

## Label-Free Assay

# Profiling Kinase Activities by Using a Peptide Chip and Mass Spectrometry\*\*

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Herein we describe a strategy that combines peptide chips with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) to evaluate kinase activities rapidly and semi-quantitatively. We have already shown that monolayers of alkanethiolates on gold are well-suited as substrates for MALDI-TOF MS, and could be used to measure enzyme activities and to perform high-throughput screenings.<sup>[1,2]</sup> A significant aspect of this approach—which we term SAMDI (*self-assembled monolayers for MALDI*) MS—is that it avoids the use of labels, greatly simplifying the formatting of assays. Herein we establish two important

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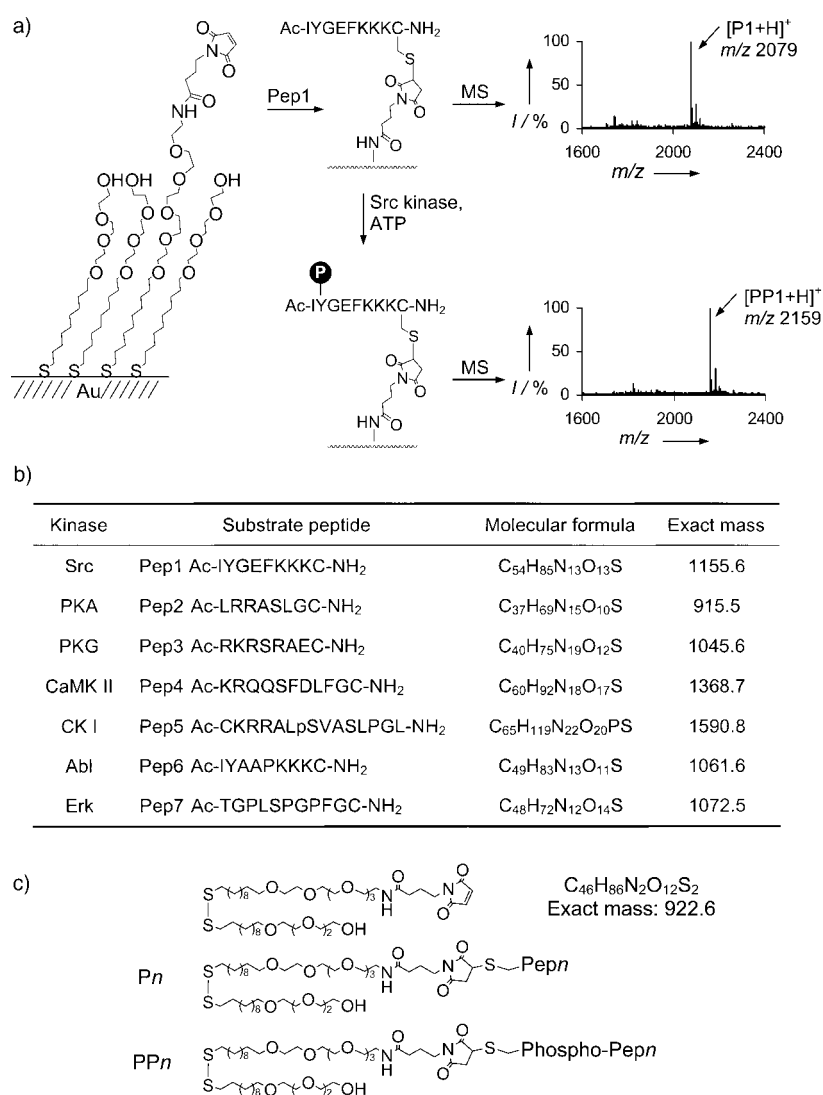
benefits that make this method well-suited to profiling multiple enzyme activities. First, SAMDI allows different classes of analytes, which may normally each require separate labeling strategies, to be evaluated by using a single method. Second, by using peptide substrates that are mass-resolved, the substrates can be immobilized on a single surface as mixtures, and do not need to be patterned into arrays.

Protein kinases are enzymes that catalyze the transfer of a phosphate group to tyrosine, serine, or threonine residues of substrate proteins. Protein kinases serve important regulatory functions in essentially all cellular processes including cell growth, migration, differentiation, and death.<sup>[3]</sup> Hence, assays that measure kinase activities are important in research and in the identification of lead compounds in drug discovery. Traditional kinase-activity assays follow one of two formats. In the first, a radioactive <sup>32</sup>P atom is used to label the phosphorylated substrates. Separation of the phosphorylated products using gel electrophoresis, phosphocellulose binding, or liquid chromatography then allows quantitation of the extent of phosphorylation and therefore of the kinase activity.<sup>[4]</sup> In the second format, the phosphorylated peptides are detected with antibodies that recognize the phosphopeptide. The assays can employ many strategies to analyze the binding of the antibody, including enzyme linked immuno sorbent assay (ELISA) formats and fluorescence polarization.<sup>[5]</sup> These techniques provide reliable methods for measuring kinase activities, but they do require substantial manipulation and are not readily extended to multikinase formats. Recent efforts have recognized that mass-spectrometric techniques could prove useful for measuring the activities because these techniques avoid labels, which leads to simpler assay formats.<sup>[6]</sup> A remaining challenge with these methods is to ease the requirement for sample enrichment, including by HPLC and affinity chromatography, prior to MS analysis.

We illustrate the SAMDI assay with the c-Src kinase.<sup>[7]</sup> We prepared mixed self-assembled monolayers (SAMs) presenting maleimide groups among a background of tri(ethylene glycol) groups.<sup>[8]</sup> A peptide substrate for c-Src (Pep1, 1 mM) was immobilized by the reaction of the terminal cysteine residue with the maleimide groups. Analysis of the monolayer by MALDI-TOF MS showed a single major peak at  $m/z$  2079 ([P1+H]<sup>+</sup>) corresponding to the mixed disulfide derived from the peptide-terminated alkanethiol and a glycol-terminated alkanethiol (termed P1 (from Pep1)). An identical monolayer was treated with c-Src kinase (0.4 U  $\mu\text{L}^{-1}$  *N*-(2-hydroxyethyl)-*N'*-(2-ethanesulphonyl)piperazine (HEPES)

buffer (pH 7.5) containing 80  $\mu\text{M}$  adenosine-5'-triphosphate (ATP), 15 mM  $\text{MgCl}_2$ ) for 1 h at 30 °C. The monolayer was then rinsed, treated with matrix (2,4,6-trihydroxyacetophenone (THAP), 2  $\mu\text{L}$ , 5 mg  $\text{mL}^{-1}$  in methanol) and analyzed by MALDI-TOF MS.<sup>[9]</sup> The original peak arising from the substrate peptide was absent and a new peak resulting from phosphorylation of the peptide was observed at  $m/z$  2159 ([PP1+H]<sup>+</sup>; Figure 1a). Importantly, all of the original peptide underwent phosphorylation, which shows that the ligands, when immobilized to the structurally well-defined monolayers, retain full activity towards the enzyme.

We assayed the catalytic subunits of protein kinase A (PKA), protein kinase G (PKG), calmodulin-dependent protein kinase II (CaMKII), casein kinase I (CKI), and Abl

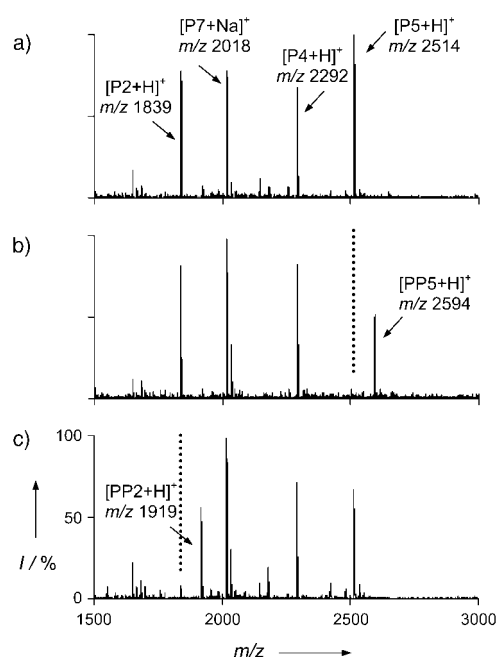


**Figure 1.** Design of monolayers for characterizing enzyme activities by MALDI-TOF MS. a) A peptide substrate for c-Src kinase (Pep1) was immobilized on a SAM presenting maleimide groups. The monolayer was treated with c-Src kinase. Both monolayers before and after the kinase treatment were analyzed by MS, which showed one major peak corresponding to the mixed disulfide containing the peptide ([P1+H]<sup>+</sup>) and phosphorylated product ([PP1+H]<sup>+</sup>), respectively. b) Sequences and masses of substrate peptides used in this work are listed. c) Molecular structures corresponding to mass peaks observed in this study are shown.

kinase by using the set of selective substrate peptides in Figure 1b.<sup>[10]</sup> The enzymatic reaction conditions were chosen to give complete phosphorylation by each kinase. Representative mass spectra are shown in Figure 2. We found that in each case, the kinase reactions resulted in the expected mass change of 80, which confirms the addition of phosphate groups to each of the peptide substrates.

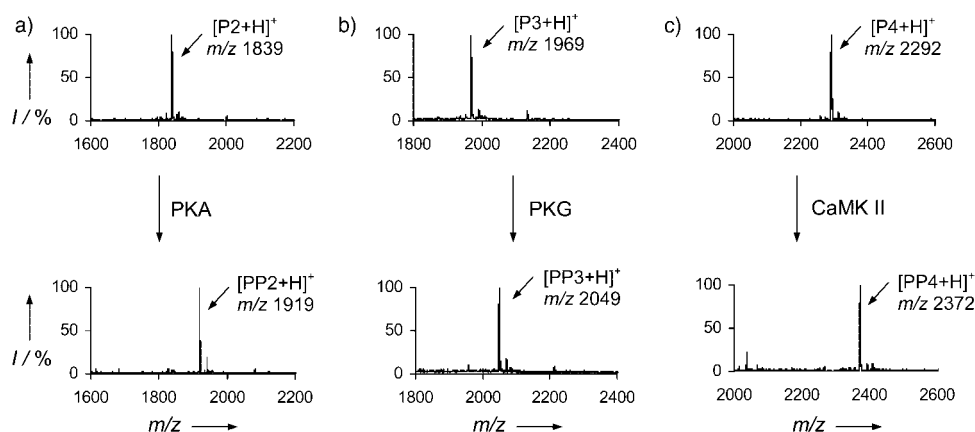
Multiple kinase activities can be measured simultaneously by using monolayers that present mixtures of kinase-specific peptide substrates. We treated a maleimide-terminated monolayer with a mixture of four peptides (Pep2, Pep4, Pep5, and Pep7). The ratio of the peptides in the mixture was adjusted to give monolayers that displayed the four peptides at similar intensities in MALDI-TOF spectra.<sup>[11]</sup> Indeed, a mass spectrum of the monolayer showed four well resolved peaks corresponding to disulfides derived from each peptide-terminated alkanethiolates ( $[P2+H]^+$   $m/z$  1839,  $[P4+H]^+$   $m/z$  2292,  $[P5+H]^+$   $m/z$  2514,  $[P7+Na]^+$   $m/z$  2018; Figure 3a). An identical monolayer was treated with the CK I kinase, which is expected to phosphorylate only Pep5. Mass analysis revealed that the original mass peak for the CK I substrate peptide ( $[P5+H]^+$ ) was absent and gave rise to a new peak corresponding to phosphorylation of this peptide ( $[PP5+H]^+$   $m/z$  2594), while the other peptides remained unmodified by the kinase (Figure 3b). In an additional example, we treated the same chip with PKA and found that the peptide substrate for this kinase was phosphorylated ( $[PP2+H]^+$   $m/z$  1919), but the other peptides were not modified (Figure 3c). These examples show that the SAMDI approach can be applied to peptide chips for rapid and easily interpretable characterization of enzymatic reactions in a multi-analyte format.

The SAMDI assay can be used to quantitatively characterize the inhibition of kinases by known inhibitors. We prepared several solutions containing CKI (1 U  $\mu\text{L}^{-1}$ ) and the inhibitor *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89) at concentrations ranging from 0.001 to 1 mM in assay buffer (pH 7.5 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer containing 10 mM  $\text{MgCl}_2$  and 0.2 mM ATP). A portion of each reaction mixture (1  $\mu\text{L}$ ) was applied onto a circular region (2 mm in diameter)

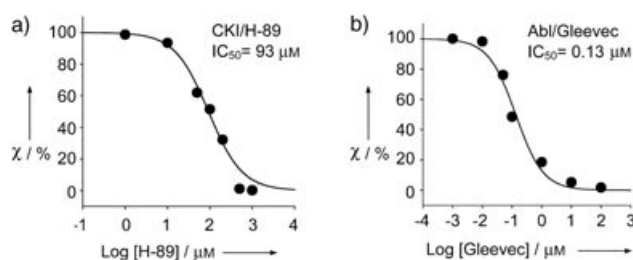


**Figure 3.** Enzymatic reactions on monolayers presenting multiple peptide substrates. a) The monolayer was prepared by applying a mixed solution of the four peptides (Pep2, Pep4, Pep5, and Pep7) to monolayers presenting maleimide groups. Molecular ion peaks corresponding to disulfides containing each substrate peptide were well resolved. b) Treatment of the monolayer with CK I resulted in phosphorylation of only the CK I substrate peptide Pep5 ( $[P5+H]^+$ ). c) Treatment of the monolayer with PKA resulted in phosphorylation of only Pep2 ( $[PP2+H]^+$ ). Dotted vertical line indicates the position of the peak for the substrate peptide.

of a monolayer presenting CKI selective substrate peptide, Pep5. The array was incubated for 30 min at 25 °C to allow the kinase reactions to proceed and then each spot was characterized by SAMDI to determine the extent of reaction. Figure 4a shows the relationship between the concentration of H-89 and the extent of the phosphorylation reaction. The data are fit well by a 1:1 inhibition model and give an  $\text{IC}_{50}$  value of 93  $\mu\text{M}$ .<sup>[12]</sup> We followed the same procedure to



**Figure 2.** Characterization of three different kinase reactions with MALDI-TOF MS. MS spectra of the monolayers presenting substrate peptides are shown before (top) and after (bottom) treatment with a) PKA onto Pep2-immobilized SAMs, b) PKG onto Pep3-immobilized SAMs, and c) CaMKII onto Pep4-immobilized SAMs. After treatment with kinases, the original peaks ( $[Pn+H]^+$ ) were absent and gave rise to new peaks corresponding to the phosphorylated product peptides ( $[PPn+H]^+$ ).



**Figure 4.** Dose-dependent inhibition curves of kinase reactions determined by SAMDI MS. a) Solutions of CKI and H-89, ranging in concentrations from 0.001 to 1 mM, were applied to the SAMs presenting substrate peptide (Pep5). The relative amount of phosphorylated peptide  $\chi$ , is plotted versus H-89 concentrations. b) Abl kinase and Gleevec also show dose-dependent inhibition.

characterize inhibition of Abl kinase by the approved drug Gleevec (also known as STI571, a 2-phenylaminopyrimidine derivative) on a monolayer presenting a selective substrate peptide of Abl kinase, Pep6.<sup>[13]</sup> A dose-dependent inhibition curve showed an  $IC_{50}$  value of 0.13  $\mu\text{M}$  (Figure 4b).<sup>[14]</sup> These data show that the SAMDI approach enables quantitative analysis of kinase activities to evaluate inhibition kinetics.

The method reported herein advances a significant body of work in which MS was employed to analyze enzyme activities. Siuzdak and co-workers, for example, have characterized enzymatic glycosylation by  $\beta$ -1,4-galactosyltransferase and showed that enzyme inhibitors could be quantitatively characterized with electrospray ionization (ESI) MS.<sup>[15]</sup> Leary and co-workers have evaluated enzyme kinetics of the carbohydrate sulfotransferase and hexokinase, and screened a mock library to identify possible inhibitors of hexokinase by using ESI MS.<sup>[16]</sup> Henion and co-workers have quantitatively evaluated the hydrolysis of dinucleotides by ribonuclease A and the hydrolysis of lactose by  $\beta$ -galactosidase using HPLC MS.<sup>[17]</sup> Additionally, phosphorylated proteins and peptides have been characterized by MS after enriching samples by affinity chromatography or liquid chromatography before MS analysis.<sup>[6]</sup> The features that distinguish our method is that the peptide substrate is immobilized on an inert surface. The immobilization simplifies the preparation of the sample for MS analysis, since the spectator molecules can be removed by rinsing. The use of an inert surface permits quantitative enzyme reactions in a solid-phase format.<sup>[18]</sup>

The high sensitivity for detecting analytes and the avoidance of labels have led many researchers to apply MS to biochemical studies, including enzyme assays, protein-ligand interactions, and biomarker characterization from biological samples.<sup>[6, 15–17, 19–21]</sup> One limitation with these practices is that the preparation of samples for MS analysis is not straightforward. Our goal is to develop relatively simple processes that can be routinely used in biological research. The use of immobilized substrates together with surfaces that provide for specific interactions, is an advance in this direction. We believe that the ability to conduct multiple assays on a single surface will prove useful in chemical biology and signal transduction research.

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