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Affinity Capture Mass Spectrometry of Biomarker Proteins Using Peptide Ligands from Biopanning

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Affinity capture mass spectrometry was used to isolate and ionize protein A from *Staphylococcus aureus* from both a commercial source and cell culture lysate using matrix assisted laser desorption/ionization (MALDI) mass spectrometry. Two surfaces are compared: gold surfaces with immunoglobulin G covalently immobilized and silica surfaces with a covalently bound small peptide discovered via biopanning. A detection limit of 2.22 bacterial cells/mL of culture fluid was determined for the immobilized peptide surfaces. This study emphasizes the ability to use peptide ligands to effectively capture a biomarker protein out of a complex mixture. This demonstrates the potential to use biopanning to generate capture ligands for a large variety of target proteins and subsequently detect the captured protein using MALDI mass spectrometry.

Detection of bacterial contamination, whether attributed to environmental outbreaks or biological warfare, has gained increasing importance over the past decade. In either situation, knowledge of the type and extent of bacterial contamination requires the use of a fast analytical technique with high sensitivity and selectivity. Traditional methods for pathogen detection require the collection and growth of microorganisms prior to biochemical assays, which is both time-consuming and, dependent on growth media, may lead to biased results because of selective cell outgrowth.[1,2] In recent years, the polymerase chain reaction (PCR) has increasingly been used to detect bacterial DNA.[3,4] However, a standard sample is often a complex mixture containing several PCR inhibitors, particularly metal chelators, and DNA from many organisms may be present. In such cases, PCR results can be ambiguous and requires the extraction of DNA, which can result in sample loss and can also be time-consuming. Furthermore, a biological warfare agent may consist of only a single toxin protein, eliminating the presence of detectable DNA.[5] It is in these situations that affinity capture mass spectrometry is ideally suited; it has the ability to extract biomarker proteins of interest and allows for rapid and sensitive detection.

Detection of bacterial contamination and strain typing by mass spectrometry is a well-known technique.[6−11] The current limitations of the application of mass spectrometry in biological studies lies in the great number of proteins and other biological molecules being ionized that may not be exclusive to one organism or strain. Additionally, some qualitative and quantitative variability is based on the media type used.[12] The use of capture ligands to extract one protein of interest from this complex mixture offers the advantage of detecting a single biomarker that would be indicative of bacterial presence without a requirement for intact cells. Several studies have shown the successful use of antibodies bound to a solid surface for isolating a protein of interest from a complex mixture.[13,14] While antibody capture is a viable technique, antibodies tend to be time-consuming to generate, have storage and stability limitations such as proper buffering and temperature sensitivity which can prove difficult in some environmental and biological samples,[15] and require additional chemistry to ensure proper orientation on a surface for solvent exposure to the epitope.[13,16−18]

Recently, a new technique involving biopanning with phage-displayed peptides offers the ability to identify small peptides that

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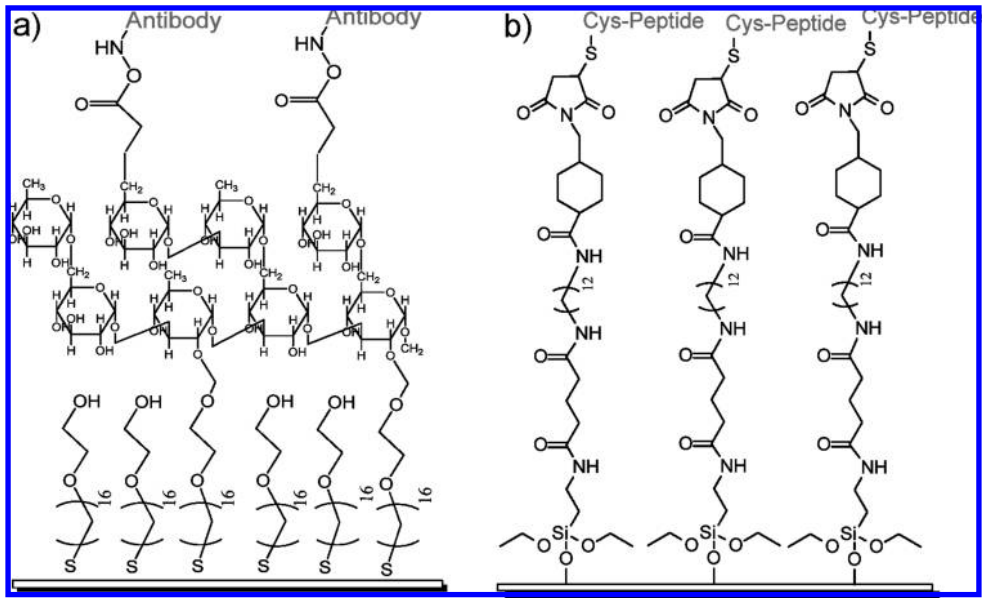

Figure 1. Chemistry of affinity capture chips. (a) IgG covalently bound to modified carboxymethyl dextran self-assembled monolayer on gold coated glass and (b) phage display peptide cross-linked by sulfo-SMCC to a diaminododecane bound to a modified silane glass surface.

can be used in a similar manner to antibodies for on-target capture of biomarkers. This technique commonly uses a library of filamentous bacteriophages displaying short peptides fused to the pIII minor coat protein.[19] Other variants utilize other microorganisms for surface display, and the displayed ligands can include small scaffold proteins, including the Z domain of protein A and antibody fragments.[20,21] By incubation of a phage library with a surface coated with the biomarker protein and with the unbound phage washed away, it is possible to isolate and amplify a phage displaying a peptide that has high specificity for the protein of interest.[22] This technique has been applied to the development of biosensors using dye labels[15] and intrinsic fluorescence.[23] The present report demonstrates the ability of a biopanning-generated peptide to capture protein A from complex mixtures. The peptide is covalently bound to a silica substrate via a linker, and detection of the target protein is achieved after capture by directly ionizing from the surface with matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This technique has the added advantage of obtaining a m/z of the protein, whereas spectroscopic assays can merely tell whether a fluorescent probe is bound without determining the extent of nonspecific adsorption.

The work presented here demonstrates a proof of concept that utilizes protein A from *Staphylococcus aureus*, a cell wall-attached protein that traditionally binds to the Fc domain of immunoglobulins.[31] A peptide ligand has also been generated for this protein by using biopanning, allowing for clear comparison to antibody based affinity capture. The results from this work illustrate the potential to use biopanning-generated peptide ligands for affinity capture mass spectrometry to detect biomarker proteins of interest from a variety of biological and environmental samples.

Table 1. Molecular Weight of Protein A Subunits Are Shown for Strains Cowan and 8325-4

	protein A IgG binding subunits MW		
	Cowan[38] (culture)	Cowan[39] (culture)	8325-4[34] (commercial)
E			6566.22
D	6655.36	7098.77	6455.12
A	6610.27	6610.27	6697.35
B	6599.32	6399.12	6599.32
C	6629.43	6615.35	6558.35

EXPERIMENTAL SECTION

Materials. Epichlorohydrin, diethylene glycol dimethyl ether, bromoacetic acid, ethanolamine hydrochloride, immunoglobulin G from human serum, protein A from *Staphylococcus aureus*, bovine serum albumin, 3-aminopropyltri(ethoxy)silane, 25% glutaraldehyde, HPLC grade toluene, and 1,12-diaminododecane were purchased from Sigma-Aldrich (St. Louis, MO). Potassium hydroxide and microslides were purchased from VWR (West Chester, PA). NaOH was purchased from J.T. Baker (Phillipsburg, NJ), dextran T5000 was purchased from Amersham Biosciences (Pittsburgh, PA), 16-mercaptohexadecanol was purchased from Frontier Scientific (Logan, UT), *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sinapic acid were purchased from Fluka Analytical (St. Louis, MO), and *N*-hydroxysuccinimide (NHS) was purchased from Acrös Organics (Morris Plains, NJ). Sodium cyanoborohydride was purchased from MP Biomedicals (Solon, OH), sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (Sulfo-SMCC) cross-linker was purchased from Pierce Biotechnology (Rockford, IL), the phage display selected peptide was synthesized by Sigma-Genosys, and the gold surfaces were produced by Evaporated Metal Films (Ithaca, NY).

Preparation of Capture Plates. *Antibody Plates.* The procedure for covalent immobilization of IgG on gold coated plates is performed using a carboxymethyldextran modified self-assembled

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monolayer as described by Löfås.[24] Briefly, a 1.35 mm × 1.75 mm gold-coated glass chip was modified with a self-assembled monolayer by immersion into 5 mM 16-mercaptohexadecanol in 80:20 (v/v) ethanol/water for 48 h at room temperature, utilizing the ability of thiols to adsorb on gold surfaces.[25] This surface was then treated with 0.6 M epichlorohydrin in 1:1 (v/v) 0.4 M sodium hydroxide with diethylene glycol dimethyl ether for 4 h at room temperature to form an epoxide. The epoxide was then modified with 0.3 g/mL dextran T5000 in 0.1 M sodium hydroxide for 20 h at room temperature. The dextran layer was then modified to a free carboxylic acid by exposure to 1 M bromoacetic acid in 2 M sodium hydroxide for 16 h at room temperature. The plates were washed with sonication in 6 × 1 min between each step.

Immobilization of IgG on this surface was described by Johnsson.[26] Briefly, the free carboxylic acid was reacted with 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) for 15 min at room temperature. This allows for the nucleophilic displacement of the NHS ester with free amines to covalently bind the antibody. A 1 μg/μL solution of IgG from human serum in phosphate buffered saline (PBS) was added to the plates and allowed to bind for 30 min at room temperature. After washing, unreacted NHS esters were capped with 1 M ethanolamine hydrochloride pH = 8.5 for 10 min at room temperature.

Peptide Plates. Standard glass microscope slides were cut into 2.5 mm × 2.5 mm chips and cleaned for 1 h in 10% (w/v) potassium hydroxide and dried for 1 h at 100 °C. A 2% (v/v) 3-aminopropyltri(ethoxy)silane solution in HPLC grade toluene was poured over the plates and reacted for 30 min with gentle shaking at room temperature. Excess silane was washed away with toluene, and a 2.5% solution of glutaraldehyde in pH 8 buffer was poured over the plates and reacted for 45 min. Excess glutaraldehyde was washed away with water, and the plates were reacted with 2% (w/v) 1,12-diaminododecane in toluene and was allowed to react with gentle shaking overnight. The plates were washed with copious amounts of toluene, and the surface was reacted with 0.1 mg/mL sodium cyanoborohydride for 1 h with shaking. A 4.8 mg/mL solution of sulfo-SMCC cross-linker was prepared and reacted by spotting on the plates for 30 min, leaving a free maleimide group for reaction with the peptide ligand, which has a cysteine residue on the C-terminus. The peptide used for this capture surface was previously described,[23] and its amino acid sequence, His-His-Lys-His-His-His, was modified at the C-terminus with Gly-Gly-Gly-Cys, where the glycine residues provide added flexibility and separation from the cross-linker, and the thiol of Cys allows for attachment to the maleimide cross-linker. A 1 mg/mL solution of this peptide reduced with TCEP was reacted with the surface for 2 h.

Cell Culture of *Staphylococcus aureus*. ATCC strain 12598 (Cowan Strain) was used to produce protein A for capture from lysed cell culture. The culture was maintained on blood agar plates (BAPs) until used. A single colony from a BAP was introduced to 25 mL of LB broth, and the culture was allowed to grow overnight at 37 °C with shaking. A 1 mL aliquot from culture was pelleted with centrifugation, culture media was decanted, and cells were resuspended in a 1 mg/mL solution of lysozyme. This suspension

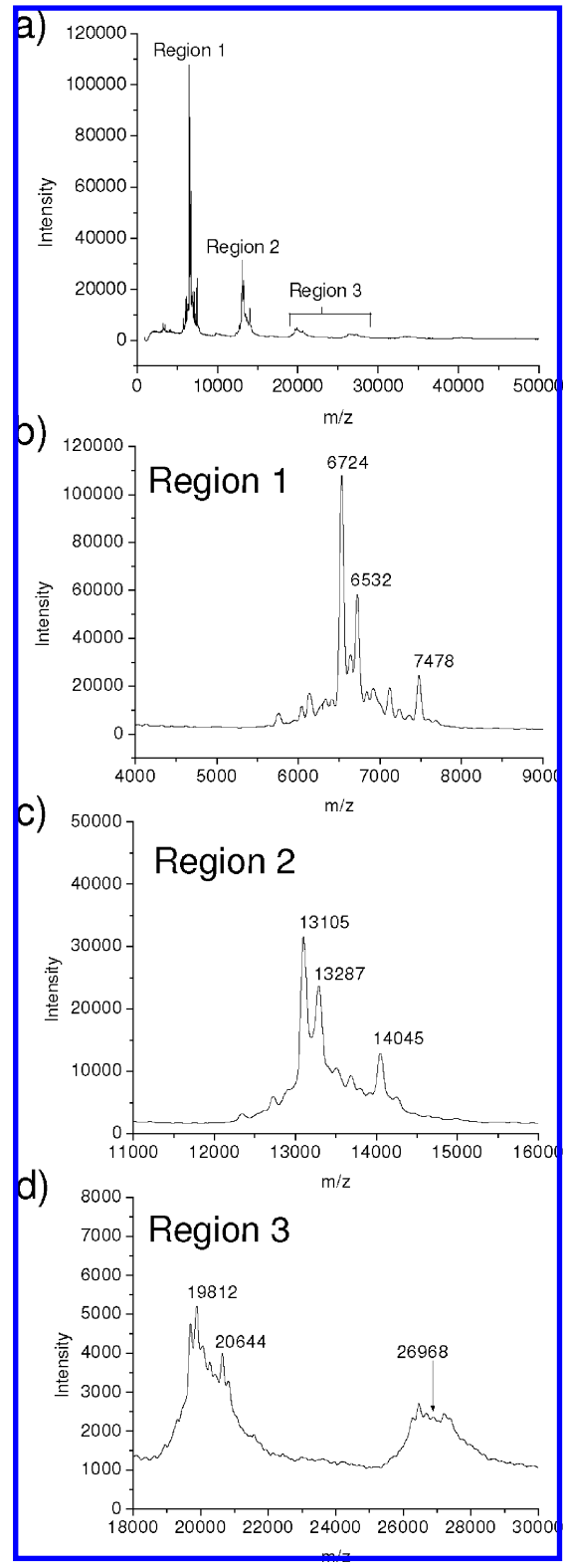

Figure 2. (a) Spectrum of protein A taken on a standard MALDI plate, (b) region 1 zoomed in, (c) region 2 zoomed in, and (d) region 3 zoomed in.

was then heated at 90 °C for 1 h to lyse the cells.[27] For the determination of the limit of detection, serial dilutions were prepared as follows. Cells were counted by light microscopy using

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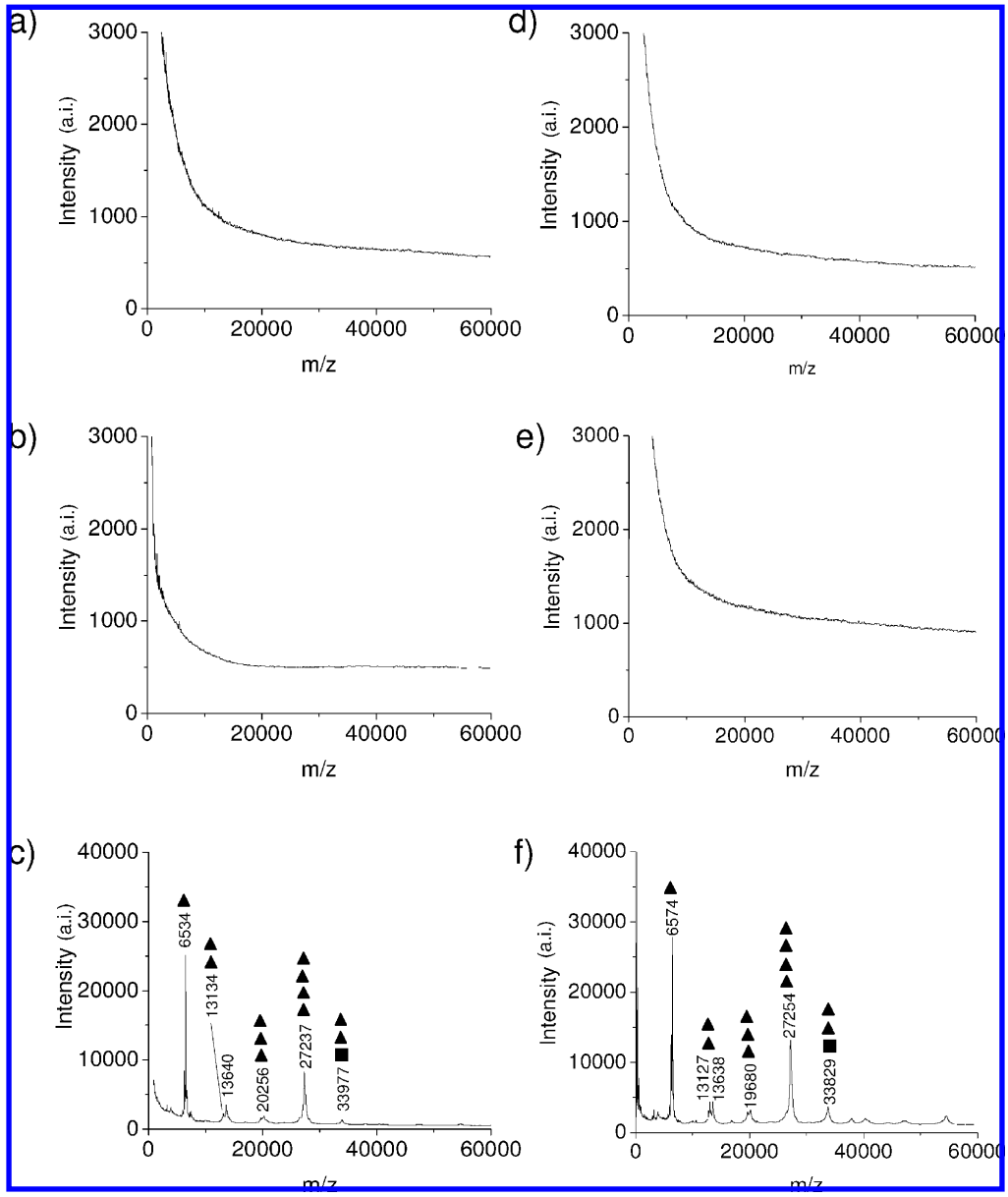

Figure 3. Capture of commercial protein A from *Staphylococcus aureus* strain 8325-4. Gold-antibody surfaces: (a) unmodified surface only, (b) surface with antibody covalently bound, and (c) surface with antibody upon protein A addition. Silica-peptide surfaces: (d) unmodified surface only, (e) surface with peptide covalently bound, (f) surface with peptide upon protein A addition. ▲ represents one IgG binding unit (E, D, A, B, or C), and ■ represents region X of EDABCX.

a Petroff−Hausser counting chamber. A series of 9 consecutive 1:10 dilutions were prepared by taking aliquots from culture media, pelleting with centrifugation, and lysing as discussed above.

Capture of Protein A from Commercial Product and Cell Lysate. Prepared plates, with antibody and peptide ligand, were treated with each commercial protein A (strain 8325-4) and cell lysate (Cowan strain). It is important to note the difference in strains used for commercial and cell culture experiments. Commercial protein A was collected from the 8325-4 strain, and the Cowan strain was grown in culture. These strains differ slightly in their protein A sequence, as well as in the number of IgG binding domains. A more thorough description of the strain differences will follow in the Results and Discussion section. A 20 μM solution of commercial protein A was prepared in PBS, and 50 μL was spotted to cover each of the plates. In the cell lysate experiment, 100 μL was spotted to completely cover the plates. Protein A was allowed to react with both surfaces for 30 min prior to washing with water.

MALDI Time-of-Flight (TOF) Mass Spectrometry. After capture by antibody or peptide, the chips were mounted on a MALDI plate (Microtiter plate (MTP) adapter for Prespotted AnchorChip Targets plate, Bruker Daltonics, Billerica, MA) with double sided tape. The surface of each plate was then spotted with 1 μL of a saturated solution of sinapic acid in 70:30:1 H_2O/acetonitrile/trifluoroacetic acid, with three different spots prepared on each surface to allow for averaging of signal across the plates. Additionally, 1 μL of 15 μM bovine serum albumin in saturated sinapic acid solution was spotted on a blank plate to allow for external mass calibration. These spots were allowed to crystallize in ambient conditions. The plate was inserted into a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (λ = 337 nm) for

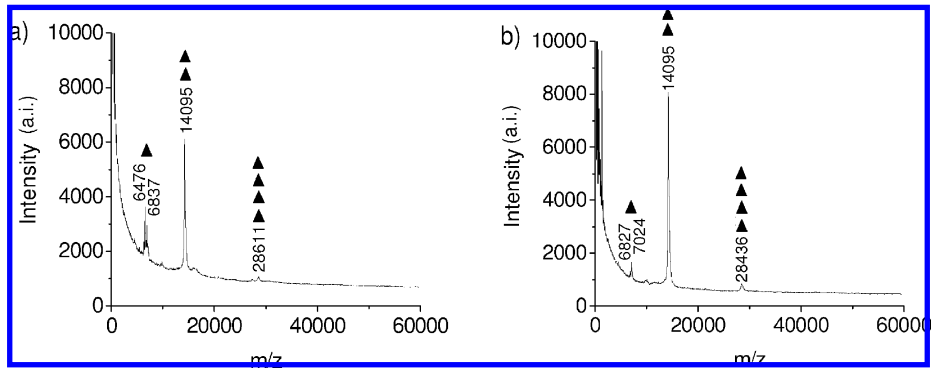

Figure 4. Protein A captured from lysed *Staphylococcus aureus* cells: (a) gold-antibody plate and (b) silica-peptide plate. Signal seen from one IgG binding unit (D, A, B, or C), two IgG binding units, and four IgG binding units.

ionization. Each spot was subjected to 300 laser shots, allowing for signal summing and averaging. Following ionization, ions are accelerated by a 20 keV electric field, travel down the drift tube, and were detected in linear detection mode, allowing for the observation of high mass/charge ions. Spectra were exported as ASCII files and were processed using Microcal Origin 6.0 (Microcal Software Inc., Northampton, MA).

RESULTS AND DISCUSSION

Surface Production for Affinity Capture Mass Spectrometry. Two different surface constructions were used: gold surfaces with a modified self-assembled monolayer for antibody attachment and silica surfaces modified with a hydrocarbon tether for peptide attachment. Each surface type was chosen based on tethering techniques used previously.[23,24,26] Figure 1 illustrates schematically the chemistry involved in binding the ligand to the surface. There are advantages to using each surface. The gold surface utilizes a carboxymethyl-dextran hydrogel layer, allowing for both spatial separation of the attached antibody from the denaturing conditions of the gold surface but also allows for the surface to be negatively charged, allowing for a "preconcentration" of protein by attraction to this surface, facilitating the kinetics of covalent attachment of the antibody.[26] Using this hydrogel layer also minimizes the contribution of nonspecific adsorption caused by interaction of solutes with the metal surface. Experiments were done without the use of this hydrogel layer with lysed cells, and results showed evidence of solute binding in the absence of a capture ligand (data not shown). While this binding was not at the same molecular weight of protein A, further experiments used this hydrogel layer to avoid any surface reactivity.

For tethering peptides, a less costly silica surface is used and the binding of the peptide is straightforward, with the surface first silanized with a free amine exposed for attachment to glutaraldehyde. Interaction with a long chain amine at the free aldehyde end of glutaraldehyde provides the spatial distance necessary to reduce steric hindrance of the tethers by increasing the distance of the tethered peptide from the surface, assuming free rotation of the tether. Reacting this free amine with an amine-reactive cross-linker that is also thiol reactive on the exposed end allows for the covalent bonding of a C-terminal cysteine containing peptide. Furthermore, binding at this C-terminal Cys allows for orientation of the peptide, leaving the N-terminal binding portion exposed.

The major advantage of using the peptide-linked surfaces over antibody-linked surfaces is stability and size. Antibodies, like proteins, have limitations in optimal temperature due to the necessity to maintain stable secondary and tertiary structure for activity. Additionally, the production of antibodies can result in a wide variety of structures. While the use of monoclonal antibodies minimizes heterogeneity, multiple isoforms are still produced due to differences in glycosylation and terminal processing which can result in antibodies with differences in binding efficiencies and specificity.[28−30]

Capture of Commercially Available Protein A. After surface preparation, a solution of protein A was spotted and allowed to react for 30 min with both antibody and peptide linked plates, followed by several water washes to remove any unbound protein. Each surface was analyzed by MALDI-TOF MS in linear mode with external calibration using bovine serum albumin.

Protein A consists of four to five homologous IgG-binding regions displayed externally on the cell with a C-terminal portion that is responsible for binding to the peptidoglycan of the cell wall, where the number of IgG binding regions varies with the strain.[31] The Cowan strain, used for cell culture experiments, has four of these units (DABC), and strain 8325-4, used for commercial protein A experiments has five (EDABC).[32] Each of these regions is approximately 6.5−7 kDa in size[33,34] and are each separated by an arginine residue that acts as a tryptic cleavage site. Protein A is bound to the cell wall via region X, which is approximately 20 kDa,[35] and consists of many octapeptide repeats that allow the protein to bind to the peptidoglycan layer.

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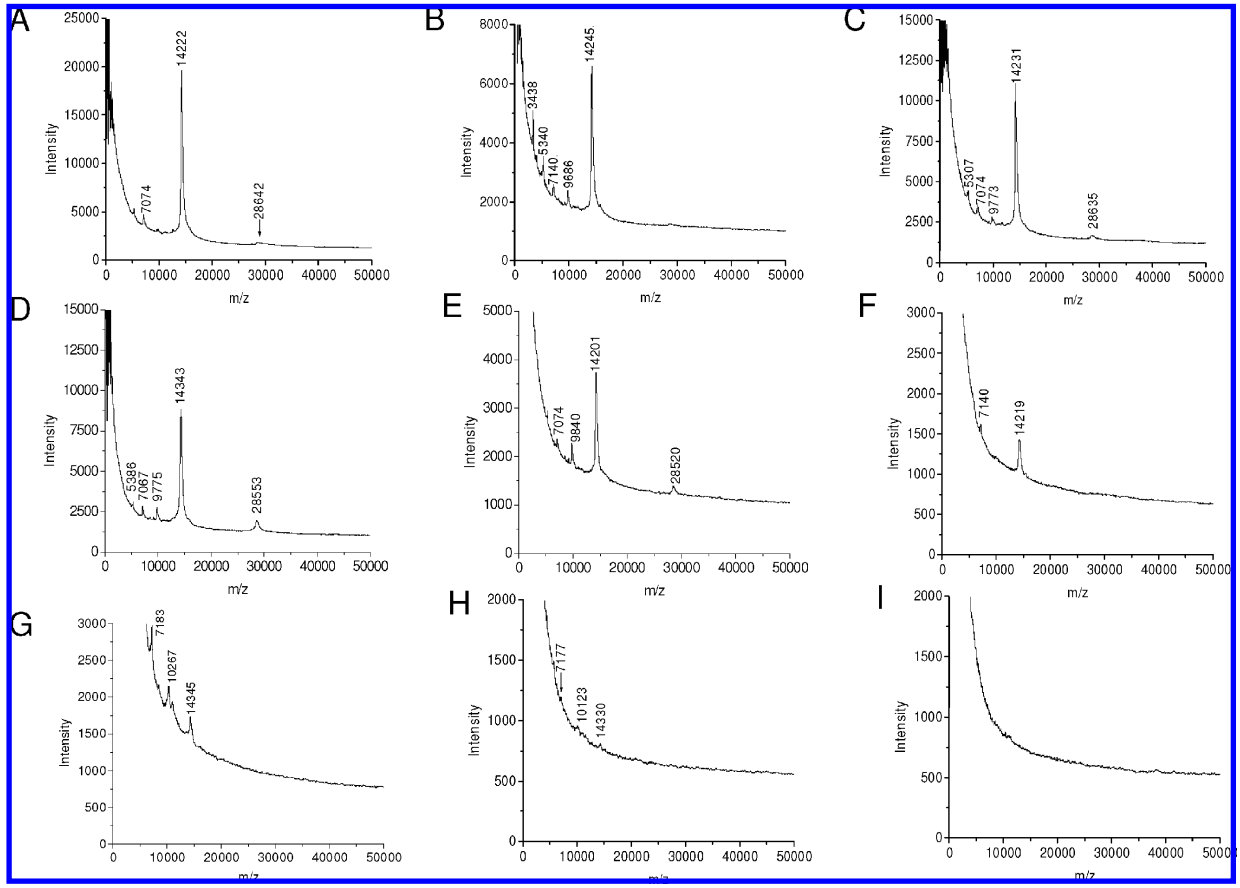

Figure 5. Cell concentration gradient for protein A capture from lysed *Staphylococcus aureus* cells: (a) 2.70×10^7, (b) 2.70×10^6, (c) 2.70×10^5, (d) 2.70×10^4, (e) 2.70×10^3, (f) 2.22×10^2, (g) 22.2, (h) 2.22, and (i) 0.22 cells/mL.

Additionally, each strain also produces slightly different protein A with varying amino acid sequence. Most of the differences seen in sequence can be attributed to point mutations.[32] The expected molecular weights have been calculated from amino acid sequences derived from genetic information and Edman degradation. Table 1 shows these molecular weights but also shows that different investigators have reported different sequences, further indicating the variability of protein A. Because the literature shows some inconsistency in the sequence, a spectrum of commercial protein A acquired for comparison using a standard MALDI plate is shown in Figure 2.

Figure 3 illustrates the ability of both the antibody and peptide surfaces to bind commercially available protein A isolated from strain 8325-4. Parts a and d of Figure 3 each shows a mass spectrum of matrix ionized from the unmodified gold-antibody and silica-peptide plates, respectively, as a negative control. Parts b and e of Figure 3 each shows ionization from surfaces that had been fully modified with the antibody and peptide, respectively, in the absence of captured protein to demonstrate that the covalent attachment of the ligands were stable under ionizing conditions. Parts c and f of Figure 3 each shows the mass spectrum of captured commercial protein A ionized from the two surface types. In these spectra, signal is produced from single IgG binding domains of protein A as well as 2, 3, and 4 intact domains and region X with IgG binding units retained. A small peak is seen at approximately 55 kDa, indicative of intact protein A, as well as a slight shoulder on the 27 kDa peak, which represents region X bound to a single IgG binding unit, both of which are unlabeled due to low signal intensity. Because the purification of protein A from culture involves intact cells versus traditional expression in *Escherichia coli*, it is likely that the truncated forms of protein A seen here, and subsequently later in culture based assay, are due to endogenous protease activity during cell lysis.

Capture of Protein A from Cell Lysate. After successful capture of protein A from commercial sources, capture from cell lysate was initiated. *Staphylococcus aureus* Cowan strain (ATCC 12598) was grown in LB medium, and cells were lysed at 90 °C with lysozyme as discussed above. This cell lysate was allowed to react with the plate surfaces for 30 min followed by several washes prior to ionization to remove any unbound or nonspecifically bound protein. Both antibody-capture and peptide-capture surfaces were analyzed by MALDI-TOF MS in linear mode with external calibration with bovine serum albumin.

Figure 4 illustrates spectra obtained for protein A capture with gold-antibody plates (a) and silica-peptide plates (b) from cell lysate. Because cell lysis was performed with lysozyme, which should disrupt the peptidoglycan layer, it is expected that region X remains bound to the cell wall. This released DABC, the IgG binding units, from the cell into the media. These spectra illustrate

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capture of a single IgG binding unit, two IgG binding units, and all four IgG binding units intact. It is important to note the lack of nonspecific adsorption or capture of other proteins from the lysate with these surfaces. If the ligands were cross-reactive with any other *S. aureus* proteins, those proteins would be detected. However, the spectra show signal corresponding to protein A only. Both gold-antibody and silica-peptide plates have high specificity for protein A and show similar signal intensity. While it was expected that the signal intensity would be higher with peptide capture, it is possible that binding kinetics play a role in the strength of signal seen for antibody plates. The K_d of the protein A−IgG interaction has been reported as 10 nM.[36] Because the dissociation kinetics of the peptide with protein A are unknown, it is not possible to assess which has tighter binding affinity. However, it is hypothesized that the antibody possibly has a lower K_d, resulting in a higher concentration of protein A on the surface when compared to peptide plates. This assay demonstrates that the silica-peptide surface has comparable utility as previously reported antibody plates.

Cell Concentration Gradient with Silica-Peptide Surfaces. The data presented thus far demonstrated that the binding capabilities of the silica-peptide plates are comparable to traditional gold-antibody plates. Also, these plates have the added advantage of having minimal buffer requirements, greater thermal stability, lower cost, and ease of preparation.[23] Additionally, the peptide is significantly smaller than an antibody, allowing for a higher loading capacity on the surface. This theoretically increases the concentration of protein able to be captured on the plate, resulting in a lower detection limit, assuming that the tethers are not tightly packed.

To demonstrate the ability of silica-peptide surfaces to detect bacterial protein at low concentrations, a gradient was set up via serial dilutions of cells prior to cell lysis and protein A detection. For this analysis, aliquots of cell media of known concentration were diluted into fresh media and cells were pelleted and resuspended in 1 mL of lysozyme. Cells were lysed at 90 °C for 1 h prior to surface capture. Each cell lysate was allowed to react with the silica-peptide surfaces for 30 min prior to MALDI-TOF analysis. The experiment was repeated in triplicate to ensure reproducibility. Spectra in Figure 5 illustrate an increase in signal intensity corresponding to an increase in cell concentration, and the minimum detectable concentration of cells was 2.22 cells/mL (S/N = 3). MALDI is inherently nonquantitative; signal intensity varies within an individual spot and is dependent on variations in crystallization on crystallization of the matrix as the laser is rastered over the spot.[37] Therefore, the limit of detection has been defined here as the minimum cell concentration at which a detectable signal is present.

CONCLUSIONS

The ability to capture and analyze protein A from *Staphylococcus aureus* cell lysate has been demonstrated by using both gold-antibody and silica-peptide plates coupled with MALDI-TOF mass spectrometry. While both surfaces have shown utility in detecting a biomarker protein out of a complex mixture, silica-peptide plates have the added advantage of ease in preparation and stability. Furthermore, these peptide plates utilize the relatively new technology of biopanning for biosensor reagents, allow for the identification of a capture ligand for virtually any protein of interest, and provide an affinity-capture method using a non-antibody-based peptide ligand. More importantly, we have shown that low concentrations of *Staphylococcus aureus* cells, approaching PCR detection limits, can be detected. Biopanning for phage-display peptides allows for the development of affinity ligands from virtually any organism, permits ease in utilizing these peptides with affinity capture mass spectrometry, and holds great potential for application to developing a diagnostic for emerging pathogens.

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