Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Lanthanide-Coded Protease-Specific Peptide–Nanoparticle Probes for Label-Free Multiplex Protease Assay Using Element Mass Spectrometry: A Proof-of-Concept Study**

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Materials. Succinic anhydride, 3-aminopropyltriethoxysiane (APTES), tetraethoxysilane (TEOS), Tris (2, 2'-bipyridyl) dichlororuthenium (II) ($Ru(Bpy)_3$), 2-morpholinoethanesulfonic (MES), 4-Morpholinepropanesulfonic acid (MOPS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), 4-(2-Aminomethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), trypsin (from bovine pancreas), and α-chymotrypsin (from bovine pancreas, type VII) were purchased from Sigma-Aldrich (St. Louis, MO). High-purity Pr_6O_{11}, Eu_2O_3, Tb_4O_7, and Ho_2O_3 were obtained from Changchun Institute of Applied Chemistry (Chinese Academy of Sciences, purity greater than 99.99%). Triton X-100, cyclohexane, n-hexanol, ammonium hydroxide, acetone, ethanol, and high-purity HNO_3 were obtained from Merck (Darmstadt, Germany). 1,4,7,10-Tetraazacyclododecane-1,4,7-trisacetic acid-10-Maleimidoethylacetamide (MMA-DOTA) was purchased from Macrocyclics (Dallas, TX). Four substrate peptides GGRGGC, GGYGGC, GEYEGC and GGEGGC were synthesized and purified by Sangon Biotech (Shanghai, China) with purity greater than 98.0 % and confirmed by ESI-MS (Figure S4). Ultrapure water (18.2 MΩ) was prepared in a Milli-Q system (Millipore Filter Co., Bedford, MA) and used throughout this study. All chemicals and reagents were at least of analytical or high grade and used without further purification.

Characterization. Lanthanides (Ln) were quantified using an ELAN DRC II ICPMS (PerkinElmer, SCIEX, Canada) equipped with a concentric pneumatic nebulizer and a cyclonic spray chamber. The ICPMS operational parameters were as follows: nebulizer gas, 0.88 L min^{-1}; auxiliary gas, 1.0 L min^{-1}; plasma gas, 15 L min^{-1}; RF power, 1200 W; dwell time, 100 ms; lens voltage, 7.2 V. Parameters such as nebulizer gas flow and lens voltage were optimized daily to obtain the best sensitivity. Mass spectrometer experiments were carried out on an ESI ion trap mass spectrometer (Esquire-LC, Bruker Daltonics, Bremen, Germany). The operational parameters were as follows: nebulizer, 75 psi; dry gas, 8 L min^{-1}; dry temperature, 300 ^oC; capillary voltage, 3.5KV; end plate offset, 0.5KV. Bare and Ln-labeled peptide conjugated SiO_2NPs were imaged on a scanning electron microscope (Hitachi FE-SEM S4800, made by Hitachi High-Technologies Europe GmbH, Krefeld, Germany). Hydrodynamic diameter and zeta potential in water were measured using the dynamic light scattering technique (BI2200SM, Brookhaven Instruments Cop., USA).

Synthesis of APTES-succinic Anhydride Conjugate. Succinic anhydride (100 mg) was dissolved in 0.5 mL of N, N-Dimethylformamide (DMF), and then 0.23 mL of 3-aminopropyl- triethoxysiane (APTES) was added to the solution. The solution was stirred at room temperature for 2 h for the reaction (Figure S1a). The resulting product was confirmed using an ESI-MS (Bruker Daltonics, Bremen, Germany) (Figure S1b, M-1 = 319.8).

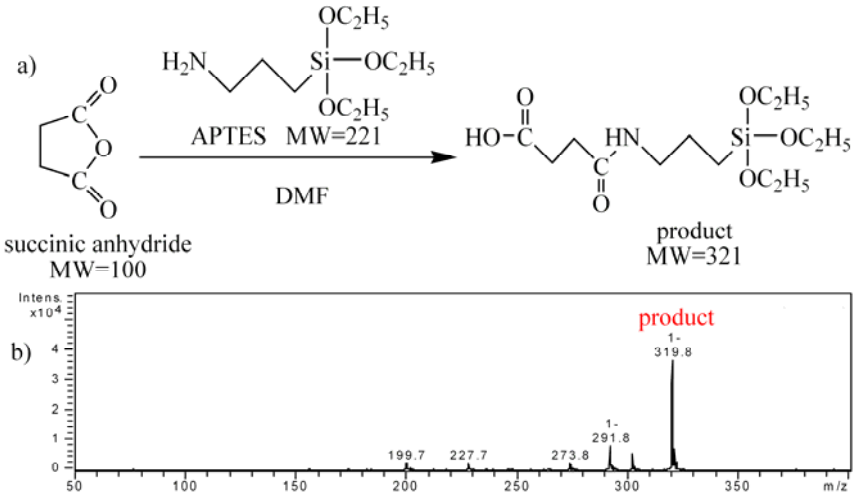

Figure S1. (a) Reaction of succinic anhydride with APTES in DMF. (b) Mass spectrum of the reaction product between succinic anhydride and APTES.

Preparation of Carboxyl Functionalized Silica Nanoparticles. Core-shell silica nanoparticles (SiO_2NPs) were synthesized in a w/o microemulsion system.[S1] The w/o microemulsion system was prepared first by mixing 17.7 mL of Triton X-100, 75.0 mL of cyclohexane, 18.0 mL of n-hexanol, 4.0 mL of water, and 0.5 mL of Tris (2, 2'-bipyridyl) dichlororuthenium (II) ($Ru(Bpy)_3$) (0.1 M). After 1h of stirring, 2.0 mL of TEOS and 1.0 mL of ammonium hydroxide were added to initiate the polymerization reaction. After 24 h of stirring, the synthesized APTES-succinic anhydride conjugate and 0.5 mL of TEOS were added to the above w/o microemulsion system stirring for another 24 h. When the reaction was complete, SiO_2NPs were isolated using acetone, and further washed with ethanol and water several times to remove any surfactant molecules. About 0.4 g of SiO_2NPs were obtained and redispersed in 40 mL of water for further use. SiO_2NPs with an approximate diameter of 65 nm were obtained, as shown in the SEM image (Figure S2).

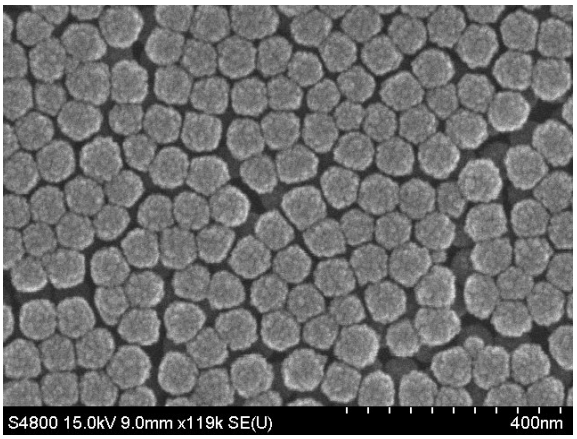

Figure S2. SEM image of carboxyl functional dye-doped core-shell SiO_2NPs.

Synthesis of Ln-coded Protease-Specific Peptides. Using the labeling methods previously optimized in our laboratory,[S2] four peptides were conjugated with MMA-DOTA-Ln in MOPS (4-Morpholinepropanesulfonic acid) buffer (100 mM, pH 7.5) at 37 ^oC for 30 min (Figure S3). It should be noted that MES and MOPS buffer which had no amino group were used in this study. Briefly, peptides GGRGGC, GGYGGC, GEYEGC and GGEGGC were labeled with MMA-DOTA-Ho, Tb, Pr and Eu, respectively. The peptide-MMA-DOTA-Ln substrate conjugates were confirmed using an ESI-MS (Figure S4).

Preparation of Ln-coded Protease-Specific Peptide-NP Probes (Figure S5). Ln-coded protease-specific peptides were conjugated onto SiO_2NPs using a cross-linker EDC. First of all, the carboxyl groups on the surface of the SiO_2NPs (0.5 mL of NPs suspensions) were preactivated with 6.4 mg of EDC and 17.6 mg of sulfo-NHS) in MES buffer (100 mM, pH 6.0) for 15 min at 25 °C. Then 5.6 μL of β-mercaptoethanol was added to deactivate excess EDC for 10 min. After centrifugation, the supernatant was removed and varying volumes (0.1, 0.2, 0.4, 0.6, 1.0 mL) of Ln-coded peptide solution (4 mM) were added to react with the sulfo-NHS activated carboxyl groups in the MOPS buffer (100 mM, pH 7.5) at 25 ^oC for 2 h. Then, 0.5 mL of Tris-HCl (100 mM, pH 7.5) was added to deactivate the residual carboxyl groups. The Ln-coded protease-specific peptide-NP probes were further washed with MOPS buffer in an ultrasonic bath several times to remove any superfluous and physically adsorbed Ln species. Finally, the obtained Ln-coded protease-specific peptide-NP probes were redispersed in 500 mL MOPS (100 mM, pH 7.5) containing $CaCl_2$ (10 mM) solution for further use. To determine the number of Ln conjugated on each carboxyl-SiO_2NP, concentrated HNO_3 was added to digest the Ln-coded protease-specific peptide-NP solution. The solution was then centrifuged and the supernatant was subjected to analysis using ICPMS (Figure S6). About 6.0×10^{13} carboxyl-SiO_2NPs were suspended in a 1 mL solution. The volume of 65 nm carboxyl-SiO_2NPs was calculated to be 1.44×10^{-22} m^3; and based on the density of SiO_2 (2.23 g/cm^3), the weight of one carboxyl-SiO_2NPs was estimated to be 3.34×10^{-16} g. Thus 1

mL carboxyl-SiO_2NPs solution with 20.1 mg carboxyl-SiO_2NPs contained 6.0×10^{13} carboxyl-SiO_2NPs, and so the number of Ln labeled per SiO_2NPs was estimated by dividing the number of loaded Ln by the number of SiO_2NPs.

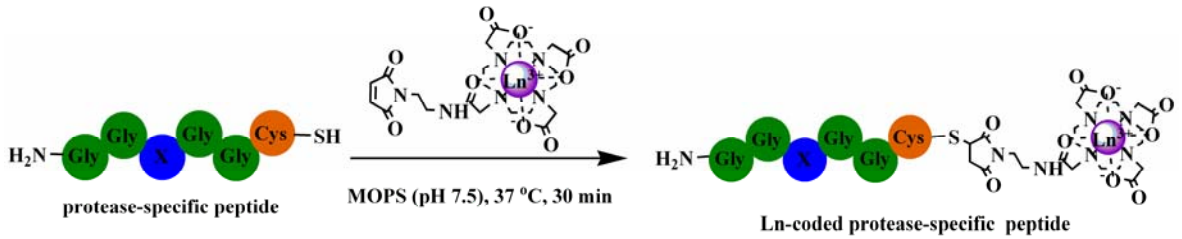

Figure S3. Reaction of the protease-specific peptide with MMA-DOTA-Ln in MOPS (pH 7.5) at 37 °C for 30 min.

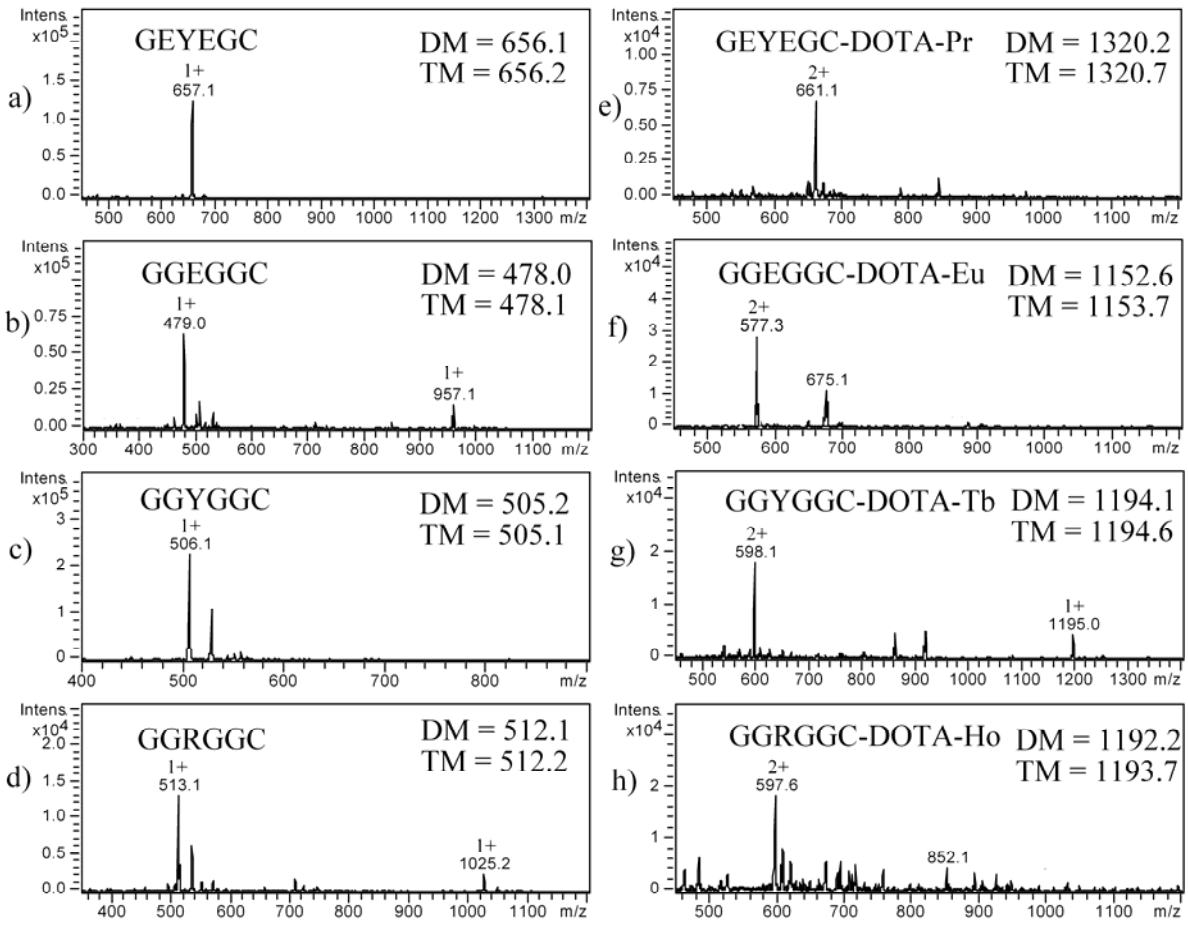

Figure S4. Mass spectra of (a) GEYEGC, (b) GGEGGC, (c) GGYGGC, (d) GGRGGC, (e) GEYEGC-MMA-DOTA-Pr, (f) GGEGGC-MMA-DOTA-Eu, (g) GGYGGC-MMA-DOTA-Tb and (h) GGRGGC-MMA-DOTA-Ho. DM and TM in the MS spectra denote deconvolution and theoretical molecular weights.

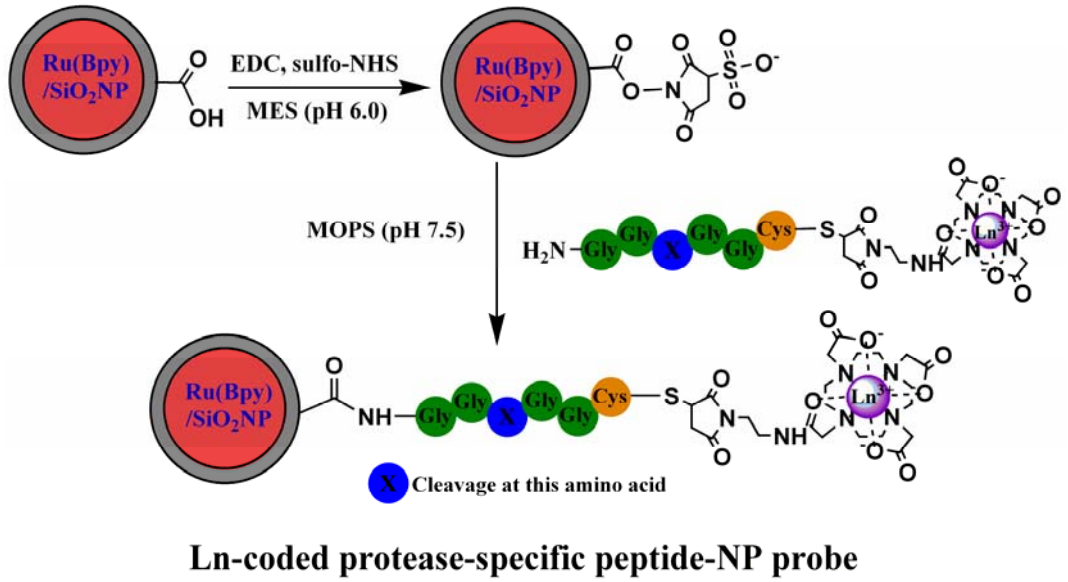

Figure S5. Conjugation of Ln-coded protease-specific peptides to the carboxyl-SiO_2NPs surface via EDC and the sulfo-NHS reaction.

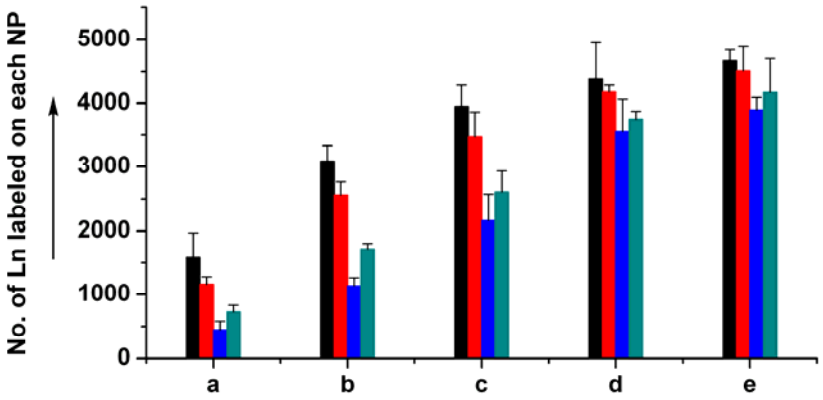

Figure S6. Number of Ln (black: Ho, red: Tb, blue: Pr, light blue: Eu) labeled on each SiO_2NP when (a) 0.1, (b) 0.2, (c) 0.4, (d) 0.6, (e) 1.0 mL of Ln-coded peptide solution (4 mM of peptide) reacted with 0.5 mL of carboxyl-activated SiO_2NPs in MOPS buffer (100 mM, pH 7.5) at 25 ^{o}C for 2 h.

Specificity of Ln-coded Protease-Specific Peptide-NP Probes to Trypsin and Chymotrypsin. To determine the specificity of Ln-coded protease-specific peptide-NP probes to trypsin and chymotrypsin, the four different probes of 2.5 μM (concentration of the peptide on the probe) were incubated respectively with trypsin (2nM) and chymotrypsin (2 nM) in MOPS (100 mM, pH 7.5) containing $CaCl_2$ (10 mM) at 25 ^{o}C for 60 min. Serine protease inhibitor AEBSF (20 mM) was added to stop the proteolytic reaction and, then the solution was centrifuged (30000g, 15 min), and the supernatant obtained was subjected to analysis using ICPMS. All experiments were carried out in triplicate.

Kinetic Analysis of Trypsin and Chymotrypsin. Initial reaction velocities of trypsin and chymotrypsin were measured for varying concentrations of P1 and P2. Briefly, 0.4, 1, 2, 3, 4, 6, 8, 12 μM of P1 and P2 were incubated with trypsin (25 nM) and chymotrypsin (50 nM). The data were fitted with the Michaelis-Menten kinetics equation. All experiments were carried out triplicates.

Detection Limits and Linearity Ranges of Trypsin and Chymotrypsin in Buffer, Human Urine and Serum. Varying concentrations (6 pM to 50 nM) of trypsin and chymotrypsin were incubated with 10 μM (concentration of the peptide on the probe) of Ln-coded protease-specific peptide-NP probes in MOPS (100 mM, pH 7.5) containing $CaCl_2$ (10 mM) or 10 × diluted urine and/or serum samples for 1 h at 25 ^{o}C. Then, AEBSF (20 mM) was added to stop the proteolytic reaction. The solution was further centrifuged, and supernatant was subjected to analysis using ICPMS. The serum and urine samples were from healthy adult volunteers. All experiments were carried out in triplicate.

References

[S1] S. Santra, R. P. Bagwe, D. Dutta, J. T. Stanley, G. A. Walter, W. Tan, B. M. Moudgil, R. A. Mericle, *Adv. Mater.* **2005**, *17*, 2165.

[S2] X. Yan, M. Xu, L. Yang, Q. Wang, *Anal. Chem.* **2010**, *82*, 1261–1269.