Atomic force microscopy examination of tobacco mosaic virus and virion RNA

Yuri F. Drygin[a,*], Olga A. Bordunova[a], Marat O. Gallyamov[b], Igor V. Yaminsky[b,c]

[a] *A.N. Belozersky Institute of Physical and Chemical Biology, Moscow State University, 119899 Moscow, Russia*
[b] *Faculty of Physics, Moscow State University, 119899 Moscow, Russia*
[c] *Faculty of Chemistry, Moscow State University, 119899 Moscow, Russia*

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Abstract Atomic force microscopy (AFM) was applied to study uncoated virus particles and RNA prepared by stripping of tobacco mosaic virions (TMV) with mild alkali or urea and dimethylsulfoxide. We found that AFM is an appropriate method to study ribonucleoprotein and free RNA structures. Images of entire tobacco mosaic virions, partially uncoated TMV particles with protruding RNA molecule from one or both ends and individual RNA molecules are presented.

Key words: Atomic force microscopy; Ribonucleoprotein; RNA; Tobacco mosaic virus

1. Introduction

Most of the information deduced from microscopy of RNA morphology and RNA-protein interactions was obtained by means of transmission electron microscope investigations in vacuo [1]. Limitations of this technique in RNA studies are: (1) it requires complexing of RNA with a basic protein; (2) spreading of the complex on the water-air surface is necessary; (3) it needs additional sample contrasting by heavy metal atom shadowing or staining and preparation of replicas, etc.

Scanning probe microscopy, which includes scanning tunneling microscopy and atomic force (or scanning force) microscopy (AFM), was proved to be a reliable, convenient technique for structure investigations of tobacco mosaic virus (TMV) [2,3]. Both methods have unique advantages in the studies of natural [4] or synthetic [5] nucleic acids and specific DNA-protein interactions in air [6,7]. Although there is sufficient information at present about sample preparation for routine DNA investigation by AFM, there are few reports, to our knowledge, on AFM of the viral high molecular weight RNA molecules [8].

The aim of this work is to examine high molecular weight RNA molecules and ribonucleoproteins by atomic force microscopy. Partial stripping and complete uncoating of TMV virions in mild alkaline conditions, dimethylsulfoxide or urea was carried out to prepare TMV ribonucleoproteins and RNA for visualization. The AFM images of entire tobacco mosaic virions, partially uncoated TMV particles with protruding RNA molecule from one or both ends and individual TMV RNA molecules are presented.

2. Materials and methods

Deionized double distilled water was used to prepare all solutions and in rinsing procedures. A sample of isolated tobacco mosaic virions purified by sucrose density gradient centrifugation was kindly provided by Dr. E.N. Dobrov. TMV RNA was isolated by SDS-phenol deproteinization. RNA concentration was estimated by absorption of the RNA solution at 260 nm (1 optical unit corresponded to 40 μg/ml of RNA, approximately). Mica sheets were from TED PELLA Co., benzyldimethylalkylammonium chloride (BAC) was kindly provided by Dr. R.C. Williams. 3-Aminopropyltriethoxysilane (APTES) was from Fluka Co. Highly oriented pyrolytic graphite (HOPG) was kindly provided by the Institute of Graphite (Moscow, Russia).

2.1. TMV stripping conditions

In all stripping experiments TMV suspension in water (2.6 mg/ml) was used.

Alkaline pH stripping [9]: TMV suspension was mixed with an equal volume of 0.2 M glycine (pH 10.4) and kept for 3 h at 0°C. One third (v/v) of 0.5 M potassium acetate (pH 5.0) was added to stop the reaction.

Urea stripping [9,10]: 6 M urea was added to TMV suspension to a final urea concentration of 4 M and kept for 2.5 h at 0°C. To stop the reaction, one and a half volume of water was added.

DMSO stripping [11]: Partial and complete stripping of virus particles was achieved in 72% DMSO solution. To uncoat virions, TMV suspension was mixed with 9 volumes of 80% DMSO and incubated for 30 min at room temperature. An equal volume of water was added to stop the reaction.

Microcolumn size exclusion chromatography on Toyo-Pearl HW-50 gel was used to separate dissociated capsid protein, free RNA, full and stripped TMV particles. In each case the column (150 ml) was equilibrated with a water solution of 0.1 M glycine, pH 10.3 or 1.6 M urea, and 36% DMSO, respectively.

2.2. Substrates

Freshly cleaved mica or HOPG was used as substrate. In some cases the mica or graphite was treated with 100 mM $MgCl_2$ [12] for 75 min or 1% BAC in formamide [13] for 45 min, or with other agents (see Section 3). Treated substrate surfaces were washed with water (0.2 ml) four times for 1–2 min each. The excess water was removed and the substrates dried on air.

2.3. Specimen preparation

3–15 ml of each sample were deposited onto the substrate surface. Adsorbed samples were washed with water and then air-dried.

2.4. Microscopy

Dried samples were investigated using Nanoscope-III AFM (Digital Instruments, USA) equipped with a 'D' scanner. We used silicon nitride sharpened probes with a cantilever spring constant of 0.06 N/m for contact mode or etched silicon probes with cantilever resonant frequency in the range of 350–380 kHz for tapping mode. In the case of the contact mode applied force was minimized to a value below 1 nN.

*Corresponding author. Fax: (7) (95) 939-31-81.
E-mail: drygin@nucleo.genebee.msu.su

Abbreviations: AFM, atomic force microscopy; APTES, 3-aminopropyltriethoxysilane; BAC, benzyldimethylalkylammonium chloride; DMSO, dimethylsulfoxide; HOPG, highly oriented pyrolytic graphite; TMV, tobacco mosaic virus; a.u., arbitrary units

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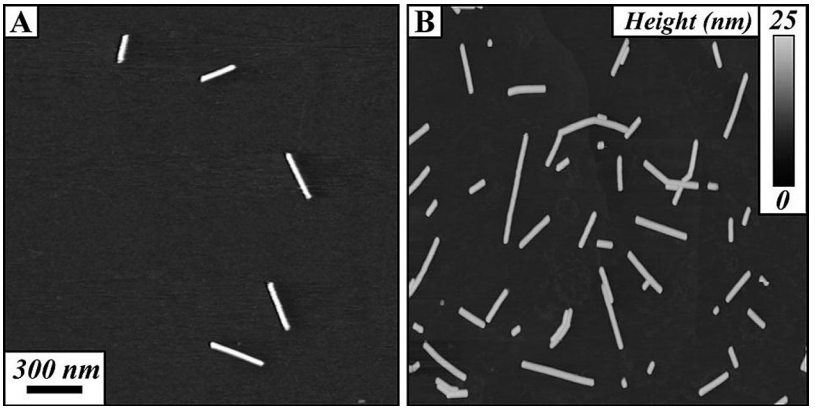

Fig. 1. AFM examination of TMV virions (0.27 mg/ml in 40% DMSO) sorbed for 7 min onto mica (A, contact mode) and for 2 min onto HOPG (B, tapping mode).

3. Results and discussion

3.1. Tobacco mosaic virion microscopy

Images of TMV particles on mica are presented in Fig. 1A. TMV virions adhere firmly to mica and remain immobile during the contact mode investigation. In contrast, TMV particles were removed by the microscope tip from the HOPG surface in the case of the contact mode, unless a very small applied force was used. This is why the tapping mode of AFM was used for TMV examination with HOPG as a substrate (Fig. 1B). One can see separate entire particles (about 300 nm length) and shorter and longer particles as well. Broken TMV particles resulted, probably, from the centrifugation process during sample purification. Longer particles could be the result of TMV virion stacking. The width of TMV particles at the virion half-height is 25–35 nm independent of the mode of investigation. The observed height of tobacco mosaic virions ranged from 17–23 nm in the case of the tapping mode to 19–23 nm in the case of the contact mode of investigation. Deposition of TMV from DMSO or formamide solution ensured a more even distribution of the particles at substrate surfaces.

We observed differences in adsorption of TMV particles and RNA onto substrates used. Much more of the virus particles was adsorbed on the HOPG surface in comparison with the mica (Fig. 1) at equal adsorption conditions (exposure time, concentration and so on). This indicates that adsorption of TMV particles onto mica is slower. The observed effect may be related to the negative charge of the mica surface in aqueous solution, because the BAC-treated mica readily adsorbed the virus. It favors the hypothesis that the negatively charged mica surface is neutralized with BAC and has taken on a hydrophobic character. On the other hand, the highly hydrophobic graphite surface retained few RNA molecules. It required a positive charge while BAC treated, and then readily adsorbed RNA (not shown).

3.2. Microscopy of uncoated tobacco mosaic virions and virion RNA

It is well known that free single-stranded RNA is difficult to examine microscopically. Until recently, the best method for RNA visualization was Kleinshmidt's modified protein monolayer technique (see for example [14,15]) which needs specimen RNA complexed with a basic protein.

It has repeatedly been demonstrated that free double-stranded DNA can be unequivocally visualized by AFM [4–6,16,17]. Recently we presented preliminary results on a scanning force microscope study of partially uncoated tobacco mosaic virions and virus RNA [8]. Here we describe the visualization of RNA and its release from the virus capsid in more detail.

To find RNA molecules deposited onto substrates, partially uncoated TMV particles were used. Here, the termini of the stripped virus particles served as a starting point for recognizing nascent RNA protruded from the well defined structure. Three methods to uncoat tobacco mosaic virions have been published. We inspected all of them to examine spreading of the RNA molecule on the substrates used.

pH 10.3 stripping: At mild alkaline conditions ([9], see Section 2), partially uncoated TMV particles of different length with RNA protruding from the ends and free RNA molecules were observed (not shown). Protruding and free TMV RNA appeared in 0.1 M glycine as a molecule with a clearly defined secondary structure, whereas the free TMV RNA tended to aggregate. Unless the specimens were extensively washed, the high background was retained.

4 M urea stripping: This treatment produced partially stripped TMV particles (presented in Fig. 2A–E). As previously, RNA tails appeared as well defined threads.

72% DMSO stripping of TMV: Partially stripped TMV particles of different length with RNA protruding from the ends are presented in Fig. 3A–E.

Actually, after dilution with water and size exclusion chromatography, the DMSO concentration dropped to 36%. It appears that an equilibrium between uncoating [11] and reconstruction [18] of TMV particles takes place in 36% DMSO solution: some short TMV particles contain RNA tails protruding from both sides of the particle whereas others reveal structures with two RNA tails extending from one side (compare Fig. 3B,C and D,E). The same was found for the urea stripped TMV (see Fig. 2B,C and D,E).

It should be pointed out that a substantial part of TMV particles was completely uncoated in 72% DMSO. Free TMV RNA molecules appeared as expanded threads (Fig. 4A,C). The length distribution of RNA molecules is presented in Fig. 4B. Reduction in DMSO concentration during size exclusion chromatography reduces RNA melting and restores its sec-

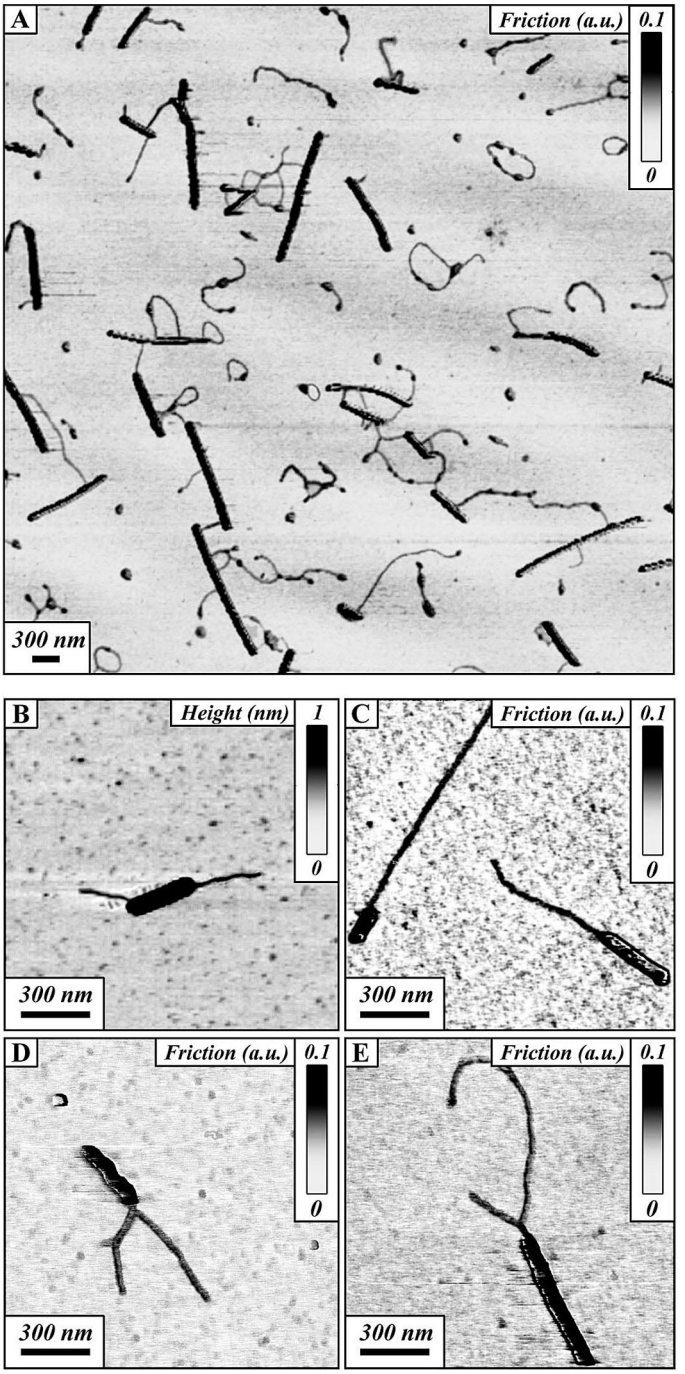

Fig. 2. Contact mode (A, C, D, E, friction images) and tapping mode (B, height image) examination of TMV particles partially uncoated by 4–1.6 M urea and deposited onto mica.

ondary structure. This may explain the wide length distribution of the RNA molecules (Fig. 4A).

Measured RNA widths at the molecule half-height ranged from 10–15 nm for the tapping mode to 15–25 nm for the contact mode of investigation. RNA heights are in the range of 0.3–1.5 nm for both the tapping and contact mode investigations.

To study TMV RNA and virus adsorption, different treatments of the mica surface were tested: 100 mM $MgCl_2$ [12], 150 mM KCl, 100 mM $MgCl_2$+150 mM KCl (for 75 min each), 1% BAC in formamide [13] and APTES [19] (for 2 h at 105°C). Mica surface treatment with Mg^{2+} and APTES improved sorption of TMV and RNA markedly, as was demonstrated for DNA [12,19]. RNA deposited onto mica treated with $MgCl_2$ reveals a coiled structure (to be published elsewhere), similar to RNA, which is structured in a water solution of the magnesium salt [20].

We examined spreading of virus RNA on the untreated substrate surfaces in a complex with the cationic surfactant BAC. It was found earlier that BAC promotes spreading of

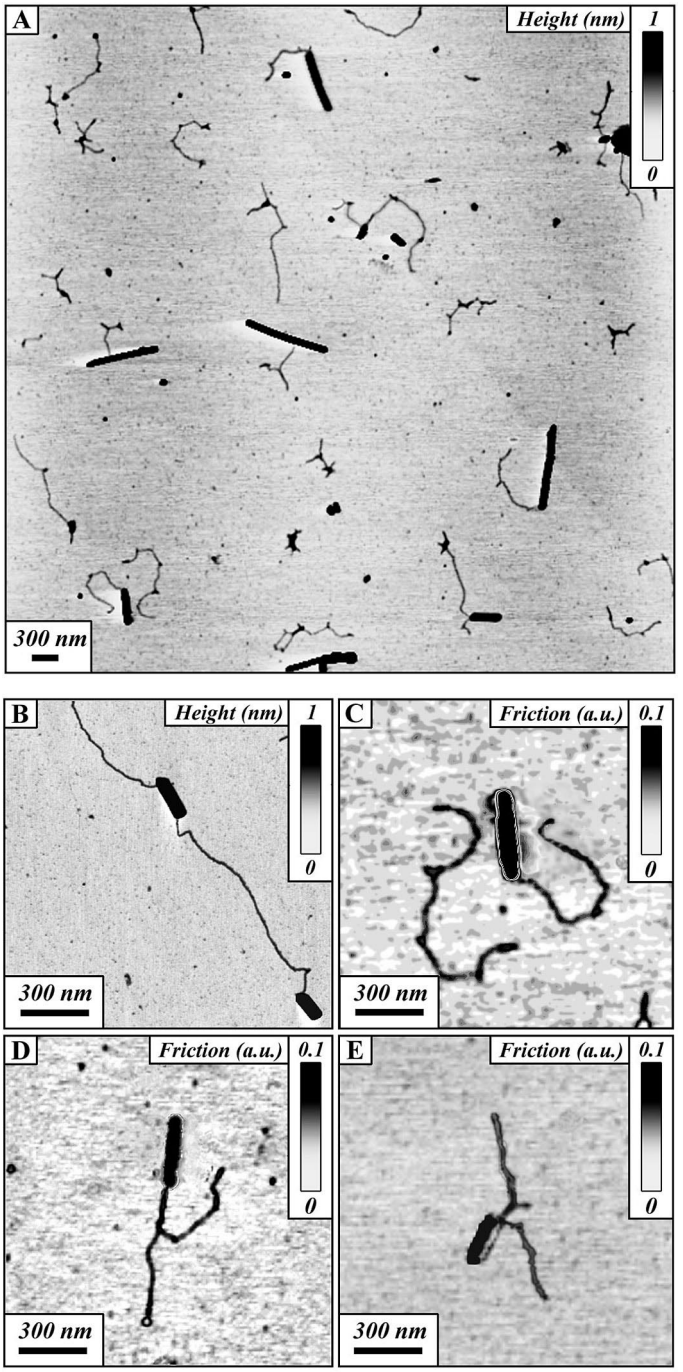

Fig. 3. Contact mode (A, height image; C, D, E, friction images) and tapping mode (B, height image) study of TMV particles partially stripped by 72–36% of DMSO.

the three-dimensional DNA molecule on the water surface accompanied by transformation of the random coil into a curved strand [13]. Complexing of DNA with BAC increased its contrast in electron microscopy study, making it possible to observe specific DNA-protein complexes. For this purpose, TMV RNA (0.138 mg/ml) in 2 mM $MgCl_2$, 200 mM KCl was exposed in 50% DMSO–3.5% formaldehyde at 45°C for 5 min to denature RNA, 1% BAC was added and the sample was deposited onto the mica surface (Fig. 4D). We found also (not shown) that the contrast of both RNA and TMV was increased by 0.1 mM uranyl acetate/acetone staining [21].Thus, AFM is an appropriate technique for investigation of RNA-protein complexes. The study performed opens new perspectives for the AFM examination of specific interactions between viral RNA and proteins.

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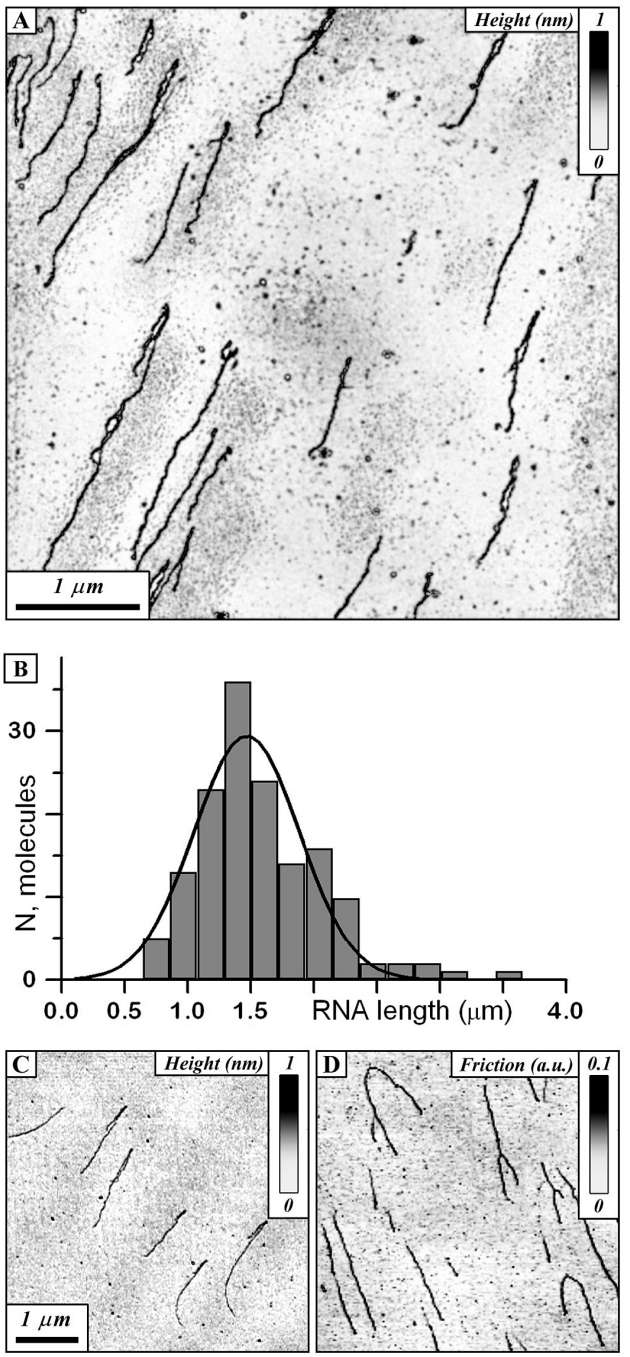

Fig. 4. A, C: Contact mode (height images) studying of TMV RNA molecules separated from TMV particles by microcolumn size exclusion chromatography in 72–36% of DMSO (expanded on mica surface). B: Histogram of TMV RNA length distribution (see A). D: Contact mode (friction image) of TMV RNA molecules spreaded onto mica surface in the presence of BAC (see text).

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