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Immobilized Antibody Orientation Analysis using Secondary Ion Mass Spectrometry and Fluorescence Imaging of Affinity-generated Patterns

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Abstract

This study assesses the capability of high-resolution surface analytical tools to distinguish immobilized antibody orientations on patterned surfaces designed for antibody affinity capture. High-fidelity, side-by-side co-patterning of protein A (antibody Fc domain affinity reagent) and fluorescein (antibody Fab domain hapten) was achieved photo-lithographically on commercial amine-reactive hydrogel polymer surfaces. This was verified from fluorescence imaging using fluorescently labeled protein A and intrinsic fluorescence from fluorescein. Subsequently, dye-labeled murine anti-fluorescein antibody (4-4-20), and antibody Fab and Fc fragments were immobilized from solution onto respective protein A- and fluorescein- co-patterned or control surfaces using antibody-ligand affinity interactions. Fluorescence assays support specific immobilization to fluorescein hapten- and protein A-patterned regions through antigen-antibody recognition and natural protein A-Fc domain interactions, respectively. Affinity-based antibody immobilization on the two different co-patterned surfaces generated side-by-side full antibody “heads-up” and “tails-up” oriented surface patterns. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis, sensitive to chemical information from the top 2-3 nm of the surface, provided ion-specific images of these antibody patterned regions, imaging and distinguishing characteristic ions from amino acids enriched in Fab domains for antibodies oriented in “heads-up” regions, and ions from amino acids enriched in Fc domains for antibodies oriented in “tails-up” regions. Principal component analysis (PCA) improved the distinct ToF-SIMS amino acid compositional and ion-

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specific surface mapping sensitivity for each “heads-up” versus “tails-up” patterned region. Characteristic Fab and Fc fragment immobilized patterns served as controls. This provides first demonstration of pattern-specific, antibody orientation-dependent surface maps based on antibody domain- and structure- specific compositional differences by ToF-SIMS analysis. Since antibody immobilization and orientation are critical to many technologies, orientation characterization using ToF-SIMS could be very useful and convenient for immobilization quality control and understanding methods for improving the performance of antibody-based surface capture assays.

Keywords

ToF-SIMS; antibody imaging; protein pattern; poly(ethylene glycol); orientation; immobilization; surface analysis

Introduction

An enormous history documents various methods to improve antibody-surface immobilization, attempting to address long-standing issues surrounding sub-optimal analyte capture and affinity binding performance on surfaces in numerous applications. Issues include antibody capture performance and validation challenges in enzyme-linked immunosorbent assays (ELISAs),¹ affinity chromatography separations,^{2, 3} lateral flow assays,⁴ antibody-based diagnostics,⁵ antibody microarray assay formats,⁶⁻⁸ and biosensors.^{9, 10} Among the many strategies proposed (i.e., better antibody-immobilized stability, sensitivity, density, and shelf-life, as well as reduced cross-reactivity), improved surface-immobilized antibody orientation is frequently a focus to improve their immobilized capture performance.^{6, 11, 12} This is intuitive since the analyte capture domains of the antibody (i.e., variable domains, Fv, within the antigen-binding Fab domains) occupy a distinct region of the antibody structure that, if immobilized against the surface, have little access to bind analyte. Previous orientation strategies provided some evidence for control of anti-hGG (hCG: human gonadotropin) antibodies at surfaces by controlling the surface charge.¹³ This approach essentially reinforced an earlier concept for improving antibody surface-immobilized bioactivity using mild acid treatment,¹⁴ seeking to exploit influences from the antibody dipole that points from its Fc region toward the F(ab')₂ fragments. However, these weak dipole interactions are neither sufficiently strong nor specific to maintain antibody orientation or conformation on surfaces in the face of many other surface forces, including hydrophobic forces, dehydration and dessication, and other surface-protein forces. For example, anti-hGG is postulated to assume mixed orientations: from more “end-on” to more “side-on” on an amine-terminated organic adlayer.¹³

Protein A and protein G, two bacterial membrane proteins with long-known specific binding affinities for the antibody Fc domain,¹⁵⁻¹⁹ have both been commercially produced and extensively used as surface affinity ligands to bind and orient antibodies for solid-phase affinity use.^{20, 21} Because both of these bacterial proteins bind the antibody Fc domain with reasonable reliability and affinity, and can be immobilized on solid supports, this produces a ready method in principle for both immobilizing and orienting antibodies on surfaces. Evidence supporting improved antibody immobilization claims in these cases often are derived from data for antigen or target binding efficiencies, indirectly asserted to result from this antibody Fc-protein A (G) interaction pairing. Rarely are direct measurements of antibody surface orientation made, nor normalized against antibody surface densities, to tease out the details of how surface orientational influences, distinct from changes in immobilization density, affect surface capture data. This is partly due to the difficulty in assessing protein-surface orientation and distinguishing orientation parameters in immobilized ensembles from effects of protein denaturation and conformational changes, as well as antibody orientational distributions. Few methods permit such discrimination.²²⁻²⁹

The current study seeks to produce new analytical methods to understand and interrogate antibody-immobilized orientations using patterned antibodies in desired orientations in adjacent regions on surfaces as a model. Recent work has served as precedent for this concept by patterning two different ligands into adjacent regions of a reactive polymer surface using an identical photolithography approach, then allowing self-selection of these surface ligands by their respective protein binding partners from a mixed aqueous solution.³⁰ High protein-surface self-selection fidelity to each surface-immobilized ligand was shown using high-resolution time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging of the patterned surfaces using protein-specific ion fragments from each immobilized protein.

This ‘ligand patterning, protein self-sorting, and surface mapping’ strategy has now been extended to antibodies to produce new immobilized antibody orientation information. Surface chemical patterns containing immobilized ligands selective for different antibody domains on a well-studied model murine-derived anti-fluorescein monoclonal antibody (MAb 4-4-20)³¹⁻³³ were exploited to capture the 4-4-20 antibody in two general postulated orientations (i.e., Fab “heads-up” vs. Fc “tails-up”). The surface amino acid compositions of the different domains exposed in the two antibody orientations is distinct. So, ToF-SIMS imaging, sensitive to the outermost 2-3 nm of the surface, was used to produce amino acid fragmentation information from the immobilized antibodies in each pattern. This surface-sensitive mass spectrometry information distinguishes immobilized antibody orientation by comparing ToF-SIMS image contrast for amino acids derived from each antibody domain captured on each ligand-patterned surface. The method should be general and useful for producing new information for other immobilized proteins on technologically and scientifically interesting surfaces.

1. Experimental

1.1 Materials

Recombinant protein A, anti-fluorescein/Oregon Green® mouse monoclonal 4-4-20 antibody, 5-carboxy-tetramethylrhodamine succinimidyl ester (TAMRA-NHS), Alexa Fluor® 647 succinimidyl ester (Alexa647-NHS) and fluorescein-5-isothiocyanate (FITC) were purchased from Invitrogen (CA, USA). Sephadex® G-50, CM Sephadex® C-25, Tween® 20, pepsin, 2-methoxyethylamine, triethylamine (TEA), dimethyl sulfoxide (DMSO), N-dimethylformamide (DMF), 11-bromoundecanoic, sodium thiosulfate, 1,4-dioxane, iodine, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), ethyl acetate, hexane, bovine serum albumin (BSA), 2-propanol, and fluoresceinamine isomer I (Sigma-Aldrich, WI, USA) were all used as received. Murine IgG-class polyclonal antibody Fab and Fc fragments were obtained from Rockland Immunochemicals (PA, USA). PEG-diamine (MW 2000) was from Nektar Therapeutics (CA, USA). All other chemicals and silica gel were obtained from Fisher Scientific (PA, USA). Gold-coated silicon pieces were obtained from SPI Supplies (USA). Carboxylic acid-terminated tetra(ethylene glycol) undecanethiol was purchased from Assemblon (WA, USA). Commercial amine-reactive polymer-coated glass microarraying slides (Slide-H™, Schott-Nexterion, Germany)³⁴⁻³⁶ were used for all surface patterning and subsequent antibody capture or immobilization studies.

1.2 Protein fluorescent probe labeling and purification

Fluorescence labeling of proteins followed protocols previously described.^{30, 36} Protein A (10mg/mL in 100 µL phosphate buffered saline (PBS)) was mixed with sodium bicarbonate (1M, 10 µL) and amine-reactive rhodamine dye derivative (TAMRA-NHS, 10mg/mL in 20 µL DMSO), and incubated with shaking for 1 hr at room temperature (RT). TAMRA-labeled protein A (protein A-TAMRA) was then purified using aqueous elution through a Sephadex G-50 column. The final concentrations of protein A and conjugated TAMRA were 1.5 mg/mL

and 92 μM (4.2 mols TAMRA/mol protein), respectively, as determined by optical absorption spectrophotometry.

Similarly, the 4-4-20 monoclonal antibody (5mg/mL in 100 μL PBS) was first mixed with sodium bicarbonate (1M, 10 μL) and Alexa647-NHS (10mg/mL in 10 μL DMSO). After 1-hour incubation with shaking at RT, the Alexa647-labeled 4-4-20 antibody (Ab-647) was purified using aqueous elution through a Sephadex G-50 column. Final antibody concentrations and conjugated Alexa647 dye were 280 $\mu\text{g/mL}$ and 20.3 μM respectively (10.9 mols/mol protein). Murine antibody Fab and Fc fragments were also similarly conjugated with Alexa647. The Fc fragment (500 μL , 1mg/mL in PBS) was incubated with Alexa647 (20 μL , 10mg/mL in DMSO) for 2 hours at RT. Concentrations of protein and Alexa647 were 220 $\mu\text{g/mL}$ and 13.4 μM , respectively. The Fab fragment (500 μL , 1.5 mg/mL in PBS) was incubated with Alexa647 (20 μL , 15mg/mL in DMSO) for 2 hours at RT. Final concentrations of protein and Alexa647 dye were 760 $\mu\text{g/mL}$ and 36.0 μM , respectively.

For Fab and Fc fragment direct covalent immobilization to patterned NHS-reactive polymer surfaces, the 4-4-20 antibody was pepsin-digested by dissolving the 4-4-20 antibody (1.5mg/mL) in 50 mM acetate buffer (pH 4.5, 20 μL).^{37, 38} Pepsin (1 μg , 2.5 w/w% vs. antibody) was then added to the antibody solution and incubated for 20 hours at 37°C. The reaction was terminated by adding 1M Tris (4.5 v/v% vs. reaction solution volume) into the reaction mixture. The Fab and Fc portions were separated using aqueous elution through a Sephadex G-50 column, and the resulting fragments (50 $\mu\text{g/mL}$ as determined by optical spectroscopy) were used for surface immobilization onto commercial polymer microarray slides as detailed below.

1.3 Preparation of fluorescein-labeled PEG-amine spacer hapten

PEG-diamine (MW: 2000, 100 mg) was mixed with FITC (20mg) in 5 mL DMF at 1:1 molar ratio with stirring for 20 hours at RT.³⁹ After DMF evaporation, the fluorescein-PEG-amine (F-PEG-NH₂) was dissolved in distilled water and purified using CM Sephadex® C-25. F-PEG-NH₂ was eluted with 25mM sodium chloride solution. After dialysis through a cellulose membrane (MWCO: 1000, Spectrum Industries, Los Angeles, CA), F-PEG-NH₂ was concentrated by evaporation and then adjusted to 15 mM in PBS containing 0.01% Tween® 20 (PBST) containing 1mM TEA, as determined by optical absorption spectrophotometry.

1.4 Preparation of protein A- and fluorescein hapten- patterned surfaces

Microlithographically patterned polymer-coated, amine-reactive commercial microarray slides were prepared to yield co-planar patterns of adjacent methoxy-capped non-reactive (inert) and NHS-bearing amine-reactive regions (NHS/MeO patterns) as previously reported.^{30, 36} Briefly, uniform commercial NHS-reactive hydrogel polymer surfaces are processed with standard photoresist and standard optical photolithographic processing methods to yield surface patterns containing repeated adjacent zones where surface methoxylation by incubation with 50 mM 2-methoxyethylamine (50 mM borate buffer, pH 9.0) for 1 hour at RT removes the NHS amine-reactivity, leaving a non-reactive methoxy-cap in place of NHS, shown to eliminate covalent immobilization and drastically limit non-specific protein capture.^{30, 36} To remove residual photoresist, slides were sonicated in DMSO, acetone and 2-propanol for 20 seconds each, producing clearly delineated adjacent micron-sized zones of NHS and methoxy-capped surface chemistry.⁴⁰ These NHS-bearing, amine-reactive, patterned polymer slides (NHS/MeO surfaces) were used for protein patterning as described.^{30, 36}

For protein A covalent immobilization, 1.5 mg/mL of protein A-TAMRA was dissolved in PBS (120 μL). The protein A solution was sandwiched between two identical NHS-patterned (NHS/MeO) slides and incubated overnight at 4°C. After rinsing with PBS and distilled water, the slides exhibited fluorescence when excited at 532 nm. Fluorescence images were scanned

and recorded using an Axon GenePix 4000B microarray scanner (Molecular Devices Corp., CA) (ex. 532 nm; em. 557-592 nm).

The F-PEG-NH₂ solution (15 mM in PBST containing 1mM TEA) was also similarly sandwiched between NHS-patterned (NHS/MeO) slides and incubated overnight at 4°C. After washing with PBST and distilled water, fluorescein patterns on slides were scanned and imaged by the identical method described above. For co-patterning of both protein A and fluorescein, F-PEG-NH₂ was first immobilized to polymer surface regions not covered by the photo-resist coated (protected) regions (i.e., native NHS-derivatized polymer surfaces without photoresist coating). After removal of photoresist by UV irradiation and resist processing, the F-PEG-NH₂ solution was then sandwiched between the NHS-patterned (photoresist/NHS) slides for fluorescein immobilization only to UV exposed regions where the photoresist had been removed (photoresist/fluorescein). After removing the remaining photoresist from the protected regions by sonication (NHS/fluorescein), protein A solution (2 mg/mL) was sandwiched between two identical NHS/fluorescein slides and incubated overnight at 4°C. The resultant co-patterned slides (protein A/fluorescein) were used for the oriented antibody immobilization study. As a control for the co-patterned samples, NHS regions of the NHS/fluorescein slide were inactivated by methoxylation to obtain MeO/fluorescein patterned surfaces.⁴⁰

1.5 4-4-20 antibody capture by protein A-patterned surfaces

Using the well-known capability of protein A to bind antibody Fc domains,¹⁶⁻¹⁸ the 4-4-20 antibody and Fc fragments were surface-captured using protein A-immobilized surfaces.^{17, 18} First, unlabeled protein A (2 mg/mL) was covalently immobilized to the NHS-patterned regions on NHS/MeO patterned slides as described for protein A-TAMRA patterning (vida supra). Then, Ab-647 solution (15 µg/mL) prepared in PBST containing 0.1% BSA was added (150 µL) onto the protein A-patterned polymer surface (protein A/MeO) and incubated for 1 hour at RT with gentle oscillatory shaking. After washing with PBST and distilled water, the slides were scanned (ex: 635 nm; em: 650-690 nm). In separate control experiments used to evaluate non-specific immobilization, Fc-647 and Fab-647 antibody fragment solutions (15 µg/mL) were also exposed to the protein A-patterned slides. For these control experiments, Fc and Fab fragments were sourced as murine polyclonal antibodies as described (vida supra).

1.6 4-4-20 antibody capture by fluorescein hapten-patterned surfaces

Anti-fluorescein antibody 4-4-20 antibody was used to generate antibody patterns on fluorescein hapten-immobilized surfaces through its known antigen-antibody interactions at different surfaces.^{41, 42} Fluorescein was first patterned on NHS/MeO patterned slides (Table 1, FM), and then fluorescent labeled Ab-647, Fc-647 or Fab-647 (each 15 µg/mL in PBST containing 0.1% BSA) were added onto separate fluorescein-patterned (fluorescein/MeO, Table 1, FM) slides for 1 hour at RT with gentle shaking. After washing with PBST and distilled water, slides were scanned to obtain both pixelated fluorescence intensities and fluorescence images.

1.7 4-4-20 antibody capture on protein A/fluorescein hapten co-patterned surfaces

Co-patterned protein A and fluorescein ligands in adjacent regions on surfaces (i.e., protein A/fluorescein) were prepared as described: F-PEG-NH₂ solution was first sandwiched between photoresist/NHS slides overnight at 4°C to immobilize F-PEG-NH₂ only to NHS-reactive regions. After removal of photoresist by sonication, protein A solution was then sandwiched between the newly exposed NHS/fluorescein patterned slides, resulting in protein A/fluorescein co-patterned slides (Table 1, PF). Then 4-4-20 antibody solution (non-labeled, 15 µg/mL) was exposed to the protein A/fluorescein slides and incubated for 1 hour at RT with gentle shaking. After washing with PBST and distilled water, these slides were dried and kept

under nitrogen at -20°C before ultra-high vacuum (UHV) surface analysis and image processing.

1.8 Synthesis of 11, 11'-dithio-bis(undecanoic acid) for control immobilization studies ⁴³

Sodium thiosulfate (9.35 g, 37.7 mmol) was added to 150 ml 50% aqueous 1,4-dioxane containing 11-bromo-undecanoic acid (10.0 g, 37.7 mmol). Then the mixture was heated under reflux at 90°C for 3 hours. The corresponding disulfide was oxidized *in situ* by adding iodine in portions until the reaction solution achieved a yellow to brown color. Iodine surplus was treated with 20% sodium pyrosulfite aqueous solution. After removal of 1,4-dioxane by rotary evaporation, the creamy suspension was filtered to yield the product 11,11'-dithio-bis(undecanoic acid). Recrystallization from ethyl acetate/tetrahydrofuran (THF) afforded 8.0 g of product (yield 89%): ^1H NMR (400 MHz, CDCl_3): 2.68 (t, 2H), 2.34 (t, 2H), 1.69-1.56 (m, 4H), and 1.40-1.29 (m, 12H).

1.9 Synthesis of 11,11'- dithio-bis(succinimidylundecanoate) (DSU) ⁴³

NHS (0.14 g, 1.2 mmol) was added to 50 mL THF containing 11,11'-dithio-bis(undecanoic acid) (0.26 g, 0.6 mmol), and 0.24 g DCC (1.2 mmol) and reacted at 0°C for 3 hours. The reaction mixture was warmed to RT and stirred for 36 hours at RT; the dicyclohexylurea was filtered off. Removal of solvent under reduced pressure, and recrystallization from acetone/hexane provided DSU as a white solid. Final purification was achieved by medium pressure liquid chromatography using silica gel and a 2:1 mixture of ethyl acetate and hexane, affording 0.29 g, (yield 75%): ^1H NMR (400 MHz, CDCl_3): 5.283 (s, 4H), 2.68 (t, 2H, $J = 7.3$ Hz), 2.60 (t, 2H, $J = 7.5$ Hz), 1.78-1.63 (m, 4H), and 1.43-1.29 (m, 12H); FAB-MS (Cs^+ , 20 keV): MALDI MS M^+ m/z Calcd. for $\text{C}_{26}\text{H}_{44}\text{NO}_5\text{S}_2^+$: 514.27, $\text{C}_{30}\text{H}_{48}\text{N}_2\text{O}_8\text{S}_2$ 628.29. Found: 514.24 and 628.28.

1.10 Formation of DSU monolayers on gold substrates

Gold-coated silicon substrates were cleaned immediately before use under oxygen plasma (5 min, 80-90W, 0.15 mbar). These treated gold substrates were immersed immediately into 1mM DSU ethanol solution for 16 hours, then removed from solution and rinsed extensively with ethanol and distilled water to remove physisorbed materials, and dried.

1.11 Covalent immobilization of protein A and fluorescein on gold surfaces

1.11.1 Protein A immobilization on gold—Protein A (1.67 mg/mL) was first dissolved in PBS and then the DSU monolayer substrates were immersed into this protein solution for 48 hours at 4°C . After rinsing with PBS and distilled water, the substrates were dried under a stream of nitrogen, and immediately used for capture of 4-4-20 antibody from solution as described above.

1.11.2 Fluorescein hapten immobilization on gold—Carboxylic acid-terminated tetra(ethylene glycol) undecanethiol was dissolved in 20 mL ethanol (0.83 mg/mL). Freshly plasma-treated gold substrates were immersed in this solution for 24 hours at RT. After rinsing with ethanol and distilled water, these substrates were dried under a stream of nitrogen and immediately immersed into a mixture of EDC (14 mg/mL) and NHS (3 mg/mL) aqueous solution for 2 hours at RT. Thereafter, the sample was rinsed with ethanol and distilled water and dried under a stream of nitrogen, and immediately used for reaction with fluoresceinamine. The NHS-adlayer-terminated gold substrates were immersed in fluoresceinamine isomer I solution (17.35 mg, first dissolved in DMSO and then into 9 mL PBS) for 12 hours at RT. After rinsing with ethanol, the substrates were dried under a stream of nitrogen, and then immediately used for capture of anti-fluorescein antibody.

1.12 4-4-20 antibody binding to protein A- and fluorescein- immobilized gold model substrates

Anti-fluorescein 4-4-20 antibody solution (50 µg/mL) was prepared in PBST containing 0.1% BSA and exposed to separate protein A- and fluorescein- immobilized surfaces on gold supports, and incubated for 2 hours at 4°C with gentle shaking. After washing with PBST and distilled water, the slides were dried under a stream of nitrogen and kept at -20°C before surface analysis.

1.13 Fab and Fc fragment immobilization on DSU monolayers on gold as control surfaces

Polyclonal murine Fab and Fc fragment solutions were prepared in PBST (1.5 µg/mL) containing 0.1% BSA. Both solutions were exposed to DSU monolayer surfaces activated with NHS groups and incubated for surface coupling reactions for 48 hours at 4°C. After washing with PBS and distilled water, these slides were dried under a stream of nitrogen and kept at -20°C before surface analysis.

1.14 Time-of-Flight Secondary Ion Mass Spectrometric (ToF-SIMS) analysis of patterned surfaces containing different captured proteins

ToF-SIMS data for all patterned ligand-, protein-, and antibody- captured surfaces were acquired on an ION-TOF 5-100 instrument (ION-TOF GmbH, Münster, Germany) using a Bi³⁺ primary ion source. Positive and negative ion images and spectra were acquired with a pulsed 25 keV, 1.3 pA primary ion beam in high current bunched mode from 500 µm × 500 µm areas on sample surfaces. All images obtained contained 128 × 128 pixels. These analysis conditions resulted in an instrumental spatial imaging resolution of approximately 4 microns.⁴⁴ Data were collected using an ion dose below the static SIMS limit of 1×10¹² ions/cm². A low-energy electron beam was used for charge compensation on the polymer-coated glass slides. The mass resolution (m/Δm) for the negative secondary ion spectra was typically between 6000 and 7500 for the m/z=25 peak. The mass resolution (m/Δm) for the positive secondary ion spectra was typically between 7000 and 8500 for the m/z=27 peak. The ToF-SIMS images were used to reconstruct spectra from a 100 µm × 100 µm area of interest. Principal component analysis (PCA) was performed on these data as described previously using a series of scripts written by NESAC/BIO for MATLAB (MathWorks, Inc., Natick, MA).^{45, 46} All ToF-SIMS data were normalized to total ion intensity prior to PCA processing. ToF-SIMS/PCA scores image maps of the surfaces were then constructed.^{40, 47} ToF-SIMS measured image line resolution from the samples was calculated to be 6.0 ± 0.4 microns as derived from multiple line scans, approximately the same resolution as the photomask.³⁶

1.15 X-ray photoelectron spectrometry (XPS) analysis of polymer surfaces

All XPS measurements were performed on a Kratos Axis Ultra DLD x-ray photoelectron spectrometer employing a hemispherical analyzer for spectroscopy and a spherical mirror analyzer for imaging.⁴⁸ Spectra were acquired with a monochromated Al-K_α x-ray source and a 0-degree take-off angle (TOA) in the “hybrid” mode. The TOA is defined as the angle between the sample surface normal and the axis of the XPS analyzer lens. A low-energy electron flood gun was used to minimize surface charging. This condition produces a sampling depth of approximately 10 nm. XPS data was collected using an analysis area of 700 µm × 300 µm. For each sample, an initial compositional survey scan was acquired, followed by a detailed elemental scans using a pass energy of 80 eV. High-resolution C1s spectra were collected using a pass energy of 20 eV and were charge-referenced to the C1s hydrocarbon peak set to 285.0 eV. Surface elemental values reported for the composition were averages of values determined from three spots on each type of surface. Data analysis was performed with Vision Processing data reduction software (Kratos Analytical Ltd.) and CasaXPS (Casa Software Ltd.).

2. Results and Discussion

Previously published ligand patterning methods were used to regionally co-immobilize both protein A and fluorescein as antibody-specific binding ligands to a planar patterned polymer surface previously shown (1) to be easily and reliably patterned, (2) to retain high surface coupling functionality in NHS-bearing, amine-reactive regions after patterning, and (3) to exhibit high resistance to non-specific binding in methoxy-capped regions.^{30, 36} These patterned polymer surfaces were used to bind specifically the Fc (to protein A) and Fab (to fluorescein hapten) domains of the known, well-published anti-fluorescein 4-4-20 antibody and its fragments^{33, 41, 49}. This strategy is intended to produce antibody surface pattern formation in adjacent surface lithographed regions, containing both antibody “heads-up” and “tails-up” patterns on protein A- and fluorescein- immobilized regions, respectively. Protein A's well-known specificity for binding antibody Fc domains and fragments¹⁵⁻¹⁹ has been frequently exploited for purifying antibodies from crude protein mixtures,⁵⁰ immobilizing antibodies to surfaces^{17, 51} or coupling with markers to detect the presence of antibodies.¹⁸ Fluorescein hapten is known to be specifically bound by the 4-4-20 anti-fluorescein monoclonal antibody through classic Fab site antigen-antibody interactions.^{32, 33, 41, 42} Thus, patterned protein A and fluorescein immobilized ligands were used to capture the 4-4-20 antibody in “heads-up” and “tails-up” orientations. This patterning success is confirmed using protein chemistry-specific, surface sensitive imaging methods. Results presented below confirm these achievements using a novel surface analytical mapping approach to imaging specific amino acids known from the antibody compositional and crystallographic data³¹⁻³³ to be enriched in either the antibody's Fab or Fc domains.

To analyze surface-immobilized biomolecules with ToF-SIMS, samples are typically air-dried and then introduced into the ultra-high vacuum (UHV) chamber. This process can lead to significant denaturation of proteins and antibodies. If significant denaturation occurred this could limit the utility of ToF-SIMS to determine the conformation and orientation of these surface-bound biomolecules. Trehalose coating^{52, 53} as well as light glutaraldehyde crosslinking⁵⁴ have been shown to preserve protein conformation and orientation for air-dried samples placed under UHV conditions. It has also been shown that surfaces with high density of PEG chains can maintain protein conformation during air drying and ToF-SIMS analysis⁵⁵. Since the polymer-coated array surface in the current study has a high density of PEG chemistry,³⁵ protein denaturation should be minimized when air-dried and analyzed under UHV with ToF-SIMS.

Detailed description of the lithographed commercial hydrogel polymer surface patterns used in this study, their generation and extensive surface characterization has been previously reported.^{30, 34-36, 40} The photomask patterning concept used for obtaining these patterns and the resulting sample identifications used for the various patterned surfaces in this study are shown in Figure 1 and Table 1, respectively.

2.1 Surface pattern fluorescence imaging

2.1.1 Protein A and fluorescein patterns on photolithographed NHS-patterned polymer surfaces—Figure 2 (a) and (b) shows fluorescence images for the PM and FM single-ligand patterned surfaces, respectively. These images clearly indicate that protein A and fluorescein are reliably immobilized into their intended patterned regions (bright signal) specifically through the NHS chemistry on the NHS/MeO patterned surface. As expected, the inactivated methoxy-capped regions (MeO, non-fluorescent, black) exhibit little chemical reactivity or non-specific binding as previously reported.^{30, 36}

2.1.2 Capture of anti-fluorescein 4-4-20 (Ab-647) antibody by protein A- and fluorescein-patterned surfaces—Anti-fluorescein 4-4-20 antibody (Ab-647) was

affinity-immobilized onto patterned protein A (PM) and fluorescein (FM) single-ligand/methoxy-capped co-patterned samples. Figure 2(c) shows antibody-generated fluorescence for the PM surface, supporting specific reaction of the full Ab-647 only on regions where protein A was immobilized. No signal was obtained when this surface was exposed to antibody fragment Fab-647 as shown in Figure 2(d). In contrast, fragment Fc-647 patterned similarly onto these same PM surfaces, similar to full antibody in Figure 2(c) (see Figure 2(e)). These images clearly indicate that, as predicted, anti-fluorescein 4-4-20 antibody (Ab-647) specifically interacts with surface-immobilized protein A through Fc recognition. Thus, when this antibody interacts with protein A-immobilized regions, a predominant “heads-up” orientation is expected in these regions (see Figure 1(b)).

Figure 2(f) shows a fluorescence image of Ab-647 patterned on FM surfaces, exhibiting selective capture of labeled 4-4-20 antibody by patterned fluorescein hapten regions. Significantly, fluorescein alone shows little detectable intrinsic fluorescence under this instrumental scanning and imaging condition, although fluorescence patterns can be observed under confocal microscopy (not shown). The FM surface was also exposed to Alexa647-labeled polyclonal murine antibody fragments (Fab-647 and Fc-647) lacking specificity for the fluorescein hapten. Absence of any antibody fluorescence in Figure 2(g) and (h) indicates that neither of these two antibody fragments bound to this surface. This validates the specificity of the anti-fluorescein 4-4-20 antibody to interact specifically with the patterned fluorescein through antigen-Fab domain interactions (and associated intrinsic non-specific binding resistance of the adjacent MeO-capped regions). This result also supports the ability to reliably orient the bound antibody's Fc domain away from the surface (i.e., “tails-up”) when anti-fluorescein antibody attaches to patterned fluorescein regions (see Figure 1(b)), an assertion to be validated with the ToF-SIMS analysis described below.

2.1.3 Patterning of anti-fluorescein 4-4-20 antibody on dual-ligand patterned surfaces (PF)—When co-patterning protein A and fluorescein onto the same sample (i.e., the PF slide), fluorescein-PEG-amine was immobilized as the PEG spacer-bound fluorescein hapten onto regions where methoxylation capping was produced in the other PM and FM samples. Previous analysis of this co-patterning has shown that NHS reactive ester chemistry is reduced in this region, attributed to lithographic processing and trace amounts of photoresist that are only detectable by ToF-SIMS analysis.^{30, 40} Use of the PEG polymer spacer for co-patterned antibody binding studies allowed the fluorescein hapten some opportunity to achieve the same capture layer dimensionality (i.e., thickness) as the neighboring protein A regions capturing antibody by Fc interactions. Figure 3(a) shows the fluorescence image for Ab-647 captured on the MF surface (complementary to the image in Figure 2(f)). Intensity of the fluorescence signal (Figure 3(b)) clearly indicates that antibody surface-immobilized amounts depend on which lithographed reactive region (i.e., unmasked or resist-masked) the fluorescein hapten is immobilized. While the full antibody was patterned on the UV exposed regions (MF slides), its intensity decreases compared to the non-UV exposed regions (FM slides), consistent with our previous work showing that the photoresist development process decreases immobilization of amine-containing molecules.⁴⁰ Also, as shown in the XPS experiments described below in section 2.2.2, Protein A layers immobilized more antibodies than fluorescein layers. So, both of these effects likely contribute to significantly higher fluorescence intensity observed when the Ab-647 was attached to MF slides compared to FM slides.

2.2 ToF-SIMS chemical imaging of surface patterns

Fluorescence imaging results directly support site-specific, high-fidelity pattern immobilization of anti-fluorescein antibody on PM and MF surfaces. Adjacent immobilization chemistries were designed to capture antibodies by known affinity interactions in reverse

orientations depending on their capture mode. This strategy should exert reliable control over surface-immobilized antibody orientation if antibody Fab domains were predominantly exposed away from the surface when immobilized on protein A regions, and Fc domains predominantly exposed away from the surface when bound to fluorescein hapten-immobilized regions. Existing commonly employed analytical methods (e.g., SPR, TIRF, AFM) have difficulties clearly distinguishing such differences in antibody conformational or orientational differences on surfaces. A newly developed ToF-SIMS amino acid fragment surface mapping/imaging technique was exploited to provide data directly from these different antibody-bound surfaces to validate this idea.^{30, 36, 40} ToF-SIMS is an exquisitely sensitive surface analytical tool: use of liquid metal ion gun (LMIG) sources in the static mode provides a rich variety of surface chemical information from the top 2-3 nm of the surface. Since the anti-fluorescein antibody is roughly 10-12 nm in any dimension,^{31, 41} ToF-SIMS methods should be capable of discerning amino acids specific to either the antibody Fab or Fc regions (i.e., the top 2-3 nm-deep zones of each antibody layer exposed to the ambient interface) if: (1) the amino acids at the surfaces of these different antibody domains were compositionally distinct,⁵⁶ (2) antibody orientation was relatively consistent across the sampling area of the ToF-SIMS analysis, and (3) the method was capable of sorting the complex surface mass spectra to provide these distinguishing features. With such performance, the strategy would be useful to distinguish “heads-up” from “tails-up” antibody orientations. Our recent work characterizing self-sorting protein mixtures on surfaces with ToF-SIMS imaging demonstrated the ability to chemically distinguish and ‘map’ subtle protein compositional differences in two different proteins in patterned surface regions analogous to the patterns used here.^{30, 36} The ability to extend this method to distinguish differently oriented immobilized protein regions in patterned surfaces would be particularly valuable for several applications, including protein-based surface-capture diagnostics (i.e., ELISA, lateral flow), antibody microarrays, lab-on-chip devices, multiplexed immobilized protein assays, and biosensors.

ToF-SIMS has been used to characterize proteins and other biological molecules on various substrates by collecting and identifying characteristic ion fragments originating from constituent amino acids.^{57, 58} However, since the overall amino acid composition of most proteins is generally restricted to the natural amino acid library, with only small differences in amino acid composition often observed between proteins, similar amino acid fragments are frequently detected from different protein ensembles on surfaces.⁵⁸ Nonetheless, compositionally distinct proteins,³⁰ or exotic recombinant mutants containing non-natural amino acids, or regio-selective interrogation of protein domains enriched in specific amino acids all provide new opportunities for ToF-SIMS to distinguish protein conformation, populations or structures at surfaces.

Amino acid compositional variation intrinsic to the 4-4-20 antibody's Fab and Fc fragments are analogously small; predictably, most ToF-SIMS data yield similar fragment patterns and intensities from these fragments from each of the two surface regions. However, as demonstrated recently,^{30, 59} careful data analysis can uniquely distinguish very similar biopolymer systems at surfaces. The multivariate statistical analysis technique, Principal Component Analysis (PCA), provides increased sensitivity for parsing similar ToF-SIMS data in such situations. PCA is used to decrease the dimensionality of complex datasets for more convenient handling, analysis and data interpretation, by transforming data sets to a new coordinate system so that the greatest variances across the dataset are identified. The highest PCA variance is contained in the first transformed variable, referred as the first principal component (PC-1); the second greatest variance is contained in PC-2, the third greatest variance in PC-3, and so on. In this case, PCA differentiates between the mass spectral amino acid fragments originating from the different regions of the patterned surfaces. Apart from antibody orientation, ion peak intensity (and hence their resulting loadings in resulting PC plots) of a particular surface-derived fragment will also depend on the region's amino acid composition.

For this reason, amino acid compositions for the two targeted antibody fragments, F(ab')₂ (4FAB) and Fc (11GT), were calculated from a public database [www.pdb.org], as shown in Table 2. One feature of these data, shown in the far right column, is that some amino acids exhibit large differences in composition between Fab and Fc fragments. These differences were exploited with PCA processing of ToF-SIMS data to distinguish control Fab and Fc immobilized imaging data and to compare to “heads-up” and “tails-up” immobilized antibody patterns.

2.2.1 ToF-SIMS and PCA analysis of surface-bound Fab and Fc fragments—PCA of the ToF-SIMS data was used to identify which specific amino acid fragments, or fragment intensity differences, distinguish differences between the control Fab and Fc antibody fragments immobilized chemically on functionalized gold adlayers and the PEG-based, hydrogel-coated glass slides. This information is essential for interpreting orientation data from the complex full antibody patterns on the PEG-based commercial hydrogel surfaces. One critical step for obtaining useful information from this analysis is the selection of m/z peaks from the immense peak sets generated from each protein surface (>10⁴ peaks in some cases). As PCA identifies the largest variations in the data set, to obtain PCA results that can be directly related to differences in the Fab and Fc fragments, it is best to only use ion fragments that are characteristic of these antibody fragments. Using this approach minimizes the influence of other differences such as surface coverage on the PCA results. A typical starting point for PCA processing of proteins is to include all major amino acid fragments in the analysis.^{60, 61} However, the underlying polymer microarray surface chemistry^{34, 35} produces interfering peaks at similar m/z values as many of the amino acid fragments, complicating this approach.³⁰

To obtain a comprehensive m/z peak list and also to understand effects of surface composition on the fragmentation pattern, two sets of control samples were analyzed by ToF-SIMS. Polyclonal model murine Fab and Fc fragments immobilized onto gold-NHS self-assembled DSU monolayers as well as the commercial polymer slides. Rigorous surface characterization of both of these organic adlayer substrates using XPS and ToF-SIMS has been reported previously.^{34-36, 62} PCA was performed on data obtained from these four samples using the full amino acid peak set from reference⁵⁶. Amino acid compositions for F(ab')₂ and Fc components from Table 2 was used to interpret the loadings data obtained from PCA. The right-hand column of Table 2 shows ratios of the two amino acid components in Fab:Fc domains: ratios greater than 1 indicate that the corresponding amino acid should load positively for the Fab sample, and ratios lower than 1 indicate that the corresponding amino acid should load positively for the Fc sample. PC-1 predictably captures the differences between the polymer and the gold substrate chemistry, but importantly PC-2 captures the difference originating from the two different antibody fragments as shown in Figure 4. Samples with Fc fragments loaded positively in PC-2, whereas samples with Fab fragments loaded negatively in PC-2. Figure 5 shows the PCA data loadings plot for PC-2. With the exception of a few amino acid fragments, the loadings plot corresponds very well to the amino acid compositional differences shown in Table 2 for Fab and Fc, supporting PCA's ability to effectively parse complex ToF-SIMS data and separate the Fab and Fc surfaces based on their slightly different amino acid compositions.

The full amino acid peak list was then carefully reduced for using PCA to discern “heads-up” vs. “tails-up” orientation effects in the patterned antibody samples. Interfering m/z peaks from the polymeric substrate background with the immobilized affinity species (protein A and fluorescein) were eliminated from the peak set. This was determined by observing and analyzing raw ToF-SIMS ion fragment images for all amino acid fragment peaks from patterned control samples (i.e., those in Table 1 before reaction with an antibody). Any fragments producing noticeable image contrast between regions on the patterned control

samples will produce image signal interference when the surface pattern is reacted with specific antibody fragments. These peaks were therefore not considered in the detailed antibody pattern analysis. The peak list of the characteristic antibody amino acid fragments used for PCA and other image analyses, along with their exact mass, are shown in Table 3. Using this approach and peak set, the ToF-SIMS peak intensity data and PCA methods used together clearly distinguish the two different antibody fragments on surfaces, irrespective of the underlying substrate. This indicates that the ion peak list is appropriate for further analysis of antibody orientation effects on patterned surfaces under the different capture conditions.

2.2.2 ToF-SIMS analysis on patterned hydrogel polymer surfaces—ToF-SIMS imaging of the co-patterned surfaces was expected to produce label-free ‘surface compositional mapping’ and confirm chemical specificity of different antibody-derived amino acid signals associated with the protein A and fluorescein patterned surface zones, respectively. The same three sets of surface patterns (PM, MF and PF) used for fluorescence imaging (Table 1, Figure 2) were also used for ToF-SIMS imaging. Each surface was exposed to the 4-4-20 antibody in a 0.1% BSA background solution as described in the experimental section.

Ion fragment images corresponding to the sum of all ToF-SIMS amino acid ion fragments listed in Table 3 from all three substrates before (control) and after 4-4-20 antibody exposure are shown in Figure 6. ToF-SIMS images from patterned control samples do not produce significant ion-derived image patterns (no antibody column in Figure 6), reflecting similar intensities for sums of amino acid fragments regardless of the surface region. This is expected based on the selection rules for the ion fragments included in the PCA peak set. After exposure to the antibody, patterned regions predicted to bind antibody exhibit significant contrast relative to non-reactive methoxy-capped regions (PM and MF rows of antibody-exposed column in Figure 6). This is consistent with previous studies that have shown the methoxy-capped regions to highly reduce non-specific protein capture.^{30, 36}

First row images in Figure 6 represent PM-patterned samples before and after antibody exposure. Antibody is predicted to attach to this surface only on protein A-patterned regions, as evident from the image in the right column. The limited image contrast present in the control PM sample is produced from the amino acid fragments originating from protein A (left image, first row). As protein A was not present on the MF sample, this amino acid-based contrast was lower for the MF sample shown in the second row, left column of Figure 6. However, after antibody exposure to this sample, clear image contrast is observed in the right column of the second row. The third row of Figure 6 shows the image for the sum of amino acid fragments for a PF sample. Since antibody is expected to attach to both the protein A and fluorescein regions, reduced contrast is expected from this image as amino acid fragments should originate equally across the entire surface, which is not evident from this image (e.g., third row, right column). We note that the raw ion fragment intensity data can be manipulated in many ways to obtain different image contrasts. Hence, we present a more analytical approach to obtain quantitative analysis of the image contrast. These calculations followed the image processing method from Tyler *et al.*,⁶³ with contrast defined by:

$$C_{1,2} = \frac{I_1 - I_2}{\sigma_{1,2}},$$

where I_1 is the average intensity in region 1, I_2 is the average intensity in region 2 and $\sigma_{1,2}$ is the pooled standard deviation of the intensity within the two regions. Here, I_1 and I_2 each refer to the intensity of a particular fragment. For this study, contrast between the normalized sums of amino acid fragments was of interest, so the above equation was modified to:

$$C_{1,2} = \frac{I_{1n} - I_{2n}}{\sigma_{1,2}},$$

where I_{1n} and I_{2n} represent the sums of intensities of amino acid fragments in regions 1 and 2 divided by the total ion intensity from the same region, respectively. For this calculation, spectra were regenerated from two different $100\ \mu\text{m} \times 100\ \mu\text{m}$ regions of the images (see Figure S1 in the supporting information for representative data analysis of these two regions). Then, ion intensities and standard deviations across each image were obtained from the IONTOF IonSpec image processing software. Normalized ion intensity for all amino acid peaks relative to total ion yield intensity was summed over each $100\ \mu\text{m} \times 100\ \mu\text{m}$ region from two different patterned regions and then the difference of the intensities between the two regions was divided by the pooled intensity standard deviation within the two regions. Figure 7 shows the calculated image contrast for each protein image in Figure 6 and asserts the validity of ToF-SIMS analysis to produce ion-specific image contrast on these patterns. Contrast for both the PM and MF samples increases after antibody exposure. However, the increase is not the same for both of these samples.

Image contrast enhancement after antibody attachment is higher in the PM sample compared to the MF sample. One possible explanation for this could be intrinsic differences in the amounts of antibody captured on protein A and fluorescein regions. To obtain relative amounts of antibody captured on protein A and fluorescein regions, XPS studies of control samples, antibody and antibody fragments immobilized on gold substrates were conducted. Organic monolayer DUS films bearing terminal NHS chemistry on gold surfaces were prepared per previous reports⁶² and reacted with protein A and fluorescein-amine to obtain the respective protein A- and fluorescein-modified control surfaces. These two surfaces were then exposed to 4-4-20 antibody and subject to XPS analysis to obtain the nitrogen content (N1s signal) reflecting relative protein surface binding.^{55, 64} Unexposed control gold-adlayer-NHS exhibits ~2 atomic% nitrogen, whereas the adlayer modified with fluorescein and then antibody shows ~3 at% nitrogen. The same samples modified with protein A and then exposed to antibody have ~6 at% nitrogen. Nitrogen content of ~10 at% is expected for complete protein monolayer coverage,⁵⁵ thus while more antibody is immobilized on protein A compared to the fluorescein regions, complete surface coverage is not obtained in either region. Increased antibody solution concentrations might be required to achieve the higher coverages observed for other proteins.

Nonetheless, XPS data confirm that protein A-modified surfaces capture more antibodies, supporting reasons for the better ToF-SIMS image contrast observed for the PM surface compared to MF surfaces (Figure 6). This is also consistent with fluorescence imaging (Figure 2): fluorescence intensity for Ab-647 captured on protein A/MeO patterns was higher than that on fluorescein/MeO and MeO/fluorescein patterned surfaces, supporting different amounts of immobilized antibody. The image contrast for the antibody exposed PF sample is intermediate to the image contrast of the antibody exposed PM and MF samples. This is consistent with the fact that Protein A binds more antibody than fluorescein and the fact that photoresist residue remaining from the UV patterning step further reduces the amount of antibody in that region. Thus the significant contrast observed in image of the antibody exposed PF patterned sample is not surprising.

PCA of ion-derived images from dual patterned antibodies would be an ideal way to isolate the specific antibody-derived fragments that produce the observed differences in the PCA loadings plot. However, the complexity of the system coupled with the varying antibody amounts on the two patterns, as evidenced by XPS and fluorescence microscopy, makes this a challenging situation for drawing direct conclusions from the PCA. For example, the PCA

loadings plot comparing antibodies attached to protein A and fluorescein modified gold surfaces (see Figure S2 in supplementary material) is similar, but not identical, to the Fab and Fc fragment loadings plot in Figure 5. The correspondence of the loadings in Figure S1 to the amino acid compositions in Table 2 is not as high as the loadings shown in Figure 5. The loadings plot for antibodies attached to co-patterned protein A-fluorescein surfaces (see Figure S3 in supplementary material) also are similar, but not identical, to the loadings shown in Figure 5. Possible reasons that the PCA loadings plots for the full antibody surfaces don't correlate as highly to the amino acid composition data (in Table 2) as the Fab and Fc fragments surfaces are (a) distribution of antibodies orientations (i.e., the antibodies are not perfectly oriented in a "heads up" or "tails up" orientations), (b) small amounts of co-adsorbed BSA is present on the surfaces (0.1% BSA is present in the antibody solution), and (c) some of the Fab fragment is sampled in the "tails up" orientation (the Fc fragment has a smaller diameter than the Fab fragment). Thus for a more direct assessment of antibody orientation, we compare image contrast of dual patterned ToF-SIMS images using amino acids found to be enriched in the Fab versus Fc domains (see Table 1). Using selected amino acids shown to be characteristic of Fab and Fc fragments provide a more direct way to examine antibody surface orientation differences proposed for inverted 4-4-20 antibody binding between fluorescein and protein A patterns. A multivariate ratio R_α , defined as the ratio of the sum of all characteristic amino acid fragments from the Fc immobilized control surfaces (Cys+Met+Asn+His) to the sum of all characteristic amino acid fragments from the Fab immobilized control surfaces (Gly+Lys+Leu+Ile), was used for determination of antibody orientation. R_α is calculated at each pixel for the dual-patterned PF samples exposed to 4-4-20 antibody and the resulting image is shown in Figure 8(a). The image contrast is produced based on differences in collective Fc versus Fab amino acid fragment intensities, consistent with the hypothesis that adjacent domains have different ion yields produced from Fc and Fab domains. For comparison Figure 8(b) shows contrast based on differences in the sum of all amino acid fragments listed in Table 3. Significantly, the R_α image in Figure 8(a) is consistent with the proposal that adjacent patterned domains produce different antibody orientations that produce these different ion yields. Since the ToF-SIMS sampling depth is 2-3 nm, the ion yields reflect only the outermost amino acids of the exposed protein regions on these surfaces. This sensitivity of ToF-SIMS to antibody surface amino acid composition instead of antibody bulk amino acid composition⁵² provides confidence that these images reflect Fc versus Fab exposures on the surface. Compositional 'mapping' of the amino acid Fab/Fc ratio (from Table 1) across the surface (Figure 8(a)) clearly indicates that even though more antibody resides on the protein A region, greater amounts of ion fragments associated with Fc domains originate from the fluorescein region (as seen from the complementarity of Figure 8(a) and Figure 8(b)).

The precision, sensitivity and accuracy of bioanalytical tools based on antibodies, including protein microarrays, biosensors, affinity chromatography and immunoassays, rely on their reproducible and optimized surface states. To obtain higher assay performance from antibody-based assays, studies have routinely focused on antibody immobilization techniques. Both surface density of immobilized antibodies and their orientation are important metrics for such performance to improve analyte detection.¹¹ While antibody orientation is reported to yield higher assay sensitivity, most studies have asserted such orientation only by showing improved assay results in controlled immobilization formats versus random immobilization.^{6, 65-68} Direct evidence of protein orientation is seldom shown because of the complexity of the problem at surfaces with immobilized proteins. In this study, a new technique for IgG orientation using well-known, site-specific interactions of protein A-Fc and antigen-antibody (fluorescein and 4-4-20 antibody) was reported. By using dual protein patterning technique reported previously by our group, "heads-up" and "tails-up" oriented antibodies could be co-patterned. Combined use of the fluorescence and ToF-SIMS imaging indicates the differences between the "heads-up" and "tails-up" antibody patterned regions corresponding to the regions where protein A and fluorescein were immobilized.

Conclusions

This study has shown that oriented murine anti-fluorescein antibody patterning is obtained and discriminated on commercially available PEG-based polymer coated glass slides with protein A/fluorescein co-patterned surfaces. Antibody was affinity-immobilized onto fluorescein- and protein A- patterned regions using two different known interactions to yield “heads-up” versus “tails-up” orientations. Resulting bound antibody patterns on protein A/MeO and fluorescein/MeO surfaces were confirmed for affinity specificity and pattern fidelity using fluorescence scanning. ToF-SIMS analysis, sensitive to surface chemistry in the top 2-3 nm of the surface, differentiates “heads-up” and “tails-up” orientations for antibodies patterned in an idealized “two-population” arrangement. PCA processing of ToF-SIMS ion fragment data for such patterned antibodies distinguishes the natural amino acid composition differences in the antibody Fab and Fc domains present and exposed differently in these two patterned regions. A multivariate ratio of amino acids characteristic of the Fab and Fc fragments was used to provide an image ‘map’ of antibody compositional variations and orientations across the patterned surfaces. This study demonstrates new analytical capabilities for discerning surface protein orientations, and in achieving this for the antibody case in point, demonstrates that antibody orientation can be induced by surface design of suitable ligand patterning for controlled antibody-surface affinity interactions. While this deliberate “up-down” affinity ligand patterning for binding antibodies may not contribute much advancement for practical antibody device utility (i.e., in the form of new technology), the analytical methods demonstrated here should prove useful in further developments to more readily understand and interrogate protein-surface states important for these technologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Optical image of the mask used for patterning protein A and fluorescein ligands into adjacent regions of amine-reactive polymer-coated slides,^{30, 36, 40} and (b) schematic showing the intended immobilized antibody orientations when attached to each specific ligand regions to selectively expose Fab and Fc domains in these regions to surface analysis methods. Scale bar for photolithographed patterns in (a): 500 μm .

Figure 2. (a, b) Scanned fluorescence images for (a) patterned protein A-TAMRA and (b) patterned fluorescein-PEG hapten on NHS/MeO patterned commercial polymer microarray slide surfaces; (c-e) fluorescence images of surface-bound Alexa647-labeled (c) 4-4-20 antibody, (d) non-specific murine Fab, and (e) murine Fc polyclonal fragment captured on protein A-patterned surfaces; (f-h) fluorescence images for bound Alexa647-labeled (f) 4-4-20 antibody, (g) non-specific murine Fab, and (h) murine Fc polyclonal fragments on fluorescein-patterned surfaces.

Figure 3.

(a) Fluorescence image of Alexa647-labeled 4-4-20 antibody (non-black zones) on the MeO/fluorescein patterned surface, and (b) relative fluorescence intensities of Alexa647-labeled proteins on various regions of the fluorescein hapten-patterned (FM and MF) surfaces.

Figure 4.

PCA analysis PC-1 and PC-2 scores plot from ToF-SIMS amino acid data for Fab and Fc fragments immobilized onto polymer and gold-NHS slides. PC-1 distinguishes the substrate surface compositional differences (i.e., gold vs. polymer) while PC-2 successfully separates the data originating between Fc fragments from Fab fragments. Such clearly distinct scoring separation allows this method to distinguish Fab vs. Fc antibody-derived amino acid fragment data on surfaces.

Figure 5. PCA loadings plot for PC-2 showing the amino acid fragments derived from ToF-SIMS analysis of Fab and Fc fragments immobilized as controls on polymer and gold-NHS surfaces. Fc fragments load in the positive direction, while amino acid fragments corresponding to the Fab fragments load in the negative direction. Results correlate well with predictions from Table 2 for comparison of bulk antibody compositional amino acid differences in each domain.

Figure 6.

ToF-SIMS surface images generated from summing all amino acid ion fragments (Table 3) for ligand-immobilized high-fidelity patterns: PM (protein A/methoxy-capped), MF (methoxycapped/fluorescein), and PF (protein A/fluorescein) patterned surfaces exposed to the 4-4-20 antibody. Image contrast results from actual ion yield contrasts across the patterns.

Figure 7.

Image contrast calculated from the ToF-SIMS images shown in Figure 6 generated by the method in Ref ⁵⁸. The samples were exposed to antibody prior to numerical image analysis to calculate contrast. Ligands immobilized in high fidelity patterns were MF (methoxycapped/fluorescein), PM (protein A/methoxy-capped), and PF (protein-A/fluorescein).

Figure 8.

Affinity-immobilized antibody PF surface images and pattern contrasts generated from ToF-SIMS surface analysis data from (a) the amino acid enrichment ratio, Ra , defined in the text for Fab domain versus Fc domain amino acid compositions for a co-patterned PF surface, and (b) sum of all ToF-SIMS amino acid fragments for the same sample (see Figure 6, row-3, second column).

Table 1

Sample identification for the various co-patterned surface samples

Patterned surface identifier	Region1 (black in Figure 1a)	Region 2 (white in Figure 1a)
PM	Protein A	2-methoxyethylamine
FM	2-methoxyethylamine	Fluorescein
MF	2-methoxyethylamine	Fluorescein
PF	Protein A	Fluorescein

Table 2Amino acid composition for F(ab')₂ and Fc fragments for murine antibody 2a.⁶⁹

Amino acid	F(ab') ₂ [4FAB]		Fc [11GT]		Ratio of Fab/Fc
	# residues	% comp	# residues	% comp	
Ala	30	3.45	14	3.31	1.04
Arg	30	3.45	12	2.84	1.21
Asn	34	3.91	34	8.04	0.49
Asp	44	5.06	11	2.60	1.95
Cys	20	2.30	14	3.30	0.70
Gln	34	3.91	14	3.30	1.18
Glu	34	3.91	28	6.62	0.59
Gly	66	7.59	18	4.25	1.78
His	12	1.38	14	3.30	0.42
Ile	26	2.99	18	4.25	0.70
Leu	58	6.67	18	4.25	1.57
Lys	48	5.52	30	7.09	0.78
Met	14	1.61	12	2.84	0.57
Phe	28	3.22	12	2.84	1.13
Pro	46	5.29	42	9.93	0.53
Ser	128	14.71	32	7.56	1.95
Thr	80	9.20	32	7.56	1.22
Trp	20	2.30	6	1.42	1.62
Tyr	44	5.06	20	4.73	1.07
Val	74	8.51	42	9.93	0.86

Table 3

Characteristic amino acid fragments and their exact masses used for ToF-SIMS analysis.

Amino Acid	Fragment	Exact mass
Alanine (Ala)	C ₂ H ₆ N C ₃ H ₆ NO	44.052 72.050
Arginine (Arg)	CH ₂ N CH ₃ N ₂ C ₄ H ₈ N	28.019 43.029 70.077
Asparagine (Asn)	CH ₂ NO C ₃ H ₇ NO	44.013 73.053
Cysteine (Cys)	CHS	44.9798
Glutamine (Gln)	C ₃ H ₆ N C ₄ H ₆ NO	56.052 84.052
Glutamic acid (Glu)	C ₄ H ₆ NO	84.052
Glycine (Gly)	CH ₂ N C ₂ H ₄ N	28.019 42.0341
Histidine (His)	C ₄ H ₅ N ₂ C ₄ H ₆ N ₂	81.0411 82.0463
Leucine/Isoleucine (Leu/Ile)	C ₅ H ₁₀ N C ₅ H ₁₂ N C ₆ H ₉ O	84.0892 86.1069 97.072
Lysine (Lys)	CH ₂ N C ₃ H ₆ N C ₅ H ₁₀ N	28.019 56.052 84.0892
Methionine (Met)	C ₂ H ₅ S	61.0139
Tryptophan (Trp)	C ₉ H ₈ N	130.0784
Threonine (Thr)	C ₃ H ₈ NO C ₂ H ₈ NO ₂	74.0697 102.0550
Tyrosine (Tyr)	C ₈ H ₁₀ NO	136.0853
Valine (Val)	C ₄ H ₈ N C ₄ H ₁₀ N	70.077 72.090