: benzoboroxole (BX) and phenylboronic acid (PBA).

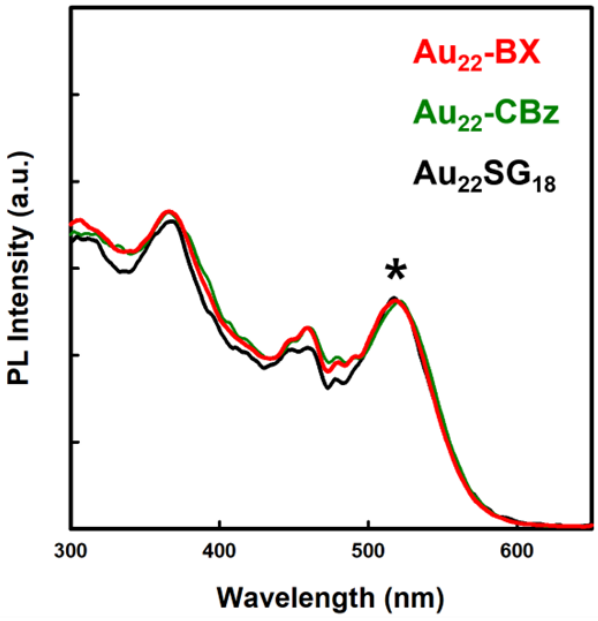

Fig. S2 PL excitation spectra (λ_{EM} = 650 nm) of $Au_{22}SG_{18}$, Au_{22}–CBz, and Au_{22}–BX in water.

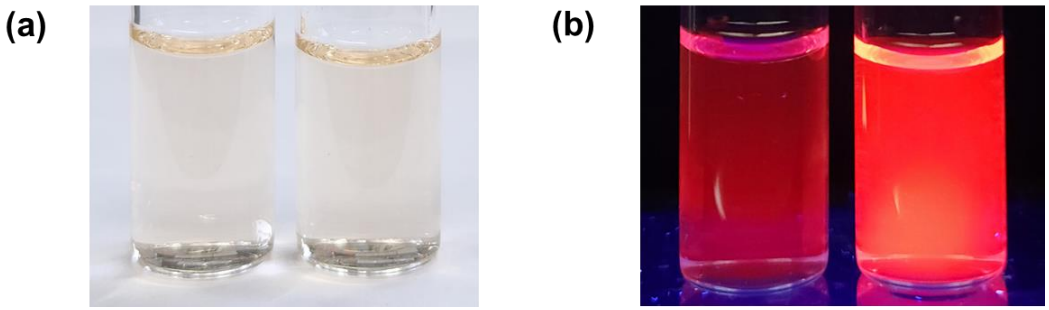

Fig. S3 Digital photographs of $Au_{22}SG_{18}$ (left) and Au_{22}–BX (right) under (a) normal light and (b) UV light irradiation (365 nm).

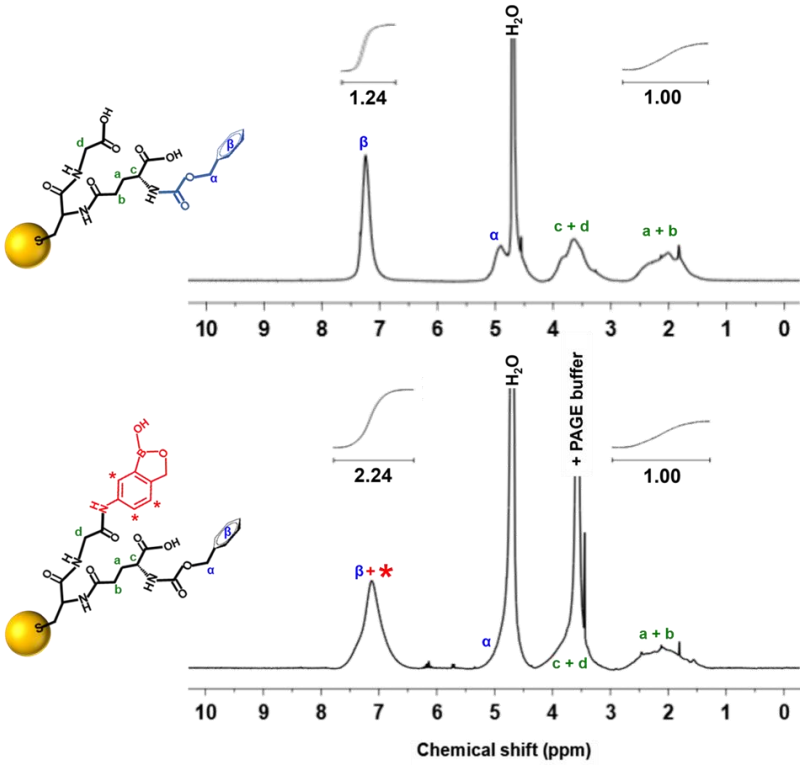

Fig. S4 ^{1}H NMR spectra of Au_{22}–CBz_{18} (top) and Au_{22}–BX_{24} (bottom) in D_2O.

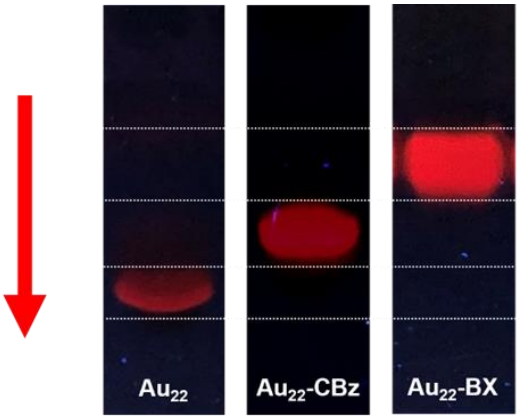

Fig. S5 Digital photographs under UV lamp irradiation (365 nm) after native PAGE separation of the synthesized NC products. The arrow indicates the sample migration direction.

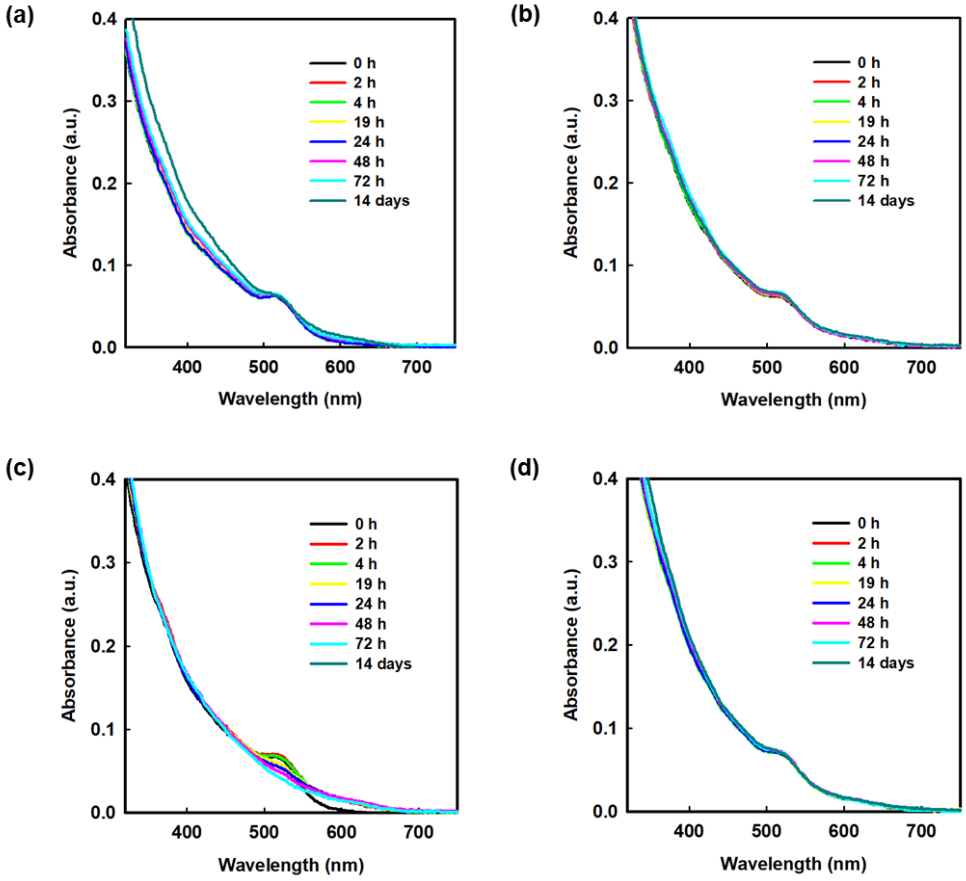

Fig. S6 Stability of $Au_{22}SG_{18}$ and Au_{22}-BX monitored over time by optical absorption spectrometry: (a) $Au_{22}SG_{18}$ in water, (b) Au_{22}-BX in water, (c) $Au_{22}SG_{18}$ under incubation conditions, and (d) Au_{22}-BX under incubation conditions.

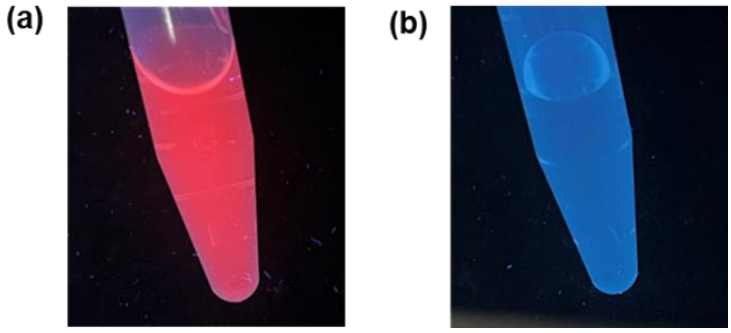

Fig. S7 Digital photographs of the samples during the elution process under UV lamp irradiation (365 nm): (a) before and (b) after the separation of Au_{22}–BA.

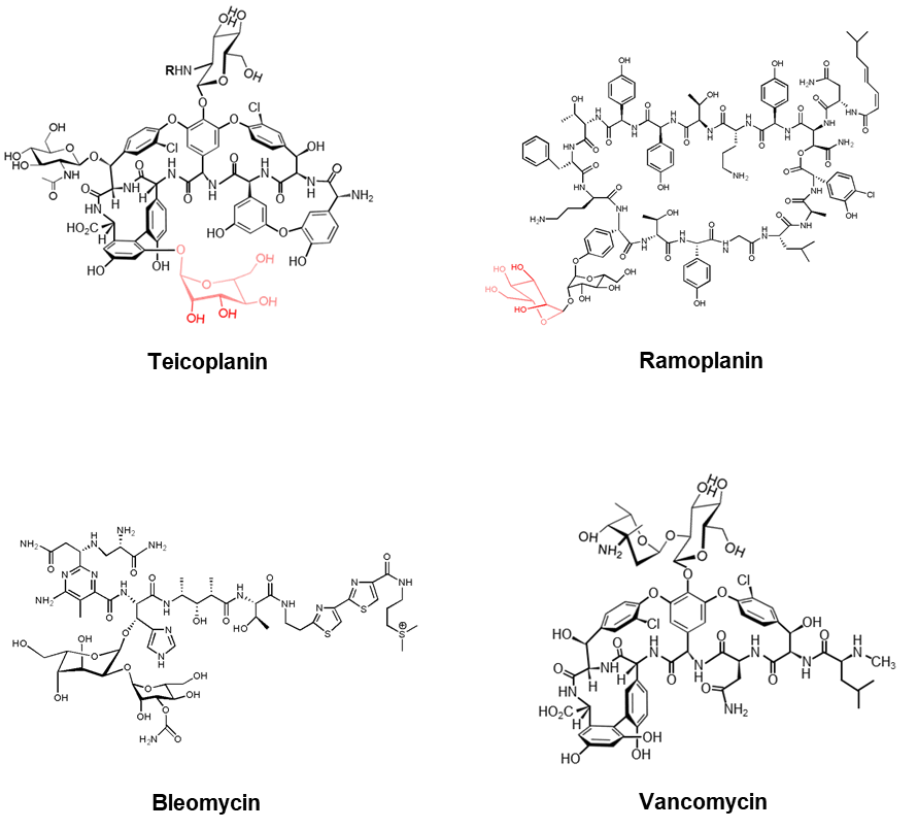

Fig. S8 Structures of standard peptides (cis-2,3-diols highlighted in red)

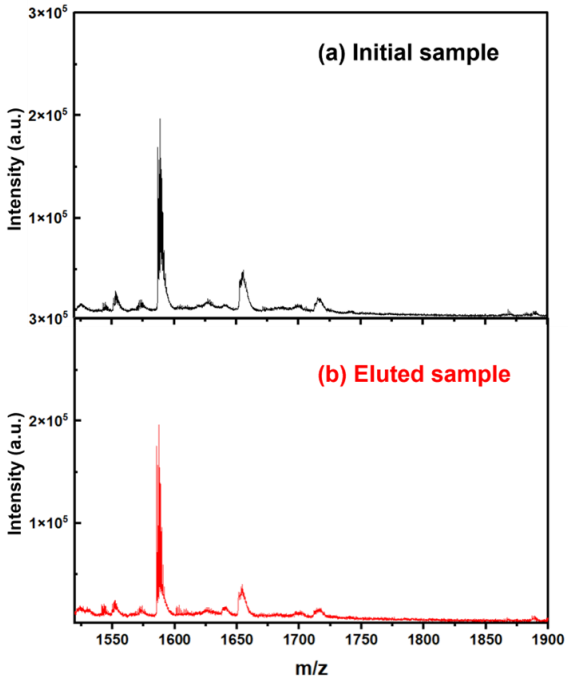

Fig. S9 MALDI mass spectra of the eluted standard peptide (Teicoplanin).

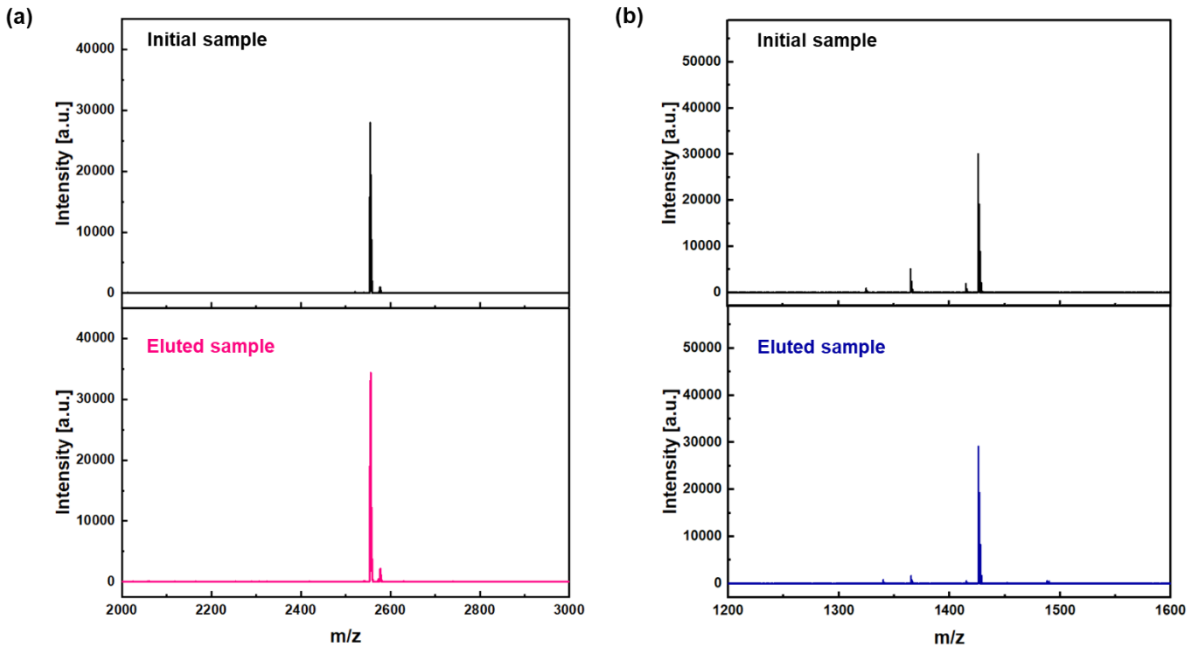

Fig. S10 MALDI mass spectra of eluted standard peptides: (a) Ramoplanin and (b) Bleomycin.

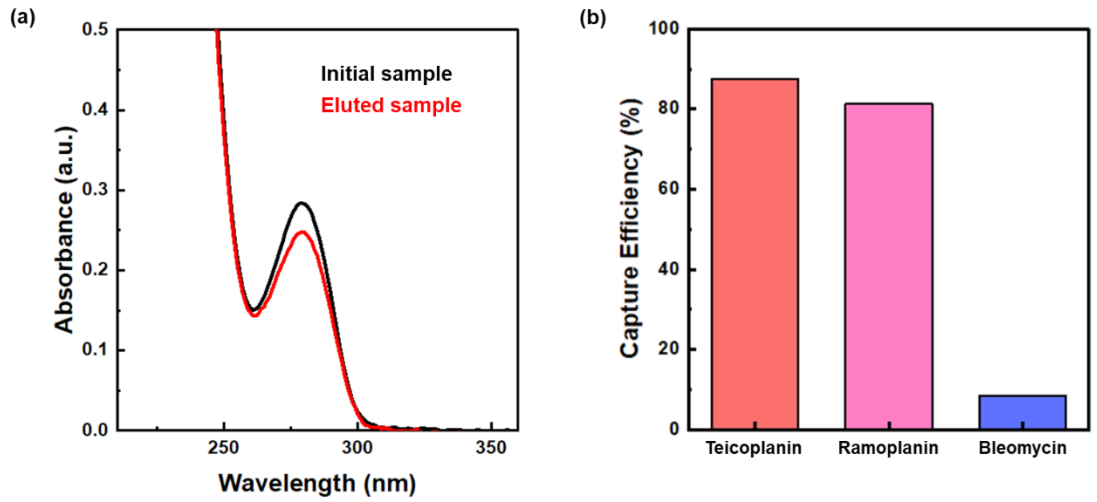

Fig. S11 Capture efficiency of standard peptides determined by optical absorption spectrometry: (a) Optical absorption spectra of the standard peptide before and after enrichment, using Teicoplanin as a representative example; (b) Capture efficiency of three standard peptides.

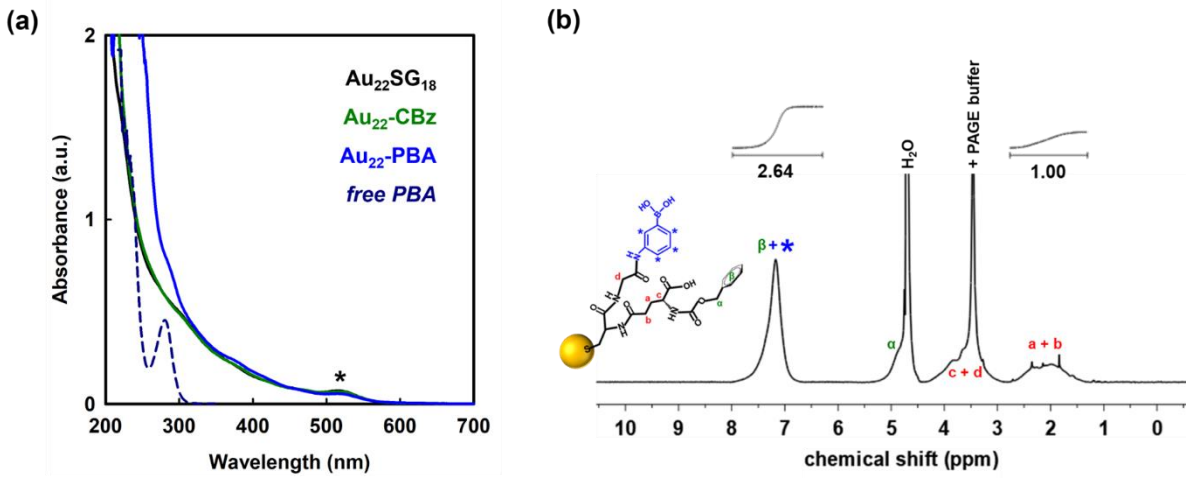

Fig. S12 Characterization of Au_{22}–PBA. (a) Optical absorption spectra of $Au_{22}SG_{18}$, Au_{22}–CBz, Au_{22}–PBA, and PBA in water. (b) ^1H NMR spectra of Au_{22}–PBA_n in D_2O ($n \approx 25$ was determined by comparing the integration numbers of the typical peaks.)

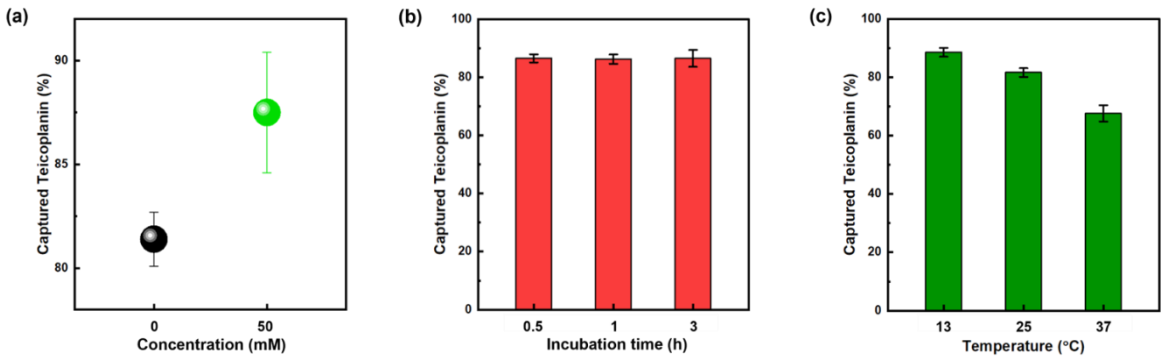

Fig. S13 Optimization of incubation conditions by standard peptides and its results: (a) effect of F^- ion addition, (b) incubation time, and (c) incubation temperature. The error bar represents the standard mean error, calculated from triplicate experiments.

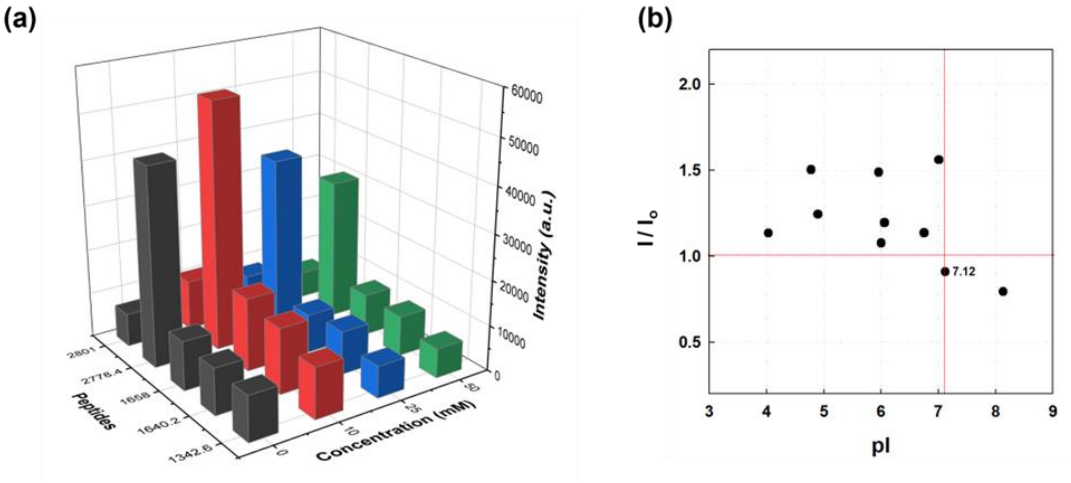

Fig. S14 Optimization of the washing conditions by glycated human serum digests and its results. (a) MALDI MS intensity of five nonglycated peptides from flow-through after adding NaF at different concentrations to the washing buffer. (The pI values of the five typical nonglycated peptides are all lower than 6.00). (b) Threshold of the applicable pI value (= 7.12)

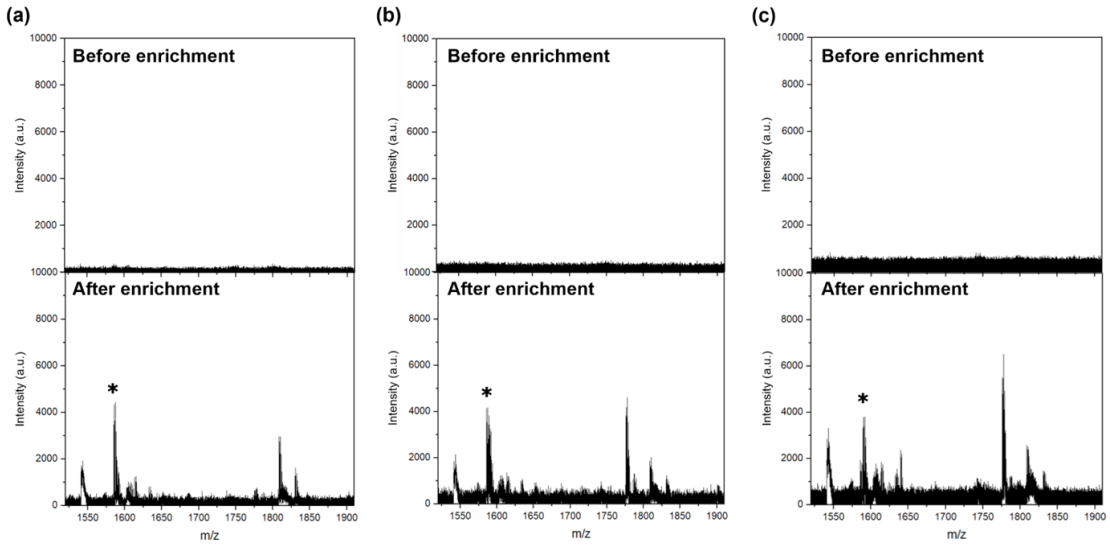

Fig. S15 Selectivity evaluation. MALDI mass spectra after enriching glycopeptides from mixtures of Teicoplanin and Vancomycin peptides at molar ratios of (a) [1:100], (b) [1:200], and (c) [1:1000]. * denotes the typical peaks of Teicoplanin. The other mass peaks originate from salt-adsorbed Teicoplanin species.

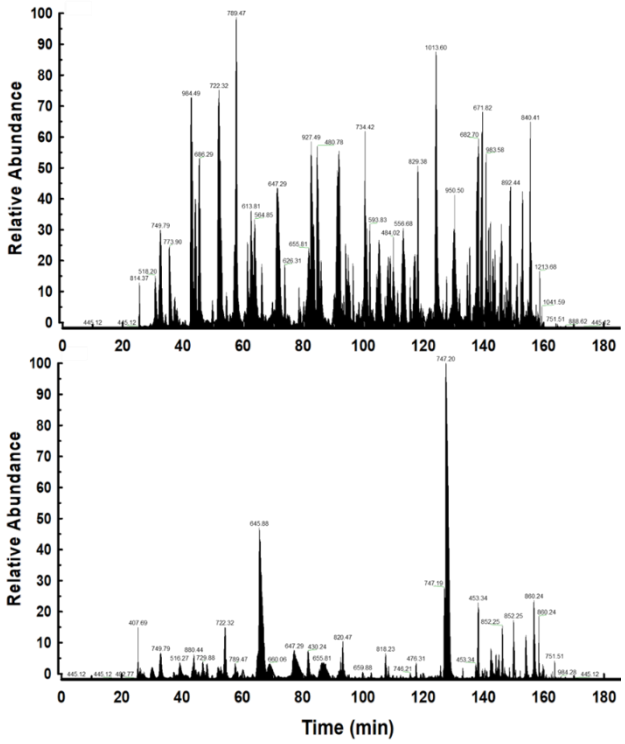

Fig. S16 Base peak chromatograms of the peptide products before (top) and after (bottom) enrichment by the optimized protocol.

Evaluation criteria	Results
Sensitivity [a]	0.01 fmol μL^{-1}
Selectivity [b]	1/1000, w/w = Teicoplanin/Vancomycin
Reusability [c]	5 cycles

Table S1 Evaluation results of enrichment performance. (a. Sensitivity was determined as the minimum concentration at which a signal with a reasonable signal-to-noise ratio could be detected in the MS spectra by progressively reducing the amount of initial Teicoplanin samples for enrichment process; b. Selectivity was determined as the highest ratio of Vancomycin to Teicoplanin at which the target Teicoplanin remained detectable after enrichment, determined by gradually increasing Vancomycin amounts in the mixture; c. Reusability was assessed by performing multiple experimental cycles with the same material and obtaining consistent result values. While the experiment was validated for up to five cycles, the potential for further reuse remains promising.)

References

1. K. Pyo, V. D. Thanthirige, K. Kwak, P. Pandurangan, G. Ramakrishna and D. Lee, *J. Am. Chem. Soc.*, 2015, **137**, 8244-8250.
2. K. Pyo, V. D. Thanthirige, S. Y. Yoon, G. Ramakrishna and D. Lee, *Nanoscale*, 2016, **8**, 20008-20016.
3. R. S. Peterson, C. G. Hill, Jr. and C. H. Amundson, *Biotechnol. Bioeng.*, 1989, **34**, 429-437.
4. R. J. Carvalho, J. Woo, M. R. Aires-Barros, S. M. Cramer and A. M. Azevedo, *Biotechnol. J.*, 2014, **9**, 1250-1258.
5. L. Martens, M. Chambers, M. Sturm, D. Kessner, F. Levander, J. Shofstahl, W. H. Tang, A. Römpp, S. Neumann, A. D. Pizarro, L. Montecchi-Palazzi, N. Tasman, M. Coleman, F. Reisinger, P. Souda, H. Hermjakob, P.-A. Binz and E. W. Deutsch, *Mol. Cell. Proteom.*, 2011, **10**, R110.000133-000137.
6. A. Keller, J. Eng, N. Zhang, X. j. Li and R. Aebersold, *Mol. Syst. Biol.*, 2005, **1**, 2005.0017.
7. E. W. Deutsch, L. Mendoza, D. Shteynberg, J. Slagel, Z. Sun and R. L. Moritz, *Prot. Clin. Appl.*, 2015, **9**, 745-754.
8. J. Jeon, J. Yang, J.-M. Park, N.-Y. Han, Y.-B. Lee and H. Lee, *J. Chromatogr. B*, 2018, **1092**, 88-94.
9. S. Milkovska-Stamenova, L. Krieg and R. Hoffmann, *Mol. Nutr. Food Res.*, 2019, **63**, 1800725.