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Joshua M. Correira, Paul R. Handali and Lauren J. Webb



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Joshua M. Correira, Paul R. Handali, and Lauren J. Webb[a]

## AFFILIATIONS

Department of Chemistry, Texas Materials Institute, and Interdisciplinary Life Sciences Program, The University of Texas at Austin, 105 E 24th St. STOP A5300, Austin, Texas 78712-1224, USA

[a]Author to whom correspondence should be addressed: lwebb@cm.utexas.edu

## ABSTRACT

Many sensors and catalysts composed of proteins immobilized on inorganic materials have been reported over the past few decades. Despite some examples of functional protein–surface and protein–nanoparticle conjugates, thorough characterization of the biological–abiological interface at the heart of these materials and devices is often overlooked *in lieu* of demonstrating acceptable system performance. This has resulted in a focus on generating functioning protein-based devices without a concerted effort to develop reliable tools necessary to measure the fundamental properties of the bio–abio interface, such as surface concentration, biomolecular structure, and activity. In this Perspective, we discuss current methods used to characterize these critical properties of devices that operate by integrating a protein into both flat surfaces and nanoparticle materials. We highlight the advantages and drawbacks of each method as they relate to understanding the function of the protein–surface interface and explore the manner in which an informed understanding of this complex interaction leads directly to the advancement of protein-based materials and technology.



## I. INTRODUCTION

Enzymes have evolved to perform chemical recognition and catalysis under mild conditions in an aqueous solution, often faster, and with greater specificity than synthetic counterparts. The catalytic function of enzymes has been used in sensors to detect small molecule substrates since invention of the electrochemical glucose sensor based on the enzyme glucose oxidase, which was first immobilized on an electrode in 1967.[1,2] Immobilization has been shown to greatly simplify sensor analysis by reducing reagents, simplifying analyte separation, and improving reusability and stability. However, despite these benefits, enzymes have long been known to inactivate to some degree, including catastrophically, following conjugation to or immobilization on synthetic materials, and limiting the general utility of this strategy for sensor design.[3] This is one of the reasons why the glucose oxidase sensor is one of the only commercially successful enzyme-based sensors for the detection of small molecules. As we will discuss in this Perspective, there have been many successful reports of enzyme-based devices in the literature; however, the investigation of fundamental properties of these systems across the literature is often inconsistent and even absent. This has made it difficult for discovering general rules and guidelines for generating successful devices based on new biological molecules or new materials and has impeded the advancement of the field beyond a few key examples. To improve the performance of enzyme-based materials, catalysts, and sensors, fundamental properties such as the structure and activity of the enzyme relative to its native non-immobilized counterpart must be investigated and understood. It is only then that informed changes to the system can be made to improve properties of interest such as activity, stability, sensitivity, selectivity, and other key performance characteristics, or to expand certain successful immobilization strategies to other systems.

Methods for integrating proteins into an inorganic or synthetic material fall into three general categories: (1) immobilization on a flat surface; (2) conjugation to a colloidal particle or bead; and (3) entrapment in a matrix. Methods for preparing these constructs have been reviewed elsewhere.[4,5] The scope of this Perspective focuses on

common analytical techniques used to characterize important properties, including specific activity, surface concentration, and protein structure, on two general types of systems: (1) enzymes immobilized on surfaces and (2) enzymes conjugated to nanoparticles. This is summarized in Fig. 1. We will also discuss the advantages and drawbacks of each method, define best practices, and describe how understanding the fundamental physical properties of the bio–abio interface provides the insight necessary for prospective and predictive design of functional and unique bio–abio materials and devices.

## II. PROTEIN–SURFACE CONJUGATES

The immobilization of enzymes onto surfaces is governed by several processes: (1) displacement of water at the surface–water interface by an equal volume of enzyme; (2) interactions between surface amino acids on the enzyme and the surface itself; (3) structural rearrangements within the enzyme to reach an energy minimized immobilized state; and (4) lateral displacement of the enzyme along the surface to reach an optimal binding site or orientation.[6–8] Additionally, there may be the formation of covalent bonds between surface molecules and amino acids or labels on the enzyme, which can anchor it in place. Clearly, each process depends heavily on the specific enzyme being used and the surface chemistry and morphology of the material it is interacting with. These processes help stabilize the enzyme, which can be both advantageous and disadvantageous. First, strong interactions between the enzyme and surface can prevent enzyme leaching, where the enzyme desorbs or otherwise detaches from the surface and is lost. Additionally, reduced motion on the surface can limit transient unfolding and denaturation, which reduce enzyme activity over time. However, in the presence of strong enzyme–surface interactions, there can be structural changes that immediately reduce enzyme activity by a large fraction (80%–90%).[9–12] This is caused by amino acid residues and chains in the protein interacting directly and favorably with the surface through noncovalent electrostatic, hydrogen bonding, and hydrophobic interactions, resulting in a new energy minimized structure. Due to the structure–function relationship of enzymes, these changes in the native structure can lead to changes in its activity. This is highly dependent on the specific enzyme and surface chemistry being used, but generally happens when there are direct protein–surface interactions that favor immobilization. There are numerous strategies to immobilize enzymes onto surfaces that have been reviewed elsewhere.[4,13–15] Here, we will discuss current methods used to characterize protein–surface conjugates and how the results relate to understanding device performance. Specifically, we will discuss methods to characterize the specific activity, concentration, and structure of immobilized enzymes. For sensor systems, the reported characterization often involves measuring sensor response as a function of (1) analyte concentration; (2) common interfering and inhibiting species; and (3) storage time, temperature, pH, ionic strength, and other conditions; and is highly practical. However, these measures do not report on *why* the integrated biological–abiological material is performing in an observed manner. To fully understand the reason of observed behavior, a baseline of enzyme performance must be obtained in solution and compared with the immobilized enzyme in a realistic and fair way. We will discuss the following metrics of surface-bound enzyme performance: specific activity, surface concentration, and structure. We will also discuss several different characterization methods that are applied to surfaces and the advantages and disadvantages of each as they relate to understanding performance.

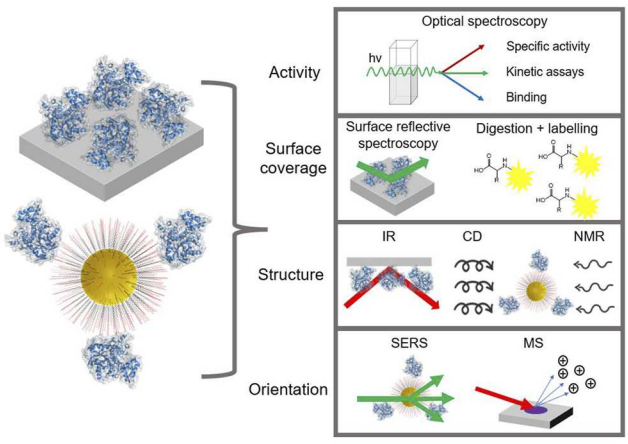


**FIG. 1.** Overview of important fundamental properties of protein-surface and protein-nanoparticle conjugates and the techniques used to characterize them.

### A. Enzyme activity

First, the most commonly reported property of surface immobilized enzymes is enzyme activity, the property that is viewed as most relevant to evaluate a biomolecular-based sensor with practical applications. In general, enzyme activity is measured by assays that monitor the rate of catalysis by the enzyme, usually by measuring changes in concentration of substrates or products as a function of time, often colorimetrically or electrochemically. Activity, when considered as just a rate of reaction, is a function of many conditions including temperature, pH, ionic strength, substrate concentration, and enzyme concentration. Thus, when comparing the activity of a surface immobilized enzyme to the native enzyme dissolved in solution, all of these conditions must be kept constant or be accounted for to understand how the molecule behaves differently in this new situation. However, this is not trivial because local concentrations of ions, protons, and substrate molecules at the surface–solution interface may be vastly different than in bulk solution. This contributes to observed changes in enzyme activity relative to the activity in the bulk solution that has nothing to do with the biological molecule itself and must be quantified. Additionally, the effects of substrate mass transport on the rate of catalysis must be identified and accounted for and the enzyme surface concentration must be known. First, we will discuss the effects of substrate mass transport.

In a traditional solution-based biochemical measurement, enzyme activity is dependent on substrate concentration, with all other variables (pH, ionic strength, and temperature) held constant, except in the case of diffusion limited systems. This is because the rate of mass transport to the enzyme, which depends on substrate concentration, is much higher than the actual catalytic rate of the enzyme, except in a few limiting cases of extremely efficient

enzymes.[16] Substrate diffusion in bulk aqueous solution to an enzyme can be modeled by spherical diffusion. However, when the enzyme is immobilized on a surface this is often no longer the case. This is mainly because mass transport to a surface is slower than it is in bulk solution and should be modeled by linear diffusion,[17] which in turn means that surface-immobilized enzymes are often diffusion limited. Higher concentrations of substrate (1–2 orders of magnitude) will, therefore, be required to compensate for the difference in mass transport, and achieve comparable rates to the native aqueous enzyme.[18] A kinetic model, most commonly based on Michaelis–Menten kinetics, must be used to understand the mass transport differences between the enzyme in aqueous solution and on a surface.[19] Doing this requires measuring enzyme activity in solution and on a surface as a function of substrate concentration and fitting the results to the Michaelis–Menten equation,

$$v = \frac{V_{Max}[S]}{K_M + [S]}. \quad (1)$$

Here, $v$ is the measured rate of reaction, $V_{Max}$ is the maximum catalytic rate of the enzyme, $K_M$ is the Michaelis constant, and [S] is the substrate concentration. $V_{Max}$ is a measure of the catalytic rate of the enzyme when it is kinetically limited under sufficiently high mass transport rates, and $K_M$ is a measure of the degree of mass transport limitations, where larger $K_M$ values indicate slower mass transport rates. Thus, $V_{Max}$ of the immobilized enzyme can be compared with $V_{Max}$ of the native aqueous enzyme to understand how the activity of the enzyme changes following immobilization, while being able to ignore mass transport effects. Under ideal circumstances, $V_{Max}$ of the immobilized enzyme should be equal to or greater than $V_{Max}$ of the native aqueous enzyme, indicating no loss of activity associated with the immobilization process; however, this is often not the case. It is common for $V_{Max}$ to be 1–2 orders of magnitude lower for an immobilized enzyme, due to surface-induced structural changes as will be discussed below, and $K_M$ to be 1–2 orders of magnitude higher, due to reduced mass transport of substrate to the immobilized enzyme. An example of this is shown in Fig. 2, which reports the Michaelis–Menten kinetics of acetylcholinesterase (AChE) adsorbed on planar gold.[11] In this example, $V_{Max}$ of immobilized AChE was about 90% lower than in the solution. Conversely, $K_M$ was also about 30-fold higher on the surface than in solution due to the decreased rate of substrate mass transport to the immobilized AChE active site. If this material was used as a sensor based on AChE activity, the limit of detection of its substrate would be 30-fold worse on the surface than in solution because of the decrease rate of mass transport to the surface. Depending on the application, this may be fatal to the success of that device, but knowing the source of that failure allows a hypothesis-based design progress to move toward success. For example, mass transport rates could be improved by stirring the solution or rotating the surface, as is the case for a rotating disk electrode.[17] Alternatively, mass transport into the active site can be improved by optimizing the orientation of the enzyme on the surface and reducing crowding.

## B. Enzyme surface concentration

As shown in Fig. 2, $V_{Max}$ should be normalized by enzyme concentration into a measure of the specific activity. This allows the activity of identical quantities of enzymes on the surface and in solution to be compared for a more detailed understanding of how the surface immobilization or environment affects the enzyme. In the above example, since $V_{Max}$ is normalized by the mass of enzyme in solution and on the surface, it shows that the activity of the enzyme itself has decreased by about 90% following immobilization. However, to do this, the enzyme surface concentration must be known. This is a commonly overlooked property of enzyme-based systems. In addition to allowing the comparison between surface and solution activities, it also grants insight into enzyme loading onto the surface (amount and packing density). This is an important property to understand because excessive loading, in the case of a densely packed monolayer, can contribute to activity losses by overcrowding and limiting substrate diffusion into the enzyme active site. Low loading (fraction of a monolayer) limits the activity of the device because fewer enzymes are available for functional activity and can also lead to enzymes unfolding and spreading to occupy a surface area.[20,21] Numerous strategies have been developed to measure enzyme surface concentration using techniques including quartz crystal microbalance with dissipation (QCM-D),[22–24] surface plasmon resonance (SPR),[25–27] attenuated total reflectance infrared spectroscopy (ATR-IR),[10,28] spectroscopic ellipsometry (SE) or single wavelength ellipsometry,[29–31] amino acid analysis (AAA),[32] solution depletion,[8,33] x-ray photoelectron spectroscopy (XPS),[34,35] and microscopy including atomic force microscopy (AFM)[36] and scanning tunneling microscopy (STM).[37,38] We will provide examples of the most common methods and discuss the advantages and

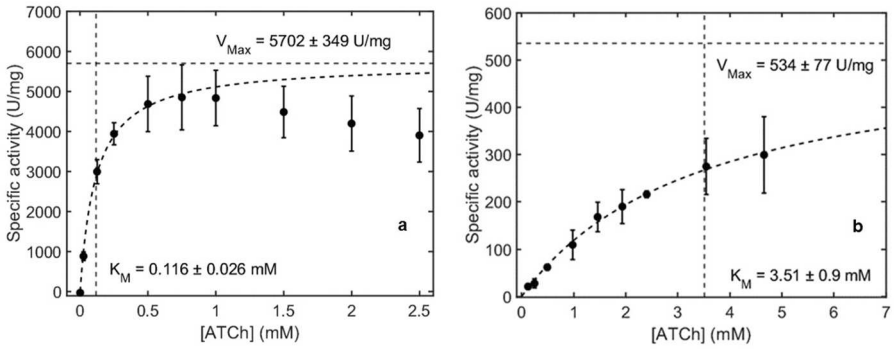


**FIG. 2.** Michaelis–Menten kinetics of acetylcholinesterase in solution (a) and adsorbed on planar gold (b). Vertical dashed lines indicate $K_M$ and horizontal dashed lines indicated $V_{Max}$. The dashed curve shows the fit to the Michaelis–Menten equation. Reprinted with permission from J. M. Correira and L. J. Webb, Langmuir **38**(11), 3501–3513 (2022). Copyright 2022 American Chemical Society.

disadvantages of each as they relate to understanding enzyme loading and resulting activity.

QCM-D is one of the most widely used techniques for measuring surface concentrations. It measures a change in the resonant frequency of an oscillating quartz crystal due to mass coupled to the surface and allows for convenient, sensitive, *in situ* analysis of proteins or other molecules immobilized on a surface. QCM-D also measures the dissipation of the quartz crystal, which other QCM techniques do not. As the crystal oscillates, molecules coupled to the surface can dampen the oscillations and dissipate the crystal's energy. This energy dissipation occurs faster when large flexible molecules, such as proteins and other polymers, are coupled to the surface of the crystal and can provide information about the viscoelastic properties of the surface film. Increases in dissipation correspond to an increase in flexibility of the film while decreases correspond to an increase in rigidity. Thus, shifts in resonant frequency and dissipation can be used to understand enzyme immobilization and layer formation on the surface.

Despite these advantages, there are numerous disadvantages that must be considered before using this technique. (1) QCM-D measures a change in resonant frequency, which is difficult to convert into the mass of immobilized material. For rigid small molecules, there is a straightforward linear relationship following the Sauerbrey equation,

$$\Delta m = -\frac{C}{n}\Delta f, \tag{2}$$

where $\Delta m$ is the change in mass coupled to the quartz crystal, $C$ is the mass sensitivity constant, $n$ is the overtone number, and $\Delta f$ is the measured change in the resonant frequency of the quartz crystal.[22,31,39] For larger molecules with increased flexibility, such as many proteins, the relationship becomes more complicated and depends on the viscoelastic properties of the immobilized biomolecule film.[40] Thus, the Sauerbrey equation should not be used if increases in dissipation are observed. (2) QCM-D measures mass coupled to the quartz crystal, not specifically immobilized to the surface, and can include any solvent molecules or ions associated with the protein. These must be considered in the model described by Eq. (2), but are usually difficult to determine quantitatively.[41] Because of this, QCM-D often dramatically overestimates the amount of immobilized protein. (3) QCM-D analysis is often performed under the laminar flow of the solvent due to the sensitivity of the measurement to vibrations. This can be very different than under stagnant solvent.[42] Under solvent flow, enzyme molecules reach the surface by diffusion and convection while under stagnant solvent, only diffusion occurs. Additionally, the bulk solution above the surface is constantly being replenished with the fresh enzyme. Thus, immobilization can occur faster and enzyme loading can differ from stagnant conditions. Considerations should be made before generalizing results obtained under solvent flow to other samples prepared under stagnant solvent where enzyme loading may be different. Additionally, oscillations of the quartz crystal itself have been shown to affect immobilization, altering the kinetics and amount of bound mass relative to a non-oscillating surface.[43] However, if care is taken to control and/or normalize for these factors, then QCM-D can be a useful technique to monitor protein immobilization and loading onto surfaces as it occurs *in situ*. An example of the adsorption of azurin onto an octanethiol-coated gold QCM sensor is shown in Fig. 3 from Fleming *et al.*[22] The decrease in resonant frequency can be seen following the addition of azurin and the adsorbed mass, and, thus, surface concentration, was determined from the Sauerbrey equation [Eq (2)]. In this case, the Sauerbrey equation could be applied because the protein film was sufficiently rigid, as exemplified by the lack of increase in dissipation ($\Delta D$) and good overlap between each overtone, as explained above. This was due to the structural rigidity of azurin but may not hold true for all proteins. It has been shown that change in resonant frequency poorly correlates with protein molecular weight from 3.5 to 150 kDa for physisorbed proteins, due to varying structural flexibility between proteins and, thus, differences in dissipation.[44] However, when the proteins were biotinylated and immobilized to a surface containing covalently bound biotin antibody, film rigidity improved and the change in resonant frequency correlated well with protein molecular weight.

SPR is another commonly used technique to monitor the immobilization of proteins *in situ* and determine the surface concentration of proteins on metals. It measures a change in the resonance angle required to optically excite surface plasmons on a metallic

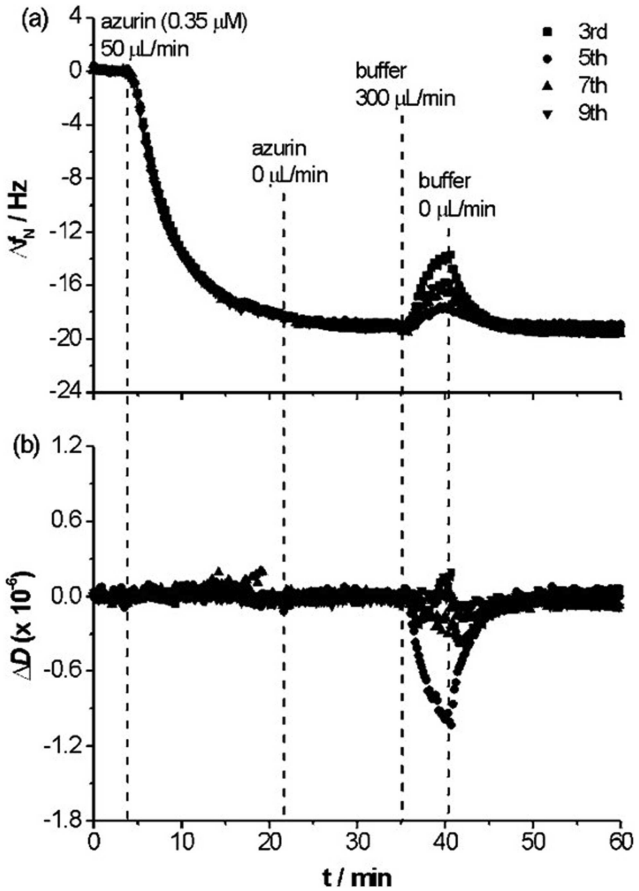


**FIG. 3.** QCM-D traces of $\Delta f_N$ (a) and $\Delta D_N$ (b) for the adsorption of azurin onto octanethiol-coated gold. Reprinted with permission from Fleming *et al.* Langmuir **24**(1), 323–327 (2008). Copyright 2008 American Chemical Society.

film, which depends on the thickness and refractive index of any films present at the metal-solution interface, such as immobilized proteins.[26,45–47] The SPR response is defined as changes in this SPR angle, which is proportional to changes of the interfacial refractive index. Unlike QCM-D, SPR is not sensitive to coupled solvent molecules, since SPR angle shifts occur when the interfacial refractive index changes relative to the ambient (often buffer); coupled solvent molecules, therefore, do not contribute to refractive index changes since they have the same refractive index as the ambient. Additionally, SPR is an optical technique and results do not depend on the viscoelastic properties of the immobilized protein film. The conversion from SPR response to surface concentration can be calibrated using complementary techniques, such as others described here.[46] Alternatively, the film refractive index or thickness can be calculated directly using a complex Fresnel calculation.[48] Once the film refractive index and thickness are known the surface concentration can be calculated. This is routinely done with ellipsometry, as will be discussed below; however, refractive index and film thickness cannot simultaneously be determined and one must be fixed at an assumed value. A classic example of monitoring protein immobilization *in situ* with SPR is from Mrksich *et al.*[26] The adsorption of four different proteins onto self-assembled monolayers on gold of varying compositions was monitored under flow conditions to determine adsorption kinetics. In this case, the results were left in terms of SPR response and were not converted to surface concentrations. Even without determining absolute surface concentrations, valuable information about immobilization kinetics and relative surface concentrations can be obtained from the raw data by comparing the magnitude and rate of change of SPR response. This is particularly useful for optimizing immobilization conditions, such as enzyme concentration, incubation time, temperature, ionic strength, and pH.

ATR-IR, or other forms of surface IR spectroscopy such as external reflection, can be very useful in terms of measuring surface concentrations and immobilized protein structure, as will be discussed later. This is because ATR-IR is surface sensitive, fast, and simple to perform. IR spectroscopy of proteins generally involves looking at absorptions of the amide backbone. Two key vibrational modes, amide I (1600–1700 $cm^{-1}$) and II (1500–1600 $cm^{-1}$), are due largely to the carbonyl stretching and N–H bending, respectively, of the amide backbone.[49] The intensities of amide I and II generally correlate with the amount of protein present in the sample and can be used as a measure of surface concentration. Our laboratory has shown that ATR-IR is useful for determining relative surface concentrations in an adsorption isotherm.[11] Determining absolute surface concentration requires relating amide absorbance to immobilized protein concentration, often through a calibration curve; an example of this is shown in Fig. 4.[10,50] There, Khaldi *et al.* reported ATR spectra collected in the air for the enzyme acetylcholinesterase (AChE) covalently bound to a self-assembled monolayer on silicon. They generated a binding curve by varying the AChE concentration during immobilization and showed that the amide I integrated area corresponded to relative surface concentration. The integrated area was then related to absolute surface concentration through a calibration curve generated from ATR spectra of solutions of varying protein concentrations. Other techniques described here, such as SPR and ellipsometry, can also be used to independently determine immobilized protein concentration and generate such a calibration curve. However, IR techniques can be limited in the presence of interfering species on the surface that have overlapping absorptions since the 1500–1700 $cm^{-1}$ region is relatively cluttered from vibrational modes. Interfering species can include molecules involved in the immobilization process such as covalent linkers, immobilized supports, and even liquid and gaseous water. Despite this, ATR-IR has been a useful technique to determine relative or absolute surface concentrations of immobilized proteins.

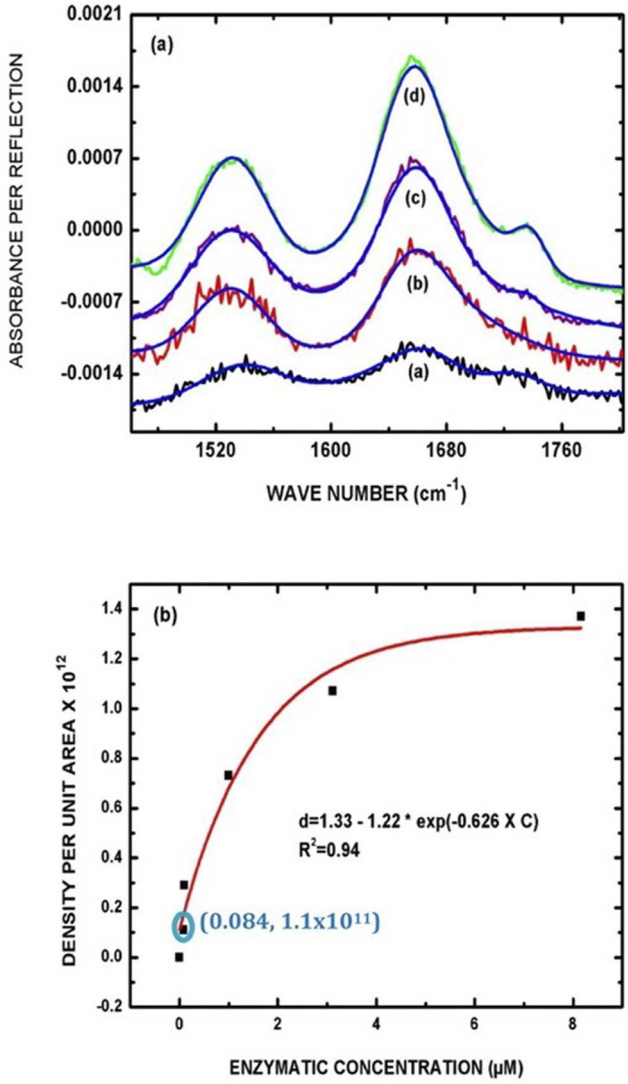


**FIG. 4.** (a) FTIR spectra of immobilized acetylcholinesterase on silicon showing amide I (1600–1700 $cm^{-1}$) and amide II (1500–1600 $cm^{-1}$) bands at different surface concentrations. (b) Binding curve relating bulk enzyme concentration to surface density per unit area, calibrated using bovine serum albumin in solution. Reprinted with permission from Khaldi *et al.* Langmuir **31**(30), 8421–8428 (2015). Copyright 2015 American Chemical Society.

Ellipsometry, specifically SE, is another optical method for characterizing the surface concentration of immobilized proteins,

similar to SPR. SE measures the change in polarization of incident light as it reflects and refracts at interfaces as a function of wavelength and incident angle.[51] The change in polarization is due to the thickness and optical functions (refractive and absorption indices) of any films present on the surface, including immobilized proteins. SE is commonly performed *ex situ*, but can be performed *in situ*. Additionally, there is a linear relationship between surface concentration and optical thickness (product of refractive index and film thickness), as seen in the numerator of the de Feijter equation,

$$\Gamma = \frac{d(n - n_0)}{M_W(dn/dc)}, \tag{3}$$

where $d$ is the film thickness in cm, $n$ is the film refractive index at 589.3 nm, $n_0$ is the ambient refractive index (commonly buffer or air) at 589.3 nm, $M_W$ is the molecular weight of the protein in units of g mol$^{-1}$, $dn/dc$ is the refractive index increment of the protein in units of mL g$^{-1}$, and $\Gamma$ is the surface concentration in units of mol cm$^{-2}$.[52] This relationship allows an absolute surface concentration to be determined by measuring the thickness and refractive index of immobilized protein films. However, in ultrathin films under 10–20 nm, as is common for layers of immobilized proteins, SE is not sensitive to both thickness and refractive index simultaneously, and they cannot be deconvoluted, similarly to SPR.[51] Instead, SE is sensitive to the optical thickness, which conveniently appears in the numerator of Eq. (3) and allows for a unique solution for surface concentration. Alternatively, other techniques, such as AFM, can be used to independently measure film thickness to overcome the thickness-index convolution.[53]

SE is commonly performed on dry samples but can be performed on samples in buffer using a liquid cell, as our laboratory has shown previously.[11] However, SE requires that the surface be flat and reflective and is usually limited to metals, semiconductors, and glasses. Additionally, any aggregates on the surface will scatter light and depolarize incident light, resulting in poor fits to the data. Data also become difficult to model for complex systems with ultrathin films and multilayers, for example, proteins immobilized on self-assembled monolayers or other films. This is because the optical functions and thicknesses of each layer must be determined.[51] Despite this, our laboratory has demonstrated that with the proper controls, SE provides an exceptionally useful means of determining absolute surface concentration with few drawbacks.[30,32,55]

### C. Enzyme structure

The last key fundamental property of surface immobilized enzymes discussed here is immobilized enzyme structure. This property is highly important to rationalize the observed surface concentration and activity. This is because properly folded enzymes can exhibit higher levels of activity and can occupy less surface area than their unfolded or misfolded counterparts, allowing for increased surface concentration and activity. Understanding the structure of immobilized enzymes in integrated biological–abiological systems can guide future work to improve system performance by tailoring surface chemistry in a way that improves the retention of enzyme structure and favors active conformations. Importantly, the structure of the immobilized enzyme should be compared with the native enzyme to identify any structural changes and possible unfolding. This is routinely investigated by two techniques, IR spectroscopy and circular dichroism spectroscopy (CD), and has more recently been investigated by vibrational sum frequency generation spectroscopy (SFG). Molecular dynamics (MD) simulations have also been used to simulate protein structure on surfaces, and the reader is directed elsewhere for more information as that is beyond the scope of this Perspective.[55–60] Additionally, the conformation and orientation of immobilized enzymes have been investigated by SFG and time of flight secondary ion mass spectrometry (ToF-SIMS).[61–67]

First, as mentioned above, IR spectroscopy of proteins is often focused on the absorptions from the amide backbone: the amide I and II vibrational modes. The frequency of amide I is highly sensitive to the secondary structural environment of each amino acid's amide bond. Fitting the broad amide I band to a sum of Gaussian peaks elucidates the secondary structural composition of the protein, where the frequency of each Gaussian corresponds to distinct features including helix (around 1660 cm$^{-1}$ in water), β-turn (1667–1685 cm$^{-1}$ in water), β-sheet (1624–1642 and 1691–1696 cm$^{-1}$), and random coil (around 1648 cm$^{-1}$ in water) (Fig. 5).[34,49,68–70] This can be done for the surface-immobilized enzyme and compared with the native enzyme free in solution, as has been shown previously for both a peptide and enzyme immobilized on gold.[11,71] However, amide I components are broad and generally overlap heavily so it may not be possible to reasonably identify more than 2 or 3 of these secondary structural elements without overfitting, depending on the intensity and clarity of the bands. Alternatively, linear regression algorithms have been built to determine secondary structure contents from amide spectra and circumvent the drawbacks of curve fitting.[72] This was recently applied to determine the secondary structure of protein films on germanium ATR elements in a high throughput microarray with good agreement with crystal structures.[73] Importantly, IR spectroscopy is often performed on dry samples due to instrumentation requirements and the strong absorption of liquid water. However, rinsing and drying surfaces containing immobilized enzymes removes salts and water that stabilize protein structure and may impact critical factors such as packing density or orientation. Thus, FTIR measurements made on dried samples are likely not representative of the sample when it is stored or operating appropriately in buffered solution. Spectra collected in nominally dry conditions, therefore, may not be representative of the enzyme conditions relevant to sensor performance and actual usage, and may not be meaningful. Care should, therefore, be taken to ensure these measurements are performed in the same medium as the functioning device. Our laboratory has shown previously that ATR-IR can be performed in a buffer composed of both $H_2O$ and $D_2O$, which is commonly done to circumvent the strong absorption of liquid water overlapping with the amide I band.[11]

Like IR spectroscopy, CD spectroscopy can elucidate structural information by looking at the differential absorption of left- and right-handed circularly polarized light by the chiral protein backbone. The absorption of each polarization, largely by electronic transitions of the amide backbone, is sensitive to the secondary structural features mentioned above due to differences in dihedral angles and hydrogen bonding. Representative CD spectra of common secondary structures have been reported and can be used as an empirical basis set to fit sample spectra as a linear combination of each type of structure using various published algorithms.[74,75]

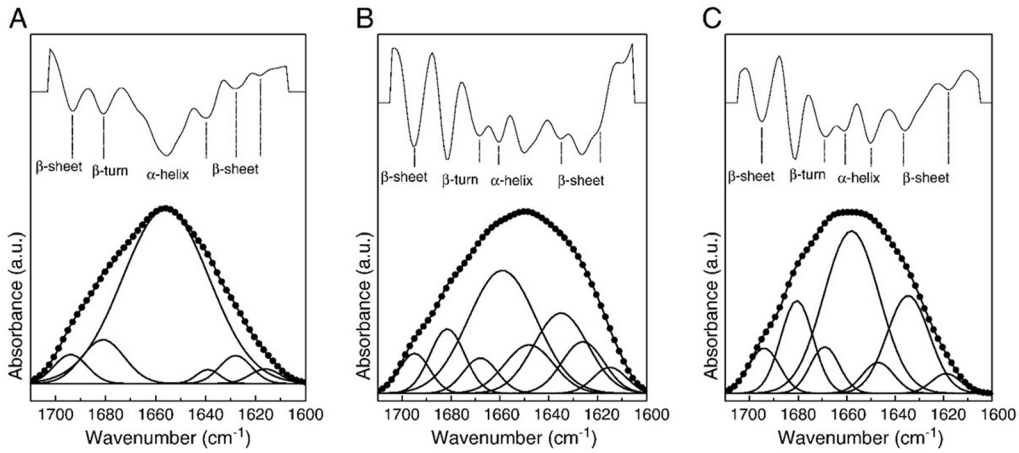


**FIG. 5.** FTIR spectra collected in the air of (a) lyophilized methemoglobin, (b) methemoglobin immobilized on bioactive glass, and (c) methemoglobin immobilized on bioactive glass functionalized with glutaraldehyde. Reprinted with permission from Gruian *et al.* Biochim. Biophys. Acta **1824**(7), 873–881 (2012). Copyright (2012) Elsevier.

This allows for the quantitative determination of secondary structure content and can be done for the surface immobilized enzyme and compared with the native enzyme free in solution. However, CD spectroscopy is generally a transmission technique and immobilized enzyme samples must be prepared on UV transparent substrates such as quartz. If metal films are present, such as gold, they should be well under 50 nm thick since most wavelengths of light are entirely absorbed beyond this. Additionally, absorption by the immobilized enzyme film is very low due to the small amount of material and low film thickness (path length), and scans may need to be accumulated for significantly longer than in solution (hours to overnight, depending on instrumentation) to obtain enough signal to appropriately fit the data; this may be challenging for unstable proteins. Multiple surfaces can also be stacked next to each other in the cuvette to improve absorption. Our laboratory and others have shown this previously for $\alpha$-helical and β-strand peptides, as well as intact proteins, immobilized on gold.[71,76–79]

Recently, vibrational SFG has been used as a unique tool to understand the structure and conformation of proteins and peptides at interfaces, including surfaces. The theory and recent history of SFG have been reviewed elsewhere and will not be discussed here.[80–82] Briefly, SFG is a second order non-linear optical spectroscopy technique where two lasers (visible and tunable IR) overlap on the sample temporally and spatially give rise to an output beam (SFG signal) that has the sum of the two input beam frequencies. The intensity of the SFG signal is enhanced when the input IR beam is in resonance with an IR and Raman active vibrational mode. Additionally, SFG signals can only be generated in media that lack centrosymmetry (e.g., at interfaces) and not in bulk solution. To understand protein secondary structures at interfaces, SFG was applied to $\alpha$-helical, β-sheet, and random coil peptides and proteins.[83,84] It was found that each type of secondary structure exhibits different absorptions in the amide I region (1600–1700 $cm^{-1}$) and N–H stretch region (3300 $cm^{-1}$) and can be resolved more clearly beyond traditional IR techniques. More recently, this has been applied to more complex interfacial protein systems.[85,86] SFG, combined with ATR, has also been a powerful tool to investigate protein conformation and orientation at interfaces, which has been challenging to study with other techniques in many cases.[21,87–90] Briefly, polarized SFG spectra and polarized ATR spectra are collected and compared with polarized spectra calculated from the known protein structure (crystal structure or from MD simulations) as a function of its orientation. This allows a heat map to be generated to determine which protein orientations match the experimental spectra the best. The combination of SFG and ATR spectra gives a narrower range of solutions than either technique independently. Thus, the use of SFG, on its own or with other spectroscopic techniques, has been shown to be a valuable tool to determine interfacial protein structures and conformations.

## III. PROTEIN–NANOPARTICLE CONJUGATES

The prospect of incorporating the functions of proteins with the materials properties of synthetic nanoparticles (NPs) has resulted in many reported successes of biological molecules being immobilized onto NPs and retaining their activity for a number of exciting applications ranging from sensing[91–94] to biomedical technologies.[95,96] However, there are three aspects of a protein–NP construct that are often overlooked when characterizing these nanoscale biomaterials: orientation, structure, and quantity of conjugated and active protein per NP unit. Indeed, there is a noticeable scarcity in the literature of reliable and routine methods to understand the protein–NP construct at the molecular level, the characterization that has long been recognized as necessary for understanding enzymes in solution. Here, we outline and discuss advances that have highlighted these specific issues and identify remaining shortcomings.

### A. Protein orientation

The ability to control the orientation of a protein as it binds to an NP is of great interest since it has been demonstrated that NPs can

be used to control the activity of the bound protein depending on its orientation.[97] Different approaches to controlling protein orientation such as site-specific labeling and electrostatic control have been applied to a number of different systems; examples include controlling conjugated antibody orientation to maximize (bio)molecular target binding and regulating protein activity by controlling the orientation of the enzyme active site relative to the NP.[98,99] Determining successful control of orientation is often done indirectly by observing the subsequent increase or decrease in protein activity and binding of the conjugated material, though it is important to note that changes in activity are not necessarily a reflection the orientation of the bound enzyme at the bio–abio interface.[99,100] In order to attribute changes in enzyme activity to orientation, it is necessary to ensure that changes in the biomolecular structure or complete unfolding are not also occurring. However, identifying simple and routine ways to directly observe the orientation of proteins immobilized on an NP has proven to be challenging. Here, we discuss some of the most commonly reported techniques for measuring biomolecular orientation within a conjugated enzyme–NP material.

Raman spectroscopy measures the inelastic scattering of an incident wave by a sample and is sensitive to vibrational modes similar to IR spectroscopy. The scattered light is either Stokes or anti-Stokes shifted, where the shift in wavelength is proportional to the energy of the corresponding vibrational mode.[101] Metallic NPs, and silver NPs (AgNPs), in particular, have been shown to enhance the intensity of the inelastically scattered light by a factor of $10^4$ through enhancement of the local electromagnetic field as a result of excitation of the localized surface plasmon resonance (LSPR), enabling the propagation of surface enhanced Raman spectroscopy (SERS) throughout this field.[102] SERS has been used to directly measure the orientation of an immobilized protein by observing the enhanced intensity of Raman bands assigned to specific amino acids or moieties within a protein. Keating *et al.* demonstrated the use of SERS to measure the orientation of cytochrome c (Cyt c) conjugated to AuNPs (Cc:AuNP) using aggregated Ag sol-gels as a SERS enhancer.[103] They compared the signal intensity of the heme group of Cyt c directly bound to the aggregated Ag (Cc:Ag) with that of Cc:AuNP bound to aggregated Ag (Ag:Cc:AuNP) [Fig. 6(a)]. They observed a decrease in the SERS enhancement of the heme Raman bands in the Ag:Cc:AuNP in comparison to the Cc:Ag, suggesting that when the Cyt c was bound to the AuNP, the heme group was facing away from the Ag and toward the AuNP since the enhancement factor would decrease as the heme group was placed further away from the Ag. Similarly, Sengupta *et al.* modified maltose-binding protein (MBP) with a silver-binding dodecapeptide (Ag4) and bound the resulting fusion protein (MBP-Ag4) to AgNPs.[104] They used SERS to observe the orientation of MBP-Ag4 on AgNPs by relating changes in the SERS spectra of the protein [specifically the bands of phenylalanine (F), tyrosine (Y), and tryptophan (W) residues] to the conformational changes that occur as a result of the protein binding to maltose. Out of the possible orientations of the protein to the surface, only an orientation with the active site facing toward the AgNP was consistent with the changes seen in the intensities of the F, Y, and W bands, which suggested then that the protein was oriented with the active site facing the AgNP. SERS is the simplest of the methods described in this Perspective, and the presence of metallic NPs provides a convenient substrate for the enhancement of the Raman bands.[102] However, its utility is still relatively limited for practical reasons. For non-metallic NPs such as SiNPs or polymer-based NPs, SERS would not be possible without adsorbing the entire protein–NP construct onto a metal substrate. Furthermore, for proteins with a more homogeneous distribution of the phenylalanine, tryptophan, and tyrosine residues throughout their tertiary structure, differentiating between orientations based on these residues would become difficult.

Mass spectrometry (MS) is a technique that analyzes molecules based on their mass to charge ratio and has seen greater usage with proteins since the development of soft ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).[101,105] MS has been used in different ways to gain insight on the orientation of an immobilized protein. Bayraktar *et al.* used amide hydrogen/deuterium exchange (HDX) to measure the solvent accessibility of residues on Cyt c immobilized on functionalized AuNPs.[106] Specifically, they dissolved Cyt c in buffered $D_2O$, then initiated HDX by diluting the sample in

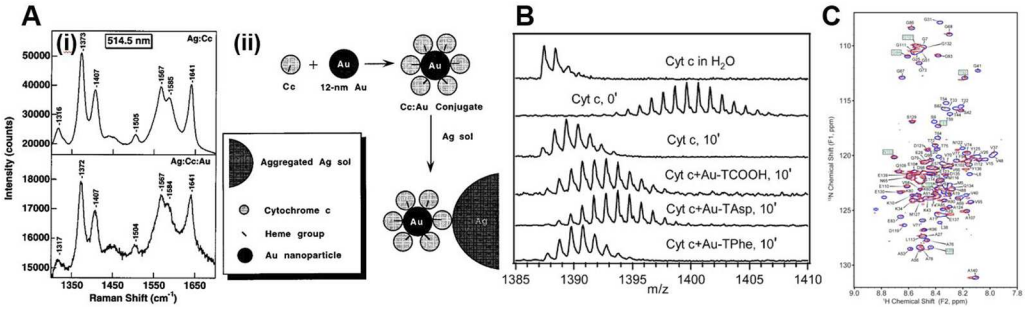


**FIG. 6.** Methods for determining the orientation of protein bound to NPs. (a) (i) SERS spectra of Cyt C bound to Ag and to AuNPs. (ii) Schematic illustration of Ag:Cc:AuNP system. Reproduced with permission from Keating *et al.*, J. Phys. Chem. B **102**(47), 9404 (1998). Copyright 1998 American Chemical Society. (b) Mass spectra of Cyt C adsorbed on NPs with different functionalization after HDX, with the numbers on the labels indicating minutes allowed for HDX before quenching. Reproduced with permission from Bayraktar *et al.*, J. Am. Chem. Soc. **129**, 2732 (2007). Copyright 2007 American Chemical Society. (c) 2D HSQC NMR spectrum of $\alpha$-syn bound to anionic citrate AuNPs (red) overlaid with NMR spectrum of $\alpha$-syn free in solution (blue). Reproduced with permission from Lin *et al.*, J. Phys. Chem. C **119**, 21035 (2015). Copyright 2015 American Chemical Society.

buffered $H_2O$. They then used pepsin to digest the protein, and the subsequent samples were analyzed with MALDI time of flight (MALDI-TOF) MS to see which peptide sequences retained the highest number of deuterium atoms [Fig. 6(b)]. This allowed them to determine which peptide sequences were most exposed to solvent, giving them an indication of the orientation of the protein on the NP. Based on these mass spectra, they reported that they were able to control the orientation of Cyt C on AuNPs by using anionic ligands with different side chains capping the AuNPs. Specifically, they tested carboxylic acid, aspartic acid and phenylalanine capped ligands. Jain *et al.* also immobilized Cyt c on AuNPs of varying functionalization and drop cast the Cc:AuNP conjugates onto microscopy glass slides.[107] These samples were subsequently analyzed with time of flight secondary ion mass spectrometry (TOF-SIMS) specifically to monitor the intensities of cysteine, glutamic acid, and leucine residue peaks in Cyt c; the relative intensities of these residues were determined to be an indication of the orientation of Cyt c with respect to the AuNP. While different varieties of MS have become well-established and standard techniques are readily available as a service in many facilities, the specific methods still usually require expertise and specialized equipment to be conducted successfully and remain unavailable to many laboratories. TOF-SIMS, in particular, is a regularly used technique to determine the orientation of bound proteins on NPs.[67,108,109] However, TOF-SIMS is performed under a high vacuum, which is not an environment representative of a protein bound to an NP while in solution.

Nuclear magnetic resonance (NMR) spectroscopy measures the absorption of radio frequency waves by spin-aligned nuclei in the presence of a magnetic field[101] and is most commonly used for small molecules in synthetic chemistry, though it is also useful for structural determination of macromolecules such as proteins and DNA.[110] Two dimensional (2D) NMR experiments, and $^{1}H–^{15}N$ coupling, in particular, have proven to be a powerful solution-based tool for structure and orientation analysis of proteins bound to NPs.[111] Lin *et al.* used heteronuclear single quantum coherence spectroscopy (HSQC) NMR to determine the change in orientation of $\alpha$-synuclein ($\alpha$-syn) bound to AuNPs depending on the charge of the capping ligand on the AuNP [Fig. 6(c)].[97] They monitored the decrease in signal intensity of amide peaks from specific amino acid residues and correlated these changes to the distance of the amino acid residue to the AuNP surface. They observed that with anionic citrate-capped AuNPs, $\alpha$-syn conjugated with the N-terminus facing toward the AuNP, while with cationic 16-mercaptohexadecyl trimethylammonium bromide (MTAB) capped AuNPs, $\alpha$-syn oriented with the C-terminus facing the AuNP. NMR as a method is well-established and is generally a readily available technique, though it suffers from low sensitivity and requires samples of high concentration to generate enough signal. NMR spectra of proteins also tend to be crowded and require extensive analysis and specialized techniques to obtain meaningful information.

### B. Amount of protein

Knowing how much protein is bound to an individual NP is crucial for understanding the efficiency of the conjugation and the effect of the conjugation on the activity of the protein through kinetics.[112,113] Without this knowledge, it is impossible to conclude whether or not a bound protein is as active as it is in solution, or how changes in surface chemistry or morphology quantitatively affect important properties of the system. Despite this, accurately measuring the amount of bound protein to NPs remains a challenging characterization step, seen in the lack of straightforward methods that can be found in the literature. The quantification of proteins in solution is usually a simple and routine measurement with a plethora of different assays available for measuring protein concentration.[113] However, the presence of NPs in solution makes this measurement significantly more challenging since NPs tend to interfere with the two standard techniques for measuring protein concentration: UV–visible and fluorescence spectroscopies. Since NPs often absorb strongly in the visible range (400–800 nm), this interferes with standard assays such as the Bradford and Lowry protein assays. The concentration of protein–NP conjugates also tends to be too low to measure the intrinsic absorbance of proteins at 280 nm; this is also complicated by the fact that many NPs also absorb in that range. Solvent depletion methods (determining the amount of bound protein by measuring the concentration of unbound protein) often overestimates enzyme amount since unbound protein can be depleted by binding to other surfaces such as the container.[112] Furthermore, NPs are often strong quenchers of fluorescence, rendering this measurement unusable in a variety of assays. For these reasons, characterizing this important property of biomolecule–NP conjugates has proven difficult. Here, we discuss a comprehensive list of methods for quantifying protein concentration on NPs.

There have been several approaches for using UV–visible spectroscopy to quantify the amount of protein on NPs, although significant disadvantages of any technique in this part of the spectrum are described above. Another common approach is to observe the LSPR shift in the absorption spectrum of the NP to determine the amount of adsorbed protein.[114] As proteins adsorb to the surface of the NP, the LSPR peak shifts in energy, which can be correlated with the amount of adsorbed protein. Belsey *et al.* used the LSPR shift, in conjunction with XPS and particle sizing techniques, in 20 nm AuNPs to determine the number of immunoglobulin G (IgG), bovine serum albumin (BSA), and a small peptide CAG4 bound per AuNP [Fig. 7(a)].[115] The advantage of using the NP absorbance in this way is that metallic NPs absorb strongly in the visible range and, thus, high concentrations are not necessary. It is also a direct measurement of the protein–NP conjugate and, thus, does not suffer from the overestimation of concentration discussed above for solvent depletion. However, it is only applicable to biomolecules in the so-called "protein corona," the thin (~10 nm)[117] area directly at the protein–NP interface where proteins are directly interacting with the NP surface, making this method unusable for NPs that have a thick ligand shell such as alkyl thiols or polyethylene glycol (PEG) chains (both common modifications and stabilization agents for metallic NPs).

Fluorescence-based methods for measuring protein concentration are attractive because of their ability to detect concentrations of proteins down to the nM range.[116] However, as mentioned earlier, NPs often quench fluorescence, thus making typical fluorescence measurements of bound proteins difficult. As a result, the reported fluorescence-based methods for determining the number of bound proteins often involve etching out the NP, leaving behind the dissolved or digested protein for measurement.[117] Filibrun and Driskell

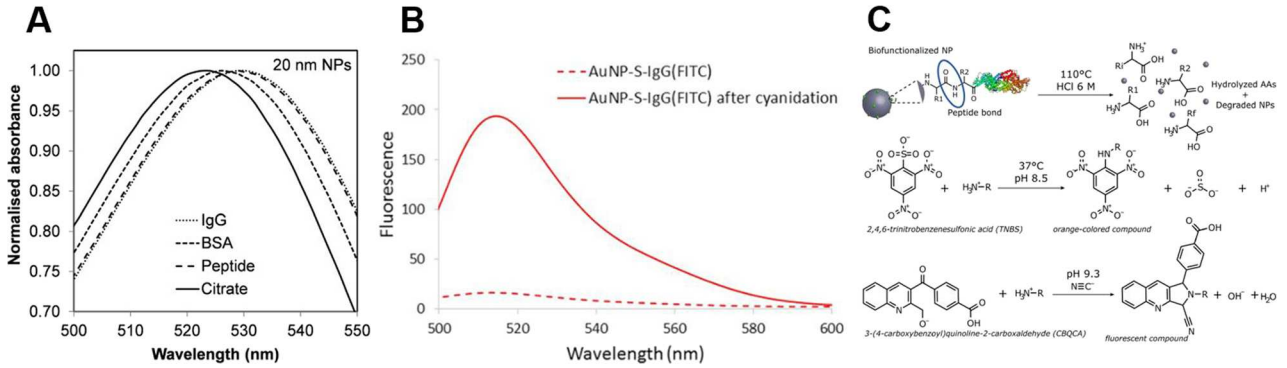


**FIG. 7.** Determining the number of proteins bound to NPs. (a) LSPR shift of 20 nm AuNPs bound with different proteins and peptides Reproduced with permission from Belsey *et al.*, Biointerphases **10**, 019012 (2015). Copyright 2015 American Vacuum Society: Science and Technology of Materials, Interfaces, and Processing. (b) Activation of fluorescence of the FITC-labeled IgG as a result of the dissolution of the NPs using NaCN. Reproduced with permission from Zhang *et al.*, Talanta **204**, 875 (2019). Copyright 2019 Elsevier. (c) Schematic representation for amino acid analysis and labeling of free amino acids with a chromophore and a fluorophore to determine protein concentration. Reproduced with permission from Oliverio *et al.*, ACS Appl. Nano Mater. **3**(10), 10497 (2020). Copyright 2020 American Chemical Society.

quantified the amount of goat anti-mouse IgG bound to AuNPs by first treating the antibody-AuNP conjugates with KI/$I_2$ etchant and subsequently using a NanoOrange assay to measure the amount of bound antibody.[118] They determined that 309 ± 93 antibodies were bound per AuNP, which indicated monolayer coverage of the 60 nm AuNPs they were investigating. Similarly, Zhang *et al.* labeled rabbit IgG with a fluorophore prior to binding to AuNPs.[117] NaCN was used to etch the AuNP core before measuring the amount of antibody per AuNP by using fluorescence. Without dissolution of the AuNP core, the fluorescence of the fluorophore was quenched by the AuNP [Fig. 7(b)]. From this method, they were able to calculate that there was 4.4 ± 0.1 IgG per AuNP. While these NP dissolution methods have been shown to work for NPs with ligands such as citrate, NPs with a dense monolayer of alkylthiol or PEG chains are resistant to such chemical digestion and can take longer than 48 h to dissolve, thus, making this approach time-consuming for NPs of that type.[119] It also requires the use of toxic cyanide ions.

Amino acid analysis (AAA) is a method that is used to determine the amino acid composition of a protein but can also be used to quantify protein concentration. Briefly, the protein is digested in 6M HCl to generate free amino acids, which are subsequently derivatized with a fluorophore and analyzed by HPLC.[112] This method has been shown to work well with proteins and peptides that are bound to AuNPs since digestion in 6N HCl causes the AuNPs to precipitate, after which they can be removed easily. Our laboratory has used AAA to quantify the number of peptides covalently bound to ~5 nm AuNPs and determined that under our preparation conditions, on average 2.6 ± 0.7 peptides were conjugated to each AuNP.[120] Similarly, Liu *et al.* used amino acid analysis to determine the number of pepsin enzymes bound to AuNPs.[112] They demonstrated that AAA allowed for detection of pepsin down to 2.89 nM, thus, allowing for the potential of AAA to be used for protein–AuNP conjugates with low surface coverage. Oliverio *et al.* recently developed a modified AAA that is high-throughput and only requires a conventional oven and a microplate reader [Fig. 7(d)].[113] They presented both a colorimetric and a fluorescent assay that measures the total amount of hydrolyzed amino acid that allows for the quantification of proteins bound to polymeric NPs, AuNPs, and SiNPs. The colorimetric assay had a linear detection range from 250 to 6.25 nmol of amino acid residues while the fluorescent assay had a linear range from 25 to 625 pmol of amino acid residues. AAA is a sensitive technique that has been shown to have low limits of detection and as such has proven to be a reliable method for determining the number of bound proteins. The biggest disadvantage to this technique is that the derivatization and subsequent HPLC analysis require instrumentation and expertise that is not available to most laboratories. Oliverio and co-workers' approach simplifies the analysis to a microplate reader, though it would not be applicable to a system with more than one type of protein as it is a bulk measurement of amino acid concentration and, thus, cannot distinguish between different proteins. It also must be considered that the digestion step may not fully cleave every amino acid bond in the protein, and as such it is heavily recommended that calibration curves be made using the actual protein of interest as opposed to a standard such as BSA.

Mass spectrometry (MS) has also been used as a method to quantify bound protein to NPs. Ju and Yeo reported a method for quantifying BSA bound to AuNPs using MALDI-TOF MS.[121] They used trypsin to digest the protein into fragments, identified one of the fragments as a reference peptide, and then synthesized an isotope-labeled peptide analog of the reference peptide to use as an internal standard. The digested proteins were then transferred to a MALDI matrix and analyzed with MALDI-TOF MS. They demonstrated that comparing the mass intensities of the reference peptide with the internal standard allowed for absolute quantification of the proteins bound to AuNPs. Schneck and co-workers also used an isotopically labeled reference peptide as an internal standard for quantification of antibodies bound to magnetic NPs after digestion with trypsin, but used isotope-dilution liquid chromatography-tandem MS (ID-LC-MS/MS) for sample analysis instead of MALDI-TOF.[122] Mass spectrometry is a sensitive technique that can measure proteins in low concentrations, though the methods described above suffer from the need for specialized equipment and expertise that are not readily available.

Microscopy encompasses the techniques that visualize samples using light, electrons, or a scanning probe and are often used in the analysis of nanomaterials.[101] There have been several reports of using fluorescence and electron microscopies to determine the number of bound proteins to NPs. Casanova and co-workers demonstrated the use of single-molecule photobleaching (SMPB) for bio-nanomaterial quantification with a fluorophore-labeled protein bound to 20 ± 4 nm NPs.[123] They observed stepwise photobleaching events, which directly correspond to the number of bound proteins, allowing them to generate a distribution of protein–QD coupling ratios as opposed to an ensemble average. Hu and co-workers demonstrated the use of negative-stain TEM and dark field STEM to image assemblies of protein–NP complexes.[124] They were able to image protein–NP complexes of discrete protein–NP ratios, distinguishing between 1:1, 2:1, and 3:1 of protein:NP. While microscopy techniques allow for individualized counting of protein–NP conjugates, their applications are limited in that both require specialized equipment and expertise. Fluorescence microscopy requires fluorophore-labeled samples in which derivatization has the potential to bias binding. It also cannot be performed for proteins bound to NPs that quench fluorescence.[113] Electron microscopy requires negative staining agents such as uranyl acetate in order to visualize light atoms such as carbon, which can potentially influence the size of the NPs. It also requires that samples be dried and placed under a vacuum.[101]

### C. Protein structure

Since a protein's ability to function is intimately related to the stability of its structure, much work has been done to functionalize NPs with biocompatible ligands that preserve the structure, and, therefore, function, of the bound protein. Determining protein structure is already a well-established field with tried and tested techniques such as x-ray crystallography, NMR, and more recently, cryo-electron microscopy (EM).[125,126] However, once again the presence of NPs can cause complications, not allowing for direct measurement of the protein structure using these same techniques. Out of the three characterizations of protein–NP conjugates discussed in this Perspective, elucidation of the structure of the bound protein is the least studied and has the fewest well developed and widely used tools. Here, we discuss the current available techniques for determining the structure of the bound protein.

CD is a technique that is frequently used as a tool to determine the secondary structure of proteins and has found much usage in the protein–NP field as it is a simple technique that can quickly give a good estimation of the structure of the entire bound protein. Lundqvist and co-workers used CD in conjunction with NMR to study the effect of NP size and curvature on the structure of an adsorbed protein.[127] Scanning in the UV range, they studied the adsorption of human carbonic anhydrase (HCA) on SiNPs and observed that larger SiNPs had more interactions with HCA, causing larger perturbations to the secondary structure of the protein. Similarly, Shang and co-workers studied the effect of SiNP size and curvature on the structure adsorbed Cyt c.[128] They measured the CD spectra of adsorbed Cyt c from the far-UV region to monitor changes in the secondary structure while also measuring the Soret region to monitor changes in the structural integrity of the amino acid residues near the heme group [Fig. 8(a)]. Similar to

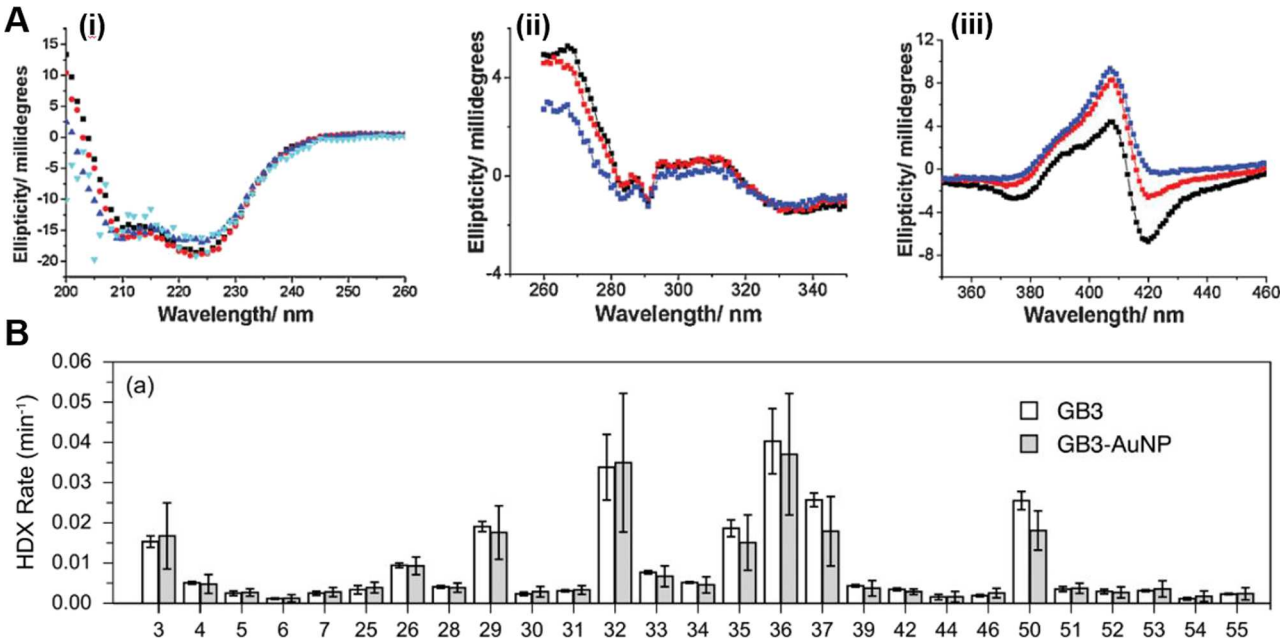


**FIG. 8.** Structure determination of proteins bound to NPs. (a) CD spectra of Cyt c free in solution (black) overlaid with Cyt c bound to 4 nm NP (blue), 15 nm NP (dark blue), and 35 nm NP (light blue) in the far-UV (i), near-UV (ii) and Soret regions (iii). Reproduced with permission from Shang *et al.*, Small **5**(4), 470 (2009). Copyright 2009 Wiley-VCH. (b) HDX rates of amino acid residues in GB3 free in solution (white) and bound to AuNP (gray) as determined with 2D $^{15}$N–$^{1}$H HSQC NMR. Reproduced with permission from Wang *et al.*, J. Phys. Chem. B **118**, 14148 (2014). Copyright 2014 American Chemical Society.

Lundqvist and co-workers, they observed that larger SiNPs had a significantly larger impact on the structure of Cyt c. CD is likely the most commonly used method to determine the structure of bound proteins for the reasons mentioned above. However, it has some limitations. Certain types and sizes of NPs absorb in the UV region, thus limiting the types of protein–NP conjugates that can be studied using CD. CD is also an ensemble measurement and thus cannot differentiate between a bulk change vs a distribution of changes to the structure. For example, CD cannot differentiate between a solution of proteins that have lost 50% of their structure and a solution of proteins where 50% of the proteins are unfolded. CD must, therefore, be used in combination with other analytical techniques to fully characterize biomolecular structure on protein–NP conjugates.

NMR has proven to be a useful technique to determine the structure of proteins in solution.[110] Wang and co-workers used solvent-exchange (SOLEXSY) NMR to determine the orientation of the third IgG-binding domain of streptococcal protein G (GB3) and ubiquitin bound to AuNPs.[129] They monitored HDX rates of protein free in solution vs protein bound to NPs to gain insight into changes in structure as a result of binding [Fig. 8(b)]. This kind of analysis allows one to monitor changes in the environment of specific amino acid residues and chains, thus allowing more detailed insight into the structural changes a protein undergoes as it binds to NPs as opposed to CD. However, as mentioned earlier NMR suffers from low sensitivity and requires high concentrations of the sample that are not always possible to achieve for NP solutions. As a result, NPs can be analyzed using solid-state NMR (SSNMR), which has proven to be a useful tool for studying ligands bound to NPs where solution-state NMR fails. Indeed, SSNMR has been used to investigate small ligands such as peptides bound to NPs,[130,131] and more recently it has been applied to entire proteins bound to NPs as well. Giuntini *et al.* demonstrated the use of SSNMR to measure the effects of binding the protein asparaginase II (ANSII) on AuNPs.[132] By observing the chemical shifts of the amide protons compared with those on unbound protein, they were able to determine that the structure of ANSII remains largely unchanged, with significant shifts only being observed on residues on the protein surface or loops, a level of detail that has not been achievable with solution-state NMR. SSNMR offers higher sensitivity of ligands bound to NPs that would normally be invisible using solution-state NMR.[133] However, a drawback of SSNMR is that the samples are not in solution, and, thus, spectra collected in these conditions may not be exactly representative of the structure of the protein when in solution.

A recent development in the field of structural biology is the emergence of cryo-EM as a method to obtain high-resolution protein structures as recent advances in sample preparation and analysis have enabled near-atomic-resolution structures.[134–137] While small NPs have been used as labels to improve the resolution structures determined by cryo-EM,[138,139] it has yet to find commonplace usage for structural determination of proteins bound to NPs. Recently, Sen *et al.* used cryo-EM to reconstruct the chaperone protein GroEL bound to platinum nanoparticles (PtNPs), obtaining a resolution of 3.93 Å.[140] This development is an exciting step toward achieving high-resolution structures of proteins bound to nanomaterials, though it is still in its infancy and new classification methods need to be developed to account for the presence of the NP.

## IV. CONCLUSIONS AND OUTLOOK

The prospect of developing protein-based materials and technology is indeed exciting for the reasons that we have described throughout this Perspective. Since the development of the blood glucose sensor, reports of successful protein-based devices for applications such as sensing and catalysis have been prevalent in the literature. However, this has resulted in an over-focus on the development of working devices with acceptable performance without characterizing the fundamental aspects of the protein–material interaction such as the efficiency of binding and the effect of binding on the structure and function of the protein. The scarcity of commercially available protein-based devices since the blood glucose meter is perhaps indicative of this oversight. Understanding these properties, in particular, can aid in an informed approach to developing methods to chemically functionalize surfaces and nanoparticles in a favorable way for proteins to bind as opposed to relying on trial and error to develop the best results. We have discussed experimental tools that are currently available in the literature to address the issues of determining binding efficiency, orientation, structure, and activity of proteins on both macroscopic surfaces and nanoparticles, considering their advantages and drawbacks. We also highlight emerging techniques such as SFG spectroscopy and cryo-EM, spotlighting recent advancements and discussing current limitations. As research continues in this field, we suggest that a concerted effort to develop and improve robust tools to understand protein–surface interfaces will be integral to the optimization of protein immobilization on surfaces that will in turn allow for an informed approach for the development of more protein-based devices.

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## AUTHOR DECLARATIONS

### Conflict of Interest

The authors have no conflicts to disclose.

### Author Contributions

**Joshua M. Correira**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). **Paul R. Handali**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). **Lauren J. Webb**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal).

## DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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