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Orientation and characterization of immobilized antibodies for improved immunoassays (Review)

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Orientation of surface immobilized capture proteins, such as antibodies, plays a critical role in the performance of immunoassays. The sensitivity of immunodiagnostic procedures is dependent on presentation of the antibody, with optimum performance requiring the antigen binding sites be directed toward the solution phase. This review describes the most recent methods for oriented antibody immobilization and the characterization techniques employed for investigation of the antibody state. The introduction describes the importance of oriented antibodies for maximizing biosensor capabilities. Methods for improving antibody binding are discussed, including surface modification and design (with sections on surface treatments, three-dimensional substrates, self-assembled monolayers, and molecular imprinting), covalent attachment (including targeting amine, carboxyl, thiol and carbohydrates, as well as “click” chemistries), and (bio)affinity techniques (with sections on material binding peptides, biotin-streptavidin interaction, DNA directed immobilization, Protein A and G, Fc binding peptides, aptamers, and metal affinity). Characterization techniques for investigating antibody orientation are discussed, including x-ray photoelectron spectroscopy, spectroscopic ellipsometry, dual polarization interferometry, neutron reflectometry, atomic force microscopy, and time-of-flight secondary-ion mass spectrometry. Future perspectives and recommendations are offered in conclusion. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>). [<http://dx.doi.org/10.1116/1.4978435>]

I. INTRODUCTION

Immunodiagnostics, protein biochips, and biosensors employed for antigen detection and quantification from biological samples often employ recognition proteins such as antibodies.^{1–4} Assay sensitivity is dependent on immobilization of the capture antibodies onto a solid support with a sufficient surface density, a conformation that is representative of their native, solution-phase state, and an orientation that maximizes their antigen capture potential.

Antibodies are biopolymers of approximately 150 kDa molecular mass and with dimensions of approximately $14 \times 10 \times 4$ nm (Refs. 3 and 5) comprised of amino acids whose sequence and composition, like other proteins, define the three-dimensional structure.⁶ An antibody is comprised of two fragment antigen binding (Fab) regions and a fragment crystallizable region (Fc). The Fab regions, joined by the hinge region, is known as $F(ab')_2$ fragment. The Fab regions are dissimilar in their composition, isoelectric point, and physical structure to the Fc region of the antibody (Fc), allowing determination of antibody orientation on the surface. Ideally, the immobilized antibody is oriented such that the Fc is substrate

facing, sometimes referred to as “end-on”; however, randomly immobilized antibodies may assume various surface orientations, including those loosely referred to as “head-on,” “side-on,” and “lying-on” (see Fig. 1).⁷

Generally, hydrophobic amino acids are internalized in a correctly folded protein structure, leaving hydrophilic residues at the antibody surface, with chemically reactive functionalities, including amine, carboxyl, and hydroxyl groups. Disulfides, such as those that contribute to the hinge region, can be reduced to make thiol species that can also be conjugated. Further, sites for targeted immobilization (including natural or non-natural amino acids) can be introduced recombinantly to antibodies.^{8–10}

Physical adsorption of antibodies onto traditional immunoassay solid supports, such as polystyrene, occurs via hydrophobic and electrostatic interactions.¹¹ While this method offers the simplest attachment pathway, it is uncontrollable, and antibodies can be immobilized in a randomly oriented manner, denatured, or displaced in later steps by washing.^{12–14} The substrate design has increased the capabilities of immunoassays by improving antibody binding capacity and reducing denaturation at the surface.¹⁵ Covalent attachment of the antibody, via its functional groups, to chemically engineered substrates has resulted in further

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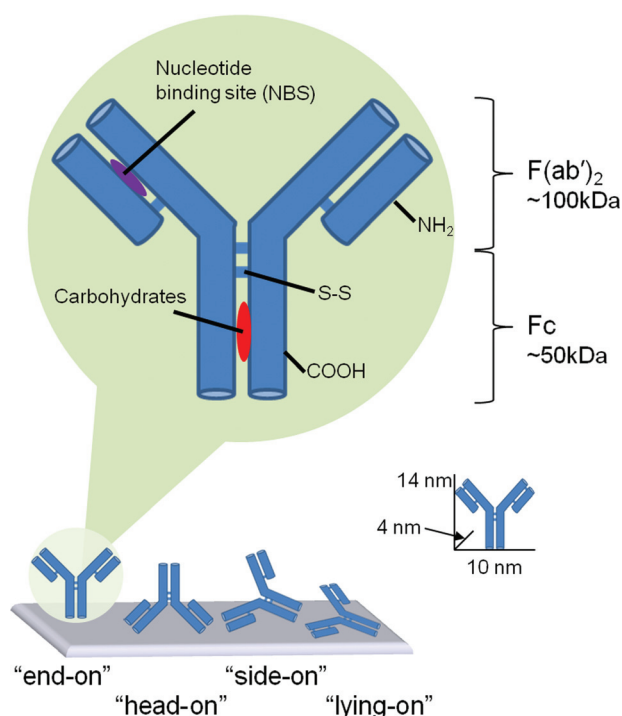


FIG. 1. Antibody orientation, dimensions, and important chemical species for targeting.

improvements in antibody density, though often these methods are not site-directed and unfavorable random orientation can occur.¹⁶ In an ideal scenario, antibodies should be immobilized in their native form, without the need for introduced functional groups, in a homogeneous arrangement such that their antigen binding sites are free from steric hindrance and are oriented so as to maximize complementary binding. A method that truly provides the ideal scenario has yet to be realized; however, recent developments, as discussed in this review, offer improved control over antibody immobilization and orientation at the interface.

In parallel with the development and evolution of immobilization methods, a significant need has emerged for surface characterization techniques that can accurately identify antibody orientation at a substrate. Current techniques that rely on indirect analysis, or inference from complex models, make definitive conclusions regarding orientation difficult.¹⁷ Advances in data processing and multivariate analysis have provided an improved level of understanding of complex surfaces, and direct surface analysis techniques, such as time-of-flight secondary ion mass spectrometry (ToF-SIMS), provide molecular information that has the potential to determine antibody orientation with confidence.

This review is structured into two parts. First, a review of current antibody immobilization strategies, including surface modification, antibody targeting, and coupling, will be presented. Second, advances in characterization techniques for investigating these systems will be explored, including indirect and direct analyses. The advantages and shortfalls of strategies and techniques will be addressed, and the review will conclude with future perspectives and recommendations.

II. IMPROVING ANTIBODY BINDING AT THE INTERFACE

A. Surface modification and design strategies

Physical adsorption is the simplest method for the immobilization of antibodies to immunoassay solid supports, such as microtiter plates. However, this method does not allow control of the antibody orientation and is typically associated with poor binding and denaturation.¹⁸ Microtiter plate manufacturers utilize polymers such as polystyrene, polypropylene, polyethylene, and cyclic olefin copolymer blends, employing surface modification methods to increase the hydrophilicity of substrates. The increase in hydrophilicity can increase antibody binding (density) and decrease the amount of denatured protein. Polymer substrates are the traditional immobilization platform for immunoassay as they provide a cheap, stable, reproducible substrate that is easy to manufacture with precision.

1. Plasma treatment and plasma polymers

Plasma treatment is a surface modification method that uses radio-frequency glow-discharge to generate a plasma of a gas or monomer vapor. Microtiter plates have been treated with oxygen, nitrogen, and other gas plasmas to create chemical functionalities at the surface, thereby reducing the hydrophobicity of the plastic.¹⁹ Similarly, nonreactive gases such as argon can be used to “activate” the surface by introducing radicals, which react with atmospheric species upon exposure to air.²⁰ Plasma treatment offers a method to functionalize existing substrates’ increasing hydrophilicity²¹ and reducing denaturation of bound proteins or to provide a starting point for further chemical treatment and covalent grafting of proteins.²² In a recent example, Pâslaru *et al.*²³ plasma treated poly(vinylidene fluoride) (PVDF) with CO₂, N₂ and N₂/H₂ (25/75) gases to attach carboxyl or amine functionality for subsequent covalent immobilization of proteins. *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) chemistry was used to attach IgG or Protein A (with subsequent IgG binding) to the PVDF treated surfaces. Possible preferential end-on orientation of IgG was achieved via the PVDF surfaces treated with N₂/H₂ and grafted with the Protein A.

Plasmas can also be used to produce polymer thin films that retain some of the chemistry associated with the monomer. The radicalized monomer fragments bind to the substrate surface, and to one another, creating a ubiquitous surface coating referred to as a plasma polymer. This methodology of creating polymers allows adherent and continuous coatings to be formed on a broad range of substrates, including microtiter plates.²⁴ Plasma polymers have been produced from a diverse range of monomers, including allylamine,^{25,26} cyclopropylamine,²⁷ bromine,²⁸ polyethylene glycol (PEG), diethylene glycol dimethyl ether (diglyme),^{29–31} and many others,^{32,33} providing a broad spectrum of chemical functionalities for subsequent protein grafting steps, including the option for patterning.^{34–36} Overall, a significant range of polymers have been used to improve biomolecule immobilization properties

of substrates for use in microarray and protein assay applications.^{33,37} Hasan and Pandey³⁸ provide an excellent review of polymers and plasma techniques for producing materials for protein immobilization.

2. Three-dimensional substrates

Three-dimensional substrates are attractive as they offer increased surface area for antibody binding and can minimize steric hindrance that may prevent antigen capture. Porous three-dimensional substrates have been prepared from a variety of materials, including various polymers, silicon, glass slides, metals, and gels. Wang and Feng¹⁵ provide an excellent review on three-dimensional substrates with a focus on the orientation of proteins.

Similarly, gels and sol-gels manufactured from agarose or dextran have found improved antibody binding due to their high surface area.^{1,39} In a recent study, Orlov *et al.*⁴⁰ employed three-dimensional immunochromatographic nitrocellulose membranes impregnated with magnetic nanoparticles for use in a strip sensor immunoassay, providing a solid phase with a large surface area for antibody immobilization. The sensor had a limit of detection of approximately 740 fM and had a strong correlation with a standard enzyme-linked immunosorbent assay (ELISA) for the detection of prostate specific antigen from serum, though with improved dynamic range (3.5 orders). One example from Feng *et al.*⁴¹ utilized repeat units of Protein A genetically fused to the nickel chelating His-tag. Adjacently immobilized (via a nickel matrix substrate) the fused proteins order as columns protruding from the substrate and could immobilize five antibodies via their Fc regions. This three-dimensional protein construct had a 64-fold increase in antigen detection sensitivity relative to standard IgG immobilization.

3. Self-assembled monolayers

The formation of a self-assembled monolayer (SAM) provides another means of modifying surface chemistry to promote antibody adsorption or to produce functional groups for subsequent covalent attachment.⁴² SAMs are typically formed from molecules that contain active functional head-groups at either end of a hydrocarbon chain and a linear carbon chain which promote self-assembly when they attach to a surface. The anchoring head-group has an affinity for the surface, while the other provides a solution-facing chemistry for protein adsorption or attachment. The central hydrocarbon chains provide stabilization of the SAM by interchain hydrophobic interactions.³⁸

The gold-thiol interaction has been exploited most commonly by utilizing alkanethiols to provide a linker that can bind gold substrates via the thiol group, and offer customizable chemistry for adsorption or coupling antibodies to SAMs.^{43,44} Lebec *et al.*⁴⁵ used alkanethiol SAMs formed from 11-mercaptoundecanoic acid (11-MUA) and 1-undecanethiol on gold substrates to produce COOH and CH₃ surface chemistries, respectively. Antibodies were adsorbed to both substrate types with an increase shown for

the CH₃ surface. However, this antibody was found to have no antigen recognition indicating denaturation or poor orientation, with the latter supported by ToF-SIMS findings. Chen *et al.*⁴⁴ demonstrated preferred orientation of mouse IgG1 (and to a lesser extent IgG2a) by exploiting the antibody dipole and the use of charged SAM surfaces. IgG1 adsorbed to a NH₂ terminated SAM produced from 11-amino-1-undecanethiol had a higher antigen/antibody ratio than a COOH terminated SAM produced from 16-mercaptohexadecanoic acid. Vashist *et al.*⁴⁶ provide a good review of antibody immobilization using silane SAMs on various substrates for improved surface densities.

4. Molecularly imprinted polymers

Molecular imprinting is a polymerization technique that uses a molecular template to produce target-specific binding regions.⁴⁷ Once formed, the target-specific binding sites in the polymer substrate may selectively immobilize the target, such as an antibody, from a complex matrix. Bereli *et al.*⁴⁸ imprinted a poly(hydroxyethyl methacrylate) cryogel with the Fc portion of anti-human-IgG to create an antibody orienting substrate. The Fc portion was then flushed from the cryogel, which was subsequently activated with carbodiimide for whole antibody binding. Anti-human-IgG was then used as a capture antibody to bind IgG from human plasma and the imprinted cryogel was demonstrated to be at least three times better than using a nonimprinted cryogel. This was despite similar amounts of the capture anti-human-IgG being immobilized, via the nonspecific carbodiimide method, indicating preferential antibody orientation. In addition to providing orientating substrates, molecularly imprinted polymers utilize gel structures with aqueous environments that are thought to reduce the probability of protein denaturation.⁴⁹

B. Covalent binding targets

While substrate design is important, the ability to reliably attach the antibody to a solid support underpins the success of an immunoassay. The covalent coupling of capture antibodies ensures robust immobilization and can improve density and orientation outcomes at the substrate. However, for oriented immobilization, site-directed attachment to the antibody is required. The covalent attachment can proceed via various chemistries dependent on the substrate functionality, target group on the antibody, and the physical restraints of the system, i.e., pH, temperature, and degree of conjugation.³³ This section will discuss functional groups on antibodies for covalent attachment, including common targets such as amine, carboxyl, thiol, and carbohydrate moieties (see Fig. 2 for overview). The covalent attachment methods of proteins are covered in good detail in reviews by Rao *et al.*⁵⁰ and Liu and Yu.¹⁶

1. Amine and carboxyl groups

Amine and carboxyl groups are ubiquitous throughout an antibody's structure and are common at the antibody's

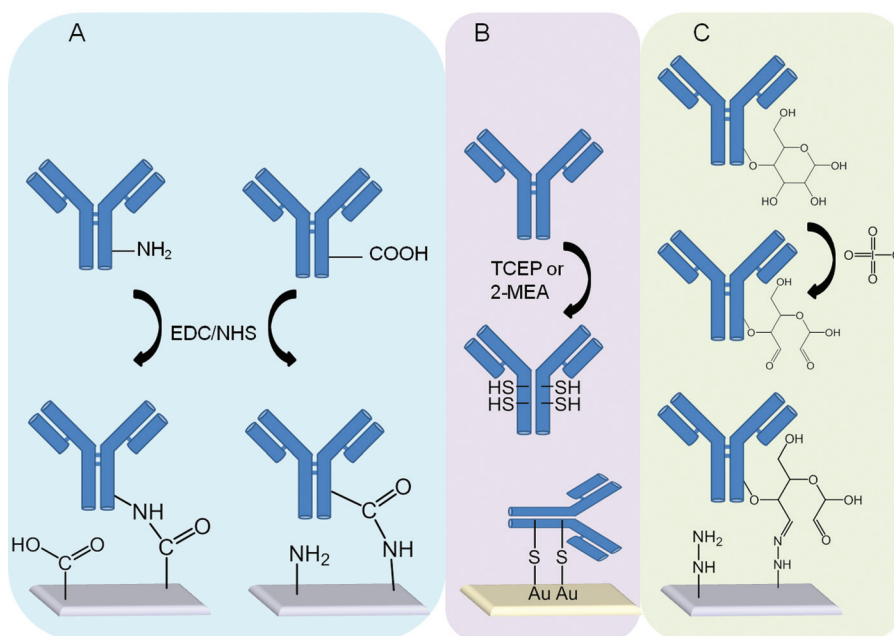


FIG. 2. Oriented antibody immobilization strategies. (a) EDC/NHS coupling of antibody amine and carboxyl groups to surface carboxyl and amine groups. (b) Reduction of antibody disulfides, with TCEP or 2-MEA, to reactive thiols for binding gold substrates. (c) Periodate oxidation of carbohydrates in the Fc region of antibodies followed by coupling to hydrazide surface chemistry.

surface due to their polar nature. Amino acids such as lysine with reactive primary amine side chains, and aspartate and glutamate with carboxyl side chains, can be targeted for covalent attachment. Due to their prevalence throughout the antibody surface, site-directed covalent antibody immobilization targeting these groups is difficult. Amine and carboxyl coupling (between the substrate and protein) is commonly attained with carbodiimide chemistry that utilizes EDC combined with succinimidyl esters (such as NHS).³³ This method is known as EDC/NHS coupling and results in robust amide bond formation. EDC/NHS chemistry has been employed as a covalent attachment methodology for immobilization of proteins and also as a method for the preparation of substrates. Carrigan *et al.*⁵¹ used EDC/NHS in two ways, (1) for the cross-linking of polyethylenimine and carboxymethyl cellulose, and (2) for activation of this substrate for protein binding targeting amine and carboxyl functionality. Sun *et al.*⁵² took a novel approach to EDC/NHS coupling by introducing NHS reactive groups to random sites on an antibody then using an electric field to preferentially orient the antibody, via its intrinsic dipole, before reaction to the free amines on cysteine immobilized to a gold electrode.

2. Thiol groups

Disulfide-bridged cysteines, such as those present in the hinge region of antibodies, can also be targeted by reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) or 2-mercaptoethylamine (2-MEA) to form reactive thiols,^{8,53} which may subsequently react with maleimide or iodoacetyl activated surfaces. However, as the cysteines are internal to the antibody tertiary structure, covalent attachment via this method can disrupt the conformation of the antibody⁵⁴ while

steric hindrance may limit antigen binding. Exploitation of the gold–thiol bond makes this immobilization strategy useful for gold substrates and nanoparticles in techniques such as surface plasmon resonance (SPR).⁵⁵ UV-excitation has also been used to initiate photoreduction of disulfide bridges in hinge regions of antibodies according to an approach known as the photonic immobilization technique.⁵⁶ Employing UV pulses at 258 nm and 10 kHz, free thiols can be produced that are then able to bind gold substrates for quartz crystal microbalance (QCM) measurements.⁵⁷ Antibody fragments, such as Fab', can also be produced with reactive thiols and used in immunosensors;^{5,42} however, this review aims to focus primarily on whole antibody immobilization and will not cover antibody fragments specifically.

Alternatively, primary amine groups can be converted to thiol functionality using, for example, 2-iminothiolane (Traut's reagent) for subsequent immobilization using the same maleimide or iodoacetyl chemistry.⁵⁸ For example, lipid PEG, functionalized with maleimide, was incorporated into lipid nanocapsules for coupling thiolated antibodies or Fab'.⁵⁹

3. “Click” chemistry

More recently, “click chemistry” exploiting 1,3-dipolar cycloaddition between an azide and an alkyne has demonstrated utility for the conjugation of single-domain camelid antibodies, known as VHH, to a dextran substrate.⁹ This technique produced an oriented system via the site-specific insertion of azidohomoalanine into the VHHs.

4. Carbohydrate groups

Antibodies are glycosylated at the Fc region and can provide a target for site-directed immobilization.^{60,61} Periodate

oxidation can be used to oxidize diols in carbohydrates into aldehyde groups that can react with amines and hydrazides.⁶² Diols can also be targeted by boronic acids to form boronate ester intermediates reversibly.⁶³ Song *et al.*⁶⁴ demonstrated end-on immobilization of antibodies using 3-aminophenylboronic acid to first couple a NHS-derivatized surface and second bind the Fc carbohydrate. Due to the reversible nature of boronate esters, Adak *et al.*⁶¹ employed a single molecule with two functional groups to first form a boronate ester with the carbohydrate, and second to covalently attach the antibody via photoinitiated cross-linking. UV exposure causes the (trifluoromethyl)phenyldiazirine functional group to form reactive carbenes that irreversibly bind the antibody. Alternatively, Huang *et al.*⁶⁵ oxidized the carbohydrate of anti- α -fetoprotein to an aldehyde and covalently linked the protein to a 3-aminopropyltriethoxysilane (APTES) modified silicon substrate. The amount of antibody immobilized increased by 32% and the antigen binding amount by 16% relative to physical adsorption. Yuan *et al.*⁶⁶ used periodate oxidation of carbohydrates on the anti-CD34 antibody to promote oriented immobilization. First, stainless steel substrates were coated with ethylene vinyl acetate, then treated with O₂ plasma, and silanated with APTES to create amine groups (labeled SCA-SS). Amines were then coupled with the oxidized carbohydrates and successful binding was assessed via cell uptake by the anti-CD34 antibody. Prieto-Simón *et al.*⁶⁷ used thiolated hydrazide SAM linkers or electrografting of diazonium salts to immobilize periodate-oxidized carbohydrates of antibodies via hydrazide chemistry onto functionalized gold substrates.

C. Affinity immobilization techniques

While covalent methods provide robust antibody immobilization and can achieve site-directed immobilization,⁶⁸ they may be unsuited due to the high prevalence of a particular functional group in the antibody (amine and carboxyl), or may cause conformation changes upon attachment (thiol), without the use protein engineering. Affinity immobilization techniques provide alternative and potentially favorable strategies to promote site-directed antibody immobilization.¹⁵

1. Material binding peptides

Peptide sequences that will preferentially immobilize to substrates, metal ions, and other biomolecules have been developed for enhanced protein orientation.⁶⁹ These peptides can be incorporated as tags, into proteins of interest, via chemical conjugation or genetic fusion. Phage screening techniques have been developed to produce peptides with specificity to a broad range of materials and proteins.⁷⁰ A large number of material binding peptides exist, including those specific to polystyrene [PS-tag,⁷¹ Lig1 (Refs. 72 and 73)] and hydrophilic polystyrene (Phi-PS) [PS19-1 and PS19-6 (Ref. 74)], silicon (Si-tag^{75,76}), glass slides or silica resin [R9 (Ref. 77)], poly(methylmethacrylate) (PMMA)

(c02,⁷⁸ PM-OMP25,⁷⁹ PMMA-tag⁸⁰), polycarbonate [PC-OMP6 (Ref. 79)], poly-L-lactide [c22 (Ref. 81)], gold [GBP (Ref. 82)], and the well known nickel and copper specific His-tag.^{83,84}

2. Biotin–streptavidin interaction

Oriented immobilization can be realized by exploiting the biotin–avidin/streptavidin interaction.⁸⁵ Antibodies can be easily conjugated to biotin using biotin-NHS chemistry that targets amines; however, this results in randomly biotinylated antibodies. Paek's group⁸⁶ compared randomly biotinylated IgG using biotin-NHS, with IgG biotinylated at the hinge disulfides via competitive maleimide chemistry for immobilization to streptavidin treated microwells and glass slides. The authors found a two-fold improvement in antigen detection for the hinge disulfide biotinylated IgG, relative to the random system. The group also demonstrated a twofold improvement for the same system by employing a gold substrate with a thiol SAM and biotin–streptavidin linker.⁸⁷

3. DNA directed immobilization

DNA-directed immobilization (DDI) of proteins is another affinity method that can produce oriented systems. This method requires the binding of short nucleotide sequences to the substrate and to the protein of interest, allowing direct binding between the two. However, to truly achieve an oriented system, care must be taken to ensure that the covalent attachment of the DNA to the antibody occurs in a site-direct manner. Wacker *et al.*⁸⁸ investigated antibody immobilization and fluorescent immunoassay performance of antibodies bound via DDI, physical adsorption, and by streptavidin–biotin interactions. IgG immobilized with DDI was found to require 100-fold less antibody for the same fluorescence detection of the analyte; however, orientation of the antibody was not independently determined, and site-directed biotinylation of the IgG was not stated. More recently, Seymour *et al.*⁸⁹ compared anti-ebola virus glycoprotein (EBOV GP) antibody immobilized directly to NHS containing copolymer or via DDI. The DDI immobilized antibody was found to be an order of magnitude more sensitive to the EBOV GP antigen. Glavan *et al.*⁹⁰ synthesized single-stranded DNA onto paper substrates and investigated antihuman C-reactive protein (hCRP) immobilized via DDI for binding hCRP from serum in a sandwich ELISA. However, DNA conjugation to the antibody utilized non-site-directed NHS chemistry.

Boozer *et al.*⁹¹ prepared mixed SAMs containing ssDNA thiols and oligo(ethylene glycol) thiols on gold SPR chips. The complementary DNA strand was cross-linked using NHS chemistry to the antibodies and immobilized by the SAM. This system generated a 50-fold improvement on their previous work⁹² using biotinylated immobilization. However, by binding DNA to the antibody nonspecifically with NHS chemistry, it is more likely that the improvement arises due to the antibody being separated from the surface, than through orientation.

4. Protein A and Protein G

Protein A and Protein G are small proteins, derived from bacteria, which can specifically bind the Fc portion of antibodies allowing oriented systems to be obtained.^{93–96} The IgG binding domain of Protein A, known as the Z-domain or ZZ-domain, is also used as a smaller synthetic option for Fc binding.⁹⁷ This technique offers a method for truly obtaining oriented antibodies as binding can only occur via the Fc portion. Due to its effectiveness, Protein A, or its derivatives, has been exploited with many surface immobilization strategies including biotin-streptavidin,⁹⁸ SAMs,^{99,100} EDC/NHS chemistry,^{100,101} glutaraldehyde,¹⁰² tyrosinase chemistry,¹⁰² non-natural amino acid insertion,¹⁰ gold binding peptide⁸² or polystyrene affinity ligand fusion,⁷³ and additional protein linkers.¹⁰³ The ZZ-domain has also been coupled to carbohydrate binding modules for selective immobilization to cellulose coated slides¹⁰⁴ and to paper¹⁰⁵ substrates, and also the metal affinity His-tag.^{106,107} It is important to note that several issues may arise with such systems: (1) the Protein A capture of the Fc is reversible, (2) Protein A has been reported to bind Fab regions and albumin (although to a much lesser extent), and (3) it is required that the Fc binding site of the Protein A is correctly oriented at the substrate to permit antibody binding.^{15,16} More recently, Yang *et al.*¹⁰⁸ engineered a photoactivatable Z-domain variant that incorporated the UV-active amino acid benzoylphenylalanine and a biotin molecule. The Z-domain preferentially bound the Fc portion of IgG that was irreversibly attached using UV-activation of the benzoylphenylalanine. When immobilized to a streptavidin coated substrate, this system had a fivefold lower antigen detection limit than randomly NHS-biotinylated IgG.

5. Fc-binding peptides and aptamers

Short peptides with specificity to the Fc domain of antibodies have been used to promote oriented immobilization. Jung *et al.*¹⁰⁹ used an Fc-binding peptide to immobilize human, rabbit, goat, and mouse antibodies, with strong selectivity for human IgG1 and IgG2. Anti-C-reactive protein (anti-CRP) antibody immobilized with the Fc-binding peptide was compared with randomly immobilized (EDC/NHS coupling) antibody immobilized to SPR chip substrates and found that a 1.6-fold increase in the CRP/anti-CRP ratio when employing the Fc-binding peptide. More recently, Tsai *et al.*¹¹⁰ demonstrated that molecular dynamics can be used to design a short peptide (RRGW) with high specificity to the mouse IgG2a antihuman prostate specific antigen (PSA) antibody. PSA binding was monitored via SPR demonstrating good antibody orientation.

Yoo and Choi¹¹¹ used a phage biopanning to screen for peptides specific to the Fc portion of rabbit anti-goat IgG. The peptide (KHRFNKD) immobilized with biotin to an avidin-QCM surface showed improved IgG binding relative to physical adsorption. Dostalova *et al.*¹¹² used the Fc binding peptide HWRGWVC to immobilize antiprostata specific membrane antigen antibodies to gold coated doxorubicin

nanocarriers with solution-phase orientation. The inclusion of the peptide gave a 1.4-fold improvement in signal during the immunoassay. Lee *et al.*¹¹³ developed photoactivatable Fc-specific antibody binding proteins (FcBPs) expressed in *Escherichia coli* that undergo photo-crosslinking (via photo-methionine) with antibodies upon UV irradiation. FcBPs were immobilized on maleimide-coated slides and the epidermal growth factor receptor (EGFR)-hAb antibody cross-linked with UV exposure. Dose-dependent antigen (EGFR) binding was observed at above 110 fmol.

Aptamers are single chain DNA, or RNA, oligonucleotides that fold to form complex three dimensional structures and can specifically immobilize proteins of interest.¹¹⁴ Miyakawa *et al.*¹¹⁵ developed an RNA aptamer that selectively binds the Fc portion of human IgG1 through IgG4, but not other nonhuman IgGs. SPR was used to assess the binding site of the aptamer on IgG, and it was found that the site was similarly positioned to that of the Protein A binding site, making it suitable for promoting antibody orientation. Ma *et al.*¹¹⁶ produced a DNA aptamer capable of binding the Fc domain of multiple mouse subclasses. This area has potential to develop aptamers capable of universal Fc binding substrates, hence promoting antibody orientation and immuno-diagnostic sensitivity.

6. Nucleotide binding site

Antibodies, even across different isotypes, contain largely conserved sequences between the heavy and light chains of the Fab region known as a nucleotide binding site (NBS). Targeting the NBS using a small molecule, indole-3-butyric acid, and UV irradiation, Alves *et al.*¹¹⁷ were able to immobilize antibodies selectively, offering a 7.9-fold increase in antigen sensitivity, compared with physical adsorption. The group also demonstrated the utility of this technique with Fab fragments.^{118,119}

7. Metal affinity

Perhaps the simplest option is to take advantage of the endogenous metal binding properties of antibodies. In addition to recombinant peptide tags for metal coordination, native tag-free IgG have been purified using metal affinity. The interaction of histidine and cysteine with metals, particularly copper and zinc, has been exploited for protein fractionation and IgG purification using immobilized metal-affinity chromatography.^{120,121} Hale¹²² then went on to demonstrate that Co(II) loaded resin could be used to irreversibly bind IgG in an oriented manner via the Fc region. Todorova-Balvay *et al.*¹²³ used computational modeling and immobilized metal-ion affinity chromatography to investigate the transition metals copper (II), nickel (II), zinc (II) and cobalt (II), to determine a native metal-binding target in the Fc portion of whole human IgG1. The histidine cluster His 433–X–His 435 was found to be surface accessible to affinity binding using these metals without the need for recombinant tags. Muir *et al.*¹²⁴ prepared metal coordinating polymer substrates and screened a large range of transition metals to

assess their antibody binding capabilities. This work covered a library of 1600 different metal immobilized surface chemistries and identified chromium perchlorate with ethylenediamine as the “lead” combination. Immobilization of antitumor necrosis factor alpha (anti-TNF α) to Luminex beads with chromium perchlorate and ethylenediamine was compared with carbodiimide coupling chemistry. The chromium-mediated immobilization has an approximately ninefold improvement in TNF α antigen sensitivity relative to the carbodiimide method. Pingarrón’s group used a metallic-complex chelating polymer (Mix&GoTM) to achieve oriented immobilization of native anti-adiponectin antibody on carboxyphenyl multiwalled carbon nanotubes¹²⁵ and graphene oxide-carboxymethyl cellulose hybrid.¹²⁶ Recently, Welch *et al.*¹²⁷ employed the chromium complex, [Cr(OH)₆]^{3−}, buffered with ethylenediamine as a wet chemical modification to ten commercial microtiter plates, postproduction, to improve antibody immobilization and ELISA performance. For an anti-EGFR, x-ray photoelectron spectroscopy (XPS) analysis indicated that the chromium modified microtiter plate bound twice the amount of antibody relative to the unmodified plate, and the ELISA signal more than tripled indicating improved antibody orientation. The chromium modification was demonstrated for use with five other antigen capture ELISAs. Welch *et al.*¹²⁸ also demonstrated optimization of the chromium complex by varying the metal salt and buffering base compounds and ratios. The optimized complex (1:1 chromium perchlorate hexahydrate to ethylenediamine) was used to improve the antigen detection limits of a bovine tumor necrosis factor alpha (TNF α) ELISA by an order of magnitude relative to untreated plates. In a recent study, Welch *et al.*²⁹ employed a traditionally low fouling diethylene glycol dimethyl ether plasma polymer

(DGpp) as a substrate for binding [Cr(OH)₆]^{3−} with subsequent antibody immobilization. When equivalent amounts of antibody were immobilized on the DGpp and the chromium functionalized DGpp substrates, a tenfold improvement in ELISA signal intensity was observed for the chromium functionalized system indicating an oriented system. ToF-SIMS analysis identified that chromium may be binding the antibody through lysine, methionine, arginine, and histidine residues.

III. IMMOBILIZED ANTIBODY CHARACTERIZATION OVERVIEW

An immunoassay is the most widely employed method to assess the state of an immobilized antibody, in that it represents the practical application of successful immobilization. ELISAs require that immobilized antibodies maintain the correct orientation, unperturbed conformation, and adequate density at the substrate surface to maximize signal production and thus antigen quantification. However, as this is an indirect analysis technique, it only allows the antibody state and orientation to be determined by inference. As a result, complementary and independent methods for assessing the antibody have been developed. While there are a number of techniques for quantifying the density of adsorbed proteins,¹²⁹ the number of techniques that can probe antibody orientation is much smaller. Table I presents an overview of current characterization techniques, and schematic representations are shown in Fig. 3.

A. X-Ray photoelectron spectroscopy

XPS is a spectroscopic technique that uses incident x-ray photons to probe the elemental and chemical composition of the top 10 nm of the sample surface. This technique can be

TABLE I. Overview of surface analysis techniques employed for investigating antibody.

	Technique	Input	Output	Information	Comments	References
A)	XPS	Monochromatic x-rays	Photoelectrons	Elemental and chemical	Quantification of antibody surface density	23, 29, 127, 128, and 131
B)	SE	Elliptically polarized light	Change in light phase or intensity	Thickness, refractive index, surface roughness	Model based analysis, inferred state of antibody	132–137
C)	Dual polarization interferometry (DPI)	Laser light	Evanescence wave change	Mass, film thickness, refractive index, density	Inferred state of antibody based on mass and film thickness	64, 138, and 139
D)	SPR	Monochromatic multiangle laser light	Change in reflected and absorbed light	Refractive index, film thickness	Inferred state based on antibody and antigen adsorption characteristics	55 and 140–142
E)	NR	Neutron beam	Change in reflection of neutron beam	Refractive index, film thickness, surface roughness	Model based analysis, inferred state of antibody	143–146
F)	AFM	Feedback driven cantilevered tip	z-height in 2D and tip/surface force	Surface roughness, phase information, imaging	Correctly oriented antibodies have 14 nm height	56, 142, and 147–149
G)	QCM	Resonance frequency of microbalance	Change in frequency and amplitude	Mass of adsorption, bioaffinity	Inferred state of antibody based on adsorption and mass	150 and 151
H)	ToF-SIMS	Ionized metal clusters, “primary-ions”	Ionized sample fragments, “secondary-ions”	Semiquantitative elemental, chemical, and molecular	Amino acid composition of F(ab') ₂ and Fc varies and can be distinguished	45, 130, and 152–157

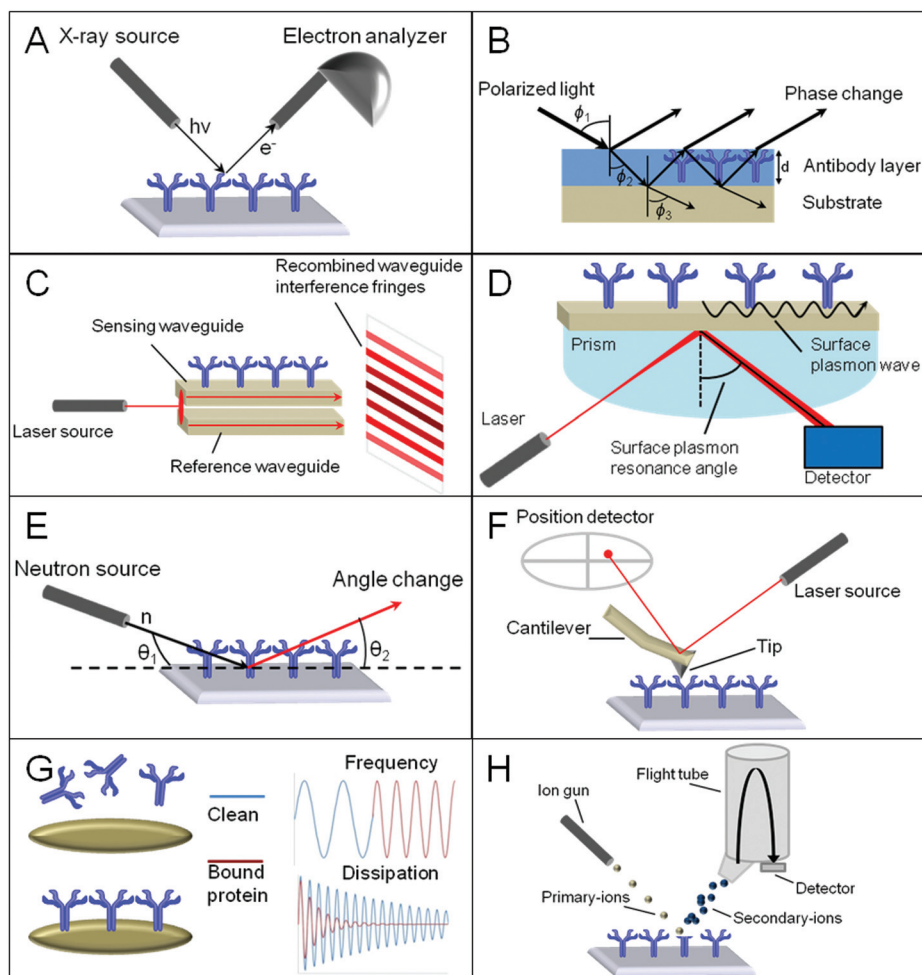


FIG. 3. Illustrative schematic of antibody orientation characterization techniques: (a) X-ray photoelectron spectroscopy, (b) spectroscopic ellipsometry, (c) dual polarization interferometry, (d) surface plasmon resonance, (e) neutron reflectometry, (f) atomic force microscopy, (g) quartz crystal microbalance, and (h) time-of-flight secondary-ion mass spectrometry.

used to quantify the amount of nitrogen present, which in turn can be correlated to the amount of immobilized antibody.^{29,127,128,130} Antibody orientation can then be inferred based on the immunoassay signal. Radadia *et al.*¹³¹ compared XPS and ELISA results to infer stability of antibody immobilized to glass and diamond films. Pâslaru *et al.*²³ prepared plasma treated PVDF membranes for adsorption or grafting of Protein A (followed by IgG) or IgG alone and used XPS to investigate sulfur and nitrogen content in the samples. The nitrogen concentration was used to quantify overall protein content, and sulfur concentration (present due to disulfide bonds in the IgG) was used to monitor IgG binding.

B. Spectroscopic ellipsometry

Spectroscopic ellipsometry (SE) is a surface sensitive optical technique that monitors the polarization change in light reflected from the sample surface (typically a metal or ceramic).^{132,133} Polarization changes occur due to variations in the dielectric or refractive index properties of the sample. Balevicius *et al.*¹³⁴ used total internal reflection ellipsometry to demonstrate that antibodies reduced with 2-

MEA, immobilized to a gold substrate, bind 2.5 times the amount of antigen as compared with their intact whole antibody counterparts immobilized randomly and covalently to SAMs. Bae *et al.*¹³⁵ compared IgG immobilized to thiolated Protein G (to represent an oriented antibody) with chemically bound IgG to an 11-MUA SAM, to represent randomly oriented binding. SE was used to estimate the IgG film thickness and together with atomic force microscopy (AFM) and SPR inference could be made regarding different orientations of the antibodies. Wang and Jin¹³⁶ first utilized Protein A adsorbed to silicon to immobilize anti-IgG and compared with anti-IgG adsorbed to the silicon. In a kinetic manner, SE was used to investigate the film thickness and found an increase in the anti-IgG bound by Protein A, inferring a preferentially oriented system. Wang then went on to investigate three different silane modifications to silicon for IgG binding and identified with SE that APTES/methyltriethoxysilane functionalized silicon covalently bound IgG using glutaraldehyde gave the largest increase in antibody and antigen binding as compared to either APTES and glutaraldehyde, or APTES alone.¹³⁷

C. Dual polarization interferometry

DPI is an optical waveguide technique that utilizes changes in the evanescent wave of the sample beam of laser light relative to a reference beam. The combined beams create an interference pattern that provides information regarding mass, film thickness, refractive index, and density.¹³⁸ Song *et al.*¹³⁹ used DPI to investigate anti-PSA antibody (anti-PSA) immobilized covalently via lysines to thiolated DPI chips, or captured via the Fc using immobilized Protein G. The thickness of the two systems was monitored as the antibody bound the PSA antigen and a detection limit of 10 pg/ml was achieved. Song went on to investigate different methods for anti-PSA immobilization with DPI, comparing boronate chelation, TCEP reduction with maleimide covalent linkage, Protein G, and random immobilization methods to PEG:Thiol or amine modified DPI chips.⁶⁴ They found that while the mass of anti-PSA loaded onto the DPI chip was lower for TCEP than boronate chelation, the antigen sensitivity was 20 times higher inferring preferential orientation with the TCEP method using amine modified chips.

D. Surface plasmon resonance

SPR is an optical technique used to monitor protein adsorption and surface interactions. Monochromatic laser light is used to stimulate a surface plasmon wave in the sensing surface and the reflected light angle is measured. Upon absorption on the sensing surface, the refractive index of the material changes causing the reflected light angle and the surface plasmon resonance angle to change accordingly. A recent review by Mauriz *et al.*⁵⁵ covers SPR-based assays in great detail.

Vashist *et al.*¹⁴⁰ utilized SPR as an assay method to investigate antibody immobilization strategies; random, covalent (EDC/NHS), oriented with adsorbed Protein A followed by antibody binding, covalent-oriented Protein A followed by antibody binding, and last covalent-CM5-dextran binding. SPR determined that the mass of antibody immobilized was greatest for the CM5 system. However, the covalent-oriented system bound the greatest amount of antigen and indicated preferential orientation. Zhang *et al.*¹⁴¹ used SPR to investigate antigen binding capabilities of gold-graphene oxide (Au/GO) composites compared with gold coated with Protein A. By tracking the resonant wavelength change as a function of antigen concentration, the authors demonstrated the oriented Protein A on Au/GO system had improved antigen sensitivity compared with Protein A on Au alone.

E. Neutron reflectometry

Neutron reflectometry (NR) is a diffraction technique used to investigate film thickness. Neutrons are reflected off the sample surface and assessed as a function of change in angle or wavelength. Lu's team have used NR to investigate several antibody binding and orientation effects.^{143–145} NR was used in conjunction with AFM to determine a flat-on orientation of anti- β -hCG antibody (specific to the β unit of human chorionic gonadotrophin) to silicon-oxide, as

observed by the film thickness.¹⁴³ Then using anti-PSA immobilized to silicon-oxide substrate, the combination of NR and DPI was used to demonstrate flat-on antibody orientation.¹⁴⁵ NR and SE were also used complementarily to assess the mass of antibody immobilized at different concentrations.¹⁴⁴ Schneck *et al.*¹⁴⁶ used NR to confirm the orientation of anti-(polyethylene glycol) (anti-PEG) antibodies immobilized to PEG polymer brushes of varying their grafting density. They noted that increased grafting density caused the distance between the two Fab regions to decrease and overall orients the antibodies such that the Fc region faced away from the PEG brush substrate into the bulk solution.

F. Atomic force microscopy

AFM is a topographic analysis technique that employs the scanning of a nanoscale tip across a sample surface. The tip is bound to an oscillating cantilever and by accurately monitoring the cantilever's change in resonance, a nanometer scale resolution image of the surface can be achieved. When surface bound, the asymmetrical dimensions of antibodies ($14 \times 10 \times 4 \text{ nm}^3$) allow changes to the surface topography, i.e., film thickness and surface roughness, to be measured and can be representative of different antibody orientations. Chen *et al.*¹⁴² used AFM and SPR to confirm end-on antibody orientation to the ProLinkerTM SAM by plotting the height profile of the immobilized antibody and monitoring antigen uptake. Coppari *et al.*¹⁴⁷ investigated a monoclonal antibody adsorbed on mica and, by combining height traces and images, were able to determine different antibody orientations with AFM. By incorporating molecular dynamic simulations with AFM images, Vilhena *et al.*¹⁴⁸ demonstrated flat-on, head-on, side-on, and end-on orientations of IgG adsorbed to graphene. Funari *et al.*⁵⁶ characterized physisorbed and irradiation-coupled antibodies on extremely smooth (root-mean-squared roughness $0.15 \pm 0.01 \text{ nm}$) gold-coated silicon wafers. The irradiation causes photo-reduction of disulfide bridges that yield free-thiols for binding gold. Using AFM, the authors found that the irradiated antibodies had a smaller contact area with the surface and a larger height distribution indicating a side-on orientation relative to the physisorbed system being flat-on. Marciello *et al.*¹⁴⁹ used AFM to investigate the orientation of antibodies at the surface of lipase-coated magnetic nanoparticles. The authors assessed the surface of the nanoparticles and after immobilization. Two immobilized antibody systems were investigated; the optimized sample, S1, with a recovered immune activity (from immunoassay) of 80%, and S2, with a lower recovered immune activity (about 3%). The peak-to-valley height determined with AFM of S1 was found to be 9 nm as compared with only 5 nm for S2, indicating preferential orientation of the antibody on S1.

G. Quartz crystal microbalance

QCM is a mass sensitive technique that monitors the change in frequency and damping of a resonant quartz

piezoelectric crystal. As the material is adsorbed to the crystal, the resonance frequency decreases, allowing the amount of material to be determined very precisely. Thus, if the mass of the antibody and its complementary antigen are known, then antibody orientation can be inferred from antigen binding. Recently, Deng *et al.*¹⁵⁰ have developed a QCM chip modification that is used to orient biotin-labeled antibodies for use in an immunoassay. QCM was used to quantify antibody binding and antigen coupling. Compared with the control surface, the biotinylated graphene oxide-avidin surface modification was found to bind slightly lower amounts of antibody, although it demonstrated improved antigen capture, suggestive of antibody orientation.

Dissipation monitoring during QCM can provide insight into the density and permeability of immobilized (bio)molecules at the interface.^{51,158} Via this method, protein orientation may be investigated; for instance, an antibody immobilized close to the surface in a flat-on orientation will have a low viscoelastic dissipation, while in contrast, an antibody in head-on or end-on orientation will have a higher viscoelastic dissipation.

Another QCM mass-based immunoassay was proposed by Akter *et al.*¹⁵¹ who employed Protein A as the antibody orienting component. Using QCM, the authors were able to monitor antibody binding, antigen selectivity, and demonstrate the benefits of their precipitation mass amplification system as applicable in immunoassay.

H. Time-of-flight secondary-ion mass spectrometry

ToF-SIMS employs a focused beam of ionized metal atoms (or clusters of atoms), large molecules such as fullerenes (C_{60}), or gas clusters, to bombard the sample and remove fragmented material from the surface. A small percentage of the fragments are ionized, known as secondary ions, which are collected as a representative sample of the elemental and molecular species from the top few monolayers of the surface (on the order of 10 Å).¹⁵⁹ The 14 nm long axis of antibodies, combined with this limited sampling depth, provides differentiation between orientations of immobilized antibody due to different amino acid compositions in the portions being analyzed.

ToF-SIMS analysis of known peptides has been used to determine most common mass fragments arising from particular amino acids.^{152,160,161} These amino acid specific lists, typically of up to 40 mass fragments, allow differentiation between protein and substrate.^{153,154} Foster *et al.*¹³⁰ analyzed bovine IgG adsorbed to gold and sodium styrenesulfonate coated gold surfaces. As the prevalence of serine is higher in the $F(ab')_2$ region of the IgG, and aspartate and valine prevalence higher in the Fc region, then a ratio of these amino acids mass fragment intensities could be used to assess the relative orientation of IgG immobilized to the substrates. Wang *et al.*¹⁵⁵ investigated anti-hCG IgG, $F(ab')_2$, and Fc immobilized on gold and also orientation promoting SAMs (COOH and NH_2). Principal component analysis (PCA) identified amino acid fragments that were more prevalent in

the $F(ab')_2$ and Fc portions, and these were correlated against the antibody amino acid composition. A ratio of the important amino acids could then be used as predictors for antibody orientation. More recently, Welch *et al.* has employed larger peak lists incorporating over 700 mass fragments for characterizing adsorbed whole antibody and antibody fragments.¹⁵⁶ The increased peak list significantly improved the ability to identify and classify the samples using multivariate analysis techniques. One potential drawback is that ToF-SIMS operates in an ultrahigh vacuum environment (UHV) that is likely to denature proteins.¹⁵⁷ One possible strategy for avoiding UHV induced denaturation is by the fixation of proteins at surfaces with trehalose. Trehalose is a disaccharide that has been used to fix the state of proteins at interfaces to preserve their conformation and minimize changes to their orientation.^{153,162} However, coating samples prior to ToF-SIMS analysis, or other surface analysis techniques such as XPS, may cause difficulties in subsequent data analysis.

I. Multivariate analysis

It is now a common practice to employ multivariate analysis techniques such as PCA to reduce the dimensionality of complex data sets, such as those derived from ToF-SIMS. PCA is used to identify the variables that contribute to the largest amount of variance in the dataset. In the case of ToF-SIMS analysis, the intensity or number of counts obtained for each mass fragment comprise the input variables for PCA. PCA has been used to investigate antibody orientation on various substrates to good effect.^{163,164} Liu *et al.*¹⁶⁵ immobilized Fab and Fc fragments to both gold and polymer-coated slide substrates and used PCA to investigate each of the four systems. Principal component 1 (PC1) separated the samples based on substrate, and principal component 2 (PC2) separated samples based on antibody fragment. The loadings plot for PC2, showing the contributions from each of the amino acid variables, correlated strongly with natural amino acid composition differences in the antibody fragments. Park *et al.*¹⁶⁴ interrogated randomly and site-directed IgG and $F(ab')_2$ with ToF-SIMS and PCA. Using known amino acid related mass fragments, PC1 showed that site-directed IgG and $F(ab')_2$ were more commonly in the end-on orientation than the randomly immobilized proteins. Kosobrodova *et al.*¹⁶⁶ investigate antibodies immobilized to untreated and plasma treated polycarbonate with ToF-SIMS and PCA and found that the $F(ab')_2$ component of the antibody was preferentially exposed on the plasma treated surface.

Artificial neural networks (ANN) are another class of multivariate analysis techniques and classify and group samples based on their similarities and differences across the input variables. Sanni *et al.*¹⁶⁷ employed ANN to differentiate between 13 different protein films, including two types of antibodies, using nominal mass values of all mass fragments available in the spectra. ANN was able to identify the key mass fragments associated with each of the proteins to

distinguish between them. More recently, Welch *et al.*¹⁵⁶ employed ANNs to discriminate between an antibody and its proteolysis fragments adsorbed to silicon substrates, based solely on their ToF-SIMS spectra. The ANN analysis method holds promise for investigating antibody orientation at interfaces due to its ability to incorporate a broad range of mass fragments and investigate complex relationships between variables.

IV. CONCLUSIONS AND PERSPECTIVES

In summary, existing and new methods for oriented antibody immobilization have been developed over the past few years with good progress made on improving the established methods. Nevertheless, some shortcomings and challenges still exist. Oriented systems based on novel surface chemistry, protein engineering, or both, require complex and time-consuming production steps, which may also be expensive. Ideally, antibodies truly representative of their native solution-phase state, i.e., without protein engineered tags, would be immobilized to a simple, cheap, and easy to produce substrate, homogeneously arranged and site-directed as to maximize their antigen capture. However, it is likely that smaller antibody fragments and aptamers may be favored over whole antibodies in the future as they can be prepared recombinantly and are easily modified genetically or chemically with the ability to maximize capture events due to increased packing and binding site density.¹⁶⁸ Additionally, camelid antibodies with one single domain for antigen binding (known as VHH) have attracted attention due to their high solubility and stability, and may provide an opportunity for incorporation into sensors.⁸ Also, peptides and aptamers provide a highly customizable method for producing covalent attachment of antibodies, or fragments thereof, either by active targeting of the protein, or the substrate, or both. In the future, it may be seen that metallic thin films are used to produce homogeneous substrates that can be targeted simply by endogenous antibody epitopes or material binding peptides coupled to Fc specific aptamers or peptides. In the case of the latter, such a system could be near universally applicable to native state antibodies.

It is clear that surface analysis techniques and multivariate analysis tools will play a prevalent role in identifying, investigating, and predicting antibody orientation at substrates of interest. Characterization of antibody orientation *in situ* or in the native “wet” environment permits a more accurate presentation of the antibody state without the potential for confirmation changes. XPS and ToF-SIMS require UHV conditions and are *ex situ*. AFM is typically performed in a dry state (however progress has been made regarding wet analysis) and is performed *ex situ*. QCM, SPR, NR, SE, and DPI can be performed in solution and with the correct apparatus *in situ* also. Additionally, complementary techniques such as sum frequency generation promise to provide *in situ* characterization of protein orientation via changes in the infrared absorption patterns; however, complex analysis is still required.^{169,170}

Nevertheless, ToF-SIMS has the potential to characterize and differentiate antibody properties including orientation and denaturation state, yielding molecularly specific information from the uppermost surface. The information-rich data greatly stands to benefit from interrogation with multivariate analysis techniques PCA and ANN. Not only can this combination offer new insight into the state of immobilized proteins, but it is also directly applicable to new material discovery and will greatly assist in the development and optimization of immunoassay performance. In parallel with this approach, recent developments in molecular modeling, and, in particular, coarse-grain modeling,^{171,172} may provide a method for assessing antibody orientation characteristics on novel substrates *in silico* before fabrication, or to aid substrate design.

In this article, we have discussed the most up-to-date and state-of-the-art methods used for the immobilization of antibodies in an oriented manner, particularly for the improvement of immunoassay performance. Further, we have discussed the current range of surface analysis techniques being used for investigating the orientation of antibody systems and have highlighted the importance of multivariate analysis tools in the interrogation and analysis of the experimental data produced.

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