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Formation of two-dimensional crystals of icosahedral RNA viruses

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This article is dedicated to the memory of Jean and Marie-Thérèse Witz.

Abstract

The formation of 2D arrays of three small icosahedral RNA viruses with known 3D structures (tomato bushy stunt virus, turnip yellow mosaic virus and bromegrass mosaic virus) has been investigated to determine the role of each component of a negative staining solution containing ammonium molybdate and polyethylene glycol. Virion association was monitored by dynamic light scattering (DLS) and virus array formation was visualised by conventional transmission electron microscopy and cryo-electron microscopy after negative staining. The structural properties of viral arrays prepared *in vitro* were compared to those of microcrystals found in the leaves of infected plants. A novel form of macroscopic 3D crystals of turnip yellow mosaic virus has been grown in the negative staining solution. On the basis of the experimental results, the hypothesis is advanced that microscopic arrays might be planar crystallisation nuclei. The formation of 2D crystals and the enhancing effect of polyethylene glycol on the self-organisation of virions at the air/water interface are discussed.

Synopsis: The formation of 2D arrays of icosahedral viruses was investigated by spectroscopic and transmission electron microscopic methods.

Keywords: Icosahedral virus; RNA virus; Tomato bushy stunt virus (TBSV); Turnip yellow mosaic virus (TYMV); Brome mosaic virus (BMV); Transmission electron microscopy; Cryo-electron microscopy; Negative staining; Polyethylene glycol (PEG); Ammonium molybdate; Air/water interface; Adsorption; Nucleation; Crystallisation; Crystallogenesis

1. Introduction

Under either normal physiological or pathological conditions, many bacterial, plant and animal cells contain crystalline protein or virus inclusions (for reviews, see *e.g.* Maramorosch (1977) and McPherson (1999), and references therein). Most of these natural 2 or 3D arrangements have too many imperfections to be suitable for *in situ* electron diffraction analyses. To overcome this limitation, methods have been implemented to produce *in vitro* arrays with a high degree of periodicity directly on the support grids used for transmission electron microscopy. When the order of the crystalline lattice encompassing large numbers of identical particles is high enough, information on the packing contacts and the architecture of the individual unit particle can be gained (see *e.g.* Harris and Holzenburg, 1989; Vonck and van Bruggen, 1990; Brisson et al., 1994; Harris, 1997; Walz and Grigorieff, 1998).

Horne and co-workers implemented a two-stage negative staining-carbon film technique with low concentrations of ammonium molybdate or uranyl acetate and noted some icosahedral viruses have a propensity to form either square or hexagonal arrays on the surface of mica sheets (Horne and Pasqualli-Ronchetti, 1974; Horne et al., 1975a,b). Subsequently, Wells et al. (1981) reported that the addition of polyethylene glycol (PEG) to the stain enhanced the formation of virus arrays. Mixtures of ammonium molybdate and PEG have also been utilised to produce 2D crystals from a number of

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proteins and enzymes (Harris, 1991, 2007; Harris et al., 1992; Harris and Holzenburg, 1995; Harris and Adrian, 1999). The combination of cryoelectron microscopy (Wilson and Glaeser, 1974) and negative staining has improved contrast and resolution of the images. It also provided the first indication that viruses can create 2D arrays within an unsupported thin-layer aqueous system (Adrian et al., 1998). Again, the addition of PEG was found to increase the tendency of viruses to form ordered 2D crystals prior to vitrification (Harris and Adrian, 1999; Harris, 2007).

We have investigated how the two components of the negative-staining solution, the electron-dense ammonium molybdate solution and the PEG contribute to the formation of 2D arrays of three small icosahedral RNA viruses. The shells of these viral particles are highly symmetric: they possess 20 equivalent facets, 15 twofold, 10 threefold, and 6 fivefold axes. The bromovirus, bromegrass mosaic virus (BMV), the tombusvirus, tomato bushy stunt virus (TBSV), and the tymovirus, turnip yellow mosaic virus (TYMV) were chosen as models because their crystallographic 3D structures are known. The association of each type of virion in aqueous solutions containing the ingredients of the stain, either separately or in mixtures, has been monitored by non-invasive spectroscopy. The effect of these compounds on the formation of 2D virus array formation has been visualised by conventional negative-stain transmission electron microscopy (TEM) and by cryo-negative staining TEM. The structural properties of *in vitro*-prepared arrays of virions have been compared to those of microcrystals made of the same virions within the leaves of infected plants. Our experimental results have led us to raise the question whether the smallest virion arrays observed on the electron microscope grids might be planar crystal nuclei. Further, the mechanism by which PEG promotes the self-association of virions into 2D and 3D crystals is discussed.

2. Materials and methods

2.1. Chemicals

Ammonium heptamolybdate tetrahydrate (M_r 1235.86, Cat. No. 1182 from Merck or A7302 from Aldrich) and uranyl acetate dihydrate (M_r 424.15, Cat. No. 8473, Merck) powders were used as received. Polyethylene glycol (PEG, M_r 1000) was from Hampton Research (50% m/v aqueous solution, Cat. No. HR2-523) or Sigma (solid, Cat. No. 81188). All solvents for light scattering analyses were prepared with sterile thrice-distilled water.

2.2. Viruses

Brome mosaic virus (BMV, common strain) was multiplied in barley *Hordeum vulgare* L., tomato bushy stunt virus (TBSV) in *Datura stramonium*, and turnip yellow mosaic virus (TYMV) in Chinese cabbage *Brassica rapa* c.v. Just Right. The three viruses were purified by isoelectric precipitation of the sap proteins at pH 4.8, followed by two or three cycles of low and high-speed centrifugation (Matthews, 1960). Concentrated virus samples were filtered through 0.2 μm pore diameter membranes (Millex, Millipore) and stored at 4 °C in 20 mM sodium acetate pH 5.8 with 0.1% (m/v) sodium azide. The quality of the virus preparations was estimated from the ratio of their absorbance at 260 nm over that at 280 nm (Table 1). Virion concentration was determined from absorbance at 260 nm (measured on 2 μl samples with a Nanodrop® ND-100 spectrophotometer) and extinction coefficient (Table 1).

2.3. Crystallisation assays

Virus preparations were tested for crystallisability in various solvents and at several virus concentrations using the vapour

Table 1
Structural and physical chemical properties of model viruses

Virus	Genome	Capsid protein		Virion with $T = 3$ geometry[a]								
	Composition and M_r	Subunit M_r (number of aa)	Copies	Total M_r[b]	$S_{20,w}$[c] (s)	D_0[d] (cm^2/s)	d_h[e] (nm)	A_2[f] (ml mol/g^2)	$E_{260\ nm}$[g] (mg/ml/cm)	$A_{260\ nm}/A_{280\ nm}$[h]	dn/dc[i] (ml/g)	pI[j]
BMV	Tripartite RNA, 1.0×10^6	20,385 (185)	180	4.7×10^6	87	1.3×10^{-7}	32	8×10^{-4}	5.1	1.7	0.2	6.8
TBSV	Monopartite RNA, 1.7×10^6	40,540 (387)	180	9.0×10^6	132	1.1×10^{-7}	37	7×10^{-4}	5.0	1.6	0.2	4.1
TYMV	Monopartite RNA, 1.9×10^6	20,088 (189)	180	5.5×10^6	116	1.4×10^{-7}	33	8×10^{-4}	8.4	1.5	0.2	3.8

[a] The stability of the virions varies with the physical chemical properties of the solvent. BMV is stable from pH 3–6 and unstable at pH > 7. The removal of calcium ions bound to TBSV induces the reversible swelling. The capsid of TYMV swells when the pH exceeds 11.

[b] Sum of the M_r of its protein and of its nucleic acid components.

[c] Sedimentation velocity of infective virions in the ultracentrifuge. Data for water at 20 °C at pH < 7 from Description of Plant Viruses at URL http://www.dpvweb.net/ (Adams and Antoniw, 2006).

[d] Translational diffusion coefficient extrapolated at zero concentration derived from dynamic light scattering measurements performed on virus in 0.1 M sodium acetate solution at pH 4.5 and at 20 °C.

[e] Hydrodynamic diameter deduced from translational diffusion coefficient assuming that particles are spheres.

[f] Second virial coefficient given by the slope of the plot representing the scattered intensity (measured by static light scattering) vs. the virus concentration. Measurements were performed on virus in 0.1 M sodium acetate solution at pH 4.5 and at 20 °C.

[g] Extinction coefficient at 260 nm.

[h] Maximal quotient of absorbance at 260 nm on absorbance at 280 nm for pure virus.

[i] Increment of refractive index determined by spectrometry and refractometry.

[j] Isoelectric point taken from Description of Plant Viruses at URL http://www.dpvweb.net/ (Adams and Antoniw, 2006).