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Probing orientation of immobilized humanized anti-lysozyme variable fragment by time-of-flight secondary-ion mass spectrometry

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Abstract

As methods to orient proteins are conceived, techniques must also be developed that provide an accurate characterization of immobilized protein orientation. In this study, x-ray photoelectron spectroscopy (XPS), surface plasmon resonance, and time-of-flight secondary ion mass spectrometry (ToF-SIMS) were used to probe the orientation of a surface immobilized variant of the humanized anti-lysozyme variable fragment (HuLys Fv, 26kDa). This protein contained both a his-tag and a cysteine residue, introduced at opposite ends of the HuLys Fv, for immobilization onto nitrilotriacetic acid (NTA) and maleimide oligo(ethylene glycol) (MEG) terminated substrates, respectively. The thiol group on the cysteine residue selectively binds to the MEG groups, while the his-tag selectively binds to the Ni loaded NTA groups. XPS was used to monitor protein coverage on both surfaces by following the change in the nitrogen atomic %. SPR results showed a 10-fold difference in lysozyme binding between the two different HuLys Fv orientations. The ToF-SIMS data provided a clear differentiation between the two samples due to the intensity differences of secondary ions originating from asymmetrically located amino acids in HuLys Fv (Histidine: 81, 82, and 110 m/z; Phenylalanine: 120 and 131 m/z). An intensity ratio of the secondary-ion peaks from the histidine and phenylalanine residues at either end of the protein was then calculated directly from the ToF-SIMS data. The 45% change in this ratio, observed between the NTA and MEG substrates with similar HuLys Fv surface coverages, indicates the HuLys Fv fragment has opposite orientations on the two different surfaces.

Keywords

surface analysis; ToF-SIMS; protein orientation; humanized anti-lysozyme variable fragment

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Introduction

Despite recent interest in the production of biomolecule-functionalized sensors and diagnostic microarrays, the widespread deployment and use of these devices has been hampered by the suboptimal binding at the interface.^{1–3} Ideally, surfaces that are based on protocols that produce reproducible, multiplexed substrates will also optimize the affinity of specific biomarkers or targets (protein, DNA oligomer, etc).^{4–6} In the case of antibody based diagnostics⁷ and microarrays sensors^{3,8} the analyte capture performance is a function of the stability and the orientation of the immobilized antibody. A wide variety of immobilization protocols have been proposed to control the orientation and activity of immobilized species, including the attachment via bioactive ligands,^{2,9–19} charge-driven orientation,^{20–22} and physical adsorption through hydrophobic interactions.^{23–25} However, there is a paucity of experimental techniques required to provide a detailed and accurate determination of protein conformation and orientation at surfaces.

One technique that has shown great promise for providing this surface conformational information is time-of-flight secondary ion mass spectrometry (ToF-SIMS). ToF-SIMS offers both extreme surface sensitivity (sampling depths typically 1–3 nm) and high chemical specificity (mass resolution of $\sim 5000\ m/\ m$ and higher) by sputtering molecular fragments from the surface with a pulsed primary ion beam.^{26,27} The small fraction of these fragments that are ionized (<1%) – positive and negative secondary-ions – are extracted through a time-of-flight mass analyzer providing a complete, and complex, mass analysis of all ions ejected from the surface.^{27,28} Despite the complexities of the resulting ToF-SIMS data, researchers have taken advantage of the shallow sampling depth provided by ToF-SIMS to elaborate on the stability and orientation of surface bound proteins.^{20,29–34} Conformational transformations were tracked by changes in intensities of secondary-ions from hydrophilic and hydrophobic amino acid side chains. Likewise, intensities of secondary-ions stemming from asymmetrically distributed amino acids provided information about orientation.^{20,31,35,36}

While most of the previous work was focused on the characterization of protein films significantly thicker than the ToF-SIMS sampling depth (e.g. 10nm versus the 1–3nm sampling depth),^{20,31–34} two recent studies tested the limits of the technique by examining a model system with a thinner protein film (the B1 domain of Protein G, 6kDa).^{37,38} Although the thickness of the resulting protein layer, 3nm, was similar to the ToF-SIMS sampling depth, these studies were able to determine differences in orientation by examining ratios of intensities of secondary ions originating from amino acid residues at opposite ends of the protein (C-terminus versus N-terminus).^{37,38}

To increase the capture performance of immobilized antibodies, researchers need to control the orientation of the analyte capture domain, which for antibodies is typically the variable domain (Fv). The work presented in this study will build upon the Protein G B1 studies and define the orientation of a surface immobilized variant of humanized anti-lysozyme variable fragment (HuLys Fv, 26kDa) by comparing the intensities of specific positive secondary ions. HuLys Fv is the smallest fragment that still retains the specific binding of the whole

antibody (lysozyme K_d binding affinity is 3nM) and consists of a two-chain heterodimer of IgG variable light (VL) and heavy (VH) domains.^{39–42}

The HuLys Fv fragment can be induced into two distinct orientations via the controlled attachment onto gold substrates covered with two functionalized alkanethiol self-assembled monolayers (SAMs). The HuLys Fv fragment used in this study has six histidine residues at the C-terminus and a cysteine residue at the opposite end (Figure 1)^{15,39} which will allow it to bind to both nitrilotriacetic acid-terminated (NTA) and maleimide-oligo(ethylene glycol) (MEG) monolayers. The NTA surface is activated by forming an NTA-metal complex that then specifically binds to the imidazole rings of a series of six histidines (his-tag).¹⁵ This induces a HuLys Fv orientation where the lysozyme-binding domain (LBD) faces away from the substrate (LBD-up). Previously, the MEG monolayers have been used to immobilize both cysteine containing proteins and thiolated DNA via covalent attachment of the sulfur to the maleimide ring.^{37,38,43} This induces an HuLys Fv orientation where the binding domain faces down towards the substrate (LBD-down). The amount of protein bound to each of these surfaces can be examined by x-ray photoelectron spectroscopy (XPS) and differences in the lysozyme binding performance to HuLys Fv as a function of the two different orientations can be tracked by surface plasmon resonance (SPR). Structural differences between the LBD-up and LBD-down orientations are then characterized by tracking intensities of secondary-ions stemming from both the his-tag opposite the LBD and the phenylalanine rich region near the LBD (Figure 1).

Experimental

Preparation of Substrates for XPS and ToF-SIMS Experiments

1×1 cm silicon substrates (Microelectronics Inc., San Jose, CA) were cleaned by sequential sonication in deionized water, dichloromethylene, acetone, and methanol. In a high vacuum electron beam evaporator (pressure < 1×10^{-6} Torr) the cleaned substrates were first coated with a thin adhesion layer of titanium (10 nm) followed by a thicker overlayer of high purity gold (99.99%, 80 nm; Washington Technology Center, Seattle, WA). Maleimide-oligo(ethylene glycol) (MEG) substrates were prepared by submerging the gold-coated substrates into a 0.1 mM maleimide-ethylene glycol disulfide (Prochimia, Sopot, Poland) ethanol solution for 1 h and then rinsing with 200 proof ethanol (Decon Labs, King of Prussia, PA). Nitrilotriacetic acid-terminated monolayers (NTA) were prepared by immersing gold-coated substrates into a 0.1mM nitrilotriacetic acid-terminated tetra(ethylene glycol)undecanethiol (Prochimia, Sopot, Poland) water solution for 1 h. Following assembly the NTA samples were rinsed with ultra-pure water and activated by resubmerging them into a 50mM NiSO_4 /water solution for 5 min. Excess NiSO_4 was removed by rinsing with ultra-pure water. Complete characterization of the packing density, composition, and molecular orientation for MEG and NTA surfaces has been published elsewhere.^{10,15,44,45}

His-tagged Protein

The gene encoding HuLys Fv (25,574 Da) was generously provided by the Jefferson Foote Lab (Fred Hutchinson Cancer Research Center, Seattle, WA) and was presented as an

insertion in the vector plasmid pAK19. Permission for use was obtained from Genentech, Inc. (South San Francisco, CA). Complete details of the plasmid structure and the His-tagged HuLys Fv expression procedure have been published elsewhere.^{15,39} From the His-tagged modified gene the codon for glutamine at position one (N-terminus) in the V_H was replaced by a codon for cysteine (Q(H1)C). This single mutation was then confirmed by DNA sequencing.³⁹

For HuLys Fv immobilization, the MEG and NTA substrates were submerged in a phosphate buffered saline protein solution (pH 7.4) at HuLys Fv concentrations of 50, 100, and 200nM to produce surfaces with varying degrees of HuLys Fv coverage. The immobilization reaction proceeded for 3 h at room temperature, after which the samples were washed first by serial dilution with PBS and then by a series of ultra-pure water baths. Samples were finally blown dry with nitrogen and stored under nitrogen.

XPS

Protein coverage was monitored by changes in the XPS determined atomic % nitrogen. XPS data was acquired on a Kratos AXIS Ultra DLD instrument (Kratos Analytical, Manchester, England) in the hybrid mode using a 0° photoelectron take-off angle (defined as the angle between the surface normal and the axis of the analyzer lens) and a monochromatic Al K $\alpha_{1,2}$ X-ray source (h ν =1486.6 eV). Atomic compositions were calculated from peak areas obtained from 0–1100 eV survey scans (C_{1s}, Au_{4f}) and selected region scans (524–544 eV for O_{1s}; 390–410 eV for N_{1s}; 155–173 eV for S_{2p}) acquired at an analyzer pass energy of 80 eV.

ToF-SIMS

Positive and negative secondary-ion spectra were collected on a TOF.SIMS 5–100 (ION-TOF, Münster, Germany) system with a pulsed 25 keV Bi₃⁺ ion source under static conditions (primary ion dose < 10¹¹ ions/cm²). For each sample, five positive and three negative spectra were collected from 100 μ m $\times$ 100 μ m regions at mass resolutions (m/m) between 4000 and 8000. Positive ion spectra were mass calibrated using CH₃⁺, C₂H₃⁺, C₃H₅⁺, and AuSCH₂⁺ while the negative spectra were mass calibrated using CH[−], C₂H[−], and AuS[−]. In both cases the differences between the experimentally measured m/z values and the expected exact mass m/z values were typically below 20 ppm. To make comparisons across spectra, each spectrum was normalized to the total ion intensity.

Preparation of Substrates for SPR Experiments

Titanium (2 nm) adhesion and gold overlayer (45 nm) films were deposited onto cleaned SF-10 glass (18 mm $\times$ 18 mm, SCHOTT Glass Technology, Inc.) by electron beam evaporation at pressures < 1 $\times$ 10^{−6} torr. Spotting masks were created by punching holes into Culture Well silicone sheets (Grace Bio-Labs, Bend, OR). The masks were then placed onto the Au-SPR chips and 1.5 μ L aqueous solutions of NTA-thiol (0.1 mM), MEG disulfide (0.1mM), and a pure OEG-thiol (hydroxyl-terminated tetra-(ethylene glycol) undecanethiol (0.1mM) were dispensed into a pattern of spots. These spotted arrays were intermediately incubated in a 75% relative humidity chamber for 16 h. Following incubation, the arrays were sequentially rinsed with ultra-pure water, 70% ethanol, and ultra-pure water. NTA

spots were activated by a NiSO_4 solution (1.5 μL , 50 mM) for 5 min and again rinsed with ultra-pure water. Both the MEG and the activated NTA spots were incubated in HuLys Fv solutions (200 nM and 50 nM, respectively) for 2 h in a 75% relative humidity chamber.

SPR

SPR images were captured on a SPRImagerII (GWC Technologies, Madison, WI) operated at room temperature using a standard flow cell and a peristaltic pump (BioRad-EconoPump) displacing fluid at 100 $\mu\text{L}/\text{sec}$. The excitation source for the SPRImagerII was *p*-polarized white light and the reflected light was focused onto a CCD detector by an 800 nm filter. All surfaces were equilibrated in PBS prior to the exposure to the lysozyme (500nM) solution. Data acquisition captured an average of 30 images/frames at each specific point and SPR signal converted to a normalized percentage change in reflectivity according to standard GWC protocols. Quantification of lysozyme binding has been described in more detail elsewhere.^{46,47} Briefly, an absolute adsorbate coverage (ng/cm^2 of lysozyme) was calculated by the following two equations.

(1)

(2)

Where d is an effective adlayer thickness, ρ_{protein} is the lysozyme density (1.43 g/cm^3),⁴⁸ l_d is the characteristic decay length, $\%R$ is the normalized percent change in reflectivity which was converted the measured SPR response using a neutral density filter, and s is the sensitivity factor. η_a and η_s are the refractive indexes of the adsorbed protein layer and buffer solution above it, respectively.

Results

In this study a small piece of antibody, HuLys Fv (3nm in height), was immobilized onto two different functionalized alkanethiol SAMs on Au. The HuLys Fv fragment is not as stable as the rigid Protein G fragment model used previously,^{37,38} since the HuLys Fv fragment consists of heavy and light chain domains that are not covalently attached.^{40–42} Other ToF-SIMS studies have examined this by tracking conformational changes of the HuLys Fv- NTA complex under vacuum conditions.⁴⁹ In the unhydrated, vacuum state there was an enrichment of secondary-ions from hydrophobic amino acid side chains. The goal of this investigation was to orient this antibody fragment in two different states, LBD-up and LBD-down, and differentiate between these two orientations with ToF-SIMS, despite the small size and instability of HuLys Fv.

ToF-SIMS data are extremely complex as each spectrum can contain thousands of mass peaks.²⁷ However, it is important to realize that different materials have distinct spectral fingerprints.²⁶ Specifically of interest for the current study, characteristic amino acid peaks are commonly used to characterize protein films.^{50–53} In this work, the complexity of the

results was reduced by the creation of a secondary-ion intensity ratio. Within the HuLys Fv fragment, there is an asymmetric distribution of phenylalanine (Phe) that is centered near the LBD (Figure 1). On the opposite side are the six histidines that make up the his-tag. A ratio based on the sum of intensities of specific secondary-ions stemming from the His and Phe (Table 1) should yield two contrasting values that correspond to the two different orientations.

The amount of protein on the surface was tracked by the nitrogen atomic % determined from the XPS N_{1s} signal. Both the MEG and NTA substrates contain nitrogen, therefore to determine the contribution of the protein layer to the N_{1s} signal, we have to subtract out the contribution from the underlying NTA or MEG layer. By examining the attenuation of the Au_{4f} signal from the Au substrate by the SAM and HuLys Fv overlayers, the nitrogen contribution from just the protein layer can be calculated using the following equation.³²

$$(3)$$

N_s and Au_s are the measured nitrogen and gold atomic %, respectively, from the MEG or NTA surfaces before HuLys Fv immobilization. N_p and Au_p are the measured nitrogen and gold atomic %, respectively, from the MEG or NTA surfaces after HuLys Fv immobilization. N_{norm} is the nitrogen atomic % corresponding to just the protein layer.

As expected, the XPS results (Figure 2) illustrate that HuLys Fv immobilized from three different solution concentrations (50, 100, and 200nM) produce surfaces with varying degrees of HuLys Fv coverage. From Figure 2, it appears that the amount surface bound HuLys Fv varies widely between the different substrates. For the MEG substrates, only at the higher adsorption concentrations (100 and 200nM) are significant amounts of nitrogen from HuLys Fv detected on the surface by XPS. This observation is also consistent with the ToF-SIMS results discussed in the following paragraph.

While the positive secondary ion spectra contain most of the characteristic amino acid peaks,⁵² the negative spectra can provide direct evidence of the MEG conjugating with the cysteine within the HuLys Fv fragment.^{37,38,43} The ratio of secondary-ions originating from reacted ($C_4H_2NO_2S^-$, $m/z = 127.98$) to unreacted maleimide rings ($C_4H_2NO_2^-$, $m/z = 96.01$) does not change until we see a sudden jump for immobilization at the highest solution concentration (Figure 3).

XPS results clearly demonstrate that the HuLys Fv immobilization rates onto the MEG and NTA substrates are different. Additionally, the nitrogen concentrations do not plateau, indicating sub monolayer HuLys Fv coverages are present on all samples. Thus, to minimize concentration based matrix effects, only secondary-ion ratios between samples with similar amounts of protein are compared.³³ In this case, the 50nM HuLys Fv-NTA sample and the 200nM HuLys Fv-MEG sample exhibit similar amounts of nitrogen, 1.6 ± 1.2 atomic % and 1.7 ± 1.1 atomic %, respectively.

The ToF-SIMS intensity ratios were calculated as the sum of intensities from the his-tag fragments (81, 82, and 110 m/z) divided by the sum of the Phe fragments (120 and 131 m/z). In the LBD-up orientation the Phe rich binding domain region is expected to be near the vacuum interface, resulting in an intensity enhancement of Phe secondary ions. In the LBD-down orientation, the his-tag is expected to be near the vacuum interface, resulting in an intensity enhancement of His secondary ions. Figure 4 shows the calculated His to Phe intensity ratios for the two samples with similar protein coverage, 200nM HuLys Fv-MEG and 50nM HuLys Fv-NTA. This ToF-SIMS intensity ratio is 45% percent higher on the 200nM HuLys Fv-MEG sample compared to the 50nM HuLys Fv-NTA sample, indicating large changes in the intensity of His and Phe secondary-ions (t-test $p < 0.0003$). These differences are consistent with expected difference in HuLys Fv orientations, LBD-down and LBD-up.

Based on the ToF-SIMS results that indicate the two different substrates successfully induced two distinct net HuLys Fv orientations, LBD-up and LBD-down, there should be a difference in the lysozyme binding performance between these two surfaces. To examine this analyte binding we used SPR to monitor the change in surface reflectivity as a lysozyme solution was flown over a substrate with four different surface chemistries: bare Au and OEG, MEG and NTA exposed to HuLys Fv solutions. The resulting SPR response curves are shown in Figure 5. A lysozyme-buffer solution was introduced at 0s and binding was allowed to proceed for 10 min. After which time the surface was purged with buffer washing away any reversibly bound lysozyme. The lysozyme coverage plotted in Figure 5 varies with the type of surface chemistry. As expected the OEG sample showed very little change in SPR response. This can be attributed to the non-fouling capabilities of the OEG monolayer, which prevented significant amounts of HuLys Fv and lysozyme from adsorbing.¹⁵ However, there was a 10-fold increase in the amount of bound lysozyme between the HuLys Fv-MEG (11.2 ng/cm²) and HuLys Fv-NTA (112.0 ng/cm²) samples. The molar ratio of adsorbed lysozyme to HuLys Fv was determined to be ~1.0 on the NTA surface, while on the MEG surface, the molar ratio of adsorbed lysozyme to HuLys Fv was ~0.1.^{15,54} This is consistent with the HuLys Fv-MEG having a LBD-down orientation and thereby making the binding site less accessible to the lysozyme. One surprising result was that, while small, the amount of irreversibly bound lysozyme on the HuLys Fv-MEG complex was nonzero. One possible explanation is that the cysteine residues present in lysozyme could be interacting with free maleimide groups on the surface. If a closed packed monolayer of HuLys Fv could be formed on the MEG surface, this should block any free maleimide groups and further decrease the non-specific lysozyme binding. Also, submonolayer coverages of HuLys Fv could result in a range of HuLys Fv orientations (i.e., each HuLys Fv molecule has sufficient space available on the MEG surface to provide a distribution of orientation angles around the nominal LBD-down orientation angle). This distribution of orientation angles could allow some access to the LBD on the HuLys Fv MEG sample.

Conclusions

The results from complementary XPS, SPR, and ToF-SIMS experiments are consistent with a HuLys Fv fragment containing a his-tag and a cysteine residue at opposite ends of the

molecule becoming immobilized in opposite orientations on NTA and MEG terminated substrates. XPS was used to monitor HuLys Fv coverage concentrations and identify immobilization conditions that produced similar amounts of HuLys Fv on the NTA and MEG surfaces. SPR showed HuLys Fv immobilized onto the NTA surface in a LBD-up orientation bound 10 times more lysozyme than HuLys Fv immobilized on the MEG surface in a LBD-down orientation. The His to Phe ToF-SIMS fragment intensities ratio was 45% higher for the HuLys Fv MEG sample compared to the HuLys Fv NTA sample. The results from this study show that ToF-SIMS can be used to determine the difference in immobilized protein orientation, even from proteins such as HuLys Fv which are similar in size to the ToF-SIMS sampling depth and possess limited stability when dried.

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Figure 1.

HuLys Fv variant with a cysteine residue inserted next to the lysozyme binding domain (LBD) and a hexahistidine tag (his-tag) introduced on the opposite side. A) The expected LBD-down orientation induced by the sulfhydryl group of the cysteine conjugating with the MEG surface. B) The expected LBD-up orientation induced by the his-tag complexing with the nickel loaded NTA surface. The locations of the histidine and phenylalanine residues are highlighted in red.

Figure 2.

Normalized nitrogen concentrations determined by XPS analysis for HuLys Fv on MEG (-o-) and HuLys Fv on NTA (-●-). Data points represent average N concentration from six analysis spots on two different samples. Error bars represent standard deviations.

Figure 3.

Direct evidence for coupling of the substrate's maleimide groups with the cysteine thiol group in HuLys Fv was obtained by comparing the average intensity of peaks from reacted and unreacted maleimide rings (reacted: $C_4H_2NO_2S^-$, $m/z = 127.98$; unreacted: $C_4H_2NO_2^-$, $m/z = 96.01$). Error bars represent the standard deviation across three analysis spots.

Figure 4.

ToF-SIMS peak intensity ratios. Peak ratios were calculated as the sum of intensities of histidine secondary ions (81, 82, and 110 m/z) divided by the sum of intensities of phenylalanine secondary ions (120 and 131 m/z). Error bars represent the standard deviation across five analysis spots (★-p value < 0.0003).

Figure 5.

SPR response for specific lysozyme binding onto bare Au (bare Au), and Huls Fv exposed OEG, NTA and MEG surfaces. The lysozyme /buffer solution was introduced at time 0 and pure buffer was reintroduced ten minutes later. The %R used to determine protein coverage was the average response over a 2mm × 2mm spot.

Table 1

Table of specific secondary ions originating from histidine and phenylalanine residues.

Source	Formula	Mass
Histidine	$C_4H_5N_2^+$	81.0395
	$C_4H_6N_2^+$	82.0561
	$C_5H_8N_3^+$	110.0787
Phenylalanine	$C_8H_{10}N^+$	120.0858
	$C_9H_7O^+$	131.0561