Assembly/disassembly of a complex icosahedral virus to incorporate heterologous nucleic acids

Elena Pascual¹, Carlos P Mata, José L Carrascosa and José R Castón²

Department of Structure of Macromolecules, Centro Nacional de Biotecnología/CSIC, Cantoblanco, Madrid, Spain

E-mail: jrcaston@cnb.csic.es

Received 2 August 2017, revised 17 October 2017

Accepted for publication 30 October 2017

Published 13 November 2017

Abstract

Hollow protein containers are widespread in nature, and include virus capsids as well as eukaryotic and bacterial complexes. Protein cages are studied extensively for applications in nanotechnology, nanomedicine and materials science. Their inner and outer surfaces can be modified chemically or genetically, and the internal cavity can be used to template, store and/or arrange molecular cargos. Virus capsids and virus-like particles (VLP, noninfectious particles) provide versatile platforms for nanoscale bioengineering. Study of capsid protein self-assembly into monodispersed particles, and of VLP structure and biophysics is necessary not only to understand natural processes, but also to infer how these platforms can be redesigned to furnish novel functional VLP. Here we address the assembly dynamics of infectious bursal disease virus (IBDV), a complex icosahedral virus. IBDV has a ~70 nm-diameter $T = 13$ capsid with VP2 trimers as the only structural subunits. During capsid assembly, VP2 is synthesized as a precursor (pVP2) whose C terminus is cleaved. The pVP2 C terminus has an amphipathic helix that controls VP2 polymorphism. In the absence of the VP3 scaffolding protein, necessary for control of assembly, 466/456-residue pVP2 intermediates bearing this helix assemble into VLP only when expressed with an N-terminal His₆ tag (the HT-VP2-466 protein). HT-VP2-466 capsids are optimal for genetic insertion of proteins (cargo space ~78 000 nm³). We established an *in vitro* assembly/disassembly system of HT-VP2-466-based VLP for heterologous nucleic acid packaging and/or encapsulation of drugs and other molecules. HT-VP2-466 (empty) capsids were disassembled and reassembled by dialysis against low-salt/basic pH and high-salt/acid pH buffers, respectively, thus illustrating the reversibility *in vitro* of IBDV capsid assembly. HT-VP2-466 VLP also packed heterologous DNA by non-specific confinement during assembly. These and previous results establish the bases for biotechnological applications based on the IBDV capsid and its ability to incorporate exogenous proteins and nucleic acids.

Keywords: virus capsid, assembly/disassembly, virus-like particle, nucleic acid encapsulation, infectious burial disease virus (IBDV)

(Some figures may appear in colour only in the online journal)

¹ Present address: Departamento de Biotecnología, Facultad de Ciencias Experimentales, Universidad Francisco de Vitoria, 28223, Pozuelo de Alarcón, Madrid, Spain.

² Dept. Estructura de Macromoléculas, Centro Nacional Biotecnología/CSIC, Campus de Cantoblanco, Darwin 3, 28049 Madrid, Spain.

Introduction

Viruses need only one or fewer proteins to build a protein shell of defined size and to package the viral genome inside this capsid to yield an infectious particle, the virion, in a biologically feasible time frame [1]. Efficient self-assembly of capsid protein (CP) is based on its built-in conformational flexibility and extensive use of symmetry (icosahedral or helical) [2]. To facilitate and direct correct intersubunit interactions, viruses can use host resources such as molecular chaperones and membrane-specific regions, or other viral components such as scaffolding, accessory, cement, and proteolytic proteins [3]. Due to their structural efficiency at the nanoscale, virus capsids are used in the laboratory extensively as protein cages to incorporate various types of materials at inner and/or outer capsid surfaces, or as nanocontainers to encapsulate proteins or other biomolecules with potential application in nanomedicine and nanobiotechnology [4, 5].

Here we analyzed the capsid assembly of infectious bursal disease virus (IBDV), a serious pathogen of poultry. IBDV is a double-stranded (ds)RNA virus with a single, non-enveloped icosahedral capsid [6]. The CP VP2 is synthesized as a precursor form, pVP2, encoded as part of the NH₂-pVP2-VP4-VP3-COOH polyprotein (1012 residues). VP4 is a viral protease that cotranslationally cleaves its own N and C termini [7, 8], and VP3 is a multifunctional protein with scaffold [9, 10] and dsRNA-binding [11] activities. pVP2 (512 residues) undergoes a series of VP4-mediated processing events at three secondary targets (residues 501, 494, and 487) [12]. The resulting pVP2 intermediates can be further cleaved by a host protease (residue 452), the puromycin-sensitive aminopeptidase PurSA [13], and/or by VP2 itself (residue 441) [14] to yield mature VP2 and several C-terminal fragments (figure 1(a)).

VP2 and a variable amount of pVP2 assemble into 260 trimers, the basic structural units that form a complex $T = 13$ capsid (protein mass 38 MDa, diameter ~ 700 Å) made up of 12 pentamers and 120 hexamers [15–17]. VP2 trimers adopt up to five distinct conformations. Although the most abundant assembly in an IBDV-infected cell is $T = 13$ particles, other minor aberrant assemblies such as tubular structures and smaller $T = 1$ and $T = 7$ icosahedral capsids (figure 1(b)) are found *in vivo*.

Expression of mature VP2 alone in a baculovirus-based system leads to spontaneous assembly of ~ 23 nm-diameter $T = 1$ subviral particles (SVP) built of 20 identical VP2 trimers, which have been resolved by x-ray crystallography [16, 18, 19]. VP2 is folded into three domains termed projection (P), shell (S), and base (B). Domains S and P are β -barrels tangential and radial to the capsid surface, respectively, whereas the B domain is formed by N- and C-terminal α -helices that line the inner capsid surface. Expression of pVP2 or pVP2 intermediates leads to formation of tubular structures with a hexagonal lattice [10, 20].

Numerous proteolytic events modulate the pVP2 71-residue C-terminal domain activity responsible for VP2 polymorphism. The inherent molecular switch that controls VP2 structural plasticity is the 443-GFKDIIRAIR-452 amphipathic

α -helix ($\alpha 5$ helix) [10, 21]. VP3 acts as a canonical scaffold protein that interacts with the basic face of the $\alpha 5$ helix through its last five C-terminal residues 255-DEDLE-259 [9, 22]. In the absence of VP3, pVP2 intermediates with helix $\alpha 5$ (VP2-466; 466 residues) assemble into virus-like particles (VLP) only when expressed with an N-terminal His₆ tag that emulates the scaffolding role of the VP3 C terminus [10], i.e. the HT-VP2-466 protein (figure 1(c)).

The chimeric HT-VP2-466 protein assembles into genuine $T = 13$ capsids and related assemblies that lack the other four viral proteins and are much larger ($\sim 78\,000\text{ nm}^3$ cargo space) than the $T = 1$ SVP (380 nm^3), making them optimal for encapsulation of much larger amounts of heterologous protein, nucleic acid and/or other material. We previously developed HT-VP2-466-based chimeric capsids as vaccine carriers, using enhanced green fluorescent protein (EGFP) or fragments derived from the influenza virus hemagglutinin and matrix proteins fused at the HT-VP2-466 N-terminal end [23]. Here we report the biochemical and structural characterization of an *in vitro* assembly system based on the HT-VP2-466 capsid and explore its suitability to package nucleic acids. Although both the *in vivo* and *in vitro* systems are relatively efficient, compared to the *in vivo* baculovirus-based assembly system, the *in vitro* single-component, reversible assembly/disassembly system can be manipulated to encapsulate specific cargoes independently of the cellular environment. In addition, HT-VP2-466 VLP bearing a DNA fragment could be used as transduction vehicles that recognize their natural receptor, the $\alpha 4\beta 1$ integrin.

Methods

Cells and viruses

QM7 quail muscle cells (ATCC CRL-1962) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C. *Trichoplusia ni* H5 insect cells (Invitrogen, ATCC, CRL-1711) were cultured in TC-100 medium (Gibco-BRL) supplemented with 10% FBS (28 °C). IBDV (Soroa strain [24]) was propagated in QM7 cells as described [6]. Recombinant baculovirus (rBV) HT-VP2-466 was used to produce HT-VP2-466 VLP in H5 cells [10].

Viral infection and purification of viruses and VLP

IBDV was purified following established procedures [11]. Briefly, supernatants of IBDV-infected QM7 cells were harvested at 72 h post-infection (hpi) and precipitated with 3.5% polyethylene glycol (PEG) 6000, 0.5 M NaCl and incubated (overnight, 4 °C, with mild shaking). The precipitate was isolated by centrifugation ($3000 \times g$, 30 min, 4 °C), resuspended in PES buffer (50 mM PIPES pH 6.2, 150 mM NaCl, 20 mM CaCl₂) supplemented with protease inhibitors (Complete Mini, Roche), and further purified by ultracentrifugation through a 25% sucrose cushion ($170\,000 \times g$, 150 min) followed by a 25–50% linear sucrose gradient ($200\,000 \times g$, 45 min).

Figure 1. Atomic structure of (p)VP2 and VP2-related icosahedral assemblies. (a) VP2 protein x-ray model (2GSY) manually bound (at residue 441) to part of the pVP2 C terminus as determined by NMR analysis (segment 442–488; 2IMU). The amphipathic helix (residues 443–452) is labeled helix $\alpha 5$. Helix $\alpha 8$ ends at residue 481, and the C-terminal-most residues are assumed to have a disordered conformation. Wavy lines indicate the conformational flexibility of the pVP2 C-terminal extension. Arrows indicate processing sites of VP2, PurSA, and VP4 protease activities. (b) Major (p)VP2 icosahedral assemblies isolated from IBDV-infected avian cells or using heterologous expression systems. Left, $T = 1$ particles (~23 nm diameter), center, $T = 7$ particles (~53 nm diameter), right, $T = 13$ particles (~70 nm diameter). (c) VP3 and pVP2 polypeptide segments involved in VP2 structural polymorphism. His₆ tag (H-tag) and VP3 C-terminal (VP3 C-ter) sequences are aligned based on polarity. The H-tag is composed of a tract of four residues (Met1-Tyr4), six His residues, a linker region (Asp11-Thr17), the TEV cleavage site (Glu18-Gly24), and Asn25-Ser28, to which the (p)VP2-based protein is bound. The VP3 C terminus includes the last 16 VP3 residues (²⁴²GRLGRWIRTVSDEDELE²⁵⁷; for simplicity, Glu257 is referred to as Glu17, and so on) and the linker region (except the sequence ¹¹DYD¹³), the TEV cleavage site (Glu18-Gly24), and Asn25-Ser28. The C-terminal (p)VP2 Phe442-Leu481 sequence (pVP2 C-ter) is shown.

To produce HT-VP2-466 VLP, H5 cell monolayers were infected with rBV HT-VP2-466 at a multiplicity of infection (moi) of 5 plaque-forming units per cell (pfu/cell) and harvested at 48 hpi. The cell pellet was lysed in PES buffer supplemented with 1% Igepal CA-630 (Sigma) and protease inhibitors (Complete Mini, Roche) for 20 min. The cell suspension was clarified by centrifugation, the supernatant loaded on a 25% sucrose cushion and centrifuged ($170\,000 \times g$, 150 min). The pellet was resuspended in PES buffer and centrifuged in a 25–50% linear sucrose gradient ($200\,000 \times g$, 45 min). Fractions (1 ml) were concentrated 10-fold by ultracentrifugation ($240\,000 \times g$, 120 min). All purification steps were performed at 4 °C.

SDS-PAGE

Concentrated gradient fractions (2–5 μ l) were mixed with Laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% glycerol, 0.012% bromophenol blue, and 2 mM dithiothreitol (DTT), pH 6.8), boiled (3 min, 100 °C) and chilled (1 min, on ice). Samples were resolved by 11% SDS-PAGE.

Agarose gel electrophoresis

Unless otherwise stated, agarose gel electrophoresis was performed in native conditions. Aliquots of purified virions or VLP, disassembled or reassembled, were incubated with 6 $\times$ blue/orange loading dye (Promega; 10 mM Tris-HCl pH 7.5, 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 50 mM EDTA). Samples were

loaded onto 0.7% agarose gels in TBE buffer (90 mM Tris-HCl pH 8.0, 90 mM boric acid, 20 mM EDTA). Where denaturing electrophoresis is indicated, 0.1% SDS was added to the gel. After electrophoresis, gels were visualized with ethidium bromide and electrotransferred to nitrocellulose membranes for Western blot.

Western blot

Samples separated on polyacrylamide or agarose gels were incubated in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 0.0375% SDS, 20% methanol) and then transferred to a nitrocellulose membrane (Protran; Schleicher and Schuell) in a semidry electroblotter (SD cell, BioRad) (1 h, 200 mA). Blots were blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and incubated with a primary rabbit anti-VP2 antibody diluted in blocking solution (2 h). Membranes were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (GE Healthcare) (1 h). Membranes were developed with ECL chemiluminescence reagent (GE Healthcare).

Electron microscopy

Samples (~5 μ l) were applied to glow-discharged carbon-coated grids and incubated (5 min). After washing, samples were negatively stained with 2% aqueous uranyl acetate and allowed to dry before observation in a JEOL 1200 EXII 187 electron microscope operating at 100 kV at a nominal magnification of X40 000.

Figure 2. Disassembly of IBDV $T = 13$ capsids is irreversible. (a)–(c) Electron microscopy and negative staining of purified IBDV $T = 13$ capsids (a): these particles were disassembled by dialysis against TE buffer (b), and dialyzed a second time against PES buffer, which resulted in assembly of $T = 1$ -like particles (c) although no $T = 13$ icosahedral particles were reassembled in these conditions. Bar, 100 nm. (d) IBDV $T = 13$ capsids (top), disassembled material (center) and $T = 1$ -like particles (bottom) were purified by ultracentrifugation on sucrose gradients, collected in 12 fractions, concentrated by ultracentrifugation, and analyzed by SDS-PAGE and Western blot using anti-VP2 antibodies. Sedimentation direction was from right to left, with fraction 12 at the gradient top.

DNase I protection assay

DNase protection assays were performed with four samples: purified dsDNA fragments obtained by treatment of plasmid pRSET-A with the restriction enzyme PvuI (New England Biolabs); HT-VP2-466 VLP; dsDNA fragments mixed with previously assembled VLP; and dsDNA fragments mixed with disassembled VLP and allowed to reassemble. The reaction mixtures (40 μ l), containing ~ 0.2 mg ml $^{-1}$ of protein, ~ 20 ng μ l $^{-1}$ of dsDNA and ~ 1 μ g ml $^{-1}$ DNase I in PES buffer were incubated (20 min, 37 °C), and stopped by addition of 10 mM EDTA. The products were separated on a 1.5% agarose gel, and detected by ethidium bromide staining.

Results

Disassembly of the IBDV mature capsid is irreversible

Dialysis of IBDV virions against TE buffer, a low-salt buffer with EDTA (5 mM Tris-HCl pH 8, 5 mM EDTA), ruptures most particles to release the ribonucleoprotein complexes (RNP, [11]). We used this treatment to characterize the IBDV assembly/disassembly pathway.

After ultracentrifugation in a sucrose gradient (figure 2(a)), purified virions were dialyzed against TE buffer (16 h, 4 °C) and the resulting structures analyzed biochemically by (negative staining) electron microscopy (EM). After dialysis, virion $T = 13$ capsids were no longer visible and the only observable macromolecular structures were filamentous RNP on a granulate background that is probably related to the individual capsomers and/or protein aggregates (figure 2(b)). To assess the ability of these components to reassemble, we further dialyzed the disassembled material against PES buffer (24 h, 4 °C), and the resulting assemblies were analyzed by negative staining. $T = 1$ -like ~ 23 nm-diameter capsids were observed, but no reassembled $T = 13$ capsid structures (figure 2(c)).

To confirm the EM analysis by independent criteria, IBDV virions (full $T = 13$ capsids), disassembled products (RNP, capsomers, and/or protein aggregates), and reassembled material ($T = 1$ capsids) were each analyzed by ultracentrifugation in

linear 25–50% sucrose gradients in appropriate buffers. Western blot analysis with anti-VP2 antibodies showed that most $T = 13$ capsids localized in the middle fractions (figure 2(d), top panel; fractions 5–7); disassembly products were detected in the top fractions (figure 2(d), center; fractions 11–12). Finally, after PES dialysis, the VP2 signal was detected at the top of the gradient (figure 2(d), center; fractions 10–12), corresponding to the mobility of $T = 1$ -sized assemblies. These results suggested that disassembly of mature IBDV particles cannot revert to the original $T = 13$ capsids, as the mature VP2 CP was able to assemble only into $T = 1$ capsids.

Disassembly/reassembly of HT-VP2-466 VLPs

As the HT-VP2-466 recombinant protein bears the two morphogenetic elements necessary for $T = 13$ VLP assembly (the amphipathic helix $\alpha 5$ and the His $_6$ tag), we carried out similar disassembly/reassembly treatments with baculovirus-derived, purified HT-VP2-466 structures [10]. HT-VP2-466 assemble relatively efficiently into $T = 13$ capsid-like particles; intermediate size $T = 7$ ~ 53 nm-diameter capsid-like structures, $T = 1$ ~ 23 nm-diameter capsids, and tubular assemblies were easily distinguished among $T = 13$ capsids (figure 3(a)). After dialysis against TE buffer, HT-VP2-466 VLP were mostly disassembled, since negative staining EM detected only small capsomers as punctate material (figure 3(b)). Contrary to what happened with IBDV capsids, after dialysis against PES buffer, we observed isometric particles whose size and morphology were similar to $T = 13$ viral particles, as well as smaller particles that probably corresponded to $T = 7$ capsids and irregular assemblies (figure 3(c)).

Native, disassembled and reassembled HT-VP2-466 structures were analyzed similarly by ultracentrifugation in a linear sucrose gradient, and resulting fractions were tested by SDS-PAGE and Western blot using VP2-specific antibodies (figure 3(d)). The VP2 signal for the original native HT-VP2-466 structures was observed from the bottom to the middle fractions, which corresponded to tubular assemblies with variable lengths, as well as isometric $T = 13$, and $T = 7$ VLP (figure

Figure 3. Disassembly/reassembly of $T = 13$ HT-VP2-466 VLP is reversible. (a)–(c) Electron microscopy and negative staining of purified HT-VP2-466 assemblies (formed by icosahedral and irregular particles) (a); these structures were disassembled by dialysis against TE buffer (b), and dialyzed a second time against PES buffer; this led to the assembly of $T = 13$, $T = 7$ and $T = 1$ -like particles and irregular particles (c). Bar, 100 nm. (d) HT-VP2-466 assemblies (top), disassembled material (center) and reassembled particles (bottom) were purified by ultracentrifugation on sucrose gradients, collected in 12 fractions, concentrated by ultracentrifugation, and analyzed by SDS-PAGE and Western blot using anti-VP2 antibodies. Sedimentation direction was as in figure 2.

3(d), top). Disassembled HT-VP2-466 structures migrated mainly to the top of the gradient (figure 3(d), center; fractions 10–12). After dialysis against PES buffer, the VP2 signal was again detected throughout the gradient, consistent with reassembly of tubules and isometric capsids. Although a fraction of HT-VP2-466 remained in the upper gradient fractions, these data show that HT-VP2-466 assembly is reversible, and thus demonstrate *in vitro* assembly of IBDV $T = 13$ capsids.

Resistance of encapsulated DNA fragments to DNase digestion

Once HT-VP2-466 VLP reassembly conditions were defined, we carried out accessibility experiments with DNase I, a nuclease specific for double-stranded DNA, to test whether DNA molecules can be non-specifically packaged during reassembly. Disassembled HT-VP2-466 VLP were dialyzed against PES buffer in the presence of two DNA fragments of ~1000 and ~1800 bp (~20 ng DNA μL^{-1}) for 24 h (~4–6 fragments per initial capsid). The resulting assemblies were analyzed in a native agarose gel with ethidium bromide staining to detect DNA, or by Western blot of the same gel with rabbit anti-VP2 serum.

When reassembled HT-VP2-466 VLP were incubated with the DNA fragments, VP2 and DNA signals did not colocalize, which indicated that they did not interact (figure 4(a), lanes 3). When disassembled VLP were incubated with the DNA fragments during reassembly, both VP2 and DNA colocalized in a single band with retarded electrophoretic mobility (figure 4(a), lanes 4, arrow), which suggested that DNA fragments were incorporated into the VLP. Band mobility differed slightly from that of IBDV virions (figure 4(a), lanes 5). To confirm that DNA fragments are packaged in the capsid interior, the reassembly products were incubated with DNase I (1 $\mu\text{g mL}^{-1}$). A single DNA band detected in the native agarose gel showed the same mobility as VLP that underwent reassembly in the presence of DNA fragments, which suggests non-specific packaging of heterologous DNA (figure 4(b), lanes 4). In a negative control, in which previously

reassembled VLP were incubated with the DNA fragments, these fragments were not resistant to DNase I treatment (figure 4(b), lanes 3). As for IBDV, with virions with 1–4 copies of dsRNA segments, HT-VP2-466 particles can contain variable copy numbers of packaged dsDNA fragments.

Finally, we verified the identity of the material in the VLP interior resistant to DNase I digestion. Reassembled VLP bearing DNA fragments were dialyzed against TE buffer to destabilize VLP and release their content. This sample and an IBDV virions positive control were incubated with 1% SDS and proteinase K (20 $\mu\text{g mL}^{-1}$; 1 h, 37 °C) and analyzed in an agarose gel with 0.1% SDS. The two bands showed the same mobility as the ~1000 and ~1800 bp DNA fragments (figure 4(c), lanes 1, 2). These results imply that HT-VP2-466 VLP can incorporate nucleic acids during *in vitro* capsid assembly, which are protected from external agents, and thus resemble the viral genome in the IBDV extracellular life cycle.

Discussion

Two general strategies are recognized for viral genome packaging mechanisms, (i) viruses that assemble a procapsid and require a nucleic acid (dsDNA) pump or portal, and (ii) those that form around their genome (mostly ssRNA). Most dsDNA viruses assemble a precursor particle, the procapsid, which subsequently expands to a mature capsid for genome packaging. Procapsid assembly requires scaffold proteins (SP), as well as a protease that cleaves SP and initiates expansion [3, 25], and a nanomotor (incorporated at the onset of capsid assembly) that pumps DNA into the capsid by ATP hydrolysis [26]. The capsid thus withstands high pressure as the genome is pumped in. By contrast, most viruses with single-stranded (ss) genomes assemble spontaneously around their genomes, that is, assembly and packaging are coupled [27]. As recently visualized by cryo-EM with the bacteriophage MS2, most ssRNA is folded into stem-loops [28, 29]. Electrostatic interactions between positive charges on the CP inner surface and negative charges on ssRNA are the driving thermodynamic

Figure 4. Encapsulation of heterologous nucleic acids during *in vitro* reassembly of HT-VP2-466 capsid and DNase accessibility. (a) and (b) Purified 1.0 and 1.8 kbp DNA fragments (lane 1), HT-VP2-466 VLP (lane 2), previously reassembled HT-VP2-466 particles mixed with DNA fragments (lane 3), and HT-VP2-466 particles allowed to reassemble in the presence of DNA fragments (lane 4) were analyzed in native agarose gel electrophoresis, alone ((a), $-$ DNase I) or pretreated with DNase I ((b), $+$ DNase I). Lane 5 is an IBDV virion control. The DNA fragments were detected by ethidium bromide staining ((a) and (b), top). The same gel was used for Western blot analysis with anti-VP2 antibodies ((a) and (b), bottom, α -VP2:). Arrows indicate the position of HT-VP2-466 VLP (VLP), 1.0 and 1.8 kbp dsDNA, 2.8 and 2.3 kbp dsRNA, and HT-VP2-466 and VP2. (c) Denaturing agarose gel electrophoresis (0.1% SDS) of purified 1000 and 1800 bp DNA fragments (lane 1), and extracted fragments after disassembly of cargo-bearing reassembled HT-VP2-466 capsids (lane 2). Lane 3 is a control of disassembled IBDV virions bearing the two genomic 2.8 and 2.3 kbp dsRNA fragments. Bands were detected by ethidium bromide staining. (d) Scheme of the HT-VP2-466 assemblies analyzed in this study (1–4, as described above).

force for capsid assembly [30]. In general terms, the stiffness and high charge density of dsDNA (or dsRNA) genomes of certain length preclude spontaneous packaging, although relatively small dsDNA fragments can be packaged spontaneously, as is the case of Simian virus 40 [31]. Viruses that package a dsDNA genome into a preformed empty capsid appear to be stronger and more rigid than viral capsids that assemble around a ss genome [32, 33].

IBDV capsid assembly is initiated with a precursor CP (pVP2) and a SP (VP3) to build a particle that resembles a procapsid-like structure. IBDV lacks a portal protein for genome translocation, and the genome is probably incorporated by VP3 (due to its RNA-binding activity) during early capsid assembly. Furthermore, IBDV dsRNA segments might be packed as positive ssRNA segments, as in other dsRNA viruses [34, 35]. VLP produced by IBDV polyprotein expression in an inducible recombinant vaccinia virus [24, 36] result in spontaneous assembly of $T = 13$ VLP, which were a mixture of empty VLP and VLP bearing nonspecific ssRNA (possibly cellular messengers) [37]. In this study, electrophoretic analysis in ethidium bromide-stained native agarose gels showed that HT-VP2-466 VLP produced with a rBV are mostly empty particles (or nucleic acid incorporation is minimal). This result indicates that, as anticipated, the His tag only emulates the SP function of the VP3 C terminus, but VP3 RNA-binding activity is entirely lost. At first glance, HT-VP2-466 VLP are a drawback for efficient packing of exogenous nucleic acids. These VLP nonetheless have advantages; they can be used to encapsulate a DNA fragment directly, as removal of nonspecific host nucleic acids is unnecessary. Here we show proof of concept for HT-VP2-466 assemblies as potential carriers of

heterologous nucleic acids in their interior cargo space. Future experiments will define the packaging of other nucleic acids (ssDNA, ssRNA, dsRNA) and the size limits for each type.

IBDV capsids can be disassembled by dialysis against a low-salt, basic pH buffer in the presence of a divalent cation chelating agent. The simultaneous effect of these factors destabilizes VP2-mediated inter-trimeric interactions. In principle, these interactions could be restored by dialysis against the original buffer, but cleavage of the C-terminal $\alpha 5$ helix in any pVP2 intermediate precursor precludes VP2 reassembly into $T = 13$ capsids or tubular structures. In contrast, HT-VP2-466 capsids and assemblies maintain the ability to establish the interactions necessary for building $T = 13$ capsids. After disassembly in TE buffer, dialysis against the original buffer in the absence of cargo leads to reassembly of these capsids and of other small particles. These experiments indicate a reversible process that assembles $T = 13$ capsids similar to those of IBDV. The development of an *in vitro* disassembly/reassembly system for HT-VP2-466 capsids allows the non-specific incorporation of macromolecules during reassembly. This system can be used to encapsulate, besides nucleic acids as described here, other proteins or organic molecules.

VLP structural integrity allows interaction with receptors and internalization in host cells, which makes them optimal nanovehicles for directed transport of macromolecules to specific cells [38]. HT-VP2-466 VLP bearing a DNA fragment can be used as transduction vehicles, to replace other, more limited techniques such as microinjection or electroporation, that are effective in cultured cells but unsuitable for whole animals or patients. HT-VP2-466 $T = 13$ and VP2 $T = 1$ capsids share very similar VP2 trimeric organization. In avian

cells, $T = 1$ capsids interact with the $\alpha 4\beta 1$ integrin as specific binding receptors [39]. This integrin is also found in mammalian lymphocytes, and HT-VP2-466 VLP might thus be used to develop new genetic therapies based on the gene-specific transport into these cell lines.

Our studies of HT-VP2-466 VLP disassembly/reassembly show the ability of these capsids to incorporate DNA fragments *in vitro*. Quantitative analyses are needed to characterize size limits and amount of nucleic acid packaged, as well as transport efficiency to the cell interior. These questions could be pursued using a reporter gene, such as that of a fluorescent protein, to detect and quantify its expression in target cells. *In vitro* packaging of the viral genome as purified RNP (or *in vitro*-reconstituted by combining viral dsRNA with VP3) could be another valid approach to generation of synthetic IBDV virions.

Conclusion

The *in vitro* disassembly and reassembly pathway of HT-VP2-466 capsids is reversible. The structural building block, the HT-VP2-466 protein, constitutively maintains the two morphogenetic elements, the $\alpha 5$ helix and the His tag, needed to construct a $T = 13$ capsid. The pVP2 amphipathic $\alpha 5$ helix is the conformational switch responsible for inherent VP2 polymorphism, whereas the His tag emulates the VP3 C-terminal segment that controls VP2 structural polymorphism and acts as a scaffold domain. During this *in vitro* HT-VP2-466 capsid reassembly, heterologous nucleic acids can be packaged in the capsid interior, which offers a broad spectrum of potential applications.

Acknowledgments

We thank C Mark for editorial assistance. EP received a PhD fellowship from the Spanish Ministry of Science and Innovation (JAE grant), and CPM was a PhD fellow of the La Caixa Foundation International Fellowship Programme (La Caixa/CNB). This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (BFU2014-54181 to JLC and BFU2014-55475-R to JRC), and the Comunidad Autónoma de Madrid (S2013/MIT-2850 to JLC and S2013/MIT-2807 to JRC). The authors declare no competing financial interests.

ORCID iDs

José R Castón <https://orcid.org/0000-0003-2350-9048>

References

- [1] Zlotnick A 2005 Theoretical aspects of virus capsid assembly *J. Mol. Recognit.* **18** 479–90
- [2] Caston J R and Carrascosa J L 2013 The basic architecture of viruses *Subcell. Biochem.* **68** 53–75
- [3] Steven A C *et al* 2005 Structure, assembly, and antigenicity of hepatitis B virus capsid proteins *Adv. Virus Res.* **64** 125–64
- [4] Yildiz I, Shukla S and Steinmetz N F 2011 Applications of viral nanoparticles in medicine *Curr. Opin. Biotechnol.* **22** 901–8
- [5] Schwarz B, Uchida M and Douglas T 2017 Biomedical and catalytic opportunities of virus-like particles in nanotechnology *Adv. Virus Res.* **97** 1–60
- [6] Luque D, Rivas G, Alfonso C, Carrascosa J L, Rodríguez J F and Caston J R 2009 Infectious bursal disease virus is an icosahedral polyplod dsRNA virus *Proc. Natl Acad. Sci. USA* **106** 2148–52
- [7] Birghan C, Mundt E and Gorbalenya A E 2000 A non-canonical lon proteinase lacking the ATPase domain employs the Ser-Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus *EMBO J.* **19** 114–23
- [8] Feldman A R, Lee J, Delmas B and Paetzel M 2006 Crystal structure of a novel viral protease with a serine/lysine catalytic dyad mechanism *J. Mol. Biol.* **358** 1378–89
- [9] Saugar I, Irigoyen N, Luque D, Carrascosa J L, Rodríguez J F and Castón J R 2010 Electrostatic interactions between capsid and scaffolding proteins mediate the structural polymorphism of a double-stranded RNA virus *J. Biol. Chem.* **285** 3643–50
- [10] Saugar I *et al* 2005 Structural polymorphism of the major capsid protein of a double-stranded RNA virus: an amphipathic alpha helix as a molecular switch *Structure* **13** 1007–17
- [11] Luque D, Saugar I, Rejas M T, Carrascosa J L, Rodríguez J F and Castón J R 2009 Infectious Bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus *J. Mol. Biol.* **386** 891–901
- [12] Sánchez A B and Rodríguez J F 1999 Proteolytic processing in infectious bursal disease virus: identification of the polypeptide cleavage sites by site-directed mutagenesis *Virology* **262** 190–9
- [13] Irigoyen N, Castón J R and Rodríguez J F 2012 Host proteolytic activity is necessary for infectious bursal disease virus capsid protein assembly *J. Biol. Chem.* **287** 24473–82
- [14] Irigoyen N, Garriga D, Navarro A, Verdager N, Rodríguez J F and Castón J R 2009 Autoproteolytic activity derived from the infectious bursal disease virus capsid protein *J. Biol. Chem.* **284** 8064–72
- [15] Castón J R *et al* 2001 C terminus of infectious bursal disease virus major capsid protein VP2 is involved in definition of the T number for capsid assembly *J. Virol.* **75** 10815–28
- [16] Coulbaly F *et al* 2005 The birnavirus crystal structure reveals structural relationships among icosahedral viruses *Cell* **120** 761–72
- [17] Böttcher B, Kiselev N A, Stel'Mashchuk V Y, Perevozchikova N A, Borisov A V and Crowther R A 1997 Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy *J. Virol.* **71** 325–30
- [18] Lee C C *et al* 2006 Crystal structure of infectious bursal disease virus VP2 subviral particle at 2.6 Å resolution: implications in virion assembly and immunogenicity *J. Struct. Biol.* **155** 74–86
- [19] Garriga D *et al* 2006 The 2.6-Ångstrom structure of infectious bursal disease virus-derived $T = 1$ particles reveals new stabilizing elements of the virus capsid *J. Virol.* **80** 6895–905
- [20] Martínez-Torrecuadrada J L, Castón J R, Castro M, Carrascosa J L, Rodríguez J F and Casal J I 2000 Different architectures in the assembly of infectious bursal disease virus capsid proteins expressed in insect cells *Virology* **278** 322–31

- [21] Galloux M *et al* 2007 Infectious bursal disease virus, a non-enveloped virus, possesses a capsid-associated peptide that deforms and perforates biological membranes *J. Biol. Chem.* **282** 20774–84
- [22] Oña A, Luque D, Abaitua F, Maraver A, Castón J R and Rodríguez J F 2004 The C-terminal domain of the pVP2 precursor is essential for the interaction between VP2 and VP3, the capsid polypeptides of infectious bursal disease virus *Virology* **322** 135–42
- [23] Pascual E *et al* 2015 Structural basis for the development of avian virus capsids that display influenza virus proteins and induce protective immunity *J. Virol.* **89** 2563–74
- [24] Lombardo E *et al* 1999 VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles *J. Virol.* **73** 6973–83
- [25] Veesler D and Johnson J E 2012 Virus maturation *Annu. Rev. Biophys.* **41** 473–96
- [26] Feiss M and Rao V B 2012 The bacteriophage DNA packaging machine *Adv. Exp. Med. Biol.* **726** 489–509
- [27] Mateu M G 2016 Assembly, engineering and applications of virus-based protein nanoparticles *Adv. Exp. Med. Biol.* **940** 83–120
- [28] Koning R I *et al* 2016 Asymmetric cryo-EM reconstruction of phage MS2 reveals genome structure *in situ* *Nat. Commun.* **7** 12524
- [29] Dai X *et al* 2017 *In situ* structures of the genome and genome-delivery apparatus in a single-stranded RNA virus *Nature* **541** 112–6
- [30] Perlmutter J D and Hagan M F 2015 Mechanisms of virus assembly *Annu. Rev. Phys. Chem.* **66** 217–39
- [31] Mukherjee S, Kler S, Oppenheim A and Zlotnick A 2010 Uncatalyzed assembly of spherical particles from SV40 VP1 pentamers and linear dsDNA incorporates both low and high cooperativity elements *Virology* **397** 199–204
- [32] Roos W H, Ivanovska I L, Evilevitch A and Wuite G J 2007 Viral capsids: mechanical characteristics, genome packaging and delivery mechanisms *Cell. Mol. Life Sci.* **64** 1484–97
- [33] Marchetti M, Wuite G and Roos W H 2016 Atomic force microscopy observation and characterization of single virions and virus-like particles by nano-indentation *Curr. Opin. Virol.* **18** 82–8
- [34] Fajardo T Jr, Sung P Y and Roy P 2015 Disruption of Specific RNA–RNA Interactions in a double-stranded RNA virus inhibits genome packaging and virus infectivity *PLoS Pathog.* **11** e1005321
- [35] Sung P Y and Roy P 2014 Sequential packaging of RNA genomic segments during the assembly of Bluetongue virus *Nucl. Acids Res.* **42** 13824–38
- [36] Castón J R, Rodríguez J F and Carrascosa J L 2008 Infectious bursal disease virus: a segmented double-stranded RNA virus with a $T = 13$ capsid that lacks a $T = 1$ core *Segmented Double-Stranded RNA Viruses Structure and Molecular Biology* ed J T Patton (Norfolk: Caister Academic Press) pp 133–44
- [37] Mertens J *et al* 2015 A protein with simultaneous capsid scaffolding and dsRNA-binding activities enhances the birnavirus capsid mechanical stability *Sci. Rep.* **5** 13486
- [38] Kaczmarczyk S J, Sitaraman K, Young H A, Hughes S H and Chatterjee D K 2011 Protein delivery using engineered virus-like particles *Proc. Natl Acad. Sci. USA* **108** 16998–7003
- [39] Delgui L *et al* 2009 The capsid protein of infectious bursal disease virus contains a functional alpha4beta1 integrin ligand motif *Virology* **386** 360–72