Measuring the Orientation of Electrostatically Immobilized Horse Heart Cytochrome C 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, horse heart cytochrome c was electrostatically immobilized onto positively and negatively charged model substrates to induce opposite end-on orientations. Previously, cytochrome c has played a prominent role in protein electrochemistry. The protein is immobilized onto a modified metal electrode and electrons pass through the heme group at one end of the protein. It has also been hypothesized that switching the orientation of this heme group will disrupt the electron pathway. Despite widespread interest, a complete description of structure at the electrode surface remains elusive, therefore, in this study we will probe the structure of these surface bound proteins with two complementary surface analytical techniques, time-of-flight secondary ion mass spectrometry (ToF-SIMS) and sum frequency generation (SFG) spectroscopy.

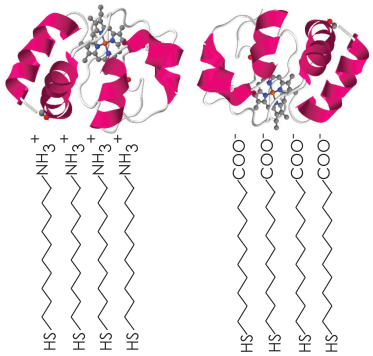

Figure 1. Schematic diagram of cytochrome c immobilized via electrostatic interactions onto positively and negatively charged substrates.

Methods. Horse heart cytochrome c was immobilized onto amine (NH_3^+) and carboxyl (COO^-) terminated self assembled monolayers (SAMs), potentially 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.

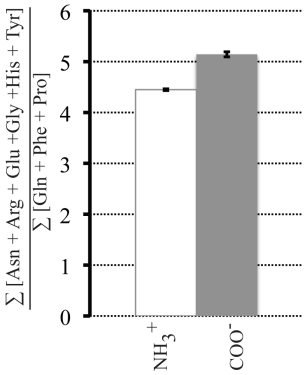

Figure 2. Peak ratios were calculated as the sum of secondary ion intensities from one end of the protein divided by secondary ions originating from the opposite end of the protein.

Results: ToF-SIMS data from a film of cytochrome c immobilized on both NH_3^+ and COO^- SAMs showed intensity differences of secondary ions originating from amino acids concentrated at opposite ends of the protein (protein surface in contact with the COO- surface: Alanine: 143 m/z, Glutamine: 84, 85 and 101 m/z, Phenylalanine: 131 m/z, Proline: 165 m/z, protein surface in contact with the NH_3^+ surface: Asparagine: 70, 74, 87, 88, 98, and 115 m/z, Arganine: 43, 59, and 73 m/z, Glutamic acid: 53 and 71 m/z, Glycine: 87 and 115 m/z, Histidine: 82, 110 and 121 m/z, Tyrosine: 136, 137 and 147 m/z). For a more quantitative examination of orientation, we developed a ratio comparing the sum of intensities of secondary ions stemming from residues at either end of the protein. A statistically significant increase in the value of this ratio, 7.5±0.2 %, was observed between the COO^- and NH_3^+ SAMs, indicating two distinct cytochrome c orientations (Figure 2). In addition, the degree of ordering of secondary structures, within the protein, was assessed by the observed SFG spectral peaks characteristic of ordered alpha-helix (1645 cm^{-1}) and beta-sheet (1624 and 1675 cm^{-1}) elements.

Conclusions: By tracking the position of asymmetric amino acids and the orientation of the secondary structures (found within cytochrome c), we have shown that ToF-SIMS and SFG can discern changes in orientation of small surface immobilized proteins.