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Review: Recent Advances and Current Challenges in Scanning Probe Microscopy of Biomolecular Surfaces and Interfaces

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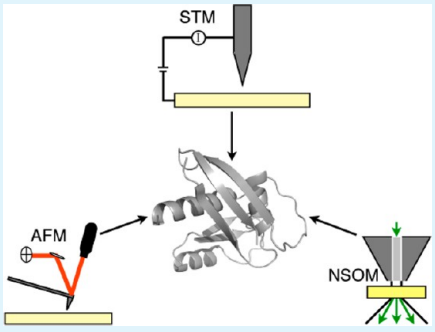
ABSTRACT: The introduction of scanning probe microscopy (SPM) techniques revolutionized the field of condensed matter science by allowing researchers to probe the structure and composition of materials on an atomic scale. Although these methods have been used to make molecular- and atomic-scale measurements on biological systems with some success, the biophysical sciences remain on the cusp of a breakthrough with SPM technologies similar in magnitude to that experienced by fields related to solid-state surfaces and interfaces. Numerous challenges arise when attempting to connect biological molecules that are often delicate, dynamic, and complex with the experimental requirements of SPM techniques. However, there are a growing number of studies in which SPM has been successfully used to achieve subnanometer resolution measurements in biological systems where carefully designed and prepared samples have been paired with appropriate SPM techniques. We review significant recent innovations in applying SPM techniques to biological molecules, and highlight challenges that face researchers attempting to gain atomic- and molecular-level information of complex biomolecular structures.



KEYWORDS: *scanning probe microscopy, STM, AFM, NSOM, protein, lipid bilayer, DNA*

INTRODUCTION

The invention of scanning tunneling microscopy (STM) in 1982[1] was a transformative event in the science of solid state surfaces and interfaces, initiating an era in which complex problems in surface and interfacial science are studied through atomic- and molecular-level resolution of the structure and chemical composition of the material.[2−7] STM, and the other scanning probe microscopy (SPM) methods it inspired, has profoundly changed areas of condensed matter science as diverse as nanolithography and surface catalysis. SPM techniques are characterized by the ability to measure a variety of properties of a surface of interest with high structural resolution, even down to the atomic scale, as seen clearly in the images of pentacene in Figure 1. In general, an SPM image is constructed by moving a sharp probe across a sample surface while using a feedback mechanism to maintain the tip−sample separation at close and constant distances, usually 1−10 nm. As the tip is scanned along the surface, electronic, topographical, force, optical, and other properties are mapped out at resolutions that range from the atomic scale up to tens of nanometers.[4,8] It has been demonstrated repeatedly that understanding the structure of materials at the atomic scale leads directly to understanding the properties of that material in ways that are simply not possible with traditional sample-averaged techniques such as crystallography or spectroscopy. SPM methods are now routine experimental tools used in diverse laboratories throughout the physical sciences.

Like any other condensed matter system, the functions of biomolecules such as proteins, DNA, and lipid bilayer membranes are a direct result of the structure of their surfaces and interfaces, and understanding the molecular-level mechanisms of how these complex systems function would be advanced considerably from the molecular- and atomic-level structural resolution achievable with SPM methods. However, applying SPM techniques to biological surfaces is not straightforward, and with a few exceptions, biomolecules have largely eluded investigation by SPM methods. This failure is due to the inherent nature of the biomaterials themselves; these

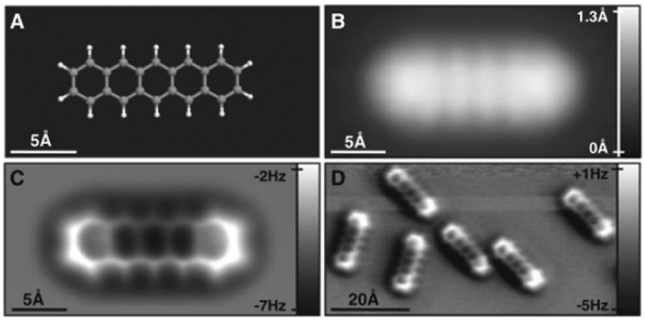


Figure 1. (A) Ball and stick model, (B) STM, and (C, D) AFM images of the pentacene, clearly demonstrating the atomic level resolution that can be attained using SPM methods. Reprinted with permission from ref 5. Copyright 2009 American Association for the Advancement of Science.

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Table 1. Comparative Advantages for Each of the Scanning Probe Techniques

	STM	AFM	NSOM
signal	electron tunneling	attractive/repulsive forces	near-field light
lateral resolution (nm)	0.1	5−10	20
vertical resolution (nm)	0.01	0.1	2−5
advantages	high lateral and vertical resolution	conductive or nonconductive surface	traditional optical imaging at higher resolution

materials display a wide diversity of chemical and structural complexity at the molecular length scale, are intrinsically dynamic, are easily deformable, and can interact strongly with a probe as it is brought close to the surface. Furthermore, the deformable and dynamic nature of many biological surfaces results in mobile structures that are only transiently displayed at a surface, requiring characterization techniques that can acquire information on a scale much faster than the mobility of the sample surface. The innovation and development of numerous scanning methods, probe choices, and feedback mechanisms for SPM developed on inorganic surfaces and substrates have therefore not been easily transferable to the investigation of biological molecules. Without the spatial resolution of SPM, proteins, DNA, lipids, and other biological molecules are most often characterized with spectroscopic and optical microscopy methods that average the behavior of a number of species and are often unable to describe the true heterogeneity of the structure, function, and organization of these materials. (Single molecule microscopy methods are capable of resolutions approaching the size of individual proteins, but are a fundamentally different experimental technique and beyond the scope of this review.)

Despite these difficulties, there is growing interest throughout biophysics, biochemistry, and biomaterials to devise experimental conditions and methods that solve these problems adequately for SPM to be useful, relevant, and widely applied, and a number of advances in this area have recently been published. By examining these successes, it is possible to understand the experimental breakthroughs that are necessary for SPM to become as important a tool for biomaterial and biointerface science as it currently is in inorganic materials research. This review has two purposes: (1) to summarize the development of SPM on biomolecular surfaces since the last significant review in 1997;[9] and (2) to define and discuss some of the most important experimental constraints that have so far prevented SPM technologies from making significant headway on the study of biomolecular surfaces. In our conclusion, we summarize how experimental challenges are spurring innovation and development of these unique methods for incorporation into traditional biophysical research.

SCANNING PROBE METHODS

The technologies and applications of SPM have been reviewed extensively elsewhere.[10,11] Before turning to a discussion of SPM on biomolecular surfaces, we briefly review the characteristics of STM, atomic force microscopy (AFM), and near-field scanning optical microscopy (NSOM) that are most relevant for understanding the successes and failures of these techniques when applied to biomolecular samples. Table 1 includes resolution information and comparative advantages of the three scanning probe techniques.

Scanning Tunneling Microscopy. Scanning tunneling microscopy (STM, Figure 2), uses a sharp, metallic probe that is positioned close enough to the surface to allow electrons to tunnel between the tip and surface. This quantum mechanical

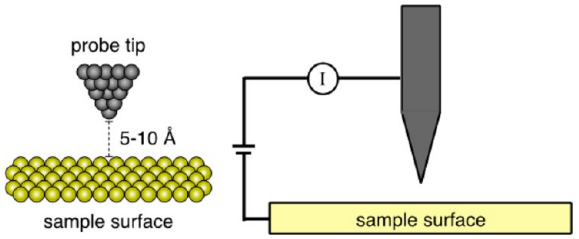

Figure 2. In STM, a metallic probe is brought close enough to a sample surface to enable tunneling between the sample and the tip. The direction of the tunneling current (I) is controlled by a bias potential that is applied between the tip and the surface. As the tip moves across the surface, the tunneling current is monitored and translated into a topographic image.

effect is possible because of the very small distances (5−10 Å) maintained between the tip and surface, allowing their electron densities to overlap. On the basis of the bias voltages applied to the tip and sample, electrons are driven from filled electronic states to empty states, generating a small current (~10−200 pA) that is used to produce an image of the topography of the sample, but also contains information about the local density of states (DOS) of the surface. The magnitude of the current is exponentially dependent on the distance the electron must tunnel and this, coupled with piezoelectric elements that control the vertical and lateral motion of the tip, give STM the sensitivity to obtain high-resolution images. By translating this change in DOS to a topographic map, vertical resolution in an STM image can reach 0.01 nm. Lateral resolution is fundamentally limited by the shape of the tip and the metal atoms that protrude from the surface, and is typically no better than 0.1 nm. Because an STM image is a convolution of the integrated topographic and electronic properties of each unique species on the surface, topography in an STM image does not necessarily relate to physical heights or lengths. Instead, the contrast observed in a topographic image relies on molecular conductivity as well as quantum tunneling, in which strong theoretical support is necessary to interpret the quantitative measurements of single molecule electronic properties. Other reviews contain detailed discussions of the factors that contribute to the contrast seen in STM images, which must be considered when coupling STM with quantitative measurements, especially when applying them to systems containing proteins, DNA, or lipid bilayers.[12,13] The contrast also yields additional pseudochemical compositional information by relating structural features obtained from STM images with physical characteristics and chemical properties of observed molecules to distinguish between unique species. Because STM measures a tunneling current, this technique requires a conductive surface that can donate or accept tunneled electrons; however, thin insulating layers, especially those with charged or highly conductive functionalities, can be imaged. Biomolecular materials are typically composed of insulating molecules with short tunneling lengths (usually <5

nm), and therefore cannot be accessed by STM if they are held even a small distance away from a conducting substrate.

Atomic Force Microscopy. Atomic force microscopy (AFM, Figure 3) monitors intermolecular interactions between

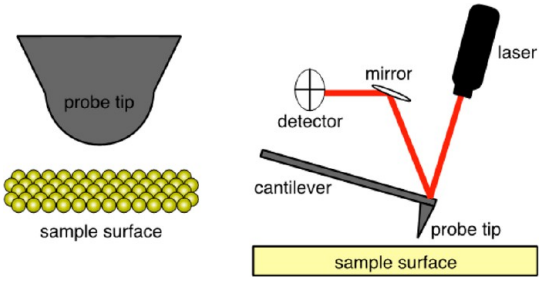

Figure 3. AFM measures noncovalent attractive and repulsive forces between the probe and the sample as a cantilever is moved across the surface and monitored through the positioning of a laser that is deflected toward a detector.

a probe tip mounted on an oscillating cantilever and the surface under investigation. The motion of the cantilever is tracked as it moves across the surface by changes in the position of a laser beam that is deflected toward a detector. Because of its reliance on noncovalent intermolecular forces, an AFM can be operated over a variety of intermolecular conditions. Three scanning modes are most commonly discussed: contact, noncontact, or tapping mode. Contact mode takes advantage of repulsive forces between the probe and the sample as the cantilever is essentially dragged across the surface, while noncontact mode uses attractive forces while the cantilever is close, but not touching, the surface. In tapping mode, the cantilever oscillates near its resonant frequency as it moves across the surface, alternating between attractive and repulsive regimes depending on the distance to the substrate. Because a portion of the tip interacts with the surface being examined, AFM resolution in the lateral *xy* direction is limited by the radius of the probe tip, typically 5−10 nm. However, higher resolutions can be reached in the vertical *z* direction (as low as 0.1 nm) because the roundness of the cantilever probe does not factor into this measurement. Because AFM relies on attractive and repulsive interactions between the tip and surface, it can be applied to any material and does not suffer from a fundamental limit of conductance like STM. AFM images are therefore truly topographical, although they are often convoluted with the size and shape of the probe tip. The use of coated or specialized tips allows for alternative measurements such as magnetic, elastic, and binding forces,[14−18] as well as surface potentials,[19,20] and is an area of much recent innovation in AFM technologies.

Near-Field Scanning Optical Microscopy. Optical microscopy techniques are fundamentally limited by the diffraction of light in the far field and are limited to resolve features on the order of half of the wavelength of the illuminating light. Near-field scanning optical microscopy (NSOM, Figure 4) is a scanning probe technique that produces topographical and optical images below the diffraction limit of light through the use of evanescent waves in the near field. NSOM typically uses metal-coated, optical fibers that have a tapered end and are fabricated with apertures significantly smaller than the wavelength of light, ∼20−100 nm. Three common methods for performing NSOM measurements can be achieved by varying the position of the illuminating source and the detector to transmit or collect light that interacts with the

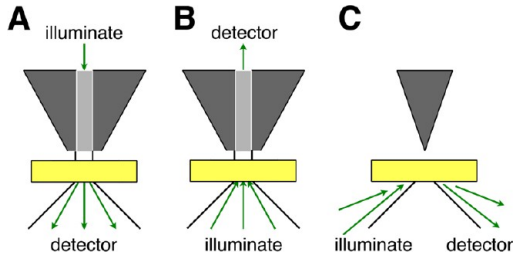

Figure 4. In NSOM, light that is transmitted through an aperture of the probe is laterally confined to the size of the opening, but only near the probe tip. Three common methods for performing NSOM measurements place the illumination source and detector at varying positions to allow for transmission or collection of light that interacts with the surface. (A) transmission, (B) collection, and (C) aperture-less probe.

surface, shown in Figure 4. Light that is transmitted through these apertures is laterally confined to the size of the opening, but only near the probe. Therefore, the probe must stay positioned close to the sample surface (<10 nm) as the illuminating field decays exponentially. Lateral resolution can reach 20 nm, but is limited by the size of the aperture and the probe distance from the sample. The working distance is also limited in the vertical direction, requiring that the probe remain close enough that the sample is still present in the near field. Vertical resolutions of 2−5 nm have been reached, however. NSOM lends itself to most conventional optical modes, typically involving illumination or collection through the probe itself.

APPLYING SPM TECHNOLOGIES TO WELL-CHARACTERIZED COMPLEX BIOLOGICAL SYSTEMS

When applying SPM methods, a comprehensive knowledge of the experimental system being investigated and its chemical environment are required, not only to interpret the acquired data accurately, but to test the accuracy of instrument capabilities. An example of this comes from the dawn of STM itself: the highly debated structure of the ultrahigh vacuum (UHV)-reconstructed Si(111) surface. Before the invention of STM, several experimental and theoretical approaches to determine the geometry of the surface had led to inconsistent results that could not rule out several reasonable candidate structures.[21] It was not until Binnig and co-workers imaged this surface directly with STM that the reconstructed 7 × 7 geometry was confirmed to be correct.[22] These results were an exciting motivation for the use of STM, but also demonstrated that a great deal of information must first be understood about the sample in order to interpret topographical images reliably. This becomes particularly challenging when considering the wide diversity in chemical composition, structure, dynamics, and environmental interactions that are known to be present in many types of biomolecules of interest.

No matter the SPM technique chosen to investigate a sample, there are a few common hurdles to overcome when probing biological systems. (I) Biological samples are inherently dynamic in their native environments and can deform and diffuse on time scales that make probe microscopy methods difficult to use. (II) Biomaterials are often described as being "soft" or "sticky," as they can be easily damaged and may interact unfavorably with the probe. (III) Biomolecules are often charged, prone to aggregation, or have semi-insulating

characteristics, all of which depend heavily on their local environment. Despite these challenges, there are an increasing number of successful examples employing SPM to characterize topography, force, optical properties, and even to provide mechanistic insight of soft, biological surfaces.[23−27] In reviewing these advances, we will emphasize how researchers have overcome these three experimental obstacles by exercising control over the substrate, local environment, sample, and tip used in their SPM measurements. Although the strategies discussed will not provide substantial protocols for preparing biological samples, such procedures have been published elsewhere.[28−31]

I. Immobilizing Biomolecules. The dynamic nature of biomolecules can make probe microscopy methods difficult to apply to some systems. To obtain resolution on the nanometer or subnanometer scale, the molecules of interest must remain immobile for the time scale of the measurement, which can take up to several minutes per frame in the case of AFM; a very mobile sample can result in blurred images or in measurements that represent an average of multiple states. A common method for managing this issue is to immobilize samples on surfaces through covalent linkages or weaker noncovalent interactions. Another way to suppress the molecular motions of some samples is to suspend them in matrices that force them to order or orient in a consistent and repeatable manner. Such repeated allows researchers to identify and measure biomolecular structures of interest more easily. As studies of proteins or peptides, bilayers, and DNA have become more common, the development of methods to immobilize and order each class of biomolecule has progressed as well. Often such methods place the molecules of interest in environments that are very different from their native surroundings. Although these deviations are important to understand and consider in any data analysis, the manufactured environment can allow for unique studies of the mechanical or electrical properties of the sample.

DNA. One of the simpler methods for attaching biomolecules to a surface is to take advantage of the fact that mica and DNA have similar surface charges. By introducing cations such as Mg^{2+} or Ni^{2+}, a layer of these ions forms on the mica surface and serves as an electrostatically adhesive layer with which DNA can be immobilized.[4,32] Furthermore, the relative surface concentration of monovalent (which alone do not promote immobilization) to divalent cations can be adjusted to mediate the strength of attachment. It has also been shown that the functionalization of a freshly cleaved mica surface with methyltrimethoxysilane has the same effect with the added benefit of straightening DNA interacting with the substrate.[33] These techniques have allowed researchers to achieve submolecular resolution of closed circular DNA plasmids as well as self-assembled DNA crystals using frequency-modulated (FM)-AFM.[34] Using this charge balancing technique for immobilization coupled with an advanced AFM method that allows for soft tip landings, thus minimizing tip-induced sample deformation, the authors were able to resolve not only the major and minor grooves of the helix but also individual phosphate groups along the backbone (Figure 5). Although the image resolution and quality of measurements made by these authors is a testament to the abilities of SPM, they would not be possible were it not for the well-understood and periodic structure of DNA.[35]

SPM techniques have also been used to characterize the structures of self-assembled nanoscale abiological DNA constructs. Winfree et al. were able to construct two-

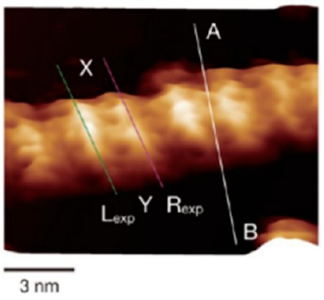

Figure 5. AFM image of plasmid DNA immobilized on a freshly cleaved mica surface in a 50 mM $NiCl_2$ solution. By coupling the well-understood structure of DNA with this immobilization technique, Ido et al. were able to resolve both major and minor grooves of DNA as well as identify topological protrusions as phosphate groups. Figure labels are described in ref 34. Reprinted with permission from ref 34. Copyright 2013 American Chemical Society.

dimensional crystal structures from strands of programmed DNA by exploiting Watson−Crick base pairing, aromatic van der Waals interactions between the nucleotide bases, and a detailed knowledge of the geometric configuration of the DNA double helix itself.[36] As the self-assembly relied on Watson−Crick base pairing, the sequence was constructed in such a way that specific nucleotide sections were complementary only to one other region of the DNA strand, thus minimizing any unintended pairing. The incorporation of DNA hairpins into the vertical columns of the lattice structure resulted in visible stripes when AFM was used to image the nanostructures on a mica surface (Figure 6). Molecularly resolved SPM images were possible in this case because of the built-in periodicity of these DNA hairpins. By tailoring this structural parameter through rational alterations in the sequence, the researchers were able to use the imaged periodicity as a benchmark for how successfully their designs formed from solution self-assembly. These so-

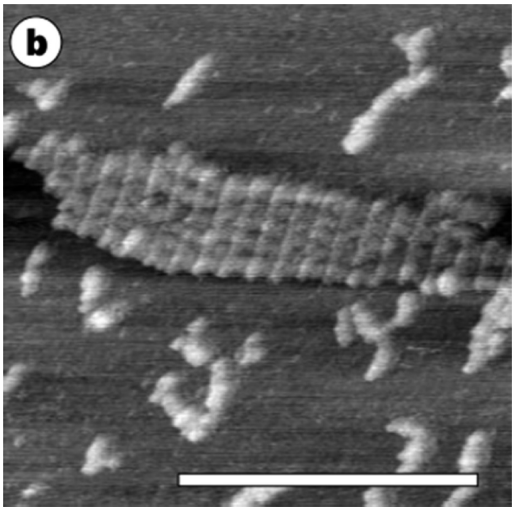

Figure 6. DNA DAO-E lattice structure designed to self-assemble through Watson−Crick base pairing and imaged by AFM. The incorporation of hairpin sequences into the vertical columns (1−2 nm) of the lattice structure give rise to the brighter stripes in the image, which act as a convenient marker calibrating the distance between structural features clearly visible in the image. The scale bar is 300 nm. Reprinted by permission ref 36. Copyright 1998 Macmillan Publishers.

called "DNA origami" methods are now being used to design complex shapes that are routinely characterized by SPM methods.[37−40]

Because the resolution of AFM allows the measurement of Watson−Crick regularity in DNA, it can also be used to observe slight deviations in structure. For example, the double-crossover, antiparallel, odd intramolecular spacing, even intermolecular spacing (DAO-E) tile motif commonly used for the self-assembly of DNA nanostructures has two connection points in its unit tiles, identified as kinked or straight.[25] Using AFM techniques similar to the imaging of the DNA plasmids, Ido et al. resolved DNA within these tiled nanostructures, identifying not only the kinked connections but two distinct connecting conformations as well.[34]

DNA has also been successfully immobilized on surfaces through modifications to include linkers that covalently bind these biomolecules to surfaces. Studies have shown that thiolated DNA binds readily to Au(111) surfaces, aiding imaging of the structures through the use of AFM. In one such study, researchers covalently bound one end of a DNA molecule through a thiol linker to a gold surface covered in a self-assembled monolayer (SAM) acting as a blocking layer to prevent surface interactions between the DNA and surface.[41] A potential was then applied to the gold surface, changing the orientation of the DNA. When a negative potential was applied, the negatively charged DNA was repelled by the surface, whereas a positive potential attracted the DNA. Using AFM, the authors observed that small defects (<2.5 nm in width) in the SAMs possibly allowed either the hydrophobic DNA grooves to interact with the alkanethiol chains at these sites or the gold surface directly. The authors hypothesized that defect sites allowed a more direct interaction between the DNA strand and the surface, which in turn made switching of the DNA orientation with respect to the surface with external inputs easier. This study demonstrates how detailed analysis of single molecules leads to striking insights into how biomolecules interact with surface structures that would be difficult, if not impossible to determine using ensemble measurements. Such results highlight once again how thorough sample characterization leads not only to the accurate interpretation of the data, but also to the discovery of new phenomena in the field of biomolecule-surface interactions.

Proteins/Peptides. Covalent attachment of a biomolecule to a surface of interest is often an attractive method for immobilization of complex samples, provided the biomolecule of interest is not altered from its biologically relevant state. Covalent chemistry can provide researchers with a great degree of control over important sample parameters such as the orientation and surface coverage of the macromolecule. An example of this was recently demonstrated by Gallardo, et al., who induced a desired secondary structure in a tethered polypeptide sequence through optimized placement of reactive functional groups dispersed throughout an alkanethiol SAM on a gold surface.[42] Alkyne groups present in modified residues of a designed peptide served as attachment points that could react with terminal azide groups on a SAM. By adjusting the surface concentration of azide groups, peptides were attached to the surface with two covalent bonds and with the helical axis oriented parallel to the surface. These peptides were structurally resolved by STM at low tunneling current.[43] This sample preparation method enabled molecular-level imaging of the tethered peptides for several reasons. The tethering points on the peptide ensured that it not only remained immobile during imaging, but also forced the sequence to maintain its helical structure (confirmed through spectroscopic measurements). The chemical reaction also allowed for control over the structured periodicity of the peptides by eliminating the presence of physisorbed peptides and leaving behind only those helices chemically bound to the substrate. Because this chemical reaction scheme resulted in a rigid and immobile surface-tethered α-helix, individual helices 2 × 3 nm in size could be imaged easily by STM under ambient conditions (Figure 7).

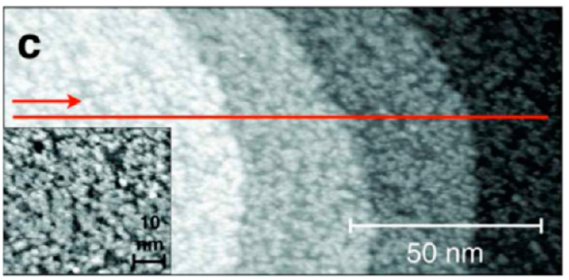

Figure 7. Ambient, low-current STM image of short helical peptides covalently bound to an underlying self-assembled monolayer of alkanethiols. By tailoring surface concentration of reactive groups, the authors were able to produce a monolayer of chemically bound α-helical peptides allowing the identification of single molecules 2 × 3 nm in size. Adapted with permission from ref 43. Copyright 2012 American Chemical Society.

Single-molecule control over a protein's position and orientation on the surface can also enable SPM measurements on a complex biomolecule. Della Pia et al. introduced cysteine into cytochrome b_{562} through amino acid mutation. These cysteines were placed either at the longitudinal or lateral extremes of the protein.[44] The protein was then exposed to a clean gold surface to form a covalent Au−S bond. The position of the cysteine mutation caused the oblong protein to orient differently on the gold substrate, with either its long axis perpendicular to the surface (for longitudinal mutations) or parallel to the surface (for lateral mutations). These orientations were confirmed by STM and AFM measurements, which were able to resolve difference in the height, length, and width of the surface-immobilized proteins depending on the location of the cysteine mutation. The authors then incorporated two cysteine residues on opposite sides of the protein either along the longitudinal or lateral axes; after one of these cysteines had reacted with the Au surface to form the gold−thiol bond, the other cysteine sulfhydryl was exposed to the platinum−iridium tip of the STM, forming a closed circuit. In this configuration, the authors performed the first single molecule, controlled orientation measurements of a protein's conductance.[23] Similar studies have been accomplished with the protein azurin by using a gold nanoparticle label to link the protein with the STM tip.[45] These experiments highlight the versatility of SPM techniques, which can be used to measure not only the topography, but also electrochemical properties of individual biomolecules. These studies illustrate the potential of SPM in answering a diverse set of questions about the structure and behavior of single biomolecules.

An alternative method for the immobilization of biomolecules to surfaces involves the use of molecular chaperons and matrices. By carefully considering how the chaperone molecules interact with the samples, predictions about the structure of the

resulting template may be made. Armed with this knowledge, data obtained from SPM methods may be interpreted based on predicted size and shape of domains as well as their periodicity on the surface. Gong et al. have employed the use of oligo-(phenylene-ethynylene) (OPE), which self-assembles to form a molecular template on highly ordered pyrolytic graphite (HOPG), to immobilize and order tripeptides.[46] The molecular network formed by OPE provided structural vacancies that were then filled by the tripeptide TGG, which was immobilized in the sites through hydrogen bonding, and was then imaged with subnanometer resolution using STM (Figure 8). Another

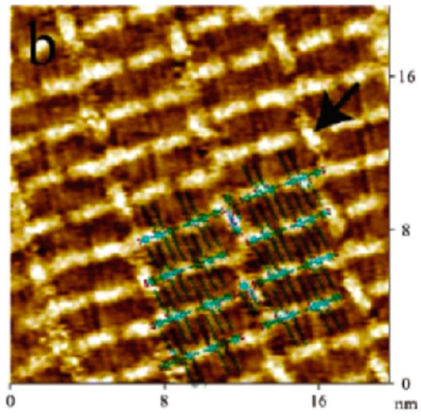

Figure 8. Polymer OPE (structure overlaid in green) was used to design a molecular template with sites of correct size to accommodate the insertion of the tripeptide TGG, indicated by the arrow. The imposed order and periodicity of the template visible in this STM image allowed researchers to probe how intermolecular interactions and geometry of the template affect the position and orientation of the peptide. Adapted with permission from ref 46. Copyright 2006 American Chemical Society.

method of chemically mediated ordering of peptides has been demonstrated through the use of the β-amyloid peptides that self-assemble into β-sheets that have been associated with Alzheimer's disease.[47] Characterization of the peptide sheets formed from the peptide fragment Aβ33−42 using STM and AFM confirmed the debated antiparallel structure of the β-sheet. Furthermore, when differently oriented linker molecules (4,4-bipyridyl (DP) or 1,2-di(4pyridyl)ethylene (DPE)) were added to the sample, the change in the pattern of self-assembly of the peptides caused by the different linker structure was easy to measure and interpret (Figure 9). Such characterization by SPM methods coupled with an accurate understanding of the modulator-peptide interactions allowed insight into the mechanics of peptide aggregation as well as possible methods for regulation.

Phospholipid Bilayers. Many in vitro measurements made on biological structures or processes suffer from physiologically irrelevant deviations induced by their non-native environments, driving the constant search and development of better model systems and more benign experimental conditions. For cell membranes, supported lipid bilayers (SLB), phospholipid bilayers constructed on a two-dimensional substrate and containing a variety of lipids, cholesterol, or even proteins, are an important model system for investigation by SPM. SLBs appropriate for SPM imaging are commonly made through well-established techniques such as vesicle deposition and Langmuir−Blodgett (LB)/Langmuir−Schaefer (LS) transfers.[30]

SPM investigation of SLBs has been an important tool to better understand the factors important in the formation of heterogeneous lipid domains, occasionally called lipid "rafts."[48] Using dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylcholine (DLPC), cholesterol, and complementary fluorescent lipid analogues, researchers were able to resolve compositional differences between microscale and nanoscale domains by NSOM. Confocal scanning microscopy (CFM) had previously shown distinct phase separations in similar systems that were several hundred nanometers in size, but the increased resolution provided by NSOM allowed the identification of nanoscale domains in 1:1 DPPC/DLPC mixtures on the order of 123−145 nm. In addition, when SLBs containing DPPC, DLPC, and cholesterol were imaged, phase separations 70−100 nm in size were found between DPPC- and DLPC-rich regions.

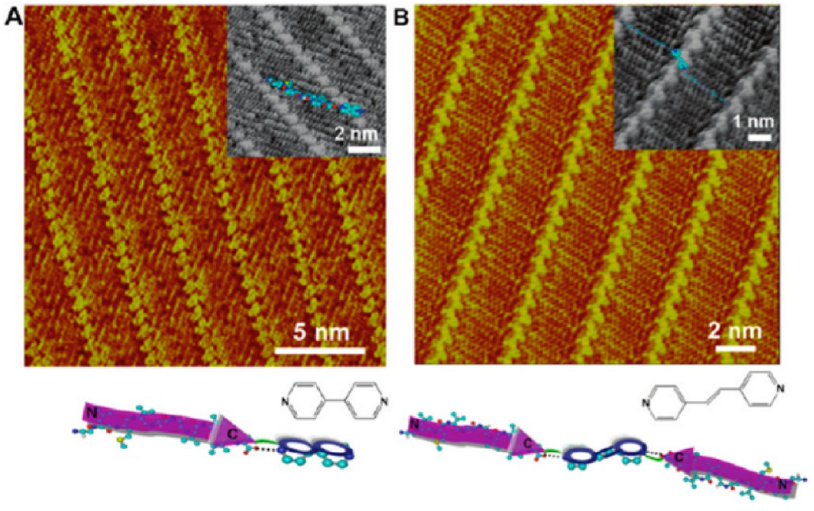

Figure 9. STM images of the assembly of a β-amyloid peptide analogue in the presence of various chaperone molecules: (A) DP, which modulates a single peptide, and (B) DPE, which is associated with two peptides. This sample preparation scheme allowed individual peptide molecules to be resolved on the subnanometer scale (see black and white insets). Reprinted with permission from ref 47. Copyright 2009 American Chemical Society.

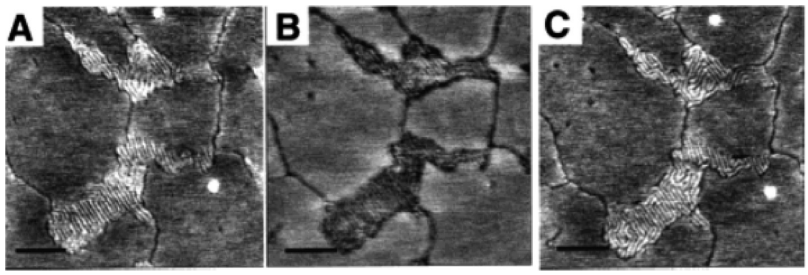

Figure 10. Supported phospholipid bilayer containing 2 mol % transmembrane peptide that induces the formation of a striated domain. When imaged at (A) <500 pN, the striated domain appears higher than the bilayer, but (B) is compressed at higher forces, >1500 pN. (C) As the force is reduced, the striated domain once again appears higher than the surrounding bilayer. Reprinted with permission from ref 56. Copyright 2000 American Chemical Society.

SLBs provide an excellent model system for studying the complex nature of the cell membrane, its components, and its interactions with other biomolecules. Pieta, et al., have used electrochemical STM to study pore formation by alamethicin (Alm), an amphipathic antimicrobial peptide.[24] Using the LB technique, a monolayer containing Alm along with the lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and egg phosphatidylglycerol (egPG) was formed on a gold surface. STM images showed the formation of barrel-stave hexameric pores composed of Alm consistent with previous literature models. Additionally, clustering of these pores was also observed. By coupling their images with the well understood nature of the peptide, Pieta, et al., demonstrated that the Alm molecules were able to participate in the formation of adjacent pores, and that only 3−4 peptides contributed to the hydrophilic interior of the channel. The information gleaned from this study helps show how STM may be used to provide mechanistic insight into the formation of structures within lipid bilayers.

When SLBs are formed through LB techniques, a lipid monolayer is produced at an air/water interface, and the solid substrate is passed through the monolayer vertically, creating the first layer. Then the second monolayer is added by repeating the process vertically (LB), or horizontally (LS). One advantage of this method is that it allows researchers to prepare bilayers where the top layer could be engineered to contain a different lipid composition than the bottom layer. Yuan and co-workers took advantage of this technique to probe the factors that influence lipid bilayer reorganization on a surface.[26] These researchers created an asymmetric SLB that contained a binary lipid bottom monolayer and a single lipid top monolayer. Fluorescently labeled lipids were also introduced for imaging experiments. By monitoring changes in topography using AFM over time intervals ranging from minutes to hours, the group was able to measure lipid "flip-flop", or the migration of lipid molecules from the bottom monolayer to the top monolayer, as well as lateral lipid diffusion. In the case of lipid flip-flop, it was found that the surface substrate contributed to the suppression of this bottom-to-top lipid migration. Again, coupling well-understood and well-defined systems with a SPM method has helped demonstrate that the substrate is rarely a passive support, but is instead an integral part of the experimental system being examined.

II. Detrimental Effects of High-Surface-Area Attractive ("Sticky") and Repulsive ("Soft") Interactions. Throughout the biomaterial SPM literature, biomolecules are typically described as "soft" and "sticky," referring to these materials' strong interactions with the probe tip through attractive and repulsive forces. The "stickiness" that authors commonly mention when discussing biomolecules is a result of charged surfaces created by amino acid side chains, the phosphate backbone of DNA, or the headgroup of a lipid. These charged surfaces create strong noncovalent electrostatic interactions with other biological and abiological macromolecules. Taken together, this means that many different kinds of biological molecules in many different environments will have strong attractive and repulsive noncovalent interactions with a SPM tip through long-range electrostatic forces. Biomolecular "softness" refers to the weak mechanical strength of globular structures held together by noncovalent interactions. A scanning probe can damage DNA,[49,50] compress or dislodge proteins,[51] or puncture, deform, or anneal lipid surfaces.[52−55] For example, Figure 10 shows how simply changing the normal force of the AFM probe during scanning a SLB can affect the height of lipids. Panels A and C were scanned with <500 pN force, whereas panel B was imaged at >1500 pN normal force. Transmembrane peptides embedded in the phospholipid bilayer disrupt the packing within the bilayer and induce the formation of striated domains, which are well-resolved under low applied force, but are compressed in the z dimension when scanned under higher normal force. At low normal forces, the striated areas were measured to be 0.2−0.4 nm above the bilayer, while at higher forces they were pushed below the bilayer surface.[56] However, the "soft" character of the material allows the lipid to resume their initial height once the force was reduced to its initial amount (panel C).

Because of these "soft" and "sticky" properties, and because biomolecules can occupy an ensemble of low energy conformations that can be easily interconverted, a probe, particularly if it is metallic or charged, will interact strongly with this surface and these interactions will alter the structure, topography, and other properties of interest in the sample being investigated during SPM characterization. With this in mind, SPM techniques must be applied in ways that minimize any damage or deformation to the sample, taking into consideration the capabilities of the instrument as well as the biomaterial imaged.

STM methods for acquiring high-resolution images of biomaterials often require low tunneling currents to maintain the tip at a distance that minimizes tip−sample interactions.[49] Although tip−surface interactions are unavoidable, it is important to monitor the images produced by the tip to infer if molecules have been physically adsorbed onto the tip, or if the tip has been damaged. For example, Deng and co-workers observed that when imaging folded and unfolded structures of cytochrome c, new tips resolved the finer structure of unfolded

areas, whereas damaged tips showed improved resolution on folded areas (Figure 11).[57]

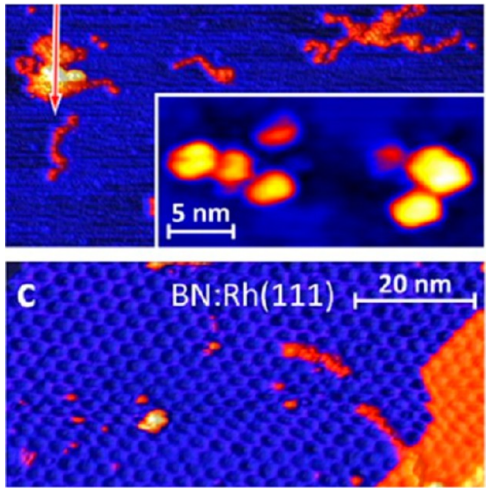

Figure 11. STM image of cytochrome c on a Cu(001) surface. The tip has been modified during scanning and shows improved resolution of the folded protein, shown in the inset image. Adapted with permission from ref 57. Copyright 2012 American Chemical Society.

The time over which an image is collected is also a significant factor when considering the damage a SPM tip may do to a biomaterial's surface. If the sample and the tip do not have time to thermally equilibrate, the mismatch in thermal properties of the two materials will result in drift and exacerbate tip-induced sample damage. Moreover, the piezoelectric elements that control the lateral and vertical motion of the scanning probe stabilize with time. Sek, et al., conducted experiments on gramicidin embedded in a lipid monolayer, and noted a significant improvement in resolution after scanning for an hour, shown in Figure 12.[58] Initially, the lipid monolayer was well resolved, but observation of the embedded gramicidin was limited by streaks and bright features. Streaking often points to the tip interacting with the surface and suggests that the feedback mechanism is not adequately controlling the tip. After 40 min, the image quality improved, and after an hour, much of the streaking had disappeared and distinct gramicidin features, as well as the lipid matrix, were observed.

One successful solution that has been demonstrated for the problem of deleterious sample-tip interactions is tapping-mode AFM (also known as amplitude-modulated AFM), a technique in which the AFM probe oscillates near its resonant frequency as it scans the surface. This instrument configuration minimizes tip−surface contact and reduces shear forces during scanning because the tip does not stay in contact with the surface at all times. Instead, the tip touches and then swings away from the surface with each oscillation. However, it can be difficult to control the force of the tip when it encounters the surface during the "tap," particularly when it is maintained in the repulsive regime, often used to obtain higher resolution imaging on inorganic substrates. Nonetheless, it has been successfully used to image double-stranded DNA and RNA on mica surfaces.[59] Frequency-modulated AFM techniques have been developed that are similar to tapping-mode AFM, but are sensitive to the shifts in resonance frequency instead of maintaining a constant cantilever tip amplitude as it moves across the surface. Because the tip amplitude ends up being much smaller than in tapping mode AFM, it imposes gentler forces on the surface.[60] Cerreta, et al., were able to resolve grooves within the DNA double-helix by maintaining the tip in the attractive, noncontact regime, ensuring that the tip did not contact the surface.[61] Furthermore, to obtain the highest resolution possible, they compensated for tip drift in the vertical direction, which helped them optimize the tip−sample separation when it was set manually. Substantially smaller cantilevers also help improve resolution, as demonstrated by Leung et al. in Figure 13, who reduced the thickness of the cantilever to 250 nm and the width to a few micrometers to resolve minor and major grooves on DNA.[62] However, it is important to note that with these small cantilevers, tip−sample interactions begin to compete in magnitude with cantilever−sample interactions, making it easier to damage the cantilever when approaching the surface, and requiring well-controlled approach settings.

Because the spatial resolution of NSOM is achieved entirely through a noncontact mechanism that also does not depend on electron tunneling lengths, in principle this method should be able to overcome the experimental challenges of working with molecules that have strong intermolecular interactions with the tip. For example, Kim et al. used a thick probe tip to minimize the amount of light that seeps out of the probe aperture to optimize the optical signal.[63] This allowed them to image strands of stretched DNA on mica substrates with 80 nm spatial resolution, shown in Figure 14. Hermann, et al. modified the aperture of their NSOM probes to display a wide taper angle approaching 90°, which dramatically increased light transmission.[27] This allowed them to employ an experimental setup that imaged the surface without the use of a feedback system. Instead, they had a photolithographically fabricated substrate with 20 μm pores over which the membrane was suspended. The NSOM probe was lowered onto the sample surface until

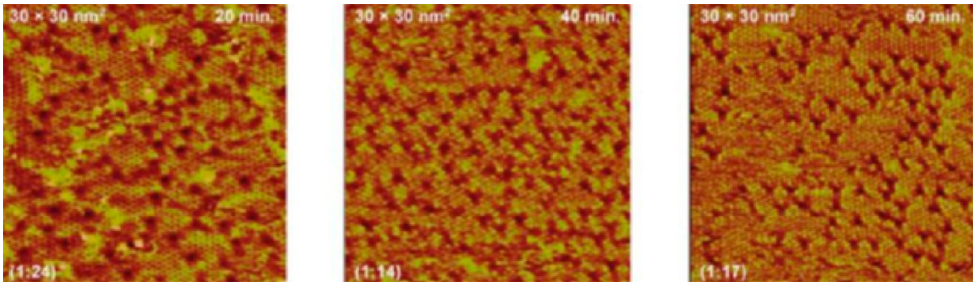

Figure 12. STM imaging of gramicidin embedded within a phospholipid monolayer over time. The images were acquired at different locations within a 200 nm × 200 nm area. Over the course of an hour, image quality improves significantly as bright features and streaking become less frequent. Reprinted with permission from ref 58. Copyright 2009 American Chemical Society.

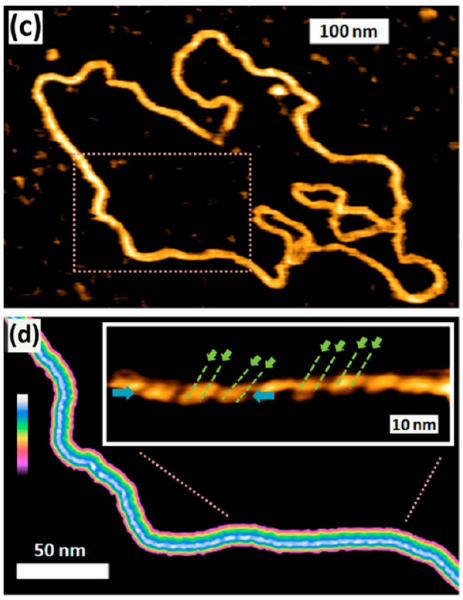

Figure 13. High-resolution AFM imaging of DNA using a miniaturized cantilever. Panel D is an image of the outlined area in panel C. In the inset image of panel D, the major and minor grooves are displayed. Green arrows point to the two strands of the DNA. Adapted with permission from ref 62. Copyright 2012 American Chemical Society.

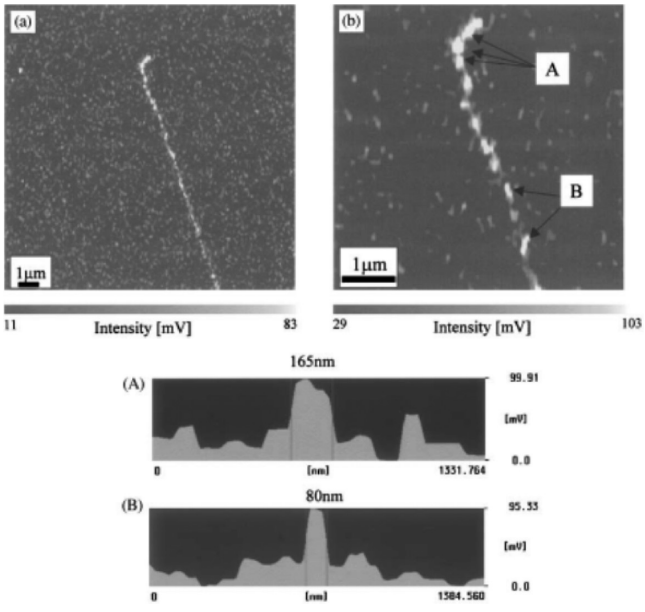

Figure 14. High-resolution imaging of stretched, fluorescently labeled DNA with NSOM. Figure labels are described in ref 63. Reprinted with permission from ref 63. Copyright 2002 Elsevier.

the membrane was under slight tension, and the transport of fluorescently labeled protein complexes and kinetic studies of their diffusion through nuclear pores was measured.[27] Sample functionalization with quantum dots and nanoparticles, which can be more photostable and brighter than molecular fluorescent probes, have been shown to reduce tip−surface interactions and still yield high-quality images.[63] For example, this sample preparation method was used to image CD44 antibodies on whole mesenchymal stem cells, which helped resolve domains that were not clear in confocal microscopy images.[64]

Although usually deleterious for SPM measurements, biomaterial−tip interactions can be taken advantage of for useful reasons, including eliminating defects within lipid domains. In Figure 15, the area outlined by the black corners has been scanned repeatedly; when a much larger region of the sample was scanned, the area within the box was significantly more ordered than the unscanned area surrounding the box.[53] It was suggested that the probe acts as a local stirrer, transporting additional lipids to areas for the improved formation of domains. This effect has been noted in other studies in which the scanning probe was allowed to penetrate the film and the order of the lipid bilayer was subsequently improved.[55] Additionally, the scanning probe can also rupture vesicles, displace bilayer domains, or induce the formation of larger bilayer domains.[54] These observations indicate that with appropriate control of the scanning probe, the tip can operate as a tool for sample preparation.

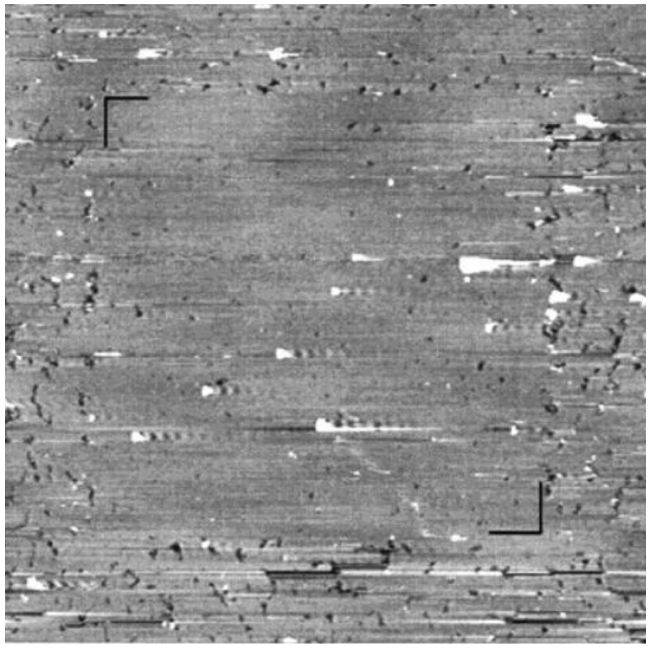

Figure 15. Supported phospholipid bilayer after repeated scanning by contact-mode AFM at 0.06 N/m, resulting in an area outlined with black corners that is relatively defect free. Image size is 1.5 μm × 1.5 μm. Reprinted with permission from ref 53. Copyright 2000 American Chemical Society.

Moreover, tip−sample interactions can be deliberately enhanced through tip-functionalization methods that coat the probe with covalently or noncovalently bound molecules that chemically interact with the surface.[65−68] Using these methods, experiments can be performed that combine high-resolution imaging with chemical information about the sample. For example, a DNA aptamer-functionalized probe was used to image the toxic protein ricin bound to a surface. Topographical images were able to resolve multiple conformations of ricin that arose from its immobilization on the Au(111) surface through various lysine residues. Molecular recognition studies were used to show the availability of binding sites for each of the ricin conformations.[69]

III. Managing Environmental Influences. Biomolecules are often charged, prone to aggregation, or have semi-insulating characteristics, all of which depend heavily on their local surface or solution environment and any of which can change the orientation and structure of the biomolecule being observed. Successfully collecting and interpreting SPM images therefore often depends on the chemical environment surrounding the biomolecule, such as solution chemistry, pH, substrate surface charge, and surface topography. The shape and composition of the probe tip can influence the magnitude of the signal obtained and can be enhanced or diminished based on the types of interactions that are desired. Substrate charge and topography can influence how biomolecules interact with surfaces by promoting or inhibiting immobilization or aggregation. The medium in which the samples are studied (ambient, solution, or vacuum) can greatly affect the quantitative analysis of data. For studies in a solution environment, ion concentration and pH can drastically influence the structure of the sample. Manipulating experimental conditions for optimum SPM signal must be done in a manner that makes the measurements relevant to the native environment of these samples in some useful way. Despite the seemingly endless variables to consider in these systems, a careful analysis of all the components along with well-designed control experiments can allow SPM to lead to new and exciting discoveries.

There are distinct benefits and challenges to applying SPM techniques to biological surfaces in ambient, ultra high vacuum (UHV), or solution environments. Conducting experiments in ambient conditions have the obvious advantages of being easily accessible, requiring little additional sample preparation, and preserving physiological hydration on biomaterial surfaces. Despite these advantages, the ambient environment is also the most difficult environment to control. Relative humidity changes constantly, and it is well-known that bringing a probe in close proximity to a sample surface can cause a hydration meniscus to form within the tip−surface contact.[70] The capillary forces thus generated can effectively pull the tip to the surface or even reorganize easily deformed biomolecules and complicate measurements. Furthermore, the scanning probe tip and substrate must be composed of materials that will not degrade or oxidize from exposure to $O_2(g)$ and water in ambient air. Therefore, STM experiments are limited to Pt/Ir tips and gold or graphitic surfaces, whereas AFM or NSOM must use these materials or mica, glass, or quartz.

Experiments in UHV solve many of these complications, but exposing biological materials to a vacuum environment is not trivial. Water is critically important to the three-dimensional structures of proteins and DNA. It is still unknown how much water is retained on the surface of biomolecules and biomaterials when exposed to UHV conditions, but it is unlikely to resemble bulk water. Despite these difficulties, high-resolution imaging of DNA and proteins or peptides have been acquired in vacuum as low as $\sim 1 \times 10^{-10}$ Torr.[49,57] It is also critically important to control how the sample is moved from ambient to UHV environments. Typical cleaning procedures for inorganic substrates call for annealing or sputtering the surface to remove atmospheric and pump contaminates after UHV has been achieved, but these methods are likely to severally damage soft biomolecular surfaces. Electrospray and pulse depositing methods have been used successfully to deposit small amounts of biological material onto a clean surface, which have been shown to retain folded and unfolded conformations.[57,71−73] It remains unclear how widely these methods may be applied.

Solution-based measurements can most closely replicate physiological or biochemical sampling conditions by controlling pH and ionic strength easily. STM, AFM, and NSOM can all be conducted in a liquid environment, but certain operational considerations have to be made. For example, it has been shown that tip−surface distances in solution are smaller than in ambient or UHV environments, and pH and ionic strength will also play a role in dictating the tunneling distance.[51] As with all STM measurements, tunneling distances are highly dependent on the medium being tunneled through—whether it is the sample or the environment—and care must be taken to ensure that the tip−sample separation is sufficient for the proposed studies. Solution environments can dampen the motion of the oscillating AFM cantilever due to the greater viscosity of the liquid with respect to ambient air. This reduces shear force between the tip and the sample and can potentially reduce damage of the surface. Unfortunately, this can actually complicate NSOM measurements, which often employ a shear-force feedback mechanism that relies on the oscillation of a tuning fork attached alongside the scanning probe. In solution, the oscillation of this tuning fork is largely dampened, which can make feedback between the tip and the control mechanism unreliable. Methods to keep the tuning fork dry include a diving bell, which allows the probe to be immersed in solution while keeping the tuning fork dry.[74]

The extent of influence of the solution not only affects instrument application, but also the measurements associated with the biomolecules being sampled. This can readily be seen in a study of charge density measurements on bacteriorhodopsin, a proton pump found within the cell membrane. The charge density data acquired by AFM were highly sensitive to the pH of the solution employed during the experiment.[75] Both the tip (silicon nitride) and substrate (alumina) had functional groups that could either donate or accept a proton; this caused charge densities calculated for bacteriorhodopsin to vary by up to 50% when changes were made to the pH of the system. Continuing work on bacteriorhodopsin, Müller and Engel systematically measured the apparent topographical height of the protein while altering pH, ionic concentration, and applied force of the cantilever probe.[76] At low applied forces (<0.3 nN), the tip is dominated by the electrostatic forces of the substrate and the sample. Although this can be overcome by increasing the applied force to the tip, this approach runs the risk of deforming the relatively soft structure of the biomaterials being imaged. Instead, measurements of sample height against electrolyte concentration may be made to find the optimum solution conditions, which screen the surface charges of the sample and substrate.

In addition to solution conditions and sample charging, careful consideration must be paid to the effect that the supporting substrate has on the behavior of samples. A study done by North et al. has demonstrated striking results that should inspire researchers to be ever mindful of the materials they use in their experiments.[77] The group used the silanization of soda-lime glass slides as a method for the immobilization of rabbit antilipid A, a procedure common to many other biomolecules. They found that glass slides they had purchased before 2008 were more efficient at immobilization than slides purchased after 2008, despite manufacturer's claims that no changes had been made to the slides. Combining AFM with a variety of other techniques, their results showed that the pre-

2008 slides had a larger magnesium content compared to the post-2008 slides. It is believed that the increased concentration of magnesium allowed for a higher surface concentration of silane, which aided in the immobilization of biomolecules. Another study demonstrating the reaching effects of supporting substrates comes from the investigation of ganglioside GM1 (GM1)-mediated formation of amyloid beta (Aβ) aggregates on lipid bilayer membranes. Using SLBs containing sphingomyelin, cholesterol, and various concentrations of GM1, the group monitored the aggregation of Aβ over time with AFM. Surprisingly, the effect of using mica as opposed to SiO_2 as a supporting substrate had more of an effect on fibril formation than GM1 concentration. The mica surface induced the formation of clusters of disordered GM1 conformations, which then led to the formation of fibril Aβ agglomerates. In the case of SiO_2, GM1 was homogenously distributed across the SLB and only globular Aβ agglomerates formed. The researchers found that the head groups of GM1 were clustering near cavities on the mica surface containing adsorbed water molecules, demonstrating once again that a carefully understood experimental system is necessary to interpret the results of SPM measurements.

SUMMARY OF CURRENT CHALLENGES IN APPLYING SPM TO BIOMOLECULAR SURFACES AND MATERIALS

The field of biophysics has been advanced by the application of tools from traditional physical and chemical sciences to the complexities of biological macromolecules. Ultracentrifugation, separations chemistry, mass spectrometry, NMR, and fluorescent spectroscopy are just a few examples of techniques that were originally conceived of and developed for description of atomic and molecular structure, but have become the backbone of modern biophysical chemistry. SPM is now one of the central methodological practices in surface and interface science, and is as important to modern surface science as spectroscopy or mass spectrometry is to the study of solution-phase small molecules. Despite this, SPM has been successfully applied to biomolecules or biomaterial surfaces only rarely. A review of the literature clearly identifies the experimental challenges that have prevented a more widespread application, originating from both sample and instrumental deficiencies.

A significant impediment to characterizing biological systems with SPM stems from the fact that biomolecules are mobile and have dynamic structures whose conformations change on time scales much smaller than SPM data acquisition times. While increasing the scanning speed of SPM could help in some systems, it is doubtful that data acquisition on the time scale of molecular motions will ever be achieved. Instead, a variety of researchers are productively addressing this problem by innovative sample preparation schemes to immobilize and induce order within their systems without sacrificing biophysical relevance. By tethering biomolecules to surface substrates through covalent linkers or electrostatic interactions, the motion of these samples can be reduced enough to allow clear imaging over the course of normal SPM measurements, up to hours long. Advances in benign sample immobilization are becoming sophisticated enough that they can be exploited to test single molecule properties on biomolecules of interest such as charge density and conductivity.[23]

Another source of the inherent difficulties of SPM measurements on biomolecular surfaces stems from the substantial dissimilarity between traditionally well-behaved inorganic samples and the diverse complexity of biomolecular structures, which can induce strong interactions with the probe as it moves across the surface. Careful consideration to instrumental parameters such as scanning mode, settings, and probe material choice can help minimize interactions with the sample enough so that the surface is not deformed, altered, or displaced. On the other hand, tip−sample interactions can be enhanced through functionalization methods that allow for complex molecular recognition or force studies.

Aside from considerations between sample−substrate immobilization and sample−tip interactions, the area governing how the sample interacts with its environment can be one of the most challenging aspects to applying SPM to these biological systems. Part of the interest in studying these systems arises from the fact that their native structures and functions are inherently sensitive to changes in their environment, which in turn allows a biological system to change quickly in response to various external stimuli. In addition, given the sensitivity of the SPM instruments, it should be anticipated that the equipment itself would also be sensitive to any environmental changes, such as tunneling distances, thermal drift, humidity, probe dampening by solvent, and tip protonation/deprotonation due to changes in pH. It is necessary to understand how all components in an SPM experiment can affect the sample as well as the measurements being taken. Researchers should be mindful of any unseen or unaccounted for interactions that can cause measured characteristics to deviate from their native states.

The examples described here clearly indicate that modern SPM is on the brink of widespread application throughout biophysical investigations of proteins, DNA, and lipid bilayer membranes as researchers are discovering innovative ways to overcome significant obstacles inherent to studying biomolecular surfaces and interfaces. Applying SPM to biological molecules pushes the limits of SPM capabilities and will require innovation in sample preparation, focusing especially on how samples behave in the manufactured environments used to immobilize them. To do this successfully, researchers with a thorough knowledge of SPM fundamentals will be called on to make important contributions to understanding how measurements are influenced by the system of interest. This will require significant collaboration at the interface of biological and materials surface science, to match the capabilities of the instrumentation with the demands of soft and sticky materials.

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Notes

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