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The Assembly Pathway of an Icosahedral Single-Stranded RNA Virus Depends on the Strength of Inter-Subunit Attractions

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Abstract

The strength of attraction between capsid proteins (CPs) of cowpea chlorotic mottle virus (CCMV) is controlled by the solution pH. Additionally, the strength of attraction between CP and the single-stranded RNA viral genome is controlled by ionic strength. By exploiting these properties, we are able to control and monitor the *in vitro* co-assembly of CCMV CP and single-stranded RNA as a function of the strength of CP–CP and CP–RNA attractions. Using the techniques of velocity sedimentation and electron microscopy, we find that the successful assembly of nuclease-resistant virus-like particles (VLPs) depends delicately on the strength of CP–CP attraction relative to CP–RNA attraction. If the attractions are too weak, the capsid cannot form; if they are too strong, the assembly suffers from kinetic traps. Separating the process into two steps—by first turning on CP–RNA attraction and then turning on CP–CP attraction—allows for the assembly of well-formed VLPs under a wide range of attraction strengths. These observations establish a protocol for the efficient *in vitro* assembly of CCMV VLPs and suggest potential strategies that the virus may employ *in vivo*.

Keywords

self-assembly; virus-like particle; cowpea chlorotic mottle virus; ssRNA; packaging

Introduction

The architecture of viruses is beautifully simple. For many single-stranded RNA (ssRNA) genome plant viruses, the virion consists of only two structural elements: the RNA carrying the genetic information, and the capsid protein (CP), many copies of which form an icosahedrally symmetric protective shell (the capsid) around the genetic cargo. During its life cycle, the virus particle must be both stable enough to protect its genome from degradation along the treacherous journey between hosts and labile enough to disassemble inside the host cell and release its genetic contents. Additionally (and remarkably), many

viruses are also capable of spontaneous self-assembly. These characteristics are made possible by a delicate balance of attractive and repulsive forces that has evolved among the viral structural units. The primary interactions within the virus particle are those between neighboring protein molecules forming the capsid (CP–CP) and those between CP and nucleic acid (CP–RNA). The present work focuses on the interplay between these key interactions and their effect on the *in vitro* assembly pathway of the plant RNA virus, cowpea chlorotic mottle virus (CCMV).

The CCMV virion consists of ~3000 nt of ssRNA packaged within a 28-nm-diameter capsid made up of 180 chemically identical copies of a 190-residue CP. We chose to study CCMV assembly for three reasons. First, CCMV is among the most robust viral self-assembly systems—it was the first icosahedral virus to be reconstituted *in vitro* from purified components [1], and CCMV CP has since been shown to package a wide range of heterologous RNAs [2], in addition to being capable of assembling into empty capsids [3,4]. Second, high-resolution reconstructions of the virion obtained by X-ray crystallography and cryo-electron microscopy (cryo-EM) [5], in conjunction with computational simulations [6], have yielded great insight into the specific molecular interactions that govern the structure, stability, and dynamic properties of the virion. Third, and most importantly, the strength of the CP–CP and CP–RNA interactions can be controlled by adjusting the pH and ionic strength (*I*), respectively [7,8]. This last point, which we exploit heavily in the present work, is described in detail in the following sections.

While much is known about the structure and intermolecular forces within the CCMV virion, the pathway of assembly remains poorly understood. In an influential paper by Zlotnick *et al.*, empty capsids of CCMV CP were shown to assemble, under acidic conditions and in the absence of RNA, by first generating a pentamer of CP dimers (the nucleation phase) followed by the stepwise association of additional CP dimers (the growth phase) until capsid completion [9]. To provide insight into the assembly pathway of RNA-containing CCMV virus-like particles (VLPs), Zlotnick *et al.* carried out further studies, involving RNA and a limiting amount of CP under physiological (neutral) pH conditions, in which they demonstrated that CP initially binds RNA with low cooperativity due to the relatively weak lateral attraction between CPs [10].

Expanding on this work, we previously investigated the co-assembly of ssRNAs of varying lengths and an excess of CCMV CP [2,11]. We reported evidence that the assembly of CCMV CP around ssRNA followed a two-step, pH-dependent, mechanism in which CP reversibly binds RNA at neutral pH to form a pre-capsid complex that, upon acidification, undergoes an irreversible reorganization into the final icosahedral capsid [11].

In the present work, we study the pathway of assembly of CCMV CP and brome mosaic virus (BMV) RNA 1, a 3200-nt heterologous RNA shown to be efficiently packaged by CCMV CP [2,11]. We choose to work with heterologous RNA in order to focus on generic features of viral assembly and avoid specific interactions that may exist between CCMV CP and CCMV RNA—specific interactions cannot be completely ruled out, however, due to the high degree of sequence homology between CCMV and BMV RNA. We monitor assembly by a combination of electron microscopy (EM) and velocity sedimentation. By controlling

the relative strength of the CP–CP and CP–RNA interactions through the pH and ionic strength of the assembly buffer, we are able to characterize the structures of disordered intermediates and kinetically trapped assembly products, as well as elucidate key features of the final capsid reorganization.

Results and Discussion

CP–CP interactions

Knowledge on CP–CP interactions in CCMV has come from decades of biophysical theory and experiment [8,12,13] and, more recently, from a high-resolution structural determination of the CCMV virion [5] and subsequent computational studies [6].

The CP exists in solution as a dimer [8] stabilized by a strong non-covalent clamp between the amino- and carboxy-terminal arms of each monomer [5]. The dimer serves as the fundamental assembling unit [8]. Lateral interactions between dimers within the capsid arise from a combination of hydrophobic, electrostatic, and divalent metal-mediated forces between amino acid residues that line the dimer–dimer interfaces. The average strength of attraction between CP dimers has been shown to be pH dependent, decreasing sharply with increasing pH [13]. This is primarily due to electrostatic repulsions that are generated by the deprotonation of adjacent acidic residues at neutral pH, in the absence of divalent metal ions [6]. This effective decrease in lateral attraction is illustrated by the decrease in thermal stability of wild-type (wt) CCMV at elevated pH values (Fig. 1). Here we see that the virion is stable to 75 °C at pH 4.5 and $I = 0.1$ M, but a sharp drop of 30 °C in melting temperature occurs upon raising the pH above 6.

CP–RNA interaction

CP interacts with RNA primarily through a disordered, highly basic, N-terminal arginine-rich motif (ARM). Studies exploiting truncated CP lacking this highly basic region have shown that RNA packaging requires the ARM [14,15]. While sequence-specific interactions have been shown to play a role in the binding of CP to RNA—both for CCMV [16] and other icosahedral plant viruses [17–20]—the predominant interaction in the case of CCMV is non-specific electrostatic attraction. Residues 1–26 contribute +10 charges that interact strongly with the negatively charged phosphate backbone of the RNA. Physical arguments based on the entropy gain associated with mobile counterion release upon polycation/nucleic acid binding estimate the free-energy benefit to be roughly kT per charge. Since there are +10 charges on the CP N-termini, we expect binding energies on the order of 10 kT per CP. Equilibrium dissociation constants that confirm this estimate have been obtained from binding assays carried out between ssRNA molecules and the N-terminal ARM of BMV CP that contains nine basic residues [21].

The electrostatic nature of CP–RNA interactions suggests that the binding should be turned off at sufficiently high salt concentrations as a result of a reduction in the entropic benefit of counterion release and charge screening—indeed, increasing ionic strength is a key step in the protocol for disassembling CCMV for the purpose of isolating CP [22]. To assay how ionic strength controls assembly, we separately labeled CP and BMV RNA 1 with different

fluorescent probes and subjected them to sucrose gradient velocity sedimentation to test for binding (Fig. 2). We see that CP and RNA co-sediment as a high-density complex in low-salt (0.1 M) buffer (Fig. 2d) but migrate as separate species at high (1 M) ionic strength (Fig. 2c)—confirming that CP–RNA interactions can be turned off by high ionic strength. On the other hand, because the positive charges on the ARM derive from arginine and lysine residues that do not titrate over the pH ranges explored in this work, the energy of binding CP to RNA is effectively pH independent.

General assembly procedure

The present work focuses on monitoring the assembly pathway of CCMV CP and BMV RNA 1 as a function of the relative strength of CP–CP and CP–RNA interactions, as well as on the order in which these interactions are “turned on”. Experimentally, this is accomplished by directing mixtures of CP and RNA through the composition space defined by the pH and ionic strength of the buffer solution. To keep track of the location in this space, we label each buffer by its pH and ionic strength, in parenthesis, according to the following format: “(pH,I)-buffer”. For example, the canonical assembly buffer described by Bancroft [50 mM NaCl, 50 mM Tris–HCl (pH 7.2), 10 mM KCl, and 5 mM MgCl₂] will be referred to as (7.2,0.1)-buffer—because it has a pH of 7.2 and an ionic strength of 0.1 M. Additionally, any dialysis step that goes from one point in composition space to another will be denoted by a lower case “d”. For example, dialyzing a solution of CP and RNA in Bancroft’s assembly buffer against the canonical storage buffer [50 mM sodium acetate (pH 4.5) and 8 mM magnesium acetate] will be written (7.2,0.1) d(4.5,0.1).

The general assembly protocol consists of direct mixing of RNA and an excess of CP (6:1 mass ratio) at neutral pH and high ionic strength, (7.2,1.0)-buffer, followed by subsequent dialysis and equilibration steps. (7.2,1.0)-buffer is chosen as the initial buffer for all assembly reactions because it allows for mixing of the reactants in the absence of attractive interactions that drive assembly; more explicitly, both CP–CP and CP–RNA interactions are turned off. After mixing, we are able to selectively turn on CP–RNA and/or CP–CP attraction by dialysis against buffers of appropriate pH and ionic strength. In the following sections, it will be useful to refer to assembly reactions as either “one-step” or “two-step” depending on the number of dialysis steps that follow the initial mixing of CP and RNA.

One-step assemblies

To test the extent of assembly under conditions of weak CP–CP attraction and strong CP–RNA attraction, we dialyzed a solution of fluorescently labeled CP and RNA in (7.2,1.0)-buffer against (7.2,0.1)-buffer and analyzed the products by a combination of velocity sedimentation and transmission EM (Fig. 3).

The fluorescence intensity profiles of fractions collected from sucrose density gradient assays show that the reaction products co-sediment as a broad band of complexed CP and RNA with a density similar to that of the CCMV virion (Fig. 3a, black curve). Analytical ultracentrifugation measurements confirm this result (data not shown). Unlike CCMV virions produced in a natural infection, or in a two-step assembly (see the following section), these nucleoprotein complexes are not stable against RNase A—the sedimentation profile

after RNase treatment shows that the complexes were completely digested into low-molecular-weight species consistent with free CP dimers and short fragments of RNA (Fig. 3a, gray curve).

Direct visualization of the reaction products by cryo-EM clarifies the basis for their sedimentation behavior and susceptibility to RNase digestion. As seen in Fig. 3b, the complexes imaged in vitrified *(7.2,0.1)-buffer* appear as disordered clumps with diameters (~30 nm) similar to that of the CCMV virion, accounting for their similar sedimentation profiles. However, unlike the native virion, the CPs in these complexes lack the regular structure that is known to protect CCMV against digestion by RNase. Closer investigation of their structure (Fig. 4a) shows that a fraction of the population exhibits regions of curvature and topography that suggest partial capsid formation. Whether these small patches of ordered CP are long-lived or transient structures could not be determined, though long-lived partial capsid shells seem unlikely given the reversible nature of CP–CP and CP–RNA binding at neutral pH [11].

Conditions of neutral pH and low ionic strength are therefore insufficient for nucleocapsid formation. While the strength of CP–RNA attraction drives all CP in solution into nucleoprotein complexes, CP–CP attractions are too weak to produce closed capsids. The attractions appear to be sufficient for the nucleation of transient partial capsid structures, but these ordered embryos are incapable of developing into closed shells. These observations are consistent with the initial stages of a generalized assembly pathway first described by McPherson [24] and later by Devkota *et al.* [25], in which CP initially binds the nucleic acid, forming a disordered complex that later rearranges to form the ordered capsid. This scenario, named the “en-masse” pathway by Elrad and Hagan [26], has been observed in coarse-grained simulations of the co-assembly of idealized CP oligomers around linear [26] and branched [27] polymers representing ssRNA under conditions when CP–RNA attraction dominates over CP–CP attraction.

The more traditional (and also less direct) imaging technique of negative-stain EM—in which sample is adsorbed onto a hydrophilic carbon-coated substrate, dried, and then treated with an electron-dense stain solution before visualization—yields higher contrast images (Figs. 3c and 4c) but suffers from a susceptibility to artifacts incurred during sample preparation. The most commonly used stain in EM, aqueous 1–2% uranyl acetate, is acidic (pH ≈ 4.5) and great caution must therefore be taken when using it to stain pH-dependent samples. Comparison of CP–RNA complexes from different regions of the same imaging sample stained with uranyl acetate (Fig. 3c and d) shows one dramatic and previously unreported artifact—*staining with uranyl acetate can induce CCMV capsid formation*.

Following the failure of the one-step neutral-pH assembly procedure to generate well-formed VLPs, we analyzed the products of one-step assembly under acidic conditions—that is, conditions of strong CP–CP attraction—by dialyzing a mixture of CP and RNA in *(7.2,1.0)-buffer* against *(4.5,0.1)-buffer*. Sucrose density gradients showed evidence of high-molecular-weight aggregates of CP and RNA (Fig. 5a)—all of the CP and RNA was found as a pellet at the bottom of the gradient. Direct visualization by cryo-EM showed micron-sized aggregates of capsid-like structures (Fig. 5b). The increased contrast of negative-stain

micrographs revealed that the capsids comprising the aggregate were often irregular in structure (i.e., non-spherical; Fig. 5c). Because the sample had been acidified before staining, potential artifacts arising from the low pH of uranyl acetate were not a concern. Treatment of these aggregates with RNase resulted in the generation of CP–RNA complexes with a sedimentation profile similar to that of closed VLPs (Fig. 5a), suggesting that the aggregated capsids are held together by RNA.

Previous studies [2] have shown that RNA can be shared between two or more CCMV capsids, the RNA serving as a tether between particles and forming small bunches of VLPs (multiplet capsids). Addition of RNase to these multiplet structures results in the digestion of the RNA tethers and liberation of the constituent capsids. The aggregated structures resulting from the low-pH assembly reaction appear to be an extreme case of such multiplet formation, with the majority of capsids sharing portions of RNA with at least two other capsids. Multiplet structures are not unique to CCMV and have been reported to occur in the *in vitro* assembly of turnip crinkle virus [20] under “conditions that favor assembly so strongly that they lock in mistakes”, as well as for bacteriophage ϕ r [28] when the RNA packaged was significantly longer than the viral genome. While the mechanism of multiplet formation has not been established experimentally, coarse-grained simulations [26,29] suggest that it is a form of kinetic trapping resulting from strong CP–CP attraction—consistent with our experimental observations and with other kinetic–thermodynamic models [30,31].

Two-step assemblies

Individually, both the neutral-pH and the low-pH one-step assembly reactions fail to generate well-formed spherical VLPs. However, a two-step assembly procedure consisting of an initial equilibration at neutral pH and subsequent acidification results in nuclease-resistant particles with closed CP capsids. This two-step assembly was achieved by mixing CP and RNA in *(7.2,1.0)-buffer*, dialysis against *(7.2,0.1)-buffer* overnight, and a second dialysis step of 6 h against *(4.5,0.1)-buffer*.

As seen by the sucrose gradient profile (Fig. 6a), the products of this two-step pH-dependent assembly procedure have an average size and density comparable to those of the complexes before acidification, with the addition of a slight high-density shoulder. However, these complexes are not converted to low-molecular-weight species upon treatment with RNase—as seen in Fig. 6a, the CP sedimentation profile is unchanged. Moreover, both negative-stain EM and cryo-EM show that acidification leads to the formation of spherical capsids that are indistinguishable from wt virions (Fig. 6b and c). The VLPs that form upon acidification persist after dialysis back to neutral pH (Fig. 7c) for at least 1 week at 4 °C (longer times were not tested).

As seen in Fig. 7a and b, the two-step procedure directs the assembly through both stages of the en-masse pathway [24–26]: disordered complex formation, followed by capsid organization. The disordered nucleoprotein complexes act as ideal pre-capsid intermediates. The initial neutral-pH equilibration period allows the system to anneal under conditions of weak CP–CP attraction into particles consisting of a single RNA molecule decorated by an excess of CP. Upon lowering the pH, the lateral interactions between nearby CP dimers

bound in the pre-capsid complex are increased and the closed capsid is formed. Comparing the products of the two-step assembly (Fig. 6) with those of the one-step neutral-pH assembly (Fig. 3), we see that acidification is necessary for generating CP–CP attractions strong enough to drive the formation of complete capsids. Further comparison against the products of the one-step acidic assembly that are shown in Fig. 5 demonstrates that the neutral-pH annealing step is crucial for avoiding kinetic traps associated with strong CP–CP attraction.

To test if capsid formation can be triggered by intermediate pH values ($4.5 < \text{pH} < 7.2$), we prepared samples of CP–RNA complexes at pH 7.2 and, in parallel, dialyzed each against assembly buffers with successively lower pH while keeping the ionic strength constant at 0.1 M. Cryo-EM and negative-stain EM (Fig. 8) of the products of each two-step, intermediate-pH reaction show that closed spherical capsids are absent above pH 6.5. At pH 6.5, a mixture of VLPs and disordered complexes is present, whereas below pH 6.0, the only structures observed are closed spherical VLPs.

CCMV undergoes numerous changes between pH 6 and 6.5. As shown in Fig. 1, there exists a sharp decrease in thermal stability of CCMV between pH 6 and 6.5. Additionally, the well-known swelling transition of CCMV is triggered by raising the pH above 6.5 in the absence of divalent metal ions [32]. Thus, the observation that the transition between disordered nucleoprotein complex and VLP occurs between pH 6.5 and 6 suggests that the lateral interactions responsible for driving capsid assembly are the same as those that are involved in maintaining capsid stability and triggering swelling. Computational simulations [6] have linked the swelling transition with the deprotonation of specific acidic residues that interact at the dimer–dimer interfaces within the capsid. We propose that the same residues play a key role in the assembly of VLPs.

One-step assemblies (revisited)

It still remained to be shown whether a one-step reaction involving moderate pH is capable of generating well-formed nucleocapsids. To test this, we directly dialyzed a mixture of CP and RNA in *(7.2, 1.0)-buffer* against *(6.0, 0.1)-buffer* (Fig. 9). The sedimentation profile generated was significantly broader and shifted toward higher density than that of the two-step reaction of Fig. 6. Both cryo-EM and negative-stain EM show that the reaction products are a mixture of well-formed single capsids and doublet capsid structures. RNase treatment sharpened the sedimentation band and, as in the case of the one-step low-pH assembly reaction, gave rise to a peak position roughly consistent with that of CCMV—the RNase-treated peak is found at fractions 7 and 8 as compared to CCMV that peaks sharply at fraction 8. We interpret the doublet particles as kinetically trapped multiplet structures similar to those produced in the one-step assembly reaction at low pH (4.5). The sedimentation profile of the RNase-treated assembly suggests that the doublets contain two RNA molecules shared between two capsids such that each constituent capsid has a composition (i.e., density) similar to wt CCMV. This interpretation suggests that reducing the absolute concentration of the assembly will reduce the presence of doublets, conceivably offering an alternative route to proper capsid formation via a one-step protocol.

Summary and conclusions

The successful self-assembly of CCMV CP and ssRNA into VLPs depends delicately on the strength of CP–CP attraction relative to CP–RNA attraction. If too weak, closed capsids do not form (Fig. 3); if too strong, the assembly suffers from kinetic traps (Fig. 5). We can control the strength of CP–CP and CP–RNA attractions *in vitro* by adjusting the pH and ionic strength, respectively: the strength of CP–CP attraction varies inversely with pH (Fig. 1), and CP–RNA attraction varies inversely with ionic strength (Fig. 2). We have determined that CP–CP attraction is too weak at neutral pH to drive VLP formation and that the pH must be decreased below 6 in order to form closed RNase-resistant nucleocapsid structures (Fig. 8). However, even under conditions that provide the weakest CP–CP attraction capable of generating closed nucleocapsids (pH 6 and $I = 0.1$ M), assembly results in kinetically trapped structures (Fig. 9).

Dividing the assembly procedure into two steps—by first lowering the ionic strength (turning on CP–RNA attraction) and later reducing the pH (turning on CP–CP attraction)—avoids the kinetic traps that are produced in the one-step reaction under conditions of moderate-to-strong CP–CP attraction. The equilibration step at neutral pH allows the system to anneal, under conditions of weak CP–CP interactions, into a homogeneous population of individual RNA molecules decorated by a high density of CP (Fig. 3). These pre-capsid intermediates resist kinetic trapping upon acidification and generate the best possible yields of spherical VLPs (Fig. 6). This two-step assembly procedure, similar to that used in packaging functionalized gold nanoparticles by BMV CP [33], should help inform the development of new virus-based nanomaterials involving self-assembled structures of CCMV CP around anionic polymer and nanoparticle cargos.

The observations that (i) the products of a one-step assembly reaction are structurally different from those of a two-step reaction carried out between identical initial and final conditions (Fig. 5c versus Fig. 6c) and that (ii) nucleocapsids that are incapable of assembling at neutral pH, once acidified, are meta-stable against dialysis back to neutral pH (Fig. 7c) demonstrate that the assembly and disassembly of RNA-containing CCMV VLPs are path-dependent processes. Unlike the assembly of pure CCMV CP [4], the assembled structures found in a mixture of CCMV CP and RNA cannot be predicted by knowledge of the pH and ionic strength alone—the buffer history is important.

The relevance of these findings *in vivo* has yet to be demonstrated. It is generally accepted that the cytoplasm of plant cells is maintained near neutral pH with ionic strength of approximately 0.1 M. Our *in vitro* results show that these conditions are insufficient for nucleocapsid formation in the absence of cellular host factors. While it has been observed that significant structural rearrangement of subcellular membranes occurs during CCMV infection [34], no measurements of variations in local conditions (e.g., pH or other ionic concentrations) within these structures have been reported. It is therefore premature to implicate acidification as a maturation pathway for CCMV *in vivo*. Likewise, while the meta-stability of closed capsids at neutral pH is an important feature of CCMV *in vitro*, *in vivo* processes exploiting these metastable states have not been directly observed.

Materials and Methods

Buffers

The following is a list of the assembly buffers used, their common name (if appropriate), and their composition: (*pH 7.2, ionic strength 1.0*)-buffer, Buffer B or Protein buffer or Dimer buffer [8], 1 M NaCl, 20 mM Tris (pH 7.2), 1 mM ethylenediaminetetraacetic acid, 1 mM DTT, and 1 mM PMSF; (*7.2,0.1*)-buffer, Bancroft's assembly buffer [7], 50 mM NaCl, 50 mM Tris-HCl (pH 7.2 or 7.0), 10 mM KCl, and 5 mM MgCl₂; (*6.5,0.1*)-buffer, 50 mM NaCl, 50 mM citric acid-NaOH (pH 6.5 or 6), 10 mM KCl, and 5 mM MgCl₂; (*4.5,0.1*)-buffer, Virus storage buffer (VSB) [35], 50 mM sodium acetate (pH 4.5) and 8 mM magnesium acetate.

CCMV purification and labeling

CCMV was purified from infected California cowpea plants (*Vigna unguiculata* cv. Black Eye) as previously described by Bancroft [12]. Alexa Fluor-647 NHS ester (AF647-NHS; Molecular Probes, USA) was covalently linked to solvent-exposed lysines on the exterior surface of CCMV by direct mixing 5 mg/mL CCMV and AF647-NHS at a mass ratio of 200:1, in 0.1 M Hepes (pH 7.2) and 5 mM MgCl₂, for 2 h at 4 °C [36], followed by overnight dialysis against VSB. Unreacted dye was removed by collecting the capsids on a sucrose cushion and resuspending them in VSB. The density of labeling (DOL), defined as the fraction of CPs labeled by AF647-NHS, was determined by UV-Vis spectroscopy according to the manufacturer.

Fluorescent labeling of CP and RNA

CCMV CP was isolated from fluorescently labeled CCMV with a DOL of ~0.05, as well as from unlabeled CCMV, as previously described by Annamalai and Rao [22]. The CP concentration and the degree of RNA contamination were measured by UV-Vis spectrophotometry; only CP solutions with 280:260 ratios greater than 1.5 (corresponding to <0.5% RNA contamination) were used in assembly reactions. SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were used to make certain that the purified protein was not cleaved.

Fluorescently labeled BMV RNA 1 was generated by *in vitro* transcription of pT7B1 plasmid [37] with T7 RNA polymerase, rATP, rGTP, rCTP, rUTP, and a fluorescently labeled rUTP-AF488 (ChromaTide® Alexa Fluor® 488-5-UTP; Molecular Probes, USA). A rATP:rGTP:rCTP:rUTP:rUTP-AF488 molar ratio of 600:600:600:5.32:1 was used and resulted in a DOL of 0.5 rUTP-AF488s per RNA. RNA was purified in TE buffer by washing the transcription products using a 100-kDa Amicon filter device.

Measuring the apparent melting curve of CCMV by differential scanning fluorimetry

The procedure was adapted from Hema *et al.* [38]. We prepared 20-mL aliquots of wt CCMV at a final concentration of 0.2 mg/mL and 2.5× SYPRO orange fluorescent dye (Molecular Probes, USA) in 50 mM phosphate buffer at pH values 5.5, 6.5, 7.5, and 8.0. An additional aliquot of identical concentrations of CCMV and dye was prepared by dilution in VSB. Differential scanning fluorimetry was performed in a 96-well plate 3200 Opticon2

(MJ Research) real-time PCR machine. All samples were heated from 25 to 95 °C, in 1 °C increments with a 15-s stabilization period at each temperature. During the stabilization period, the fluorescence intensity was measured against a blank containing the appropriate buffer and 2.5× SYPRO orange. Excitation/emission wavelengths of 470/550 nm were used. Increased fluorescence emission derives from the binding of SYPRO orange to hydrophobic regions of the CP that are exposed upon capsid melting. The apparent melting temperature is defined as the temperature at which the derivative of the fluorescence emission signal ($-dI/dT$) is maximal.

***In vitro* assembly reactions**

It has been previously reported [2] that a relative 6-fold mass excess of CP to RNA is required for complete *in vitro* packaging of ssRNA by CCMV CP. To avoid complications arising from unpackaged RNA, we carried out all assembly reactions by direct mixing 18 µg of CP and 3 µg of BMV RNA 1 in 100 µL of (7.2,1.0)-buffer, followed by subsequent dialysis and equilibration steps. All dialyses were equilibrated for at least 6 h at 4 °C.

Electron microscopy

Negative stain: 6 µL of assembly reaction was deposited on glow-discharged copper grids (400 mesh) that previously had been coated with Parlodion and carbon. After 1 min, the grids were blotted and stained with 6 µL of 2% uranyl acetate for 1 min followed by complete stain removal and storage in a desiccator overnight.

Cryo: 3 µL of assembly reaction was deposited on a Quantifoil holey carbon grid (200 mesh; R2/1) that had been previously glow discharged. The grids were then blotted and flash-frozen by rapid plunging into liquid ethane cooled to liquid nitrogen temperature. Micrographs were acquired using an FEI Tecnai G2 TF20 microscope operated at an accelerating voltage of 200 kV. Images were recorded at 3–4 microns underfocus with a TIETZ F415MP 4000 × 4000 pixel CCD camera. Total beam exposure was maintained between 20 and 40 e/Å² for cryo-EM samples.

Velocity sedimentation by sucrose density gradient centrifugation

We made 10–40% (% w/v) sucrose gradients by subjecting 3.5 mL of 25% sucrose in the desired buffer to repeated (3×) freeze–thaw cycles. The gradients were loaded with 0.2 mL of sample and centrifuged for 3 h at 30,000 rpm at 4 °C in a SW50.1 rotor (Beckman Coulter) and manually fractionated by pipetting 0.2-mL aliquots from top to bottom. The 19 extracted fractions were loaded into a 96-well flat-black flat-bottom plate. The fluorescence of the labeled CP (AF647; excitation/emission, 650/668 nm) and of RNA (AF488; 495/519 nm) were measured for each fraction using a Tecan M1000 plate reader.

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Abbreviations used

CCMV	cowpea chlorotic mottle virus
ssRNA	single-stranded RNA
BMV	brome mosaic virus
VLP	virus-like particle
CP	capsid protein
wt	wild-type
EM	electron microscopy
cryo-EM	cryo-electron microscopy
ARM	arginine-rich motif
DOL	density of labeling

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Fig. 1. Strength of CP–CP attraction is pH dependent. The apparent melting temperature of wt CCMV decreases sharply as a function of pH at $I = 0.1$ M.

Fig. 2.

Strength of CP–RNA attraction depends on ionic strength. Solutions of fluorescently labeled CP and RNA in either high-salt assembly buffer (pH 7.2, $I = 1$ M) or low-salt assembly buffer (pH 7.2, $I = 0.1$ M) were analyzed by sucrose density gradient centrifugation. Fractions were collected from top (fraction 1) to bottom (fraction 15), and the fluorescence intensity was measured. (a) Purified CP has a salt-independent sedimentation profile that peaks at the top of the gradient. (b) Purified RNA also showed a salt-independent profile. (c) CP and RNA equilibrated in high-salt assembly buffer for 4 h at 4 °C do not interact. (d) CP and RNA equilibrated in low-salt assembly buffer for 4 h at 4 °C co-sediment as a high-density complex of CP and RNA.

Fig. 3.

Assembly under conditions of weak CP-CP attraction results in disordered complexes of CP and RNA. Fluorescently labeled CP and RNA were mixed in $(7.2,1.0)$ -buffer, dialyzed against $(7.2,0.1)$ -buffer, and equilibrated overnight at 4 °C. (a) The sedimentation profile of the assembly products with CP is plotted as black squares and that of RNA is plotted as black triangles. An aliquot of product that was treated with RNase A was analyzed in parallel, and the CP profile is plotted as gray squares. The arrow shows the peak fraction of CCMV virions when analyzed under identical conditions. Fractions were collected from top (fraction 1) to bottom (fraction 19). (b) Cryo-EM micrograph of the reaction products. (c and d) Uranyl acetate negative-stain EM micrographs—in (c), the stain was carefully and completely removed from the grid before storage; in (d), excess stain was not completely removed. Scale bars represent 28 nm.

Fig. 4.

Disordered CP-RNA complexes display some capsid features. (a–c) Regions of electron micrographs that have been enlarged to enable the examination of single particles. (a) Cryo-EM image of a CP-RNA complex visible in the upper left corner of Fig. 3b. (b) Cryo-EM image of wt CCMV. (c) Negative-stain EM image of wt CCMV. (d) Cryo-EM reconstruction of the CCMV virion taken with permission from Fox *et al.* [23]. Note that the right-most edge of the largely disordered complex shown in (a) has curvature and “knobbiness” that are consistent with the CCMV capsid and its morphological units.

Fig. 5.

Aggregated structures of irregular capsids result from one-step low-pH assembly. Fluorescently labeled CP and RNA were mixed in *(7.2,1.0)-buffer*, dialyzed against *(4.5,0.1)-buffer*, and equilibrated overnight at 4 °C. (a) The sedimentation profile of the assembly products with CP is plotted as black squares and that of RNA is plotted as black triangles. An aliquot of product that was treated with RNase A was analyzed in parallel and the CP profile is plotted as gray squares. The arrow shows the peak fraction of CCMV virions when analyzed under identical conditions. Fractions were collected from top (fraction 1) to bottom (fraction 19). (b) Cryo-EM micrograph of the reaction products at low magnification; note that the aggregates are microns in size. (c) Negative-stain EM micrograph. The scale bar represents 280 nm in (b) and 28 nm in (c).

Fig. 6.

Two-step pH-dependent assembly generates capsid structures from disordered complexes by turning on CP-CP attraction. Fluorescently labeled CP and RNA assembled into disordered complexes as in Fig. 3 was further dialyzed against low pH $(4.5, 0.1)$ -buffer and equilibrated for 6 h at 4 °C. (a) The sedimentation profile of the assembly products with CP is plotted as black squares and that of RNA is plotted as black triangles. Aliquot of product that was treated with RNase A was analyzed in parallel and the CP profile is plotted as gray squares. The arrow shows the peak fraction of CCMV virions when analyzed under identical conditions. Fractions were collected from top (fraction 1) to bottom (fraction 19). (b) Cryo-EM micrograph. (c) Negative-stain EM micrograph. Scale bars represent 28 nm.

Fig. 7.

Nucleocapsid formation shows strong hysteresis. Cryo-EM micrographs (negative-stain images are shown in insets) of assembly reactions with different buffer histories. (a) Images of disordered CP–RNA complexes, prepared as in Fig. 3, in neutral pH $(7.2, 0.1)$ -buffer. (b) Spherical VLPs formed by dialyzing the complexes in (a) against low pH $(4.5, 0.1)$ -buffer. (c) The spherical particles formed in (b) maintain their structure after dialysis back to neutral pH $(7.2, 0.1)$ -buffer. Note that the samples imaged in (a) and (c) are composed of chemically equivalent mixtures of CP and RNA suspended in identical buffer solution; they differ only in their buffer history either previously acidified (c), or not (a). Scale bars represent 28 nm.

$(7.2, 1.0)d(7.2, 0.1)d(\pi, 0.1)$

Fig. 8.

The transition between disordered complexes and spherical particles occurs between pH 6.5 and 6. CP and RNA were assembled into disordered complexes, as in Fig. 3, and aliquots were dialyzed, in parallel, against buffered solutions of constant ionic strength ($I = 0.1$ M) and successively lower pH. Negative-stain EM (top) and cryo-EM (bottom) micrographs are shown for final pH values $\pi = 7.2, 7.0, 6.5$, and 6.0 . Disordered complexes dominate at pH values above 6. A sharp transition occurs between pH 6 and 6.5 in which all complexes are converted to spherical VLPs. The scale bar represents 28 nm.

Fig. 9.

Singlet and doublet capsid structures result from one-step moderate-pH assembly. Fluorescently labeled CP and RNA were mixed in $(7.2, 1.0)$ -buffer, dialyzed against $(6.0, 0.1)$ -buffer, and equilibrated overnight at 4 °C. (a) The sedimentation profile of the assembly products with CP is plotted as black squares and that of RNA is plotted as black triangles. An aliquot of product that was treated with RNase A was analyzed in parallel and the CP profile is plotted as gray squares. The arrow shows the peak fraction of CCMV virions when analyzed under identical conditions. Fractions were collected from top (fraction 1) to bottom (fraction 19). (b) Representative cryo-EM micrograph of the reaction products. (c) Representative negative-stain EM micrograph. Note the presence of doublet particles. Scale bars represent 28 nm.