Protein quantification on dendrimer-activated surfaces by using time-of-flight secondary ion mass spectrometry and principal component regression

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1. Introduction

Many efforts have been made to quantify immobilized proteins since determining the surface density of the proteins is essential to various fields of biosensor and biomaterial engineering [1]. By and large, photometric or fluorescent methods have been used to do this, but these methods are intrinsically limited, as manifested in reagent-induced instability, sample contamination, and unsuitable spectral properties [2]. More accurate methods are needed to determine the surface density of proteins immobilized on bio-interfaces such as polyamidoamine (PAMAM) dendrimers, a new class of polymers with branched amidoamine groups.

Recently, time-of-flight secondary ion mass spectrometry (ToF-SIMS) has been recognized as a useful tool for investigating the surface density of proteins due to its chemical specificity and surface sensitivity [3]. Indeed, it was revealed that ToF-SIMS spectra offered valuable information on the uppermost protein layers (10–15 Å) when combined with other techniques such as radiolabelling, X-ray photoelectron spectroscopy (XPS) or surface plasmon resonance (SPR) [4–6]. ToF-SIMS combined with ellipsometry is of particular interest, but no quantitative study has been done for this combination.

The present work describes a quantitative ToF-SIMS study in which the surface protein density was systematically varied as a function of protein concentration and independently quantified using the ellipsometry technique. Principal component analysis (PCA) and principal component regression (PCR) were used to identify a correlation between the intensities of the secondary ion peaks and the surface protein densities. From the ToF-SIMS and ellipsometry results, a good linear correlation of protein density was found. Our study shows that surface protein densities are higher on dendrimer-activated surfaces than on SAMs surfaces due to the spherical property of the dendrimer, and that these surface protein densities can be easily quantified with high sensitivity in a label-free manner by ToF-SIMS.

2. Experimental

2.1. Sample preparations

Gold substrates were prepared by successively evaporating a 20 Å thick film of Ti and a 400 Å thick film of gold onto a Si wafer. The gold substrates were cut into 10 mm x 10 mm pieces and
2.2. ToF-SIMS and PCA

ToF-SIMS was carried out using a ToF-SIMS V instrument (IONTOF GmbH, Germany) with 25–keV Au$^+$ primary ions (average current of 0.8 pA, pulse width of 16.8 ns, repetition rate of 5 Hz). The analysis area of 500 $\mu$m $\times$ 500 $\mu$m was randomly rastered by primary ions for the spectrum analysis. The primary ion dose was maintained below $10^{12}$ ions/cm$^2$ to ensure static SIMS condition. A bunching system was used to reduce the pulse width, resulting in a mass resolution exceeding $M/\Delta M = 10^4$ (full width half maximum [FWHM]) at $m/z$ 0–300 in the positive mode, and positive ion spectra were internally calibrated by using H$^+$, H$_2^+$, CH$_3^+$, CH$_2^+$, and C$_2$H$_4^+$ peaks. A principal component analysis was preprocessed using a PLS_Toolbox (version 3.5, Eigenvector Research, Manson, WA) for MATLAB (version 7.0 MathWorks, Inc., Natick, MA). Each peak was normalized to the sum of the selected characteristic peaks, to eliminate systematic differences between spectra. The characteristic peaks of streptavidin and biotin for PCA were then performed using a mean-centering method. Afterward, the first principal component (PC1) was used to establish a principal component regression between sample scores and the surface thickness (optical thickness) of streptavidin. To clarify, the quantified sample scores were plotted as a function of surface thickness, and then fitted to calculate determination coefficient ($R^2$) using the linear regression procedure with 95% confidence intervals by using a graphic software of Sigmaplot (version 9.0, SYSTAT Software).

2.3. Ellipsometry

Ellipsometric measurements were used to determine the optical thicknesses of the SAMs and the films of proteins. The optical thickness of each sample was the average of 2–3 measurements performed at different locations on each substrate by using a spectroscopic ellipsometer (VU-302, J.A. Woollam Co., USA) at a wavelength of 633 nm and an angle of incidence of 70°. The gold substrates used for the ellipsometric measurements were 40 nm in thickness (Au) and were deposited without a preferred direction or angle of incidence. The ellipsometric thicknesses of SAMs, PAMAM dendrimers, biotin, and immobilized proteins were estimated using a three-layer model and by assuming a refractive index of 1.46 [8,9]. The thickness of the protein was calculated by subtracting the thickness of the underlying surface. The ellipsometric analyses were performed using commercial software (WWASE32, J.A. Woollam Co., USA). The surface density was derived from the ellipsometric thickness and the refractive index ($n$) through the equation proposed by DeFeijter et al. [10].

$$\Gamma = n_1 - n_2 \frac{dn}{dc}$$

where $\Gamma$ is the surface density of protein per unit area, $d$ is the average ellipsometric thickness of the protein layer, $n_1$ is the average refractive index of the protein layer (1.46), $n_2$ is the refractive index of PBS buffer (1.33), and $dn/dc$ is the variation of the refractive index with protein concentration in solution, which was found to be a constant of 0.18 cm$^2$/g for a variety of proteins.

3. Results and discussion

Streptavidin was immobilized via biotinylation onto two different monolayers to compare their surface densities: a G3-PAMAM dendrimer monolayer and a self-assembled monolayer (Scheme 1). Among numerous peaks from the ToF-SIMS spectra in the range of $m/z$ 0–300, characteristic peaks of streptavidin were commonly observed for both surfaces at $m/z$ 59 (CH$_2$N$_2^+$, Arg), 110 (C$_4$H$_8$N$_2^+$, His), 120 (C$_6$H$_{12}$N$^+$, Phe), 130 (C$_7$H$_7$N$^+$, Trp), 136 (C$_8$H$_{11}$NO$^+$, Tyr), 159 (C$_{10}$H$_{11}$N$_2^+$, Trp), and 170 (C$_{11}$H$_9$NO$^+$, Biotin). Biotin peaks were also observed for both surfaces at $m/z$ 76 (C$_9$H$_8$NS$^+$), 97 (C$_{10}$H$_{14}$N$_2^+$), 114 (C$_{12}$H$_{22}$N$_4^+$), 227 (C$_{10}$H$_{23}$O$_2$S$_2^+$), and 272 (C$_{12}$H$_{16}$O$_2$S$_2^+$) [11]. To determine the quantitative relationship between the SIMS spectra and streptavidin concentration, the scores data for principal component 1 (PC1) from a principal component analysis of the two different surfaces were monitored as a function of the solution concentration of the streptavidin. The characteristic peaks of streptavidin and biotin were used for PCA calculation as loadings data. The PCA results in Fig. 1 show that the PC1 scores on both surfaces increase as streptavidin concentration increases. Here, positive scores mean stronger signal from peaks representing streptavidin (positive loadings in the loadings plot, see Fig. 1b) and weaker signal from peaks representing biotin (negative loadings). In addition, the dendrimer-activated surfaces

![Scheme 1](image-url)
show higher scores on PC1, compared to SAMs. Taken together, these results indicate that PCA scores are directly related to the amount of streptavidin on a single surface and/or on different surfaces. As previously reported, multivariate PCA allowed for a more accurate quantitative ToF-SIMS study than a single peak-based analysis [6,11].

To establish the surface protein densities of the samples as independent variables, the thickness of the streptavidin was converted into protein density of the protein layer on the basis of an empirically determined relation between the refractive index ($\frac{C_2}{2}$) and protein density. We calculated the surface density per unit area (mm$^2$) of streptavidin immobilized on either SAMs or dendrimer monolayers using Eq. (1). Consistent with previous literature [12], the dendrimer monolayer displayed a loading capacity of streptavidin that was higher than the SAMs, resulting in a thicker protein layer. With the assumption of the molecular size of streptavidin to be 45 Å ($\frac{C_2}{2}$) and 50 Å, the surface density of the protein layer ($\frac{C_2}{2}$) was attainable to ~50% of maximum coverage.

We further performed a principal component regression, a general regression method with two variables [13], to find a linear correlation between scores from ToF-SIMS spectra and surface protein density from ellipsometric thickness. As seen in Fig. 2, the scores on PC1 showed a good linear relationship ($R^2 = 0.9508$) with the surface density of streptavidin, indicating that PCA scores from the ToF-SIMS spectra are suitable for a quantification study of surface-immobilized proteins. In particular, PCR revealed that the scores from the G3 dendrimer monolayer produced a higher surface density of streptavidin in the linear regression than scores from SAMs. This shows that three-dimensional structures of dendrimers allow for increased protein immobilization, as documented elsewhere [7,12]. Based on these results, we have shown that PCA of ToF-SIMS data is useful to the study of surface protein density and that a ToF-SIMS-based approach can be easily applied with high sensitivity in a label-free manner to a quantitative study of proteins.

4. Conclusions

We presented a quantitative ToF-SIMS study of immobilized proteins on PAMAM dendrimer monolayers and on SAMs. PCA results from ToF-SIMS spectra of streptavidin showed a linear regression with ellipsometric thickness when systematically studied as a function of streptavidin concentration. From the PCR-based quantitative analysis between ToF-SIMS and ellipsometry, our study shows that a dendrimer-activated surface led to a higher density profile of proteins, as compared to SAMs.

Acknowledgements

This work was supported by the Bio-Signal Analysis Technology Innovation Program (M10645010002-06N4501-00210), the Nano Science & Technology Program (M10503000868-07M0300-86810) and the Nano/Bio Science & Technology Program (M10536090002-07N3609-00210) of MEST/KOSEF, and the Next-Generation New-Technology Development Program for MKE.

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