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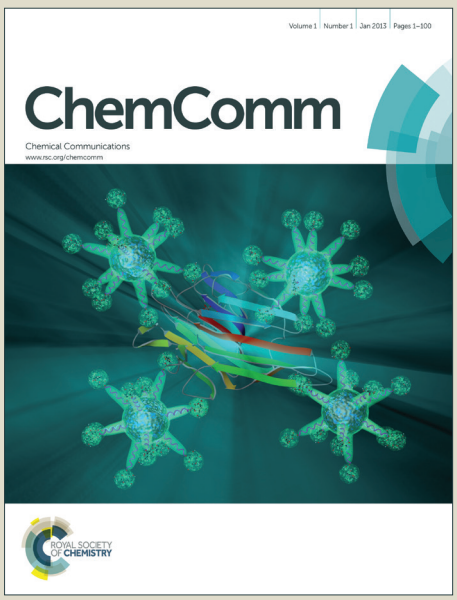

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Multiplex Isotope Dimethyl Labeling of Substrate Peptides for High Throughput Kinase Activity Assay via Quantitative MALDI MS

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A simple, cost-effective and high throughput method was developed for multiplexed kinase activity assay based on the multiplex isotope labeling of designed substrate peptides. This strategy was successfully applied to monitor the time-dependent consumption of substrates and generation of products in the single and multiple substrate systems.

Protein kinases play a key role in regulating many cellular processes, such as glucose metabolism, gene transduction, cell proliferation and apoptosis.[1] As a number of diseases, such as Alzheimer disease,[2] diabetes[3] and cancer,[4] are linked to the aberration of protein kinase activities, protein kinases become the targets for treatment of these diseases, and great emphasis has been paid on developing protein kinase inhibitors as drugs for clinical use.[5] Thus, sensitive, high-throughput and widely applicable approaches for monitoring protein kinase activities is of great importance in the discovery of protein kinase inhibitors, and the development of drug therapies.

Many approaches including radiometric assay,[6] immunoassay,[7] fluorometric assay,[8] have been developed to monitor enzyme activities. However, these methods often suffer some disadvantages. For example, radiometric assay suffers the disadvantages of radioactive waste and hazardous operation. Immunoassay has some limitations, including cross reactivity with nontarget substrates and time-consuming preparation of the antibodies. The fluorometric assay, suffers from complicated derivatization of substrates with florescent tag, which may influence the properties of the substrates. More importantly, all these methods are only best fitted to detect one reaction product from the enzymatic reaction. They are typically unable to simultaneously detect multiple substrates and their products in an enzyme-catalyzed reaction.

Recently, mass spectrometry (MS) has become an indispensable tool in proteomics,[9] and efforts have been made to expand its potential applications in other field. Electrospray ionization (ESI) and mass-assisted laser desorption/ionization (MALDI) are the most commonly used techniques for MS analysis of proteins and peptides, whereas MALDI-MS represents an ideal platform for monitoring enzyme activities. This is because of its simplicity and speed of analysis, high sensitivity (detection of femtomole levels of peptides), excellent mass accuracy, much less restriction in the use of salts and buffers that are typically found in enzyme reaction mixtures. For monitoring the process of enzyme catalyzed reactions, multiple samples derived from the reaction with different enzyme concentrations or different reaction times should be analyzed. The throughput for enzyme activity assay will be significantly improved if multiple samples could be analyzed on the same spot.

Stable isotope dimethyl labeling is recognized to be simple, cost-effective, highly efficient, and is very popular for quantitative proteomics analysis.[10] In addition to the conventional dual or triple dimethyl labeling, five-plex isotope labeling of peptides could be achieved by combining different types of isotopic formaldehyde and cyanoborohydride (Fig. S1).[11] This labeling reaction targets the primary amines on peptides. When only one primary amine group is presented on the peptide, e.g. LRRASLG which has one N-terminal amine, only one dimethyl group is introduced and the mass difference between adjacent isotopic labeled peaks is only 2 Da. While if the peptide has more than one primary amine, e.g. LRRASLGGK, SHKQIYYSDK where additional amine groups are presented on side chains, multiple dimethyl groups could be introduced and mass difference over 4 Da could be achieved (Fig. 1). Due to the overlap of isotope clusters, quantification of isotope labeled peptides with mass difference of 2 Da is not accurate. Therefore, to accurately determine their concentrations change during an enzymatic reaction by multiple isotope dimethyl labeling, the substrates and their products must have at least two primary amine groups. Kemptide, the well-known PKA substrate peptide (LRRASLG, 957.1 Da), has only one N-terminal primary amine. To enable the multiplexed assay, we designed a new

peptide substrate LRRASLGGK (1037.1 Da) by adding glycine and lysine to the C-terminal.

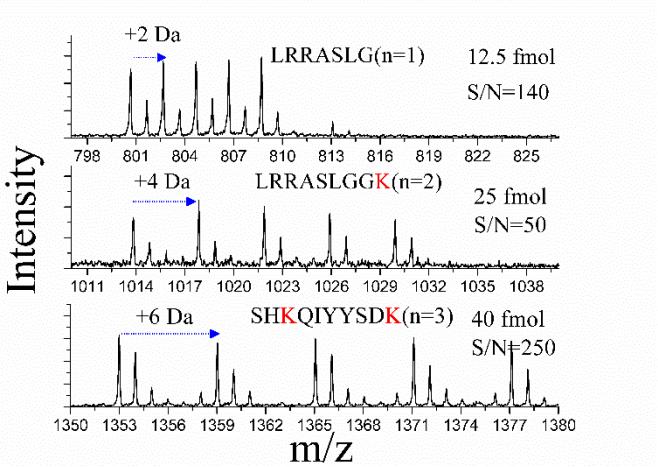

Fig. 1 MALDI MS spectrum for isotope dimethyl labeled peptides with different numbers of primary amines.

We first tested if the designed substrate peptide could be phosphorylated by PKA. The substrate peptide was incubated with PKA and ATP. After reaction for 20 min, the sample was diluted and analyzed by MALDI MS immediately. A control experiment was done in the same way except no PKA was added. As shown in Fig. S2, a new peak with 80 Da increase of mass was observed in the experiment indicating one phosphate group was introduced to the peptides. While the same peak was not observed in the control experiment. These results indicated this peptide could be phosphorylated by PKA. This is not surprising since the section of sequence recognized by the PKA is still presented on the peptide. For the enzyme activity assay, the enzymatic reaction must be quenched as soon as a certain time interval is reached to keep the concentration of the substrates and their products unchanged. The enzymatic reaction could be quenched by addition of inhibitors or by change the pH of the buffer. However, these approaches may not be compatible with the following dimethyl labeling. Because the covalent modification of enzymes may result in the loss of their natural catalytic activity, we suspected that PKA activity may be abolished by the addition of isotope dimethyl labeling reagents. To test this assumption, 2 μL formaldehyde (CH_2O, 4%, vol/vol) and 2 μL cyanoborohydride ($NaBH_3CN$, 0.6 M) were added to the PKA solution. Immediately after the addition, the substrate peptide was added to monitor the enzyme activity. As can be seen in Fig. S2c, a dominant peak with mass increase of 56 Da was observed. This is because the substrate peptide was labeled with two dimethyl groups. However, the product peptide, i.e. the phosphorylated peptide, was not found. This means the PKA activity is lost immediately when dimethyl labeling reagents were added. Thus, the quenching operation can be simply merged into the labeling reaction. If not otherwise stated, the labeling reagents were added immediately after the enzymatic reaction in the following experiments.

This multiplex labeling strategy was then applied to monitor the consumption of substrate and generation of product during the PKA catalyzed phosphorylation (see ESI† for details about the quantification performance of this labeling strategy). The substrate peptide was first phosphorylated in the reaction mixture with fixed PKA concentration, after every certain time intervals of 1.0 min, a fraction of the solution was taken and labeled with dimethyl groups from lightest to the heaviest in turn immediately. After the labeling, the five samples were mixed together and analyzed by MALDI MS. Fig. S3 gives the spectra of this analysis, five time course points with consumption of substrate and generation of product can be monitored on MALDI shot. While in conventional quantitative MADLI MS with internal standards, analysing five different spots are required for analysis of five samples. Compared with the fluorometric assay, this MAIDL MS based assay allows for the measurement of both substrate and its product.

As this approach is quantitative, it can be used to determine the kinetic constants for the kinase catalyzed reaction. Because the absolute concentration of the product should be determined, one of the five channels is used for internal standards with known concentration. Thus maximum of four samples can be quantitatively analyzed simultaneously by this labeling strategy. If conventional triple-plex labelling approach is used, then only two samples can be quantified. Clearly this new five-plex labelling approach is more fitted to determine the kinetic constants. In this study, the time course experiment with four time points was applied to determine the kinetic constants. The enzymatic reaction was initiated by adding PKA into the solution with the substrate peptide and ATP, and the same volume of the solution was taken after reaction of 0.5, 1, 3, and 5 min. These four fractions were labeled with dimethyl groups from the lightest to the last but one heaviest in turn immediately after taken. The synthesized product, LRRApSLGGK, with known amount was labeled with the heaviest dimethyl group and used as the internal standard. The above five samples were pooled together for MALDI MS analysis. As shown in Fig. 2a, the intensities of the products increased with the increasing of reaction time. Their concentrations could be easily determined by comparing with the intensity of internal standard. The change of product concentration during the time course study was given in Fig. 2b.

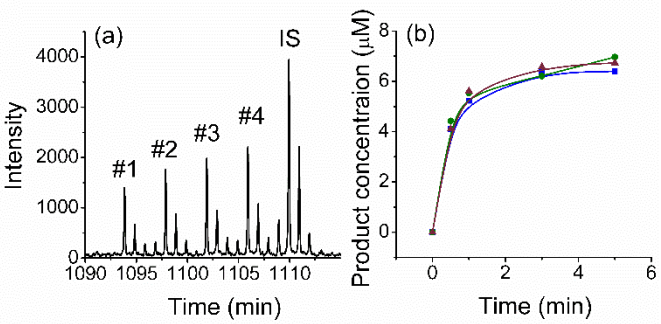

Fig. 2 Time dependent formation of phosphorylated product LRRApSLGGK in time course studies. (a) Representative MALDI MS spectrum of the five-plex isotope labeled products. The signals labeled #1-4 represent the formed products after certain time intervals and that labeled and IS represents the peptide for internal standard. (b) The dependence of product formation on the reaction times for three experiments.

To accurately determine the kinetic values, three independent assays were performed, respectively (Fig. 2b). The kinetic constants were determined from the time course results directly according to the Michaelis-Menten derivative equation.[12] Values of K_m and k_{cat} for the phosphorylation reaction were determined to be 6.16 ± 0.43 μM and 4.38 ± 0.34 min^{-1}, respectively. The RSD for the K_m and k_{cat} was about 6.94 % and 7.68 % for triplicate analyses. Above results demonstrated the good reproducibility of this approach.

The capability of this method for kinase inhibitor screening was also investigated. The protein kinase inhibitor H-89, a selective inhibitor of PKA, was tested. Different concentrations of H-89 were added in the reaction system under the fixed PKA concentration for enzymatic reaction. As expected, contrast to the experiment without addition of any inhibitor, the intensity of the products corresponding to the same time point decreased gradually with the increasing concentration of H-89, indicating the reduction of peptide phosphorylation and inhibition of PKA

(Fig. 3a). The inhibitor curves of H-89 yield an inhibition constant Ki of 45.7 nM, which is consistent to the reported value of 48 nM.[13] The values of apparent Km were 8.23 μM (15 nM H-89) and 6.82 μM (5 nM H-89), which are higher than the control value 6.16 μM, indicating the affinity of the substrate to the enzyme decreased with the increasing of inhibitor concentration. These results and high speed of the MALDI readout clearly demonstrated that the MS-based labeling assay is of great potential in high throughput protein kinase inhibitor screening.

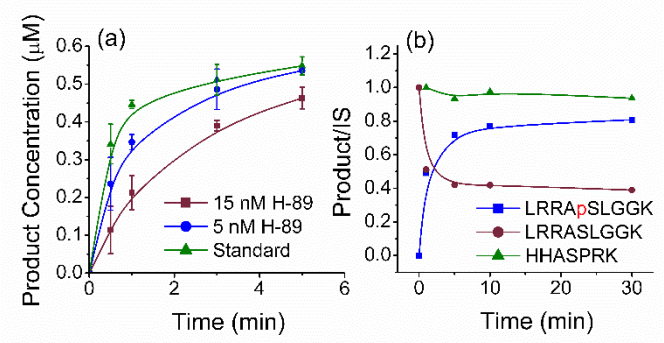

Fig. 3 (a) Inhibition of kinase activity by using different amounts of H-89: without H-89 (-▲-), with 5 nM H-89 (-●-), with 15 nM H-89 (-■-). The concentrations of substrates, PKA and IS are fixed to be 7.5 μM, 0.5 unit/μL and 12 μM respectively. All values represent the average from two replicates. (b) Time dependent formation of phosphorylated product LRRApSLGGK (-■-) and consumption of substrate LRRASLGGK (-●-) and HHASPRK (-▲-) by the multiplexed labeling strategy.

In the simplified system, the kinase only phosphorylates one substrate. However, in a real biological system a kinase often coexists with multiple substrates. And incubation of a kinase with multiple proteins/peptides in vitro is often used to screen optimal substrates.[14] The conventional flurometric assay is unable to monitor multiple substrates and their products. While this MALDI MS based method is able to achieve this goal. To demonstrate this capability, another peptide HHASPRK, which is the substrate of cyclin-dependent kinase-2 (CDK2), was also added to reaction system. Therefore, two substrate peptides were incubated with PKA. Because the peptide HHASPRK also has two primary amines, multiplex labeling with 4 Da mass difference can be achieved for this peptide. The PKA catalyzed phosphorylation, quenching, labeling and pooling of the labeled samples were sequentially performed as above. The changes of the concentrations for the two peptides and their phosphorylated peptides in the time course study were measured as shown in Fig. 3b. The decreasing of LRRASLGGK concentration and increasing of the phosphorylated product, LRRApSLGGK, concentration indicated LRRASLGGK was phosphorylated and consumed by PKA. However, no significant change in the concentration of HHASPRK was observed and no phosphorylated product was observed indicating HHASPRK cannot be phosphorylated by PKA. Above example clearly demonstrated that multi-substrate assay can be achieved by this multiplex labeling method.

Conclusions

In conclusion, we have developed a MALDI MS based approach for multiplexed protein kinase activity assay. This multiplexed kinase activity assay was exemplified by using PKA as the model kinase and the LRRASLGGK as the substrate peptide. Monitoring of PKA activities for different reaction times in both single and multiple substrate system were achieved by analyzing only one MALDI spot. In addition, kinetic constants can be accurately obtained benefiting from the complete labeling reactions and accurate quantification. Furthermore, this approach is also successfully applied to screen kinase inhibitors. As a consequence, this proposed approach allows high-throughput, simple, quick, cost-effective and accurate quantitative kinase/inhibitor profiling, we believe this MALDI MS-based labeling strategy will be a useful tool for kinase activity monitoring, and kinase inhibitor screening.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and supplementary figures. See DOI: 10.1039/c000000x/

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