Sensor Issues

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Beyond Simple Cartoons: Challenges in Characterizing Electrochemical Biosensor Interfaces

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ABSTRACT: Design and development of surface-based biosensors is challenging given the multidiscipline nature of this enterprise, which is certainly the case for electrochemical biosensors. Self-assembly approaches are used to modify the surface with capture probes along with electrochemical methods for detection. Complex surface structures are created to improve the probe-target interaction. These multi-component surface structures are usually idealized in schematic representations. Many rely on the analytical performance of the sensor surface as an indication of the quality of the surface modification strategy. While directly linked to the eventual device, arguments for pursuing a more extensive characterization of the molecular environments at the surface are presented as a path to understanding how to make electrochemical sensors that are more robust, reliable with improved sensitivity. This is a complex task that is most often accomplished using methods that only report the average characteristics of the surface. Less often applied are methods that are sensitive to the probe (or adsorbate) present in non-ideal configurations (e.g., aggregates, clusters, non-specifically adsorbed). Though these structures may compose a small fraction of the overall modified surface, they have an uncertain impact on sensor performance and reliability. Addressing this issue requires application of imaging methods over a variety of length scales (e.g., optical microscopy and/or scanning probe microscopy) that provide valuable insight into the diversity of surface structures and molecular environments present at the sensing interface. Furthermore, using in-situ analytical methods, while complex, can be more relevant to the sensing environment. Reliable measurements of the nature and extent of these features are required to assess the impact of these non-ideal configurations on the sensing process. The development and use of methods that can characterize complex surface based biosensors is arguably required, highlighting the need for a multi-disciplinary approach towards the preparation and analysis of the biosensor surface. In many ways, representing the surface without reliance on overly simplified cartoons will highlight these important considerations for improving sensor characteristics.

Electrochemical biosensors are now embedded technologies used for point of care diagnostics[1,2] since they are simple, easy to miniaturize, have low power requirements and can be easily integrated with mobile devices.[3,4] Advancing beyond blood glucose analysis, these sensors are now being developed to detect not only small molecules but also more complex biomolecules (e.g., nucleic acids, peptides and proteins). These biosensor surfaces are prepared via immobilization of the capture probe onto the electrode surface. Significant research effort has focussed on improving the sensor performance by maximizing probe-target binding and minimizing issues related to non-specific target-surface interaction, nonbinding target-probe interactions and preventing fouling of the sensor surface.

Typically, probe decorated biosensing surfaces are described using simple cartoons[5] which only include the idealized probe-surface interaction and arrangement. The problem with such representations, especially for novice biosensor researchers, is the omission of the many other probe-surface interactions that result in heterogeneous surface modification. These arrangements are not part of the sensor design, and are usually undesired and in many cases ignored. This leads to a focus on optimizing the desired probe-target interaction to achieve the greatest sensitivity and specificity, but with less attention being paid to optimizing probe immobilization and the environment of the surface bound probe.

Nevertheless, electrochemical biosensors are being successfully used in biological fluids and therefore 'work'.[6] As the principle focus of these biosensor studies is on the probe-target interaction, the sensor performance is evaluated through various analytical figures of merit (limit of detection, sensitivity, etc). Unfortunately, this does not reveal the underlying surface science involved in probe-surface interactions, making rational sensor improvement difficult.[7] Therefore interest in more carefully defining the nature of the many possible interactions between the probe and surface and measuring the local environment experienced by the probe is increasing. To address this issue and accurately describe all the nuances of the modified surface requires an integrated surface science characteri-

zation approach. This can be achieved by designing and using surface analytical methods to answer questions such as:

- how homogeneous (or heterogeneous) is the environment experienced by the probe molecule?
- is the whole surface modified as expected or is non-ideal or unwanted probe immobilization or adsorption present?
- to what extent does surface heterogeneity or non-ideality influence the transduction event?
- how to accurately characterize the condition of the surface-bound probe molecules as well as those in non-ideal configurations/environments?

These questions address the non-ideal nature of the biosensor surfaces created. The presence of heterogeneity on a surface should be expected. It can result from both the substrate and the modifying molecular layer. Unfortunately, its potential impact on biosensor performance has been seldom a focus of research. Herein, we provide a viewpoint that adopts in-situ or operando surface characterization as a guiding paradigm for understanding the influence of probe environment diversity on performance metrics, sensor stability and robustness.

Adsorption	General term to describe interaction of a molecule (e.g., capture probe, target, fouling agent) with a surface.
Physisorption or Physical Immobilization	Typically used to describe the weak attachment of molecules (e.g., capture probe or target) to a surface, often randomly oriented
Chemisorption or Immobilization	Covalent attachment of capture probes with better control over the orientation than physisorption
Covalent Immobilization	Chemical modification of an immobilized or chemisorbed monolayer of capture probes
Specific adsorption	Capture probes bound to the surface as designed or anticipated
Non-specific adsorption	Can describe the non-ideal adsorption of the capture probes to the surface Can also be used to describe the adsorption of other solution species that foul the surface or degrade the analytical signal

Table 1 Terminology commonly used to describe the variety of surface modifications

A thorough surface analysis approach is certainly costly in terms of time and resources because no single characterization tool can provide the needed microscopic characterization at the molecular length scale resolution over the macro-sized spatial dimensions characteristic of biosensing surfaces. Furthermore, these methods are limited in terms of their sensitivity to the variety of probes (adsorbates) or species on the surface. This is also a limitation when time resolved measurements are needed such as exploring changes in the surface during the sensing process itself. Nevertheless, modern surface analytical tools have matured to the point that much more robust characterization of the surface are feasible by using multiple (preferably in situ) techniques. An accurate characterization includes not only the averaged properties but also a measure of the non-ideal nature of these sensor surfaces. Enhanced surface characterization studies will provide important feedback enabling development and evaluation of surface modification chemistries thus advancing and optimizing sensing performance through design-characterize-test cycles.

Since the generally used term "adsorption" has many meanings for the interdisciplinary communities that work on biosensors, definitions of the terms used in this article to describe the surface modification are provided in Table 1.

HETEROGENEITY ON SURFACES

In a simplified cartoon representation of the transduction process, it is implicitly assumed that the biosensor surface is homogeneous in all aspects. In a real sensor, this is an oversimplification and non-idealities inevitably exist at the microscopic scale. As an illustrative example, we consider a biosensor relying on a metal substrate for the formation of a mixed SAM (self-assembled monolayer) of a thiol-modified single stranded DNA probe (receptor molecule) and a diluent thiol molecule chemically bound to a gold electrode. Thiolated DNA is a capture probe that can be chemisorbed onto the gold surface via the formation of a Au-S bond (immobilization) or through base pair interactions (non-specific adsorption)[8]. Ideally, the former is overwhelmingly dominant and a mixed monolayer results with uniformly distributed and appropriately diluted DNA tethered to the surface (Figure 1a). As argued previously[9,10], this ideal case is only part of the interfacial story. Non-ideal probe-surface interactions will result in undesirable or unwanted probe configurations on the electrode surface and need to be explicitly considered in the design, development and use of biosensors. To do so first requires investigation of the origin of these non-ideal characteristics.

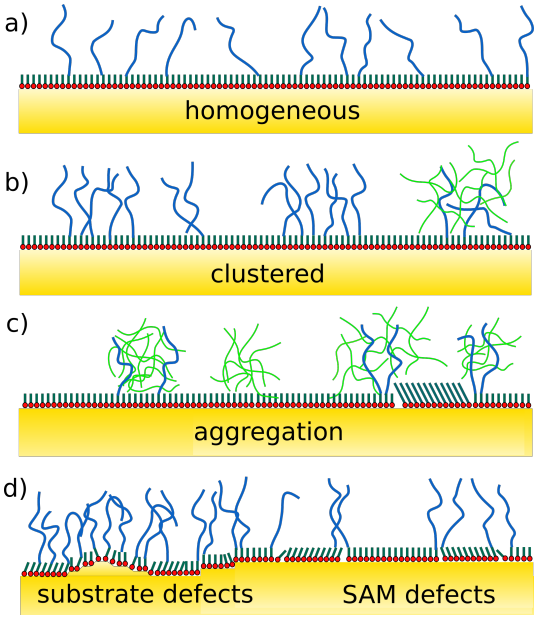

Figure 1 Cartoons depicting a SAM-based biosensor surface comprised of a diluent (colored black) and single-stranded, thiolated DNA capture probe (colored blue and green). a) ideal probe coverage and distribution, b) non-uniform clustered distribution of immobilized (specifically adsorbed, blue) probes and examples of non-specifically adsorbed probes (colored green), c) aggregation of capture probes on the surface (non-specifically adsorbed colored green), via interaction with the immobilized capture probes, or near defects in a multi component SAM, d) clustering caused

by high local capture probe density located in/near defects in the substrate or the SAM.

The monolayer: Surface based sensing is believed to be most efficient if the analyte can access bound capture probes with identical target binding or interaction energetics. Compared to processes in solution, the presence of the interface has been shown to significantly change the affinity or kinetics of such an interaction[11]. For example, efficient hybridization of bound DNA probes to the complementary strands (targets) in solution requires the tethered DNA probes to be arranged sufficiently far apart.[8,12,13] However, local clustering of immobilized probes can exist (Figure 1b) even on atomically featureless single crystal metal surfaces. These localized areas of high DNA density favour non-specific adsorption (physisorption) of the probes through base pair interactions and can lead to aggregate formation (Figure 1c).[14–16] In addition, these regions will not effectively hybridize the target strands, reducing sensitivity in the simplest manifestation.

In addition, the SAM may have differing domain structures thereby producing molecular grain boundaries (Figure 1d).[17,18] Annealing these structures can be achieved through careful preparation conditions, typically long immersion times allowing the alkylthiolate SAM to reorganize and decrease the density of this type of defects. The prevalence of multi-component monolayers (e.g., prepared to prevent non-specific adsorption of proteins in biological fluids[5,19,20]) further complicates this picture (Figure 1c) given that phase segregation is known to occur in these cases.[21]

The substrate: Practitioners of electrochemical studies know well the challenges in preparing metal substrates. Even single crystal gold substrates have defective regions, ranging in dimension from micron-sized scratches to atomic scale steps. These can be clearly distinguished through careful electrochemical measurements[22] typically performed under ideal conditions (e.g., using flame annealed single crystal electrodes in hanging meniscus arrangements). Conscientious of cost, biosensor devices are typically prepared via vapour deposited noble metal films on glass, on-top of adhesive layers such as Cr or Ti. This results in a variety of surface features, from large <111> facets to small randomly oriented facets distributed throughout the surface creating many grain boundaries.[23] In the quest for enhanced signals, nanostructured metal surfaces have been used to increase the surface area and accessibility to the bound probes.[24] However, nanostructures inherently introduce many high surface energy features.[10,25] Like grain boundaries, these highly localized areas of the surface are sites that will promote non-ideal specific and non-specific probe conformations (Figure 1c&d).

The above example is representative of heterogeneities likely to be encountered when employing molecular modification of metal substrates for biosensing applications. Although it may be unclear how biosensor sensitivity and selectivity is affected by surface-based heterogeneities, we contend that they should not be assumed absent. Therefore it is important to determine the existence and extent of these features on biosensor surfaces which requires measurements that are sensitive to these imperfections. It is equally important to evaluate and identify methodologies that can be used to specifically detect and measure the extent of these non-ideal probe environments so as to either determine their influence on the measurement, or to provide insight into their remediation or elimination.

SURFACE ANALYSIS CHALLENGES

Ultimately, the analytical performance of the electrochemical biosensor will rely upon the nature of the molecular layer decorating the electrode surface. Better understanding and control over the probe-interface characteristics should improve the sensitivity and selectivity of the measurement. This assumption can be tested only through the use of methods that reliably and accurately assess the nature of the sensor interface including quantification of the possible surface heterogeneities[7]. Ex-situ surface analysis methods (e.g., XPS) are often used, but removal from the aqueous environment complicates the interpretation of these results. Many in situ methods that are sensitive to these non-idealities and are able to map out the surface in terms of the chemical environment and probe availability, distribution and configuration must be used. In the opinion of the authors, a bias towards more in-situ surface analysis is a wholly worthwhile strategy, especially given the breadth and variety of sensor designs in current literature. It is certainly challenging and time consuming, but it is necessary for the development of approaches that eliminate undesired configurations and, ultimately, lead to more reliable, robust and better performing biosensors.

Ideally surface analysis methods should;

- report on the distribution of immobilized (specifically adsorbed) probes and their local surface concentration as well as differentiate them from non-specifically adsorbed species.
- be compatible with electrochemical methods ensuring control over the electrode potential.

These are challenging requirements for the study of an interface buried in electrolyte and will normally require using more than one method. In situ methods that are often used to study the electrode interface can be grouped into electrochemical and spectroscopic methods that measure ensemble averages, and optical or scanning probe based microscopy methods which provide spatially resolved measurements of the surface. Both approaches can provide access to kinetic or time domain studies. Within the context of the questions posed in the Introduction, an evaluation of the advantages and disadvantages of these methods is provided below.

ELECTROCHEMICAL METHODS

Electroanalytical methods are inherently sensitive to the interface and provide in situ measurements, typically averaged over the whole electrode surface. Probing the biosensor interface electrochemically can be performed in the absence or presence of redox species, resulting in the measurement of non-faradaic or faradaic responses, respectively.

Non-faradaic transduction. The absence of a redox species implies a genuinely "label-free, marker-free" method. At modified electrodes, the measured capacitance is to a very good approximation, proportional to the dielectric permittivity of the interface and inversely proportional to its characteristic thickness. In principle, the determination of the capacitance is thus well-suited to the characterisation of the biosensor interface, and hence to the transduction of the recognition event. In practice, however, the variation of capacitance upon analyte recognition is usually modest (though measurable) resulting in rather low sensitivities.[26,27] This can be explained by the rela-

tive contribution, in the total capacitance, of the different species present in the interfacial region.

Faradaic transduction. For electrochemical sensors, the addition of redox active species in solution (acting as a "marker" or "indicator"), or using redox labelled adsorbates (probes), enables facile measurement of Faradaic electrochemical signals.[28–32] A number of redox species have been evaluated for use in the SAM based analytical determinations.[33–35] These redox markers can also report on the local molecular environment.[36–38] Determining redox characteristics are useful not only for characterizing the environment of the probes, but also for transduction if the target binding affects the microscopic environment to a measurable extent. This holds true when the capture probe is directly labeled with a redox active moiety.

Faradaic responses are extremely sensitive to the state of the sensing interface, because they rely on electron transfer across the adsorbed layer.[19] The thickness, permeability, charge density or hydration of the layer can all have a tremendous influence on the rate of electron transfer between the electrode and a given redox marker.[35,39,40] The measured current, which is an average of the charge flow over the entire surface area, is most sensitive to the areas of lowest faradaic impedance. In this context, defects can have a strong impact on the faradaic response if their presence results in the formation of "pathways" whose impedance is lower (e.g., Figure 1d) than that of the defect-free areas of the sensor layer. Those regions with higher impedance (e.g., aggregates in Figure 1c) will act to block the electrode surface from the redox species in solution. A similar effect may not be realized when using redox labelled probes. If the aggregates are composed of the redox probes arranged in a formation in which target binding cannot occur, then e.g., for a signal off sensor, a larger redox signal may be expected, which would only contribute to the background signal that is typically measured.[33] The opposite may be true for a signal on sensor configuration. Defects in the SAM (Figure 1d) also act to increase the redox signal measured in this case by providing low impedance pathways for the probe to undergo electron transfer.[39,41] Apparently, using other approaches to measure the extent of heterogeneity on these modified surfaces are needed so as to correlate the electrochemical signals to the actual physical nature of the modified interface.

OPTICAL SPECTROSCOPIES

Characterizing the metal surface in situ has involved the use of UV/visible and infrared spectroscopic methods for many years. Recent advances in instrumentation and surface-enhanced techniques have enabled characterization of molecularly modified surfaces. These measurements provide important molecular specific information such as the composition of the interface and the orientation of the adsorbed species. Typically, to improve signal to noise (S/N), large illumination spots are required which limits the measurements to ensemble averages. As discussed above, averaging over large spatial length scales is problematic if the biosensing surface provides polydisperse microenvironments.

Electroreflectance (ER) methods are useful for in situ study of chromophore containing monolayers.[42] With measurement timescales that match well with electrochemical timescales (e.g., cyclic voltammetry) ER measurements can discriminate the different components on the surface based on electron transfer kinetics which are coupled to optical changes. ER can also detect changes in the interface that are amperometrically silent, as well as detecting small faradaic signals connected to optical changes that are buried in a large non-faradaic signal.

In situ infrared spectroscopy (IR) can provide chemically specific information detailing the molecular composition of the interface at different potentials. SNIFTIRS, PM-IRRAS and SEIRAS have been used to study SAMs and physisorbed mono or multilayers.[43–45] As a non-destructive and label-free method, IR spectra can reveal molecular orientation, protein conformation, functionality with excellent surface sensitivity. Detailed interpretation of peak positions can provide key insight on the relative state of organization of SAMs used to support molecular sensing motifs (*viz* Figure 1c). Potential modulation techniques can reveal changes in the molecular adsorbate characteristics with potential or charge on the electrode surface. However, IR techniques suffer from significant averaging effects due to the requirement of large optical throughout for high S/N measurements.

Vibrational spectra can also be measured using SERS, SHINERS or TERS[46–48]. Compared to IR, these methods are more compatible with aqueous electrolyte environments due to negligible solvent absorption. However they rely on the enhancement of the scattering via structured interfaces[46] which result in an overweighting of the signal from 'hotspots'. Furthermore, SERS methods are significantly more destructive *cf*. IR as they require laser excitation sources that may damage adsorbed biomolecules.

SPR has been used extensively for the study of the probe immobilized onto surfaces, in particular for evaluating binding kinetics and evaluating non-specific adsorption in real solutions.[49,50] SPR methods using thin films of gold can be coupled with electrochemistry to examine SAM surface preparation, adsorption, probe reorientation, and hybridization.[12,20,51,52] The surface composition defines the sensing specificity and with flow chambers, it can be used to examine specific and non-specific adsorption onto the sensor surface. SPR is also sensitive to regions that are away from the electrochemical surface (at ~100 nm depending on the evanescent wave decay length) providing an approach to measure surface bound aggregates (Figure 1c).

In-situ spectroscopic methods may be able to detect the presence of non-ideal probe conformations or structures (e.g., Figure 1b-d), provided that the spectral signature is sufficiently different than the expected ideal probe immobilization. Unfortunately, the large area being analyzed restricts these measurements to averages that are inherently less sensitive and rely on subtle changes in the optical response.

OPTICAL MICROSCOPIES

A number of recent advances in instrumentation and particularly camera sensitivity have allowed the molecular specificity of spectroscopy to be coupled with imaging capabilities. The achievable spatial resolution is generally diffraction limited and is around the wavelength of light used. IR microscopy has been applied to an electrode surface using a synchrotron source realizing 10 μm spatial resolution.[53,54] Nevertheless, even greater resolution is required to assess aggregation and molecular heterogenieties that exist on sub-micron length scales at the surface. AFM coupled IR (often called nanoIR or near-field IR) breaks the diffraction limit and offers great promise as an imaging tool for molecular characterization of

biosensor surfaces.[55] At present a major limitation is very poor compatibility of nanoIR with liquid environments that essentially precludes in situ measurements.

Imaging has also been reported in electrochemical SPR studies of the deposition and desorption of SAMs in addition to studying electrochemical redox reactions on the surface. These methods offer an in-situ surface specific view of the surface at a resolution that is similar to optical microscopy.[56,57]

Fluorescence microscopy as applied to the electrochemical interface has been used to study biomolecule modified electrodes[14–16,58–60]. In these studies, the molecule of interest must contain a fluorescent tag. Spatial resolutions below 0.5 μm can be realized, and the use of confocal microscopes will provide excellent images free from interferences originating from out of focus regions. The fluorescence intensity strongly depends on the distance of the fluorophore from the electrode surface. The fluorophore in the excited state is quenched by energy transfer to the metal and quenching can be very efficient up to 20 nm from the electrode surface (depending on the metal and fluorophore[58]). This property results in measurements that are sensitive to probes immobilized onto long SAMs used to modify the surface or other arrangements that place the probe far from the metal surface. The field of view is compatible with sensor sizes and with the use of super-resolution microscopy, nanometer resolution can be achieved thus making it a powerful tool for mapping molecular heterogeneity of biosensing interfaces.[7,61] Fluorescence microscopy was used to examine the density and heterogeneity of the DNA probe surface coverage that resulted from changing the order of surface preparation.[14]

SCANNING PROBE MICROSCOPIES

Studying the monolayer covered electrode surface has been investigated extensively using in-situ AFM[57,62–64] and STM[18,64–67]. These methods provide atomic level resolution that is compatible with many electrochemical methods. The field of view is typically larger in AFM than STM but far smaller than optical methods. The time to record an image of the surface can be slow, which limits measurement of fast processes over large areas. This prevents complete imaging of the biosensor surface and facilitates reporting of 'representative' images. A natural bias for selecting highly ordered or well-defined images should be avoided to prevent oversimplification and underrepresentation of heterogeneity. Under typical imaging conditions, scanning probe methods can detail the coordination of the first adsorbed layer of probe molecules to the surface atoms. Carefully controlling the imaging conditions may result in more information from subsequent layers, but typically, the AFM or STM tip tends to move weakly adsorbed species. This is clearly demonstrated when trying to image immobilized DNA SAMs[68–70] where only the first few bases near the surface are resolved since they are not as mobile as the rest of the probe strand. This in situ AFM approach can analyze the clustering of probes on a length scale of many probe molecules (e.g., Figure 1b). Careful design of the coupling of DNA to the surface using DNA nanostructures like tetrahedrons was used to control surface density and was analyzed by AFM confirming the electrochemical results.[71] In many cases, especially complex multicomponent interfaces, the molecular specificity of what is imaged can be difficult to identify unambiguously. Flat, well-defined substrates are preferred for imaging and rough polycrystalline surfaces are largely incompatible with high resolution probe microscopy, further separating it from biosensor surfaces.

At a slightly larger length scale, similar to optical methods, is SECM or SICM imaging using electrochemical currents, impedance or ionic current[72]. This in-situ method has a large field of view and is linked to electrochemical activity of the surface, making it a useful mapping tool. The imaging is as slow as AFM or STM, but ultrafast scanning methods are in development.[73] It is not clear if the weakly adsorbed, or non-specifically adsorbed probe aggregates on the surface would be imaged or if they would be displaced during imaging.

CONCLUSIONS

Surface based biosensors encompass many possibilities, from large planar surfaces of metal or glass, to highly curved surfaces such as nanoparticles or quantum dots. The presence of a surface enables many possibilities for advanced detection strategies but also brings with it many challenges: a significant one being the ability to accurately measure the composition, configuration and environment of the adsorbed molecules. For applications such as biosensing, surface analysis is most reliable when performed in-situ (e.g., in aqueous buffered environment), which introduces further experimental /technical challenges. The multi-component make-up of surfaces containing immobilized biomolecules, the variety of local environments and of immobilized or adsorbed species, whether desired or unwanted (e.g. non-specifically adsorbed) demands development of new surface analytical methods. The methods need to distinguish between specifically (immobilized) and non-specifically adsorbed species but do not perturb the surface or disrupt the adsorbed layer; and more importantly, they should map the surface so as to measure the distribution and orientation of capture probes, their local surface environments and concentrations.

The need for expanding the scope of interfacial characterization makes this a challenging approach to designing and building biosensing surfaces. Many methods (electrochemical and spectroscopic) produce ensemble averages that can be used to evaluate the uniformity of the sensor surface, but only for those signals that are sensitive to local environments. In-situ imaging or mapping of the interface using optical or probe microscopy can provide nanometer resolved surface analyses but are prone to subjective selection. Ultimately the use of more than one approach will be required to carefully study the modified surfaces. New advances in in-situ surface analysis will contribute to developing and verifying the surface preparation strategies so as to ameliorate the undesired modifications. Developing a capacity to reliably perform these measurements will prove useful in creating surfaces that are like the ideal cartoons imagined, moving beyond analytical performance metrics, towards a more realistic and complete picture of the surface.

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Author Contributions

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ABBREVIATIONS

PM-IRRAS, Polarization Modulation Infrared Reflection Absorption Spectroscopy; SNIFTIRS, Subtractive Normalized FTIR Spectroscopy, SEIRAS, Surface Enhanced Infrared Absorption Spectroscopy; SPR, Surface Plasmon Resonance; SERS, Surface Enhanced Raman Spectroscopy; SHINERS, Shell-Isolated Nanoparticle-Enhanced Raman Spectroscopy; TERS, Tip-Enhanced Raman Spectroscopy; AFM, Atomic Force Microscopy; STM, Scanning Tunneling Microscopy; SECM, Scanning Electrochemical Microscopy; SICM, Scanning Ion Conductance Microscopy

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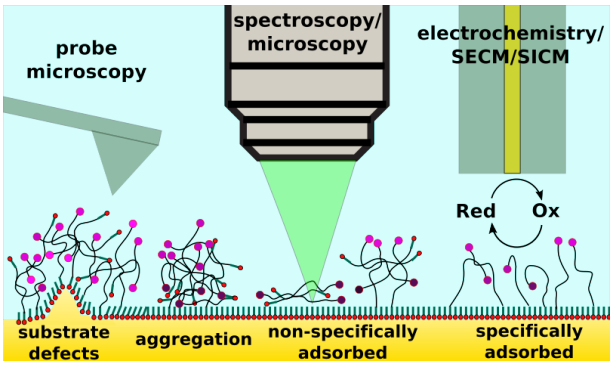
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