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Quantitation of Surface-bound Proteins on Biochips Using MALDI-TOF MS

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We report on a novel method for the quantitation of proteins specifically bound on a ligand-presenting biochip by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The bound protein was digested by trypsin, and the resulting peptide fragments were analyzed by MALDI-TOF MS in the presence of an isotope-labeled internal standard (IS). The IS has the same sequence as a reference peptide (RP) of the target protein digest, but a different molecular weight. The absolute amount of the specifically bound protein on a biochip is then quantitated by comparison of mass intensities between the RP and the IS. Because they have the same molecular milieu, the mass intensities of these two analytes represent the real amounts of analytes on the chip. As a model system, we tested glutathione *s*-transferase (GST) and a GST-fusion protein, which were captured on glutathione-presenting biochips. We observed that the glutathione densities on biochips showed a good correlation with the absolute quantity of the proteins. We believe that our method will provide an alternative to currently existing tools for the absolute quantitation of surface-bound proteins.

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Introduction

The protein chip has been an important analysis format for the study of biochemical functions of proteins and interactions of proteins with other proteins, substrates, and small molecules.[1-4] Unlike other types of biochips, such as peptide chips, the fabrication of protein chips requires several considerations in order to conserve protein functions on a substrate surface. For example, the orientation of proteins should be predictable, and protein folding should be maintained when proteins are immobilized on a surface.[5,6] Most of all, the amount and density of proteins on a substrate surface are important parameters that need to be calculable in most applications.

Various analytical tools, including surface plasmon resonance (SPR),[7,8] quartz crystal microbalance (QCM),[9] X-ray photoelectron spectroscopy,[10] amido black assay,[11] amino acid analysis,[12] fluorometric assay,[13] and enzyme-linked immunosorbent assay (ELISA),[14] have been used in an effort to quantitate surface-immobilized proteins. Although the use of these methods can provide valuable quantitative information on proteins on solid surfaces, these techniques require large amounts of proteins and materials, and some techniques cannot provide any information on protein identities.

In this paper, we report on a novel method for the quantitation of proteins that are immobilized on a ligand-presenting biochip *via* a specific interaction by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Surface-bound proteins are digested by trypsin, and the resulting peptide fragments are analyzed by MALDI-TOF MS after the addition of an internal standard (IS). The absolute amount of the specifically bound protein on a biochip is then quantitated by comparison of the mass intensities between the reference peptide (RP) of the target protein digest and the IS (Fig. 1a). The quantitative character of MALDI-TOF MS is the point at issue among active users, and it is generally accepted that MALDI-TOF MS is not inherently quantitative, because the peak intensities are strongly influenced by the molecular milieu of the analytes.[15] Thus, a comparison of the intensities may not represent the real amounts of analytes. In this respect, we adopted the strategy of AQUA (absolute quantitation of proteins), in which determinations of the absolute levels of proteins and posttranslationally modified proteins are achieved by comparison of the mass signals of an externally added stable isotope labeled standard peptide to the endogenous peptide in the sample.[16,17]

In our strategy for the absolute quantitation of a protein that is specifically bound to ligand presenting biochips using MALDI-TOF MS, the IS is designed to have the same sequence as the RP, except for one residue, which is replaced by the same residue containing stable isotopes, such as 13C and 15N. Therefore, the IS has the same molecular milieu as the RP of the target protein proteolysis, but a different molecular weight, and is clearly distinguishable by MS analysis. In this context, the behavior of the IS would be identical to that of the RP with respect to desorption/ionization, crystallization with a matrix, and other handling procedures. Taken together, the IS, which is precisely added to samples in a known quantity, allows for absolute quantitation of proteins on biochips.

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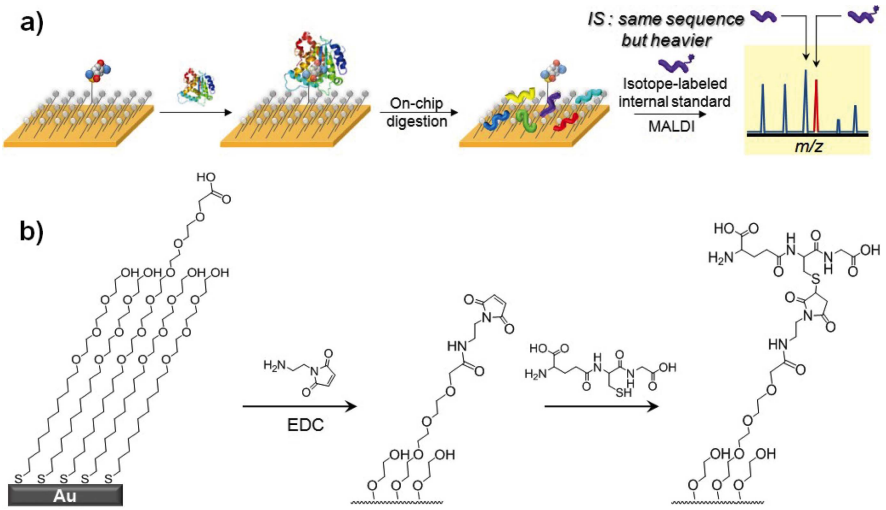

Fig. 1 Schematic presentation of our strategy for the absolute quantitation of surface-bound proteins. a) The target protein on the ligand presenting chip is digested by trypsin. The resulting peptide fragments are analyzed by MALDI-TOF MS with an isotope-labeled IS. A comparison of mass intensities between a resulting peptide from the target protein and the IS enables absolute quantitation of the target protein. b) Structure of SAMs on gold and chemical modifications for the preparation of glutathione-presenting biochips. The carboxylic acid-presenting monolayer was coupled to *N*-aminoethyl maleimide, followed by glutathione.

Experimental

Reagents and chemicals

Gold-coated coverslips were prepared by the evaporation of titanium (5 nm) and then gold (15 nm) using an electron beam evaporator (Korea Vacuum, KVE T-C500200).[18] Isotope labeled internal standard peptides, LERPHR*D and YEEHLYER*, were custom-synthesized by AnyGen Co., Ltd. (Kwangju, Korea). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), acetonitrile, trifluoroacetic acid (TFA), dithiothreitol, ammonium bicarbonate, calcium chloride, and glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol was obtained from Merck (Darmstadt, Germany). α-Cyano-4-hydroxycinnamic acid (CHCA) was from Bruker Daltonics (Germany). Trypsin was from Promega Co. (USA).

Preparation of carboxylic acid-presenting monolayers[18]

Gold-coated coverslips were cleaned in a Piranha solution (sulfuric acid:30% hydrogen peroxide = 70:30. Warning: the Piranha solution is highly corrosive and reactive. Handle with caution.) for 2 min, washed with deionized water and ethanol, and dried under a nitrogen stream. The coverslips were incubated in a mixed solution of tri(ethylene glycol)-terminated alkanethiol and acid penta(ethylene glycol)-terminated alkanethiol solution at ratios ranging from 1:99 to 20:80 for 12 h (the total concentration of the alkanethiol solution was 1 mM). Monolayers were washed with absolute ethanol and dried under a stream of nitrogen.

Preparation of GSH-presenting monolayers

This carboxylic acid-presenting monolayer was treated with EDC (20 mg/mL in pH 7.4 phosphate-buffered saline (PBS)) and *N*-aminoethyl maleimide (7 mg/mL in PBS) for 2 h, washed with PBS and absolute ethanol, and dried under a stream of nitrogen. GSH (1 mM in 10 mM Tris, pH 7.5) was then covalently anchored on the monolayer by incubating for 2 h.[19]

Protein immobilization and on-chip digestion

GSH-presenting monolayers were treated with 5 µL of 15 µM proteins (glutathione *s*-transferase (GST) and glutathione *s*-transferase human catechol-*O*-methyltransferase (GST-hCOMT) in 10 mM Tris, pH 7.5, 1 mM dithiothreitol) for 2 h, rinsed with tri-distilled water, dried under a stream of nitrogen, and stored at 4°C. For on-chip tryptic digestion, 5 µL of a trypsin solution (10 ng/µL in 20 mM ammonium bicarbonate, 0.5 mM calcium chloride, and 10% acetonitrile) was applied to protein-bound chips and incubated at 37°C for 6 h; chips were then dried under ambient conditions.[20,21]

MALDI-TOF analysis

One microliter of isotope-labeled IS was added to the tryptic digestion mixture on chips and mixed by pipetting. After the chips were dried, 1.5 µL of CHCA (1 mg/150 µL in 50% acetonitrile, 0.05% TFA in distilled water) was applied on the chips. The resulting mixtures on the chips were analyzed by MALDI-TOF MS, and then quantitated by comparison of mass intensities. Mass analysis was performed using an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a smartbeam laser as an ionization source. All of the spectra were acquired with a 19-kV accelerating voltage, a 50-Hz repetition rate, and a positive mode with an average of ~300 shots.

Results and Discussion

To test the fidelity of our strategy, we examined two proteins, glutathione *s*-transferase (GST) and a GST-fusion protein, glutathione *s*-transferase human catechol-*O*-methyltransferase (GST-hCOMT). The target proteins were captured specifically on self-assembled monolayers (SAMs) on gold which presented the ligand, glutathione (GSH). SAMs were prepared by a mixed solution of tri(ethylene glycol)-terminated alkanethiol and acid penta(ethylene glycol)-terminated alkanethiol. This carboxylic

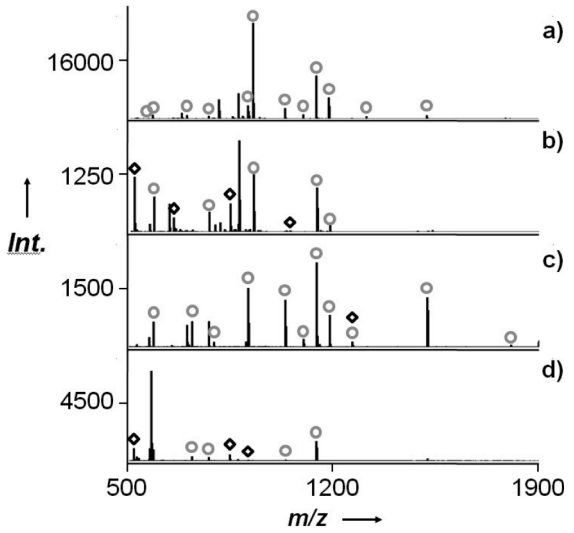

Fig. 2 Mass spectra of the resulting peptide fragments of a) in-solution digested GST, b) on-chip digested GST, c) in-solution digested GST-hCOMT, and d) on-chip digested GST-hCOMT (○, peptides from digested GST or GST-hCOMT; ◇, peptides from auto-digested trypsin).

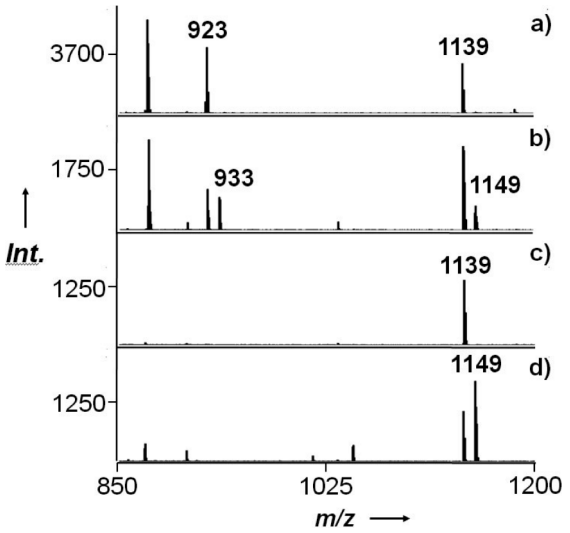

Fig. 3 MS spectra of tryptic peptides from (a) on-chip digested GST, (b) on-chip digested GST with IS, (c) on-chip digested GST-hCOMT, and (d) on-chip digested GST-hCOMT with IS. Peaks at *m/z* 923 and 1139 correspond to RPs from digested GST/GST-hCOMT, and peaks at *m/z* 933 and 1149 correspond to ISs for RPs.

acid-presenting monolayer was treated with EDC and *N*-aminoethyl maleimide to afford maleimide functionality on the surface. Glutathione was then covalently anchored on the monolayer by way of Michael addition (Fig. 1b). The oligoethylene glycol groups ensure that the monolayer is inert to nonspecific protein adsorption, which is a strict requirement for study of protein-ligand interactions at interfaces.[22]

First, we performed on-chip digestion of GST and GST-hCOMT for the determination of RPs. We prepared GSH-presenting monolayers with a surface density of 10%. Monolayers were treated with GST or GST-hCOMT (15 μM in 10 mM Tris buffer, pH 7.5, 1 mM dithiothreitol). Following incubation for 2 h, monolayers were rinsed with deionized water, and subsequently subjected to on-chip digestion. For on-chip digestion, 5 μL of a trypsin solution (10 ng/μL) was applied to protein-immobilized chips, which were then incubated at 37°C in a humidified chamber overnight. Chips were dried, treated with the 1.5 μL of CHCA (1 mg/150 μL) as a matrix, and analyzed by MALDI-TOF MS without further sample-preparation steps. On-chip digested peptide fragments (Figs. 2b and 2d) were verified by comparison with in-solution digested peptide fragments (Figs. 2a and 2c). The identities of the resulting peptides were obtained by the Biotools program from BRUKER (for details and full analysis, see Tables S1 and S2 in Supporting Information). Two peptides (LERPHRD (M_w, 922), YEEHLYER (M_w, 1138)) were chosen as RPs, which satisfy the following criteria: i) to give distinctive peaks with high intensity, ii) to consist of 7 – 8 amino acids, iii) not to contain chemically reactive residues, such as cysteine/methionine/tryptophan (oxidation) and asparagine/glutamine (deamination) (Figs. 3a and 3c). Two different isotope-labeled ISs were then synthesized in order to represent tryptic peptides: LERPHR*D (M_w, 932), YEEHLYER* (M_w, 1148). Internal standard peptides were prepared with isotope-labeled arginine, which has six 13C and four 15N. Figures 3b and 3d show the MS spectra of on-chip protein digests for GST and GST-hCOMT in the presence of ISs. Note that the peaks in Fig. 3 were observed as a proton adduct $[M+H]^+$. The tryptic peptide LERPHRD is a partially cleaved product located at the C-terminal end of GST; therefore, in the current study, YEEHLYER was used for the absolute quantitation of surface-bound proteins, since the quantity of partially cleaved LERPHRD might not be in agreement with the real quantity of the bound protein on the chip.

We next optimized the experimental procedure in order to ensure that: i) the protein quantitated on the biochip is bound to the monolayer through a specific interaction with the surface-presented ligand; ii) the bound protein is completely digested through on-chip tryptic digestion, so that the quantity of the RP is in agreement with the amount of the bound protein. We prepared GSH-presenting monolayers with a surface density of 5%. Monolayers were treated with two different samples, GST and GST premixed with GSH (74 fold molar excess). Following incubation for 2 h, monolayers were rinsed with deionized water 1 – 4 times, and subsequently subjected to on-chip digestion. MS analysis and quantitation with the IS indicated that rinsing two times was suitable for the removal of non-bound GST and non-specifically bound GST from the monolayers. We repeated the experiment with 10% GSH-presenting monolayers and obtained similar results. Next, quantitation was performed using the same conditions with various digestion times, ranging from 2 to 12 h. MS analysis showed that the maximum efficiency of trypsin was afforded at 6 h incubation, while incubation for a period of time shorter than 6 h resulted in incomplete digestion of the surface-bound GST, and an incubation period longer than 6 h resulted in increasing tryptic autolysis by-products and non-specific cleavage of the target protein, both of which could affect the quantitative analysis using our strategy. Taken together, for all experiments in this report, protein-treated monolayers were rinsed two times and digested for 6 h, assuming that this procedure ensures complete digestion of specifically bound proteins on biochips.

Finally, we quantitated the surface-bound GST on ligand-presenting monolayers with various ligand densities. The densities of GSH on the surface with a size of 3×3 mm^2

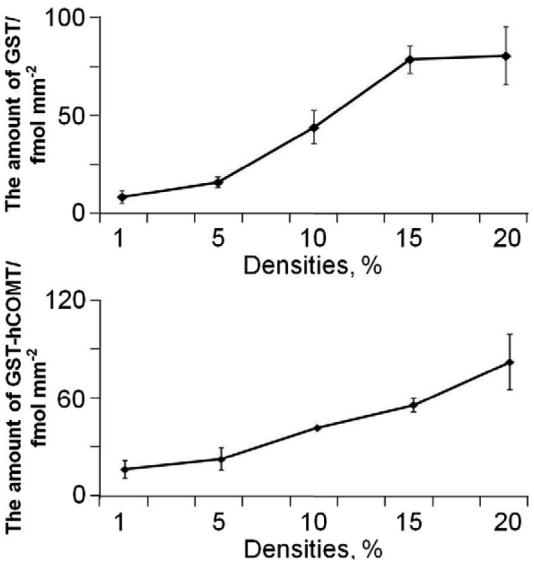

Fig. 4 Amount of GST and GST-hCOMT on glutathione-presenting chips with various ligand densities ranging from 1 to 20%. The obtained quantities of proteins using our strategy increased with the ligand densities.

were prepared ranging from 1 to 20%.[23] The monolayer was treated with GST or GST-hCOMT for 2 h, rinsed with deionized water 2 times, and on-chip digested for 6 h with 5 μL of trypsin (10 ng/μL). For the quantitation of protein, 1 μL of IS in tri-distilled water (0.112 pmol, amino acid analysis was carried out to determine the exact quantities) was added on the monolayer and thoroughly mixed by pipetting. The monolayer was dried and analyzed with MALDI-TOF MS using 1.5 μL of CHCA (1 mg/150 μL). The RP fragment and IS were analyzed and the quantity of the proteins was obtained by comparison of the mass intensities of RP and IS. Figure 4 shows the amount of GST and GST-hCOMT on the ligand-presenting monolayers with various densities, indicating that the amount of bound proteins was almost linearly increased with the ligand density. We observed poor sample-to-sample reproducibility at densities over 20%, indicating that monolayers were not inert, and that a non-specific adsorption of proteins was severely involved at a ligand density higher than 20%. Note that the results shown are the average of five separate determinations. The amount of bound proteins obtained using our strategy is consistent with the estimation by considering the size of GST.[24] By assuming no spacing between GST and single-layer adsorption, the area of one GST molecule, ~14 nm^2, gives the theoretical maximum density of ~120 fmol/mm^2. The current study afforded ~70 fmol/mm^2 as the maximum density for specifically bound GST on ligand presenting monolayers with a nominal size of 3×3 mm^2. The observed result is ~60% of the theoretical maximum density, and could be attributed to repulsion or steric hindrance between the adsorbed neighboring GST. This observation is in good agreement with another report in which the adsorption of BSA on gold nanoparticles was quantitatively investigated using several spectroscopic methods.[25]

Conclusions

We have demonstrated that specifically bound proteins on a ligand presenting monolayer can be quantitated using on-chip digestion combined with MALDI-TOF MS analysis in the presence of an isotope-labeled internal standard. Using the method described here, GST and a GST fusion protein bound specifically to GSH presenting monolayers were quantitated. Compared with other quantitation methods, our strategy provides straightforward, simple, and reproducible experimental protocols. Besides the quantitation of specifically bound proteins on ligand presenting monolayers, this approach can also be expanded to other surfaces; for example, we are currently performing systematic quantitation of proteins immobilized on several types of nanomaterials through specific/nonspecific interactions or covalent bond formation. We believe that our strategy will be a useful tool for providing quantitative information regarding proteins on solid surfaces including both 2- and 3-dimensional materials.

Acknowledgements

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Supporting Information

Additional information as noted in the text. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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