Encapsulation of DNA, a protein and a fluorophore into virus-like particles by the capsid protein of cucumber mosaic virus

Xiaoyun Lu, Jeremy R. Thompson and Keith L. Perry

Department of Plant Pathology and Plant–Microbe Biology, 334 Plant Science, Cornell University, Ithaca, NY 14853, USA

An important property of some spherical plant viruses is their ability to reassemble in vitro from native capsid protein (CP) and RNA into infectious virus-like particles (VLPs). Virions of cucumber mosaic virus (CMV) are stabilized by protein–RNA interactions and the nucleic acid is essential for assembly. This study demonstrated that VLPs will form in the presence of both ssDNA and dsDNA oligonucleotides, and with a lower size limit of 20 nt. Based on urea disruption assays, assembled VLPs from CMV CP and RNA (termed ReCMV) exhibited a level of stability similar to that of virions purified from plants, whilst VLPs from CMV CP and a 20mer exhibited comparable or greater stability. Fluorescent labelling of VLPs was achieved by the encapsidation of an Alexa Fluor 488-labelled 45mer oligonucleotide (ReCMV-Alexa488-45) and confirmed by transmission electron and confocal microscopy. Using ssDNA as a nucleating factor, encapsidation of fluorescently labelled streptavidin (53 kDa) conjugated to a biotinylated oligonucleotide was observed. The biological activity and stability of ReCMV and ReCMV-Alexa488-45 was confirmed in infectivity assays and insect vector feeding assays. This work demonstrates the utility of CMV CP as a protein cage for use in the growing repertoire of nanotechnological applications.

INTRODUCTION

The capacity for biological macromolecules to self-assemble into structurally well-defined aggregates is of particular interest to technologists in the development of nanostructured devices and to molecular biologists in the development of drug delivery and imaging systems (Lowe, 2000; Wu & Payne, 2004). As such, plant virus capsid proteins (CPs) have been studied for their ability to form multilayered arrays (Steinmetz et al., 2008), nanotubes (Wang et al., 2008), light-harvesting systems (Miller et al., 2007) and diagnostic imaging devices (Gonzalez et al., 2009), and for epitope display (Smith et al., 2009). (For more general reviews, see Douglas & Young, 2006; Fischlechner & Donath, 2007; Manchester & Singh, 2006; Ren et al., 2010; Singh et al., 2006; and Young et al., 2008).

Plant viruses have played a critical role in our understanding of molecular self-assembly: the first example of in vitro self-assembly of a biological particle was for the rod-like particles of tobacco mosaic virus (TMV) by Fraenkel-Conrat & Williams (19555). Since then, research has concentrated mainly on exploiting the structural properties of TMV, and the icosahedral particles of brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV) and cowpea mosaic virus (CPMV) (Young et al., 2008, and references therein).

One aspect in this latter group of viruses that has received particular attention has been the encapsidation of non-viral cargos with various potential objectives. For CCMV, the production of vessels for nanomaterial synthesis has been demonstrated where the porosity of the virions and their pH-dependent capacity to gate was utilized to regulate the encapsidation of an anionic organic polymer (Douglas & Young, 1998). Virions of BMV assembled around gold nanoparticles using citrate (Dragnea et al., 2003) and polyethylene glycol (Sun et al., 2007) as an intermediate have also been successfully obtained. The possible application of these virus-like particles (VLPs) in imaging and the increasing flexibility in design is evidenced in the conjugation of fluorophores both externally to the surface of virions for CPMV (Lewis et al., 2006) and turnip yellow mosaic virus (Barnhill et al., 2007), and internally by non-covalently anchoring EGFP via a linker to the N terminus of the CP of CCMV (Minten et al., 2009).

Among the first plant viruses in which in vitro reassembly was demonstrated was cucumber mosaic virus (CMV), a member of the family Bromoviridae to which CCMV and BMV also belong. CMV virions have T=3 symmetry and their structure has been determined by X-ray crystallography to a resolution of 3.2 Å (Fraenkel-Conrat, 1970; Kaper, 1969; Kaper & Geelen, 1971; Palukaitis & García-Arenal, 2003; Palukaitis et al., 1992; Smith et al., 2000). Remarkably, the CMV structure is nearly identical to that of CCMV,
although their CPs share only 19% amino acid sequence identity and 34% similarity (Smith et al., 2000; Wikoff et al., 1997). Key structural features of the CMV CP that influence virion formation are the positively charged N terminus, a proximal domain-forming part of a unique inter-subunit bundle of six amphipathic helices, and amino acids involved in inter-subunit base pairing (Smith et al., 2000; Wikoff et al., 1997). Unlike CCMV, CMV virions do not undergo swelling at pH 7.0, whereas CCMV virions exhibit a pH-dependent swelling from ~280 to 300 Å diameter (Speir et al., 1995). The permanently ‘swollen’ state of CMV virions and their porosity is thought to explain the sensitivity of the viral RNA to RNases (Wikoff et al., 1997). CMV also differs from CCMV and some other members of the Bromoviridae in its requirement for a nucleic acid to stabilize virions. By contrast, CCMV can reassemble in vitro into empty VLPs in the absence of a nucleic acid (Zhao et al., 1995). Whilst BMV CPs can assemble into VLPs in vitro under low-pH conditions in the absence of a nucleic acid, assembly under physiological conditions is dependent on tRNA-like structures of a nucleic acid (Choi et al., 2002). After the reversible dissociation of CMV was first demonstrated in 1969 (Kaper, 1969), subsequent reassembly work showed that hybrid virions containing the CPs of both CMV and the cucumovirus tomato aspermy virus could be reassembled in the presence of viral RNA and that insect vector transmission specificity was a function of the CP (Chen et al., 1995).

In this study, we have demonstrated the utility of CMV in encapsidating a variety of non-viral molecules. First, we showed that both ssDNA and dsDNA of less than 45 nt were sufficient for virion reassembly to occur, although single-stranded 15mers failed to produce any VLPs. We then internally labelled virions by coupling a fluorophore to an oligomer and showed the applicability of such a construct in the context of its biological activity. Lastly, we demonstrated the encapsidation of fluorescently labelled streptavidin molecules coupled to biotinylated oligomers. This work as a whole demonstrates the potential of CMV CPs to be exploited in the production of VLPs for biological and nanotechnological studies.

RESULTS AND DISCUSSION

CMV CPs encapsidate ssDNA and dsDNA of 20 nt but not of 15 nt

The strategy for the reassembly of VLPs is shown in Fig. 1. The in vitro reassembly system with CMV viral RNAs was initially confirmed by gel electrophoresis of native reassembled CMV particles (ReCMV). In an agarose gel, the reassembled virions migrated to the same position as the purified wild-type CMV (Fig. 2). The integrity of the reassembled products was validated further by transmission electron microscopy (TEM) of purified CMV and ReCMV, where no differences were observed in virion size or shape (Fig. 3a, b). Reassembly was also achieved for ssDNA oligonucleotides of 70, 45 and 20 nt (Table S1, available in JGV Online) but not for an oligonucleotide of 15 nt (Figs 2 and 3c). Importantly, other CMV oligonucleotide sequences of this size (one additional oligonucleotide for each size) were also tested, yielding the same results (data not shown), showing that the requirements for virus reassembly were not sequence specific, a finding supported by earlier studies (Fraenkel-Conrat, 1970) and work carried out using short plasmid-derived DNA fragments (Chen, 1991). In addition a dsDNA (CMV-20-duplex) was also successfully encapsidated (Fig. 2), and wild-type-like VLPs observed by TEM (Fig. 3d). The specific oligonucleotide band observed in gels corresponding to unincorporated DNA for ReCMV-70, ReCMV-45 and ReCMV-20 (Fig. 2) could be removed from the reassembly products by adding more CP in the reassembly process (data not shown). An additional observation was that the DNA-containing VLPs consistently migrated slightly slower than the native virions and ReCMV, suggesting there may be slight differences in the structure or particle stability of the VLPs or, more likely, that they contain less total nucleic acid and therefore have a lower negative charge.

VLPs encapsidating a 20mer oligonucleotide have comparable stability to that of native or reassembled CMV

To test whether there was a difference in particle stability, ReCMV and particles encapsidating a 20mer CMV ssDNA
oligonucleotide (CMV-20) were treated with a range of urea concentrations to assess their stability (Fig. 4). The electrophoretic migration of native CMV virions was altered at urea concentrations above 2 M, indicative of virion disruption (Ng et al., 2000). Similarly, for ReCMV, disruption was observed at urea concentrations of 3–4 M. The VLP products of ReCMV-20 migrated as a more uniform and discrete band in the gel. A reduction in staining intensity was observed at 3 and 4 M urea, and eliminated at 5 M. The fact that there was still a band visible at 4 M urea suggested that ReCMV-20 VLPs had a stability comparable to that of the native virions. Treatment of the native and ReCMV virions with 5 M urea resulted in altered electrophoretic migration, indicating virion disruption but not disruption of the ribonucleoprotein complexes. By contrast, the absence of any visible staining at 5 M for ReCMV-20 suggested that the nucleic acid–protein complexes were completely disrupted.

Disassembly of assembled VLPs encapsidating a dsDNA confirms the nature of their contents

In a previous study, dsDNA was not observed to be encapsidated by CMV CPs (Chen, 1991). The dsDNA 20mer used here was obtained commercially as an HPLC-purified molecule (Integrated DNA Technologies). We wanted to confirm that the encapsidated CMV-20-duplex form was visible following disassembly. CMV-20 and CMV-20-duplex were encapsidated as described previously, disassembled and their contents separated by centrifugation followed by electrophoresis on an agarose gel alongside the original ssDNA and dsDNA oligonucleotides (Fig. 5). The CMV-20-duplex recovered from the VLPs migrated to the same position as the original dsDNA and was easily distinguishable from the single-stranded molecule of the same sequence (CMV-20), thereby confirming that both ssDNA and dsDNA can function to nucleate VLP assembly.

Fig. 2. Non-denaturing agarose gel electrophoresis of 10 μg CMV CP reassembly products encapsidating DNA oligonucleotides. ReCMV-70, ReCMV-45, ReCMV-20, ReCMV-15 and ReCMV-20-duplex refer to the reassembly products of reactions with single-stranded 70-, 45-, 20-, 15mer and double-stranded 20mer oligonucleotides, respectively. ReCMV indicates reassembly products with CMV RNAs, whilst CMV indicates native, purified virions. Unincorporated oligonucleotides are visible at the bottom of the gel. Lane M, GeneRuler DNA Ladder Mix (Fermentas); thin arrow, 100 bp size marker; thick arrow, 3 kb size marker.

Fig. 3. TEM images of native, purified CMV virions (a), reassembled virions with CMV RNAs (ReCMV) (b), VLPs with a single-stranded 20mer oligonucleotide (ReCMV-20) (c), VLPs with a double-stranded 20mer oligonucleotide (ReCMV-20-duplex) (d), VLPs with a single-stranded 45mer oligonucleotide labelled with Alexa Fluor 488 (ReCMV-Alexa488-45) (e) and VLPs with a biotinylated 70mer oligonucleotide and streptavidin labelled with Alexa Fluor 488 (ReCMV-Bt-70+Str-Alexa) (f). The virion and VLP preparations were negatively stained with 1 % uranyl acetate. Bar, 100 nm.
CMV capsids encapsidate fluorophore-labelled DNA

In order to label virions, a DNA oligomer of 45 nt conjugated to the fluorophore Alexa Fluor 488 was subjected to reassembly with CMV CP using the same procedure as for the DNA oligonucleotides. The resultant VLPs were analysed on an agarose gel (Fig. 6a, b) and by TEM (Fig. 3e). From the gel, it was apparent that almost all of the fluorophore-labelled DNA was incorporated into particles.

Encapsulation of a 53 kDa protein conjugated to DNA and a fluorophore

Based on the use of DNA as a nucleation factor for encapsidation, we wanted to test whether a protein as large as streptavidin could be encapsidated in vitro. Streptavidin conjugated to Alexa Fluor 488 was bound to a biotinylated DNA oligonucleotide (CMV-Bt-70), reassembly was performed and migration of the products was compared in an agarose gel. As controls, we used reassembly reactions containing the CP and (i) an unbiotinylated oligonucleotide of the same sequence (CMV-70) in the absence of streptavidin, (ii) the unbiotinylated oligonucleotide CMV-70 with the streptavidin fluorophore conjugate (CMV-70 + Str-Alexa), (iii) the streptavidin fluorophore conjugate in the absence of an oligonucleotide (Str-Alexa), and (iv) the biotinylated oligonucleotide in the absence of streptavidin (CMV-Bt-70). Only the reassembly reaction initiated with the biotinylated oligonucleotide conjugated to fluorescent streptavidin (CMV-Bt-70 + Str-Alexa) resulted in a product showing a significant pre-ethidium bromide fluorescent signal, indicating incorporation of the protein (Fig. 6c, d). The integrity of these VLPs was confirmed by TEM and shown to have the size (Fig. S1) and shape (Fig. 3f) of native virions. VLPs formed with streptavidin were additionally analysed spectrophotometrically, and the spectra for ReCMV-Bt-70 + Str-Alexa showed an intermediate absorption profile characteristic of both CMV virions and the oligonucleotide–streptavidin complex (CMV-Bt-70 + Str-Alexa; Fig. 6e).

Reassembled virions maintain their biological activity and stability

In order to evaluate the biological activity of ReCMV, infectivity assays were performed. Two independent preparations of ReCMV were infectious at a mean dilution of 2^11, whilst purifications of native CMV showed a statistically significant higher level of infectivity at a mean dilution of 2^12 (total of 13 infectivity experiments, P=0.013 by t-test). The biological activity of ReCMV was also evaluated by testing for its transmission by an aphid vector. When aphids were fed on a solution of the virus through a Parafilm membrane, the transmission of ReCMV was not significantly different from that of purified CMV (for experimental design, see Ng et al., 2005; five experimental replicates, P=0.22 by t-test). The fluorescently labelled ReCMV-Alexa488-45 particles were also tested for their ability to be used as a tracer in vivo. Imaging of a solution of the VLP preparation by confocal microscopy with excitation at 488 nm showed uniform fluorescence with some indication of aggregation (Fig. S2). ReCMV-Alexa488-45 was fed to aphid vectors in a sucrose solution and the mouthparts visualized by confocal microscopy.
Although no specific interaction in the mouthparts was consistently observed, the aphid feeding tube (stylet) was clearly visualized (Fig. S3).

In comparison with other virus systems that require a nucleic acid for assembly, the nucleating factor required for CMV is relatively simple. For example, in the red clover necrotic mosaic virus system, the assembly of wild-type virions requires an origin of assembly consisting of two interacting nucleic acids (Loo et al., 2006, 2007). Similarly, BMV virion in vitro reassembly has been optimized by using synthetic DNA to mimic the capsid-binding site of native RNA (Chen et al., 2005). In addition, there is an apparent degree of flexibility in the type and size of cargos that can be encapsidated. To our knowledge, streptavidin at 53 kDa is the largest globular protein to date to have been encapsidated. Other large molecules include anionic polyacid chains of 450 kDa in hibiscus chlorotic ringspot virus (Ren et al., 2006) and gold particles of 18 nm in BMV (Sun et al., 2007). Although not determined, a relatively low number of streptavidin molecules would be expected to be encapsidated; this was observed for CCMV.
encapsulation of an enzyme (Comellas-Aragonès et al., 2007). In considering the size constraints, the internal core of CMV has been estimated to have a diameter of 110 Å (Wikoff et al., 1997), whilst the dimension of the tetrameric form of streptavidin is estimated to be 54 × 58 × 48 Å (Hendrickson et al., 1989), indicating that there could be a limited capacity beyond two molecules per virion without any additional swelling.

Taken together, these results demonstrate: (i) the relative simplicity of seeding the assembly of CMV CPs into VLPs using nucleating factors of non-specific ssDNA or dsDNA as short as 20 nt, and (ii) the ability, using such a nucleating factor, to stably encapsidate different molecule species conjugates, including a fluorophore and a large globular protein. A number of properties of this experimental system will facilitate in vitro assembly with non-viral cargos for nanotechnological applications; these include one of the broadest host ranges of any virus (>1000 species), allowing a flexibility in the choice of host plant used for virus propagation, high levels of virion accumulation in the host (making the purification of milligram quantities of purified coat protein from plants a routine operation) and the relative purity and stability of the virions over a range of pHs.

**METHODS**

**Virus maintenance and purification.** CMV Fny strain (subgroup I) was maintained in Nicotiana tabacum after mechanical inoculation and purified by a standard CMV procedure (Ng et al., 2000). The purified virions were stored at 4 °C in 5 mM sodium borate buffer (pH 9.0) containing 0.5 mM EDTA and their concentration determined spectrophotometrically assuming an extinction coefficient of CMV at a wavelength of λ = 260 nm of ε = 5.0 ml mg⁻¹ cm⁻¹ (Francki et al., 1966).

**Dissociation of purified CMV and preparation of CP.** CMV CP was dissociated from purified CMV by a LiCl dissociation procedure (Kaper, 1969) with the following modifications. Purified CMV (4 ml) was mixed with an equal volume of 6 M LiCl, incubated at −70 °C for 20 min and thawed on ice. One millilitre of the mixture was removed and subjected to an overnight reassembly (described below) as a reassembly control. From the remaining 7 ml, viral RNAs were removed by centrifugation at 15 000 g for 10 min and stored on ice for use as a reassembly control. The supernatant containing viral CPs was successively dialysed for 1 h against the following buffers: 2 M LiCl, 1 M LiCl [both in 0.5 M Tris/HCl (pH 8.4), 2.5 mM DTT] and 0.5 M LiCl [in 0.2 M Tris/HCl (pH 8.4), 2.5 mM DTT]. The resulting preparation was clarified by ultra centrifugation at 180 000 g for 90 min and the protein concentration determined spectrophotometrically by Warburg–Christian analysis (BioMate 3; Thermo Scientific) as described by the manufacturer.

**In vitro assembly of CMV virions.** The clarified CMV CPs were mixed with CMV RNAs obtained from the dissociation process (the treatment designated ReCMV), DNA oligonucleotides or DNA oligonucleotide conjugates (Integrated DNA Technologies) at a ratio of RNA (w/w) of 4:1 in an autoclaved Spectra/Por dialysis membrane tube (MWCO 12–14 000 Da; Spectrum Laboratories). The oligonucleotides selected corresponded to regions of CMV RNA 3 (Table S1). Dialysis was carried out against reassembly buffer TCMg [20 mM Tris/HCl (pH 7.2), 80 mM KCl, 1 mM DTT, 1 mM MgCl₂] with gentle stirring at 4 °C overnight (Kaper, 1969). The reassembled CMV virions (ReCMV) were collected by ultracentrifugation at 180 000 g for 90 min and resuspended in 5 mM sodium borate buffer (pH 9.0). For streptavidin encapsidation, biotinylated primer CMV-Bt-70 was first incubated with streptavidin–Alexa Fluor 488 conjugate (Invitrogen) at a ratio (w/w) of 1:5:1 in 100 mM Tris/HCl (pH 8.0) for 1 h at room temperature to allow conjugation. The conjugate was then introduced directly to the CP mix.

**Microscopy.** For TEM, samples were prepared by mounting 5 μl virion sample onto a 300-mesh Formvar- and carbon-coated copper grid and air drying for 30 s. The sample was stained with 2% (w/v) aqueous uranyl acetate solution and examined with a JOEL 100CXII transmission electron microscope. Confoval laser-scanning images were taken with a TCS SP5 laser-scanning confocal microscope (Leica Microsystems). Focal planes were scanned with a 488 nm argon laser using a 550 nm (FVX-BA550RF) barrier filter with a 40× immersion objective. Images were processed using the software (LAS AF) provided by the manufacturer.

**Urea disruption assay.** Approximately 50 μg virion sample was treated with 0–6 M urea in 1 × TAE buffer [40 mM Tris/HCl (pH 7.4), 20 mM sodium acetate, 1 mM EDTA] in a total volume of 50 μl and incubated at 20 °C for 1 h. Tracking dye (6 × DNA loading dye; Fermentas) was added to each sample at a ratio of 1:3 (tracking dye:sample). Fifteen microlitres of the mixture was subjected to electrophoresis under non-denaturing conditions in a 1.2% (w/v) agarose/TAE gel. The gel was stained with 1 μg etidium bromide ml⁻¹, washed with water and photographed on an UV transilluminator.

**Biological assays.** Non-viruliferous Aphis gossypii nymphs were reared on healthy cucumber seedlings. Before acquisition, leaves with aphids were detached and placed in plastic dishes with tight-fitting lids. The aphids were starved for 48 h by storing the plastic dishes at 4 °C, after which they were shaken off onto a Petri dish and held for 1 h at room temperature. An artificial diet for membrane feeding on Parafilm (Pechiney Plastic Packaging Co.) was prepared by suspending virions at 1 mg ml⁻¹ in 20 mM sodium phosphate buffer (pH 7.0) containing 5% (w/v) sucrose. One hundred and fifty microlitres of the artificial diet was sandwiched between two Parafilm membranes stretched over one end of a plastic tube (15 mm internal diameter). Starved aphids were transferred with a camel-hair brush to the membrane and allowed 30–90 s of feeding for acquisition probes. Probing was observed under a dissecting microscope, and groups of three actively feeding aphids were transferred to the leaves of 2-week-old healthy tobacco plants and allowed to feed overnight. The plants were sprayed with an insecticide (5%, w/v, Orthene; Valent), placed in a greenhouse for 14–21 days and scored for symptom development. For confocal microscopy imaging, styllets of actively feeding aphids were cut under a dissecting microscope and examined under a confocal microscope; the process took on average 2 min. For infectivity assays, mechanical inoculation of carbonodium-dusted leaves of 4–6-week-old N. tabacum plants was performed with 5 μl of a twofold dilution series of a 1 mg ml⁻¹ virion stock solution diluted to 2⁻¹⁵. Symptoms were scored at 14–21 days post-inoculation.

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