Measuring the Orientation of Chemically and Electrostatically Immobilized Protein G B1 by Time-of-Flight Secondary Ion Mass Spectroscopy and Sum Frequency Generation

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Introduction. The ability to orient proteins on surfaces to control exposure of their biologically active sites will benefit a wide range of applications including protein microarrays, antibody-based diagnostics, affinity chromatography, and biomaterials that present ligands to bind cell receptors. As methods to orient proteins are developed, techniques are required to provide an accurate picture of the orientation of these proteins. Since no single technique provides a high-resolution image of surface-bound proteins, combinations of surface analytical and spectroscopic techniques are required. In this study, we have developed two model systems based on the chemical and electrostatic immobilization of a small rigid protein (Protein G B1 domain, 6kDa) to further develop the capabilities of time-of-flight secondary ion mass spectrometry (ToF-SIMS) and sum frequency generation (SFG) spectroscopy as tools to probe the orientation of surface immobilized proteins.

Methods. Two Protein G B1 variants (T11C and V21C) with a cysteine introduced at either end of the protein were immobilized onto both maleimide-oligo(ethylene glycol)-functionalized (MEG) and bare gold substrates via the cysteine thiol, thus inducing opposite end-on orientation. Two different charged mutants (D4 and D4') exhibiting net positive and negative charges at either end (for pH 6-8) were produced by neutralizing four negatively charged residues closest to the end of the protein (Asp to Asn or Glu to Gln mutations) and immobilized onto amine (NH_3^+) and carboxyl (COO^-) terminated self assembled monolayers (SAMs). Again, inducing opposite end-on orientation. All SAMs were assembled onto Au via a thiol end group and the quality of these layers were determined by x-ray photoelectron spectroscopy. ToF-SIMS is well suited for characterizing the composition and structure of protein films due to its high chemical specificity and surface sensitivity. ToF-SIMS provides high mass resolution ($m/\Delta m$ ~ 5000) and high sensitivity (10^7-10^{11} atoms/cm^2), with a sampling depth of ~2nm that only samples the top portion of the protein (height of the protein ~3nm). Thus, yielding a collection of secondary ions from amino acids from the upper portion of the protein (Figure 1). The vibrational SFG spectra provided complimentary information about the orientation of the secondary structures within the proteins.

Results: ToF-SIMS data from the T11C and V21C variants showed an enrichment of secondary ions originating from asymmetric amino acids (Asparagine: 70, 87, and 98 m/z; Leucine: 86 m/z; Phenylalanine: 120 m/z; Tryptophan: 130 m/z; Methionine: 61 m/z; Tyrosine: 107 and 136 m/z) concentrated in the opposite end of the protein from the cysteine, indicating end-on orientations for the two variants on both substrates. These spectral intensities were also compared with 50:50 mixtures of the variants and with nonspecifically immobilized proteins in random orientations. On the MEG substrates, orientation was enhanced by increasing both the pH (7.0 to 9.5) and salt concentration (0 to 1.5 M NaCl) of the protein-buffer solution (Figure 2). Comparing the ToF-SIMS results for the D4 and D4' variants on both NH_3^+ and COO^- SAMs, we again saw a difference between the intensities of secondary ions from amino acids at opposite ends of the protein. Thus, indicating that the two different charged substrates induced opposite end-on orientations. In addition, SFG spectral peaks characteristic of ordered alpha-helix (1645 cm^{-1}) and beta-sheet (1624 and 1675 cm^{-1}) elements were observed for all variants, with a phase that indicated a predominantly upright orientation for the alpha-helix, consistent with an end-on protein orientation.

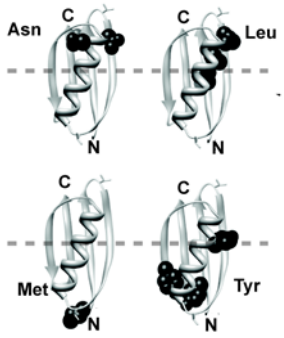

Figure 1. Map of asymmetric amino acids within the T11C & V21C variants. Leu (Ile) and Asn are near the C-terminus (C) while, Met and Tyr are near the N-terminus (N). The dotted line represents the predicted SIMS sampling depth.

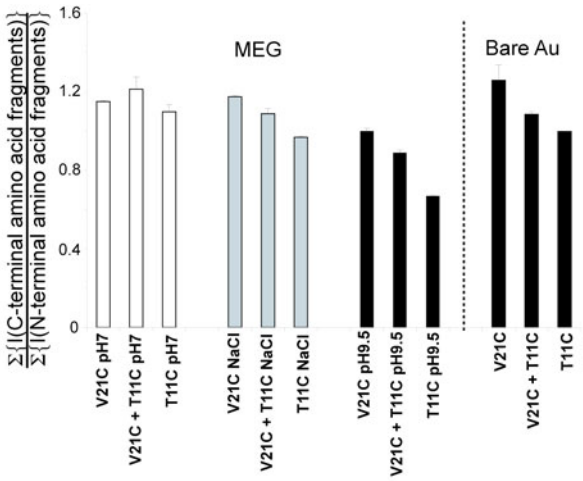

Figure 2. Peak intensity ratios were calculated as the sum of secondary ion intensities of C-terminal amino acids, divided by the sum of secondary ion intensities of N-terminal amino acids.

Conclusions: By tracking the position of asymmetric amino acids and the orientation of the secondary structures (found within the Protein G B1 variants), we have shown that ToF-SIMS and SFG can be used to probe end-on orientations of small surface immobilized proteins.