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

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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)

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**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 nanocarriers 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-icosahedral capsid [6]. The CP VP2 is synthesized as a precursor form, pVP2, encoded as part of the NH2-pVP2-VP4-icosahedral capsid [6]. The CP VP2 is synthesized as a precursor form, pVP2, encoded as part of the NH2-pVP2-VP4-icosahedral capsid [6]. The CP VP2 is synthesized as a precursor form, pVP2, encoded as part of the NH2-pVP2-VP4-icosahedral capsid [6]. The CP VP2 is synthesized as a precursor form, pVP2, encoded as part of the NH2-pVP2-VP4-icosahedral capsid [6].

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 (~78,000 nm³ cargo space) than the T = 1 SVP (380 nm³), 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 α4β1 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 × g, 30 min, 4 °C), resuspended in PBS buffer (50 mM PIPES pH 6.2, 150 mM NaCl, 2 mM CaCl₂) supplemented with protease inhibitors (Complete Mini, Roche), and further purified by ultracentrifugation through a 25% sucrose cushion (170,000 × g, 150 min) followed by a 25–50% linear sucrose gradient (200,000 × g, 45 min).

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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 \( \mu l \)) 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 × 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 \( \mu l \)) 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 X40000.
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 (16h, 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 granule 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 (24h, 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 disassembled 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 α5 and the His6 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...
To confirm that DNA fragments are packaged in the capsid, we carried out 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 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.

Resistence of encapsulated DNA fragments to DNase digestion

Once HT-VP2-466 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 µg 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 µg 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 ~1800bp 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 forces for the assembly process.
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 are 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 encapsidate, 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.
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.

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