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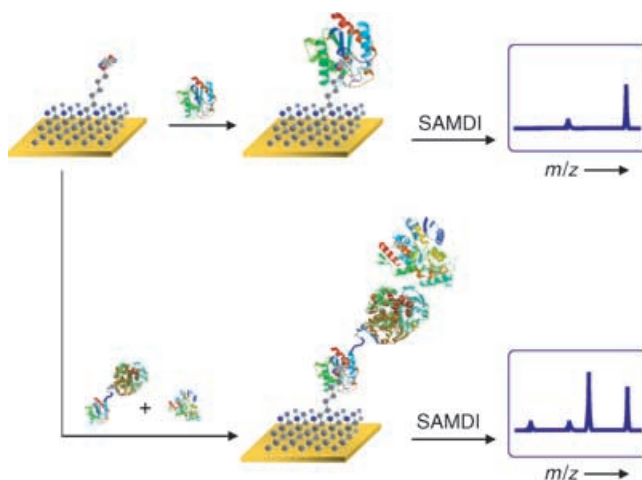
Label-Free Detection of Protein–Protein Interactions on Biochips**

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The recent introduction of protein microarrays is now providing tools for the global profiling of biochemical activities.^[1] Snyder and co-workers, for example, used yeast protein chips to identify kinase substrates and lipid-binding proteins.^[2] Labaer and co-workers reported self-assembling protein microarrays to map pairwise interactions among 29 human DNA replication proteins.^[3] Any application of a protein chip must involve a suitable labeling strategy that will permit the observation of activities. Common strategies include the use of radioisotopes to follow phosphorylation reactions^[4] and fluorescent tags to identify protein–protein interactions.^[5] The use of labels has limitations, including the need for additional steps in an assay, the difficulty in detecting certain biochemical activities, and the inability to identify unanticipated activities. These limitations have motivated the development of label-free formats for identifying the full range of biochemical activities on protein chips. Herein, we demonstrate that MALDI-TOF MS can be used to identify protein–ligand and protein–protein interactions on biochips that are prepared from self-assembled monolayers (SAMs) on gold. This work is significant because it extends the capacity of the SAMDI (self-assembled monolayers for MALDI) method from observation of low-molecular-weight species to proteins that are 50 kDa in size.

Our approach begins with a SAM of alkanethiolates on gold that presents maleimide groups amongst a background of tri(ethylene glycol) groups in a ratio of 1:20. The maleimide groups serve as a handle to immobilize thiol-derivatized small

molecules,^[6] and the tri(ethylene glycol) group ensures that the monolayers are inert to nonspecific protein adsorption.^[7] Two model systems were used in the examples that follow: the binding of carbonic anhydrase (CA) to a benzenesulfonamide ligand and the binding of glutathione S-transferase (GST)-tagged protein complexes to a glutathione ligand (Scheme 1).



Scheme 1. Strategy for the label-free detection of protein–ligand (top) and protein–protein interactions (bottom) using MALDI-TOF MS on SAMs presenting capture ligands.

To immobilize the small molecules, monolayers were treated with either glutathione thiol (1 mM in tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 7.5) or benzenesulfonamide thiol (2.5 mM in phosphate-buffered saline (PBS) containing 10% methanol, pH 7.4) (Figure 1a).^[8] MS analysis of these monolayers showed peaks corresponding to the immobilized ligands and confirmed that the reactions proceeded in high yield (Figure 1b).^[9]

Herein, we report several examples that establish the use of SAMDI for the direct observation of proteins bound to surface-immobilized ligands. A monolayer presenting glutathione groups was treated with GST (10 μM in Tris buffer containing 1 mM dithiothreitol (DTT), pH 7.5) for 1 hr, rinsed with distilled water, and dried under a stream of nitrogen. MS analysis of this monolayer showed two major peaks corresponding to the singly and doubly charged protein at m/z 28.0 kDa ($[M+H]^+$) and m/z 14.0 kDa ($[M+2H]^{2+}$), respectively (Figure 2a). A control experiment showed that the addition of soluble glutathione (1 mM in Tris buffer, pH 7.5) to the solution containing GST prevented binding of the protein to the monolayer. Indeed, MS data from the monolayer showed no major peaks, verifying the effectiveness of the monolayer in preventing nonspecific interactions (Figure 2b). Furthermore, no protein peaks were observed when a maleimide-presenting monolayer was treated with a cysteine-containing hexapeptide (CGRGDS) followed by incubation with GST (data not shown).

This experiment was repeated with several fusion proteins. A fusion between GST and a focal adhesion targeting domain (GST–FAT) bound to the monolayer, as evidenced by two major peaks corresponding to singly and doubly charged

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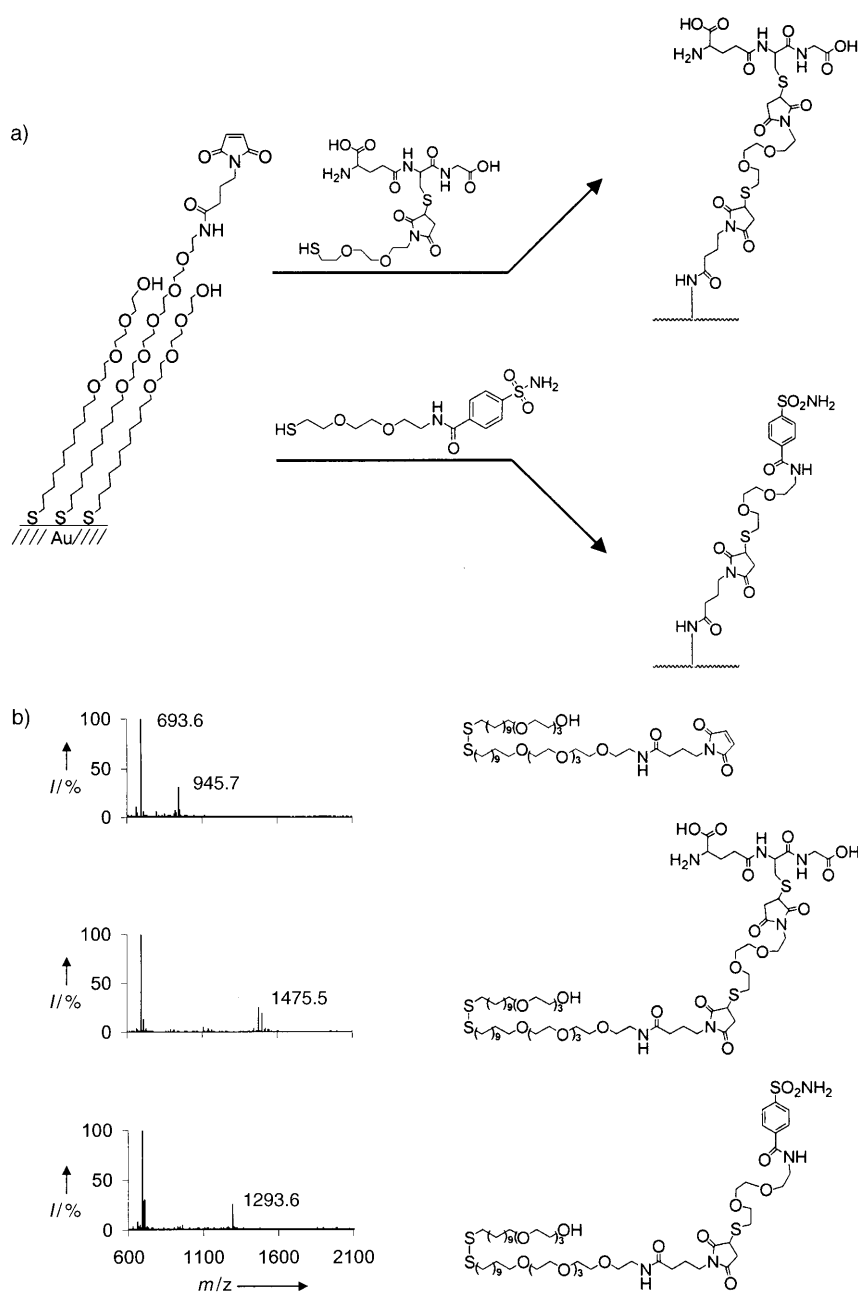


Figure 1. a) Protein-capture ligands were immobilized onto a maleimide-terminated SAM by reaction of the thiol-tagged ligands. b) MS data for the initial monolayer (top) displayed a peak at m/z 945.7, which corresponds to the mixed disulfide derived from a maleimide-terminated alkanethiolate and a tri(ethylene glycol)-terminated alkanethiolate. After treatment with ligands, the original peak disappeared and gave rise to a new peak at m/z 1475.5, which corresponds to the glutathione-conjugated product (middle) or at m/z 1293.6, which corresponds to the benzenesulfonamide-conjugated product (bottom).

bound GST-FAT at m/z 44.2 kDa ($[M+H]^+$) and m/z 22.1 kDa ($[M+2H]^{2+}$), respectively (Figure 2c).^[10] We also found that a monolayer treated with a crude lysate of *E. coli* that had been transformed with the GST-FAT plasmid gave major peaks identical to those obtained from a purified GST-FAT protein (Figure 2d). This result gives further evidence that the immobilization of GST proteins is specific, and that the monolayer is inert to the nonspecific adsorption of proteins. In a separate experiment, a monolayer presenting

the benzenesulfonamide ligand was treated with CA ($5 \mu\text{M}$ in Tris buffer, pH 7.5). MS analysis of this monolayer revealed peaks corresponding to the bound protein, including a doubly charged monomer ($[M+2H]^{2+}$, m/z 14.5 kDa), a singly charged monomer ($[M+H]^+$, m/z 28.9 kDa), and a singly charged dimer ($[2M+H]^+$, m/z 57.9 kDa) (Figure 2e). Pre-incubation of CA with soluble benzenesulfonamide ($500 \mu\text{M}$) inhibited the binding of protein to the monolayer (data not shown). This latter example is significant because it suggests that SAMDI can be applied to the use of small-molecule arrays to identify inhibitors of target proteins.^[11] Indeed, small arrays of carbohydrate ligands have been prepared on SAMs^[12] and chemistries have been demonstrated for the immobilization of small molecules that have amine or thiol functional groups.^[6,13]

This method is also well-suited for characterizing protein-protein interactions on biochips. We demonstrate this application with the interaction between estrogen receptor (ER) and steroid receptor coactivator (SRC).^[14,15] ER serves as a molecular switch; its binding to SRC requires a small-molecule agonist.^[16] The MALDI MS spectrum of a glutathione-presenting monolayer that was exposed to GST-SRC gave peaks corresponding to the singly and doubly charged fusion protein at m/z 44.7 kDa ($[M+H]^+$) and m/z 22.4 kDa ($[M+2H]^{2+}$), respectively (Figure 3a).^[17] A solution containing GST-SRC ($10 \mu\text{M}$), ER ($10 \mu\text{M}$), and estradiol (E2, agonist, $50 \mu\text{M}$) was applied to the monolayer and analyzed by MS. The analysis revealed peaks derived from both GST-SRC ($[M+H]^+$ at m/z 44.7 kDa, $[M+2H]^{2+}$ at m/z 22.4 kDa) and ER ($[M+H]^+$ at m/z 30.4 kDa, $[M+2H]^{2+}$ at m/z 15.2 kDa) (Figure 3b). However, a repeat of this experiment in which the E2 agonist was replaced with an antagonist (4-hydroxytamoxifen, OHT, $50 \mu\text{M}$) that disrupts the protein-protein interaction, resulted in major MS peaks derived from GST-SRC and only minor peaks derived from ER (Figure 3c). As expected, the presence of the antagonist significantly diminished the recruitment of ER. These results indicate that the combination of SAMs presenting capture ligands and MALDI-TOF MS allows the detection of protein complexes without any

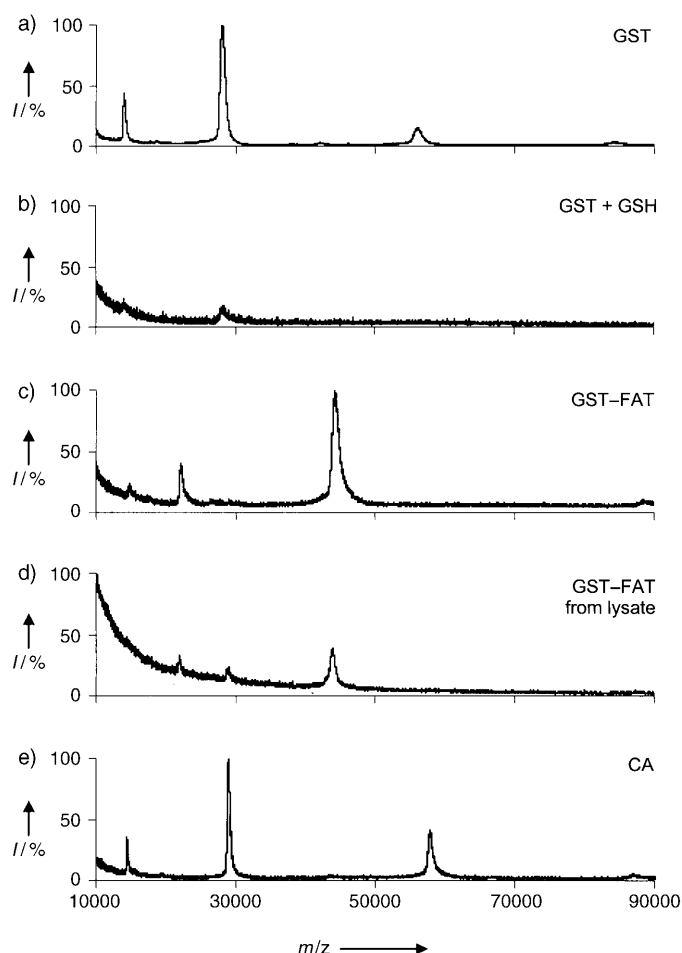


Figure 2. MS analysis of the binding of target proteins to monolayers presenting capture ligands. a) A monolayer presenting glutathione was treated with GST; the data show two major peaks corresponding to GST ions at m/z 28.0 kDa ($[M+H]^+$) and m/z 14.0 kDa ($[M+2H]^{2+}$), respectively. b) Pre-incubation of GST with soluble glutathione gave rise to no major peaks. c) An identical experiment was performed with GST-FAT, and the data show two major peaks corresponding to GST-FAT ions at m/z 44.2 kDa ($[M+H]^+$) and m/z 22.1 kDa ($[M+2H]^{2+}$), respectively. d) The monolayer was treated with a crude lysate from *E. coli* transformed with the GST-FAT plasmid and analyzed. The MS data show major peaks identical to those obtained from purified GST-FAT. e) A benzenesulfonamide-presenting monolayer was treated with CA; the resulting peaks correspond to the CA ions at m/z 14.5 kDa ($[M+2H]^{2+}$), m/z 28.9 kDa ($[M+H]^+$), and m/z 57.9 kDa ($[2M+H]^+$).

labels. Furthermore, it differentiates agonist-induced and antagonist-blocked protein-protein interactions.

The combination of biochips and MALDI-TOF MS offers a powerful and flexible method for identifying protein-protein interactions on biochips and should be extendible to arrays. Importantly, the performance of this method is directly related to the use of self-assembled monolayers of alkanethiolates on gold as the substrate. The structural regularity and synthetic flexibility that are intrinsic to monolayer surface chemistries allow the properties of the interface to be optimized for biochip applications. In particular, the use of oligo(ethylene glycol)-terminated alkanethiols is very effective at controlling unwanted protein adsorption at the inter-

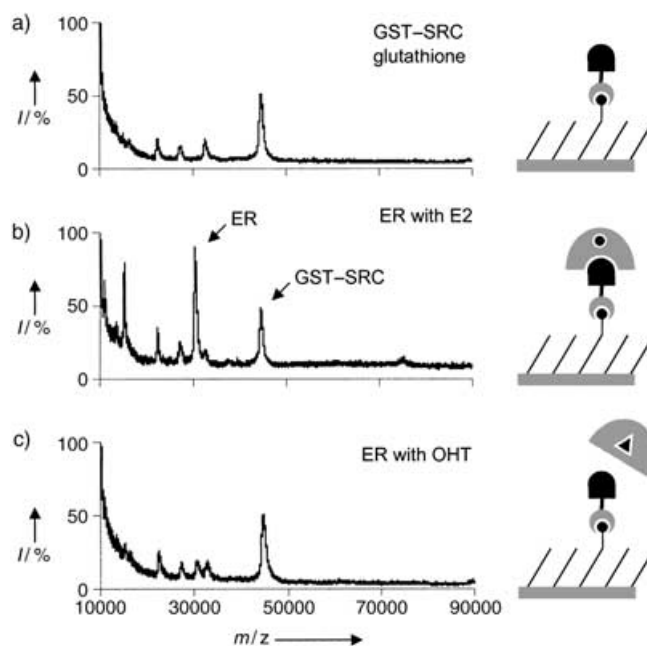


Figure 3. SAMDI analysis of protein-protein interactions on biochips. a) MS data from a glutathione-presenting monolayer treated with GST-SRC show peaks that correspond to bound-protein ions at m/z 44.7 kDa ($[M+H]^+$) and at m/z 22.4 kDa ($[M+2H]^{2+}$). b) A solution containing GST-SRC, ER, and E2 (agonist) was applied to a glutathione-presenting monolayer; the observed peaks derived from both GST-SRC ($[M+H]^+$ at m/z 44.7 kDa, $[M+2H]^{2+}$ at m/z 22.4 kDa) and ER ($[M+H]^+$ at m/z 30.4 kDa, $[M+2H]^{2+}$ at m/z 15.2 kDa). c) MS analysis of an identical experiment with OHT (antagonist) instead of E2 gave major peaks derived from GST-SRC and only minor peaks derived from ER.

face. This reduces false-positive signals and eliminates the need for usual blocking procedures.^[7] Furthermore, a range of immobilization chemistries gives good control over the orientation, density, and activity of small molecules and proteins that are presented on the surface.^[18] These factors make monolayers useful for quantitative assays of biochemical activities.^[4b] The monolayers are also well-suited to analysis by MALDI-TOF MS. The gold substrate facilitates the release of alkanethiolates and bound proteins, and gives clean spectra that are readily interpreted. Whereas alternate methods based on porous silicon substrates share some of these properties,^[19] only SAMDI allows MS detection of the interaction of both low-molecular-weight molecules (as in the case of immobilized peptides that report enzyme activities) and high-molecular-weight proteins, and is based on surface chemistries that are tailored for biochip applications.^[20]

The work described herein offers a new opportunity for applying protein arrays to identify biomolecular interactions. The ability to identify protein-protein interactions without the need for labels will be particularly relevant for the observation of unanticipated activities. The SAMDI format also allows different classes of protein-protein (and enzyme-protein) interactions to be studied with a single array. Current methods, by contrast, often use labeling strategies that allow only a specific class of activity to be identified. These characteristics also suggest that SAMDI will be useful for

chemical screenings that identify small-molecule inhibitors (or promoters) of protein–protein interactions.^[20e]

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