Enzyme Assays

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Protein Kinase Assay on Peptide-Conjugated Gold Nanoparticles by Using Secondary-Ion Mass Spectrometric Imaging**

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Kinases serve as key regulatory components that mediate signaling pathways with respect to various cellular functions,[1] and they are considered one of the major targets for drug discovery.[2] Significant efforts have been made to assay these enzymes and their inhibitors for the development of potent therapeutic reagents against many human diseases,[3] but most studies have relied on labor-intensive radioactivity- or fluorescence-based techniques. As a label-free approach, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been employed to determine the kinase activity and to screen inhibitors.[4] Recently, time-of-flight secondary-ion mass spectrometry (TOF-SIMS), as an alternative to MALDI-TOF MS, has been drawing much attention due to its potential for the label-free analysis of biomolecule modifications with high sensitivity and chemical specificity.[5] We previously reported that the use of gold nanoparticles (AuNPs) significantly enhanced the secondary-ion (SI) emission of peptides in TOF-SIMS.[6]

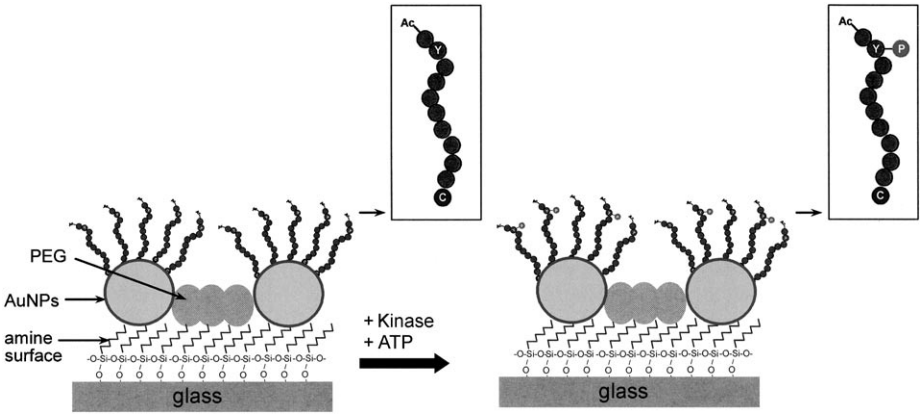

Figure 1. Schematic representation of the kinase assay by using SIMS on peptide-conjugated AuNPs on a glass surface. Y = tyrosine, Ⓟ: HPO_3^{2-}.

Here we describe a label-free assay of protein kinase on peptide-conjugated AuNPs by using secondary-ion mass spectrometric imaging. The AuNPs act as both signal enhancers and target concentrators (Figure 1). To endow the peptide substrate for Abl kinase with surface orientation, two cysteine-terminated peptides with different lengths (P_{Abl}, Ac-IYAAPKK$(G)_4$C; P_{Abl-s}, Ac-IYAAPKKC) were conjugated to AuNPs that had been attached onto a positive amine layer on a glass surface (see the Supporting Information). The resulting surface was treated with a succinimidyl poly(ethylene glycol) (PEG) to block the remaining amine groups. Fourier transform infrared (FTIR) spectra support the fact that the functional groups of the peptides remained intact even after PEG blocking (see the Supporting Information). By using an atomic primary ion, quasimolecular secondary-ion signals were most strongly observed for $[MH-SH-COOH]^+$ (m/z 1085.61 for P_{Abl} and m/z 857.52 for P_{Abl-s}) instead of $[MH]^+$, as shown in Figure 2a and b. This result indicated that the cysteine sulfhydryl group (SH) was strongly attached onto the surface of the AuNPs by a thiol bond,[7] and molecular ion signals with the subtraction of a carboxylic acid group reflect one of the sputtering features in amino acid analysis with SIMS.[8]

The kinase reaction resulted in a straightforward change in the mass of the peptide substrates (Figure 2c and d). Specifically, the reaction with Abl kinase generated positive ion signals with the signals of the two peptides (P_{Abl} and P_{Abl-s}) shifted by a mass equivalent to that of HPO_3 (80 Da), thereby causing a decrease in the original unphosphorylated signal intensity. In the positive and negative modes, the signals of $[Tyr-COOH+HPO_3]^+$, $[PO_2]^-$, $[PO_3]^-$, and $[H_2PO_4]^-$ were additionally observed after the kinase reaction (see the

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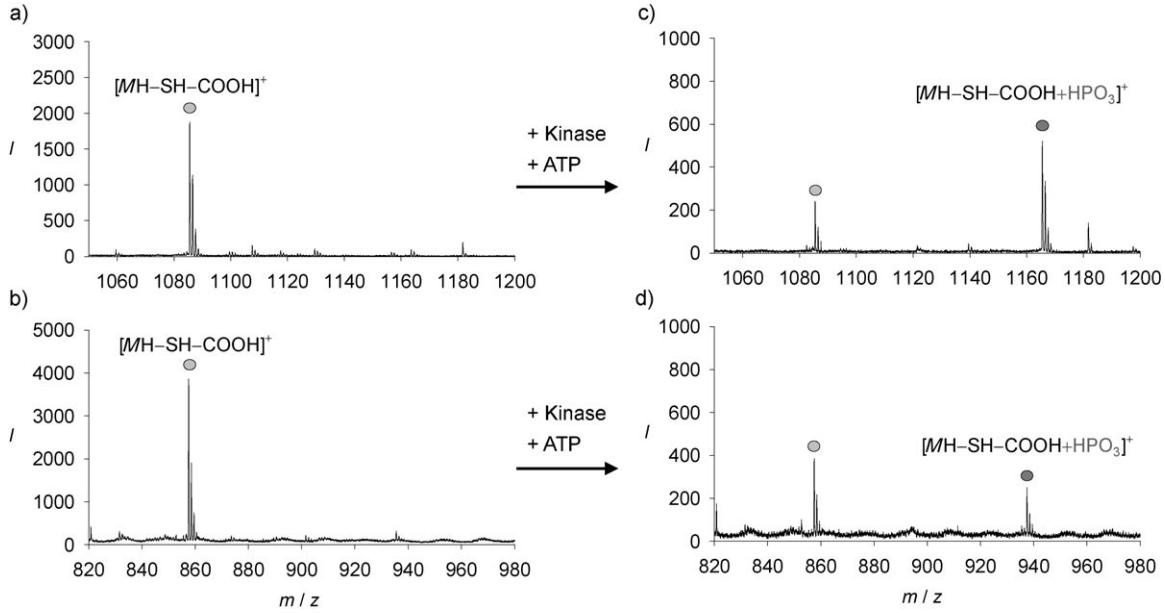

Figure 2. Positive TOF-SIMS spectra generated by the Abl kinase reaction for different peptide substrates. a) P_{Abl} (Ac-IYAAPKK$(G)_4$C; $M_r = 1162.58$); b) P_{Abl-s} (Ac-IYAAPKKC; $M_r = 934.49$); c) and d) SIMS spectra from P_{Abl} and P_{Abl-s}, respectively, after the kinase reaction.

Supporting Information). The phosphorylation efficiency (E_P) was determined by SI ratiometry between the original unphosphorylated signal and the phosphorylated one within a single spectrum (see the Supporting Information). When a spacer of $(Gly)_4$ was introduced to the end of peptide substrate (in P_{Abl}), the E_P value was estimated to be 70%, whereas the E_P value for P_{Abl-s} without a spacer was only 29%. The use of a peptide substrate without a cysteine residue at the terminus (that is, Ac-IYAAPKK) gave rise to a significantly lower E_P value, at a level of about 10% (data not shown). This result strongly implies that the surface orientation and length of the peptide substrate are crucial for an efficient kinase reaction on the AuNPs. The AuNPs are likely to induce a high loading capacity of peptides[6] and easy accessibility of the enzyme as a result of their three-dimensional structure when compared to a flat bare gold surface.[9] We previously observed that the binding capacity of a three-dimensional dendrimer layer for ligands is much higher than that of self-assembled monolayers (SAMs).[10]

From surface plasmon resonance (SPR) analysis, the PEG-blocked surface was revealed to show significantly lower binding of nonspecific antibody, which leads to improved binding of phosphopeptide-specific antibody (Figure 3). Reduction of nonspecific protein binding is expected to give rise to a more sensitive inhibition assay for the kinase. The peptide-ion signal from the kinase reaction was notably lower when no PEG blocking was attempted (see the Supporting Information).

For the inhibition assay, Abl and PKA kinase activities were measured in the presence of different concentrations of the respective inhibitors (staurosporine for Abl and CMIQ for PKA) by using AuNPs containing an appropriate substrate. The peptide P_{PKA} (Ac-LRRASL$(G)_4$C) was additionally used as a substrate for PKA. As a result, a dose-dependent decrease in kinase activity was clearly observed (Figure 4), and the IC_{50} values of staurosporine and CMIQ

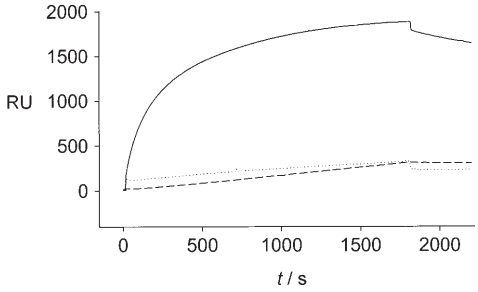

Figure 3. SPR sensorgrams for binding of phosphotyrosine antibody (anti-pY; ——) and mouse immunoglobulin G (mIgG; -----) on the surface of P_{Abl}-conjugated AuNPs with PEG blocking; RU = response unit. Anti-pY or mIgG (10 μg mL^{-1} in phosphate-buffered saline + tween 20 (PBST)) was injected after the kinase reaction. As another control, anti-pY was flowed over the surface without the kinase reaction (•••••).

were estimated to be 141 and 92 nM, respectively, values that were higher relatively than those reported in solution.[11] Based on this result, it seems that our approach can be effectively used for the quantitative assay of kinase activity and its inhibition.

To check the utility of SIMS imaging for the kinase assay in a high-throughput manner, two peptides (P_{Abl} and P_{PKA}) were independently spotted onto the AuNP-attached glass surface by using a microarrayer (spot 1 and spot 2, Figure 5a). Kinase solution (Abl or PKA) with or without inhibitor was loaded onto each spot area. As shown in Figure 5b, a change in the mass caused in the kinase reaction was distinctly revealed by SIMS chemical imaging. A control surface without the kinase reaction yielded no quasimolecular-ion images in the phosphorylated mass region, whereas kinase-treated spots (lines 1 and 2) yielded strong ion mass images (that is, $[P_{Abl}H-HS-COOH+HPO_3]^+$ or $[P_{PKA}H-SH-COOH+HPO_3]^+$ as appropriate) with a decrease in the image intensity at the original peptide-ion

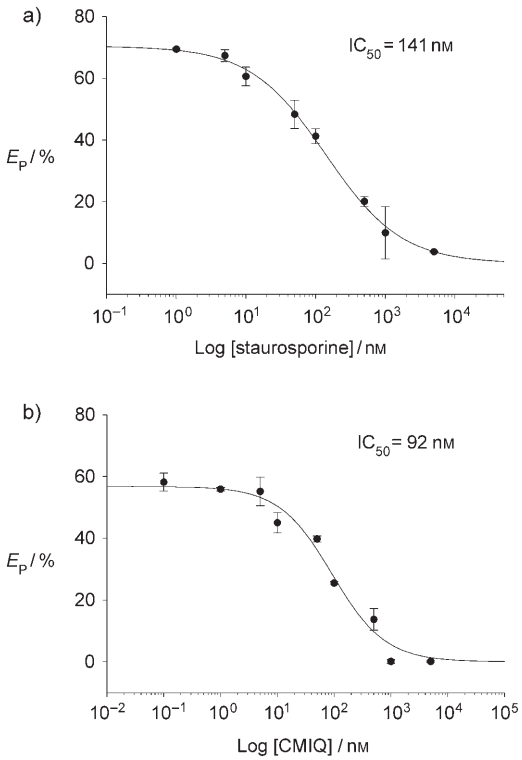

Figure 4. Inhibition assay for kinases as a function of inhibitor concentration. a) Abl; b) PKA. The error bar indicates the standard deviation in quadruplicate experiments.

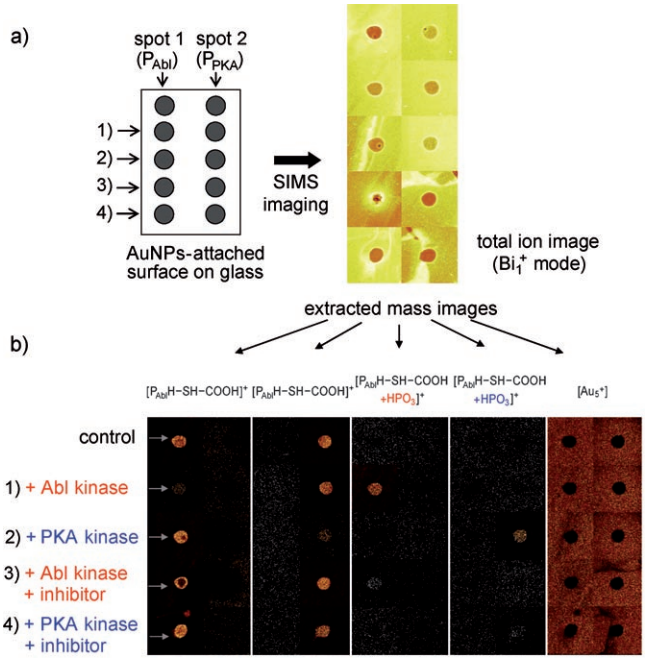

Figure 5. a) Microspotting of peptides onto an AuNP-attached glass slide for kinase assays. b) SIMS imaging for the detection of phosphorylation. Kinase (Abl or PKA) was loaded onto the spot area in the absence and presence of the respective inhibitor (staurosporine or CMIQ). The concentration of inhibitor was 10 μM. Imaging data for mass regions of interest were extracted from the total ion image.

mass (that is, $[P_{Abl}H-HS-COOH]^+$ and $[P_{PKA}H-SH-COOH]^+$, respectively). Addition of inhibitor dramatically reduced the intensity of the ion mass images (lines 3 and 4). No cross-reaction images for kinases against the opposite peptides were observed. It was also possible to obtain multiplexed SIMS images by using a mixture of two peptides for the detection of phosphorylation (see the Supporting Information). This result indicates that SIMS-based chemical imaging enabled kinase assaying with high specificity in a high-throughput manner because all images for ions with differing masses could be collectively extracted from a single measuring image. SIMS is known to provide a greater resolution sensitivity in chemical imaging than MALDI MS.[12]

In addition to the high-throughput and multiplexed imaging, the SIMS-based approach (spectrum and imaging) offers several advantages over conventional assays. First, mass-based detection permits a straightforward identification in a label-free manner for enzyme-induced subtle modifications of peptides without the need for phosphopeptide-specific antibodies as reported elsewhere.[13] A number of short peptide substrates can be applied for this purpose, since they have been specifically recognized by numerous enzymes in vivo and in vitro as stable substrates.[14] Secondly, this approach provides versatile applicability of the easy-to-prepare AuNPs to various solid surfaces (glass, Si, Au, etc.). Although this approach is limited to the mass range of analytes less than ≈1300 Da,[15] an increased surface area through control of the size and shape of the AuNPs could improve the SI yield of peptides up to a relatively high mass range. Most importantly, matrix-free SIMS analysis can generate reproducible ion signals compared to MALDI MS. Several SIMS imaging techniques have been applied to various fields, but most of them have been focused on tissue imaging[16] or element-level analysis,[17] rather than chip-based protein assays. We anticipate that the AuNP-enhanced SIMS approach (spectrum and imaging) can be effectively applied to the assay of various protein kinases and the screening of their inhibitors with high sensitivity in a high-throughput and multiplexed manner.

Experimental Section

Synthesis of AuNPs: The AuNPs were synthesized by the citrate-stabilization method, as described elsewhere.[18] Briefly, a stock solution (100 μL) containing 300 mM $HAuCl_4 \cdot 3H_2O$ was added to distilled water (100 mL) to give a final concentration of 300 μM; this was followed by vigorous stirring. 30 mM Sodium citrate dihydrate (1.5 mL) was added to this solution, at a final concentration of 450 μM (molar ratio of tetrachloroaurate to sodium citrate 1:1.5), and the mixture was stirred. For fast reduction and formation of gold colloids, a stock solution (100 μL) containing 300 mM $NaBH_4$ was quickly added to the reaction solution, and this was followed by stirring. The clustering of AuNPs was checked by UV/Vis spectroscopy (UV-2550, Shimadzu), and the average size of the AuNPs on the glass surface was estimated to be 10.7 ± 2.7 nm by using a field-emission scanning electron microscope (FE-SEM; Sirion, FEI, Netherlands; see the Supporting Information).

Kinase and inhibition assay: The kinase was assayed in wells formed by overlaying a chambered silicon coverslip (50 wells, 3 mm in diameter and 1 mm in height) onto an amine-functionalized glass

slide (Slide A+, Schott Nexterion, Germany). A solution of 10 nM AuNPs (10 μL) in double-distilled water was added to the wells, and this was followed by incubation for 30 min for attachment of the AuNPs onto the amine–glass surface. The wells were washed with double-distilled water three times and then dried. Three kinds of peptides (Ac-IYAAPKK$(G)_4$C and Ac-IYAAPKKC for Abl, and Ac-LRRASL$(G)_4$C for PKA) were then dissolved in 10 mM phosphate-buffered saline (PBS; 50 μg mL^{-1}, pH 7.4) and directly attached onto AuNPs in the wells. This was followed by thorough washing with water and drying under a stream of N_2. Prior to the kinase reaction, methoxy poly(ethylene glycol)succinimidyl α-methylbenzoate (*m*PEG-SMB; Nektar Inc.) dissolved in a 50 mM bicarbonate buffer (pH 8.5) was added to the wells at a final concentration of 2 mM to block the remaining amine groups; the wells were then rinsed with water and dried. The kinase reaction was carried out by addition of a reaction mixture (10 μL) containing either 2 nM Abl or 50 nM PKA with 100 μM adenosine triphosphate (ATP) at 30 °C for 60 min. For the inhibition assay, inhibitor (staurosporine or CMIQ) dissolved in dimethylsulfoxide was diluted to different concentrations in distilled water and added to the reaction mixture. After the kinase reaction, the wells were rinsed with double-distilled water and treated with 0.5 % diammonium citrate dibasic solution for 10 min to enhance the emission of phosphorous ions. After removal of the silicon coverslip, the resulting surfaces were subjected to SIMS analysis. The phosphorylation efficiency (E_P) and IC_{50} values were calculated as described in the Supporting Information.

Microarray of peptide substrates for SIMS imaging: Two peptides (Ac-IYAAPKK$(G)_4$C and Ac-LRRASL$(G)_4$C) were dissolved in 10 mM PBS (pH 7.4) containing 5 % glycerol (v/v) to give a final concentration of 50 μg mL^{-1}. The peptide solution was arrayed on the AuNP-attached surface. Microspotting was performed by using a robotic arrayer (Microsys, Cartesian Technologies, Irvine, CA, USA) equipped with CMP 3 spotting pins (Telechem International, Sunnyvale, CA, USA). For the kinase reaction, a multiwell-type chambered silicon coverslip was overlaid onto the glass surface with the exposure of a spotting area in each well. A reaction mixture (10 μL) containing either 2 nM Abl or 50 nM PKA with 100 μM ATP was added to each well; this was followed by incubation at 30 °C for 60 min. Inhibitor-containing kinase solutions (staurosporine or CMIQ, final concentration of ca. 10 μM) were compared under the same conditions. Spotting areas on the glass surface were subjected to SIMS imaging immediately after they had been washed with distilled water and 0.5 % diammonium citrate dibasic solution.

TOF-SIMS analysis: Ion spectra measurement by TOF-SIMS was carried out with a TOF-SIMS V instrument (ION-TOF GmbH, Germany), by using 25 keV Bi_1^+ primary ions. The ion currents were measured to be 0.5 pA (Bi_1^+) at 5 kHz by using a Faraday cup located on the grounded sample holder. A bunching system gives pulse durations of 18.0 ns with a mass resolution exceeding $M/\Delta M = 10^4$ (full width half maximum (FWHM)) at m/z values larger than 500 in both the positive and negative modes. The analysis area of 500 × 500 μm^2 was randomly rastered by primary ions to obtain the SIMS spectra, and the primary ion dose was maintained below 10^{12} ions cm^{-2} to ensure static SIMS conditions. Positive and negative ion spectra were internally calibrated by using the H^+, H_2^+, CH_3^+, $C_2H_3^+$, and $C_3H_4^+$ signals or the H^-, C^-, CH^-, C_2^-, and C_2H^- signals, respectively. For TOF-SIMS imaging, the Bi_1^+ beam (25 kV) was randomly rastered over each spot near the static limit of a primary-ion influence. All images were taken in the positive mode at 256 × 256 pixels. Mass images of interest were collected from the raw data in the range of m/z below 1500.

SPR: SPR analysis was performed by using a BIAcore-3000 instrument and gold sensor chips (BIAcore; see the Supporting Information).

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