Core-controlled polymorphism in virus-like particles

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This study concerns the self-assembly of virus-like particles (VLPs) composed of an icosahedral virus protein coat encapsulating a functionalized spherical nanoparticle core. The recent development of efficient methods for VLP self-assembly has opened the way to structural studies. Using electron microscopy with image reconstruction, the structures of several VLPs obtained from brome mosaic virus capsid proteins and gold nanoparticles were elucidated. Varying the gold core diameter provides control over the capsid structure. The number of subunits required for a complete capsid increases with the core diameter. The packaging efficiency is a function of the number of capsid protein subunits per gold nanoparticle. VLPs of varying diameters were found to resemble to three classes of viral particles found in cells (T = 1, 2, and 3). As a consequence of their regularity, VLPs form three-dimensional crystals under the same conditions as the wild-type virus. The crystals represent a form of metamaterials with optical properties influenced by multipolar plasmonic coupling.

Results and Discussion

Symmetric VLPs may provide a technology for therapeutic or diagnostic agent delivery that is improved over amorphous shell nanoparticles that are already known to be efficient in similar applications (21). The advantage of a regular surface protein motif is that the binding domains are functionally identical by virtue of their equivalent environment. It has been shown in several situations that receptor-mediated targeting can be achieved even when using amorphous coatings (22). However, the principle challenges for nanoparticle delivery currently include: limited life-time in body fluids, nanoparticle transduction across the cellular membrane, avoidance of the exocytotic pathways, and target specificity. To optimize their infectivity, viruses have evolved to overcome these challenges. We still must learn how to apply virus strategies to targeted delivery. A simple question is central to this objective. What is the minimum set of requirements that a cargo must satisfy to promote encapsidation without interfering with its carrier? Answering this question for a model system should provide novel benchmarks for future development of nanoparticle delivery platforms. The present paper is a step in this direction.

Major factors that could affect the efficiency of VLP formation include the chemical and physical properties of the core surface. We have determined that a gold core functionalized with a coating of carboxylated polyethylene glycol (PEG) can allow efficient assembly of VLPs (18). Here, we report some first steps in a systematic examination of how these cores influence the capsid structures and functional properties. The ultimate goal is to determine the minimal set of properties that an artificial core has to possess to promote the assembly of a symmetric viral protein structure around it. The main question to be addressed is then: what is the relation between the core surface properties and the capsid morphology? In this paper, we are specifically focusing on the gold core size.

We used brome mosaic virus (BMV) as a model system to provide the protein cage for our VLPs because it can yield an abundance of capsid. BMV is a small icosahedral virus (~280 Å) that infects Poaceae species (23). The crystal structure of the native BMV has been solved and is composed of 180 identical proteins of 20 kDa that form pentameric or hexameric subunits in a T = 3 lattice (24). The basic subunit of assembly is a dimer. The BMV capsid is compact at low to moderate ionic strength buffers with pH below 5.0, but experiences a profound structural transition when the pH is increased from 5 to 7 (25). Mg2+ stabilizes the capsid at pH levels close to neutrality, where reversible expansion occurs without dissociation. At pH above 7.5 and ionic strength higher than 0.5 M, the capsid dissociates and the viral RNA precipitates. Upon reestablishment of low pH and ionic strength, reassociation occurs. Wild-type BMV is composed of three nearly identical classes of particles that contain separate RNA1, RNA2, or RNA3, and RNA4. In plants and in a replication-competent host for BMV replication, Saccharomyces cerevisiae, empty particles of the T = 3 geometry and a smaller pseudoT = 2 capsid have been observed (26–28). A T = 1 geometry forms in vitro after cleavage of the N-terminal 63 or 35 amino acid residues of the capsid protein (CP) by trypsin or endogenous protease (29). The interaction between RNA and protein is proposed to mediate the transition between the pseudoT = 2 and the T = 3 forms of BMV, and the length of the RNA could mediate a switch from T = 1 to pseudoT = 2 capsid (28). The relatively nonspecific association between the negatively charged nucleic acid core and the dense positive charge of the internal capsid surface make BMV an ideal system for nanoparticle encapsidation. The ability to produce structurally distinct viral particles suggests that BMV capsids can be used to produce structurally and functionally distinct constructs in a regulated manner.

Supporting Information.

For further details, see supporting information (SI) Figs. 6–11.

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Abbreviations: VLP, virus-like particle; PEG, polyethylene glycol; BMV, brome mosaic virus; CP, capsid protein; AFM, atomic force microscopy.

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Incorporation Efficiency. When gold particles are absent from the assembly mixture, a larger number of empty capsids are observed in TEM micrographs than in the presence of Au (18). Incorporation of gold nanoparticles is therefore the result of the competition between the formation of empty capsids and other aggregates and VLPs. In the absence of a kinetic model to quantitatively describe this competition, we define efficiency of the VLP synthesis as the ratio between the final number of complete VLP particles formed and the total number of Au particles.

For all gold core sizes, the relative efficiency of encapsidation depends on the initial CP to gold nanoparticle ratio (CP/Au) with a threshold value above ~100 protein subunits per gold nanoparticle (Fig. 1A). At low CP/Au ratios, the efficiency of VLP₆ is greater than VLP₁₂. At the ratio of 270:1 used throughout this paper, efficiency peaks at ~70% for VLP₁₂, in agreement with earlier findings, suggesting that the Au cores act as nucleating centers for capsid self-assembly (Fig. 1B). For VLP₁₂, there are typically <5% empty capsids and very few naked gold particles (<1% of the total amount of gold). VLP₆ and VLP₉ also yielded preparations homogeneous in size and shape (SI Fig. 6). However, the assembling efficiencies were noticeably lower, mainly due to an increase in the number of empty particles seen in electron micrographs. For VLP₁₅ and VLP₁₈, the gold particles became heterogeneous in size and shape (SI Fig. 6). Fewer than 45% of the gold particles had a complete capsid. Correspondingly, we observed an increase in the proportion of empty particles, naked gold particles, gold cores that were incompletely coated with protein, and VLPs with irregular protein coats.

Mean VLP diameters correlated well with gold core diameters (Fig. 1C). However, analysis of VLP size histograms indicates that there are at least three subpopulations of particles: 25, 29, and 32 nm (dots in Fig. 1C and SI Fig. 8). The VLPs measured for 3D image reconstruction also yielded the three basic diameters, although somewhat smaller: 21–22, 26, and 28 nm. The source of the difference in these measurements is not clear. It may come from sample preparations or digitization procedures. Au cores of 15 nm and larger were more heterogeneous, in terms of size and shape, than the smaller cores. However, when the VLPs obtained from 15-nm cores were sorted by diameter, similar distributions of VLP and VLP₃ were incompletely coated with protein, and VLPs with irregular protein coats.

Two-Dimensional Crystal Analysis of R3BMV and VLP. The high degree of homogeneity of the VLP samples with cores of 6, 9, and 12 nm encouraged us to attempt to grow 2D crystals for TEM analysis. Two-dimensional crystals of all three preparations and R3BMV (control) were obtained with the lipid monolayer method using negatively charged lipid (Fig. 2A). Fourier transforms reveal reflections that go readily out to the eighth order, suggesting a resolution of ~3 nm. Each virus capsid in the averaged 2D projection map of the crystal (space group p3) has six similar sized densities around a central mass. The arrangement of the densities is consistent with the pattern of pentamers and hexamers on the
The capsid of \( T = 3 \) BMV. However, the unit cell parameters of different crystals of R3BMV varied from 25 to 27 nm, which may be the result of variations in the size of individual capsids or in the packing density. Note that the average diameter of R3BMV from single-particle analysis is 28 nm, which is larger than the dimension measured in the 2D crystals. This might be due to the capsids being more densely packed in the crystal when compared with the situation in solution. The changes in 2D lattice dimensions may also reflect different crystal forms that serendipitously have similar lattice constants.

The unit cell dimension of VLP12 crystals is \( \approx 25 \) nm, smaller than the R3BMV and the average diameter of VLP12 in solution. Due to readily discernable gaps in the density going from the center toward the periphery, three separate zones, i.e., the Au core, the PEG shell, and the protein capsid, can be identified (Fig. 2B). The density in the gap between the PEG and the protein is not entirely homogeneous and appears to delineate putative contact points between specific capsid sites and the PEG shell. Hexagonally packed VLP6 crystals have a unit cell size of 22 ± 1 nm.

**Single-Particle Reconstructions.** To elucidate additional structural features of VLP12, VLP9, and VLP6, we performed single-particle analysis with 3D reconstruction. The samples were spread on a carbon grid, along with R3BMV (Fig. 3). We noticed that the single-particle spread of the VLP12 contained more empty particles in comparison to the particles in the 2D crystal, suggesting that the core-containing VLPS are preferentially incorporated into the 2D lattice.

Classification and averaging of >2,000 R3BMV particles show that individual capsids (Fig. 3A *Inset*) have the same arrangement of hexamers and pentamers as the crystal projection (Fig. 2A), although the crystal projection had higher resolution. The PEG shell of the PEG-Au can be observed in the original electron micrograph (Fig. 3B) and is much clearer in the class averages (Fig. 3B *Inset*).

The 3D reconstruction of R3BMV (Fig. 4A) resulted in a model that corresponds well to the published CryoEM structure of \( T = 3 \) BMV, with 12 pentamers and 20 hexamers per capsid (total of 180 subunits) (28), demonstrating the validity of this approach. To compare the similarity between our 3D reconstruction and the structure from the CryoEM database, we calculated the Fourier shell correlation between the two structures (SI Fig. 7, thick line). The two models are correlated up to 3 nm (with 0.5-nm threshold), which is satisfactory if one considers the differences in sample preparation and imaging conditions.

The 3D projection of VLP12 contains 12 pentamers (Fig. 4D). There is clear evidence of density at the hexamer position, with a quasi-sixfold symmetry. However, it is not as prominent as the fivefolds, which may be due to structural variation related to the cores and to negative stain and drying. The small diameter (25 nm) seems to suggest that VLP12 may have a 120-unit capsid; however, it is possible that the viral capsids shrunk slightly during sample processing for TEM imaging (31). Therefore, it is likely that VLP12 is a \( T = 3 \) particle in solution.

The VLP9 and VLP6 3D reconstructions clearly lack hexamers and therefore are associated with a different structure than \( T = 3 \). Thus, VLP9 is similar to a pseudo\( T = 2 \) arrangement previously determined for the RNA-controlled 120-unit BMV capsid (24) (Fig. 4C). VLP9 has a characteristic latticework of holes penetrating the surface, which renders it similar to the previously reported \( T = 1 \) structure obtained by treatment of the wild-type \( T = 3 \) virus with CaCl2 and crystallized from sodium malonate (29) (Fig. 4B).

Interestingly, a section through the PEG layer in all three of the VLPS appears to have a hexagonal shape around the gold core. In the VLP12, the spaces are less obvious, suggesting a tighter packaging within these particles. Because the PEG is attached uniformly around the gold, the altered distribution may be imposed by the capsid subunits, possibly the flexible N terminus, which intercalates into the RNA (25). The hexagonal shape results in notable spaces where no apparent contact was observed between the PEG and the capsid. These results demonstrate that the conformation of the VLPS can be manipulated simply by changing the diameter of the core. Furthermore, the conformations observed are good molecular mimics of structures of BMV and BMV assembly intermediates found in cells.

Given that there are clear structural differences between VLP6 and VLP12, a different ratio of the capsid to core is expected to be required for efficient formation of the two VLPS. Indeed, Fig. 1A indicates that, at low CP/Au ratios, VLP6 forms with greater efficiency than VLP12.
capsids to form cocrystals. In support of this idea, both R3BMV and VLP crystallize in the rhombohedral group (Fig. 5 B and C). Therefore, cocrystallization of VLP₁₂ with R3BMV was attempted. Co-crystals were obtained for every concentration ratio tested (see SI Fig. 9). The mixed crystals are red colored with a color intensity proportional to the relative concentration of VLP₁₂, which excludes the possibility of segregation during crystallization. These results provide a strong indication that BMV and VLP₁₂ capsids are sufficiently similar in solution to grow crystals. Because the concentration of VLPs in the initial crystallization drop is lower than that of R3BMV, pure VLP crystals are usually significantly smaller than R3BMV crystals. Their small size (typically <10–20 μm, laterally) makes them difficult to handle. Therefore, we used absorption spectroscopy to obtain more information on these 3D crystals.

Because one would expect a significant deviation of the optical spectrum for small gold interparticle distances due to multipolar effects on coupling (33), we analyzed the transmitted light through VLP₁₂ crystals (Fig. 5D). The absorption spectrum shows a double hump that can be obtained by superposition of two Gaussian lineshapes, one centered at 481 nm and the other centered at 608 nm. In contrast, a dilute VLP₁₂ solution exhibited a single peak due to the well known surface plasmon resonance at 526 nm (Fig. 5D).

Capsid Assembly Control. Insight into empty capsid formation has been obtained in recent years from theoretical (33–37) and in vitro experimental studies (31, 38, 39). In this case, a sequential assembly principle has been proposed in which either preformed intermediates or individual structural subunits adhere to a proto-capsomer or initial nucleation center to form a capsid (33, 40). However, when the nucleic acid core is present, capsid nucleation experiments follow different kinetics (41). The sequential model does not provide an explicit role of the nucleic acid in the assembly process nor does it show how identical protein subunits form a pentamer or a hexamer.

A recent alternative model posits that a disordered nucleoprotein aggregate resembling a reverse micelle assemblies initially and is followed by subsequent condensation and conformational change of the protein on the micelle surface to yield the capsid quaternary structure (42, 43). In this context, capsid assembly around artificial cores opens new possibilities for experimental virus assembly modeling because the thermodynamic properties of the core can be controlled.

The isotropic experimental core model should provide an accessible departure point to test the most general aspects of virus self-assembly modeling that include contributions of the nucleic acid core. We propose that the VLP system described here, with its structurally uniform core, is an experimental example of the model. By varying a single parameter, namely the core radius, we obtained a strong correlation with assembly efficiency. In the following, we analyze several of the features of this VLP assembly from the perspective of the two models.

The protein concentration at which the assembly experiments have been carried (30 μM) is well above the critical concentration for empty capsid assembly (~10 μM) (44). Therefore, the existence of a threshold CP/Au ratio for VLP formation, even when the protein concentration is larger than the critical concentration for empty capsids, signifies that there is a rapid initial association step between protein subunits and the negatively charged cores that is independent of the structural requirements for capsid formation. In support of this view, an increased number of empty capsids was observed in the absence of Au cores. Although protein association with Au-PEG cores also occurs for nanoparticles larger than 12 nm, the resulting VLPs do not form crystals and therefore are likely to possess a disorganized protein shell, with low incorporation efficiency. It thus appears that icosahedral symmetry is somehow critical to the process of assembly or to particle stability.

If the number of CP subunits per core is insufficient to form a
complete shell, larger aggregates will form by coalescence of the incomplete nanoparticle/protein aggregates (19). In turn, this results in low encapsidation efficiencies and the presence of a threshold CP/Au ratio.

Smaller CPs result in smaller capsids (Fig. 4). However, in the absence of a perturbation from the core, empty capsid growth results almost exclusively in a \( T = 3 \) structure. \( T = 1 \) capsids similar to VLPs are observed when the N termini of the CP are cleaved between amino acids 35 and 36 (30). The cores are thus screening the N termini interactions normally occurring in empty capsids made of intact protein. With the N termini interaction screened, the VLP capsid assembles from the minimum number of subunits needed to enclose the core inside a closed shell. In the view of the micelle model, smaller cores would recruit less protein subunits at the initial step so that a smaller \( T \) number is more likely to occur. Interestingly, in the \textit{in vitro} assembly of BMV, \( T = 3 \) particles will assemble from intact proteins in the presence of the natural BMV genome, but pseudo \( T = 2 \) particles can be obtained using engineered RNA (28). We note that pentamers are clearly present in all combinations with the effective medium approximation does not reproduce, however, the double spectral feature in Fig. 5 so that its origins are not related to multiple beam interference.

We propose that the observed double peak is the signature of multipolar coupling between the gold cores that leads to plasmonic band formation. This feature cannot be accounted for by the Maxwell-Garnett theory (48) used in calculating the effective medium dielectric constant because it does not include contributions beyond those from dipolar. However, plasmon hybridization occurs when coupling between adjacent gold particles becomes significant and introduction of multipolar terms becomes required (49). The expected result is two absorption bands, one shifted to the blue and the other to the red of the surface plasmon peak of the single particle, similar to our observations from VLP\(_{12}\) crystals (Fig. 5). Such plasmonic band splittings in metallo-dielectric materials have been predicted theoretically, but never demonstrated experimentally in 3D structures (46, 49).

By this argument, the VLP crystal qualifies as an optical metamaterial having properties determined by both structure and material composition. Note that, for concentrated VLP solutions, the splitting is not observed because of a much broader spectral response. Therefore, crystals obtained from VLPs represent a convenient way to fabricate highly regular 3D structures with lattice constants in the sub-100-nm range. As suggested here, the lattice period can be varied by using different core sizes and possibly, in the future, by using engineered CPs and anisotropic cores.

**Materials and Methods**

**VLP Assembly on Au-PEG with Different Diameters.** Functionalized gold cores (Au-PEG) with different diameters (6, 9, 12, 15, 18, and 20 nm) were synthesized as reported (19). The Au-PEG nanoparticle solutions are routinely screened by dynamic light scattering (Malvern, Mastersizer 2000) and UV-Vis spectroscopy for correct average size and polydispersity before encapsidation and transmission electron microscopy characterization. To form VLPs, Au-PEG particles are added to BMV CP at molar ratio of 1:270. VLP assembly has been carried out at neutral pH following the same protocol used in Chen et al. (18), but with a protein concentration of 30 \( \mu \)M. The efficiency of encapsulation was measured as the ratio of VLPs having an Au core completely encapsulated by a capsid to the total number of gold nanoparticles (irrespective of level of encapsulation) present in a given area of the electron micrograph. Throughout the paper, VLP, will designate a virus-like particle prepared with a gold core of “\( x \)”-nm diameter (\( x = 6, 9, 12, 15, \) or 18 nm). Native BMV containing RNA3 and RNA4 that serve as controls for the structural analysis of VLPs are extracted from tobacco plants. Briefly, plants were infiltrated with \textit{Agrobacterium} to express the BMV 1a and 2a replication proteins (no RNA1 or RNA2 are produced in this case), and also RNA3. The RNA3 will replicate and result in the transcription of RNA4, which can be translated to produce the CP. Because there are no BMV RNA1 and RNA2 in the cells, the only RNAs packaged are RNA3 and RNA4. This preparation is called R3BMV.

**Transmission Electron Microscopy.** A JEOL 1010 TEM operated at 100 kV was used for routine negative staining and direct size measurements. Two-dimensional crystals were grown according to the lipid monolayer method as described by Sun et al. (50). Briefly, 15 \( \mu l \) of the R3BMV or VLP solution at 0.6 mg/ml concentration in buffer A [50 mM Mes, pH 6.0/1 mM MgCl\(_2\)/40 mM Na\(_2\)SO\(_4\)/50 mM (NEt\(_2\))\(_2\)SO\(_4\)] were added to a custom-designed Teflon well of 4 mm in width and 1 mm in depth. A total of 0.3 \( \mu l \) of mixed lipid solution of 30 \( \mu g/ml \) DOPS and 90 \( \mu g/ml \) egg phosphotidylcholine (Avanti Lipids, Alabaster, AL, catalog nos. 850375 and 840051) in chloroform/hexane (1:1, vol/vol) was layered on top of the protein solution and incubated overnight at 4°C in a humidity chamber. The 2D crystals were transferred to a carbon-coated copper grid, washed with distilled water, and negatively stained with an aqueous solution of 1% uranyl acetate. Two-dimensional crystals imaging...
has been done at a calibrated magnification of \( \times38,900 \) using a JEOL (Tokyo, Japan) 1200EX transmission electron microscope operated at an acceleration voltage of 100 kV. Electron micrographs were digitalized using an Epson (Long Beach, CA) Projection 3200 scanner at 1,200 dpi, corresponding to 5.1 Â/pixel at the specimen level. Computer-aided image processing was carried out with the CRISP software package (51).

Three-Dimensional Reconstruction of R3BMV and VLP. A total of 2.6 Â/3RBMV or VLP at 50 Â/µm in 100 mM Mes (pH 6.0) was adsorbed to a glow-discharged carbon-coated copper grid, negatively stained, imaged, and digitized as described above. The EMAN software package was used for single-particle image analysis and 3D reconstruction (52). For all preparations, >1,000 particles were selected from the digitized electron micrographs using EMAN’s boxer routine. The images were then filtered to remove high- and low-frequency noise, translationally and rotationally aligned, classified, and averaged without applying symmetry.

For 3D reconstruction, an initial model was produced from the centered particles with EMAN’s starticos program and subjected to eight cycles of refinement by projection matching. The final 3D reconstruction was visualized with the University of California, Santa Cruz, Chimera software (53). The 3D CryoEM structure of BMV was downloaded as CCP4 format from VIPER-NMD (54) (VIPER ID: em.2bmw and em.3bmw), changed to MRC format with Imagic’s em.c2m program, scaled to 5.1 Â/pixel with EMAN’s proc3d, and aligned to our 3D reconstruction using the align3D routine in EMAN (52).

Three-Dimensional Crystallization and AFM. Crystals were grown from BMV (24). Three-dimensional crystals of BMV and VLP have been prepared by the hanging drop vapor diffusion method. BMV and VLP solutions have been concentrated to 2 mg/ml, and adsorbed to a glow-discharged carbon-coated copper grid, negatively stained, imaged, and digitized as described above. The EMAN software package was used for single-particle image analysis and 3D reconstruction (52). For all preparations, >1,000 particles were selected from the digitized electron micrographs using EMAN’s boxer routine. The images were then filtered to remove high- and low-frequency noise, translationally and rotationally aligned, classified, and averaged without applying symmetry.

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Three-Dimensional Crystallization and AFM. Crystals were grown according to a protocol modified from the one outlined by Lucas et al. for BMV (24). Three-dimensional crystals of BMV and VLP have been prepared by the hanging drop vapor diffusion method. BMV and VLP solutions have been concentrated to 2 mg/ml, and adsorbed to a glow-discharged carbon-coated copper grid, negatively stained, imaged, and digitized as described above. The EMAN software package was used for single-particle image analysis and 3D reconstruction (52). For all preparations, >1,000 particles were selected from the digitized electron micrographs using EMAN’s boxer routine. The images were then filtered to remove high- and low-frequency noise, translationally and rotationally aligned, classified, and averaged without applying symmetry.

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Conclusion

Nanostructures can be functionalized to act as nucleating centers efficiently promoting self-assembly of symmetric capsids. The VLP ensembles thus obtained are homogeneous and can be crystallized for high-resolution structural studies. Such crystals may represent a new venue for plasmic metamaterials. With a single parameter, the gold core diameter, it is possible to switch between several polymorphs that have been already observed for RNA-controlled BMV packaging. The incorporation efficiency is also a function of the gold core diameter, and it reaches a maximum for a gold core of 12 nm. The ability to regulate the formation of specific structures should be important in designing nanoparticles with desired functions. Due to its versatility and possibility of control of the interaction between the anionic core and the protein shell, the nanoparticle VLP concept provides for a future template for self-assembly studies of protein cages.

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