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Peptide Code-on-a-Microplate for Protease Activity Analysis via MALDI-TOF Mass Spectrometric Quantitation

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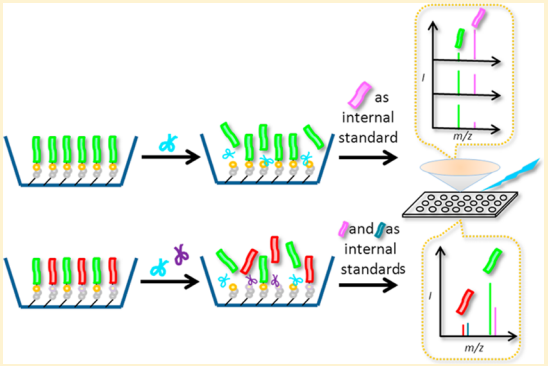
Supporting Information

ABSTRACT: A peptide-encoded microplate was proposed for MALDI-TOF mass spectrometric (MS) analysis of protease activity. The peptide codes were designed to contain a coding region and the substrate of protease for enzymatic cleavage, respectively, and an internal standard method was proposed for the MS quantitation of the cleavage products of these peptide codes. Upon the cleavage reaction in the presence of target proteases, the coding regions were released from the microplate, which were directly quantitated by using corresponding peptides with one-amino acid difference as the internal standards. The coding region could be used as the unique "Protease ID" for the identification of corresponding protease, and the amount of the cleavage product was used for protease activity analysis. Using trypsin and chymotrypsin as the model proteases to verify the multiplex protease assay, the designed "Trypsin ID" and "Chymotrypsin ID" occurred at m/z 761.6 and 711.6. The logarithm value of the intensity ratio of "Protease ID" to internal standard was proportional to trypsin and chymotrypsin concentration in a range from 5.0 to 500 and 10 to 500 nM, respectively. The detection limits for trypsin and chymotrypsin were 2.3 and 5.2 nM, respectively. The peptide-encoded microplate showed good selectivity. This proposed method provided a powerful tool for convenient identification and activity analysis of multiplex proteases.



Proteases, known as proteolytic enzymes, can catalyze the breakdown of proteins by hydrolysis of peptide bonds with high selectivity.[1] More than 500 proteases or protease homologues are produced by human cells, and some play fundamental roles in a series of biological processes, including tissue remodeling, blood clotting, protein metabolism, and immune defense.[2−5] Alterations in protease activities have been implicated in many diseases, such as arthritis, inflammation, and Alzheimer's disease, as well as cancer.[6−8] Therefore, effective methods for protease assay, especially for the analysis of multiple proteases, are necessary and may contribute to uncover the roles of proteases in different physiological processes. Furthermore, a sensitive and easy-to-use protease assay strategy is critical to evaluate the drug candidates that target the proteases.[9]

A traditional immunoassay method has been used for sensitive detection of protease concentrations, but it often suffers from the risk of false-positives due to cross-reactions and the interference of inactive proteases.[10,11] Thus, this method is unsuitable for protease activity assay because the protease concentration is not necessarily proportional to its activity, which is modulated by post-translational mechanisms.[12] As an alternative, the peptide cleavage has been considered to be a suitable technique for the analysis of multiplex proteases by synthesizing specific peptides for certain proteases.[13−15] After cleavage by the corresponding target proteases, the released or reserved peptides can be detected with optical[13,14] or electrochemical[15] techniques. The fluorescence detection of caspase-3, MMP-7, and MMP-2 shows the detection limits of 0.4 nM,[13] 0.5 pg mL^{-1}, and 4.8 pg mL^{-1},[14] respectively, and the electrochemical method can detect trypsin and chymotrypsin down to 2.5 and 1.6 ng mL^{-1}.[15] However, the possible signal overlapping limits their application, and the chemical labeling reagents used for obtaining the signal are also susceptible to disruption of intrinsic catalytic activity of proteases.[16,17] Compared with these detection techniques, the measurement of molecular mass can overcome the obstacles and provide a label-free analysis of enzyme activities. The elimination of the labeling regents is significant to remain the biologically relevant activities of the proteases and improve assay selectivity by distinguishing nonspecific proteolysis at different amino acid residues without background interference.[18]

Recently, lanthanides have been adapted as elemental tags to multiplex protease assays using inductively coupled plasma mass spectrometry (ICP-MS), which show satisfactory results.[19,20] However, the preparation and purification of the lanthanide probes still keep time-consuming. Although liquid chromatography−tandem mass spectrometry (LC-MS/MS) combined with a multiple reaction monitoring (MRM)

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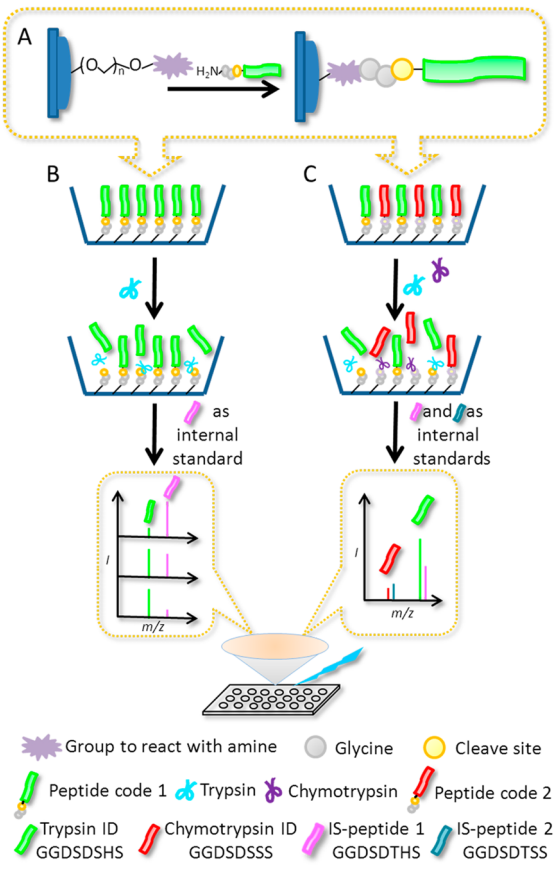
technique has also been used to quantitatively characterize enzyme activities and considered to be a "gold standard method", this technique requires tedious pretreatment of the samples, such as separation and desalting protocols,[21,22] and a corresponding method for activity analysis of proteases has not developed yet. Considering the fact that the MALDI-TOF mass spectrometric (MS) method has special advantages for analysis of multiplex peptides due to its high throughput and tolerance for complex samples and can be used for peptide quantitation with an internal standard,[23] this work developed a peptide encoding technique for multiplex proteases and designed a peptide-encoded microplate for MALDI-TOF MS analysis of protease activity.

The molecular weights of peptides can be simply adjusted by changing its length and sequence, and these molecules can be released by enzymatic hydrolysis for subsequent MS quantitation. Thus, they are ideal codes for multiple target proteases. Inspired by these properties, the peptide codes were designed to contain a coding region as the "Protease ID" and the substrate of protease for enzymatic cleavage respectively, which were immobilized on a microplate for protease activity analysis (Scheme 1). Upon their cleavage by different target proteases, the "Protease ID" was specifically released, identified and quantified with the proposed internal standard method (Scheme 1B,C). As a proof of concept, trypsin and chymotrypsin were as the model proteases to achieve a duplex protease assay, with "Trypsin ID" and "Chymotrypsin ID" at m/z 761.6 and 711.6, respectively. This technique was capable of distinguishing different kinds of proteases with good selectivity and precision. Owing to the simplicity, quick speed, and high throughput, it is a powerful tool for effective assay of protease activity.

Scheme 1. Schematic Illustration of (A) Fabrication of Peptide-Encoded Microplate, (B) Single Peptide Modified Microplate for Detection of Trypsin, and (C) Peptide-Encoded Microplate for Multiplex Protease Activity Assay Using MALDI-TOF MS



EXPERIMENTAL SECTION

Materials and Reagents. Immobilizer amino plates were purchased from Thermo Scientific Nunc (Vedbaek, Denmark). Peptides GGRGGDSDSHS (peptide code 1, M_w 1030.96), GGDSDTHS (IS-peptide 1, M_w 774.70), GGYGGDSDSSS (peptide code 2, M_w 987.89), and GGDSDTSS (IS-peptide 2, M_w 724.64) were synthesized and purified by Sangon Biotech (Shanghai, China) with purity greater than 95.0%. Trypsin (from bovine pancreas), α-chymotrypsin (from bovine pancreas, type VII), horseradish peroxidise (HRP), bovine serum album (BSA), human serum immunoglobulin G (human IgG), glucosamine oxidase (GO_x), phenylmethanesulfonyl fluoride (PMSF, ≥99.0%), 4-(2-aminomethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, ≥97%), 4-morpholinepropanesulfonic acid (MOPS), trifluoroacetic acid (TFA, ≥90%), and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (U.S.A.). Acetonitrile was obtained from Merck (Darmstadt, Germany). All these reagents were used as received without further purification and aqueous solutions were prepared using ultrapure water (≥18.2 MΩ, Milli-Q, Millipore).

Fabrication of Peptide-Encoded Microplate. Peptide codes were dissolved in 2 mM sodium carbonate buffer (pH 9.6) with a final concentration of 50 μM, respectively. The peptide-encoded microplate was fabricated by dropping 100 μL of the solution in each well of the plate and incubating at room temperature (20−25 °C) overnight. After the wells were gently washed with water and dried with nitrogen steam, the microplate was ready for use.

Single Protease Assay. For identifying the protease, 500 nM of trypsin in 10 mM MOPS (50 μL, pH 7.8) was added to a peptide 1-encoded well of the plate and incubated for 30 min at 37 °C. A tota of 1 μL of the reacted solution was then deposited on the well of the MALDI target plate and dried at room temperature. For monitoring the reaction process, 100 nM trypsin (50 μL in 10 mM MOPS) was incubated in peptide 1-encoded well for 5, 10, 20, 30, and 40 min at 37 °C. After mixing the solutions with 10 μL of IS-peptide 1 at 50 μM in acetonitrile as the internal standard, MALDI-TOF MS analysis was carried out.

To perform the quantitative detection, trypsin (50 μL) at different concentrations (2 nM to 2 μM) in 10 mM MOPS or 10× diluted human urine samples was incubated in peptide 1-encoded microplate. While for trypsin inhibition assay, different concentrations of inhibitors (0.01, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM) were mixed with 100 nM trypsin, and 50 μL of the mixtures were added to peptide 1-encoded wells to incubate at 37 °C for 30 min. Afterward, 10 μL of 50 μM IS-peptide 1 in acetonitrile was mixed with the solution and submitted to MALDI-TOF MS analysis. The urine samples were collected from a healthy adult volunteer with an informed consent, which was approved by the local ethical committee.

Duplex Protease Assay. To demonstrate the application of this method in multiplex analysis of proteases, a series of concentrations (5−500 nM) of trypsin and chymotrypsin in 10 mM MOPS containing 2 mM $CaCl_2$ were added to the peptide

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1 and peptide 2-encoded microplate and incubated for 30 min at 37 °C. A total of 10 μL of a mixture containing 50 μM IS-peptide 1 and 50 μM IS-peptide 2 as internal standards was then added to each well and submitted to MALDI-TOF MS analysis.

The lysates of HeLa, MCF-7, PANC-1, and PC-3 cells were used as practical samples to verify the application of the duplex protease assay, respectively. These cells were grown in cell culture media in a humidified atmosphere containing 5% CO_2 at 37 °C. After suspending with trypsin, they were washed thrice with phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4 to remove the culture media and the used trypsin. The cell number was determined using a Petroff-Hausser cell counter (U.S.A.). These cells were dispersed in 10 mM MOPS containing 2 mM $CaCl_2$, sonicated for 15 min, and centrifuged to collect the supernatant. Finally, 50 μL of the 10× diluted supernatant was subjected to MALDI-TOF MS analysis.

MS Analysis. MALDI-TOF MS experiments were performed on a 4800 *Plus* MALDI TOF/TOF Analyzer (AB Sciex, U.S.A.) with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV. After 1 μL of the solution was deposited on the well of MALDI plate and dried, 1 μL of the saturated CHCA in the mixture of 50% acetonitrile, 0.1% TFA, and 49.9% water (V/V/V) was induced as matrix. The MS data were acquired in positive reflective mode. For each spectrum, 50 shots from different positions of the target spot (automatic mode) were collected and analyzed. Data analysis was performed with a Data Explorer Software from AB Sciex. The presence of corresponding protease could be rapidly identified from one mass spectrum.

To assess the accuracy of the proposed method, the activities of trypsin and chymotrypsin were also detected with BAEE (*N*-α-benzoyl-L-arginine-ethylester at 253 nm) and BTEE (*N*-α-benzoyl-L-tyrosine-ethylester at 256 nm) methods, respectively.[24−26] Briefly, the assay was carried out in 0.1 M Tris-HCl buffer (pH 8.0) containing 6 mM $CaCl_2$, 10 μM substrate (BAEE or BTEE), and trypsin or chymotrypsin at different concentrations, or 10× diluted samples. The absorbance was measured at 25 °C on a UV-3600 spectrophotometer (Shimadzu, Japan).

RESULTS AND DISCUSSION

Peptide Code for Trypsin. The proposed coding technique was demonstrated using peptide code 1 with sequence GGRGGDSDSHS designed as the substrate of trypsin. A portable peptide 1-encoded microplate could be prepared by binding peptide code 1 to the immobilizer amino plate. The capacity of the obtained microplate could be examined by the mass spectra after corresponding reaction. No significant peak was detectable in the absence of trypsin (Figure 1A), while a strong peak at *m*/*z* 761.6 was observed on the mass spectrum after the microplate was exposed to 500 nM target trypsin (Figure 1B), indicating low background and the cleavage of peptide code 1 by trypsin, which occurred at the carboxyl side of arginine of peptide code 1. The signal peak at *m*/*z* 761.6 corresponded to the protinated form of the coding region, GGDSDSHS, which was verified with MS/MS analysis (Figure S1A). As a marker the occurrrence of peak at *m*/*z* 761.6 coded the information on trypsin. Thus, it served as the "Trypsin ID".

Feasibility of Internal Standard Method. Inhomogeneous crystallization of analytes in MALDI matrices often produces "sweet spots", which results in poor reproducibility and severely hinders the quantitative application of MALDI-TOF MS. Thus, an internal standard was mixed in the sample solution for the quantitation of the cleaved peptide. For trypsin activity assay, IS-peptide 1 (GGDSDTHS, *m*/*z* = 775.7) with one-amino acid difference from the "Trypsin ID", in which one serine was changed to threonine, was used as the internal standard. In different measurements, the absolute intensity of the "Protease ID" with the same concentration greatly changed (Figure S2A−C), however, the peak intensity ratio of "Trypsin ID" at *m*/*z* 761.6 to IS-peptide 1 at *m*/*z* 775.7 remained around 0.69 with the relative standard deviation (RSD) of 1.4% (Figure S2D−F). This result demonstrated the feasibility of the internal standard method for quantitative analysis of cleaved peptide.

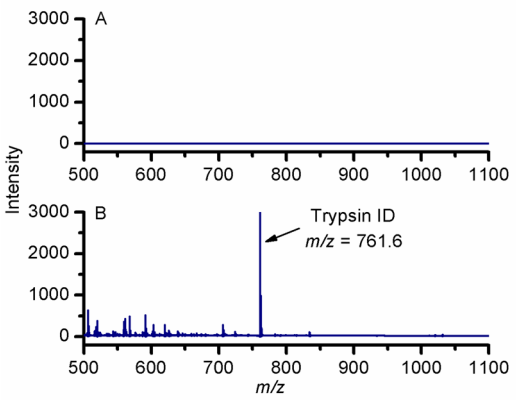

Figure 1. Mass spectra of the solutions obtained from peptide code 1 modified microplate after reaction with MOPS in absence (A) and presence (B) of 500 nM trypsin for 30 min.

Quantitative Detection of Protease Activity. In order to ensure the complete cleavage of the peptide codes by target proteases, the cleavage dynamics was examined with the internal standard method. With the increasing incubation time of 100 nM trypsin in peptide 1-encoded microplate, the peak intensity ratio of "Trypsin ID" at *m*/*z* 761.6 to IS-peptide 1 at *m*/*z* 775.7 increased and trended to the maximum value at the time of 30 min (Figure S3). Thus, 30 min was selected as the cleavage time for quantitative analysis of cleaved peptides.

After various concentrations of 50 μL of target trypsin were incubated in the wells of peptide 1-encoded microplate, the MS spectra of the mixtures of reaction solutions and 10 μL of 50 μM IS-peptide 1 showed the increasing peak intensity ratio of "Trypsin ID" at *m*/*z* 761.6 to IS-peptide 1 at *m*/*z* 775.7 with the increasing concentration of trypsin (Figure 2A). The logarithm values of the ratio and trypsin concentration showed a linear relationship in the concentration range from 5.0 nM to 1000 nM with R^2=0.992 (Figure 2B). The limit of detection (LOD) was estimated to be 2.0 nM by using the calibration curve and 3 times standard deviation of blank measurements. The LOD was lower than that of 4 nM for graphene oxide-quenched fluorescence assay,[27] and comparable with that of 2 nM for phosphorescence turn-on or label-free colormetric assay.[28,29] The specifically cleaved product with unique "Trypsin ID" rather than optical or electronic signals was more specific and convenient for the identification.

According to the previous reports,[30−32] the trypsin level is associated with some pancreatic diseases such as acute pancreatitis and cystic fibrosis. Unfortunately, this work did not get the samples from patient, and only the urine sample was

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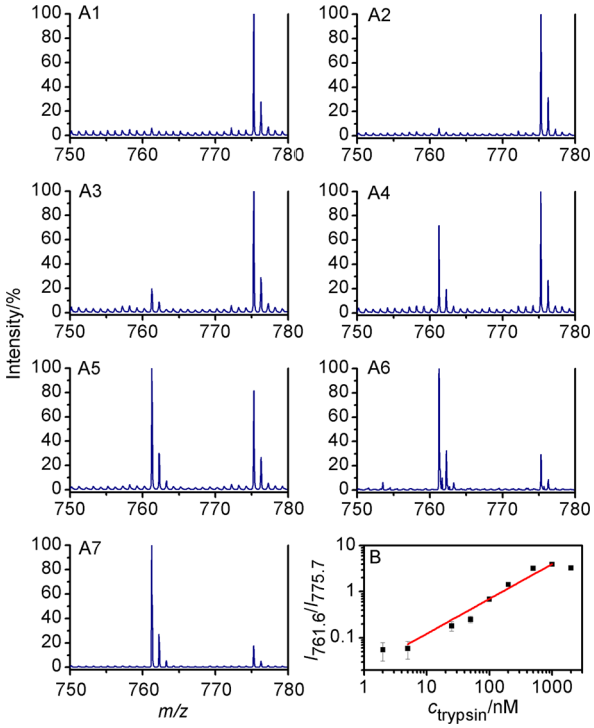

Figure 2. MALDI-TOF MS quantitation of trypsin using peptide code 1 modified microplate and IS-peptide 1 as internal standard. (A1−A7) Mass spectra for 5, 25, 50, 100, 200, 500, and 1000 nM trypsin, and (B) linear calibration curve. The data in (B) were obtained by three independent experiments.

collected from an adult healthy volunteer as a biological sample to test practical application of the proposed encoding strategy. It did not show the peak of "Trypsin ID" at m/z 761.6, indicating there was no trypsin or trypsin-like protease in the sample, which was consistent with that measured with BAEE method.[24−26] To obtain the calibration curve, various concentrations of trypsin were spiked in 10-fold diluted urine samples. After incubating the spiked samples in the wells of peptide 1-encoded microplate and mixing with the internal standard, the MS spectra showed obvious peaks of "Trypsin ID" and the IS-peptide 1 (Figure 3A), indicating that the components of urine sample did not obvoiusly affect the hydrolysis and MS analysis. The calibration curve showed a linear range from 10 to 1000 nM with a LOD of 4.0 nM (Figure 3B).

Although there is virtually no trypsin in urine of healthy humans, the level of trypsin is high in the urine of some patients. For example, pancreas transplant patients show the average concentration of 84.4 μg mL^{-1} for trypsin, corresponding to 3.4 μM.[33,34] Thus, the proposed microplate could be implemented for direct detection of trypsin in clinical urine samples after the samples were diluted for 10 times. After 10× fold diluted urine sample from healthy human was spiked with 50 nM trypsin, the recovery for three detections was (105.7 ± 5.8)%, indicating acceptable accuracy.

Screening of Protease Inhibitors. The evaluation of protease inhibitors is significant in drug discovery.[35−37] Here the peptide-encoded microplate was tested against two frequently used small molecular inhibitors, PMSF and AEBSF. The inhibition efficiency (%) could be calculated with $[1-(I_{761.6} \times I_{0\ 775.7})/(I_{0\ 761.6} \times I_{775.7})] \times 100\%$, where I_0 and I are the MS peak intensity in the absence and presence of

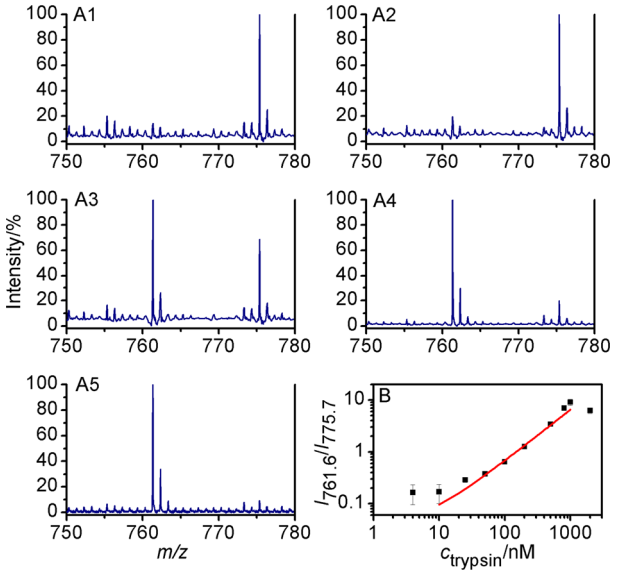

Figure 3. Quantitative detection of trypsin in spiked human urine samples with the proposed method. (A1−A5) Mass spectra for urine samples spiked with 25, 50, 200, 500, and 1000 nM of trypsin, and (B) linear calibration curve.

inhibitor, respectively. Figure 4 shows the plots of the inhibition efficiency toward trypsin versus the concentration of two

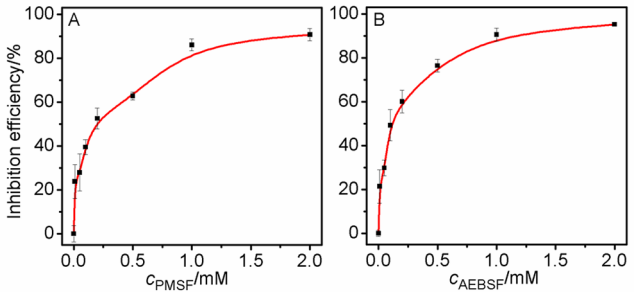

Figure 4. Plots of inhibition efficiency of (A) PMSF and (B) AEBSF to 100 nM trypsin vs their concentrations.

inhibitors. The addition of both PMSF and AEBSF could reduce the activity of trypsin and thus reduce the cleavage of peptide code 1. The IC_{50} (defined as 50% inhibition efficiency) for PMSF and AEBSF were estimated to be 0.19 and 0.12 mM, respectively. These results demonstrated the application of the coding assay in the screening of protease inhibitors.

Duplex Protease Assay with Peptide-Encoded Microplate. Trypsin can specifically cleave peptides at the C-terminal of Arg/Lys residue, while chymotrypsin specifically cleaves the peptides at the C-terminal of Tyr/Phe/Trp residue. Considering the difference between their specific cleavage sites, these enzymes were used as model targets for the duplex protease assay. To demonstrate the feasibility of the proposed assay for distinguishing these proteases, both peptide code 1 GGRGGDSDSHS and peptide code 2 GGYGGDSDSSS were coupled to immobilizer amino plates. In the absence of targets the MS spectrum did not show any typical peak of "Protease ID" (Figure 5A). The presence of 500 nM trypsin led to the appearance of only one strong peak at m/z 761.6, while a peak at m/z 711.6 was specifically detected when the microplate was exposed to 500 nM chymotrypsin (Figure 5B and C). When

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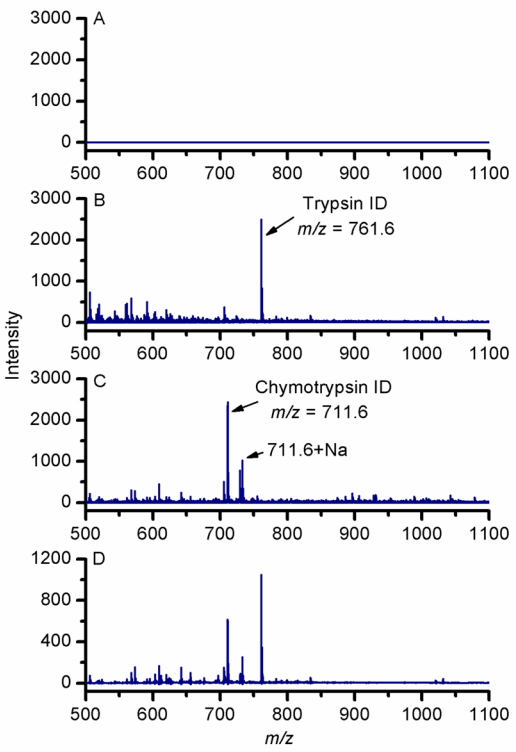

Figure 5. Mass spectra of the solutions obtained from duplex peptide-encoded microplate after reaction with MOPS in the (A) absence and presence of (B) 500 nM trypsin, (C) 500 nM chymotrypsin, and (D) 500 nM trypsin and chymotrypsin for 30 min.

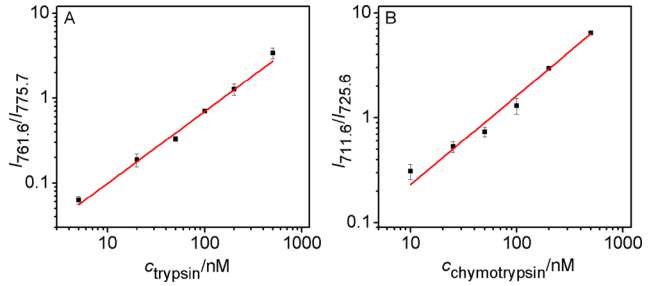

Figure 6. Plots of logarithm values of the intensity ratios of (A) trypsin ID to IS-peptide 1 and (B) chymotrypsin ID to IS-peptide 2 vs trypsin and chymotrypsin concentrations, respectively.

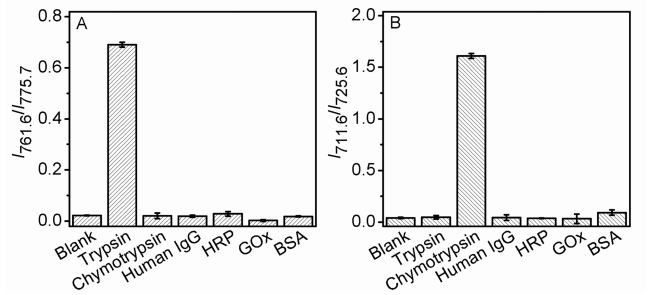

Figure 7. Intensity ratios of (A) trypsin ID to IS-peptide 1 and (B) chymotrypsin ID to IS-peptide 2 obtained with duplex peptide-encoded microplate for blank control, 100 nM trypsin, 100 nM chymotrypsin, 1 μM human IgG, 1 μM HRP, 1 μM GO$_x$, and 1 μM BSA, respectively.

the microplate was exposed to 500 nM trypsin and chymotrypsin, two cleavage products were observed on the MS spectrum (Figure 5D). These phenomena indecated that both trypsin and chymotrypsin only cleaved their corresponding peptide codes at predicted sites. The cleavage products were just the coding regions GGDSDSHS (m/z = 761.6) and GGDSDSSS (m/z = 711.6), which were further verified with a positive-ion MS/MS method (Figure S1, Tables S1 and S2). Therefore, this microplate could be used for identification of the types of proteases with the unique "Protease ID" peaks.

For quantitative assay of the protease activity, a mixture of IS-peptide 1 and IS-peptide 2 was introduced as the multiple internal standards. Similarly, the logarithm value of the peak intensity ratio of "Protease ID" to IS-peptide ($I_{761.6}/I_{775.7}$ for trypsin and $I_{711.6}/I_{725.6}$ for chymotrypsin) was proportional to the logarithm value of protease concentration. The linear ranges for trypsin and chymotrypsin were in 5.0−500 and 10−500 nM (Figure 6), respectively. Their detection limits (3σ) were 2.3 and 5.2 nM, respectively. This implied the effectiveness of the proposed method for duplex protease assay. Besides, more delicate design of peptide codes will benefit more specific proteases (such as caspase) by employing the peptide encoding strategy.

Specificity and Stability of Peptide-Encoded Microplate. The specificity of the peptide-encoded microplate was investigated by comparing the peak intensity ratio of "Protease ID" to IS-peptide toward the model target proteases and other proteins such as BSA, human IgG, HRP, and GO$_x$. The blank test showed negligible background, while the target proteases showed obvious responses on corresponding peptide-encoded microplates (Figure 7). When other proteins existed in the reaction solution, the response was also negligible, even at a concentration 10-fold higher than that of trypsin and chymotrypsin, suggesting outstanding specificity of the proposed method, which avoided the problem of "false signal".

The reproducibility of the proposed method was examined by testing 20, 100, and 200 nM trypsin and chymotrypsin for 5×, respectively. The peak intensity ratios of "Protease ID" to IS-peptide showed the RSDs of 3.6, 5.3, and 2.6% for trypsin, and 4.5, 3.5, and 4.6% for chymotrypsin, indicating acceptable precision and fabrication reproducibility of the peptide-encoded microplates. When the microplate was stored at 4 °C when not in use, both the absolute intensity and peak intensity ratios did not obviously change after 4 weeks. All the tests proved that the peptide-encoded microplate could be a robustness tool for protease analysis.

Detection of Proteases in Cell Lysates. The proposed method was further assessed with cell-related samples including two nonpancreatic cells (HeLa and MCF-7) and two pancreatic carcinoma cells (PANC-1 and PC-3). The detection results are listed in Table 1, which indicated that the proposed method could differentiate nonpancreatic and pancreatic cells. The measurement accuracy for trypsin and chymotrypsin was also examined by comparing the results with those obtained from reference method,[24−26] which showed the relative errors less than 6.2%. Thus, the proposed peptide encoding strategy possessed potential application in clinical diagnosis.

CONCLUSIONS

This work proposes a concept of peptide code-on-a-microplate and a peptide encoding technique for simple and rapid analysis of protease activity using MALDI-TOF MS. The peptide codes contain a coding region and a cleavage site for different proteases, respectively. The proteases can be identified with the

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Table 1. Detection Results of Cell Lysates Using the Proposed and Reference Methods[a]

	trypsin (μM)			chymotrypsin (nM)		
sample	proposed method	reference method	relative error (%)	proposed method	reference method	relative error (%)
HeLa	ND	ND		ND	ND	
MCF-7	ND	ND		ND	ND	
PANC-1	1.20 ± 0.09	1.28	−6.2	137.31 ± 4.42	131.20	4.7
PC-3	1.28 ± 0.07	1.34	−4.5	115.58 ± 5.23	112.33	2.9

[a]Cell concentration: HeLa, 7.5 × 10^5 cell mL^{-1}; MCF-7, 5.0 × 10^5 cell mL^{-1}; PANC-1, 4.5 × 10^5 cell mL^{-1}; PC-3, 4.8 × 10^5 cell mL^{-1}.

specific m/z on MS spectra of the cleaved encoding regions. By using another peptide with difference of one amino acid as the internal standard, the MALDI-TOF MS quantitative detection of protease activity can be performed. With more sophisticated design of the peptide sequences, multiplex protease assay with high selectivity has been demonstrated. This proposed strategy shows excellent analytical performance with good convenience, high specificity as well as acceptable precision. It could be anticipated that the peptide encoding technique and this method provide a powerful tool for multiplex sensing and investigation of numerous protease-related processes.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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