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A homogeneous mass-encoded strategy for mass spectrometric biosensing of multiplex proteins

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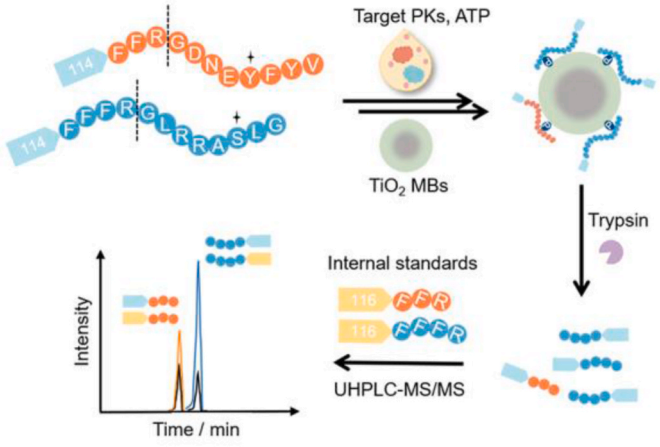
HIGHLIGHTS

- A homogeneous mass-encoded strategy was presented for multiplex kinase activity assay.
- The activity assay could be performed in homogeneous solution without substrate immobilization.
- The proposed mass spectrometric biosensing demonstrated sensitive and specific detection of kinase activities.
- The work advanced the application of mass spectrometric biosensing in clinical research.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Protein kinases play important roles in fundamental biological processes. Aberrant activities may result in many diseases, thus the detection of multiplex kinase activities is important in clinical diagnosis. Recent progress has focused on the mass spectrometric biosensing technique to enable highly sensitive detection of multiplex targets. However, the application of the developed methods in multiplex enzyme analysis is greatly challenged due to the substrate immobilization on solid interfaces, which affect the contact between substrates and enzymes to reduce enzyme reaction efficiency.

Results: This work developed a homogeneous mass-encoded method for biosensing of multiplex proteins, which was performed using designed peptides containing the coding sequences and substrate regions. With the assistance of titanium dioxide coated magnetic beads (TiO_2-MBs) to capture the phosphopeptide products, followed by trypsin to cleave the products for releasing the coding sequences, the kinase assays were achieved by

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submitting the supernatant for ultrahigh performance liquid chromatography-tandem mass spectrometric (UHPLC-MS/MS) analysis. Using protein kinase A (PKA) and human epidermal growth factor receptor 2 (HER2) as model targets, the peak area ratios of the coding sequences to the internal standards showed linear relations of 1.0–100 ng mL^{-1} and 0.2–20 U mL^{-1}, with the detection limits of 0.46 ng mL^{-1} and 0.033 U mL^{-1} for HER2 and PKA, respectively. The proposed strategy also demonstrated great practicability in inhibition analysis and kinase activity assays in cell lysates.

Significance: A homogeneous mass-encoded method for multiplex detection of kinase activities could simplify the assay procedure and reveal the enzyme activities with free substrates. The strategy enabled multiplex kinase activity assays with convenience, high sensitivity, and high specificity, demonstrating promising applications in clinical fields.

1. Introduction

Protein kinases are known as enzymes to catalyze protein phosphorylation, one of the most frequent post-translational modifications (PTMs) [1–4]. They play significant roles in fundamental metabolic processes and cellular signal communications [5–8]. Research has demonstrated that aberrant kinase activities may result in many diseases [9,10], and specific protein kinases can serve as biomarkers for certain types of cancer, also as molecular targets for the discovery of novel inhibitor drugs in regard to treatments [11–13]. Thus, accurate detection of kinase activities, especially for multiplex targets, is of great importance for biochemical research, clinical diagnosis, and classification of diseases.

Generally, traditional kinase activity assays adopt two formats. The first one depends on antibodies that can specifically recognize phosphopeptides, and the proposed strategies such as the enzyme-linked immunosorbent assay (ELISA) and fluorescence detection are subsequently applied for the detection of binding antibodies [14,15]. In another format, the labeling substances/nanostructures are employed to link with the phosphate group of substrate peptides for colorimetric, bioluminescence, or electrochemical biosensing and ensuring the kinase activity [16–19]. Capillary electrophoresis (CE) is also a recognized analytical technique for miniaturizing kinase assays, which allows for the separation and analysis of enzyme substrates and products based on their electrophoretic mobility, and presents several advantages such as low sample consumption, automation of analysis, and compatibility with various detection modes [20,21]. All these techniques provide reliable methods for target activity measurements, but most of them have not been readily extended to multi-activity analysis. Because mass spectrometry (MS) allows high-throughput analysis without using extra labels (optical-, electrochemical-, etc.), research has focused on the mass-resolved technique for multiplex enzyme activity assay [22,23]. However, negatively charged phosphate groups transferred to the substrates by kinases may reduce the ionization efficiency of the substances for mass spectrometric measurements, which results in dramatic decrease in MS signals of the products for kinase activity assays. Besides the decreased ionization efficiency of the phosphopeptide, the lowered stoichiometry in the presence of large amounts of unphosphorylated peptides also results in MS signal suppression [24,25]. Thus, a mass-encoded system and the related MS-based method need to be developed with mass-tag probes to improve the sensitivity for kinase activity assay.

Recently, our group have proposed the concept of "mass spectrometric biosensing", which combines the advantages of MS and biosensing techniques, and can transfer the information of target analytes into MS signals using mass-tag probes [26–29]. So far, mass spectrometric biosensing has become a powerful tool for multiplex detection of bio-molecules such as protein markers, miRNAs, and metal ions, showing great potential for clinical diagnosis [30–35]. These developed methods have also been applied for the detection of multiplex protease activities with the designed mass-encoded suspension array and the phospholipid-structured microplate [28,29]. Nonetheless, the immobilization of peptide substrates, as well as enzymes, whether on the solid electrode/plate [18,19,29], magnetic beads [28], nanomaterials [16], or elsewhere, may affect the contact between enzymes and peptides, which changes the reaction efficiency between substrates and target enzymes due to the conjugation between biomolecule and the solid interface via the covalent bond or other interactions. Even though the immobilization of the target/kinase may limit the reaction to a certain area, simulating some situations with enzymes and receptors immobilized onto cell or organelle membranes in real matrices, and the magnetic nanoparticles with immobilized enzymes can be conveniently separated and reused for scientific research, many strategies for the enzyme assays cannot reflect the target activities in the full sense with substrates in the free states, and the reactants (enzymes and substrates) with the free states show higher reaction velocities for enzyme reactions [28,29,36–38]. To dissolve this problem, here we developed a homogeneous mass-encoded strategy for the detection of multi-kinase activities (Scheme 1), which eliminated the need for substrate immobilization to ensure the amount of peptide substrate and the full contact between the free-state substrates and kinases to increase the efficiency for enzyme reactions by utilizing the elaborately designed peptide sequences as mass codes. Using human epidermal growth factor receptor 2 (HER2) and protein kinase A (PKA) as model kinases, the phosphorylation catalyzed by target kinases was directly performed in the homogeneous buffer. The phosphorylated products were captured by titanium dioxide-coated magnetic beads (TiO_2-MBs), which are commercially available and can offer stable enrichment and convenient magnetic separation method for the phosphorylated peptide products [39]. With the assistance of trypsin for subsequently releasing the peptide mass coding regions, the kinase types and activities were qualitatively and quantitatively analyzed with ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), permitting multiplex analysis with high precision and sensitivity, and providing the analytical basis from two dimensions of retention time and ion transitions, respectively. This work provided an optimized avenue for kinase activity assay.

2. Experimental section

2.1. Preparation of peptide-iTRAQ conjugates

Firstly, the iTRAQ reagents (iTRAQ114 and iTRAQ116) were balanced to room temperature, and 70 μL ethanol was added to each vial and vortex for 1 min. To synthesize the peptide-iTRAQ conjugates, 10 μL 2 mM P1 or P2, while 10 μL 2 mM P3 or P4 in 0.5 M triethylammonium bicarbonate (pH 8.5, supplied by iTRAQ reagents multiplex kit, which contained iTRAQ-114, -115, -116, and -117, and the chemical reagents for iTRAQ labeling, including dissolution buffer, ethanol and so on) were respectively mixed with a vial of iTRAQ114 or iTRAQ116. The reactions between peptides P1–P4 and the iTRAQ reagents were carried out at room temperature for 1 h with gentle shaking. The products of equal volumes of P3-iTRAQ116 and P4-iTRAQ116 were mixed as the internal standard peptide solutions (IS-peptides). The obtained P1-iTRAQ114, P2-iTRAQ114, and IS-peptides were diluted to 10-fold volumes with ultra-pure water and stored at −20 °C for further use.

2.2. Procedure for kinase activity assays

After TiO_2-MBs were thoroughly suspended, 400 μL of 25 mg mL^{-1} TiO_2-MBs was transferred to a centrifuge tube to discard the suspension with a magnet. The TiO_2-MBs were re-dispersed with 10 mL acetonitrile (ACN) to 1.0 mg mL^{-1}. The amount of TiO_2-MBs for quantitative analysis of kinase activity was optimized by mixing 5 μL of the as-prepared P1-iTRAQ114 and P2-iTRAQ114 with 80 μL of 50 mM tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 200 μM ATP, and 10 μL of the mixture of 50 ng mL^{-1} HER2 and 20 U mL^{-1} PKA as kinase sample to incubate at 37 °C for 2 h, followed by adding 5.0, 10, 20, 50, 100, and 200 μL of the as-prepared TiO_2-MBs suspension to capture the phosphorylated peptide products for 1 h at room temperature with gentle shaking, respectively. After washing and magnetic separation, 50 μL of 100 ng mL^{-1} trypsin in 25 mM NH_4HCO_3 was added in the MBs to incubate for 30 min. The concentration of trypsin was optimized to ensure the complete release of the coding regions by adding 50 μL of trypsin at 0, 10, 20, 50, 100, and 200 ng mL^{-1} in 25 mM NH_4HCO_3 in phosphorylated peptide products treated MBs to incubate for 30 min. The investigation of kinase reaction dynamics was performed at 37 °C for different reaction times of 0, 30, 60, 90, 120, 150, and 180 min. After trypsin cleavage for 30 min, the obtained supernatants were diluted to a five-fold volume with methanol containing 1 % fomic acid, mixed with 2 μL of IS-peptides, and subjected to UHPLC-MS/MS analysis.

Quantitative kinase assays were performed under the optimized conditions. Briefly, 5 μL of the as-prepared P1-iTRAQ114 and P2-iTRAQ114 were mixed with 80 μL tris-HCl buffer (pH 7.5, 50 mM, containing 10 mM $MgCl_2$ and 200 μM ATP), and 10 μL of the kinase sample, including single HER2 or PKA, the mixture of HER2 and PKA at a series of concentrations (0, 1.0, 2.0, 5.0, 10, 20, 50, 100, and 200 ng mL^{-1} for HER2, and 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, and 50 U mL^{-1} for PKA), kinase with different inhibitors, or the lysates from different cell lines, were added to incubate for 2 h at 37 °C for feasibility evaluation, quantitative analysis, inhibitor screening, and real sample analysis, respectively. Afterward, the reaction solution was mixed with 100 μL of TiO_2-MBs suspension (1.0 mg mL^{-1} in ACN) for capturing the of phosphorylated peptide products for 1 h at room temperature with gentle shaking. The supernatant was discarded by magnetic separation, with TiO_2-MBs washed twice with 100 μL of 50 % ACN aqueous solution to remove the non-specifically adsorbed substances. Finally, 50 μL of 100 ng mL^{-1} trypsin in 25 mM NH_4HCO_3 was added to the MBs and incubated for 30 min under vigorous shaking to release the coding regions for each kinase substrate. The supernatant was diluted to a five-fold volume with methanol containing 1 % fomic acid, mixed with 2 μL of IS-peptides, and subjected to UHPLC-MS/MS analysis.

2.3. UHPLC-MS/MS analysis

Quantitative analysis was performed on UHPLC-MS/MS system which consisted of an LC30A UHPLC system (Shimadzu, Japan) and a QTRAP 5500 mass spectrometer (AB Sciex, USA). 2 μL of the sample was injected into the instrumental system and separated with the Kinetex Biphenyl 100 Å column (100 × 3.0 mm, 2.6 μm, Phenomenex, USA). Mobile phase A was 5 mM NH_4OAc containing 0.1 % formic acid, and mobile phase B was methanol containing 0.1 % formic acid. The following gradients were applied at the constant flow rate of 0.3 mL min^{-1} for a total program of 5.0 min: 0.0−0.5 min 5 % B, 0.5−0.6 min 5 %–20 % B, 0.6−1.5 min 20 %–95 % B, 1.5−4.2 min 95 % B, 4.2−4.3 min 95 %–5 % B, and 5.0 min stop. The mass spectrometer with electrospray ionization (ESI) source was operated in the positive multiple reaction monitoring (MRM) mode, with optimized acquisition parameters of curtain gas (30), ion-spray voltage (5500), ion source gas 1 (45), ion source gas 2 (30), source temperature (550 °C), collision gas (High), declustering potentials (90), entrance potential (10), and collision cell exit potential (12). The ion transitions and collision energies (CEs) for the analytes of coding peptides and their respective internal standards are shown in Table 1. For each iron transition, the dwell time was set as 100 ms. Data analyses were processed with Analyst and MultiQuant (AB Sciex, USA).

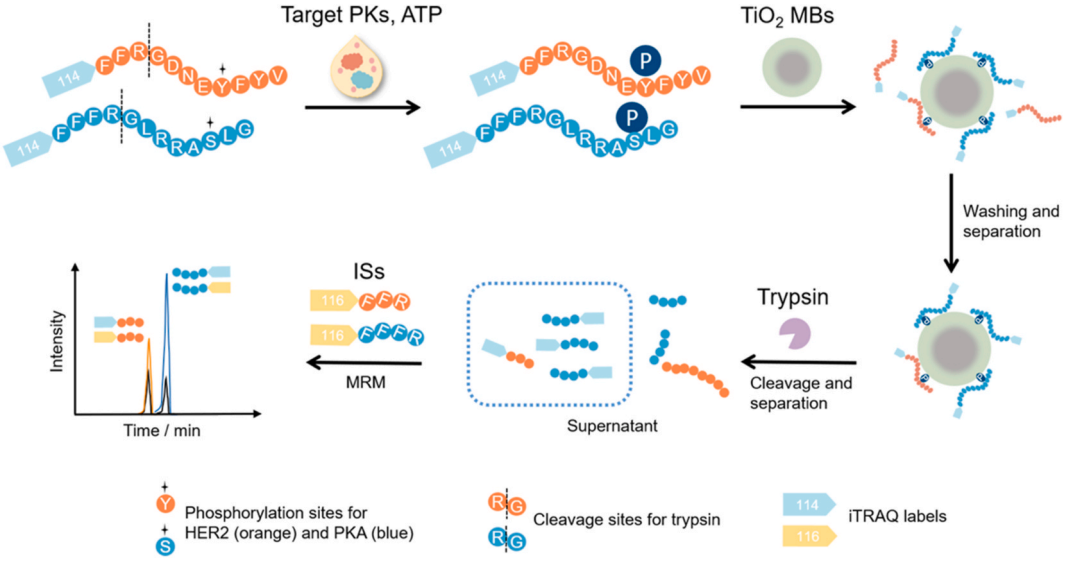
3. Results and discussion

3.1. Design principles of the proposed strategy

The peptides were elaborately designed to contain the coding sequences and substrate regions for phosphorylated reactions catalyzed by

Table 1
Sequences, optimized ion transitions and CEs for coding peptides of phosphorylation products and the internal standards.

Analyte	Sequence	Charge	Transition	CE
pP_{HER2}	iTRAQ114-FFR	2	307.4/114.1	35
IS-pP_{HER2}	iTRAQ116-FFR	2	307.4/116.1	35
pP_{PKA}	iTRAQ114-FFFR	2	380.9/114.1	35
IS-pP_{PKA}	iTRAQ116-FFFR	2	380.9/116.1	35



Scheme 1. Schematic illustration of kinase activity assay with HER2 and PKA as model targets using the proposed homogeneous mass-encoded strategy.

specific kinase, with an amino acid arginine (R) inserted between the two parts for trypsin cleavage. The coding sequences consisted of different numbers of phenylalanines (FF for P1 and P3, while FFF for P2 and P4), which could be better retained and separated by the reversed-phase column through hydrophobic interactions, while iTRAQ114 and iTRAQ116 were applied for labeling the integrated peptides (P1 and P2) and the internal standards (P3 and P4), respectively. Compared with protein substrate, the peptide substrate provided more specified recognition regions for phosphorylation reaction the kinase target. In addition, the specifically designed coding sequences and iTRAQ labels consisted in the peptide substrate could be released for qualitative and quantitative analysis of the targets, which constituted the core factor for the mass spectrometric biosensing strategy. In this proof-of-concept study, HER2 and PKA, which have been proved to be associated with many diseases, were selected as model kinases to demonstrate the proposed strategy. Unlike cytoplasmic kinases such as PKA, HER2 is primarily a transmembrane receptor involved in cellular signaling, and is classified as a receptor tyrosine kinase (RTK) rather than a strictly conventional kinase. It belongs to the EGFR receptor family, and has intrinsic tyrosine kinase activity, which means that HER2 can phosphorylate tyrosine residues both on itself and other proteins when activated [40]. The detailed information for sequences of peptides and peptide-iTRAQ conjugates was listed in Table S1, which were verified by MALDI-TOF MS with a mass shift of 144 Da (Fig. S1).

As illustrated in Scheme 1, the enzyme assay was directly performed in the homogeneous sample solution, during which the substrates P1-iTRAQ114 and P2-iTRAQ114 could be recognized and phosphorylated at specific sites by their respective targets of HER2 and PKA, respectively. TiO_2-MBs were subsequently employed to enrich the phosphorylated products, along with non-specifically adsorbed substances removed by separation and washing to obtain better signal responses for MS analysis. Finally, the tool enzyme trypsin was added to cleave the phosphopeptide products that captured onto the surface of TiO_2 MBs to release the coding sequences. By submitting the supernatant that was mixed with the IS-peptides for UHPLC-MS/MS analysis, the kinase activities were assessed via the peak area ratios of released coding peptides to the internal standards under the MRM mode.

3.2. Feasibility of the proposed multiplex kinase activity assay

The feasibility was demonstrated by using blank buffer, HER2, PKA, or their mixture as samples for kinase activity assay. The coding peptide sequences labeled with iTRAQ116 (iTRAQ116-FFR and iTRAQ116-FFFR) were introduced as internal standards before submitting for UHPLC-MS/MS analysis to monitor the instrument status and present relative changes of the peak intensities. Internal standard analytes that related with IS-pP_{HER2} and IS-pP_{PKA} and were used for normalization appeared at the respective retention time of 3.1 min and 3.2 min on each extracted ion chromatograms (XICs) (Fig. 1A–D). The kinase assays were performed for five independent batches, and the results showed the mean intensities of $(2.42 \pm 0.21) \times 10^5$ and $(1.93 \pm 0.12) \times 10^5$ with the coefficient of variations (CVs) of 8.7 % and 6.4 % for IS-pP_{HER2} and IS-pP_{PKA} respectively, suggesting acceptable stability and satisfactory instrumental conditions suitable for measurements. As expected, almost no coding sequences of pP_{HER2} and pP_{PKA} were observed on the XIC relating with the blank buffer solution (Fig. 1A), while the peak intensity of pP_{HER2} or pP_{PKA} individually increased when using single kinase of HER2 or PKA as the sample (Fig. 1B and C), and both peaks dramatically

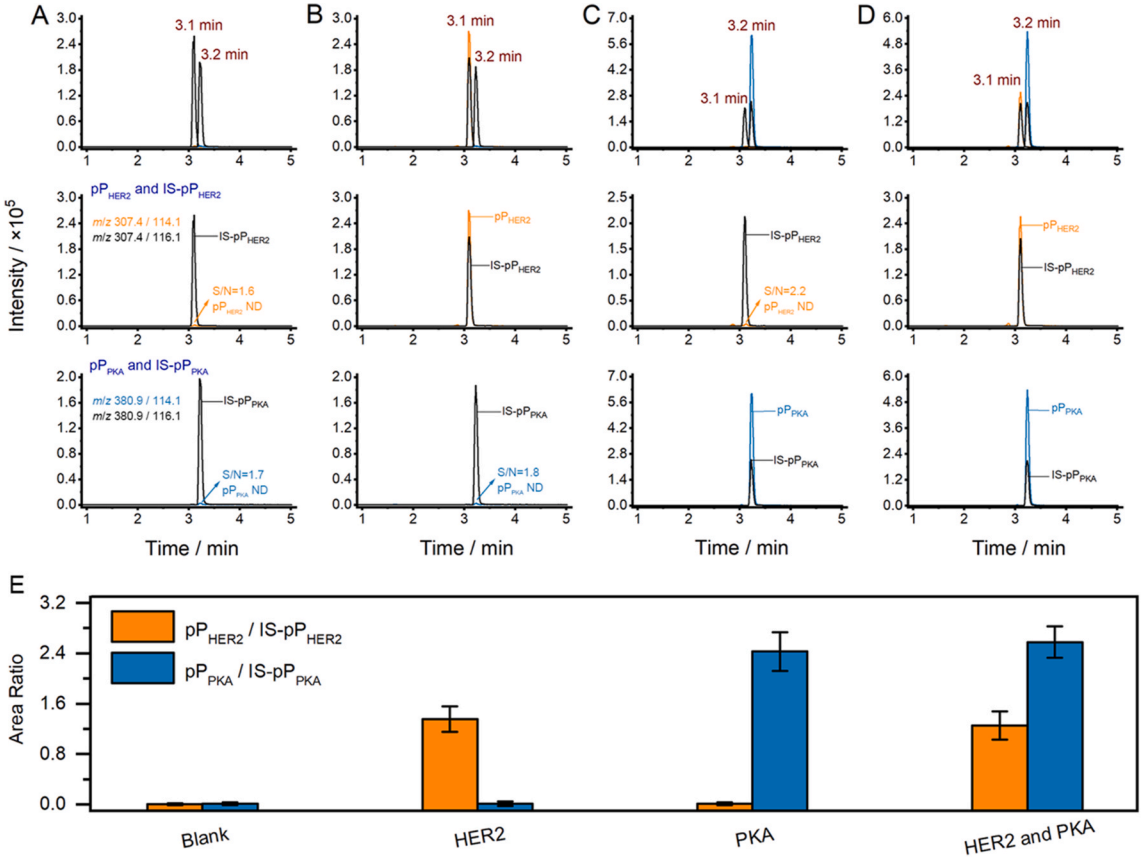


Fig. 1. Extracted MRM chromatograms (XICs) of the coding regions (*m/z* 307.4/114.1 and *m/z* 380.9/114.1) and their corresponding internal standards (*m/z* 307.4/116.1 and *m/z* 380.9/116.1) with the (A) blank buffer, (B) 50 ng mL^{-1} HER2, (C) 20 U mL^{-1} PKA, and (D) the mixture of HER2 and PKA as samples for kinase activity assays, with signal-to-noise ratios (S/Ns) labeled for coding regions of blank samples, proving that relevant products were not detected (S/N < 3). (E) Peak area ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} calculated from the XICs of (A)–(D).

increased for the sample of HER2 and PKA mixture (Fig. 1D), with the MS/MS spectra of the detected tags of iTRAQ114 and iTRAQ116 for respective coding peptides and their respective internal standards (Fig. S2). The peak area ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} showed obvious increase with increasing HER2 and PKA, respectively, and displayed little cross influence between two targets (Fig. 1E), which was also confirmed with MALDI-MS analysis with more intuitive and concrete presence of the substrate and phosphorylated product *m/z* values (Fig. S3), demonstrating the feasibility of the homogeneous mass-encoded strategy for multiplex kinase activity assay.

3.3. HER2 and PKA activity assays

To obtain better performance for quantitative analysis, the amount of TiO_2-MBs was optimized to ensure the complete capture of phosphopeptide products. Different amounts of TiO_2-MBs (5.0−200 μg) were added to the aqueous solutions after the reaction between kinases and substrates, respectively, followed by magnetic separation, addition of trypsin and IS-peptides, dilution and submission of the supernatant for MS measurements. Obviously, the peak area ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} increased with the increasing amount of TiO_2-MBs, with the plateaus reached when the amount of TiO_2-MBs was 100 μg, indicating the equilibrium and saturation for the capture of phosphorylated products (Fig. S4). From the size of TiO_2-MBs (diameter 1.2 μm) and the density of MBs (around 2.0 g cm^{-3}), the weight of one bead was calculated to be 1.81×10^{-12} g, and 100 μg of MBs totally contained 5.52×10^{7} beads. According to the average peak area ratios of pP_{HER2}/IS-pP_{HER2} (1.768) and pP_{PKA}/IS-pP_{PKA} (2.407) corresponding to 100 μg of TiO_2-MBs (Fig. S4) and the dilution processes, the amount of phosphorylated peptide products of HER2 and PKA were estimated to be 4.42×10^{-11} mol and 6.02×10^{-11} mol. Thus the quantity of phosphorylated peptide products of HER2 and PKA immobilized on per bead were 8.01×10^{-19} mol and 1.09×10^{-18} mol, respectively.

Next, a series of concentrations of trypsin (0−200 ng mL^{-1}) were respectively added to the TiO_2-MBs after the capture of phosphorylated peptides to obtain the optimal trypsin concentration for guaranteeing the overall release of coding peptide sequences. The peak area ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} reached the maximum at 100 ng mL^{-1}, suggesting the maximum release of the coding peptides for respective targets (Fig. S5).

Finally, the reaction dynamics of HER2 and PKA were checked by incubating P1-iTRAQ114 and P2-iTRAQ114 with the target mixture for different reaction times to obtain the ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA}. With the increasing reaction time, the ratios increased and reached the plateau at 120 min, afterward the values did not increase, demonstrating the completion of phosphorylated reactions (Fig. S6). Therefore, the experimental conditions of 100 μg TiO_2-MBs, 100 ng mL^{-1} trypsin, and the incubating time of 120 min were used for quantitative assay.

Under optimal parameters, the mixtures of HER2 and PKA at series of concentrations were applied for activity assay. The peak intensities of the coding peptides pP_{HER2} and pP_{PKA} increased with the increasing concentrations of HER2 and PKA, respectively, while the intensities of IS-pP_{HER2} and IS-pP_{PKA} little changed (Fig. S7). Plots of the peak area ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} displayed acceptable linearity in the ranges of 1.0–100 ng mL^{-1} for HER2 and 0.2−20 U mL^{-1} for PKA, respectively, with both *r* values more than 0.99 (Fig. 2). The detection limits for HER2 and PKA were assessed to be 0.46 ng mL^{-1} and 0.033 U mL^{-1} at the values of 3 times S/N (5.2 and 3.3) with the confidence intervals of 95 % by student's t-test, respectively, which were comparable to those reported with fluorescence, electrochemical, and mass spectrometric methods (Table S2).

3.4. Analysis of kinase inhibitors

Many drugs aim to adopt the kinase activities, thus the evaluation of inhibition effect is of great significance for drug screening [41,42]. Here, we pre-mixed HER2 and PKA with a commonly and commercially used kinase inhibitor mixture containing multiplex components of highly efficient small-molecule inhibitors in pH 7.2 buffer for the activity analysis [43]. The peak intensities of the coding peptides pP_{HER2} and pP_{PKA} in the XIC showed obvious declines for the sample in the presence of Cocktail (Fig. 3B) compared with that in the absence of the inhibitor (Fig. 3A), which illustrated the declined release of coding regions, and could be inferred that the inhibitor Cocktail dramatically reduced the activities of HER2 and PKA.

To further quantitatively assess the efficiency for specific inhibitors, different concentrations of CP-724714 and H89 that were known as specific inhibitors for HER2 and PKA, respectively, were mixed with the corresponding target to perform the activity assays under optimized conditions. The values of IC50, defined as 50 % inhibition efficiency, were calculated to be (17 ± 3.6) nM and (46 ± 8.7) nM for CP-724714 and H89 with the standard deviations calculated according to the fitting errors of the curves, which showed little significant deviations from the reported values of 10 nM and 48 nM, respectively (Fig. 4) [44,45]. To confirm the type of kinase inhibitors, ATP, one of the key factors and substrates for kinase activity, with increasing concentration from 200 μM to 500 μM was added in the mixtures to re-evaluate the inhibition kinetics (Fig. S8), which showed increased IC50 values of CP-724714 for HER2 and H89 for PKA, indicating that both CP-724714 and H89 were ATP-competitive kinase inhibitors for HER2 and PKA, respectively. The conclusion was in accordance with those reported previously [46,47]. The results demonstrated the feasibility and reasonableness of the strategy for qualitative and quantitative assessment of inhibitors.

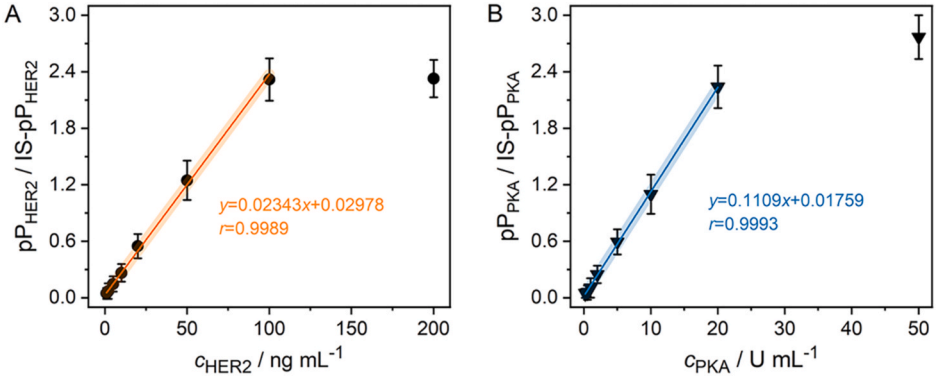


Fig. 2. Plots of peak area ratios of (C) pP_{HER2}/IS-pP_{HER2} and (D) pP_{PKA}/IS-pP_{PKA} *vs* the concentrations of HER2 and PKA, and the linear equations fitting at the confidence bands of 95 %.

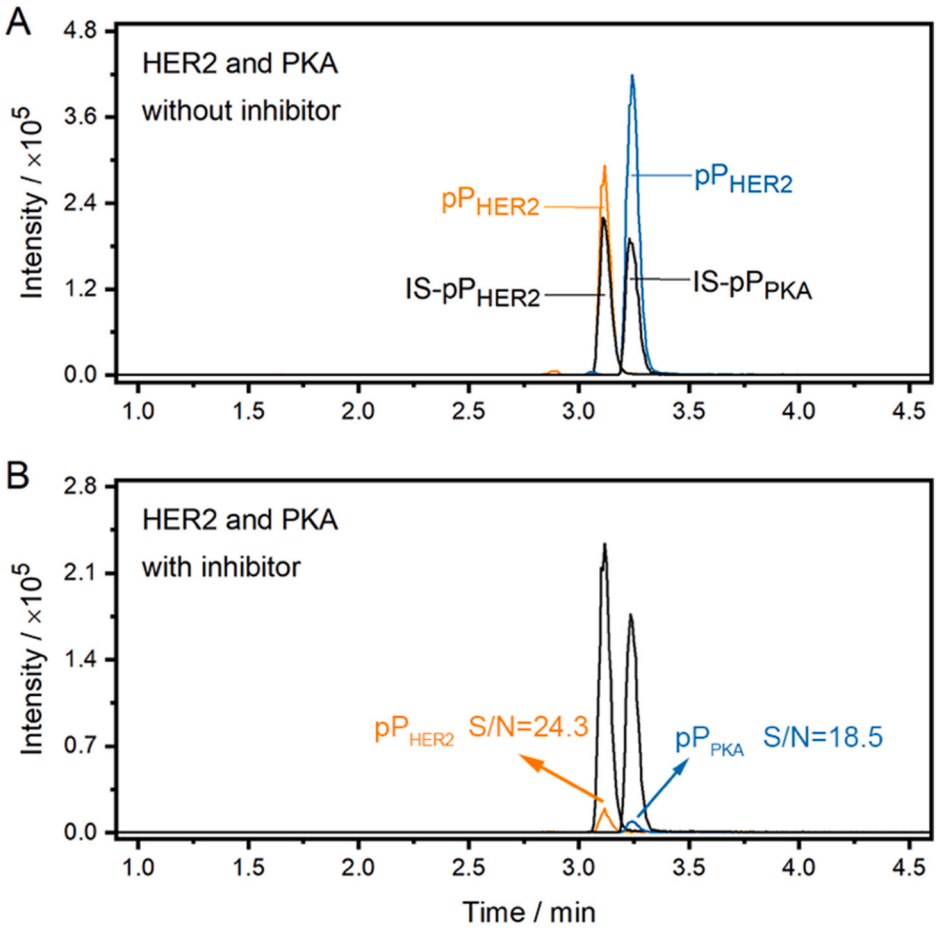


Fig. 3. Extracted MRM chromatograms of coding regions pP_{HER2} and pP_{PKA} and intertal standards IS-pP_{HER2} and IS-pP_{PKA} by using 50 ng mL^{-1} HER2 and 20 U mL^{-1} PKA (A) without and (B) with the commercially obtained kinase inhibitor pre-mixed with HER2 and PKA as the sample. The S/N values for coding regions dramatically decreased in the presence of inhibitor.

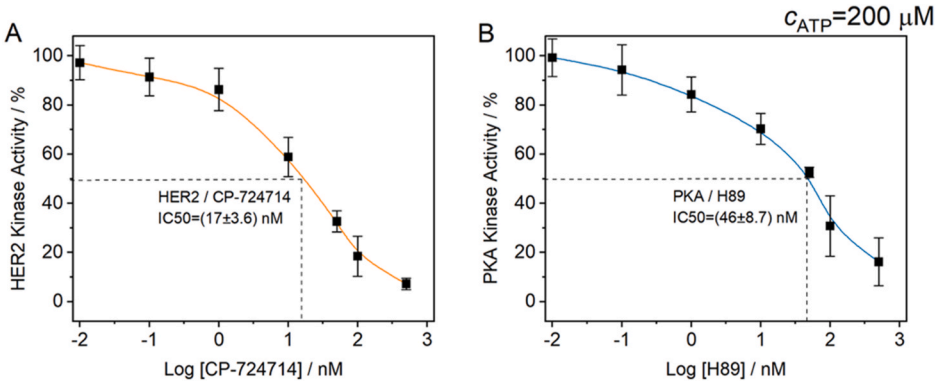


Fig. 4. Plots of kinase activities of (A) 50 ng mL^{-1} HER2 *vs* logarithm of the concentration of CP-724714 and (B) 20 U mL^{-1} PKA *vs* logarithm of the concentration of H89 under the optimized experimental conditions.

3.5. Selectivity, reproducibility, and stability

The specificity of the proposed method was tested by using some possible interfering enzymes (PKC, PKG, MMP-2, and Casp-3) for the activity. The peak area ratios pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} for HER2 and PKA were 10–40 folds higher than those of interfering substances, which were quite close to that of blank buffer, indicating good specificity of the strategy (Fig. 5).

The reproducibility was investigated with inter- and intra-assay tests, which were operated with the sample of 50 ng mL^{-1} HER2 and 20 U mL^{-1} PKA to perform six parallel experiments for activity assays, and submitting one of the six samples to measure the mass spectrometric responses for six times, respectively, and CVs for both inter- and intra-assays were less than 5 % (Fig. S9). The intraday tests were performed by analyzing three different concentrations (low, medium, and high) for HER2 and PKA with five replicates in the same day, while the interday precision was evaluated in five separate days. The intraday and interday tests respectively showed the CVs of 5.19 %–9.88 % and 3.87 %–8.17 %, indicating good reproducibility of the method (Table S3). The stability was tested by performing the assay with the prepared P1-iTRAQ114 and

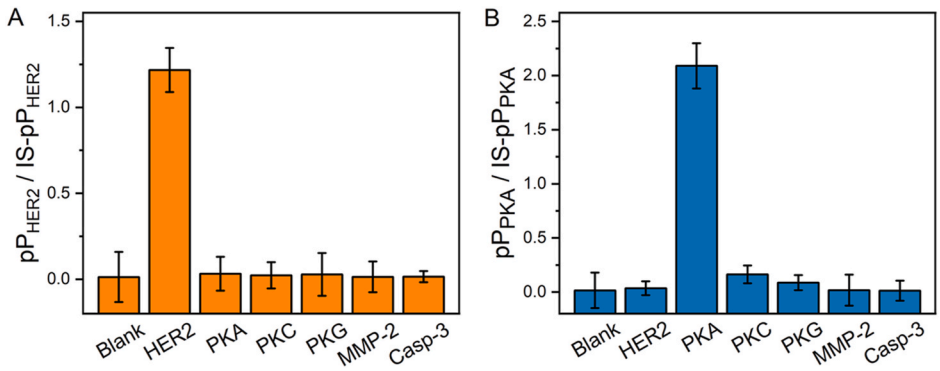


Fig. 5. Peak area ratios of (A) pP_{HER2} to IS-pP_{HER2} and (B) pP_{PKA} to IS-pP_{PKA} for blank buffer, 50 ng mL^{-1} HER2, 20 U mL^{-1} PKA, 50 ng mL^{-1} PKC, 50 ng mL^{-1} PKG, 100 ng mL^{-1} MMP-2, and 100 ng mL^{-1} Casp-3.

P2-iTRAQ114 stored for 7 and 14 days, respectively, with both ratios of pP_{HER2}/IS-pP_{HER2} and pP_{PKA}/IS-pP_{PKA} at three different concentrations of HER2 and PKA for 7 and 14 days, which showed little obvious changes (Fig. S10). Thus, the proposed method was of satisfied reproducibility and good stability.

Compared with the mass spectrometric method focused on kinase assay [22], this work upgraded the qualitative and quantitative analysis platform to offer reliable results with good sensitivity, high specificity, and acceptable reproducibility. According to the slopes of linear equations, the proposed internal standard method showed the improved sensitivity for enzyme assays, justifying the priority of this work to a certain extent [28,29].

3.6. Application in cell lysates

To demonstrate the practical application of the proposed method, certain amounts of HER2 and PKA were added to the MCF-10A cell lysates for kinase assays. Table S4 shows the results of kinase activities from three parallel experiments, with recoveries ranged from 93.8 % to 102.6 % compared with the reference values. Thus the proposed method could serve as a reliable tool for multiple kinase assay in biological samples such as cell lysates.

The matrix effects (ME) were assessed with the freshly extracted cell lysates of MCF-10A, MCF-7, T47D, and MDA-MB-453 after heated to 80 °C to inactivate the kinases, cooled down to room temperature, and spiked with three different concentrations of HER2 and PKA as samples to perform kinase activity assays (Table S5). The MEs (%) were evaluated to be −12.8 %–6.0 %, and $|ME| \leq 20$ % demonstrated that matrix did not significantly affect the quantification. This was contributed from the washing and magnetic separation steps involved in the assay to purify the detection solution.

HER2 has been found to be over-expressed in about 20 % of breast cancer, while PKA could be used as a biomarker for early diagnosis and progression assessment of cancer. Here, the cell lysates of non-tumorigenic breast epithelial cell line of MCF-10A and three breast cancer cell lines of MCF-7, T47D, and MDA-MB-453 with different levels of HER2 were chosen as samples for HER2 and PKA activity assays with the proposed method to demonstrate the feasibility to differentiate breast cancer cells [48]. The activity of PKA in cell lysates from MCF-7, T47D, and MDA-MB-453 were higher than that of MCF-10A (Fig. 6, orange columns), implying high PKA activity in cancer cells. However, it should be noticed that HER2 activity was slightly higher in MDA-MB-453 than that in other breast cancer cells, indicating different HER2 activities in breast cancer cells (Fig. 6, blue columns), which were in accordance with previous reports [49]. Though the results showed different average values of PKA and HER2, the specificity between PKA and HER2 was enough for different cell lines, because this method detected kinase activities of HER2 and PKA, rather than expression levels of the proteins. Only parts of HER2 and PKA were activated in these cells, thus the differences of HER2 and PKA kinase activities were insignificant [50,51]. Therefore, the mass spectrometric biosensing strategy showed great potential for cell differentiation, clinical diagnosis and prognostic assessment.

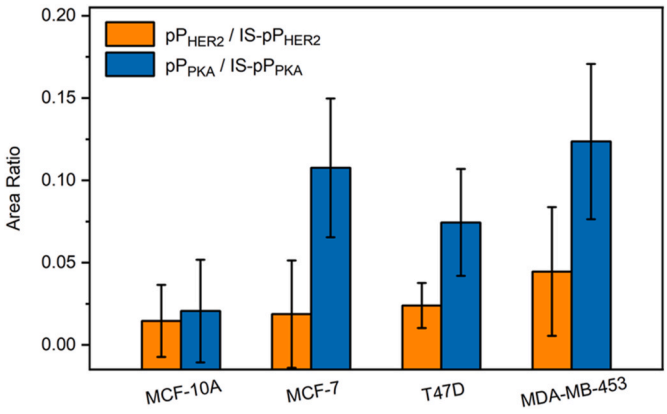


Fig. 6. Activity assays of HER2 (orange) and PKA (blue) in cell lysates of MCF-10A, MCF-7, T47D, and MDA-MB-453 with the proposed homogeneous mass-encoded strategy, respectively. All experiments were carried out in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

In summary, a homogeneous mass-encoded strategy has been proposed for the detection of multiplex kinase activities. The phosphorylated reactions between kinases and the designed peptide substrates can be directly performed in the aqueous buffer solution without the need of substrate immobilization, thus simplifying the assay procedure and revealing the enzyme activities with peptide substrates in the free state as the in-vivo environment. By using TiO_2-MBs to capture the peptide products with phosphate groups, and trypsin to release the coding regions for quantitative UHPLC-MS/MS analysis, this method achieves HER2 and PKA activity assays with satisfied linear relations and detection limits. It has been applied for inhibitor analysis and kinase activity assays in different cell lysates, showing great potential for kinase-target drug screening, discrimination of cancer cells, and cancer diagnosis. The homogeneous mass-encoded strategy complements and improves enzyme biosensing technique. It can be expanded for more kinase-target assays and is hopeful to be a powerful tool for clinical applications and kinase-related biomedical research, despite the drawbacks of high cost, equipment availability, and sample preparation complexity, which means that greater efforts are still needed to develop the assay methods in the future.

CRediT authorship contribution statement

Junjie Hu: Writing – original draft, Validation, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation. **Fei Liu:** Validation, Investigation, Data curation. **Yunlong Chen:** Writing – review & editing, Validation, Funding acquisition, Formal analysis. **Xinxin Xie:** Writing – review & editing. **Huangxian Ju:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2025.344219.

Data availability

No data was used for the research described in the article.

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