Quantitative Analysis of Surface-Immobilized Protein by TOF-SIMS: Effects of Protein Orientation and Trehalose Additive

Young-Pil Kim,1 Mi-Young Hong,1 Jinmo Kim,† Eunkeu Oh,† Hyun Kyong Shon,† Dae Won Moon,† Hak-Sung Kim,*,† and Tae Geol Lee*,†

Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Korea, and Nanobio Fusion Research Center, Korea Research Institute of Standards and Science (KRISS), Daejeon 305-600, Korea

We demonstrate the effects of protein orientation and trehalose on a quantitative analysis of surface-immobilized proteins by using time-of-flight secondary ion mass spectrometry (TOF-SIMS). As our model protein, streptavidin (SA) was quantitatively immobilized on a solid surface at different configurations by random or oriented immobilization and subsequently treated with trehalose. The resulting surface was analyzed by using TOF-SIMS and surface plasmon resonance (SPR) spectroscopy, where the secondary ion spectra from SA were compared with the surface density of the protein. In the case of oriented immobilization, the ion peak intensities measured by TOF-SIMS were correlated well with the SPR data, regardless of the presence of trehalose. Alternatively, trehalose significantly increased correlation between TOF-SIMS and SPR data for the randomly immobilized SA. It is likely that a trehalose-treated surface is less vulnerable to denaturation, thus leading to a reliable quantification of surface-immobilized proteins by TOF-SIMS. Our results show that TOF-SIMS can be used for understanding biophysical states such as orientation and denaturation of surface-immobilized proteins as well as for quantifying proteins within the field of biosensors and biochips.

A quantitative analysis of surface-immobilized proteins is crucial for the understanding of binding events between capture biomolecules and target analytes in a variety of biochip applications as well as for the construction of a more efficient biosensing system.1 A number of analytical methods employing labeling and non-labeling detection strategies have been developed for quantification,2 but they are still challenged by the minimal quantitative sensitivity.3 Indeed, it has already been demonstrated that TOF-SIMS spectra of the uppermost atomic layers (10À15 Å) yield valuable information regarding the biophysical state of proteins or polypeptides that are adsorbed to various types of surfaces.4 Along with these studies, quantitative studies of adsorbed proteins using the TOF-SIMS technique, in conjunction with the 125I-radiolabeling or X-ray photoelectron spectroscopy techniques for tissue engineering applications, have been reported.5 Despite these studies, a TOF-SIMS analysis has not been a straightforward method for the quantification of proteins, especially given the fluctuating biophysical states of the proteins (such as their orientation, conformation, or both). For example, significant discrepancies were reported between a TOF-SIMS analysis of adsorbed proteins and 125I-radiolabeled proteins determined by using radioactivity measurement,6 and there was very little correlation between secondary ion intensity and the number of amino acids.7 It was suggested that these results were mainly caused by the changes in orientation and conformation of adsorbed proteins on the surface.


Efforts have been made to resolve this problem, but no detailed investigations were attempted with respect to the biophysical effects on the quantification of surface-immobilized proteins. Unlike quantifying surface-adsorbed proteins, we previously reported that TOF-SIMS can be effective in quantifying surface-immobilized proteins. In the present study, we demonstrate the effects of protein orientation and trehalose treatment on a TOF-SIMS quantitative analysis of surface-immobilized proteins. In addition to protein orientation, another important consideration is the prevention of protein denaturation. In general, the preferred additives for reducing protein denaturation in protein chip applications have been trehalose and glycerol. Trehalose in particular is known to effectively stabilize the function and structure of proteins subjected to drying-induced stress, by linking multiple hydrogen bonds between the hydroxyl groups of trehalose and the surface polar residues of proteins. Castner and co-workers have demonstrated that a trehalose coating on absorbed proteins would effectively maintain conformational stability without reducing the secondary ion intensity of the protein. Consequently, trehalose as a preservation agent should be seriously considered for quantitative analysis.

Streptavidin (SA), as a model protein, was immobilized by two different methods onto a solid surface, namely, random and oriented immobilization, and then subjected to a TOF-SIMS analysis in the presence and absence of a trehalose additive. Specifically, a principal component analysis (PCA) for secondary ion peaks of proteins was carried out as a multivariate analysis. The resulting data were compared with surface plasmon resonance (SPR) data for insight into how protein orientation and the addition of trehalose affect quantification of surface-immobilized proteins in a TOF-SIMS analysis.

**EXPERIMENTAL SECTION**

**Sample Preparation.** Related reagents were all purchased from commercial sources: 99% α-D-glucopyranoside (α-D-Glc, Sigma), bovine serum albumin (BSA, Sigma), and biotinylated BSA (b-BSA, Sigma). A gold substrate was prepared by sequentially evaporating a 20Å-thick film of Ti and a 400Å-thick film of gold onto a Si wafer. Prior to the deposition of the monolayer, the substrate was cut into 10 mm × 10 mm pieces and cleaned for 5 min by immersion into a piranha solution (1:4) 30% H₂O₂: concentrated H₂SO₄ (v/v). Caution: the piranha solution reacts violently with most organic materials and must be handled with extreme care. For the formation of self-assembled monolayers (SAMs), the substrates were immersed in a 2 mM ethanolic solution of AUT for 2 h, followed by a thorough rinsing with absolute grade ethanol and distilled water. The resulting substrates were then subjected to further modification in two different ways: one was immersed in a 2 mM BS³ solution in 50 mM bicarbonate buffer (pH 8.5) for 30 min, and the other in a 2 mM sulfo-NHS-LC-Biotin solution in 50 mM bicarbonate buffer (pH 8.5) for 30 min.

For binding of SA on the modified surfaces, the BS³-functionalized and biotinylated surfaces were treated with serially diluted SA solutions (0.2, 1, 5, and 20 µg·mL⁻¹) in 10 mM PBS (pH 8.5) and 10 mM PBS (pH 7.4), respectively, for 30 min, followed by a thorough washing with PBS and water. In the case of the BS³-modified surface, a slightly alkaline buffer solution (pH 8.5) was used to facilitate a more efficient reaction between SA (qL = 6.5) and the NHS groups of BS³. To investigate the effect of trehalose on the quantitative analysis, SA-bound surfaces were incubated with 0.1% (w/v) trehalose in PBS for 20 min, dried under a stream of nitrogen, and subjected to TOF-SIMS analysis.

**TOF-SIMS and Principal Component Analysis.** Ion spectra measurement by TOF-SIMS was carried out with a TOF-SIMS V instrument (ION-TOF GmbH), using 25-keV Au⁺ primary ions. The ion currents were measured to be 0.8 pA (Au⁺) at 5 kHz by using a Faraday cup located on the grounded sample holder. A bunching system gives pulse durations of 16.8 ns with a mass resolution exceeding M/ΔM = 10⁴ (full width half-maximum) at m/z 0–300 in the positive mode. The analysis area of 500 × 500 µm² was randomly rastered by primary ions, and the primary ion dose was maintained below 10¹² ions·cm⁻² to ensure static SIMS condition. Positive ion spectra were internally calibrated using H⁺, H₂⁺, CH₃⁺, CH₄⁺, and C₂H₅⁺ peaks. To verify homogeneous and reproducible conditions of the samples, three points per sample were randomly analyzed, and sample preparation was run in duplicate. A PCA was performed using a PLS_Toolbox (ver. 3.5, Eigenvector Research, Manson, WA) for MATLAB (ver 7.0 MathWorks, Inc., Natick, MA). For the quantitative PCA, the total data set from each condition (Chart 1a or b) consisted of 30 spectra (5 different SA concentrations (0, 0.2, 1, 5, 20 µg·mL⁻¹) × 6 spots (duplicate sample, 3 random spots/sample)). Each peak was normalized by using the total intensity for each spectrum. Normalization helps to eliminate systematic differences (i.e., variations in the instrument at each time of data acquisition) in total secondary ion yield between spectra. Unless otherwise stated, the characteristic peak list of SA, biotin, and BS³ for PCA were then performed using a mean-centering method. An autoscaling method was also carried out in order to give equal weight to each of the selected characteristic peaks of the random or oriented SA. Afterward, the first principal component (PC 1) was used to establish a principal component regression (PCR) between sample scores and the surface density of SA. To clarify, the quantified sample scores were plotted as a function of surface density and then fitted to calculate determination coefficient (R²) by the linear regression procedure with 95% confidence intervals by using a graphic software of Sigm Apart (ver 9.0, SYSTAT Software).

**SPR Spectroscopy.** SPR spectroscopy was performed with a Biacore-3000 instrument and gold sensor chips (Biacore). The chip surface was first cleaned with 0.1 N NaOH containing 0.1% Triton-X for 5 min. The modification steps preceding the immobilization process for SA were similar to those described for the gold chip. Following the modification of a sensor chip with sulfo-NHS-LC-Biotin, the resulting sensor chip was locked into
RESULTS AND DISCUSSION

Effect of Protein Orientation. To investigate the effects of protein orientation on a quantitative analysis by TOF-SIMS, SA was immobilized by two different ways, random immobilization onto SAMs of AUT on a gold surface (Chart 1a) or oriented immobilization by using a biotinylated surface (Chart 1b). SA with a molecular mass of ~60 kDa consists of four identical subunits, and each monomer bears a binding site for biotin. SA is thought to be “oriented” on a biotinylated surface due to the strong interaction with biotin, unlike in the amine-coupling method. Prior to the quantitative analysis by TOF-SIMS, modified sensor chips were treated with solutions of varying concentrations of SA (0.2, 1, 5, and 20 μg·mL⁻¹) and then subjected to SPR analysis. Based on the assumption that a mass change of 1 ng·mm⁻² in a SPR sensorgram corresponds to a resonance angle shift of 0.1° (1000 RU in a BIACore instrument) at a sensing surface, the surface density of SA could be calculated from the experimentally measured value (ng·mm⁻²). The binding density by SPR was varied as a function of SA concentration at a given time. As expected, the binding level of SA increased with increasing SA concentration for both immobilization methods (Figure 1). However, the biotinylated surface resulted in a higher binding level of SA than the BS3-modified one when the same concentration of SA was injected for a predetermined time. The maximum surface densities of SA for oriented and random immobilization methods were observed to be 231 and 113 ng·cm⁻², respectively, when incubated with a SA solution of 20 μg·mL⁻¹.

When the two different SA layers were subjected to a TOF-SIMS analysis, numerous peaks were generated with molecular ion masses of up to 200. Of them, seven characteristics peaks were selected for a quantitative analysis of SA as shown in Table 1. BS3- or biotin-related peaks were also assigned. We previously described that cluster ion beams showed a higher signal-to-background ratio for characteristic secondary ions of SA, compared to monoatomic ion beams. Accordingly, for our quantitative analysis of SA, a cluster Au⁺ primary ion beam was chosen over a monoatomic Au⁺ ion beam. Characteristic peaks of SA were distinct in the range of m/z between 100 and 200. Even though there were many peaks of m/z <100, they were indistinguishable from the overlapped underlying layers (AUT, BS3, and biotin-related peaks) that also contained nitrogen-related moieties. Only the m/z 59 peak was distinct in the range of m/z <100, whereas six dominant peaks (m/z 110, 120, 130, 136, 159, and 170) were observed in the range of m/z 100–200 on both random and oriented SA surfaces (Figure 2). The difference in immobilizing methods affected the intensity of generated secondary ion peaks.

---

Concentrations (0.2, 1, 5, and 20 µg mL\(^{-1}\)) were sequentially loaded over each surface through four flow channels. Protein binding is expressed in response unit (RU), and the binding densities of an immobilized protein were determined to be 28, 82, 103, and 113 ng cm\(^{-2}\), respectively. The reaction was maintained for 30 min at a flow rate of 5 µL min\(^{-1}\) at 25°C. These results are representative of duplicate experiments performed on different sensor chip surfaces.

**Table 1. All Characteristic Peaks Selected for Analysis of Principal Component Regression**

<table>
<thead>
<tr>
<th>characteristic peaks</th>
<th>center mass</th>
<th>surface origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH(_3)N(^+) (Arg)</td>
<td>59.05</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)NS(^+)</td>
<td>76.02</td>
<td>biotin</td>
</tr>
<tr>
<td>C(_2)H(_4)N(^+)</td>
<td>97.06</td>
<td>biotin</td>
</tr>
<tr>
<td>C(_2)H(_4)N(^+) (His)</td>
<td>110.07</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(^+) (Lys)</td>
<td>114.10</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(_3) (Phe)</td>
<td>120.08</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)NO(^+) (Tyr)</td>
<td>130.07</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(_2) (Trp)</td>
<td>130.07</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(_2) (Trp)</td>
<td>136.08</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(_2) (Trp)</td>
<td>136.08</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)N(_2) (Trp)</td>
<td>136.08</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)NO(^+)</td>
<td>170.06</td>
<td>SA</td>
</tr>
<tr>
<td>C(_2)H(_4)SO(^+)</td>
<td>224.99</td>
<td>BS(^3)</td>
</tr>
<tr>
<td>C(_2)H(_4)O(_2)N(_3)S(^+)</td>
<td>227.09</td>
<td>biotin</td>
</tr>
<tr>
<td>C(_2)H(_4)S(_2)</td>
<td>242.94</td>
<td>BS(^3)</td>
</tr>
<tr>
<td>C(_2)H(_4)SO(^+)</td>
<td>268.95</td>
<td>BS(^3)</td>
</tr>
<tr>
<td>C(_2)H(_4)O(_2)N(_3)S(^+)</td>
<td>272.14</td>
<td>biotin</td>
</tr>
</tbody>
</table>

Without a change in the peak list of characteristic secondary ions, which may be indicative of the homogeneous distribution of compositional amino acid residues over the entire protein structure. Similar TOF-SIMS spectra for differently oriented SA were also observed for wild type SA and its mutant (his-tagged or cysteine-substituted form at each monomer) adsorbed on the gold surface. In contrast to our observation, ion spectra of proteins like BSA, IgG, and fibrinogen generally were reported to yield different secondary ion peak profiles, depending on their orientation, conformation, or both triggered by underlying substrates when directly adsorbed on surfaces.\(^{15}\)

**Correlation between TOF-SIMS and SPR Data in the Absence of Trehalose.** To quantify surface-immobilized proteins with TOF-SIMS, a PCA and PCR\(^2\) were performed for sensor chips with different surface densities of SA. BS\(^3\) or biotin-related peaks were also added to respective PCA data for easy separation of spectra. Figure 3 shows a PCR quantification of surface-immobilized SA by the two different methods. In the case of oriented immobilization using a biotinylated surface, the scores display a linear evolution with respect to the surface density of SA ($R^2 = 0.9634$) as can be seen in Figure 3b, indicating the usefulness of PCR for quantification of surface-immobilized SA. On the other hand, random immobilization shows a relatively poor correlation between the TOF-SIMS scores and surface density of SA ($R^2 = 0.7387$) (Figure 3a). This poor correlation may be caused by the denaturation of proteins under high-vacuum conditions during the TOF-SIMS measurement.

In both oriented and random immobilizations, samples with positive scores were dependent on the variables with positive loadings of SA peaks such as Arg (m/z 59), His (m/z 110), Phe (m/z 120), Trp (m/z 136), and Tyr (m/z 130, 159, and 170), whereas samples with negative scores were dependent on the variables with negative loadings of BS\(^3\) or biotin-related peaks (Figure 3c and d). In the PCR model, the strong dependency of the PCA score on the surface protein density implies a strong correlation between the TOF-SIMS and SPR data, which in turn indicates the reliability of a quantitative analysis using TOF-SIMS. We previously observed that the quantification procedure could be simplified with a PCR model of the correlation between the characteristic SIMS peaks and surface densities obtained from the SPR data.\(^9\) To check the effects of nonspecifically adsorbed proteins on our study, we tested nonspecific binding of SA on the AUT SAM (data not shown). The resulting SPR data showed a very low surface density (less than 2 ng cm\(^{-2}\)) even with a high concentration of SA. Similarly, the TOF-SIMS data also showed very low intensities of related secondary ion peaks of SA, as opposed to the two immobilization methods. In particular, the binding signals of the biotin- and BS\(^3\)-linked SA were unchanged after PBST washing with a high salt concentration (1 M NaCl) in the SPR experiment. In the study, therefore, nonspecific binding was negligible and did not affect correlation between SPR and TOF-SIMS data.

**Effect of Trehalose.** In order to obtain a more reliable quantification by minimizing protein denaturation, surfaces with various densities of SA were treated with a 0.1% trehalose solution in PBS buffer for 20 min and subjected to a TOF-SIMS analysis. Interestingly, a significant improvement in correlation with SPR analysis was observed for random immobilization, showing $R^2 = 0.9016$ as shown in Figure 4a, compared to that without trehalose ($R^2 = 0.7387$, Figure 3a). Notably, the percentage of captured proteins by the denaturant of proteins under high-vacuum conditions during the TOF-SIMS measurement.


the same samples was much reduced. This result indicates that the addition of trehalose is critical to a quantitative analysis of randomly immobilized proteins using TOF-SIMS. As for oriented immobilization, a marginal improvement ($R^2$ from 0.9634 to $R^2 = 0.9831$) in correlation with SPR data was observed when compared to the correlation in the absence of trehalose (Figure 4b).

It should be noted that the background peaks from trehalose or a chemical cross-linker would contribute to the peak intensity, causing some fluctuation in the quantitative analysis, but their interference was found to be negligible. In practice, the ratios between the summation of seven characteristic peak intensities of SA to the total intensity were proportional to the surface density of SA (from 1.4 to 3.3% in BS-SA, and from 2.8 to 6.9% in biotin-SA), and no significant difference in the ratios occurred due to the trehalose treatment. Therefore, the intensities of the characteristic peaks of SA measured by TOF-SIMS can be effectively used for quantitative analysis of surface-immobilized SA. Even when random and oriented SA layers with various surface densities were mixed, a good linearity between the TOF-SIMS and SPR results could be retained in the presence of a trehalose additive (Figure 5). In addition, an autoscaling method was more useful for quantitative analysis than a mean-centering method ($R^2 = 0.9497$, Figure 5a) because it is performed by dividing mean-
centered data by the standard deviation of each variable, thus maximizing sample differences ($R^2 = 0.9605$, Figure 5b). The underlying peaks (BS and biotin-related peaks) were removed from the variances because autoscaling increases the differences in peak intensities by maximizing the noise between similar spectra. In other words, if all of the peaks from Table 1 are used in a combined quantitative PCA of SA, the BS and biotin-related peaks could influence the quantitative analysis of SA. Since the aim of this PCA was to perform a quantitative analysis of SA under different surface conditions, the peaks from each substrate were removed in the scores set to interpret only SA-related peaks for quantification.

Effects of Protein Orientation and Trehalose Treatment on Denaturation of Proteins. The changes in peak intensities of the amino acids in the SIMS spectra were verified before and after trehalose treatment (Figure 6). To remove the effect of surface protein density, the surface densities were adjusted to similar levels for two differently immobilized SA molecules by using SPR: 112 and 125 ng cm$^{-2}$ for random and oriented immobilizations, respectively. Although orientation, in spite of the similar surface density, may affect the ion intensity of the protein due to the differently exposed position of the two surfaces, randomly immobilized SA in the presence of trehalose produced ion intensities similar to biotin-coupled SA (lower spectra of Figure 6a and b). This result seems to be caused by a homogeneous distribution of peak-generating amino acid residues over the entire homotetrameric structure of SA, allowing the amino acid residues to be evenly and equally detected by TOF-SIMS. However, with a similar surface density, the characteristic peaks of biotin-coupled SA showed less change in intensities with the trehalose treatment, compared to randomly immobilized SA (Figure 6). To identify which characteristic peaks of SA caused the most significant changes due to trehalose treatment, a PCA of the characteristic peaks of SA was carried out for trehalose-treated and untreated samples in the respective surfaces (Figure 7). In the random immobilized SA, sample scores were distinctly separated into two groups, namely, trehalose-treated and untreated ones (Figure 7a). The positive scores corresponding to untreated samples were mainly due to arginine (Arg) and tryptophan (Trp)-related peaks as the positive loadings data (Figure 7b). This would indicate that trehalose treatment inhibits the secondary ion intensity of Arg and Trp, as shown in Figure 6a. On the other hand, sample variances in the oriented immobilization were not fully separated, and the effect of trehalose on the scores was irrelevant to the corresponding loadings data (Figure 7c and d). The large differences in PC 1 scores between trehalose-treated and untreated SA indicates that trehalose treatment caused significant changes in the detected amino acids of the SA. 

![Figure 4](image.png)

**Figure 4.** Effect of trehalose on quantitative PCR for SA immobilized by amine-coupling (a, c) and biotin-coupling methods (b, d), respectively. Scores plots (a, b) and the corresponding loadings plots (c, d) are presented for comparison with those in Figure 1.

![Figure 5](image.png)

**Figure 5.** Combined quantitative PCR with TOF-SIMS data of two different SA layers. All combined scores of PC 1 with (a) mean-centering and (b) autoscaling were plotted in the presence of 0.1% trehalose treatment with respect to the surface density SA measured by SPR. The PCA scores were obtained by using only SA characteristic peaks in the peak list as shown in Table 1.
Since the Arg-related peak \( m/z 59 \) is normally positioned at the exterior region of SA, unlike Trp, trehalose could easily react with the polar amino acid residue via hydrogen bonding,\(^{18}\) resulting in the reduction of secondary ion generation of the Arg-related peaks on both surfaces (Figure 6a and b). However, Trp-related peaks \( m/z 130, 159, \) and 170) exhibited more striking changes in the randomly immobilized SA with trehalose treatment than in the biotin-coupled SA. As seen in the SIMS spectra, in the randomly immobilized SA, the ion intensities from Trp were higher in the absence of trehalose than those in the presence of trehalose (Figure 6a, upper and lower spectra), whereas biotin-coupled SA showed little trehalose effect on Trp ion intensity.

Figure 6. Trehalose effect on positive SIMS spectra of SA immobilized by two different methods: (a) amine-coupled and (b) biotin-coupled methods, respectively. The surfaces having similar surface densities of SA (a, 112 ng·cm\(^{-2}\); b, 125 ng·cm\(^{-2}\)) were treated with trehalose and analyzed by TOF-SIMS. The \( m/z \) values are indicated for distinct characteristic peaks of SA.

Figure 7. PC 1 scores and loadings plot from PCA of positive ion spectra from amine-coupled (a, b) and biotin-coupled SA (c, d), respectively, at similar surface densities (a, 112 ng·cm\(^{-2}\); b, 125 ng·cm\(^{-2}\)). Each set of 6 sample scores (2 replicates x 3 points per sample) was compared in the absence (open circle) and presence (filled circle) of 0.1% trehalose treatment.

Considering that Trps are located near the biotin-binding site of SA,\textsuperscript{19} the TOF-SIMS sampling depth does not normally allow for sufficient ion generation from Trp residues. It has been reported that the distance from the top position to Trp residues near the biotin-binding site is estimated to be 15–20 Å.\textsuperscript{20} Denaturation of SA, however, can cause the nonpolar amino acid residues including Trp to be exposed toward the exterior part of SA, and a significant difference in the intensities of Trp ion peaks could be observed for conditions in the absence and presence of trehalose treatment.\textsuperscript{21}

Therefore, our PCA results indicate that protein denaturation is more likely to be prevalent for randomly immobilized SA than for biotin-coupled SA.

Denaturation of surface-immobilized proteins generally reduces their binding capacity, lowering the performance of biochips in terms of the sensitivity and reproducibility.\textsuperscript{22} To compare the denaturation effect on proteins with similar surface densities, binding densities of b-BSA were monitored using SPR between a BS3 and a biotin-coupled SA surface (Figure 8). The maximum binding density of b-BSA was observed in the case of trehalose treatment for both surfaces: 463 RU (±46.3 ng cm\textsuperscript{-2}) for random SA and 752 RU (±75.2 ng cm\textsuperscript{-2}) for oriented SA. On the other emission of 350 nm, native SA generated no emission wavelength of Trps buried within the hydrophobic protein core, whereas denatured SA resulted in a high emission peak of Trps.\textsuperscript{21}

![Figure 8. SPR binding response of b-BSA on the surfaces with two differently immobilized SA in the absence (black line) and presence (red line) of trehalose treatment: (a) amine-coupled SA and (b) biotin-coupled SA. Specific interaction with b-BSA (solid line) was performed under conditions with similar surface densities of SA (a, 112 ng cm\textsuperscript{-2}; b, 125 ng cm\textsuperscript{-2}). As a control, BSA without a biotin group (dotted line) was used to compare the SPR response.](image-url)


hand, when BSA without biotin was used as a control, the binding density was relatively low regardless of trehalose treatment: 110–148 RU in BS\textsuperscript{3}-coupled SA and 56–75 RU in biotin-coupled SA. Despite similar surface densities of SA for the biotin-coupled and BS\textsuperscript{3}-coupled surfaces, the higher binding density of b-BSA on the biotin-coupled SA, as opposed to the BS\textsuperscript{3}-coupled SA, indicates that there are more available biotin-binding sites on the biotin-coupled surface. This is likely due to the minimal variations in the surface position of oriented SA, unlike random SA. This homogeneity of oriented SA would allow for high binding accessibility of b-BSA.

In the case of randomly immobilized SA, the binding level of b-BSA increased with trehalose treatment (385 RU in the absence of trehalose and 463 RU in the presence of trehalose). But no significant change was observed for oriented immobilization (721 RU/752 RU). Considering that randomly immobilized SA unlike biotin-coupled SA entraps relatively large amounts of water,\textsuperscript{23} it was expected that randomly immobilized SA underwent denaturation by losing its binding capacity more extensively, compared to biotin-coupled SA. Therefore, the role of trehalose, namely, preventing dehydration, was more pronounced for randomly immobilized proteins than for those immobilized with orientation during the TOF-SIMS analysis, which explains the difference in linear correlation between the SIMS and the SPR data as shown in Figures 3 and 4. This result strongly suggests that treatment with trehalose is essential for a reliable TOF-SIMS quantitative analysis of surface-immobilized proteins. It has been also reported that a significant decrease in the binding capacity of surface-bound SA layer can occur when the layer is dry; treatment of the layer with trehalose prevented the loss of binding capacity.\textsuperscript{24}

Quantification by TOF-SIMS. Despite the advantages of TOF-SIMS, there are limits to its applicability in the quantification and analysis of protein orientation or denaturation. The main drawback in current TOF-SIMS technique is the difficulty of generating a distinct characteristic peak or larger fragments of a respective protein in a mixture of proteins. Effective methods to increase the secondary molecular ion yield of proteins, which include the use of cluster ion analysis beams\textsuperscript{25} or the incorporation of various signal enhancers,\textsuperscript{26} would allow multiple quantifications of various proteins on surfaces. Most importantly, since SPR alone offers very little information on the orientation or denaturation of surface-immobilized proteins, a combination of TOF-SIMS and SPR data would provide a deeper understanding of the relationship between the biophysical state of a protein and its surface density. We anticipate that our approach will be useful not only for the quantitative analysis of surface-immobilized proteins but for understanding their biophysical states on a surface in the field of biosensors and biochips.

CONCLUSIONS

We have demonstrated the effect of trehalose treatment and protein orientation on a quantitative analysis of surface-immobilized proteins by using TOF-SIMS. When SA was immobilized with orientation on a surface, the secondary ion peak intensities measured by TOF-SIMS were well correlated with SPR analysis regardless of trehalose treatment. On the other hand, in the case of randomly immobilized SA, a significant improvement in correlation with SPR analysis was observed only with trehalose treatment. It is likely that the trehalose acted to prevent the surface-immobilized proteins from dehydration, which enabled proteins to become less vulnerable to denaturation, thus allowing for a reliable quantification of surface-immobilized proteins. TOF-SIMS can be used for understanding the biophysical states of surface-immobilized proteins such as orientation or extent of denaturation as well as quantitative analysis in the field of biosensors and biochips.

ACKNOWLEDGMENT

This work was supported by the R&D Program of Fusion Strategies for Advanced Technologies of the Ministry of Commerce, Industry and Energy (MOCIE), the KIBB Inititative Research Program (KIBB, Korea), the Bio-Signal Analysis Technology Innovation Program (M106450100002-6N4501-00210), the Nano/Bio Science & Technology Program (M105360000205-N3609-00210) and the Nano Science & Technology Program (M10503000218-05M0300-21810) of the Ministry of Science and Technology (MOST), and the Korea Health 21 R&D Project (0405-MN1-0604-0007) of the Ministry of Health & Welfare (MHW).

Received for review August 28, 2006. Accepted December 14, 2006.

AC0616005