Evaluation of Proteins Immobilized on Glass Substrates of Biosensor with TOF-SIMS

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Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is capable of chemically visualizing proteins on insulated samples. Measurement of protein distribution on material surfaces is required for developing high performance biosensors. In this study, an immobilized probe protein, protein A immobilized on a glass plate, and that of a sample protein, immunogloblin G (IgG) in solution, reacting with protein A on the biosensor surface were measured by means of TOF-SIMS. Each protein immobilized on the glass plates were measured with TOF-SIMS to obtain reference spectra for data analysis using mutual information, calculated as subtraction of *a posteriori* information entropy from *a priori* information entropy indicates specific secondary ion peaks related to a certain protein in spectra. Then, fragment ions specific to protein A and IgG, respectively, were selected to obtain the TOF-SIMS image of the proteins.

INTRODUCTION

Evaluation and control of the biosensor surface is required for developing high performance sensing systems, and evaluation of biosensor surfaces is important for development of sophisticated biosensors. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) measurement of distribution of immobilized probe protein on the biosensor surface and sample protein reacted with the probe protein contribute to evaluation of reaction occurring on the sensor surface. However, TOF-SIMS is not suitable for ionization of intact large molecules such as proteins. Especially, it is difficult to select specific peaks of secondary ions related to each protein for obtaining chemical imaged of proteins, respectively, because every protein consists of the same 20 amino acids and therefore fragment ions from proteins are very similar. Therefore appropriate data analysis techniques are required for characterization of TOF-SIMS spectra with fragment ions from large molecules. Multivariate analysis techniques, such as principal component analysis (PCA) and linear discriminant analysis (LDA), have been employed to interpret TOF-SIMS spectra using fragment ions related to proteins [1-3]. Information theory [4] has been also employed to analyze TOF-SIMS data of biomaterials containing proteins to select specific peaks for chemical imaging [5-8]. Mutual information [4-9], defined by information theory, describes the specificity of every peak in TOF-SIMS spectra of a sample comparing the other sample such as reference sample. With the mutual information important and specific peaks can be selected out of numerous candidate peaks.

In this study, the model biosensor sample was fluorescence-labeled protein A-immobilized glass plates, which has specific reaction with immunogloblin G (IgG) [10, 11]. The protein A-immobilized glass plates were examined by TOF-SIMS with mutual information. Also immunoglobulin G (IgG)-immobilized glass plates were measured as reference samples in order to discriminate protein-related peaks from protein A and IgG.

MATERIALS AND METHODS

Protein Immobilization

A slide glass was cut into pieces measuring 9 mm square, and these pieces were aminosilanized with aminopropyltrimethoxy-silan (Tokyo Kasei,

Tokyo, Japan). The aminosilanized glass was activated by glutaraldehyde and then soaked in a 2 ml of 2.5 μg/ml Qdot655TM-labeled protein A (Quantum Dot Corporation, Pittsburgh, PA), FITC-protein A (Zymed Lab. Inc., San Francisco, CA) and IgG (Zymed Lab. Inc.) solutions, respectively, and allowed to react in the dark for 30 hrs at 227 K. After the glass plate was washed in pH 7.4 phosphate buffer saline (PBS) and then in pure water with sonic wave for 1 min to remove adsorbed proteins. Then, these glass plates were rinsed with pure water and dried before TOF-SIMS measurement.

TOF-SIMS measurement

Positive ion spectra by TOF-SIMS, TFS-2100 (Physical Electronics, Eden Prairie, MN) using 12 keV Ga+ primary ion source, were acquired up to 1000 m/z while maintaining the primary ion dose less than 10^{12} ions/cm^2 to ensure static conditions [12]. A pulsed low-energy electron flood gun was used for charge neutralization for all samples.

Data analysis

Values of the mutual information comparing the samples were calculated by subtracting *a posteriori* entropy from *a priori* entropy. Information entropy before the estimation of peak intensities based on an appropriate threshold is defined as "*a priori* entropy" S(A), and that of after the estimation is "*a posteriori* entropy" S(A|B).

$$S(A) = - \sum p(ai)\log_2 p(ai) \quad (1)$$

The probability $p(ai) = n(ai)/N$ (i =1, 2)

n(a1) is the number of spectra belonging to the sample, n(a2) is that belonging to the reference sample, and N is the total number of TOF-SIMS spectra.

With a certain peak threshold V, the set of spectra are split into two subsets B1 and B2. The peak intensity greater than V is classified to B1 and the number of the spectra containing these peaks is n(b1), and that less than V is classified to B2 and the number of the spectra containing these peaks is n(b2). Therefore the information entropy of splitting induced by V, S(B) is defined by equation (2).

$$S(B) = - \sum p(bj)\log_2 p(bj) \quad (2)$$

The probability $p(bj) = n(bj)/N$ (j =1, 2)

The calculation steps of mutual information I(A;B) [5, 6] were the followings:

$$I(A;B) = S(A) - S(A|B) \quad (3)$$

$$S(A|B) = - \sum \sum p(bj)\, p(ai|bj)\log_2 p(ai|bj) \quad (4)$$

The probability $p(ai|bj) = n(ai|bj)/n(bj)$

n(ai|bj) is the number of spectra belonging to sample category "i" out of the spectra containing peaks greater than V. The best value of V is chosen to provide the largest I(A;B). When I(A;B) = S(A), the peak intensity of each spectra is completely classified to the right category.

Thus values of the mutual information were calculated on comparison of the protein A-immobilized glass, the IgG-immobilized glass and the aminosilanized glass plates without protein. Peaks of secondary ion at m/z = 40 to 270 were chosen for calculation.

RESULTS AND DISCUSSION

In terms of comparing values of the mutual information of peaks of secondary ions from the glass plates with or without protein A, there are little differences (data are not shown). On the other hand, the values of the mutual information of peaks from glass plates with or without IgG show differences between those with and without IgG as shown in Table 1. The reason why different results are shown depending on an immobilized-protein is mainly because of size of the proteins. Since there are the same rate for immobilization of protein molecules

Table 1 Mutual information (MI) of aminosilanized glass plates with or without IgG

Sample	MI (-)	Peak of secondary ion (m/z)
IgG	1	70, 72, 74, 84, 86, 87, 91, 103, 107, 110 ,130, 131, 147, 159, 166, 170, 171, 184, 185
Glass (without IgG)	1	40, 45, 46, 47, 48, 127, 182, 213, 270

Table 2 Mutual information (MI) of protein A or IgG-imobilized on aminosilanized glass plates

Sample	MI (-)	Peak of secondary ion (m/z)
protein A	0.95	40, 45, 46, 47, 48, 127, 182, 213, 270
IgG	0.95	184
	0.50	72, 74, 159

using hydroxyls on a glass surface, the rate of uncovered area with a protein depends on the size of the proteins. Molecular weights of protein A and IgG are approximately 45,000 and 155,000, respectively. In other words, it is clearly shown by means of TOF-SIMS measurement that when IgG is immobilized on glass plates there are much less uncovered area than when protein A is done because of their sizes.

In order to select specific peaks to each protein, the values of the mutual information comparing protein A and IgG of the TOF-SIMS spectra were calculated, and peaks of secondary ions with large values of mutual information are shown in Table 2. Comparing Table 1 and 2, a peak of secondary ion at m/z = 184 ($C_9H_{16}N_2S$) is specific fragment ion from IgG. In addition, peaks at m/z = 72, 74, and 159 are also able to be used for chemical mapping of IgG when there are no protein A molecules are co-existing, and even when protein A molecules are co-existing the chemical mapping would be possible by means of considering influence of protein A. There seems to be no difference between the selected peaks from glass plates shown in Table 1 and those from protein A-immobilized glass plates shown in Table 2. However, when chemical structures of the selected peaks are considered it is found that there are somewhat differences.

There are two peaks around 182, peaks of secondary ions at m/z = 182.1 ($C_{13}H_{12}N$) and at m/z = 182.2 ($C_{12}H_{24}N$), respectively. Since the peak at m/z 182.2 is hardly observed on proteins-immobilized glass plates as shown in Fig. 1, though it is observed on both native and aminosilanized glass plates it would be from unspecific adsorption of contaminants. Therefore the peak at m/z = 182 (182.1) is able to be applied to obtain chemical mapping of protein A. (a) spectrum of aminosilanized glass plate (b) spectrum of protein A-immobilized glass plate In order to clarify protein immobilization, TOF-SIMS secondary ion images of IgG-immobilized glass plates patterned with chromium are shown in Figure 2. Images in Figs. 2 (a) and (b) show a glass area and a chromium pattern area, respectively. The shape like a scale bar of a ruler appears in them. Since IgG molecules bond with amino groups on a aminosilanized glass surface, IgG distributions are expected to be shown in the same areas in Fig. 2 (a). Fig. 2 (c) and (d), both are images of IgG, show the same distribution as that shown with Si+ shown in Fig. 2 (a), though intensities are different depending on ionization rates of chemicals. Since fragment ions of higher mass tend to be produced less than lower mass fragment ions in this TOF-SIMS measurement condition, the secondary ion image of the peak at m/z = 72 shows clearer image than that of the peak at m/z =184. However, fragment ions of lower mass are often

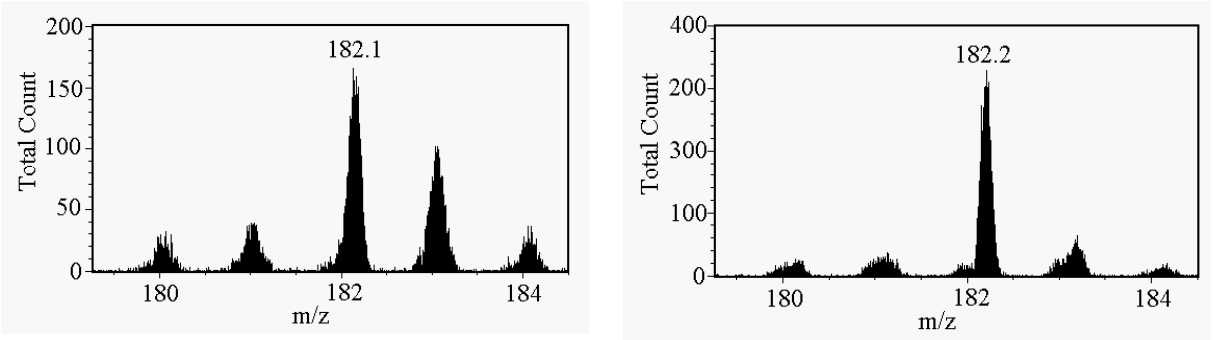
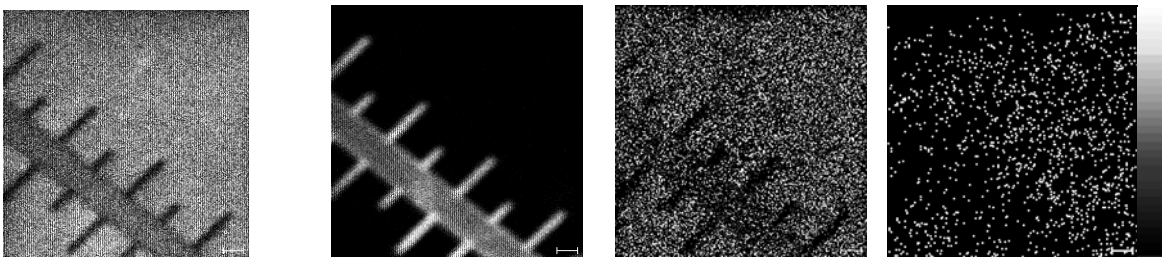


Fig. 1 Comparison of the peak at m/z = 182 between aminosilanized glass and protein A-immobilized glass plates



(a) Si+ (b) Cr+ (c) 72 (d) 184

Fig. 2 TOF-SIMS imaging of IgG on a glass plate (scale bar: 10μm)

related to other substances such as co-existing proteins. Therefore novel techniques for producing large fragment ions at higher ion yields are required to estimate much more complicated biomaterials.

CONCLUSIONS

TOF-SIMS spectra of the protein immobilized glass plates were analyzed with mutual information. This analysis method characterizes the TOF-SIMS spectra, and provides specific peaks from each protein to obtain chemical mapping. Thus the analysis of protein immobilization on the biosensor surface with TOF-SIMS evaluates immobilization processes of proteins on biosensor surfaces and clarifies phenomena on the surface to contribute to the improvement and the development of sophisticated biosensors. Moreover, IgG reaction with the protein A-immobilized glass plates is also able to be estimated with TOF-SIMS chemical mapping.

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