Preparation of Isolated Biomolecules for SFM Observations: T4 Bacteriophage as a Test Sample

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ABSTRACT The T4 bacteriophage has been used to investigate protocols for the preparation of samples for scanning force microscopy in air, in order to obtaining reproducible images. The resolution of images and the distribution of bacteriophages on the substrate depends on the buffer type, its concentration, the surface treatment of substrate, and the method of deposition. The best imaging conditions for the phages require dilution in a volatile buffer at low ionic strength and adsorption onto hydrophilic surfaces. When imaging with the scanning force microscopy the quality of the images is influenced by the vertical and lateral forces applied on the sample and by the tip geometry.

INTRODUCTION

The development of the scanning tunneling and scanning force microscopy (STM and SFM)[1] has opened new opportunities in the study of the structure of organic and biological molecules. These techniques give information on the surface profile of molecules and are complementary to electron microscopy (EM) images, which give electron density information. Scanning probe microscopes (SPM) can observe biomolecules in the nanometer range either in air or under liquid, allowing such high resolution imaging to take place in more physiological conditions than those used previously (Guckenberger et al., 1988; Haeberle et al., 1992; Jahanmir et al., 1992).

SPM observations of biological molecules reported in the literature can be divided in two types. First, the observation of ordered monolayers with a periodic or crystalline structure, such as bacterial membranes, some proteins, and organic monolayers (Guckenberger et al., 1989; Apell et al., 1992; Fuchs, 1992). The stability of the molecular network often allows a higher resolution than with isolated molecules. Second, individual molecules have been imaged. This has been much more difficult, because often the molecules do not form stable interactions with the surface and can be displaced or damaged by the tip during scanning (Nawaz et al., 1992). In spite of this, since the pioneering observations of isolated viruses (Baro et al., 1985) many images of isolated molecules have been published, especially of nucleic acids (Arscott and Bloomfield, 1990; Thundat et al., 1992a, 1992b).

In the field of SPM imaging of biomolecules, most of the effort to date has been to optimize the scan parameters in order to reduce the damage done to the molecule (Engel, 1991; Nawaz et al., 1992). However, the central problem which has arisen is the reproducibility of the sample preparation. This difficulty has meant that most of publications show only a single image with few molecules rather than a field of molecules or a montage, a standard practice in EM (Coombs and Eiserling, 1977). Little work has been reported on the preparation of biological samples for SPM studies. Some investigations have been undertaken specifically to image DNA molecules (Allison et al., 1992; Thundat et al., 1992a, 1992b), but the same conditions are not suitable for all biomolecules.

We have used T4 bacteriophage as a test system to optimize the conditions for SPM studies of biomolecules, because its structure has been intensively described and contains a wide range of clearly discernible structural features of different sizes and, finally, it is relatively easy to purify (Mathews et al., 1983). T4 consists of a head, containing its DNA, a rod-shaped tail sheath consisting of 24 annuli with a pitch spacing of 4.1 nm and which terminates in a basal plate that has six short spikes and six long fibers (Fig. 1,

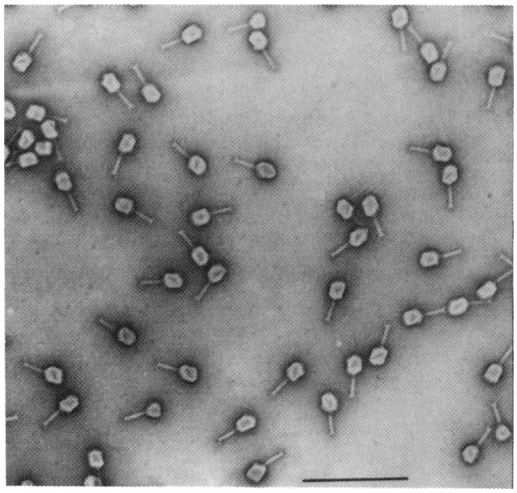

FIGURE 1 TEM image of T4 (10^{10} particles/ml) diluted in 200 mM ammonium acetate, adsorbed on glow-discharged carbon-coated grid and stained with 2% uranyl acetate. The scale bar corresponds to 0.5 μm.

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[1]*Abbreviations used:* SPM, scanning probe microscopy; SFM, scanning force microscopy; STM, scanning tunneling microscopy; EM, electron microscopy; TEM, transmission electron microscopy; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HOPG, highly oriented pyrolytic graphite; PMMA, polymethylmethacrylate.

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Table 1). To date, studies of this bacteriophage by STM have concentrated on a mutant form known as polyhead (Steven et al., 1976; Stemmer et al., 1989). This mutation causes an aberrant extended tubular structure of the head, and these particles form paracrystalline arrays. The wild type bacteriophages have been imaged by SFM only once as individual particles (Kolbe et al., 1992). A related phage, the slightly smaller bacteriophage T7, has been observed by STM (Garcia et al., 1989; Keller et al., 1990).

Since T4 shows a wide range of dimensions in its structural details, it allows us to test the imaging capabilities of the SFM over two orders of magnitude. This enables us to verify the influence of the sample preparation methods on the resolution of the images and thus to develop a method which gives reproducible SFM images of biomolecules. We have systematically investigated the parameters involved in adsorption of the phages. Moreover, to facilitate the interpretation of SPM images and avoid possible artefacts (Clemmer and Beebe, 1991) it is important to carry out parallel observations using well established techniques, like transmission electron microscopy (TEM, Arakawa et al. (1992)). We have made parallel observations with TEM whenever possible.

MATERIALS AND METHODS

Preparation of T4 bacteriophages

Our first SFM observations used bacteriophages suspended in M9 bacterial growth medium and showed the importance of purification. Consequently, all T4 were purified by CsCl gradient centrifugation (Coombs and Eiserling, 1977), and dialyzed against 200 mM ammonium acetate buffer at 4°C overnight. The final concentration of the stock solution of T4 is 10^{12} particles/ml, and a 1:100 dilution was used in all experiments.

Surface modification

In order to increase the hydrophilicity of the substrate we used glow discharge (Gombotz and Hoffman, 1987). This consists of exposing the surface of the substrate to a gas discharge obtained by applying a potential difference of 800 V between two electrodes. The air pressure was 10^{-2} mbar and a current of 2–6 mA was applied for 20 s.

TEM

The TEM images were obtained on a Zeiss EM109 and were used to characterize T4 bacteriophages before the SFM investigations. Ten μl of diluted bacteriophages (10^{10} particles/ml) in 200 mM ammonium acetate buffer was adsorbed on glow-discharged, carbon-coated grid and stained with 2% uranyl acetate (Balzers Union, FL).

TABLE 1 Comparison of T4 dimensions measured by TEM and SFM

	TEM	SFM
	nm	
Entire virus length	200	260
Head diameter	80	130
Tail diameter	20	50
Fibers diameter	2	20

SFM

Instruments and imaging

A commercial SFM (Park Scientific Instruments, Mountain View, CA) with a maximum scan width of 125 μm was used. Imaging at high resolution was performed with a 10-μm scan size. Microfabricated Si_3N_4 cantilevers with integrated pyramidal tips and spring constants of 0.21, 0.032, and 0.064 N/m were used. The tip with 0.064 N/m gave best results for the imaging of the T4 phage, with a typical loading force of 10^{-8} N in air. The scan frequency was typically 2 Hz or 0.5 Hz for higher resolution imaging.

No image processing was carried out except for linear background subtraction.

Force calibration

The calibration of the force applied on the sample has been carried out on T4 adsorbed on titanium film. The cantilever was calibrated by withdrawing it using the piezoelectric driver and measuring the displacement required to retract the tip from the surface. From the spring constant of the lever (0.064 N/m) we can calculate the applied force of 1×10^{-8} N/m.

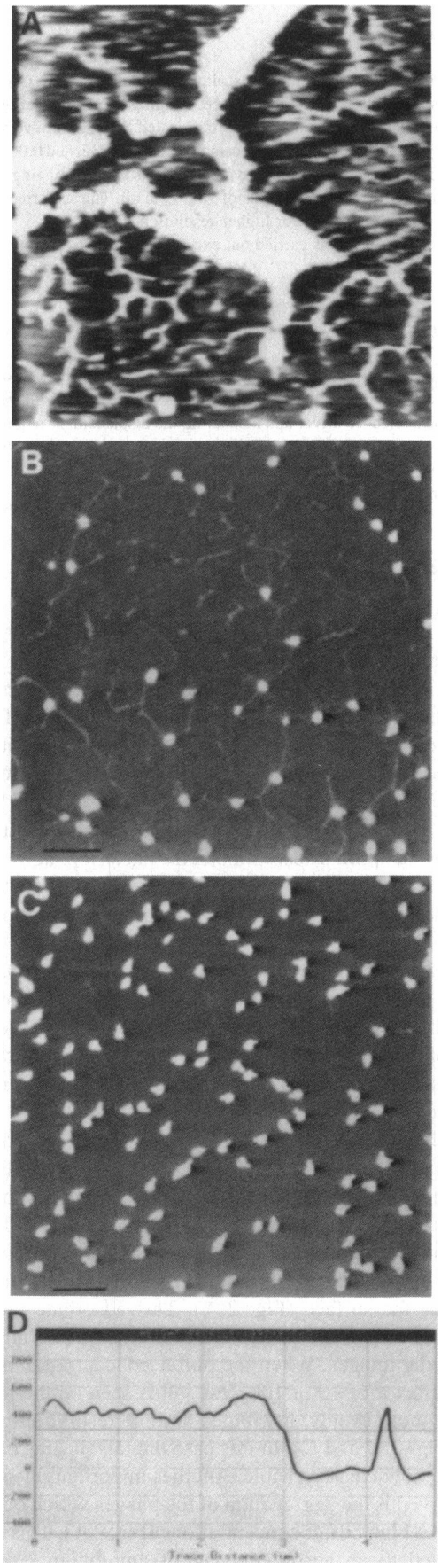
All the images are taken in repulsive contact mode.

RESULTS AND DISCUSSION

Effect of buffer and ionic strength

Preliminary observations of T4 by SFM used phages suspended in *Escherichia coli* growth media such as M9 minimal medium. The images displayed a large amount of undesired background components which reduce the quality of the images. These are due to the salts and sugars in the growth media, therefore, in this study we have used purified bacteriophages. First, we wanted to find an appropriate buffer which allowed optimal adsorption of the bacteriophages. Parallel TEM and SFM experiments have shown that nonvolatile buffers, like Hepes or Tris-HCl, influence the adsorption of the molecules by precipitating and crystallizing on the surface even when present at low concentration. They also favor aggregation of the viruses on the surface (data not shown). On the other hand, volatile buffers like ammonium acetate have the advantage that the majority of the buffer evaporates, leaving less salt on the surface. Consequently, this buffer has been chosen for all subsequent sample dilutions.

To test the influence of the ionic strength on adsorption and distribution of the molecules, T4 solutions at three different buffer concentrations (200, 20, and 2 mM ammonium acetate) have been adsorbed on freshly cleaved mica and air-dried at room temperature. Deposit of salts are clearly seen at the highest buffer concentration as long filaments meandering over the surface (Fig. 2 *A*). This adsorbate moves under the tip during the scan, adheres to it and lowering the quality of the images. When the buffer concentration is diluted by a factor of 10 or 100, less salt is seen on the mica, the T4 are more evenly distributed and the resolution is increased (Fig. 2, *B* and *C*). In this case the tail sheaths of the phages are now clearly visible. Another important phenomenon observed is the aggregation of the phages which occurs especially at high buffer concentrations (Fig. 2 *A*). This may be attributed to an adsorbed layer of ammonium acetate which modifies the polarity and the adsorption properties of the original cleaved surface leading to a close packed ad-



sorption of phages. The profile of this image measured between the large aggregate and an isolated virus, shows that the aggregate height corresponds to one layer of T4 (Fig. 2 *D*). The resolution on the few isolated T4 is very low due to an embedding of the particles in the salt. Such buffer artefacts at the same ionic strength, are not observed on TEM images (Fig. 1), because this technique is sensitive to a combination of the sample thickness and the electron density. These experiments show that volatile buffer must be used at low concentration to achieve a good spreading of the bacteriophages in order to avoid salt artefacts and also to improve the resolution of images.

Substrates used to image T4 phages with SFM

The surface used as a substrate for adsorption of T4 and the SFM observations needs to be as flat as possible, in other words to have a roughness significantly smaller than the dimensions of the bacteriophage. We observed T4 on a wide range of surfaces (Table 2) and the results show differences in their adsorption behavior. In general, on surfaces such as HOPG and Silicon wafer, they appear aggregated and the majority are displaced during the scan. Such problems have already been described for HOPG (Clemmer and Beebe, 1992; Amrein and Gross, 1992). Our results demonstrate that similar phenomena are seen for a wide range of hydrophobic surfaces. Thus, in order to improve the adsorption properties of the various surfaces, they have all been treated by glow discharge.

Surface modification

Glow discharge is widely used by electron microscopists to clean the surface of the substrate and thereby facilitate the spreading of the molecules on carbon-coated grids. We have shown by Auger spectroscopy on titanium and vanadium thin films (Taborelli et al., 1993), that this treatment removes the adsorbed hydrocarbons and oxidizes the surface making it more hydrophilic. Images on bare substrates like HOPG or silicon wafer after glow discharge treatment show that the surface structure is modified by the presence of adsorbates which increase the roughness of the surfaces but do not prevent identification of bacteriophages. To quantify the hydrophilicity we have measured the contact angle between a 1-μl droplet of distilled water and the surface before and after treatment by glow discharge. We observed a better spreading of the droplet and a decrease in the contact angle is clearly visible after glow discharge (Fig. 3).

FIGURE 2 SFM images of T4 adsorbed on freshly cleaved mica. The vertical scale (z) has been compressed to give more information about the surface adsorbates, which renders the phages oversaturated white. The buffer concentration is (*A*) 200 mM, (*B*) 20 mM, (*C*) 2 mM ammonium acetate for the three samples. (*D*) The profile of *A*, between the aggregate and the isolated T4 on the right side, shows that only one layer of viruses are packed together. The scale bar corresponds to 1 μm, and the vertical black to white scale is 12 nm.

TABLE 2 Surfaces used as substrate for T4 adsorption and observation by SFM

	rms roughness, 1 μm × μm area	Composition	Structure
	nm		
Mica	0.10	$KA1_2(Si_3A1)O_{10}(OH,F)_2$	Crystalline layer
Silicon wafer	0.15	Si (100) + SiO_2	Amorphous surface
PMMA	0.15	$(CH_2\text{-}CH_3\text{-}COOCH_3)_n$	Organic 3-dimensional crystal
HOPG	<0.10	C	Crystalline layer
WSe_2	<0.10	WSe_2	Crystalline layer
Titanium/mica	0.70	Ti + TiO_2	Thin film (25 nm)
Vanadium/mica	0.60	V + V_2O_3/VO_2	Thin film (50 mn)
Carbon/glass	0.25	C	Thin film (5 nm)

Titanium and vanadium thin films are evaporated on freshly cleaved mica as previously described (22). The carbon is evaporated on glass coverslip in a freeze etching system (Balzers, BAF400) at room temperature; the thickness is monitored by comparison with a quartz crystal.

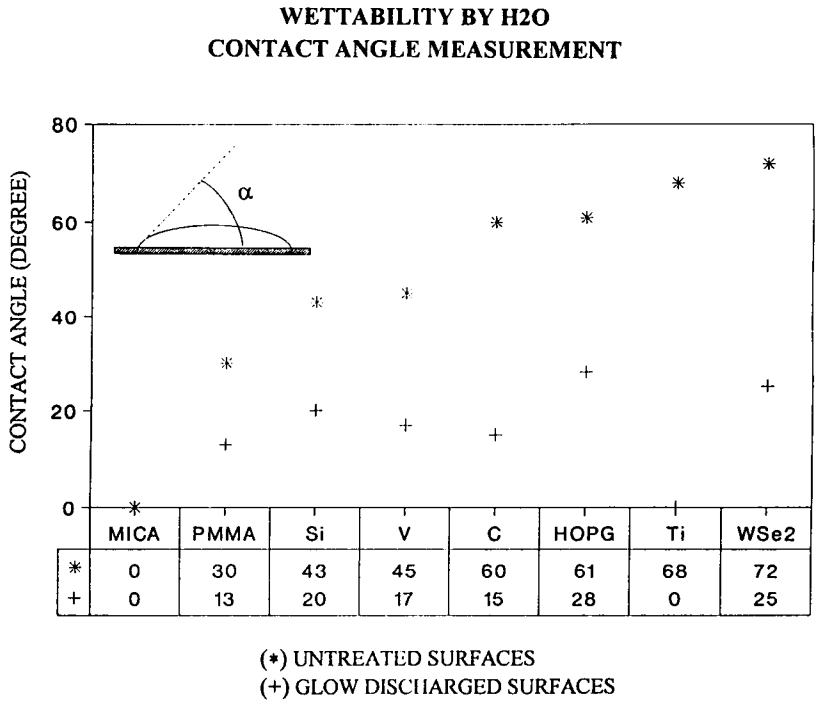


FIGURE 3 Plots of the contact angle measurement, by optical microscopy, of a drop of water (1 μl) on the various substrate surfaces presented on Table 2, before (*) and after (+) the glow discharge treatment (800 V; 5 mA; 3×10^{-2} mbar; 20 s).

The untreated surfaces are classified by increasing order of wettability. According to the spreading of T4 on these surfaces, untreated Mica is the most hydrophilic substrate, where only stable and isolated particles are observed (Fig. 1 *C*). PMMA shows intermediate hydrophilicity, and a combination of isolated particles and aggregates of stable phages adsorbed on the surface are seen (Fig. 4 *A*). Finally silicon wafers, HOPG and WSe_2 are considered as hydrophobic, and the images display only aggregates of unstable phages on these untreated surfaces. On Fig. 4 *B* we can see a SFM image of T4 adsorbed on silicon wafer which has not been treated by glow discharge showing aggregates which move during the scan (Fig. 4 *B*).

In contrast, SFM observations using PMMA and silicon wafers substrates treated by glow discharge gave stable images of isolated molecules, observed in a reproducible way (Fig. 4, *C* and *D*).

On evaporated films of titanium, vanadium, and carbon, only a few aggregates were observed on untreated surfaces and the effect of glow discharge was less pronounced. This is due to the higher roughness of the evaporated surfaces which influences the spreading of the molecules even on the untreated substrates.

To test the reproducibility and the efficiency of the treatment, the glow discharge has been carried out at 2 and 6 mA on silicon wafers. Compared with untreated surfaces, both surfaces show an increase of roughness. No significant change is seen in the contact angle between the surface and water, but a better spreading of the viruses is obtained after treatment with a current intensity of 6 mA.

We conclude that, for all surfaces, glow discharge improves the quality of images for large particles such as T4, by enhancing hydrophilic interactions between the phage and the surface, reducing the probability that the phage will bind to each other and form aggregates. This increase in image quality clearly outweighs any problems caused by the increase in surface roughness after glow discharge.

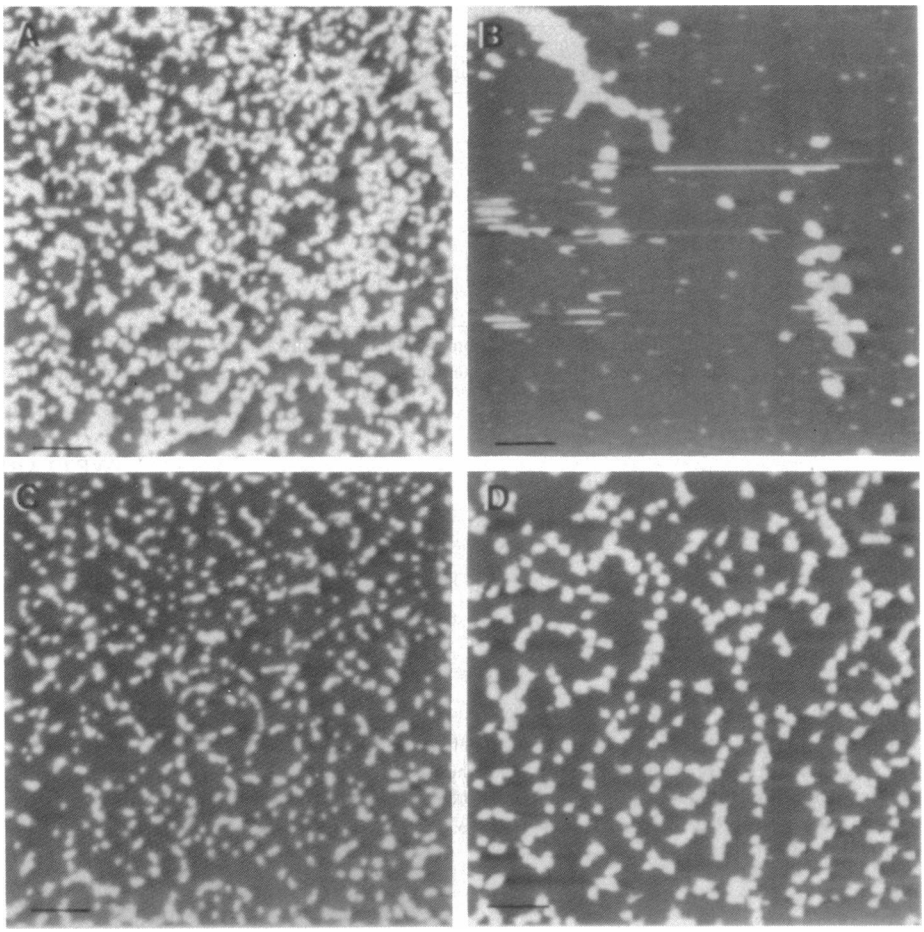


FIGURE 4 SFM images of T4 at the same molecule and buffer concentration as Fig. 2 *C* and dried in ambient conditions, adsorbed on untreated PMMA (*A*) and silicon wafer (*B*), on glow-discharged PMMA (*C*), and on glow-discharged silicon wafer (*D*). The scale bar corresponds to 1 μm, and the vertical black to white scale is 70 nm.

Deposition method

The stabilization of bacteriophages is an important requirement for SFM observations. Molecules can be imaged if they are not displaced during scanning. A correct deposition method must not significantly alter the structure of the phage and promote a good distribution of isolated and stable adsorbed molecules. The critical step of the deposition method is the drying, since removal of the hydration shell may change the conformation of the component of the phage and hence produce artefactual images due to samples preparation.

We have tested various deposition methods taken from TEM techniques adapted for SFM observations. Initially, spraying and droplet deposition were compared. For all deposition method described below, T4 was always adsorbed at the same concentration on glow discharged silicon wafer, in ammonium acetate buffer.

Spraying is often used for the adsorption of viruses in TEM, but with large and complex phages such as T4, the impact of the bacteriophages on the surface of the substrate may cause their damage. Indeed, in samples where the phage was sprayed, T4 with distorted heads are often obtained. The reduced thickness of the T4 heads in some images is caused by the breakage and results in the expulsion of phage DNA, which can be seen as a blurred stain beside the head (Fig. 5 *A*). In an attempt to minimize this damage 25% of glycerol was added before spraying, but no improvement was observed in the SFM images. The resolution was low and problems were encountered with the tip sticking to the surface. Spraying does not give a reliable distribution of intact and isolated T4 viruses for SFM observations.

The second deposition method used was direct application of a drop of suspended bacteriophages on the surface. Here, we used optical microscopy to enable a correct positioning of the tip inside the dried drop. Usually, this drop is composed of three distinct regions: the edge of the drop, an aggregate of salt, and a free region. The first region determines the outline of the dried drop, and viruses may be found on the outer edge of this border line. There, the viruses appear close-packed with the tail sheaths oriented toward the outside due to the surface tension during drying (Fig. 5 *B*). In the second region salt precipitates are visible as clusters on the surface. Images in these regions display compacted and embedded particles. The roughness in these adsorbates is often sufficiently pronounced that the tip cannot follow the surface topography and snaps away. The final region is generally a few millimeters wide and thus large enough to position the tip. It contains well spread isolated viruses with few artefacts and is the preferred region for scanning. The images presented have been obtained in such region.

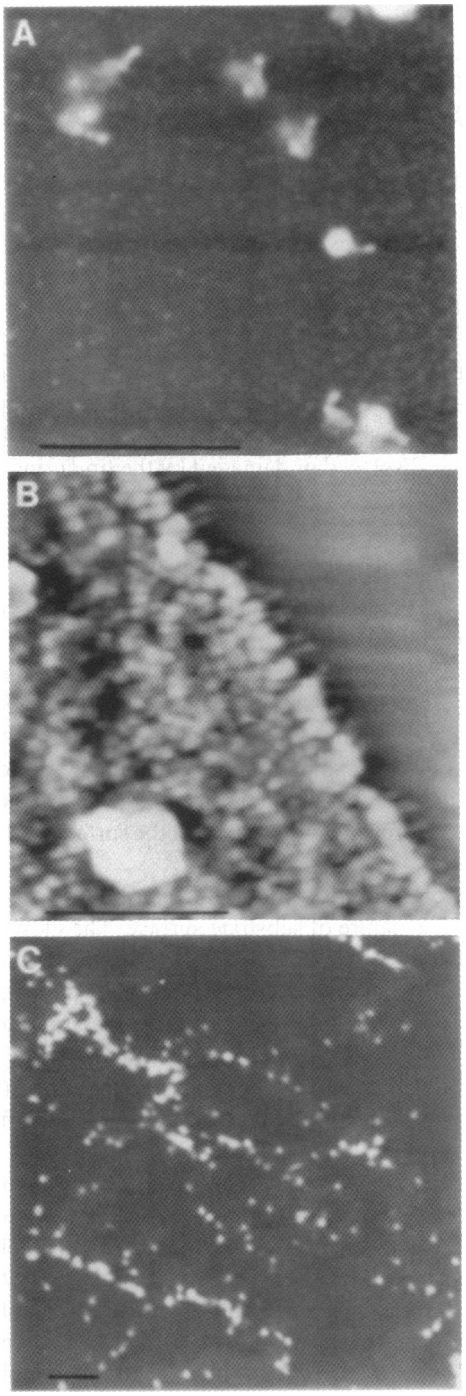


FIGURE 5 SFM images of the different deposition methods investigated with T4. (*A*) Spraying method: The intact T4 in the middle right of the image shows a normal head thickness of 70 nm. However, the four phages on the top of the image show a reduced head thickness of 15 nm, due to its breaking. The head content can be seen expelled at the side of the head. The scale bar corresponds to 1 μm, and the black to white scale is 30 nm. (*B*) SFM image of T4 adsorbed on a glow-discharged silicon wafer. At the drop edge, all the tail sheaths are oriented toward the outside edge of the drop, due to the surface tension effects. The scale bar corresponds to 1 μm, and the black to white scale is 210 nm. (*C*) Droplet deposition method. The surface is floated during 30 s on a 70-μl drop, and the excess of water remaining on the surface is withdrawn with a filter paper. The image shows the ridges caused by the drying process. The scale bar corresponds to 1 μm, and the black to white scale is 70 nm.

The droplet deposition method is the simplest and more reproducible method. Three major variations were carried out in the method, which had a significant effect on the images. First, a suspended solution of diluted particles was deposited as a large drop (60 μl) on a Teflon plate and the substrate was floated on the drop. After 30 s of adsorption the excess solution was removed with a filter paper (Whatmann 3 MM) and the sample was air dried at ambient temperature. With this method a nonuniform distribution was obtained (Fig. 5 *C*). Large areas (> 10 μm) were observed with no adsorbed T4 and traces of aggregated phages. When we withdrew the excess liquid, weakly bound T4 were removed or oriented by the capillary action of the filter paper.

Second, the solution of phage was deposited as a droplet (3 μl) on the surface and the sample dried in ambient conditions. When a large scan size was used, the images showed that the bacteriophages were more evenly distributed and also exhibited less structural damage (Fig. 4 *B*).

Third, the deposited drop was exposed to a filtered stream of nitrogen, which enables the sample to be dried in a few minutes rather than half an hour. If the observations are carried out immediately after the drying process, the bacteriophages are not immobilized on the surface and they may be still surrounded by a film of water and are pushed away by the tip during the scan, as shown by the clear stripes on the images (data not shown). One hour after drying these artefacts disappear, indicating that the particles are now tightly bound to the surface. This time delay means that we were not able to directly compare this last variant with the other deposition methods. In all three techniques the shape and dimensions of bacteriophages are always similar, provided that the head is not broken. The particles generally exhibited a nonuniform distribution, which is explained by a nonuniform surface energy of the substrate which creates microdomains on the surface. Nevertheless, the 3-μl droplet deposition is the easiest and the most reliable technique to observe isolated viruses on a large area.

Influence of vertical and lateral forces

The vertical force applied by the lever in air is essentially given by the action of capillary forces between the tip and the sample, mediated by the layer of contaminants which is always adsorbed on the surface and especially after glow discharge. We have measured a vertical force applied on the sample of 1×10^{-8} N, which is close to the lower limit for working in air. This force seems to avoid major damage to the molecules, but an increase up to 4×10^{-8} N on the same region already provokes typical stripes on the image which indicates a displacement of phages when scanning (Fig. 6).

Lateral forces or friction effects have already been mentioned in SFM imaging of polymers or other biomolecules (Leung and Goh, 1992; Zenhausern et al., 1992a). This effect can also be observed on T4 samples. This phenomenon induces inversion of the image contrast by changing the scan direction with respect to the symmetry axis of the lever (Zenhausern et al., 1992a) or by increasing the vertical force

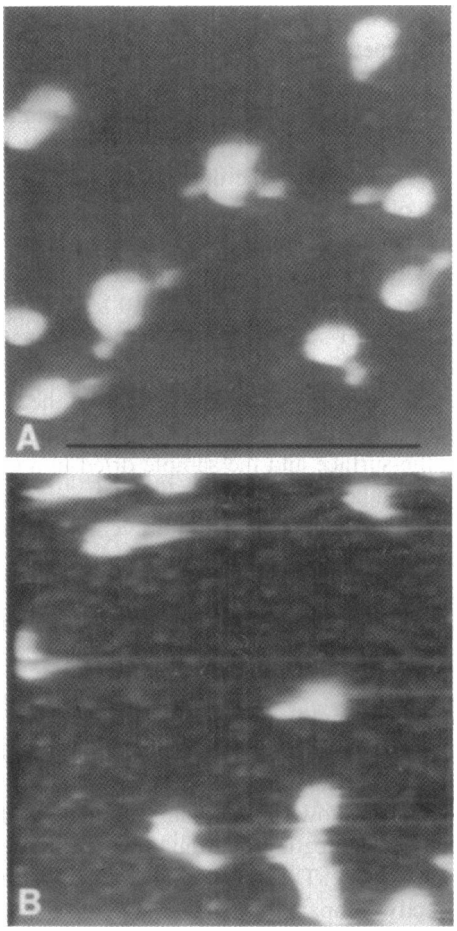


FIGURE 6 SFM image of T4 adsorbed on glow-discharged titanium film evaporated on mica, scanned with a vertical force of 1×10^{-8} N (*A*) and after successive increase of force until 4×10^{-8} N (*B*). The scale bar corresponds to 1 μm, and the black to white scale is 70 nm.

applied on the sample. In such cases the entire bacteriophage can appear completely black on a white surface, it is flattened on the substrate, and consequently the resolution does not allow to observe structural details.

The sensitivity to friction depends on the choice of the spring constant of the lever. The inversion of contrast is more pronounced when using a lever with a lower spring constant (0.032 N), because it has a lower torsional spring constant. Observations carried out with levers of higher spring constant show that the viruses are damaged by these tips.

Influence of the tip geometry and resolution

All the images presented have been obtained with pyramidal tips. As already mentioned these affect the resolution on the molecule (Zenhausern et al., 1992b). The outline of T4 is principally the combination of the tip shape and the molecular shape. With such a tip the outline of large features like the T4 head appears always enlarged (Table 1), due to the tip radius (40 nm nominally). Moreover, the steepest slope observed in the images is often an angle of 45°, which corresponds to the angle of the pyramidal tip. It is probable that observation with a sharper tip will lead to a better resolution. However, tips with high aspect ratio are also more fragile and the detection of lower forces is necessary in order to use them. Moreover, the contamination of the tip during the scanning enlarged the tip radius and therefore lowered the resolution.

When the sample is prepared according to the optimal parameters, the head and the tail sheath of T4 are always observed. Unfortunately, the tail fibers are not visible in a reproducible way. We could distinguish them only once at high buffer concentration, but the other structures were embedded in salts. Clearly, the high buffer concentration may help to maintain tightly such fine and fragile structure on the surface substrate. At low ionic strength, they appear only blurred: obviously they are not stably adsorbed on the surface and can be displaced or damaged by the tip during scanning.

CONCLUSIONS

We have investigated various preparation parameters to obtain reproducible images of isolated molecules by using the T4 bacteriophage as a test sample. The SFM images show large differences on the adsorption and distribution of the molecules depending on sample preparation conditions. Volatile buffer as ammonium acetate at low ionic strength and drying in ambient conditions are the best parameters to observe T4 viruses well spread over the surface with only few artefacts.

The molecule adsorption and distribution is also influenced by the nature of substrate surface. The glow discharge is a suitable technique to favor firm adsorption and avoid aggregation phenomena encountered with the hydrophobic surfaces, in spite of the increase of roughness. For investigations with smaller biomolecules, untreated freshly cleaved mica would be preferable, since the surface is hydrophilic by itself and atomically flat, thus avoiding misidentification of molecules.

A survey showed that the simplest droplet deposition method, i. e., droplets air-dried under ambient conditions, is the most reliable technique for observing isolated phage over a large surface area. The problems encountered with the imaging conditions are comparable with those mentioned by other authors. The tip geometry, the vertical forces and especially lateral forces, are the most important conditions that need careful investigations for optimal and reproducible images. Thinner tips with a cantilever of a lower spring constant should lead to a better resolution on the images, but will require that the experiments are performed with lower applied forces, for instance in liquid. These experiments are now in progress.

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