Multiplex Protease Assay

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

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At least 569 proteases play fundamental roles in human natural physiological processes and progress their functions in a precise and complicated molecular network.[1,2] Thus, their abnormality is associated frequently with many diseases, such as cancer,[2] cardiovascular diseases,[3] and Alzheimer's disease.[4] Fluorogenic and colorimetric substrates, which in general contain a protease-specific peptide and a conjugated fluorogenic or chromophoric group, have been widely used for a protease assay.[5] However, to the best of our knowledge, these suffer from several drawbacks including necessary different wavelength excitation for corresponding fluorogenic groups, or possible emission spectra overlapping of even strictly size-controlled quantum dots when using a single-wavelength excitation, besides possible background spectral interferences and fluorescence bleaching and quenching. As a result, these methods are mainly aimed at simplex protease assay, and are difficult, to a great extent, to apply to simultaneous multiplex protease assay in one biological sample. To better understand the network working mechanism, it is crucial to perform a multiplex correlative protease assay simultaneously, efficiently, and accurately. Developing novel methods is very desirable to meet such a requirement.

Element mass spectrometry (inductively coupled plasma mass spectrometry, ICP-MS) is a highly sensitive mass-based element determination technique that can determine most of the elements below the ppb level with broad dynamic range and excellent mass resolution, and is used traditionally for element quantification and speciation. These features have been recently extended by combining element-labeling strategies with effective separation techniques, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and polyacrylamide gel electrophoresis (PAGE), to quantify biomolecules including antigens and peptides as well as proteins, in which naturally occurring heteroelements,[6] artificially labeled elemental tags, and nanoparticles were used.[7,8] More recently, ICP-MS was used to measure protease activity via biotinylated lanthanide (Ln)-labeled substrates,[9] and to absolutely quantify Cu,Zn superoxide dismutase by postcolumn isotope dilution analysis,[10] thereby showing unique advantages compared with the fluorogenic and colorimetric methods reported.

Herein, we report the design and synthesis of an Ln-coded protease-specific peptide-conjugated nanoparticle (peptide–NP) probe for the first time (Scheme 1). It comprises three parts: 1) a specific peptide substrate designed and synthesized for a target protease; 2) bifunctional 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid-10-maleimidoethylacetamide (MMA-DOTA) for coding Ln in DOTA and conjugating the peptide substrate through the reaction between MMA and the sulfhydryl of the cysteine residue in the peptide, which results in an Ln-coded protease-specific peptide; and 3) a carboxyl-SiO_2 NP doped with $[Ru(bpy)_3]$ (orange) for conjugating the Ln-coded protease-specific peptide through the reaction of the N-terminal NH_2 of the peptide. The obtained Ln-coded protease-specific peptide–NPs were used as a probe together with ICP-MS for label-free multiplex protease assay. Cleaved and uncleaved probes (orange) could be separated directly by ultracentrifugation without any further separation such as HPLC and CE or PAGE.

Different from the fluorogenic and colorimetric probes, the coded Ln could be easily distinguished and determined upon ICP-MS and so overcame the bottlenecks encountered in multiplex protease assay when fluorogenic and colorimetric methods were applied. Ln, including 14 nonradioactive elements, can be coded into the DOTA in the probes, which implies that 14 proteases existing in one biological sample can be determined simultaneously in theory. In this proof-of-concept study, trypsin and chymotrypsin were used as model proteases to demonstrate this proposal, to achieve duplex protease activity assay. Trypsin cleaves peptides specifically at the carboxyl side of lysine or arginine, while chymotrypsin cleaves peptides at the carboxyl side of tyrosine, tryptophan, and/or phenylalanine. Based on these principles, four peptide substrates (P1 to P4) were designed and synthesized (Table 1), in which arginine-containing peptide GGRGGC was the substrate for trypsin, tyrosine-containing peptides GGYGGC and GEYEGC for chymotrypsin, and GGEGGC for control experiments.

First, SiO_2 NPs were synthesized in the water/oil microemulsion system (for detailed procedures, see the Supporting

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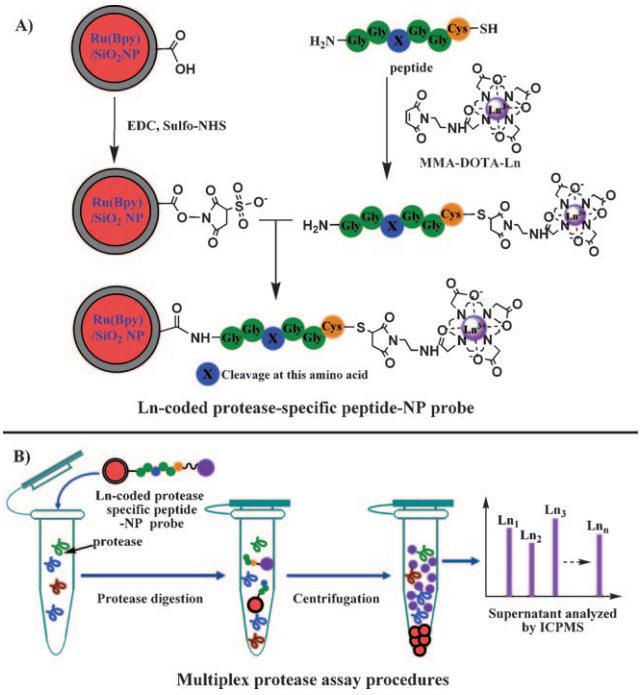

Scheme 1. A) Synthesis of an Ln-coded protease-specific peptide–NP probe. Protease-specific peptides with C-terminal SH in cysteine were conjugated with the maleimide group of Ln-coded MMA-DOTA-Ln, and then the N-terminal NH_2 in glycine of the peptides was conjugated to the carboxyl groups on the surface of the dye-doped silica NP (orange) through an EDC reaction. B) Protease assay procedures. First, Ln-coded protease-specific peptide–NP probes were added to a biological sample. In the presence of proteases, the peptides were cleaved and the Ln released from the probes. After ultracentrifugation, uncleaved Ln-coded protease-specific peptide–NP probes were removed and the protease-cleaved Ln harvested in the supernatant was subjected to analysis using ICP-MS, thereby realizing multiplex protease assay. bpy = 2,2′-bipyridine, EDC = *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride, NHS = *N*-hydroxysuccinimide.

Table 1: Bare SiO_2 NPs and four Ln-coded protease-specific peptide–NP probes with their corresponding hydrodynamic diameter (D_h) and zeta potential (ξ) values.

	D_h[a] [nm]	ξ[a] [mV]
bare SiO_2 NPs	73.6 ± 1.2	−50.08 ± 4.93
GGRGGC–DOTA-Ho-labeled SiO_2 NPs (P1)	80.4 ± 1.3	−40.68 ± 0.61
GGYGGC–DOTA-Tb-labeled SiO_2 NPs (P2)	77.7 ± 1.0	−43.72 ± 2.57
GEYEGC–DOTA-Pr-labeled SiO_2 NPs (P3)	79.4 ± 1.2	−51.20 ± 0.94
GGEGGC–DOTA-Eu-labeled SiO_2 NPs (P4)	78.9 ± 1.7	−46.80 ± 0.11

[a] The values were measured using the dynamic light scattering technique.

Information). The surface of the SiO_2 NPs was functionalized with carboxyl groups via 3-aminopropyltriethoxysilane (APTES)–succinic anhydride conjugate (see Figure S1 in the Supporting Information). Figure S2 in the Supporting Information shows the scanning electron microscopy image of the carboxyl-functionalized SiO_2 NPs. The carboxyl-SiO_2 NPs were uniform with an average diameter of about 65 nm, and the hydrodynamic diameter (D_h) was determined to be (73.6 ± 1.2) nm (Table 1). The carboxyl-SiO_2 NPs were highly dispersed in water and stable for months, which was consistent with the strongly negative zeta potential (ξ) of (−50.18 ± 1.93) mV measured in water.

The sulfhydryls in the cysteine residue of the peptides (GGRGGC, GGYGGC and GEYEGC, as well as GGEGGC) were conjugated with the MMA in MMA-DOTA-Ln under the reaction conditions optimized in our previous work (Figure S3 in the Supporting Information).[7j] Successful conjugation of one peptide with one MMA-DOTA-Ln unit was confirmed by using ESI-MS (see Figure S4 in the Supporting Information). The carboxyl-SiO_2 NPs were further conjugated with the Ln-coded protease-specific peptides by using the zero-length coupling reagent EDC (see Figure S5 in the Supporting Information). D_h and ξ (Table 1) in the case of P1 were (80.4 ± 1.3) nm and (−40.68 ± 0.61) mV, respectively. These increased D_h and ξ values relative to the bare carboxyl-SiO_2 NPs were ascribed to the conjugation of GGRGGC–MMA-DOTA-Ho to the surface of carboxyl-SiO_2 NPs. Similar phenomena were observed in the cases of P2 and P4. However, in the case of P3, its D_h value [(79.4 ± 1.2) nm] increased but its ξ value [(−51.20 ± 0.94) mV] decreased compared with the bare NPs. This decrease in ξ was because of the negative charges from the two Glu residues (pK_a 4.15) in GEYEGC. The peptide-dependent properties of the Ln-coded protease-specific peptide–NPs confirmed the conjugation of Ln-coded protease-specific peptides to the NPs. Under the optimized reaction conditions, 3900–4500 Ln-coded protease-specific peptides were conjugated to each carboxyl-SiO_2 NP as determined by ICP-MS (see Figure S6 in the Supporting Information), which resulted in a concentration effect on the probes and thus an improvement in sensitivity.

To investigate the specificity of the four Ln-coded protease-specific peptide–NP probes to trypsin and chymotrypsin, equal amounts of the probes were mixed and incubated with the proteases in 3-(*N*-morpholino)propanesulfonic acid (MOPS; for detailed procedures, see the Supporting Information). The results obtained (Figure 1)

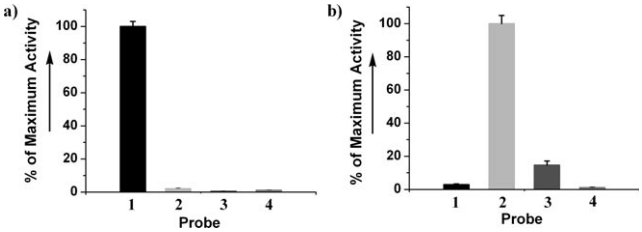

Figure 1. Specificity of probes 1–4 (see Table 1 for probe details) to a) trypsin and b) chymotrypsin.

indicated that P1 and P2 were very specific to trypsin and chymotrypsin, respectively, while no signal was observed in the case of P4 (the control). Compared with P2, which has two smaller Gly residues adjacent to Tyr, the peptide in P3 has two Glu residues adjacent to Tyr. Although chymotrypsin could cleave the peptide in P3 at the carboxyl side of Tyr, the efficiency was much lower because of the larger steric hindrance and the negative charge from adjacent Glu

units,[11] which suggests the importance of protease-specific peptide designation for selective and sensitive protease assay.

Subsequently, we characterized the kinetics of the proteases to the corresponding probes by using ICP-MS. Varying concentrations of P1 and P2 were incubated respectively with trypsin (25 nM) and chymotrypsin (50 nM) to measure the corresponding initial velocities. The Michaelis constant (K_M) and maximum velocity (V_{max}) were estimated according to the Michaelis–Menten kinetics equation ($V_0 = (V_{max}[S])/(K_M+[S])$), where V_0 is the initial proteolytic velocity and [S] is the substrate concentration (in this study, the concentration of the conjugated protease-specific peptide in the probe). In the cases of P1 and P2, the K_M and V_{max} values were estimated to be (18.34 ± 6.27) μM and (2.53 ± 0.43) μM min^{-1}, and (14.54 ± 3.67) μM and (2.21 ± 0.31) μM min^{-1}, respectively (Figure 2), and the catalytic

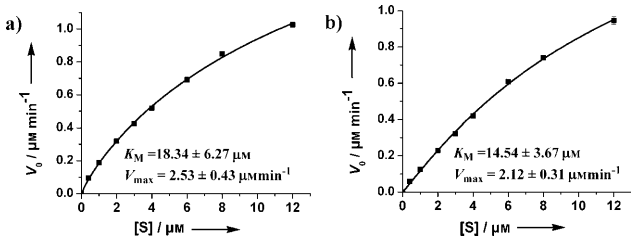

Figure 2. Plots of initial proteolytic velocity (V_0) of a) trypsin against the concentration of GGRGGC in P1 and b) chymotrypsin against the concentration of GGYGGC in P2, and the corresponding estimated K_M and V_{max} values (means of three experiments).

constants ($K_{cat} = V_{max}$/[protease]) were (1.69 ± 0.29) and (0.71 ± 0.1) S^{-1}, respectively. K_{cat}/K_M indicates the ability of an enzyme to capture its substrates,[12] and the values for P1 and P2 were calculated to be 9.2×10^4 and 4.8×10^4 M^{-1} S^{-1}, comparable to the reported values of some low-molecular-weight substrates,[13] and thus implying the effectiveness of the designed and synthesized probes. Moreover, there was a static interaction between the Ln-coded protease-specific peptide–NPs and the positively charged trypsin (pI 10.1) and chymotrypsin (pI 8.8) under the experimental pH 7.5 conditions, which resulted in an enrichment effect of the proteases on the probe[14] and further improved the sensitivity of the assay.

Although trypsin and chymotrypsin are normally secreted by pancreatic acinar cells for food digestion, elevated levels of these two proteases in blood and/or urine are reported to be associated with pancreatic disease and invasion by some types of cancer.[15] We therefore applied this proposed method to assay trypsin and chymotrypsin in serum and/or urine, and demonstrated the feasibility of its use for duplex protease assay in one biological sample. Varying and known concentrations of trypsin and chymotrypsin were spiked into serum and/or urine from healthy volunteers, and appropriate amounts of P1 and P2 were added to initiate the proteolytic reaction. The cleaved Ho and Tb concentrations determined by using ICP-MS were plotted against the protease concentration (Figure 3). Compared with the detection limits (DLs) (3σ) of 0.5 and 1.3 pM for trypsin and chymotrypsin, respectively, and linear ranges from 6 to 60 pM obtained in the

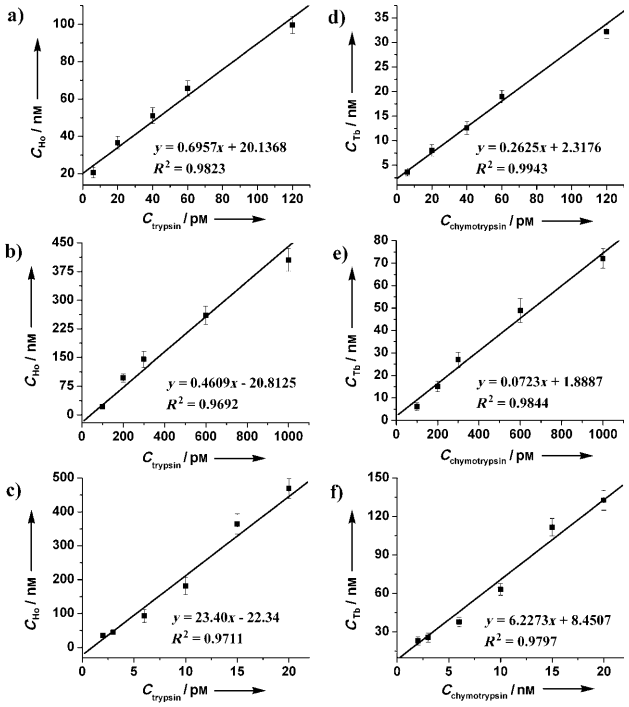

Figure 3. Linear relationship between the concentrations of trypsin (a–c) or chymotrypsin (d–f) and cleaved Ho or Tb in a, d) buffer, b, e) urine, and c, f) serum.

MOPS-containing $CaCl_2$ buffer solution, the DLs of trypsin and chymotrypsin in urine were 6 and 30 pM, respectively, and the linear range was from 100 to 1000 pM; in serum, the DLs were 0.12 and 0.42 nM, and they ranged from 2 to 20 nM. The decreased DLs in urine and serum probably resulted from endogenous proteases and protease inhibitors in these fluids. It should be noted, however, that the DLs obtained were still compatible with the determination of trypsin (reference level, 212 ng mL^{-1} or 9.1 nM) and chymotrypsin (37.5 ng mL^{-1} or 1.47 nM) in serum,[16,17] and lower than the reported DLs of ^{125}I-labeled antibody radioimmunoassay (DL of trypsin, 0.5 nM) and a commercially available chymotrypsin ELISA kit (DL of chymotrypsin, 0.56 nM).[16,18] The determined concentrations of trypsin in the serum and urine samples were estimated to be (6.9 ± 0.2) and (2.5 ± 0.1) nM, respectively, and for chymotrypsin (313.6 ± 12.0) and (763.2 ± 6.8) pM. It should be pointed out that these values are the contributions of not only endogenous trypsin and chymotrypsin but also of trypsin- and chymotrypsin-like proteases and their inhibitors in the samples. More sophisticated designation of probes will benefit more specific protease (such as caspase) assay.

In summary, we have developed a novel strategy for label-free multiplex protease assay using newly designed and synthesized Ln-coded protease-specific peptide–NP probes and ICP-MS. The characteristics of solid Ln-coded protease-specific peptide–NPs allowed the easy separation of specifically cleaved and uncleaved probes. More importantly, the different types of protease-specific peptides and the correspondingly coded lanthanides guaranteed multiplex protease assay in one biological sample when using ICP-MS, without

any subsequent laborious separation procedures. Integrating the lanthanide-coded protease-specific peptide with functional NPs together with ICP-MS will open a new door and pave the way to more efficient and accurate multiplex protease assay. This novel strategy can be extended easily by designing various protease-specific peptide substrates and coding corresponding ICP-MS detectable elements (not limited to lanthanides) in the functional nanoparticle probes for simultaneous, efficient, and accurate multiplex protease assay in various biosystems. Such research is ongoing in our laboratory.

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