Optical Chemical Imaging of Tobacco Mosaic Virus in Solution at 60-nm Resolution

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ABSTRACT Scanning near-field optical microscopy can provide images with a resolution less than the wavelength of light, and therefore ought in principle to be of great value in studies of biological structures. In this work we show how for the first time images have been obtained of tobacco mosaic virus particles at 60-nm resolution, combined with chemical imaging using monoclonal antibodies under in vitro conditions.

INTRODUCTION

Optical microscopy is widely used in the biosciences to probe molecular structure. With the use of fluorescent labels, both spatial and functional mapping can be performed. Optical microscopy is a well-established and low-cost method, but it is fundamentally limited in its lateral resolution by the diffraction limit. The ultimate limit is 250 nm for an instrument using visible light. Scanning electron microscopy overcomes this problem and offers a lateral resolution of 5–50 nm, but the technique suffers from requiring fixed specimens and from the possibility that dry preparation and gold coating may introduce artefacts. The resolution of transmission electron microscopy is ~10 nm, but again freeze fracture methods, required to study most surface structures, may introduce artefacts. Finally, spectroscopy and functional mapping are difficult with either form of electron microscopy. Scanning near-field optical microscopy (SNOM) overcomes the diffraction limit of optical microscopy and allows optical images with a resolution of 10–100 nm to be obtained (Dürig et al., 1986). Briefly, the technique is based on the use of an "optical stethoscope," which places a small near-field light source, typically a tapered optical fiber, a very short distance from the sample (less than the wavelength of light). Images are obtained by scanning the light source over the sample, and resolution is limited only by the diameter of the light source, typically 10–100 nm. All mechanisms used to obtain contrast in conventional microscopy have been successfully applied to SNOM (for example, imaging by light scattering, refractive index, polarization, and fluorescence) (Pohl and Courjon, 1993).

One major hurdle in the application of SNOM is the ability to image in liquids. Control of the distance between sample and near-field probe is more difficult because of damping of oscillations due both to the presence of the liquid and, in buffer, to electrostatic interactions between the ions on the surface of the near-field probe and ions in solution. Furthermore, in imaging biological material there is an additional difficulty in distance control due to the softness of the material compared with the probe. In the last year several groups, including our own, have published images produced in liquids with SNOMs with different distance control mechanisms (Keller et al., 1997; Moyer and Kammer, 1996; Muramatsu et al., 1995). This development of imaging in liquids opens up the possibility of in vitro chemical microscopy at nanometer resolution.

In this paper we demonstrate 60-nm resolution of tobacco mosaic virus (TMV) in buffer, and chemical imaging of this virus using monoclonal antibodies. Chemical imaging is performed by using molecules that have a specific interaction with certain chemical functionalities in the sample. Thus by imaging the spatial distribution of these molecules on the sample, it is possible to directly determine the spatial distribution of these chemical functionalities. In this paper we present the first example, to our knowledge, of chemical imaging of a biological sample in solution, at this resolution, and hence another step in developing SNOM for in vitro imaging.

MATERIALS AND METHODS

Imaging technique

Details of the developed instrumentation (SNOM) have been published previously (Keller et al., 1997); therefore only the key features will be mentioned here. The unusual feature of our instrument is that the sample is mounted on an atomic force microscope cantilever (L.O.T.-Oriel, Surrey, England), which vibrates at its resonance frequency. In this combined AFM-SNOM, an inverted AC-mode configuration is used, in which the AFM cantilever is driven by exciting acoustic modes of the liquid cell (Putman et al., 1994). As the near-field probe approaches a direction normal to the sample, the amplitude of vibration is damped because of the attractive coupling between the cantilever and probe. The interaction between the cantilever and probe is not always attractive, depending on the pH of the solution (Arai et al., 1996). This interaction is used to control the sample-probe distance during scans. This method of distance control contrasts with the more commonly used shear-force control mechanism for SNOM. The light emitted from the probe and scattered from the sample is collected by using a multimode fiber (3M, Poole, England) held in close proximity to the sample and probe and then is passed to a silicon avalanche

Received for publication 21 August 1997 and in final form 13 December 1997.

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0006-3495/98/04/2076/04 $2.00

photodiode (EG&G, Vaudreuil, Québec) for photon counting. The near-field probe consists of a tapered single-mode fiber (Fibercore, Hampshire, England), with its sides coated in aluminum (~150 nm), down which 5 mW of light from a YLF laser at 527 nm is launched.

Sample preparation

To establish the usefulness of liquid SNOM for imaging of biological structures, we decided to study the TMV (Namba and Stubbs, 1986). The TMV is one of the best studied viruses; its structure has been determined with atomic resolution. TMV is a rod-shaped particle 300 nm long and 18 nm wide. It consists of 2130 identical protein subunits arranged as a helix with a RNA molecule in the center. In addition, the antigenic structure of TMV has been studied thoroughly by van Regenmortel and co-workers (Saunal and van Regenmortel, 1995). They studied monoclonal antibodies raised against the TMV coat protein and discovered 10 monoclonal antibodies that bind to the 5′ end of the virus. These antibodies have been imaged by immunoelectromicroscopy (Dore et al., 1988). Because of the wealth of structural information about TMV and the availability of metatope antibodies (Molecular Biology, Strasbourg, France), we selected this as a test system for imaging at nanometer resolution.

The principal experimental challenge in these experiments has been sample preparation. We require the sample (TMV obtained from the Laboratory of Molecular Biology, Cambridge, England) to be deposited as single isolated virus particles with sufficient adherence that the particles are not displaced by the tip surface forces that are part of the process of imaging. It is well known that virus particles aggregate naturally on surfaces and in solution. Bovine serum albumin (BSA) binds to TMV at pH ~7, and hence it is particularly suited as a cross-linking protein between the virus and cantilever. In addition, BSA is a known colloid stabilizer and hence was used to prevent aggregation of the TMV (Wadu-Mesthrige et al., 1996). The following method was used to immobilize a single TMV on a modified substrate surface for imaging in liquid (Fig. 1). The TMV was attached to the cantilever by labeling the surface with BSA (Sigma, Poole, England). Cantilevers were cleaned and degreased and then coated with ~30 nm gold by vacuum evaporation at 10^{-6} mbar. The freshly coated gold surfaces were irradiated with ultraviolet light (Ar-ion laser, 244 nm, 10 mW) for 45 min and incubated in a 50-μl drop of BSA (c_{BSA} = 21 mg/ml), previously resuspended in phosphate saline buffer (PBS) (pH 7.4) and heat inactivated (50°C), for 24 h at a temperature of 35°C in a humidified camber. After the adsorption of the BSA, the cantilever was rinsed three times with PBS and dipped in a fresh 20% solution of glutaraldehyde (Sigma) for ~30 s to stabilize the protein film. The modified cantilever was washed with PBS. Attachment of TMV was carried out just before a SNOM experiment by incubating the cantilever in a 40-μl drop of TMV (c_{TMV} = 200 μg/ml) at room temperature for 5 min. BSA in high concentration was added to the TMV solution before adsorption on the modified cantilever surface. The particle ratio of BSA:TMV was 1800:1.

The image shown in Fig. 2 was obtained by a modified method of sample preparation to increase the adhesion of the TMV to the cantilever. The nanosphere labeling of the antibodies was carried out by the sandwich method (Fig. 2 *d*). In this process the TMV is first labeled with a metatope monoclonal antibody that binds only to the 5′ end. A biotin-labeled polyclonal antibody was attached to this monoclonal antibody and was then bound by an avidin-labeled nanosphere of 30-nm diameter. The method used was as follows. Twenty microliters of monoclonal antibody was added to 3 μl TMV solution (21 mg/ml) for 20 min at room temperature. To this solution was added 60 μl of a biotin-labeled polyclonal antibody (Biotin-XX-goat-anti mouse IgG (H+L), 2 mg/ml; Molecular Probes, Leiden, the Netherlands) for 20 min at 19°C. Nanospheres were attached by the addition of 5 μl of 30-nm-diameter avidin-labeled latex spheres (Molecular Probes) to 40 μl of the previous solution for 60 min at room temperature. Aggregation of the labeled TMV (TMV labeled with nanospheres by use of the sandwich method) was controlled by the addition of 10 μl of labeled TMV to 40 μl of BSA solution (9 mg/ml). The solution was left overnight at room temperature. The final concentration was 135 μg/ml TMV. A 40-μl drop of the nanosphere-labeled TMV (135 μg/ml) solution was placed on a BSA-biotin-labeled cantilever for 30 min at room temperature. The labeled TMV is attached to the substrate by two mechanisms. The first mechanism is the physisorption of the TMV to the BSA-biotin functionalized substrate, and the second mechanism is the chemisorption of the avidin-labeled nanospheres (attached to the TMV) to the biotin on the cantilever via a biotin-avidin bond. The surface treatment of the cantilever with BSA-biotin was carried out in exactly the same way as described above for the BSA labeling of the cantilever surface.

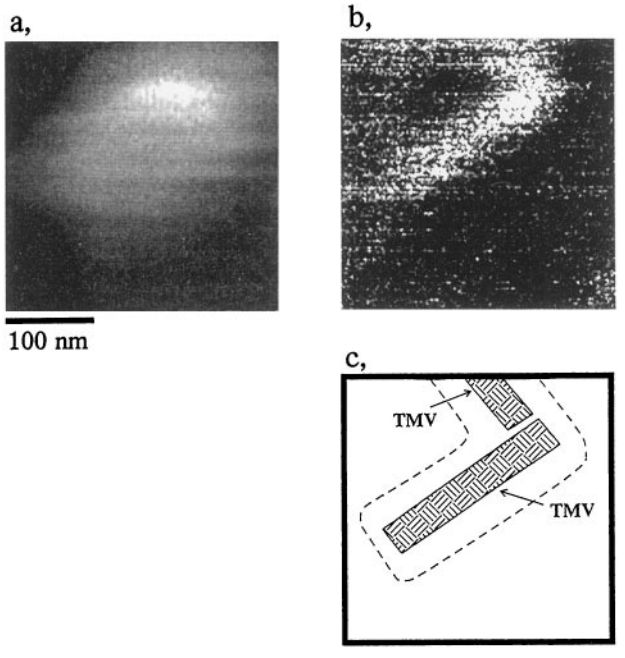

FIGURE 1 Combined AFM/SNOM image of tobacco mosaic virus on a BSA-labeled cantilever in PBS buffer. (*a*) Topographic image. (*b*) Optical image collected with 80 nm probe. (*c*) Interpretation of optical image. Imaging parameters: scan size 320 nm × 320 nm, input power 6 mW, integration time 22 ms/pixel, count rate 500-5500 Hz

RESULTS AND DISCUSSION

Fig. 1 *b* shows an optical image of TMV deposited in the manner described above collected in PBS. In the absence of BSA, aggregation of the TMV occurs, resulting in clusters. This gives images (not shown) that display a rippled surface, which is expected for an array of particles that aggregate end to end and side to side. Even in the presence of BSA, we still observe end-to-end interaction between the TMV rods. In the equivalent topographic image (Fig. 1 *a*), the resolution is 200 nm, because as shown in Fig. 1 *c*, this is determined by both the diameter of the fiber at the taper and the thickness of the coating. In the optical image (Fig. 1 *b*) the resolution is determined by only the diameter of the fiber and is 80 nm in this case. We measure the correct length of the TMV to be 300 nm, but the width is determined by our optical resolution of 80 nm, as explained in Fig. 1 *c*. The dashed line in Fig. 1 *c* represents the convolution between sample and probe (aperture size). There is excellent correspondence between the optical and topographic images.

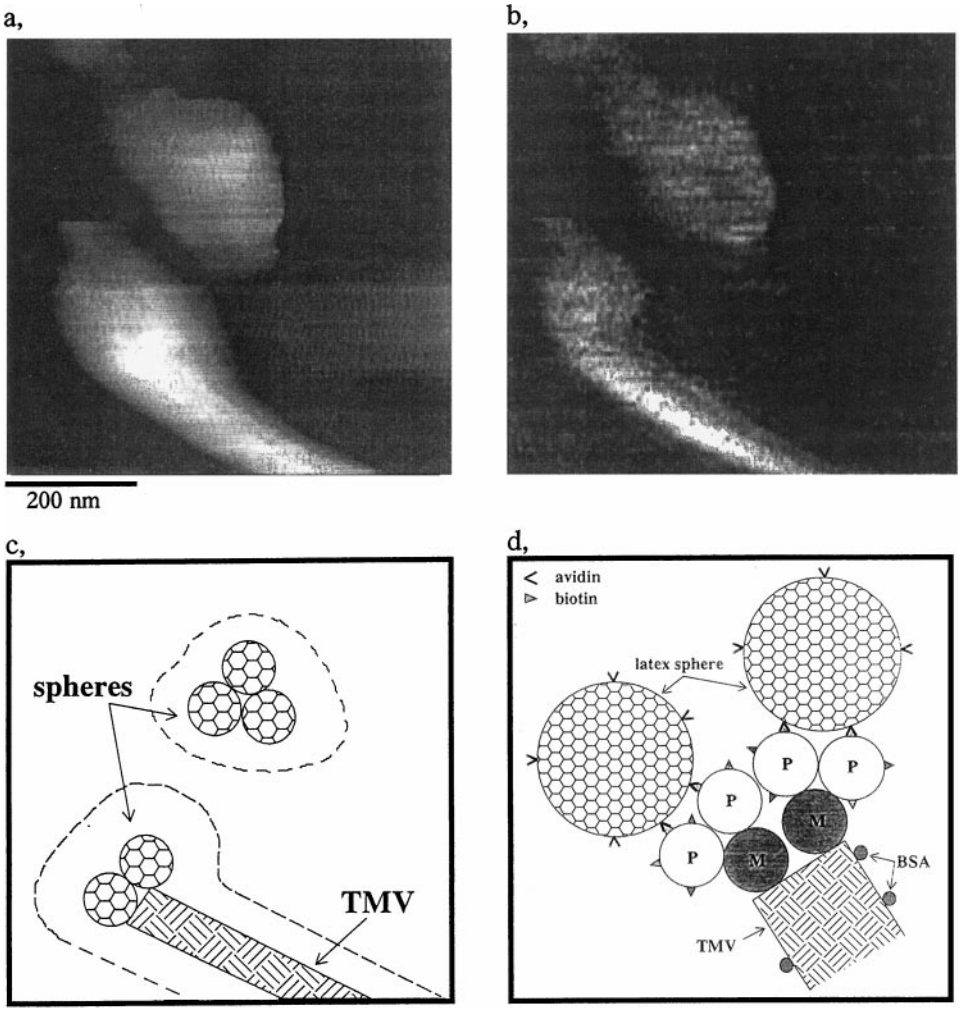

FIGURE 2 AFM/SNOM images of TMV labeled at the 5′ position by a monoclonal antibody and deposited on the cantilever surface via the sandwich method. (*a*) Topographic image. (*b*) Optical image collected with 60-nm probe. (*c*) Interpretation of optical image. The dotted line shows the outline resulting from the dimensions of TMV convoluted with the finite probe size. (*d*) Schematic diagram of the labeling procedure showing TMV, nanospheres, and monoclonal (M) and polyclonal (P) antibodies drawn to scale. Imaging parameters: scan size 700 nm × 700 nm, input power 12 mW, integration time 22 ms/pixel, count rate 4,500–27,000 Hz

A resolution of 80 nm is insufficient to permit the direct imaging of the position of antibodies on the surface of a virus particle. However, the position of antibodies can be found by mechanical amplification with the sandwich method as described above. The results of this process are shown in Fig. 2.

In the center of the optical image (Fig. 2 *b*) is a circular feature that, although it is 120 nm in diameter, probably contains an arrangement of three nanospheres, the apparent size being a result of the 60-nm aperture size. These nanospheres appear in the image a by-product of the immobilization technique used to attach the TMV. This is because nanospheres that are not attached to TMV during the labeling procedure can be bound to the surface over the biotin-avidin bond.

Of much more interest is the feature obtained in the lower half of the optical image (Fig. 2 *b*). The feature is a rodlike structure partially connected to a cluster at the end of the rod. The measured length of the rod in the optical domain is 400 nm, indicating that end-to-end aggregation has taken place, because the normal length of a single TMV is ~300 nm as shown in Fig. 1. The width of the TMV is limited by the optical resolution of 60 nm, as explained in Fig. 2 *c*. The dotted line in Fig. 2 *c* again represents the convolution between probe and sample in the optical domain. The cluster attached at the end of the TMV consists of nanospheres. The diameter of the cluster of nanospheres at the end of the TMV is 120 nm. The diameter of the two nanospheres would appear in the image to be 120 nm (60 nm per nanosphere), because of the optical resolution, so we assign the cluster to two spheres. Furthermore, the nanosheres at the end of the TMV indicate the 5′ end and therefore distinguish this end from the other end of the TMV. It should be mentioned that the other end of the TMV is bound to another TMV through end-to-end aggregation. However, only the 5′ end of the TMV will bind to the monoclonal antibodies and thus can be mechanically amplified by the sandwich method. We have therefore imaged the TMV at 60-nm resolution in buffer and chemically imaged the 5′ end of the virus by using monoclonal antibodies and 30-nm nanospheres.

The topographic image (Fig. 2 *a*) shows excellent correspondence with the optical image, but has a poorer resolution, because of the coating on the fiber. The topographic image was obtained simultaneously with the optical image. As shown in Figs. 1 and 2, the resolution in the topographic

and optical domains depends on the structure of the probe. The optical resolution (10–100 nm) depends on the fiber diameter. The topographic resolution is determined by the fiber diameter plus the metal coating around the tapered region of the tip, and will always therefore be poorer than the optical resolution. The metal film is needed to prevent light leakage from the tapered region of the tip and thus to produce a point light source.

CONCLUSION

The images here clearly show that chemical imaging of viruses, bacteria, and cells is possible in vitro by the use of antibodies coupled with labeling by nanospheres at 60-nm resolution. This should have widespread application in molecular biology and immunology. At present there are two principal experimental limitations, distance control mechanism and sample preparation. In practice it is difficult and time consuming to find the optimum conditions that will lead to isolated entities on a clean, smooth substrate. Only experience will make this process more efficient. Distance control is very important, because if the probe goes out of control and collides with the surface, it is invariably broken in these experiments. There are a number of ways in which this problem might be solved. Among the most promising and applicable in buffer solution is electrochemical control of the potential between the probe and surface, so as to null the tip-probe interactions. Finally, planned improvements in the instrumentation available should make it possible to perform experiments at 20-nm resolution in liquids. This will make it possible to follow dynamical processes in vitro, such as viral entry into cells, T cell activation, or bacteriacides acting on bacteria. We therefore believe SNOM imaging will become a powerful tool for probing biological structures and dynamics in the next few years.

We are grateful to Dr. Jo Butler and Dr. Brad Amos at the Laboratory of Molecular Biology, Cambridge, for invaluable advice and discussions and for providing the TMV used in these experiments. We thank Prof. van Regenmortel for generously providing the antibodies.

We are grateful to the EPSRC for a Ph.D. studentship and to Unilever Research for support of this project, particularly Dr. Peter Doyle and Dr. Phil Cummins. We also are grateful to the Newton Trust for funding to purchase a fiber puller.

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