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Molecules Under the Microscope*

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The scanning probe microscopes represent a novel class of instrumentation which provides an entirely new method of pharmaceutical analysis. This generic group of instruments, which includes the scanning tunnelling microscope and the atomic force microscope, measure surface-related properties at the nanometre level using a sharp probe. In the case of the scanning tunnelling microscopy local conductivity is recorded, whereas the atomic force microscope is essentially an atomic resolution profilometer.

The scanning tunnelling microscope was developed through the Nobel Prize winning studies of Binnig and Rohrer in the late 1970s and early 1980s (Binnig et al 1982). For the instrument to operate successfully, the material to be examined (the substrate) must be electrically conducting. The instrument scans a metallic probe, normally Pt/Ir or W wire (although even pencil leads can provide surprisingly good results), within a few Å of a surface while applying a voltage bias between the substrate and the probe. This bias causes a small tunnelling current, of the order of nA, to flow between the tip and the substrate (Fig. 1). Since the magnitude of this tunnelling current is exponentially dependent on the gap size between the tip and the substrate, very small changes in sample height cause large changes in tunnelling current. Through the use of a piezo-ceramic crystal, which expands and contracts on the application of voltages, the scanning tunnelling microscope can maintain the tunnelling current at a constant level and hence the probe at a constant height above the surface. It may, therefore map out the topography of a surface with nm resolution.

The atomic force microscope was developed in the mid 1980s; importantly it is an instrument that can record high-resolution images on both conducting and non-conducting materials (Binnig et al 1986). Here the probe, which is mounted on a soft spring, is brought into contact with a surface such that it experiences a very small interaction force, usually of the order of nN. The probe is then scanned across the surface, while conserving a constant force between the tip and the sample. The contact force is maintained using a feedback loop controlled by signals generated from the deflections of the supporting spring, or cantilever (Fig. 2). These deflections, which are caused by changes in surface stiffness or topography, are most commonly monitored optically by following the deflection of a laser that is reflected off the back of the cantilever. The atomic force microscope is thus capable of recording the contours of a surface generated by the close proximity of tip and surface atoms.

While the scanning tunnelling microscope was originally constructed to image semiconductor and metallic materials, it has now been found to have many biophysical applications. However, as with many analytical procedures, adequate specimen preparation is critical. As the probe exerts a force on the sample, the molecules under examination are normally immobilized to prevent them from being swept from the field of view by the tip. In addition, careful image interpretation and data processing are essential to distinguish between the observation of artifactual features on substrates and biomolecules (Roberts et al 1994). Molecular level images have now been obtained on a range of proteins, carbohydrates and nucleic acids (Roberts et al 1994).

Since its development the atomic force microscope has been utilized mainly for biological applications; images have been obtained on a range of samples from proteins and nucleic acids to whole living cells. While the forces exerted on the sample by the probe are significant, they may be reduced by imaging under a solution to alleviate capillary forces. Alternatively non-contact modes of imaging may be utilized where minimal tip-sample contact occurs (Roberts et al 1993a).

Sample Preparation

Due to the high forces exerted between the probe and the sample, biological materials may be easily swept outside of the imaging area by the probe. Many days or weeks may be spent searching for molecules that are always just a few nm away! To overcome this problem, rigorous sample preparation methods have been developed. These techniques normally involve immobilizing the molecules under examination to the substrate.

Using the well developed electron microscopy method of metallic coating, a thin layer of Pt/C can be evaporated onto a sample. This is normally undertaken after the sample has been sprayed onto the substrate to ensure uniform distribution (Wilkins et al 1993a). The Pt/C overcoat is normally robust enough to prevent the molecules being swept by the probe and also advantageously provides an adequate electron conduction route for high quality imaging by the scanning tunnelling microscope. The limiting factor in this method is the grain size of the coating (normally 4 nm) which effectively precludes ultra-high resolution imaging of the molecules themselves. However, the resolution is certainly similar to high resolution electron microscopy techniques and unlike the two-dimensional data

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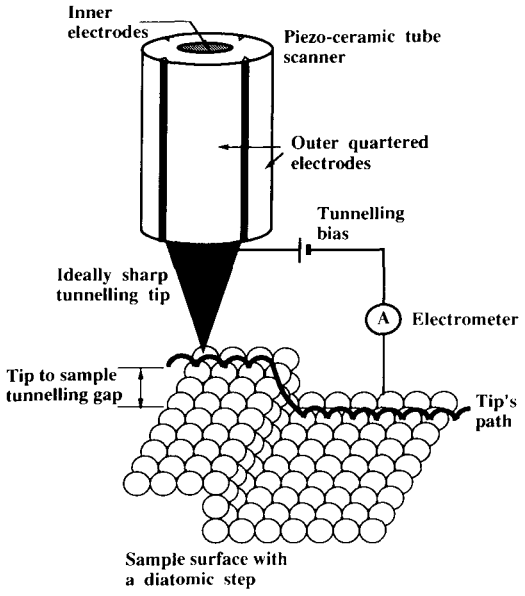

FIG. 1. A schematic representation of a scanning tunnelling microscope. A tiny current is made to flow across a small gap between a metallic probe and a conducting surface. A high resolution image of the surface is built up by scanning the probe over the surface whilst keeping the current and hence height constant.

provided by electron microscopy, the scanning probe microscopy topographical information is obtained in three-dimensions (Wilkins et al 1993b). Fig. 3 shows a scanning tunnelling microscope image of a single molecule of porcine gut mucin that has been prepared using this method, the image clearly shows both the protein backbone and the carbohydrate side chains (Roberts et al 1995).

Direct chemical modification of either the substrate or the biological macromolecule allows uncoated materials to be directly imaged without the use of a metal coating. Within

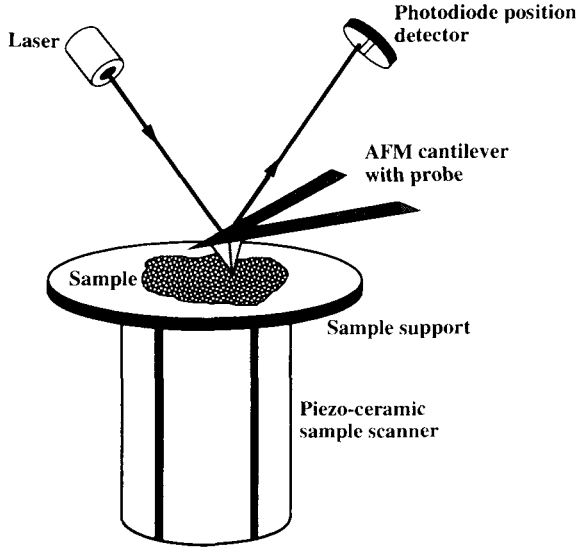

FIG. 2. A schematic diagram of an atomic force microscope. A sharp probe is brought into contact with a sample surface. The force of interaction between the two is monitored via the deflection of a reflected laser beam. An image of the sample surface is built up by scanning the sample under the probe whilst keeping the force of interaction constant.

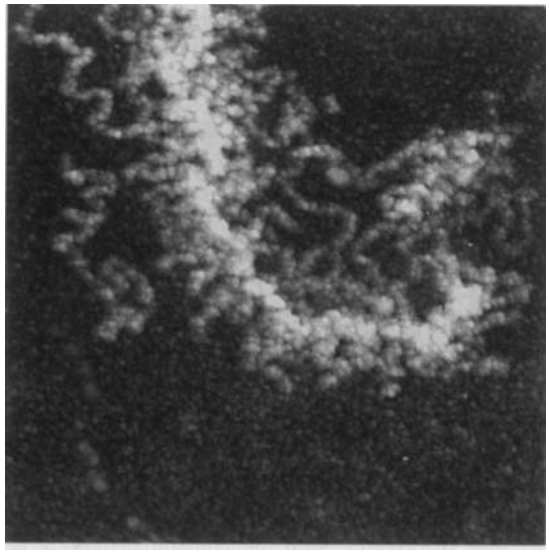

FIG. 3. A 360 × 360 nm scanning tunnelling microscope scan of an individual mucin molecule (black to white, 6.9 nm). The image clearly shows the protein backbone and carbohydrate side chains of the glycoprotein.

surface science, alkane thiols are known to spontaneously assemble on gold surfaces to produce a covalently-bound self-assembled monolayer (SAM). Through the use of alkane thiols with defined functional groups, such as mercaptoproprionic acid, the SAM will have carboxylate functionality which allows bio-molecules to be immobilized using conventional carbodiimide chemistry (Scheme 1)

$$\mathrm{Au + HSCH_2CH_2COOH}$$

$$\downarrow \quad \text{in } \mathrm{H_2O}$$

$$\mathrm{Au\text{-}SCH_2CH_2COOH}$$

$$\downarrow \quad \begin{matrix}\text{1. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide} \\ \text{2. protein}\end{matrix}$$

$$\mathrm{Au\text{-}SCH_2CH_2CONH\text{-}[protein]}$$

SCHEME 1. Preparation of SAM-immobilized protein.

(Leggett et al 1993a). Through the use of photolysis it is possible to pattern these surfaces to create multi-protein arrays of defined tertiary architecture.

Alternatively the macromolecule can be activated to bind the substrate. Treating protein with Traut's reagent directly produces proteins with free thiol groups. These thiols datively bind to gold, constructing biomolecular coated surfaces that are essentially identical to those produced utilizing the SAM method (Leggett et al 1993a). A more biochemical approach to molecular immobilization involves taking advantage of the high binding affinity that streptavidin has for biotin (Davies et al 1994a). Here biotinylated molecules may be bound to passively adsorbed streptavidin creating a robust three-dimensional molecular array (Fig. 4). This method of sample preparation has allowed the functionality of both passively and actively

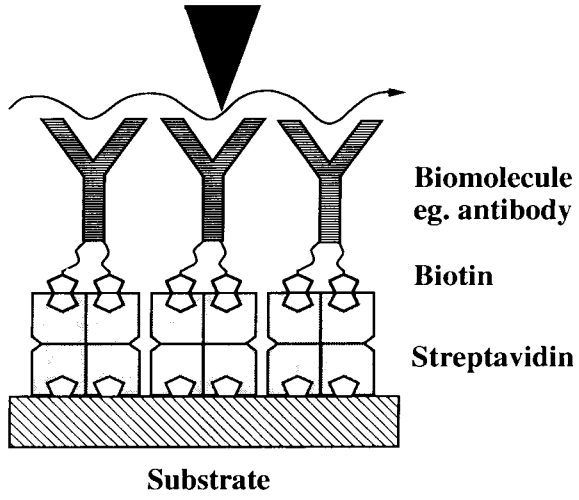

FIG. 4. Biotinylated molecules may be immobilized to surfaces coated with streptavidin. Such immobilization allows a probe microscope to image the biomolecules with minimal disruption.

immobilized antibodies to be compared. It has been clearly shown that antibodies that are passively adsorbed onto polystyrene surfaces (Fig. 5a) are less able to bind their antigen than are antibodies that are actively bound utilizing the streptavidin/biotin system (Fig. 5b) (Davies et al 1994b).

Image Validation

While the surfaces of the popular scanning probe microscopy substrates, highly oriented pyrolytic graphite (HOPG), ^{111}Au and mica, are flat at the nm level, many surface and sub-surface features exist which have similar topographical features to biological macromolecules. In the literature many of these artifactual features have previously been mistaken for proteins and nucleic acids. To fully utilize scanning probe microscopy data, robust validation strategies are required.

One of the milestones in the development of the biophysical applications of scanning probe microscopy has been the comparison of scanning probe data sets with those obtained from complementary biophysical techniques including electron microscopy and X-ray crystallography. Studies on a range of molecules including the carbohydrate xanthan (Wilkins et al 1993b) and Alzheimer's disease-related amyloid fibrils (Shivji 1995) indicate that the scanning probe microscopes are capable of recording topographical information on the macromolecules with similar molecular dimensions to that obtained by electron microscopy. As previously noted, unlike electron microscopy, the scanning probe data provides information in three dimensions. In addition, crystal structures of proteins, such as catalase, have been directly compared with scanning tunnelling microscopy images to validate the data sets (Fig. 6) (Williams et al 1991; Leggett et al 1993a). While the molecules investigated are anticipated to have similar molecular dimensions to structures from complementary techniques, the scanning probe data are expected to show larger dimensions; when imaging with a probe microscope the data obtained is a convolution of the shape of the probe and the surface of the substrate (Williams et al

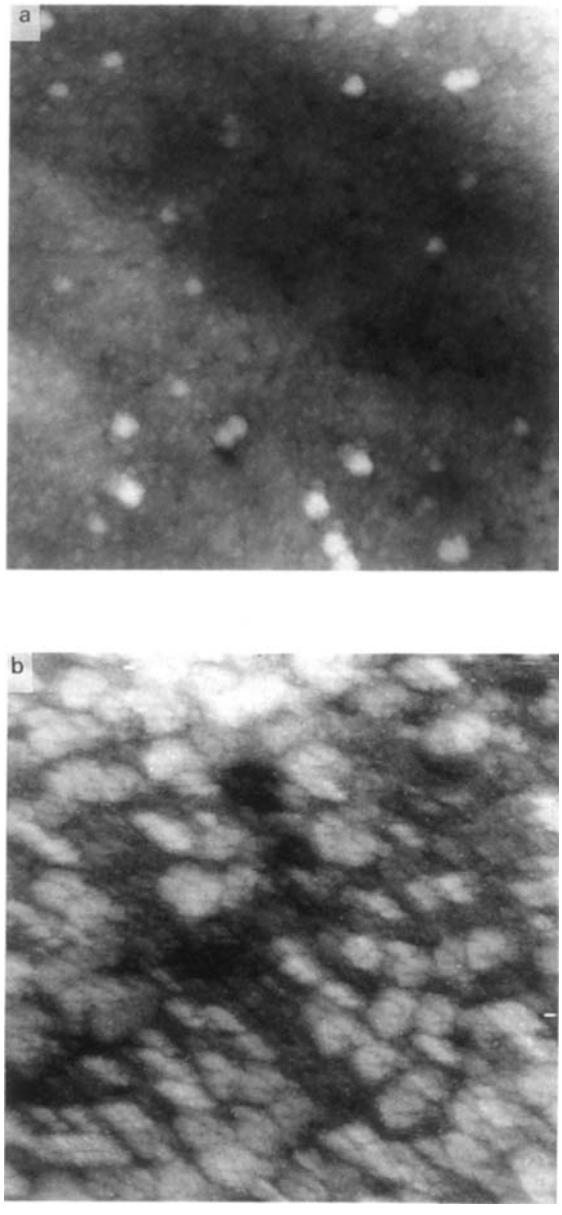

FIG. 5. Scanning tunnelling microscope topographs (250 × 250 nm) of microtitre well surfaces showing (a) ferritin molecules bound by passively adsorbed anti-ferritin antibody and (b) ferritin bound by a biotinylated anti-ferritin antibody linked to the well surface via streptavidin. The images display ferritin molecules randomly distributed on the substrate surfaces. The surface coverage of the ferritin is approximately 5% in the passively adsorbed antibody case and 60% in the biotinylated antibody streptavidin example.

1995). Indeed if the tip used was blunt relative to sharp features present on the substrate then the image obtained would be that of the topography of the probe. Through the analysis of the surface of the sample image it is now possible to calculate the dimensions of the bluntest possible probe that could have produced that image (Fig. 7). Importantly, once this theoretical probe has been calculated it may then be used to identify those areas of the image that may be tip-induced and those areas that may be real (Williams et al 1996). In addition, the calculated probe dimensions also reveal information concerning the compliance and physical properties of the substrate under investigation.

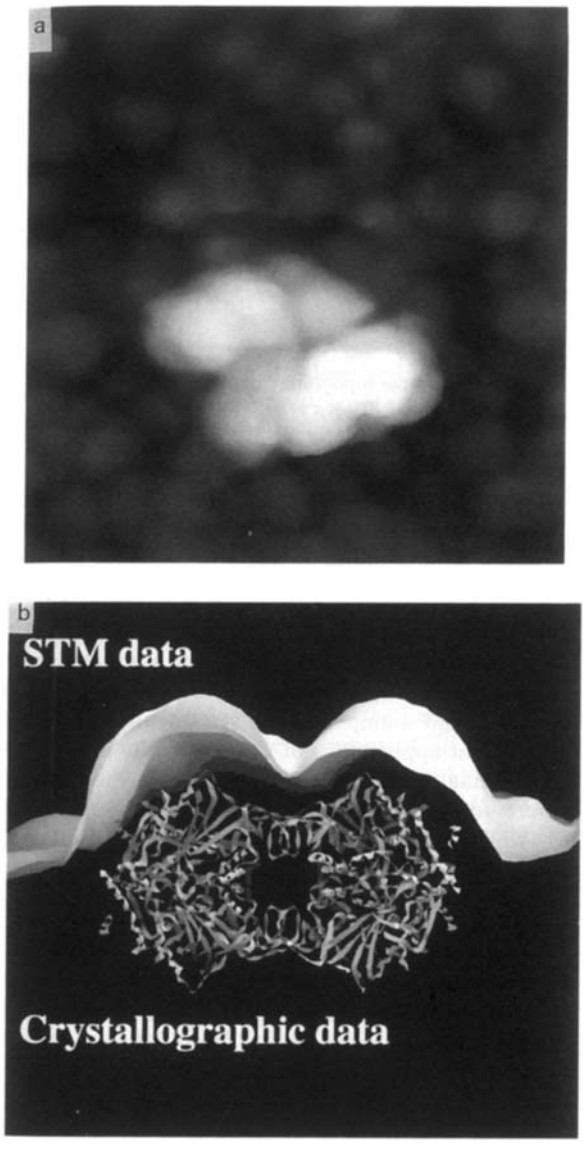

FIG. 6. a. A 15 × 15 nm scanning tunnelling microscope image of a single catalase molecule; b. a comparison of scanning tunnelling microscope and crystallographic catalase data. A ribbon model of the protein has been placed beneath a cross-section of the scanning tunnelling microscope data. There is a clear correlation in terms of dimensions and overall shape.

There are presently a number of algorithms that provide analysis of the contents of the images. When investigating samples with distinct quantized features, such as globular proteins, cluster analysis may be utilized to identify the number of molecules present and whether they are monomers, dimers or trimers. This method has been successfully used to identify the aggregation of the protein ferritin (Williams et al 1994) and recently to quantify the presence of IgG and IgM immunoglobulin molecules bound to polystyrene wells used for immunoassays (Roberts et al 1995a).

One method of indicating whether a feature observed on the substrate is a biological molecule is to allow the probe to come into contact with it and perturb it. This method of image validation has been utilized in the study of tumour-associated polymorphic epithelial mucin. Here the glycoprotein molecules were successfully manipulated on graphite by a scanning tunnelling microscope probe (Roberts et al 1992a).

With the probe close to the surface it is possible to electrochemically modify the substrate at the nm level. An example of such a reaction is shown in Scheme 2 where carbon, under ambient conditions, is converted to hydrogen and carbon monoxide through the application of voltage pulses utilizing the scanning tunnelling microscope (Roberts et al 1992b).

$$C_{(s)} + H_2O_{(l)} \xrightarrow[\text{Voltage pulse}]{} CO_{(g)} + H_{2(g)}$$

SCHEME 2. Modification of a carbon surface by a scanning tunnelling microscope.

As shown in Fig. 8, this reaction enables the scanning tunnelling microscope to lithographically etch lines and symbols on the surface of graphite with nm precision. It also allows practitioners to distinguish between biomolecules and sub-surface features. If a feature is cut-through with the scanning tunnelling microscope with no disturbance then it is probably an artifactual feature; real molecules are normally repulsed by the applied voltage. This lithography method has been extended to examine a wide range of inorganic substrates including gold and platinum (Roberts et al 1993b).

Modes of Imaging

A critical issue in the scanning tunnelling microscopy analysis of biomolecules is how the images of macromolecules are produced; conventional wisdom tells us that most proteins are insulators which should not be visible in the images. It has been found that water has a critical role in scanning tunnelling microscopy image formation, providing a means of current flow between the probe and the substrate. Images of the protein catalase show a marked increase in contrast as the protein molecules are hydrated (Leggett et al 1993b; Parker et al 1995).

In the case of the atomic force microscope, image contrast is normally achieved through van de Waals contact between the probe and the material under analysis. In this case the presence of a water layer on the surface of the material is problematic as capillary forces cause the probe to be forced into the surface thereby applying a higher force. The easiest way to circumvent this problem is to immerse the probe and cantilever into a solution thereby removing any capillary forces (Roberts et al 1993a). Advantageously this allows the imaging of samples in physiological solutions. To minimize sample sweeping, the atomic force microscope can also be operated in a modified imaging mode, such as the tapping mode; here the probe is tapped onto the sample at discrete points to minimize sample damage. The atomic force microscope may also operate in a number of so-called non-contact zero-force modes where the probe is oscillated above the sample to provide topographic information.

The ability of the atomic force microscope to image in aqueous environments allows it to be used to follow biochemical and chemical events in real time in solution.

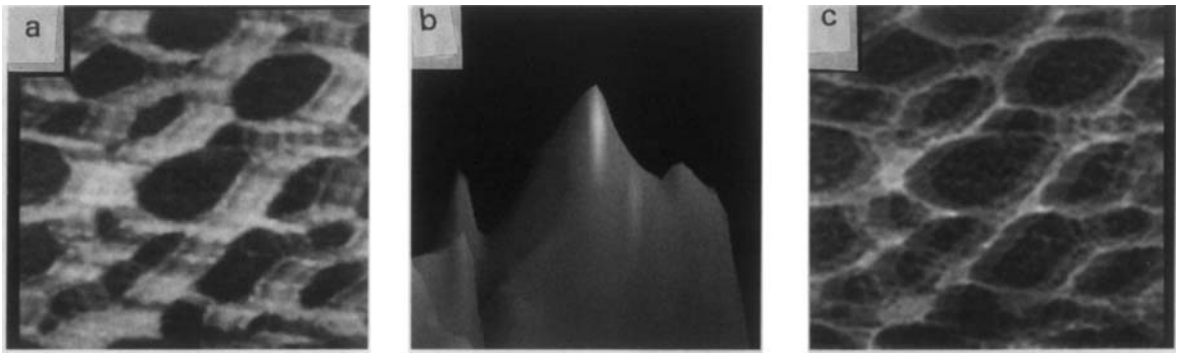

FIG. 7. All scanning probe microscopy image data results not only from the sample topography but also that of the probe. Using novel routines this probe information can largely be removed from the image. We demonstrate this here for the case of a multiple imaging probe. a. A scanning tunnelling microscope image of a polymer supra-molecular network-each part of the network appears to be duplicated a number of times; b. a calculation of the probe that could have formed the image in a-the probe has three principle asperities, and it is these that has given the overlay effect apparent in the image data; c. having calculated the probe topography this can then be removed from the image data, resulting in a truer representation of the actual sample.

Examples of such experiments include a number of studies of polymer degradation where heterogeneous events have been observed (Shakesheff et al 1994, 1995a), as well as an elegant example of protein release from polymeric materials that is shown in Fig. 9 (Shakesheff et al 1995b). Such experiments will inevitably be extended to the study of drug-receptor interactions, in solution, in real-time. The studies utilizing aqueous environments have very recently been extended through the construction of a combined atomic force microscope/surface plasmon resonance instrument. The surface plasmon resonance allows the continuous monitoring of the bulk substrate properties while the microscope provides detailed topographical information (Chen et al 1995).

In addition to the direct recording of three-dimensional data, the atomic force microscope is also capable of measuring forces at the nN level. As shown in Fig. 10, this is undertaken through the immobilization of pairs of biomolecules onto the probe and the substrate, respectively, and measuring the adhesion forces when they are being pulled apart. The method has recently been utilized to measure the forces of adhesion between complementary strands of DNA (Lee et al 1994a) as well as that between streptavidin and biotin (Lee et al 1994b; Moy et al 1994).

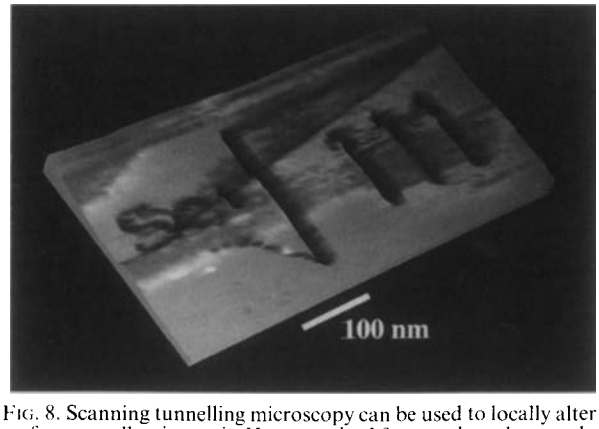

FIG. 8. Scanning tunnelling microscopy can be used to locally alter a surface as well as image it. Here nm-sized features have been etched into a graphite surface.

Conclusions

The strengths of the scanning probe microscopes in the biophysical arena are their ability to not only resolve features at the nm level but also to be able to physically interrogate and manipulate the system under investigation. Current applications of these versatile instruments, such as imaging samples in solution and the ability to measure forces of biomolecular adhesion, suggest that they will be essential tools in both pharmaceutical analysis and research. Combining the scanning probe microscopes with other analytical technologies will open untold new horizons.

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We should like to acknowledge the major contribution in these studies of our colleague Dr David Jackson who was

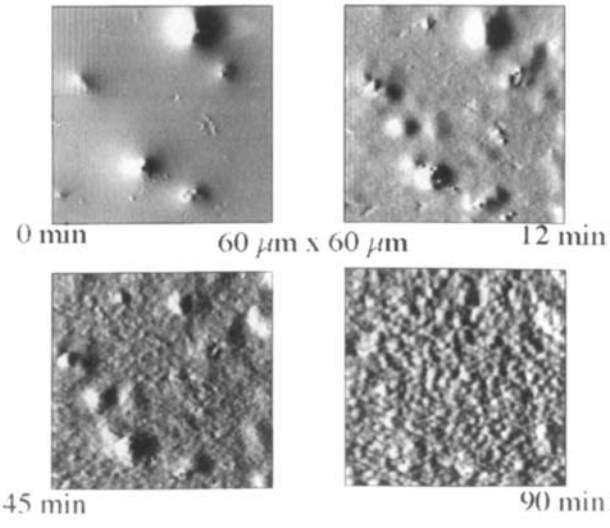

FIG. 9. A sequence of in-situ atomic force microscopy images showing the release of albumin particles from a poly (ortho ester) matrix in a pH 6 environment. As the bioerodable polymer degrades small albumin particles are exposed to the aqueous environment and are released from the polymer, eventually leaving only the rough polymer surface.

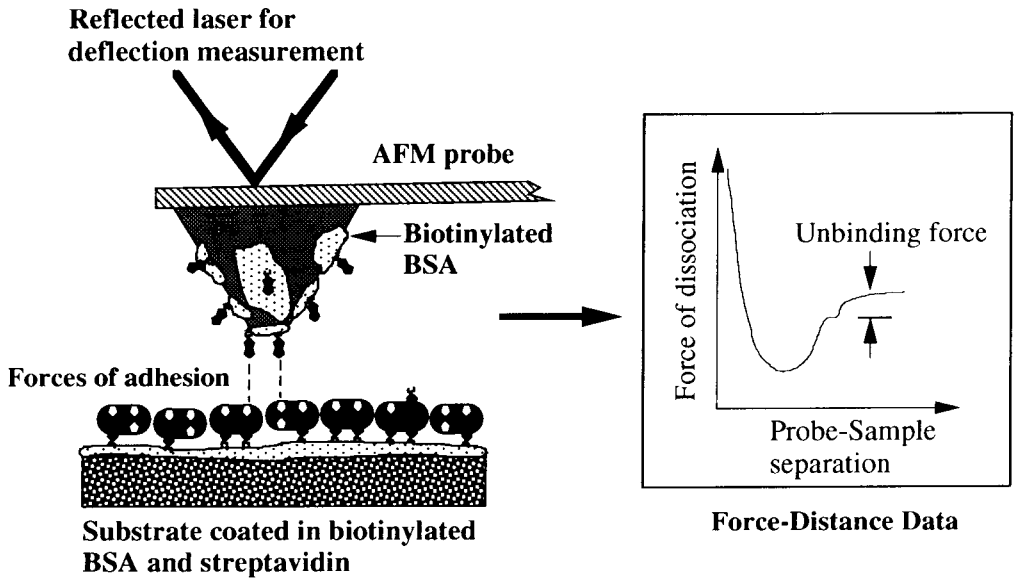

FIG. 10. Schematic diagram demonstrating the ability of the atomic force microscope to monitor forces of adhesion between biomolecules e.g. streptavidin and biotin.

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