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

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ABSTRACT

Interaction between streptavidin and biotin on poly(amidoamine) (PAMAM) dendrimer-activated surfaces and on self-assembled monolayers (SAMs) was quantitatively studied by using time-of-flight secondary ion mass spectrometry (ToF-SIMS). 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.

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 density of streptavidin immobilized on a biotinylated G3 PAMAM dendrimer surface is compared to that of streptavidin immobilized on a biotinylated self-assembled monolayer (SAM). Since dendrimer activation is known to increase the number of proteins immobilized on the surface [7], we attempted to observe a quantitative difference between the two surfaces (i.e., dendrimer-activated and SAM surfaces) with the use of ToF-SIMS spectra. The streptavidin thickness was concurrently determined by ellipsometry (i.e., the surface density of streptavidin) for a more accurate quantitative ToF-SIMS analysis. In particular, principal component analysis (PCA) and principal component regression (PCR) were performed on secondary ion peaks of the two sample surfaces to minimize the complexity of the ToF-SIMS data sets and to obtain a correlation between the ToF-SIMS data and ellipsometric thickness, respectively.

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 × 10 mm pieces and

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cleaned for 5 min in a piranha solution (1:4) 30% H_2O_2: concentrated H_2SO_4 (v/v). (*Caution*: the piranha solution reacts violently with most organic materials and must be handled with extreme care.) The cleaned gold substrates were washed sequentially with distilled water and absolute grade ethanol. For the SAM formation, the substrates were immersed for 2 h in a 2 mM ethanol solution of either 11-mercaptoundecanoic acid (MUA, Aldrich) or 11-amino-1-undecanethiol (AUT, Dojindo). For the monolayer of dendrimer, the MUA SAM-forming gold surface was activated by immersing it in an ethanol solution of EDAC (0.1 M) and PF_5 (0.2 M) for 30 min. The PF_5-activated surface was rinsed with ethanol and water, and transferred into a methanol solution of PAMAM G3 dendrimer (18 mM, based on the primary amine concentration), followed by incubation for 2 h at room temperature. The AUT SAM and G3 dendrimer-modified substrates were then biotinylated by immersing them into a solution of sulfo-NHS-LC-Biotin (Pierce, USA) in 0.05 M bicarbonate buffer of pH 8.5 for 30 min. To hydrolyze the remaining reactive esters, the substrate was immediately subject to a solution of sodium bicarbonate buffer of pH 9.5 for 20 min, followed by washing with water. For quantitative analysis, the biotinylated substrates were immersed in varying concentrations of streptavidin (Sigma) (0.5, 1, 5, 10, and 20 μg/mL) in a 10 mM phosphate buffer saline (pH 7.4) for 40 min, followed by a thorough washing in PBS and water, dried under a stream of nitrogen, and used immediately thereafter.

2.2. ToF-SIMS and PCA

ToF-SIMS was carried out using a ToF-SIMS V instrument (ION-TOF GmbH, Germany) with 25-keV Au^+ primary ions (average current of 0.8 pA, pulse width of 16.8 ns, repetition rate of 5 kHz). The analysis area of 500 μm × 500 μm 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^+, $C_2H_3^+$, and $C_3H_4^+$ 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

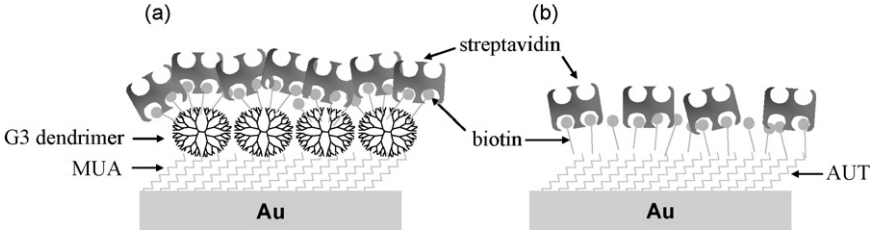
Ellipsometric measurements were used to determine the optical thicknesses of the SAMs and the films of proteins. The optical thickness of each sample was the average of 2–3 measurements performed at different locations on each substrate by using a spectroscopic ellipsometer (VU-302, J.A. Woollam Co., 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 by 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 (WVASE32, 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],

$$\Gamma = d\frac{n_1 - n_2}{\mathrm{d}n/\mathrm{d}c} \quad (1)$$

where Γ 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^3/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 ($CH_5N_3^+$, Arg), 110 ($C_5H_8N_3^+$, His), 120 ($C_8H_{10}N^+$, Phe), 130 ($C_9H_8N^+$, Trp), 136 ($C_8H_{10}NO^+$, Tyr), 159 ($C_{10}H_{11}N_2^+$, Trp), and 170 ($C_{11}H_8NO^+$, Trp). Biotin peaks were also observed for both surfaces at m/z 76 ($C_2H_6NS^+$), 97 ($C_4H_7N_3^+$), 114 ($C_5H_{12}N_3^+$), 227 ($C_{10}H_{15}O_2N_2S^+$), and 272 ($C_{12}H_{22}O_2N_3S^+$) [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. Schematic of streptavidin immobilized on a (a) G3 PAMAM dendrimer monolayer and (b) self-assembled monolayer of 11-amino-1-undecanethiol (AUT) onto gold.

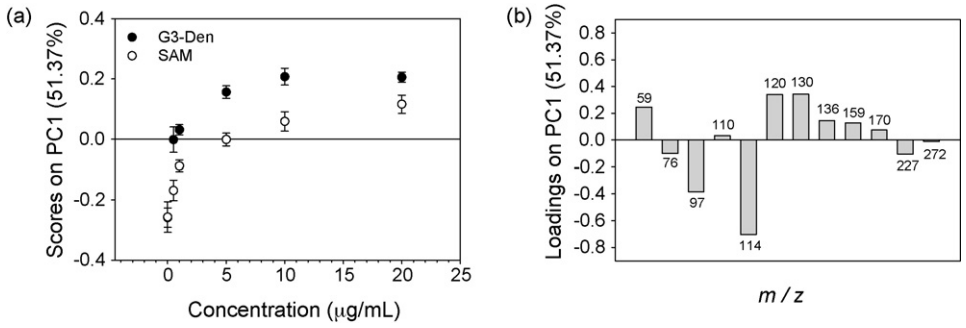


Fig. 1. (a) Scores plot and (b) corresponding loadings plot of PC1 from PCA of positive ion spectra of streptavidin. Quantitative scores for streptavidin immobilized on biotinylated G3-dendrimer (black circles) and on biotinylated SAMs (white circles) are represented as a function of streptavidin concentration. The loadings plots are labelled *m*/*z* value with characteristic peaks of biotin and streptavidin.

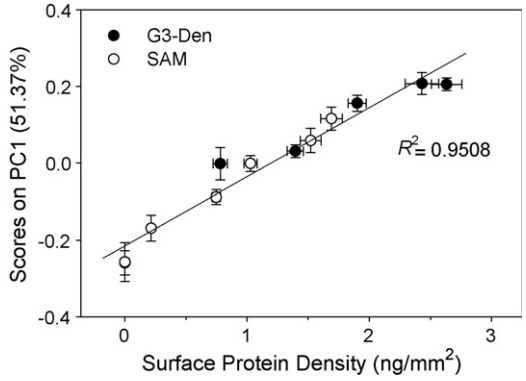


Fig. 2. Quantitative principal component regression (PCR) between scores of PC1 from ToF-SIMS spectra of streptavidin and surface protein density from ellipsometric thickness.

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, a 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 (~1.46) 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 × 45 Å × 50 Å, the surface density of the protein layer (~2.6 ng/mm^2) on the dendrimer monolayer 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.

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