Scanning Force Microscopy (SFM) has opened doors to fundamentally new kinds of biopolymer characterization. Three-dimensional structural imaging down to the molecular scale in important fluid environments is now possible. Dynamic phenomena such as protein crystal growth, and the response to nanomanipulations by the SFM probe, have been imaged in real time. Biologically specific adhesion measurements at the level of single bonds have been reported, and two-dimensional adhesion images obtained. Viscoelastic and frictional behavior in condensed biopolymer systems have been probed down to the \( \approx 10 \text{nm} \) scale. This article provides an overview of these novel SFM capabilities, as well as state-of-the-art structural imaging methodologies.

The advent of scanning probe microscopy (SPM) marked a renaissance in surface characterization. The first SPM invented, scanning tunneling microscopy (STM), has been used to probe the structural and electronic characteristics of electrically conductive materials down to the atomic scale, via a localized tunneling current between STM tip and sample. The later invention\(^2\) and ongoing development\(^3,4\) of scanning force microscopy (SFM, also known as atomic force microscopy or AFM) broadened the range of characteristic materials, physical phenomena and sample environments by probing tip-sample interactions of great variety and spatial range\(^5\). In the last four years in particular, there has been an explosion of SFM studies of biomaterials ranging in size from single molecules up to cells and tissues\(^6,7\). Although imaging the structure of such systems down to the molecular scale is not a new achievement (much has been accomplished with electron microscopy), doing so without sample modification and in relevant fluid environments, as well as probing adhesion, viscoelasticity and other properties on the nanometer scale, are novel SFM capabilities\(^5,8\).

The purpose of the present article is to review some of the most notable advances in SFM characterization of biopolymers reported in the last two years. Brief attention will be given to improvements in the ‘art’ of obtaining good topographic images of biopolymer structures. This is a more mature SFM topic that has received much attention (dating back to 1988) and has been reviewed very recently\(^5,6\). The present article focuses instead on fundamentally new types of SFM research into biopolymeric systems.

**Instrument fundamentals**

The essentials of the most common and commercially available type of SFM are illustrated in Fig. 1a. The sample base is mounted on a piezoelectric \( xyz \) translator (not shown). Imaging is performed by raster-scanning the sample in the \( xy \) plane while monitoring localized forces between the sample and a sharp stylus attached to a soft, microfabricated cantilever (triangular piece in Fig. 1a). The forces are gauged by focusing a laser beam near the end of the cantilever and directing the reflected light onto a two- or four-diode photodetector array. Vertical cantilever displacements, i.e. vertical forces, are measured via the voltage difference between top and bottom photodiodes. Cantilever twisting is monitored with the left and right photodiodes; this provides a gauge of lateral forces when the fast-scan direction is chosen normal to the soft axis of the cantilever, as shown in Fig. 1a. (The depicted photodetector array pertains to a four-diode instrument used in the Center for Interfacial Engineering at the University of Minnesota; in more recent commercial versions the diode junctions lie parallel and perpendicular to the sample surface, i.e. the detector is rotated in-plane by \( 45^\circ \) with respect to the orientation in Fig. 1a.)

For standard topographic imaging, feedback displaces the sample in the \( z \)-direction to maintain an approximately constant vertical cantilever deflection (‘constant force’ mode); the topographic image then is a plot of vertical sample displacement during \( xy \) raster-scanning (or during one-dimensional scanning in the \( xy \) plane for 1D-trace analysis). The finite response time of the feedback circuit results in slight deviations in the vertical cantilever deflection, particularly at locations where the surface topography is steep. Images of these deviations (‘error signal’ or ‘deflection’ mode) can be used to accent surface steps or boundaries between surface regions, etc., and are often illuminating.

Fictional or lateral force microscopy (FFM or LFM) measures lateral force during \( xy \) scanning. In general, both nonconservative (energy-dissipative) and conservative lateral forces exist; the latter derive from off-vertical components of the tip-sample normal force\(^8,9\), and in a closed cycle do no net work (i.e. are not energy-dissipative)\(^9\). The ‘true’ frictional component (energy-dissipative) is obtained to first approximation from the difference between lateral forces measured while scanning in opposite directions\(^8\). Frictional forces on steep surface regions can couple with the vertical force sensing (e.g. exert a downward drag on the cantilever), thereby distorting the imaged topography\(^9\). This can be identified as a dependence of the imaged topography on the scan direction.

Force-versus-distance measurements (also termed ‘force curves’, ‘force-displacement measurements’, etc.), are collected by monitoring vertical cantilever deflection while cycling the sample up (approach) and down (withdrawal). The information obtained allows one to both determine the operating force during imaging and...
Topographic imaging methodology

Since the first studies, continual developments have improved the spatial resolution of SFM of biopolymeric and other 'soft' structures, reduced the perturbative nature of the measurements, and aided in identifying the imaged molecules. Most of the difficulties encountered are related to the bluntness (relative to molecules) and ill-defined characteristics of the imaging asperity, the large mechanical compliance of the imaged materials, the perturbative effects of shear forces, and the intrinsic instability of many surface-adsorbed species in fluid environments. Methodological improvements that surmount or reduce these problems are chronicled in recent reviews\textsuperscript{5,6} and will not be listed in detail here. They can be grouped into methods to construct sharper tips\textsuperscript{12}, elucidate tip shape\textsuperscript{13} and contamination\textsuperscript{14}, reduce the normal imaging force\textsuperscript{14,15}, remove or reduce shear forces\textsuperscript{15-18}, stiffen the sample\textsuperscript{17,19}, anchor sample to substrate\textsuperscript{16,20}, and affix identifying objects to molecules of interest\textsuperscript{21,22}. The references cited here are relatively recent papers that can direct readers to a larger set of related studies.

Some of the most important developments focus on reducing the intrinsically perturbative character of the tip-sample interactions. It was realized early on that, in air, capillary effects of adsorbed water introduce strong attractive forces between tip and sample, thus yielding a large contact area and damaging many samples; this was avoided by imaging in a dry gas environment or in a fluid. It was also apparent that shear forces would often move or stretch the sample, degrading image quality dramatically. Elegant methods that largely eliminate shear forces are the tapping and non-contact modes of imaging described in the instrumental section. Two research groups have published biopolymer SFM studies combining two of the above strategies, that is, imaging in fluid with tapping mode, to achieve enhanced resolution on stable structures in important environments\textsuperscript{16-18}.

Another new and rapidly developing methodology is the use of functionalized substrates or samples to anchor strongly the investigated molecules in desired patterns or orientations\textsuperscript{20,23-25}; in particular this strategy has been applied to lithographically prepared substrates\textsuperscript{20,25}. Specific functional interactions have also been utilized to label DNA\textsuperscript{21} and protein\textsuperscript{22} molecules with colloidal gold particles. Ongoing efforts to combine topographic imaging with other local characterizations (e.g. near-field optical and friction force microscopies) are providing further means of identifying imaged structures.

Dynamic process imaging in situ

Imaging important biopolymer processes in situ in real time is one of the most exciting capacities of SFM. On the molecular level, enzymatic degradation of DNA has been imaged in real time\textsuperscript{15}. Studies characterizing time-dependent changes in extended biological structures have generally focused on complex multicomponent systems such as cells\textsuperscript{26}. An interesting study of a simpler dynamic system is the imaging of three-dimensional lysozyme crystal growth by Dubin et al.\textsuperscript{27} An example of their results is shown in Fig. 2, a four-minute sequence taken in the 'deflection' mode. The sequence of 9 × 9 μm images depicts the competition between two modes of growth of a lysozyme crystal in its native solution environment: (1) random two-dimensional nucleation and
facilitated growth at the outcrop of a screw dislocation. The first frame shows the surface immediately following a period of active inhibition of growth at the dislocation, achieved by rapid scanning of the SFM at high force over a small area. New layers were continually added to the crystal by nucleation, spreading and merging of islands. During and after the time period shown, the creation of a growth spiral at the dislocation re-established a large hillock, which eventually dominated layer formation in this region. The images were obtained while scanning at a low force that had no apparent effect on the surface.

Molecular-scale images of static, two-dimensional protein arrays in solution have improved markedly, invoking the advances listed earlier. A good example of state-of-the-art imaging is the work of Schabert and Engel, who resolved grain boundaries as well as periodic lattices of OmpF porin, a major protein of the outer membrane of *Escherichia coli*. Molecular resolution has also been obtained on nonbiological organic crystals whose real-time growth was imaged on a larger scale. Thus in the near future the mechanisms of biopolymer crystal growth may be revealed in real-time SFM images at the level of individual molecules. This might mirror some of the fascinating atom-by-atom growth of inorganic films imaged in vacuum with STM.

**Adhesion measurements and mapping**

In SFM the force-versus-distance relationship generally differs between approach and withdrawal; this hysteresis reflects energy dissipated in breaking tip-sample adhesive bonds. Three research groups have demonstrated two-dimensional mapping of tip-sample adhesive forces by measuring force versus distance at each image point along the surface. In a study by Radmacher et al., adhesive forces were imaged in water on lysozyme molecules adsorbed on mica, and on uncovered mica. A one-dimensional trace from these measurements is presented in Fig. 3. The topographic trace is shown in Fig. 3a and the adhesion trace in Fig. 3b; the corresponding surface regions are denoted at the top of the figure. The authors also measured the time elapsed between the initial reduction in magnitude and final vanishing of the attractive force during withdrawal, to obtain the 'lift-off speed' (Fig. 3c). This quantity was thought to reflect the amount of viscous damping of the cantilever deflection during withdrawal. The distinct differences in adhesion and lift-off speed on lysozyme and mica, seen in Fig. 3, possibly reflect differences in surface forces and viscoelasticity of the near-surface regions. Imaging spatial variations in adhesion is such a new concept that one can only speculate about its general implications. Perhaps two-dimensional correlations in adhesive forces will elucidate in-plane interactions, for example.

Because SFM force measurements have resolution of the order of discrete hydrogen bonds, the technique can be used to study specific biological adhesion (lock-and-key molecular-recognition interactions) on the scale of single bonds. Three research groups have published the first studies addressing this capability. Figure 4 shows force-versus-distance results from one study by Lee et al., investigating the biotin–streptavidin ligand–receptor interaction in buffer. In particular, the authors measured the interaction between cantilever-attached glass microspheres covered with biotin-functionalized bovine serum albumin (BBSA) and mica surfaces functionalized with either streptavidin (Fig. 4a) or biotin-blocked streptavidin (Fig. 4b). Comparison reveals larger adhesion (negative forces during withdrawal) in (a). Histograms of numerous biotin–streptavidin adhesion measurements revealed a narrow and somewhat skewed distribution of forces of much larger mean value than the biotin-blocked streptavidin case. Analysis led to the conclusion that a single molecular biotin–streptavidin interaction predominated.

In a similar study by another group, the SFM tip itself was avidin-functionalized and brought into contact with individual polystyrene spheres, each functionalized with a particular biotin. In some cases histograms of the measured adhesive forces exhibited multipeaked distributions, which upon statistical analysis implied integer multiples of single molecular interactions. The first SFM measurement of forces between complementary strands of DNA was also reported recently; and interchain forces associated with Watson–Crick base pairing identified. Major challenges in all of these studies are, firstly, in distinguishing biologically specific forces from non-specific (e.g. van der Waals), and, secondly, in interpreting a succession of partial adhesive failures during withdrawal.

An exciting prospect is the integration of the two new capabilities described in this section, specific biological adhesion measurement and adhesion mapping, with the new methods for functionalizing lithographically patterned substrates. The ability to prepare and characterize such structures could have important applications in biosensor technology.
results are shown in Fig. 5, a plot of vertical cantilever deflection (force) versus sample height (vertical displacement towards the tip). The large water content in water-swollen films results in very low elastic modulus; increasing propanol content strongly increases stiffness. The authors explicitly demonstrated a decrease in tip–sample contact area, and resulting improvement in imaging resolution, with increasing gel stiffness\(^9\).

The capacity to image and characterize locally solid–liquid composite systems may contribute to biological tissue and cell research, and provide better molecular-scale understandings of technological gels. The latter could have ramifications for the photographic, pharmaceutical and many other industries.

**Frictional characterization**

Friction force microscopy was discovered to be a means of distinguishing materials with extraordinary spatial resolution\(^{5,4}\). Two research groups have applied the method to biopolymeric systems. To characterize patterned biopolymer arrays, Frediani et al. imaged friction on fibronectin selectively adsorbed onto micron-scale aminosilane strips, between strips of bovine serum albumin (prepared lithographically)\(^{35}\). Friction images verified the presence of open aminosilane regions (i.e. incomplete wetting by adsorbed fibronectin). Haugstad et al. studied nominally dry gelatin films adsorbed onto mica, and in frictional images distinguished two phases of gelatin with sharply differing frictional characteristics\(^{8,42}\).

The two studies by Haugstad et al.\(^{8,42}\) revealed the power of FFM to probe phase behavior in condensed biopolymeric systems. Measurements and analysis indicated that FFM scanning of gelatin films could induce a glass-to-melt transition on the low-friction, highly crystalline phase, and a glass-to-rubber transition on the high-friction phase\(^{42}\). On the latter, scanning at the highest forces attainable yielded a complete phase transformation: the high-friction gelatin film was disrupted, then reassociated on the mica surface into the low-friction phase\(^3\). This result is displayed in Fig. 6a, showing topography and frictional force images (left/right) of a 2 × 2 μm region following disruptive raster-scanning of a 1 × 1 μm region that originally contained the high-friction component. This region was completely transformed to molten liquid biopolymer (plus water) after disruptive raster-scanning. The higher topography/friction regions are rendered brighter in contrast in these images. The top, middle and bottom images were collected 1.0, 2.5 and 6.0 min after termination of the disruptive process and record the appearance and lateral growth of a highly crystalline, low-friction film on mica, formed as free molecules (that are invisible to imaging) continually join the imaged condensed phase. Small moieties of the same phase, but originally present in the film, can be seen to the left and right of the modified region. Topographic and frictional images of newly transformed films indicated both solid-like and fluid-like characteristics.

In Fig. 6b the 1.5 × 1.5 μm topography and friction images (following disruptive scanning of a 2 × 2 μm region) were collected sequentially at 6.0, 25 and 60 min total loading force (top to bottom) during a 3 min interval immediately following the transformation process. At 60 min the discontinuous film supported the tip and the gelatin nanostructure was resolved\(^\circ\). Tip pen-
eration at 25 nN is seen as an absence of contrast in the
topography images (tip in contact with the mica substrate)
and inversion of film/mica frictional contrast (reflecting
viscous drag as the tip scanned through the film). Gross
film morphology is not altered by the scanning tip under
penetrating conditions; however, small holes =10–100 nm
in diameter are created and/or enlarged. In both Figs 6a
and 6b the time-resolved lateral growth of the film was
not related to the scanning process and continued for
about 30 min after the transformation.

In sum these first FFM studies indicate that molecular
relaxation and topology, intermolecular coupling, phase
transformations, self-association and quasi-fluid charac-
teristics can be probed locally in condensed biopolymeric
systems. The difficulties in such studies derive from the
large number of physical phenomena (adhesion\(^{31}\), visco-
elasticity\(^{32,42}\), electrostatic charging, etc.) that in general
may affect frictional behavior. Understanding the roles
of these phenomena will be aided by the controlled vari-
ation of external parameters such as humidity and tem-
perature, as well as the chemical and structural com-
position of biopolymers.

Outlook
There is every reason to expect a continuation of the trend
of improvements in topographic SFM imaging seen in the
last five years. We should expect enhancements in tip
preparation, spatial resolution and sample identification
and stability, as well as a better understanding of scan-
ing-related perturbations; such developments may
become more specific to the class of biopolymer system
investigated. This progress will also allow more careful
studies of dynamic behavior in important fluid media, a
subfield of biopolymer SFM that is still in its infancy.

The methodology required to identify and separate
specific from nonspecific adhesive forces will probably
develop rapidly, judging from the rate at which new stud-
ies are being published. The ability to prepare patterned
substrates of desired functionality, and image tip–sample
adhesion in two dimensions, clearly would aid in this
endeavor. Improved understandings of biological ad-
hesion, as well as development of biosensor and other
technologies, are anticipated. An important issue in mea-
surements of force versus distance or penetration (to
classify adhesion, elasticity, etc.) is the location of the
'zero' of the vertical scale, i.e. the position at which
tip–sample contact occurs. Appreciable uncertainty in
this quantity is considered by some to be a major defi-
cency of SFM relative to the surface forces apparatus.
As with all SFM methodologies, we should expect con-
tinued progress in understanding tip–sample contact:
where it occurs, what initiates it (e.g. cantilever instabil-
ity versus interfacial peak), the role of mechanical
compliance in tip and sample, and so on.

The full complexity of viscoelastic behavior on
biopolymeric systems has only begun to be explored with
SFM. Undoubtedly the ability to characterize these
properties on a molecular or near-molecular scale on
biopolymers will yield new insights into the link between

Fig. 5 Force curves of thin gelatin films in different fluid media. The curve in propanol
is almost indistinguishable from the force curve taken on a hard substrate. The more
water added to the propanol, the softer the gelatin appears to be. (Reproduced, with
permission, from Ref. 19.)
molecular characteristics and the related condensed matter properties. Frictional behavior is the most complex, incorporating issues of interfacial chemistry and viscoelastic, i.e. relaxational, properties. The importance of both aspects is not a new realization, as witnessed by the adhesion versus deformation debate in engineering studies of polymer friction dating back more than 30 years. What is novel and especially exciting is probing friction to identify and characterize biopolymeric materials on the =10 nm scale.

The more physical phenomena simultaneously probed, the more powerful a technique becomes, provided the effects can be identified and separated. This is why hybrid SPMs, e.g. combining SFM and local spectroscopies, are so enticing and will provide continued developments in SPM capabilities in the coming years. Combined scanning force and fluorescence microscopes now exist in the form of commercial near-field scanning optical microscopes, and scanning NMR microscopy may be possible in the not-so-distant future. Combined with SFM, the latter would provide an unprecedented capability to probe biopolymeric systems on the nanometer scale.

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