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# Surface Plasmon Resonance Imaging Measurements of Antibody Arrays for the Multiplexed Detection of Low Molecular Weight Protein Biomarkers

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**This paper describes a simple methodology for the creation of high-density multiplexed antibody arrays on gold surfaces that can be used to detect low molecular weight protein biomarkers with surface plasmon resonance imaging (SPRI). A one-step carbonyldiimidazole (CDI) surface reaction was utilized to attach antibodies onto alkanethiol-modified gold surfaces and characterized with polarization modulation FT-IR reflection absorption spectroscopy. The CDI chemistry was then employed to create an antibody microarray with array element sizes varying from 750 $\mu$m down to 200 $\mu$m. As a demonstration, a three-component antibody array was employed to detect two clinically important protein biomarkers, $\beta_2$-microglobulin (11.8 kDa) and cystatin C (13.4 kDa). SPRI measurements could simultaneously detect both of these small unlabeled proteins with no cross talk at solution concentrations from 300 nM down to 1 nM. In addition, the adsorption strengths of these biomarkers onto an antibody array were measured with SPRI and compared to those obtained from the kinetic analysis of single-channel angle shift SPR measurements.**

The multiplexed detection and quantitation of low molecular weight protein biomarkers in biological samples via binding to antibody arrays is emerging as an essential tool for researchers interested in gathering information for either biomedical research (e.g., proteomics, drug design, and evaluation)[1−3] or medical diagnostics (e.g., disease prognosis, drug eligibility, and therapeutic monitoring).[4−7] To create an array for biomarker detection, antibodies are typically attached to transparent substrates such as plastic microwell arrays,[8,9] nitrocellulose membranes,[10,11] silicon nitride,[12] hydrogel polymers,[13] and optical glass.[14,15] Antigen binding is then detected by the specific adsorption of a second antibody that is either fluorescently labeled or coupled to an enzyme that reacts with either a fluorogenic or a chromogenic substrate.[16,17]

While these methods are very sensitive, the sandwich assay or ELISA format requires a second antibody−antigen interaction for each component in the array. An alternative method is to use competitive adsorption experiments with fluorescently labeled antigens,[7,18,19] but these experiments also require a different fluorescently labeled molecule for each array element. A simpler methodology would be to use an optical method that can directly detect antigen binding.

Surface plasmon resonance (SPR) is a technique that can be used to detect the direct binding of protein biomarkers onto gold surfaces via changes in the local index of refraction upon adsorption. Moreover, SPR can be used in a quantitative fashion to measure the strength of adsorption (Langmuir adsorption coefficient) as well as the kinetics of antigen adsorption to the surface.[20] SPR measurements are typically performed in a flow

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cell format that can handle only a few antigens simultaneously;[21] however, the technique of SPR imaging (SPRI) is a method that can potentially offer multiplexed detection of hundreds to thousands of surface bioaffinity interactions. SPRI has been applied to a wide variety of bioaffinity interactions, including DNA−DNA, DNA−protein, peptide−protein, and protein−protein binding.[20,22−31]

SPRI measurements of antibody arrays have been reported recently: Koga et al. developed antibody arrays to detect mouse KIAA proteins[32] (MW ∼130 000) and proteins in cell lysates,[24] and McDermott et al. used a microfluidic approach to create antibody arrays to monitor bovine serum albumin (BSA; MW ∼69 000) and bovine IgG adsorption (MW ∼150 000).[27] Difficulties in these measurements include the stability of the antibody array when dried and attachment chemistries that result in the fabrication of inhomogeneous array elements with only a subset of bioactive antibodies. No SPRI measurements of small protein biomarkers (MW ∼ 10 000) have been reported to date.

In this paper, we report our progress in the fabrication of antibody arrays for the multiplexed detection of low molecular weight protein markers. Specifically, we describe the fabrication of antibody arrays that can detect $\beta_2$-microglobulin ($\beta_2$m; MW 11 800) and cystatin C (cysC; MW 13 400). $\beta_2$m and cysC are two clinically important biomarkers present throughout the body. $\beta_2$m is associated with the outer membrane of many cells including lymphocytes and has been used as a biomarker for renal function,[33] viral meningitis,[34] and multiple sclerosis.[35] cysC is an extracellular cysteine protease inhibitor that has been suggested as a marker for a number of inflammatory ailments (e.g., periodontal disease and joint disease) and tumors.[36] cysC levels have also been used as a reliable measure of the glomerular filtration rate, which has been linked to renal failure.[37] For each of these biomarkers, we use SPRI to measure their adsorption strength onto an antibody array and demonstrate that SPRI has sufficient sensitivity (nanomolar detection limits) to detect these biomarkers in buffer samples.

In addition to the detection of low molecular weight proteins, we also demonstrate two pieces of array fabrication methodology for antibodies: (i) a one-step carbonyldiimidazole (CDI) surface reaction for the attachment of antibodies onto alkanethiol-modified gold surfaces that we characterize with polarization modulation FT-IR reflection absorption spectroscopy (PM-FTIRRAS)[38,39] and (ii) the creation of antibody microarrays with array element sizes varying from 750 $\mu$m down to 200 $\mu$m. These fabrication methods are essential for the future creation of high-density multiplexed antibody arrays.

## EXPERIMENTAL CONSIDERATIONS

**Materials.** 11-Mercaptoundecanoic acid (MUA, Aldrich) and 1,1′-carbonyldiimidazole (CDI, Aldrich) were used as received. Polyclonal rabbit anti-human $\beta$-2-microglobulin (anti-$\beta_2$m, 3.9 g/L) and rabbit anti-human cystatin C (anti-cysC, 16 g/L) were purchased from Dako A/S. The antibody to Staphylococcal enterotoxin B (SEB, 1 g/L) was purchased from Toxin Technology. Two protein biomarkers extracted from human urine, $\beta$-2-microglobulin ($\beta_2$m) and cystatin C (cysC), were purchased from Calbiochem. The antibodies used for arraying were diluted in 10 mM sodium acetate (pH 5.0), a five-fold dilution for anti-$\beta_2$m, 20-fold for anti-cysC, and 2-fold for anti-SEB were used to bring them to approximately the same concentration (∼0.8 g/L). All rinsing steps were performed with either Millipore filtered water or absolute ethanol. All experiments were performed at room temperature unless stated otherwise.

**Antibody Array Fabrication.** Thin gold films (45 nm) with a 1-nm underlayer of chromium were deposited onto SF-10 glass (Schott Glass) using a Denton DV-502A metal evaporator. The gold chips were then immersed in 1 mM ethanolic MUA solution overnight to form a well-packed self-assembled monolayer. The MUA monolayer was then reacted overnight with CDI in acetone solution (10 g/L). Immediately prior to arraying, the CDI-modified chips were rinsed in acetone and dried under a nitrogen stream. The chips were arrayed using a SpotBot Protein Edition Personal Microarrayer (Telechem International Inc.), equipped with a humidity control apparatus and megasonic wash station. During the array fabrication, the humidity was controlled at 40−55% and the platen temperature was set at 15 °C using a thermal control bath. The four-pin print head of the arrayer was fitted with a Stealth microspotting pin (Telechem International Inc.). Various pin sizes were utilized to produce spots with diameters ranging from 135 (pin model SMP4) to 400 $\mu$m (model SMP12). CDI-modified chips were positioned in a predetermined area on the platen and fixed using adhesive tape. Ten microliter aliquots of each antibody solution were placed in individual wells of a 384-well microtiter plate located within the arrayer enclosure. The antibodies were arrayed on a single chip, utilizing alternating spotting cycles (i.e., in the first cycle, the pin aspirated anti-cysC solution to create two rows of anti-cysC spots; in the second cycle, the next two rows were spotted with anti-$\beta_2$m; etc.). Following antibody immobilization, the CDI-modified background was inactivated by washing thoroughly with water or buffer for 1 h before drying under a $N_2$ stream and storing at room temperature overnight. The inactivation process was verified via PM-FTIRRAS measurements; the intensities of the imidazolide bands gradually decreased with time (see peak assignments listed in Table 1).


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**Table 1. PM-FTIRRAS Band Assignments for Surface Modification with CDI and Antibodies**

| Surface | Frequency ($cm^{-1}$) | Assignment | Figure |
|---|---|---|---|
| MUA | 1738 | free carboxylic acid stretch | 2A |
| | 1718 | H-bonded carboxylic acid stretch | |
| | 1465 | $CH_2$ scissors deformation | |
| | 1410 | symmetric carboxylate stretch | |
| imidazolide | 1745 | asymmetric stretch of the CDI carbonyls | 2B |
| | 1512 | ring mode | |
| | 1475 | $CH_2$ scissor deformation | |
| | 1395 | C−N stretch | |
| | 1246 | C−N stretch in aromatic | |
| antibody | 1660 | amide I | 2C−E |
| | 1540 | amide II | |

The antibody arrays created using the CDI chemistry can be reused by regenerating the antibody surface with 60 mM HCl and can be stored at room temperature for more than 2 days.

**Patterned Gold Array Fabrication.** SF10 glass substrates were first spin-coated with a solution of 0.9% Cytop (Asahi Glass Co.) dissolved in CTL-180 solvent (Asahi Glass Co.).[40] An initial spin rate of 500 rpm was applied for 5 s before manually ramping to 5000 rpm and spinning for a further 50 s. The Cytop-coated slide was then baked at 190 °C for 1 h. A thin gold film (45 nm) with a 1-nm underlayer of chromium were then vapor-deposited onto the Cytop-coated slide through a stainless steel mask featuring a pattern of circles of 750-$\mu$m diameter with a center-to-center separation of 1360 $\mu$m. This provides a pattern of gold spots surrounded by a hydrophobic Cytop background. Next, the patterned gold array was placed into an ethanolic MUAM solution for 2 h, which was then allowed to react with CDI solution for at least 4 h. Various antibody solutions were then spotted onto the patterned gold and kept in a humidity chamber for 30 min to react. Finally, the remaining CDI-modified array elements were inactivated as described above and used as a background. The array was stored at room temperature until use.

**PM-FTIRRAS Measurements.** All samples used in PM-FTIRRAS experiments were prepared on commercial gold slides (5 nm of Cr, 100 nm of Au from Evaporated Metal Films). Grazing angle PM-FTIRRAS measurements were performed using a Mattson RS-1 spectrometer and optical layout described elsewhere.[38,39] Before taking each ex situ spectrum, the gold slides were reacted with desired reactants, then thoroughly washed with water, and dried under a $N_2$ stream. Spectra were collected using a HgCdTe detector, averaging 1000 scans acquired at a resolution of 4 $cm^{-1}$.

**SPR Imaging.** For SPRI measurements of low molecular weight biomarker interactions with surface-immobilized antibodies, an SPR imager (GWC Technologies) was employed. Briefly, collimated p-polarized light impinges onto a prism/thin gold film/flow cell assembly at a fixed incident angle near the SPR angle. The reflected light is then passed through a narrow band-pass filter (830 nm) and collected with a CCD camera. All SPR images were collected using the software package Digital Optics V++ 4.0 and further analyzed using the software package NIH Image V.1.63. All SPRI experiments of biomarker bindings onto antibody microarrays were performed under equilibrium conditions. Various concentrations of biomarker solutions buffered in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 were injected and allowed to react for 10 min before washing with the same HEPES buffer to obtain SPRI difference images.

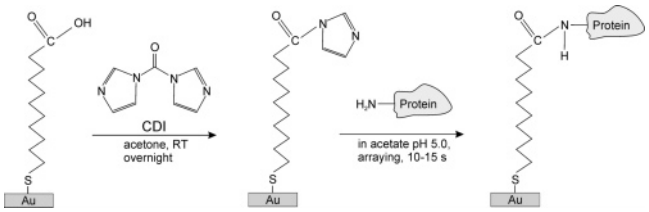


**Figure 1.** Schematic showing the covalent attachment of antibody onto an alkanethiol-modified gold surface via CDI linking chemistry. The reaction of a MUA monolayer with CDI forms an imidazolide monolayer that couples with a lysine residue on an antibody to create an amide linkage to the surface.

**Single Channel Angle Shift SPR Measurements.** A two-channel Biacore X SPR biosensor instrument was used to independently determine the kinetic constants of the antibody−antigen interactions. Antibodies to $\beta_2$m and cysC were separately immobilized in one of two-channel flow cells, and 50-$\mu$L aliquots of $\beta_2$m and cysC biomarkers at different conditions were injected at 50 $\mu$L/min over both flow cells. Following signal referencing and baseline correction, the binding curves were analyzed with BIAevaluation Version 4.1 software and fitted using a 1:1 Langmuir association and dissociation model (see also Adsorption Strength Measurements for $\beta_2$m and cysC).

## RESULTS AND DISCUSSION

**CDI Attachment Chemistry for Creating Antibody Arrays.** The reliable and reproducible immobilization of active antibodies onto gold surfaces at a sufficiently high surface density is a fundamental first step in the multiplexed detection of low molecular weight protein biomarkers with SPRI. With this aim in mind, we have chosen a simple, well-established, one-step CDI reaction to attach antibodies onto alkanethiol-modified gold surfaces. CDI linking chemistry has been used to chemically modify proteins[41] or immobilize proteins on various substrates including cellulose membranes, gel matrixes, and glass/silica surfaces.[41−45] The reaction scheme illustrating the surface CDI linking chemistry for antibody immobilization on gold thin films is shown in Figure 1. CDI first reacts with the terminal carboxylic acid functional groups of a self-assembled MUA alkanethiol monolayer, resulting in the formation of an amine-reactive imidazolide monolayer. This imidazolide layer is then exposed to a particular antibody and reacts with a randomly positioned lysine residue in the antibody to form an amide attachment onto the surface array element.

Both the CDI surface reaction and antibody coupling steps were characterized with PM-FTIRRAS. The PM-FTIRRAS spec-

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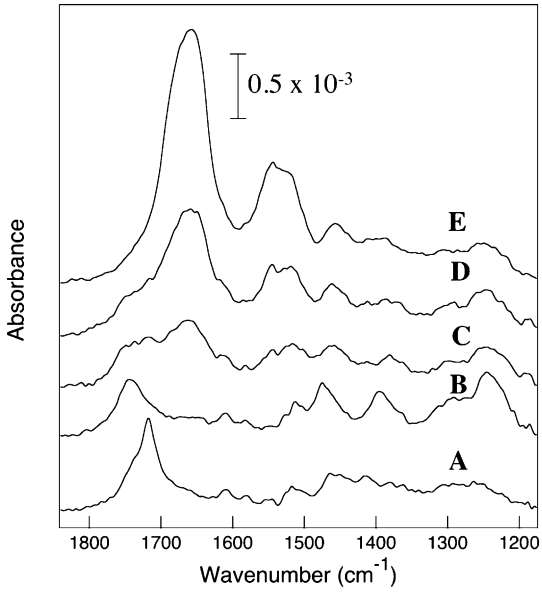


**Figure 2.** Series of PM-FTIRRAS spectra characterizing the CDI and antibody attachment chemistries described in Figure 1. (A) Formation of a self-assembled MUA monolayer overnight. (B) Reaction of CDI with a MUA-modified surface overnight resulting in the formation of an amine-reactive imidazolide monolayer. (C−E) Reaction of a primary amine group randomly present in the antibody with the imidazolide monolayer formed in (B) at anti-$\beta_2$m concentrations of 50 pM (C), 5 nM (D), and 50 nM (E). At each concentration, the antibodies were reacted with the CDI-modified surface for 30 min, thoroughly washed with water, and dried under a nitrogen stream before taking PM-FTIRRAS spectra. The assignments of all the PM-FTIRRAS bands in (A−E) are summarized in Table 1.

trum of a self-assembled MUA alkanethiol monolayer formed on a gold surface is shown in Figure 2 (curve A). The most prominent features in the spectra are the carbonyl stretch bands of the hydrogen-bonded carboxylic acid groups (COOH) at 1738 and 1718 $cm^{-1}$.[38] Following the surface reaction of CDI with the carboxylic acid groups of the MUA monolayer, (curve B in Figure 2), these MUA bands are replaced by a strong band at 1745 $cm^{-1}$. This new carbonyl band is assigned to the carbonyl stretching frequency of the imidazolide monolayer on the surface and differs substantially from both the carbonyl stretching frequencies of the MUA monolayer and the symmetric carbonyl stretch of CDI molecules in solution (1758 $cm^{-1}$). The other bands observed at 1512, 1475, 1395, and 1246 $cm^{-1}$ are associated with $CH_2$ scissor deformations and C−N stretches. The frequencies of all of the vibrational bands observed for both the CDI surface reaction and subsequent antibody coupling steps are listed in Table 1 along with band assignments.

Figure 2 also presents a series of PM-FTIRRAS spectra (labeled C−E) acquired after the surface reaction of the active imidazolide groups with antibody solutions with various concentrations (curves C for 50 pM, D for 500 pM, and E for 50 nM anti-$\beta_2$m). At low concentrations, the intensities of the amide I and amide II bands in the PM-FTIRRAS spectra increase as the antibody concentration in solution is increased. At concentrations above 1 mM, the amide band intensities level off to a maximum value, indicating the formation of a full antibody monolayer on the CDI-modified surface.

The surface density of active antibodies on the gold surface is a critical parameter that affects the detection sensitivity of low molecular weight biomarkers with SPRI. A very rough estimate of the surface density of the antibody monolayer created with the CDI linking chemistry can be obtained by comparing the intensity of the amide bands in PM-FTIRRAS spectra with those observed from short peptide monolayers having a known, measured surface density of $1.5 \times 10^{13}$ molecules/$cm^2$.[31] The intensity of the amide bands from the anti-$\beta_2$m monolayer (MW ~150 000) is ~7−8 times more intense than the amide bands observed previously from a monolayer of short peptides (11-mer MW ~1500).[31] Assuming that the integrated amide band intensity of an absorbed protein is proportional to its molecular weight, we estimate that the surface density of antibodies on the gold thin films was ~$10^{12}$ molecules/$cm^2$. These numbers are similar to those calculated for a packed monolayer of antibodies based on the physical size (~10 nm × 10 nm) of IgG molecules (~$1 \times 10^{12}$ molecules/$cm^2$). In previous SPRI measurements, we have been able to detect protein adsorption onto peptide monolayers with similar or lower surface densities;[31] thus, the adsorption of low molecular weight biomarkers should be detectable at this antibody surface coverage.

**SPRI Detection of Low Molecular Weight Biomarkers Using Antibody Arrays.** Using the CDI attachment chemistry for antibodies onto the gold surface, we now are in a position to create antibody arrays and assess the feasibility of using surface immobilized antibodies for the multiplexed detection of low molecular weight biomarkers with SPRI. Figure 3 demonstrates a three-component patterned gold array containing 750-$\mu$m array elements of anti-cysC, anti-$\beta_2$m, and inactivated CDI-modified background. The anti-cysC and anti-$\beta_2$m array elements were created with the CDI attachment chemistry described in the previous section, and the inactivated CDI-modified background array elements were formed by prolonged rinsing of the CDI-modified surface with water/buffer (The conditions required for inactivation were determined from PM-FTIRRAS measurements and are stated in the Experimental Considerations section).

SPRI was then used to monitor the specific adsorption of the two biomarkers ($\beta_2$m and cysC) onto the antibody array. Figure 3a shows a representative SPRI difference image after the adsorption of $\beta_2$m from a 50 nM solution onto the array. Specific binding of $\beta_2$m onto the anti-$\beta_2$m array elements was observed; the upper line profile in Figure 3b shows a $\Delta R$ increase of ~1%. The uniformity of the $\Delta\%R$ in the line profile indicates good reproducibility in the spotting process. Nonspecific binding of $\beta_2$m onto the incorrect (cysC or CDI-modified background) array elements was not observed at these concentrations. At higher concentrations (above 75 nM), a small increase in the background SPRI signal was observed but was always below 5% of the signal observed for biomarker binding.

Figure 3c shows a representative SPRI difference image after exposing the array to a 100 nM solution of cysC. As in the case of $\beta_2$m, the specific adsorption of this 13.4 kDa biomarker onto the anti-cysC array elements is easily measured at this concentration. These SPRI experiments for the adsorption of $\beta_2$m and cysC were repeated at various nanomolar solution concentrations in order to determine a detection limit of ~1 nM for both of these biomarkers. This limit of detection is well below the concentrations of these biomarkers observed in clinical samples, which are

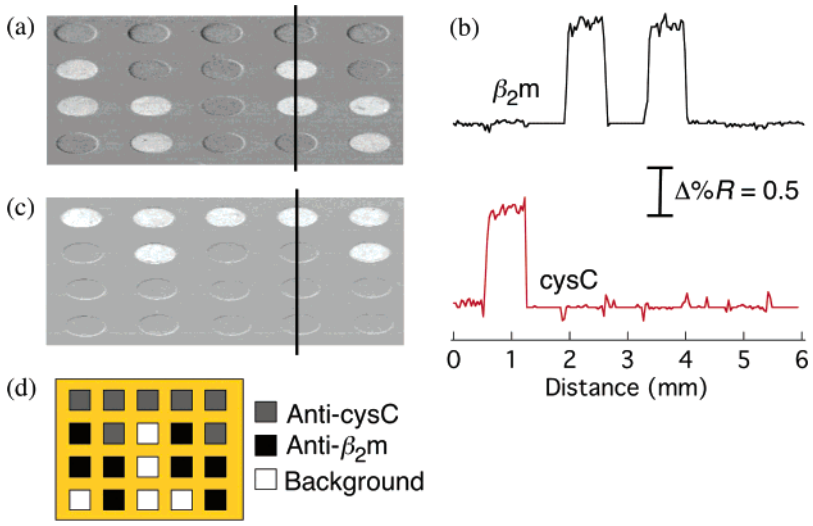


**Figure 3.** SPRI difference images obtained for the specific adsorption of 50 nM $\beta_2$m (a) and 100 nM cysC (c) onto a three-component antibody microarray. (b) The corresponding line profiles obtained for the adsorption of $\beta_2$m onto anti-$\beta_2$m (upper) and cysC onto anti-cysC elements (lower). The array consists of anti-$\beta_2$m, anti-cysC, and inactivated CDI-modified background elements with the pattern of the array shown in (d). The difference image was obtained by subtracting images taken before and after biomarker exposure. Upon exposure of 50 nM $\beta_2$m solution to the array, changes in $\Delta\%R$ were observed only at the anti-$\beta_2$m elements, with the line profile taken across the difference image (a) showing about a 1% increase in $\Delta R$. When 100 nM cysC was injected onto the array, analysis of the difference image (c) shows a 1% increase in $\Delta R$ only at the anti-cysC elements. Each array element has a diameter of 750 $\mu$m with a center-to-center separation of 1360 $\mu$m.

typically in the micromolar concentration range.[34,46,47] We therefore conclude that SPRI has ample sensitivity for the detection of these small protein biomarkers. The concentration dependence of the SPRI signal from $\beta_2$m and cysC is characterized further in the next section.

These SPRI experiments were repeated on a second microarray created with 200-$\mu$m elements formed on a continuous gold surface. Figure 4a shows a representative SPRI difference image from the adsorption of $\beta_2$m from a 10 nM solution onto a three-component array containing anti-cysC, anti-$\beta_2$m, and anti-SEB. The line profile in Figure 4b demonstrates that the $\beta_2$m binds specifically onto the anti-$\beta_2$m array elements with an increase in $\Delta R$ of ~0.7%. Again, negligible nonspecific binding was observed onto the other biomarkers or the inactivated CDI-modified background at these concentrations. As expected, the signal-to-noise ratio of the line profile from the smaller 200-$\mu$m array elements in Figure 4b is not as good as that observed with the 750-$\mu$m array elements (Figure 3b).

For array elements smaller than 200 $\mu$m, physical adsorption due to drying effects was observed. These drying effects included the formation of "coffee rings" in the elements similar to those observed previously during the formation of antibody arrays on amine-terminated glass and silicon nitride surfaces.[12,48,49] For these small array elements, monolayer coverages could only be achieved by ensuring that the number of antibody molecules in the spotting solution corresponded to a surface density of no more than $10^{12}$ molecules/cm$^2$. For antibody arrays with 200-$\mu$m elements or larger, drying effects were not observed. This was confirmed with control experiments (not shown) comparing antibody array elements that did not undergo the CDI activation process to antibody array elements that were created with the covalent CDI attachment chemistry. Therefore, to avoid antibody physisorption, we only used antibody arrays with element sizes ranging from 200 to 750 $\mu$m.

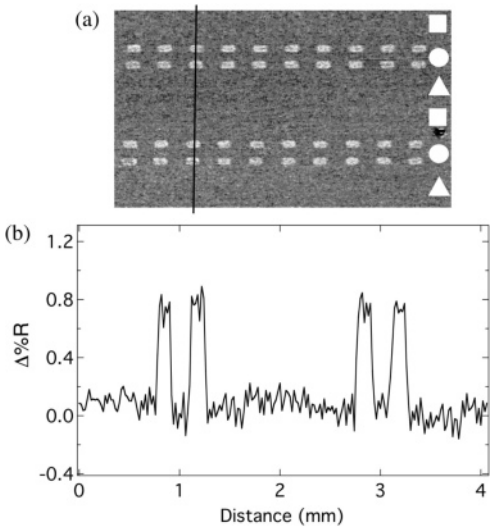


**Figure 4.** (a) Representative SPRI difference image and (b) corresponding quantitative line profile obtained for the specific interaction of 10 nM $\beta_2$m with anti-$\beta_2$m array elements. The array was patterned in alternating double rows of three different antibodies, anti-cysC (■), anti-$\beta_2$m (●), and anti-SEB (▲). Each array element was 200 $\mu$m × 200 $\mu$m with a center-to-center separation of 450 $\mu$m. The difference image was obtained by subtracting images acquired before and after $\beta_2$m adsorption. When the array was exposed to 10 nM $\beta_2$m solution, an ~0.7% increase in $\Delta R$ was observed only at the anti-$\beta_2$m elements from the line profiles taken across the difference images.

**Adsorption Strength Measurements for $\beta_2$m and cysC.** The quantitative analysis of the adsorption strength of a biomarker to

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(48) Gong, P.; Harbers, G. M.; Grainger, D. W. *Anal. Chem.* **2006**, *78*, 2342−2351.
(49) Gong, P.; Grainger, D. W. *Surf. Sci.* **2004**, *570*, 67−77.

a specific antibody is important both for understanding the limits of detection in real biological samples and for the development of better antibodies for these biomarkers. If the target biomarker (A) and the surface-bound antibody probe (B) interact in a simple 1:1 ratio, in the absence of bulk transport, the surface reaction can be written as

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \quad (1)$$

where AB is the surface-bound biomarker−antibody complex and $k_a$ and $k_d$ are the Langmuir adsorption and desorption rate constants, respectively. If $\Gamma$ is the surface coverage of this complex and $\Gamma_{max}$ is the total number of surface sites available to the biomarker, then the relative surface coverage ($\theta = \Gamma/\Gamma_{max}$) at equilibrium is given by the Langmuir adsorption isotherm:[20]

$$\theta = \frac{K_{ads}C}{1 + K_{ads}C} \quad (2)$$

where $C$ is the concentration of biomarkers and the Langmuir adsorption coefficient $K_{ads}$ is defined as $K_{ads} = k_a/k_d$.

The adsorption strengths of the two low molecular weight biomarkers $\beta_2$m and cysC onto two- or three-component antibody arrays (anti-cysC, anti-$\beta_2$m, and anti-SEB) were measured using equilibrium SPRI measurements. Figure 5 shows plots of the fractional surface coverage ($\theta$) obtained from $\Delta\%R$ measurements versus biomarker concentration for the adsorption of (a) cysC onto anti-cysC and (b) $\beta_2$m onto anti-$\beta_2$m array elements. These plots assume a linear relationship between $\Delta\%R$ and $\theta$, and the data points were fit to a Langmuir isotherm (eq 2). The best fit was obtained for cysC by letting $\Delta R = 1.0\%$ correspond to $\theta = 1$; for $\beta_2$m, the best fit was obtained by letting $\Delta R = 1.1\%$ correspond to $\theta = 1$. Each data point was obtained at equilibrium and is the average of five different SPRI chip measurements with a chip-to-chip variation of ~10%. The fit of the data to eq 2 also determined two Langmuir adsorption coefficients ($K_{ads}$); 1.0 (±0.11) × $10^8$ $M^{-1}$ for cysC and 1.4 (±0.15) × $10^8$ $M^{-1}$ for $\beta_2$m. The $K_{ads}$ values were then used to ascertain the antibody concentration corresponding to a 50% fractional surface coverage ($C_{0.5\theta}$). A $C_{0.5\theta}$ of 9 and 6 nM for cysC and $\beta_2$m, respectively, were obtained. These $C_{0.5\theta}$ values can be compared with the equilibrium dissociation constant ($K_D$) for the antibody−antigen interaction in homogeneous solution. Although the $K_D$ for these specific antibody−biomarker pairs have not been measured, values of 0.3 and 15 nM have been reported for the $K_D$ of these biomarkers with anti-cysC[47] and anti-$\beta_2$m[50] prepared by different methods.

For comparison, a separate set of biomarker binding experiments was performed using Biacore angle shift SPR measurements and antibodies bound to a dextran thin film. A kinetic analysis of the Biacore measurements gave similar values for $K_{ads}$: for cysC binding, $K_{ads} = 4.7 \times 10^8$ $M^{-1}$ ($k_a = 2.17 \times 10^5$ $M^{-1}\cdot s^{-1}$; $k_d = 4.61 \times 10^{-4}$ $s^{-1}$) and for $\beta_2$m, $K_{ads} = 5.6 \times 10^8$ $M^{-1}$ ($k_a = 9.97 \times 10^4$ $M^{-1}\cdot s^{-1}$; $k_d = 1.77 \times 10^{-4}$ $s^{-1}$). These nanomolar binding constants determine the limits of detection for the two biomarkers with both SPR and SPRI.

(50) www.abcam.com.

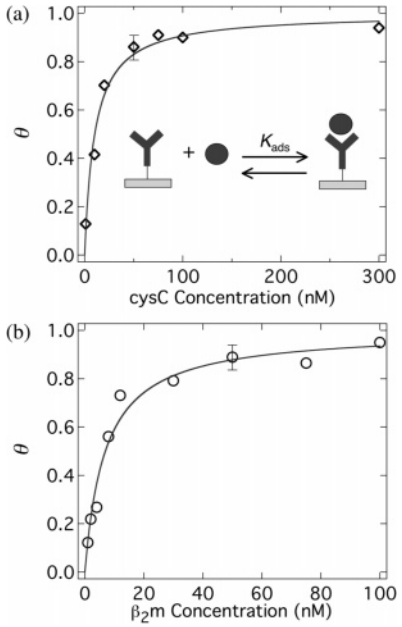


**Figure 5.** Fractional surface coverages ($\theta$) versus concentration for the adsorption of (a) cysC and (b) $\beta_2$m onto two- or three-component antibody arrays (anti-cysC, anti-$\beta_2$m, and anti-SEB). The fractional surface coverages were obtained from $\Delta\%R$ values for the adsorption of cysC and $\beta_2$m onto anti-cysC and anti-$\beta_2$m array elements, respectively. Concentrations ranged from 1 to 300 nM for cysC and 1 to 100 nM for $\beta_2$m. All measurements were performed at equilibrium and each data point is an average of $\Delta\%R$ measurements from 5 different chips. The solid lines in each figure are fits of the data to Langmuir isotherms. The figure inset in (a) shows a schematic of antigen adsorption onto surface-attached antibodies and the definition of $K_{ads}$.

## CONCLUSIONS

We have demonstrated in this paper the application of SPRI for the multiplexed direct detection of two low molecular weight protein biomarkers, $\beta_2$m and cysC. To detect these biomarkers with antibody arrays in real serum or urine samples, additional surface chemistries need to be developed in order to suppress the nonspecific adsorption of other proteins onto the array background. The reaction of amine-terminated poly(ethylene glycol) molecules with an unreacted CDI-modified surface is a potential solution.

The nanomolar binding affinities of these antibodies place the limits of detection for SPRI at ~1 nM. This detection limit demonstrates that the SPRI analysis of antibody arrays prepared on gold surfaces should be sensitive enough for the multiplexed detection of these clinically important biomarkers in real samples.[34,36] Antibodies with picomolar binding constants would allow substantially lower detection limits for biomarkers that need to be measured at lower concentrations. In addition, we will pursue the development of more sensitive SPRI methodologies—a nanomolar detection limit is sufficient for these two biomarkers, but there are many other low molecular weight protein biomarkers that are present in plasma samples at picomolar concentrations.

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