Self-assembly approaches to nanomaterial encapsulation in viral protein cages†

Stella E. Aniagyei,[a] Christopher DuFort,[a] C. Cheng Kao[b] and Bogdan Dragnea*[a]

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A perspective on abiotic material encapsulation inside virus capsids is provided. The emphasis is on the physical principles of virus assembly relevant to packaging, strategies for encapsulation and capsid modification, and on emerging applications.

Introduction

At the border of living matter, viruses represent a class of biological supramolecular entities with no metabolism of their own but are apt at subverting the machinery of the cell for their own replication. In their most basic form, viruses consist of a segment of nucleic acid (either RNA or DNA) packaged inside a protein cage (the capsid). Capsid formation occurs by self-assembly from individual protein subunits. This apparent simplicity may be deceptive: there are close to 4000 known viruses today, spanning all kingdoms and classified in more than 90 families—a number that grows at a rate of 2 new families per year.[1]

Viruses excel at the packaging and delivery of nucleic acids, which is reflected by the fact that they account for the largest reservoir of genetic material on the planet.[2] The main focus of this feature article is on the issue of whether the virus' ability of adapting and responding to a variety of chemical environments in order to target and gain access to a host's interior could be preserved while creating a new class of bio-inspired architectures for *non-genomic* materials packaging and transport. Such complexes could find their way into biomedical applications, for instance in high-contrast functional imaging or as oncolytic virus-like agents

Beyond delivery applications, virus-like particles containing abiotic cores could be arranged, by virtue of their symmetry, into hierarchical materials with special optical or magnetic properties. Due to the small characteristic distances involved, well within the optical or magnetic coupling range, the new material properties will be the result of both the physical characteristics of the encapsulated cores and the way these cores are positioned in a regular lattice by well-defined biomolecular interactions, similar to those occurring in protein crystals. In this particular instance, tailoring of the material physical properties could be achieved by genetic engineering of the protein cage. Conversely, the nature of biomolecular interactions could be investigated by measuring the lattice-dependent physical properties of these hybrid biotic/abiotic materials.

A final application concerns various forms of templated chemistry,[3] which take advantage of the well-defined structural (and therefore functional) properties of external and internal capsid interfaces. For example, single-crystal nanoparticles nearly monodisperse in size could be grown inside the capsid cavity through processes reminiscent of biomineralization[4] and gold nanoparticles could be attached in a symmetric array at fixed locations on the outside of the capsid using chemically addressable amino acids on the capsid external surface.[5] Approaches to capsid modification utilizing a pre-assembled capsid as a template for subsequent chemistry have made the topic of an excellent recent review[3] and we shall not attempt to

[a]*Department of Chemistry, Indiana University, Bloomington, IN 47405, USA. E-mail: dragnea@indiana.edu*
[b]*Department of Biochemistry & Biophysics, Texas A&M University, College Station, TX 77843-2128, USA*
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Stella Aniagyei

Stella Aniagyei was born in Tema, Ghana and received her B.S. in chemistry from Bates College. Her doctoral work focuses on the development of virus–nanoparticle hybrids as novel bioanalytical tools. She currently investigates the role of the nanoparticle scaffold in virus-like particle (VLP) assembly as compared with protein–protein interactions via *synthesis, characterization and elucidation of VLP structures.*

Bogdan Dragnea

Bogdan Dragnea is an Associate Professor of Chemistry at Indiana University in Bloomington. He joined the faculty in 2001 after post-doctoral studies in near-field optics at JILA (advisor S. R. Leone), Boulder, CO. He has a PhD in physics from Université de Paris-Sud, Orsay, France (advisor B. Bourguignon). Besides bio-inspired materials and self-assembly, his research interests include nanophotonics.

cover them here. Instead, in this feature article, a distinction is made between synthesizing the cargo inside a pre-assembled capsid and the case of assembling the protein cage around an existing cargo, a process called *encapsidation*. The two approaches are complementary: in the former method, the capsid itself plays the role of a nano-reactor with catalytic walls of well-defined surface chemistry. Moreover, some viruses exhibit a pH-induced swelling or phase transition of the capsid, which can be used for gating molecules in and out during a reaction. In the latter method, the cargo is synthesized separately and therefore the synthesis conditions are not restricted by biocompatibility requirements. Also, prior to encapsulation, the core can be functionalized with molecules that help to control the interaction between the protein shell to be assembled and the core. This is important in experiments where maintaining the protein fold integrity is crucial, as in the growth of crystals for X-ray diffraction from virus-like particles (VLPs) encapsulating functionalized gold cores.[6]

The self-assembly approach to encapsulation has not been previously reviewed. The present feature article thus seeks to provide a perspective on the general principles of foreign material encapsulation by self-assembly, as well as on recent contributions and milestones of this emerging field. Emphasis will be on theoretical and experimental insights into the physical principles of virus assembly relevant to packaging, various strategies for encapsulation and capsid modification, and finally on emerging applications. In conclusion, some open questions and promising future directions will be highlighted.

General structural aspects of icosahedral viruses

The idea of diverting viruses from pathogenic agents into biotechnological resources has been long used in gene therapy—an approach to manage disease through gene replacement.[7] Virus-based delivery platforms have been adopted in 75% of the total number of gene therapy clinical trials between 1989 and 2004, while the rest used DNA-plasmids and non-viral vectors.[8] The main challenges associated with virus vectors for gene therapy come from the activation of the innate immune response to viral molecules and from the possibility of genetic disruption with long-term secondary effects.[9] In addition to being potentially recombinogenic, genome-carrying virus-like particles are potent elicitors of the nucleic-acid sensing receptors.[10] Therefore, some of the challenges of virus-based delivery in gene therapy actually come from the presence of genetic material. A virus-based delivery platform of *non-genomic material* would then present the benefits of vector capabilities while avoiding genome-related challenges.

Since the vast majority of the functional attributes directly related to the vector abilities of a virus reside in the protein shell encapsulating the genome (the capsid), we will start with a brief presentation of the most general structural aspects of virus capsids.

Depending on capsid composition, viruses can be divided into two main classes: enveloped viruses, which have the protein capsid wrapped in a lipid membrane (envelope) derived from the host, and non-enveloped viruses, which lack such a lipid membrane, Fig. 1. In terms of composition, both enveloped and non-enveloped viruses can have attachment proteins in addition

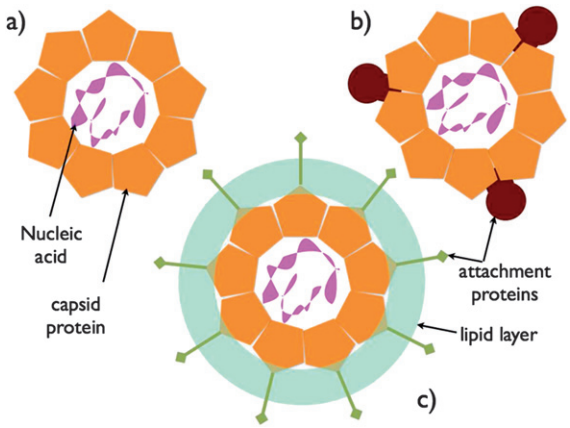


Fig. 1 General architectures of quasi-spherical viruses. a) Non-enveloped virus. b) Non-enveloped virus with attachment proteins. c) Enveloped virus with glycoproteins playing the role of viral entry mediators.

to the structural proteins of the capsid. Regarding morphology, both enveloped and non-enveloped viruses can have capsids with helical symmetry (rod-like appearance) or icosahedral symmetry (quasi-spherical appearance).

As with most biological systems, there are many exceptions and some viruses have morphologies and compositions that differ from those of the main groups. For example, geminiviruses have prolate capsids formed from two icosahedral capsids joined together while poxviruses are brick-shaped, with a complex structure including the largest known viral genomes and over a hundred proteins.[11]

Icosahedral virus capsids contain an integer multiple of 60 structural subunits. The integer factor is called the *T*-number in the quasi-equivalence theory of Caspar and Klug[12] which describes the subunit tiling pattern of viruses with concepts borrowed from crystallography. As a consequence of the quasi-equivalence theory, the relation between the *T*-number and the size of the capsid is approximately described by:

$$T^{1/2} = \frac{0.618D}{d}$$

where D is the diameter of the icosahedral particle and d is the center-to-center distance between the basic structural units.[12] Numerous icosahedral virus structures have been elucidated by means of cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction methods and there exist several excellent reviews on this and other structural methods.[13–16] A gallery of icosahedral capsids is presented in Fig. 2.

Following the general characteristics path from quaternary to tertiary structure at the subunit level, prototypical viral protein folds can often be observed with the prevalent motif being that of an eight-stranded anti-parallel β-barrel plus external loops.[1] From virus to virus, the least variable motif is the β-barrel and the most variable are the external loops. The reason for the β-barrel motif prevalence is not known. With few exceptions, the capsid proteins of icosahedral viruses have arms at their N-termini and often also at their C-termini. These arms are often partially disordered, which is thought to be important for their interaction with the nucleic acid upon encapsulation, Fig. 3. This interaction will be the subject of discussion in subsequent sections.

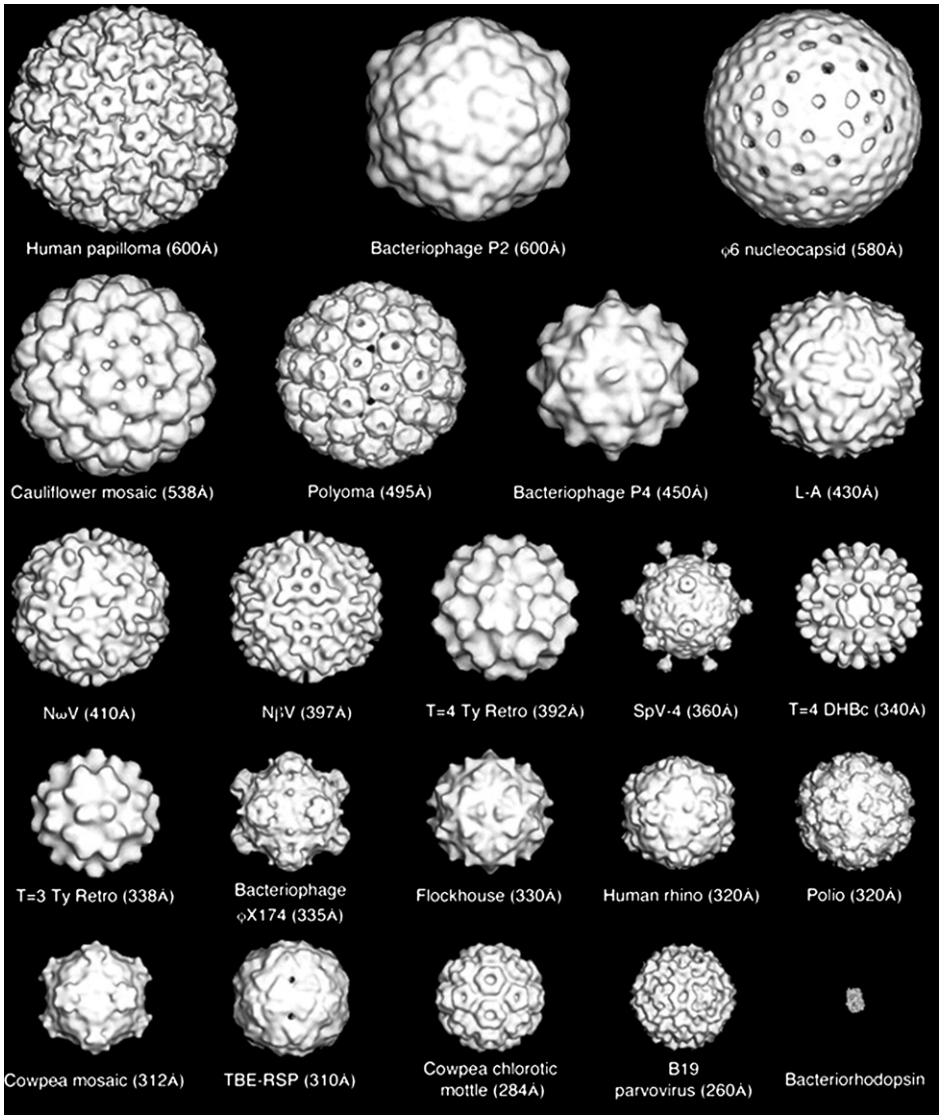


Fig. 2 Representative icosahedral virus capsid structures obtained by cryo-electron microscopy and 3D image reconstruction methods. The bacteriorhodopsin protein is shown for comparison in the lower right corner (from ref. 13, © 2007 American Society for Microbiology, USA).

Specific and non-specific interactions in genome packaging

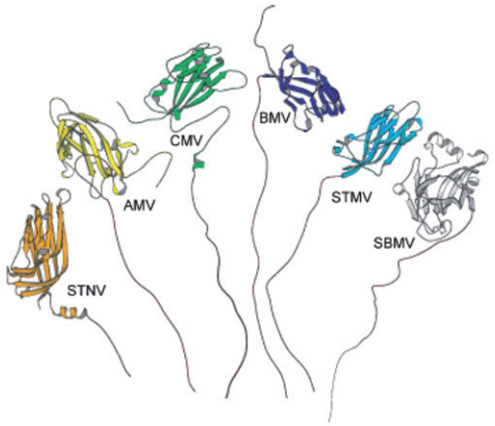
Mechanisms of genome packing

To operate as a delivery vector, a virus-like system should package the cargo, take advantage of the extracellular and intracellular transport pathways, and finally disassemble in the presence of desired conditions. Engineering vector properties for this three-step sequence starts with controlling the packaging of non-genomic materials.

The size of the packaged genomes varies from the smallest encapsidated virus known, the satellite panicum mosaic virus[18,19] with a 826 nt genome packaged in a 160 Å capsid, to the giants of the *Iridoviridae* family which package 380×10^3 nt long genomes inside 3500 Å diameter capsids.

There are three main ways through which packaging of the viral nucleic acid is believed to proceed upon *in-vivo* assembly of native virions.[20] The question is, which of them is adaptable to foreign materials packaging? The first requires input of chemical energy: double stranded DNA phages have developed a motor complex to stuff the nucleic acid strand into a pre-assembled capsid in the presence of ATP,[21–24] Fig. 4(a). Up to now, this mechanism has not been diverted towards packaging of non-genomic materials.

The second mechanism is through cooperative, simultaneous self-assembly of the nucleic acid and its capsid. For example, rigid helical viruses, such as tobacco mosaic virus, use this mechanism to selectively package viral RNA. Probably because of key assembly steps requiring nucleic acid–protein *specific* interactions, which are hard to control, there are only few examples of using it to encapsulate foreign materials. However, it provided inspiration for a recently reported construction of an artificial light harvesting system,[25] a templating approach for the self-assembly of monodisperse organic structures,[26] and for the encapsulation of nanoparticles in capsids that would not assemble in the absence of the packaging signal analogs, such as



VIRUS	SEQUENCE
STMV	MGRGKVKPNRKSTGDNSNVVTMIRAGSYPKVNPTPTWVRA
STNV	MAKQQNNRRKSATMRAVKRMINTHLEHKRFALINSGNTNA
BMV	MSTSGTGKMTRAQRRAAARRNRWTARVQPVIVEPLAAGQGKAIKAIAGYS
CMV	MDKSESTSAGRNRRRRPRRGSRSASSSADANFRVLSQQLSRLNKTLAAGR
SBMV	MSGLFHHRTKPREIRAFVMATRLTKKQLAQAIQNTLPNPPRRKRRAKRRA
SV40	MKMAPTKRKGSCPGAAPKKPKEPVQVPKLVIKGGIEVLGVKTGVDSFTEV
BBV	MVRNNNRRRQRTQRIVTTTTQTAPVPQQNVPKQPRRRRNRARRNRRQGRA

Fig. 3 The capsid proteins of six icosahedral viruses (including plant, mammalian, and insect viruses) shown in the ribbon format and composed of two generic domains: a beta barrel (ribbons) and a positively charged floppy amino terminal (lower panel, sequence) (from ref. 17, © 2005 Wiley Periodicals, Inc., UK).

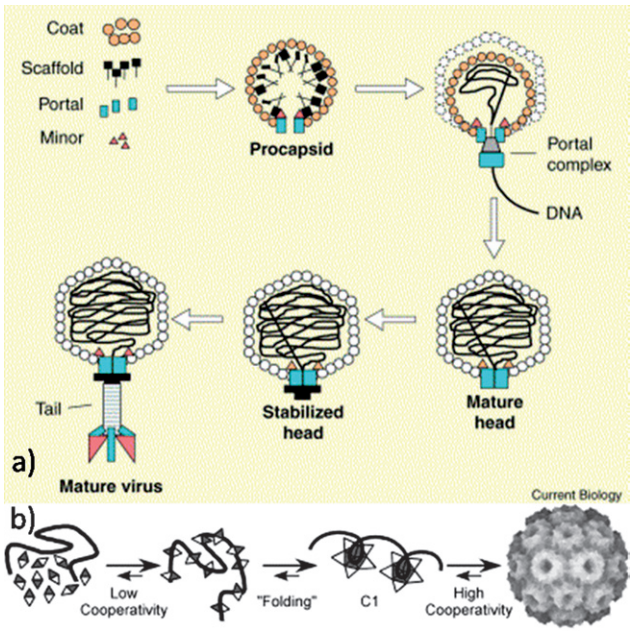


Fig. 4 a) Assembly path of a tailed double-stranded DNA bacteriophage. The structural proteins of the phage head are first form a procapsid with icosahedral symmetry. Located at one vertex in the procapsid is a portal complex, which stuffs DNA into the head through an ATP-powered reaction (from ref. 24, © 2003 Elsevier Science Ltd, USA). b) Proposed mechanism for CCMV: the initial step is formation of a nucleoprotein complex followed by refolding and growth (from ref. 32, © 2003 Elsevier Ltd).

the red clover necrotic mosaic virus[27] and the nucleocapsid of an alphavirus.[28]

A third type of assembly mechanism occurs through pre-condensation of the nucleic acid followed by addition of the surrounding protein subunits.[20] Small, non-enveloped icosahedral viruses containing single-stranded DNA or RNA, such as the adeno-associated virus[29] (AAV), the brome mosaic virus[30] (BMV) or the cowpea chlorotic mottle virus[31] (CCMV), have adopted this packaging strategy. For these viruses, the nucleic acid is believed to recruit the capsid protein through mostly *non-specific* electrostatic interactions followed by capsid formation as a result of steric and solvation constraints[32,33] (Fig. 4b). In general, virus assembly may include both specific and non-specific interaction stages and thus there is no net delimitation between the second and the third mechanisms.

As a consequence of their lack of *in-vitro* specificity for packaging and because of their symmetry, which allows for simplifications in modeling and placement of desired chemical moieties, icosahedral (quasi-spherical) and helical (tubular) capsids have been the most studied in relation to the possibility of loading them with non-genomic materials.

Driving forces in packaging

The use of available space for viral genome packaging is done with remarkable efficiency. For example, in some bacteriophages, the genome is packaged by a motor protein complex using chemical energy from ATP at internal pressures of tens of atmospheres.[23] However, no ATP-dependent activity is involved in the packaging of RNA by single stranded RNA (ssRNA) icosahedral viruses, which still manage to reach packaged nucleic acid densities as large as those for hydrated RNA crystals.[34] In the case of ssRNA viruses, the thermodynamic driving force for compressing the RNA to such densities is provided by the electrostatic attraction between the capsid and the genome.

Following the pioneering work of Bancroft, Fraenkel-Conrat, Caspar and Klug,[12,35–37] a variety of phages, plant, and animal viruses can been assembled *in vitro* from their molecular components. The spontaneous assembly of an infectious virus from its constituent parts: nucleic acid, proteins, and sometimes lipids, under the proper thermodynamic conditions is miraculous in its own right. Bancroft *et al.* were the first to show that the driving force for packaging and efficient assembly includes a non-specific electrostatic interaction, at least for some ssRNA viruses, by encapsulating polyanions instead of nucleic acid.[35] Specific interactions could also be required, at least for *in vivo* assembly, as suggested by the prevalence of a hairpin packaging signal in the viral RNA. The hairpin loop is believed to recognize and selectively bind protein oligomers upon the initial phases of virus assembly. In MS2, an icosahedral bacteriophage, the driving force for complex formation is the stacking of the hairpin loop base -5 onto a tyrosine side chain[38] although hydrogen bonding may also play a role.

It is worth noting here that some *in vitro* assembly reactions are sufficiently homogeneous to allow structural features of the reconstituted viruses to be delineated by standard X-ray diffraction or cryo-EM. These properties have generated considerable interest in using viruses as models to understand the nature of nucleic acid–protein interaction and the assembly of

supramolecular structures.[20] With the current progress in packaging foreign materials inside virus capsids, this view may extend in the future to virus-like particles as convenient models for the study of abiotic/protein interfaces.

Non-genomic material encapsidation

Approaches to non-genomic material encapsidation can be classified into three categories as well,[39] Fig. 5. The first approach, Fig. 5a, takes advantage of the chemically well-defined local environment of the viral cavity to synthesize foreign material directly inside the capsid.[3,4] In this case, reagents or cargo molecules to be encapsidated have to be transported and then sequestered inside the capsid.

The second approach, encapsidation by assembly, mimics the natural interaction between the capsid proteins and nucleic acid to promote the assembly of a virus-like capsid around a foreign core. In this case, one starts with a previously prepared cargo, for example with nanoparticles functionalized with anionic moieties[40] or specific nucleic acid packaging signals[27,41] and separated protein subunits. Encapsidation occurs spontaneously by mixing the protein subunits with cargo particles at specific ionic strength, temperature, and pH ranges. Functionalized polymers,[42,43] enzymes,[44] single or multiple nanoparticles,[6,27,40,41,45–47] and even liquid nanodroplets[48] have been encapsidated by this method. A related strategy, associated with Bromoviridae in particular, is to reversibly manipulate the pH to induce swelling and contraction of the viral particles to facilitate the entry and sequestering of the foreign materials.[4,49]

The third approach, encapsidation by synthesis, is based upon the covalent attachment of the cargo molecules to site-specifically engineered residues on the capsid protein.[50,51] The cowpea mosaic virus is a good example of functional versatility achievable through the chemical coupling of peptides with virus capsids,[5,50,52–54] Fig. 5c. Similar to the other approaches, *en route* to the target, the cargo can be kept from interacting with the outside environment or the cell until the capsid is directed to release it.[55]

Comprehensive reviews already exist on the chemical coupling of biologically active moieties to virus capsids[3,39] and its applications.[56,57] The self-assembly route to encapsidation has not been yet been reviewed. Nevertheless, the self-assembly route is particularly interesting because:

1. It operates on an already prepared cargo and therefore does not impose biocompatibility limitations on the synthesis conditions.

2. Empty capsids generally assemble and disassemble in different conditions than infectious viruses. By adding a core with tunable physical and chemical properties,[6,42,45] the virus-like particle stability can be, in principle, adjusted to respond in a desired way to *in vivo* conditions. As an illustration of this point, an alphavirus nucleocapsid laden with an anionic gold nanoparticle was found to be more stable than the native nucleocapsid during long-term storage and freezing/thawing cycles.[28]

3. The physical properties of the artificial core have an important role in determining the size and the shape of the surrounding capsid.[6,42,58] This fact implies that there is flexibility in the size of a cargo that can be accommodated by a virus capsid. As a consequence, the same protein building blocks and thermodynamic parameters could be used to obtain a variety of virus-like containers.

4. Based upon prevalent structural motifs in icosahedral capsids, there should be general principles of encapsidation by self-assembly which are conserved across different virus families and promise the possibility for a platform-independent approach. It is worth noting here, however, that even the most general features of icosahedral virus self-assembly remain a matter of debate.[17,59] Apparent controversies may be solved as more models for encapsidation become available and bridge the existing insights from assembly of empty capsids and those of native virions.

Charge, size, and shape constraints in encapsidation

Efficient production of a virus-like particle (VLP) requires that we understand the interplay between the physical and chemical properties of the core material needed to promote the spontaneous formation of a capsid-like structure. It is here assumed that the capsid protein has been obtained *via* any of the conventional molecular biology routes, *i.e.* either through recombinant expression or purified from disassembled viruses extracted from

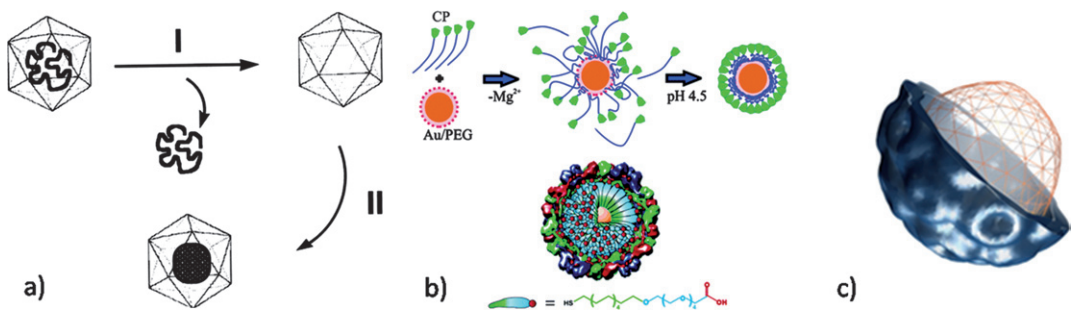


Fig. 5 Three approaches to encapsidation of non-genomic cargo. a) Synthesis inside the capsid: step I involves the removal of viral RNA and purification of the empty virus particle by ultracentrifugation on sucrose gradients. Step II involves the selective mineralization of an inorganic species (the cargo) within the confines of the virus particle (reprinted with permission from ref. 4, © 1998 Macmillan Publishers Ltd, USA). b) Assembly of the capsid around its cargo: First, a core having an anionic surface charge associates with the positively charged N-termini of capsid proteins. Then, solvation and steric constraints organize the protein subunits into a symmetric capsid (reprinted with permission from ref. 45, © 2006 American Chemical Society, USA). c) Chemical coupling of therapeutic moieties to the inner surface of the capsid.

their hosts. An alternative approach would be to start with a given core state and interrogate routes of protein modification, which may lead to self-assembly and encapsidation. However, to the best of our knowledge, there are no reports yet along these lines.

We have seen that depending on the type of virus, the native interactions between the genomic cargo and the surrounding protein vary from mainly non-specific, in which case encapsulation of foreign materials can be achieved without help from nucleic acid, to highly specific, in which case oligonucleotides of a minimum length and specific sequence may be required for proper assembly as is the case of the Sindbis virus.[60] Specific RNA/DNA interactions with the capsid do not preclude encapsidation of foreign materials. However, in this case, cargo functionalization with the specific oligonucleotides required for promotion of assembly may be necessary.[27,28]

Incorporated macromolecules, droplets, and particles all share the same size scale, which is comparable with, if not identical to, the capsid cavity of the wild type virus. The question of how much flexibility in accommodating different core sizes there is has not been definitively answered yet for any type of capsid, but examples of *in vitro* capsid adaptation to accommodate different cargo sizes and shapes exist.[6,42,58]

The appeal of ssRNA virus capsids as paradigms for VLP nanocontainers comes from the fact that many features of their self-assembly can be understood from basic thermodynamic principles. For instance, the origin of icosahedral symmetry can be understood using minimal thermodynamic models for equilibrium capsid structure.[61,62] At the same time, the capsid proteins of these viruses expose free amino terminal tails, which are highly positively charged and many of them will bind nucleic acid at least partially through non-specific long-range electrostatic interactions, Fig. 3.

Belyi and Muthukumar[63] have shown that, even if these interactions were completely non-specific, control over both the length and the conformations of the genome is likely to occur. Within the framework of an electrostatic model for genome binding analogous to continuum theories for polymer brushes, despite their non-specificity, these interactions lead to the prediction that the total genome length Λ of a virus should be proportional to the net charge Q on its capsid peptide arms,

$$\Lambda = \eta Q$$

If the non-specific interaction assumption holds, the proportionality coefficient is expected to be conserved across different viruses. A compilation of the genome size dependence on the net charge of the polypeptide arms is given in Fig. 6.

The ratio of genome length to the charge of the capsid peptide arms has been found indeed to be conserved, with $\eta = 1.61 \pm 0.03$. Non-specific electrostatic interactions are therefore dominant in the assembly of, at least, those ssRNA viruses that served for the data compilation in Fig. 6.

The remarkable ability of such models to describe the general features of viruses as thermodynamic systems is a key ingredient in attracting interest for materials science applications.

Until now, we have seen that genomes of a certain length could be selected upon spontaneous assembly. As polydispersity in the dimensions of a core is a likely scenario, the question is what happens when there is a mismatch between the size of the core to be encapsulated and the native capsid? Will the capsid tolerate variations in the core size? What is the span of the mismatch tolerance? Does the assembly stop when a significant mismatch exists or will a new quasi-equivalent structure form?

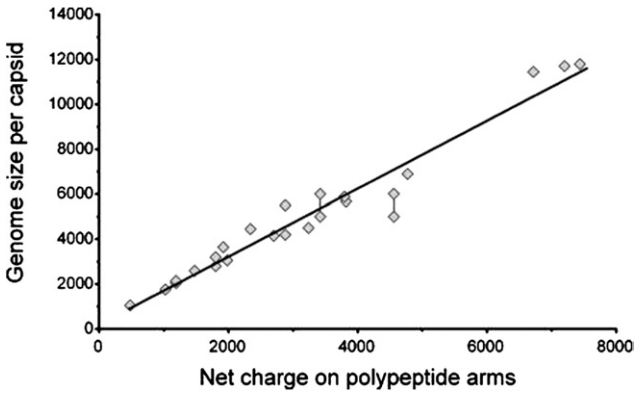


Fig. 6 Correlation between the viral genome length and the net charge on the flexible N-termini from viral capsids. Each point represents a different ssRNA virus (from ref. 63, © 2006 National Academy of Sciences, USA).

Using nanoparticles of nearly monodisperse size distribution can help in answering these questions. Moreover, metal nanoparticle cores can be easily identified under a transmission electron microscope and their diameters accurately measured.

BMV is the first icosahedral capsid to be studied with respect to nanoparticle encapsidation.[40] The inner surface of its capsid is uniformly decorated with a positively charged arginine-rich motif.[64] BMV RNA is smeared on the capsid inner surface in a structureless distribution except for some radial layering (reminiscent of polyelectrolyte films) as observed in X-ray and neutron scattering studies.[64] The average diameter of the inner cavity hosting the RNA is 16 nm.

To study nanoparticle encapsidation as a function of mismatch between the cargo and the available capsid volume, one needs to somehow characterize the efficiency of the process. Thus, for an efficiency of 100%, one would expect all the particles to be encapsidated when 1E (one equivalent) of proteins have been mixed together with the particles. When TEM is used for analysis, encapsidation efficiency can be defined as:

$$\eta = N_{\mathrm{VLP}}/N_{\mathrm{NP}}$$

where N_{VLP} = the number of virus-like particles that have a complete virus coat around the nanoparticle core, N_{NP} = total number of particle cores observable in the TEM pictures.

In the early studies of colloidal gold nanoparticle encapsidation in BMV, the negative charge of the core was carried by layers of citrate,[40] phosphine, or short DNA analogs of the RNA packaging signal[41] directly bound to gold by covalent linkers. Encapsidation efficiencies were low, reaching a maximum of 1–3%, for all nanoparticle diameters tested. Low efficiencies precluded studies of encapsidation as a function of size mismatch between the capsid cavity and the nanoparticle core. However, a dramatic improvement in the encapsidation efficiency was observed when the gold nanoparticle to be incorporated was coated with a monolayer of carboxy-terminated polyethylene glycol (PEG) (Fig. 5b).[45] The PEG molecule does not specifically

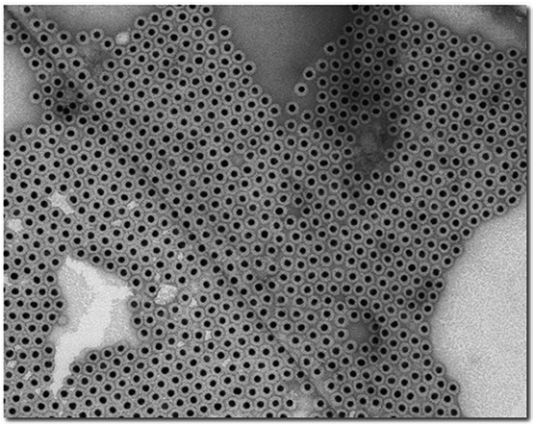


Fig. 7 Transmission electron micrograph of virus-like particles (VLPs) containing gold nanoparticle cores (12 nm diameter) instead of RNA. VLPs can be efficiently assembled if the nanoparticles are pre-coated with a covalently bound carboxy-terminated PEG layer.

interact with the protein, instead it prevents capsid protein denaturation that may occur on an unprotected gold surface. Fig. 7 shows the homogeneous character of assembly products, the result of PEG functionalization of nanoparticle cores, which opened the way to studies of nanoparticle core packaging as a function of size.

In addition to high efficiency, cryo-electron microscopy observations of individual VLPs indicated the presence of regularly distributed capsomeres, Fig. 8.

Sun *et al.*[43] varied the PEG-coated nanoparticle diameter between 6 nm and 20 nm and found that changes in the nanoparticle size affected the efficiency of encapsulation by BMV capsids, with a maximum efficiency occurring for 12 nm gold nanoparticles. This result was confirmed by Loo *et al.* for a different virus, the red clover necrotic mosaic virus, which failed to encapsidate DNA-functionalized nanoparticles larger than the capsid cavity, but yielded VLPs for smaller particle sizes.[27] Therefore, like genomic RNA, assembly of nanoparticle cores is also dependent on the size of the core. However, in addition to a change in encapsidation efficiency, the number of protein subunits surrounding the nanoparticle has been also found to change within the limits of the quasi-equivalence model, Fig. 9.[6] Thus, $T = 1$ BMV capsids (60 protein subunits) have been

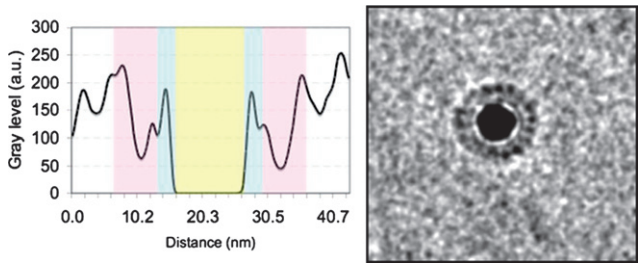


Fig. 8 Cryo-TEM picture of a VLP containing a 12 nm, PEG-coated gold nanoparticle and cross-section through the center with different grey levels corresponding to different layers (gold, PEG, capsid protein) approximately marked in color. Note that despite the facetted character of the nanoparticle, the protein coat preserves a regular pattern of capsomers and a quasi-spherical aspect.

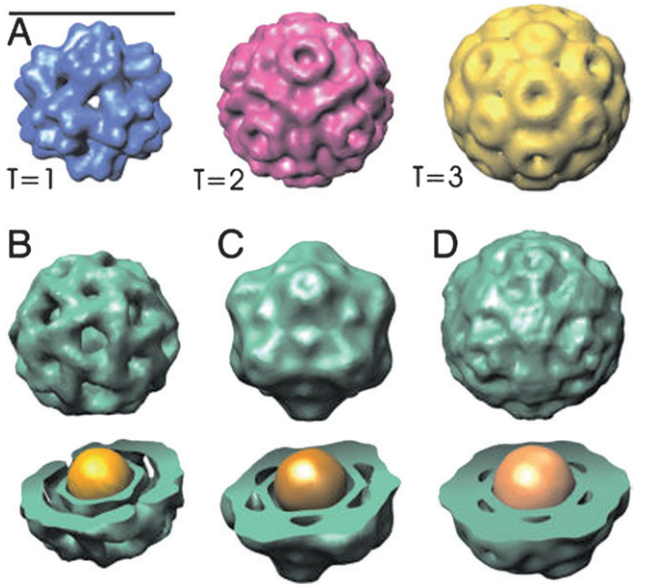


Fig. 9 3D single particle reconstruction of recombinant viruses and VLPs from negative stain TEM data. (A) $T = 1$, 2, and 3 models of BMV capsids. (B) $T = 1$ VLP structure formed around a 6 nm nanoparticle core, (C) pseudo-$T = 2$ VLP structure formed around a 9 nm core, (D) $T = 3$ VLP structure formed around a 12 nm gold core. (Reproduced from ref. 6, © 2006 National Academy of Sciences, USA.)

obtained for 6 nm nanoparticle cores, while 12 nm particle cores have promoted the formation of $T = 3$ (180 protein subunits).

An interesting feature in the sections of Fig. 9 is the variation in the "void space" present in the layering of the molecular coat as the particle size changes from 6 nm to 12 nm. It seems like the growth of the particle is followed by the capsid but not at the same rate—the empty layer becomes increasingly thinner as the particle size increases. Interestingly, $T = 1$, pseudo $T = 2$, and $T = 3$ capsids have been observed before, but not larger T-numbers. The above observation of progressive reduction in the void space may suggest that there is a limit in the dihedral angles between capsomeres, which hinders the formation of larger icosahedral capsids. Core–shell complexes larger in diameter than the 28 nm of the $T = 3$ capsid have been obtained,[47] albeit at low efficiencies, and until now it has not been possible to obtain reliable structures from them by EM and single particle reconstruction. It is likely that these VLPs have a partially disorganized protein shell.

Polymorphic adaptation to the size of the cargo is not an exclusive characteristic of nanoparticle encapsidation. Hu *et al.*[47] have studied the *in vitro* assembly of VLPs formed by the capsid protein of CCMV and the anionic polymer poly(styrene sulfonate) for five molecular masses ranging from 400 kDa to 3.4 MDa (the native RNA has a mass of ~1 MDa). Similar to the encapsidation of nanoparticles, even though the size and the charge of the polymer cargo increased monotonically over a broad range, only two discrete sizes of VLPs were observed, corresponding to pseudo $T = 2$ and $T = 3$ structures.

Therefore, both nanoparticle and polymer encapsidation studies indicate that although there might be an optimum charge ratio for encapsidation as suggested by ref. 63, the core size constraint is also a critical factor.

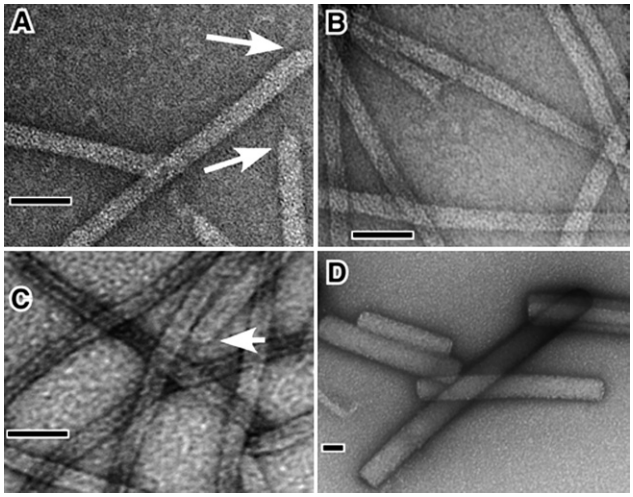


Fig. 10 Negative stain transmission electron microscopy of *in vitro* assembled tubular structures from CCMV capsid proteins and double-stranded DNA. Different micrographs represents assembly results at different DNA bp : CP dimer ratios. Tube diameter is uniform, 17 nm, but lengths vary; arrows identify capped ends (from ref. 58, © 2006 American Chemical Society, USA).

Up to now, the discussion has revolved around quasi-spherical particles, but core-templated capsid assembly is not restricted to quasi-spherical shapes. The assembly of the coat protein of CCMV has been redirected to form tubular structures capped with $T = 1$ hemispheres around rigid linear double-stranded DNA scaffolds,[58] Fig. 10.

How can spherical virus proteins assemble into a quasi-cylindrical structure? Nguyen *et al.*[65] have provided a generalization of the Caspar–Klug classification[12] of icosahedral viruses to arbitrarily shaped capsids (Fig. 11). Capsid shapes are determined by isometric construction, *i.e.* replacing hexagons by pentagons at certain locations to help folding the planar hexagonal sheet into a closed shell. For icosahedra, the pentagon positions are determined by two template vectors, while for non-icosahedral shells four template vectors are necessary to index their location. This generalization was able to explain the occurrence of non-icosahedral nucleocapsid shapes in retroviruses and promises to also be useful in describing template-induced polymorphism.

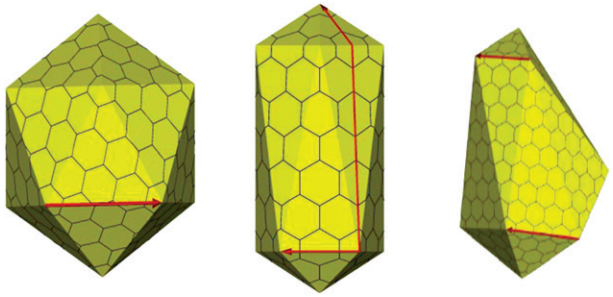


Fig. 11 Icosahedral and non-icosahedral isometric shells obtained by generalizing the quasi-equivalence theory of Caspar and Klug (from ref. 65, © 2006 American Physical Society, USA). The spherocylinder in the middle is believed to represent the VLP structures obtained by Mukherjee *et al.*[58] by redirecting the assembly of a spherical virus capsid.

While a significant body of structural information on self-assembled VLPs has already emerged, much less is known about the differences between the dynamics and the assembly mechanisms of VLPs and empty capsids. These differences could be quite significant and potentially shed light on the virus assembly mechanisms, as recently pointed out by Hagan.[66] His coarse-grained computational models predict that for strong core–subunit interactions, subunits adsorb onto core surfaces *en masse* in a disordered manner, and then undergo a cooperative rearrangement into an ordered capsid structure, in agreement with the main tenet of the McPherson proposal.[17] This assembly mechanism is unlike any of the pathways identified for empty capsid formation. The question remains whether they might be similar to intact virus assembly.

Functional self-assembled virus-based materials

While still in its early stages, the field of virus-encapsulated nanomaterials currently experiences accelerated growth in several applied directions. These include magnetic[47,67] and optical imaging,[56,67,68] development of therapeutic vectors,[69–71] "bottom-up" fabrication of hierarchical structures,[72–75] and enzymatic reaction control.[44,76] In the following, we discuss several representative examples from each of these areas.

In vivo imaging

Virus-based nanoparticles have several unique properties when compared with other technologies such as functionalized colloidal particles, micelles, *etc*. First, self-assembled virus cages are mono-disperse to a degree that is very difficult to reproduce by nanoparticle synthesis. This may be very important for cellular attachment and entry, as suggested by Jiang *et al.* who found that nanoparticle-mediated cellular response strongly depended on the size of the nanoparticle.[77] Second, virus cages can be modified to present targeting molecules or peptides, which have the same local environment, hence the same structure and function, by virtue of virus symmetry. While it is reasonable to think that a homogeneous chemical environment for the target moiety presentation is advantageous, there are no direct comparisons yet between the efficiencies of VLPs and other, more heterogeneous, vectors. Third, specificity to a certain target or other properties desired for the capsid can be achieved by site-directed mutagenesis. These advantages made *in vivo* imaging one of the first applications to be explored and there are already a number of excellent reviews on the topic.[56,78,79]

Drug delivery

Another application area of interest is the use of engineered capsids as carriers for therapeutic molecules[55,70,80] like anti-cancer drugs[81] or the use of modified viruses for viral oncolysis (the destruction of cancer cells by replicating viruses).[82] Central issues to be considered here are the mechanisms of cellular targeting and entry. Viruses have evolved a variety of sophisticated entry mechanisms. Viral entry mechanisms make topics of intense current research themselves.[83] VLP delivery technologies

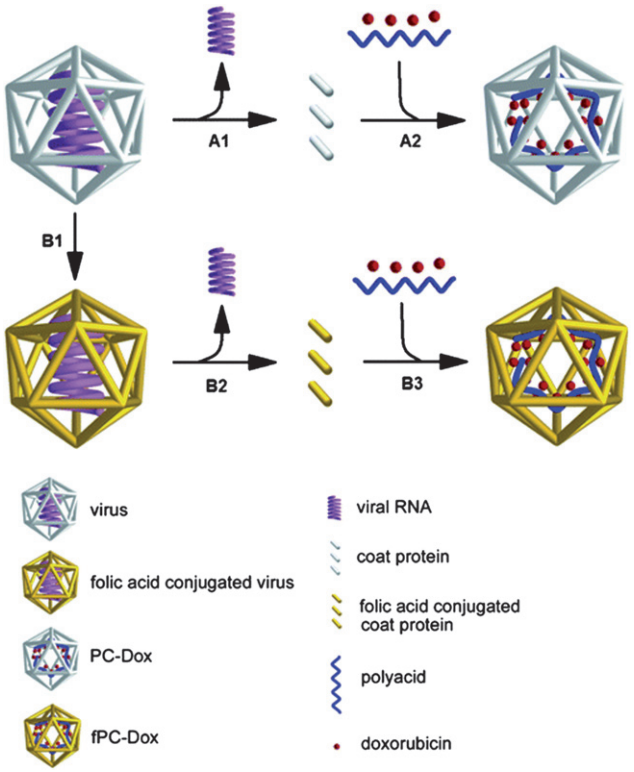


Fig. 12 Schematic of the preparation of doxorubicin-loaded capsids with (fPC-Dox) and without (PC-Dox) folic acid conjugation (from ref. 81, © 2007 American Chemical Society, USA).

will improve as knowledge about these mechanisms emerge. All virus entry mechanisms begin with attachment to cell surface receptors, which can be carbohydrates, lipids, or proteins. After that, there are two general strategies for crossing the plasma membrane: the endocytotic and the non-endocytotic routes. The non-endocytotic route involves direct crossing of the plasma membrane by fusion at the cell surface, for example, HIV. Some viruses, like the Semliki Forest virus, will cross the cell membrane in seconds with an efficiency of 80% (*i.e.* 80% of the cell-surface attached virus will penetrate the membrane).[83] It is not known why some viruses are so efficient and what structural features are responsible for it. It would be interesting to know how human-made delivery vectors compare with this efficiency. It is important to address the issue of efficiency because a less efficient platform would require higher concentrations of sample, which may induce adverse secondary effects.

In a recent example, Ren *et al.*[81] have used the hibiscus chlorotic ringspot virus (HCRV) to simultaneously encapsulate polyprotic acid-associated doxorubicin (a small molecule anti-cancer drug), with an encapsulation efficiency for doxorubicin of 7% (Fig. 12). To make a drug-loaded capsid that specifically targets cancer cells, folic acid conjugated capsids have been prepared, an approach that has also been used for the cowpea mosaic[69] and sindbis viruses.[84] (Normal cells express low levels of folic acid receptors, while cancer cells over-express them.) Upon uptake in ovarian cancer cells, the HCRV VLPs exhibited sustained release of doxorubicin and *in vitro* cytotoxicity. While these results show promise, questions remain concerning the immune response and *in vivo* efficacy of the plant virus-based formulations.

Single-enzyme nanoreactors

Virus capsids have a dynamic structure. Many capsids are formed from a network of interacting proteins that are involved in entry and important conformational changes will be induced by the entry process as well. These changes may provide for the exchange of small molecules such as in the doxorubicin example discussed above. The permeability of the capsid is a function of the external environment, in general. For example, CCMV has a pH-dependent capsid structure, characterized by a "swelling" transition occurring upon an increase of pH from pH 4.5 to pH 7.0. This transition has been used to gate the entry of polyanions in the capsid.[4] In an elegant experiment, this gating behavior has been recently used to demonstrate control of substrate access to single horseradish peroxidase enzymes confined inside CCMV capsids.[44] The work promises to open the way for studies of single enzymes in well-defined, genetically adjustable, chemical environments characteristic of viral capsids.

It is conceivable that, in the future, self-assembled nano-reactors made from viral capsids may become useful when organized as lattices or patterns on extended surfaces with the purpose of controlled release or chemistry. A few reports on virus capsid patterning and even hierarchical forms of organization have already emerged.[73,75,85,86]

"Bottom-up" fabrication of hierarchical structures

It is well known that viruses can spontaneously organize in certain conditions into three-dimensional crystals, by a variety of growth mechanisms.[87] The propensity of BMV capsids to crystallize has been used by Sun *et al.* who found that 3D rhombohedral crystals of VLPs containing 12 nm gold nanoparticles can be grown in the same conditions as native BMV crystals,[6] Fig. 13. Preliminary X-ray diffraction studies from

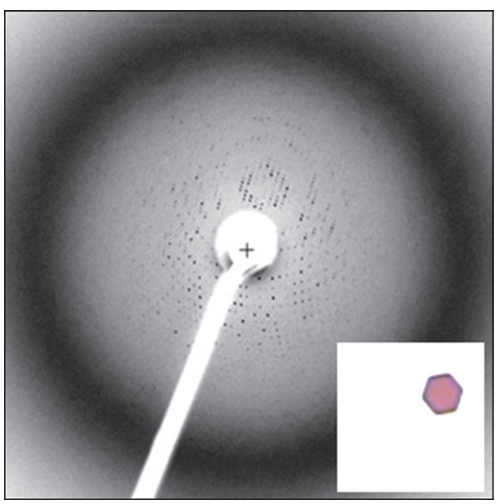


Fig. 13 X-Ray diffraction pattern from a VLP crystal (inset) at 5 Å resolution. The red color comes from the surface plasmon resonance of the encapsidated 12 nm Au nanoparticles. The lattice constant was 275 Å (very close to the expected diameter for intact BMV).

these gold VLP crystals suggest a lattice constant identical with that for BMV. Moreover, BMV and VLPs will co-crystallize upon mixing together at any ratio of VLP : BMV.[6] These results demonstrate that capsid surface features of VLPs and BMV capsids must be very similar at the molecular level. An interesting feature of the diffraction pattern is its remarkable sharpness with diffraction spots at 5 Å, Fig. 13. We deduce that the virus crystallization approach, through its well-defined molecular interactions, leads to a remarkable accuracy in positioning the Au nanoparticles in the nodes of the lattice, much better than any of the currently available lithographic methods. This positioning accuracy probably comes from the fact that the capsid structure is not influenced by, but rather "absorbs", the slight imperfections of the particle inside. Thus, location errors due to variances in the particle size or shape will not accumulate and the long-range order of the lattice is preserved. It is easy to note in the inset of Fig. 13 the color of the VLP crystal, which is due to the surface plasmon resonance of the gold nanoparticles. Since the gold cores are separated by distances comparable to their diameters, the optical properties of the crystal are, in part, determined by the lattice constant. Surface plasmon-assisted spectroscopies such as surface-enhanced infrared absorption[88,89] (SEIRA), surface-enhanced fluorescence,[90,91] and surface-enhanced Raman spectroscopy[92,93] (SERS) have met with great interest recently for their ability to reveal chemical signatures of small amounts of unlabeled molecular compounds.

It is likely that metallodielectric biomaterials like the one presented here would harbor optical properties amenable to spectroscopic applications like SEIRA and SERS. It is reasonable to expect that selection rules and enhancement factors would be different from the traditional roughened metal films or 2D surface-supported nanostructures by virtue of the 3D symmetry and equivalence in the emitter (biomolecular analyte) conformation at any lattice point. The field is open now for experimental and theoretical testing of these expectations.

Finally, to transform virus-based nanomaterials into functional devices, the problem of interfacing them with other structures needs to be addressed. Cheung *et al.*[94] have recently demonstrated that CPMV can be engineered to bind specifically and reversibly at nanoscale chemical templates with ~30 nm spatial resolution. Hierarchical ordering on the surface of droplets, beyond 3D crystallization or planar 1D–2D templating, has been also demonstrated by using the interface between two immiscible liquids to induce the spontaneous formation of CPMV assemblies.[75]

Conclusion

Virus-based materials exhibit a set of unique properties that open vast possibilities for exploration in relation with better drug delivery and *in vivo* imaging agents, or as new paradigms for optical spectroscopy and controlled enzymatic reactions. These unique characteristics include: self-terminated assembly into strictly mono-dispersed shell structures, innate biocompatibility, possibility of genetic engineering, and a set of universal physical principles operating across vast areas in the virus realm, which has recently started to emerge and promises to stimulate researchers' interest for many years to come.

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