Development of the dodecahedral penton particle from adenovirus 3 for therapeutic application

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The subviral dodecahedral particle of adenovirus 3, which assembles spontaneously in insect cells expressing the viral penton base protein, shows promise as a vector for drug delivery. Its ability to gain cell entry has been demonstrated and recent structural analysis has outlined details of the interfaces between penton bases and the importance of proteolysis of the penton base N terminus for assembly, providing a basis for understanding particle assembly and stability. Here, work in manipulating the assembly status of the dodecahedron by changing buffer conditions and subsequent success in passively encapsidating a marker molecule is described. This represents an important stage towards development of the dodecahedral particle for use as a delivery vehicle capable of targeting therapeutic molecules to specific cell types.

Therapeutic intervention targeted to specific cell types has enormous potential for treatments involving toxic drugs by avoiding problems of systemic delivery. Suitable vectors should target and enter cells efficiently, contain sufficient cargo to be efficacious while protecting it during passage and elicit a minimal immune response. Ideally, such a vehicle should be capable of retargeting as desired. The capsids of non-enveloped viruses have evolved many of these features and some, including adenovirus, have been employed for delivery of genes. However, some complications can arise from the use of such recombinant adenoviruses that harbour viral coding sequences. The subviral dodecahedral particle was proposed as an alternative to the whole adenovirus capsid, as it is devoid of any viral genetic sequences and so cannot promote infection and its smaller complement of protein may illicit a weaker immune response (Fender et al., 1997).

Dodecahedra of adenovirus serotype 3 (Ad3) are observed in infected cells and assemble spontaneously upon expression of the 60 kDa penton base protein in insect cells as 12 pentameric penton bases (Fender et al., 1997; Norrby, 1966; Pettersson & Höglund, 1969; Schoehn et al., 1996) that are either partially or fully N-terminally proteolysed (Fuschiotti et al., 2006). Co-expressed fiber protein binds to the penton bases as in the virus, and combining fibers and penton bases from different adenovirus serotypes allows targeting of specific cell types (Mizuguchi & Hayakawa, 2004). The penton base protein is multifunctional: in addition to its structural role and binding the fiber, it is involved in viral cell entry through binding to cellular sH integrins (Wickham et al., 1993) and in viral release from endosomes on the pathway towards the cell nucleus (Greber et al., 1993). Cell entry has been demonstrated by immunological localization of penton bases within target cells (Fender et al., 2003).

As structural analysis of the dodecahedron reveals that the internal cavity of 80 Å (8 nm) diameter is too small to accommodate a gene (Fuschiotti et al., 2006; Schoehn et al., 1996), we have pursued an alternative use for targeted drug delivery. Our first goal was to control assembly status of the dodecahedral particle, as production of intact particles was not reproducible. Our recent structural characterization (Fuschiotti et al., 2006) determined the importance of proteolysis of the N-terminal 37 residues, 60 copies of which cannot fit into the interior volume of the dodecahedron and are absent in assembled particles. We explored various conditions for promoting assembly, as listed in Table 1. Assembly status was screened by negative-stain electron microscopy (EM) (Fig. 1) and by native agarose-gel electrophoresis.

Ad3 base dodecahedra were prepared in the baculovirus expression system as described previously (Fender et al., 1997) and purified by anionic-exchange chromatography (Econo-Pac High Q Cartridge; Bio-Rad) as described previously (Fuschiotti et al., 2006). Disassembly was readily
Table 1. Conditions tested for promoting assembly of dodecahedra from expressed and purified Ad3 penton bases

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer conditions</th>
<th>EM observation</th>
<th>Fig. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sucrose gradient 30–35 %</td>
<td>10 mM Tris (pH 7-4), 150 mM NaCl, 2 mM EDTA, 10 % glycerol</td>
<td>Fragile dodecahedra and penton bases</td>
</tr>
<tr>
<td>2.</td>
<td>Dialysis (to remove sucrose and glycerol)</td>
<td>75 mM NaCl, 2 mM EDTA</td>
<td>Fragile dodecahedra</td>
</tr>
<tr>
<td>3.</td>
<td>Gel filtration</td>
<td>As for #2; Superose-6 column</td>
<td>Mostly penton bases</td>
</tr>
<tr>
<td>4.</td>
<td>Anionic exchange</td>
<td>Tris (pH 8-0), NaCl gradient</td>
<td>Fragile dodecahedra and penton bases</td>
</tr>
<tr>
<td>5.</td>
<td>+DNA</td>
<td>As for #4</td>
<td>Fragile dodecahedra and penton bases</td>
</tr>
<tr>
<td>6.</td>
<td>+Heparin</td>
<td>As for #4</td>
<td>Few, fragile dodecahedra</td>
</tr>
<tr>
<td>7.</td>
<td>Sodium acetate</td>
<td>20 mM sodium acetate, 150 mM NaCl (pH 6), 4 °C</td>
<td>Mostly penton bases</td>
</tr>
<tr>
<td>8.</td>
<td>Mg²⁺</td>
<td>10 mM MgCl₂, Tris buffer (pH 7-4)</td>
<td>Mostly penton bases</td>
</tr>
<tr>
<td>9.</td>
<td>Mg²⁺</td>
<td>50 mM MgCl₂, Tris buffer (pH 7-4)</td>
<td>Some dodecahedra and penton bases</td>
</tr>
<tr>
<td>10.</td>
<td>Mg⁺³</td>
<td>50 mM MgCl₂, 50 mM phosphate (pH 6-6)</td>
<td>Few irregular particles</td>
</tr>
<tr>
<td>11.</td>
<td>(NH₄)₂SO₄</td>
<td>Tris/HCl (pH 7-4), 750 mM (NH₄)₂SO₄</td>
<td>Irregular particles and penton bases</td>
</tr>
<tr>
<td>12.</td>
<td>(NH₄)₂SO₄</td>
<td>Acetate buffer (pH 6), 750 mM (NH₄)₂SO₄, 4 °C</td>
<td>Mostly well-formed dodecahedra and few penton bases</td>
</tr>
<tr>
<td>13.</td>
<td>(NH₄)₂SO₄</td>
<td>750 mM (NH₄)₂SO₄, 50 mM phosphate (pH 6-6)</td>
<td>–</td>
</tr>
</tbody>
</table>

Various conditions were tested for promoting assembly of dodecahedra from expressed and purified Ad3 penton bases, as listed. Visualization by EM is given for some samples as indicated (Fig. 1). In general, samples appeared fragile and often promising biochemical characterization yielded EM images that included free pentons in the background and varying degrees of assembly and disassembly.

We established that pH and ionic strength are the major factors affecting assembly status. Titration in phosphate buffer at different pH values identified the limits between which the dodecahedra remained stable. Under conditions of high pH (8-9) and low ionic strength, penton bases were unassembled and most of the protein was present in the lighter fractions of the sucrose gradient (Fig. 1k) and observed by EM as free bases or irregular clusters (Fig. 1l).

Assembly into dodecahedra was observed after removal of chelating agents (EDTA) at high ionic strength and low pH. After dialysis against buffer containing 750 mM ammonium sulphate at pH 6-6, the preparation was recovered in the low part of the sucrose-density gradient (Fig. 1m), corresponding to heavier, assembled particles, as confirmed by EM (Fig. 1n). Control of particle assembly through manipulating pH promises an effective method for encapsidating therapeutic molecules, as well as establishing the feasibility of using this system at physiological conditions.

The effects of pH on particle status may be explained by examining the structural elements that maintain particle integrity. The subunit interactions within each penton base are relatively strong, as is evident from visualization of free penton bases in all conditions under which dodecahedra were not formed. For the Ad2 penton base, approximately 26 % of the subunit surface area is buried at this interface (Zubieta et al., 2005). Most of the interface residues are the same or substituted conservatively, suggesting that the specific interactions will be similar. These interactions were narrowed down in the Ad2 crystal structure to four residues in each of the three regions, suggesting that the specific interactions will be similar.
corresponding in the Ad3 sequence to aa 99–102 (NDFT) contacting aa 425–428 (RSTR), and aa 58–61 (SDVS) contacting a copy of itself. Of these in the Ad3 sequence, five are substituted conservatively compared with the Ad2 protein and six are identical. Two that are identical, D100 and R425, appear to make a salt bridge between pentons in the Ad2 structure (PDB id, 1X9P), the strength of which may be modulated by pH. Mutations at these sites will be necessary to establish the residues critical to dodecahedron stability, as well as to explore modifications that enhance particle integrity.

Our second goal was to demonstrate passive encapsidation of ‘cargo’ within the dodecahedron by shifting buffer conditions from disassembly to assembly after incubation with a marker molecule and subsequent imaging by cryo-EM to allow visualization of internal density, performed as described previously (Fuschiotti et al., 2006). The marker chosen, Nanogold (Nanoprobes Inc.), has a small core of gold atoms, 14 Å (1.4 nm) in diameter, surrounded by an organic shell of triphenylphosphines and is sufficiently electron-dense to be visible at the magnification used (×38 000). Unlike larger colloidal gold, Nanogold does not adhere spontaneously to protein, but is often used to specifically label thiols, such as the cysteine side chain, through maleimido derivitization of one of the phosphines and an appropriate incubation process. In this experiment, it is a passive marker chosen because it is small enough to fit inside the dodecahedron cavity. Dodecahedra were disassembled at basic pH (8.9) and mixed with Nanogold particles. Subsequently, the sample was dialysed at 4 °C into ‘assembly’ buffer. Several approaches were used to remove free gold particles, including filtration by 100K Ultrafree Millipore 0.5 centrifugal filters, dialysis (100 000 MWCO membrane; Perbio) and sucrose density-gradient separation.

For cryo-EM, samples were applied to holey carbon on copper Quantifoil grids, blotted and plunge-frozen into

![Fig. 1. Examples of conditions tested for assembly and screened by negative-stain EM using sodium silicotungstate as the contrasting agent. Panels (a–j) are described in Table 1 and include samples from gel filtration, anionic exchange and incubation with negatively charged polymers (DNA and heparin). Symbols in panel (a) indicate well-formed dodecahedra (black arrow), poorly formed or disassembling dodecahedra (white arrow), dodecahedra disassembled into a loose collection of penton bases (white arrowhead) and free penton bases (open arrowhead). The particles appearing the most regular are in (j) and only a few free penton bases are evident in the background. Bar, 500 Å (50 nm). (k–n) Disassembly and reassembly of dodecahedra as analysed by sucrose density-gradient separation followed by negative-stain EM. Buffer conditions of higher pH and low ionic strength yield lighter material (k) that appears to be disassembled penton bases by EM (l). Reassembly is achieved by lowering the pH and increasing ionic strength to give material in the heavier fractions (m) that is visualized in (n) as assembled dodecahedral particles. Bar, 250 Å (25 nm).](http://vir.sgmjournals.org)
liquid ethane according to standard methods (Fuschiotti et al., 2006). Frozen grids were transferred onto a Gatan 626 cryoholder for imaging in an FEI CM200 microscope operating at 200 kV and a nominal magnification of ×38 000. Low-dose techniques were used to focus and expose suitable areas onto Kodak SO163 film with an estimated dose of 6–12 e Å⁻². Film was developed in full-strength Kodak D19 developer for 12 min.

Micrographs reveal gold particles that are not encapsidated by dodecahedra readily visible on the support film (Fig. 2a). Fortuitously, these free gold clusters are scavenged by the carbon film and none were observed free in the holes of frozen buffer, thus avoiding coincidental co-projection of gold particles and empty dodecahedra. The carbon-attached clusters are variable in size, suggesting that they have a tendency to aggregate, and we conclude that the smallest of the black dots probably represents one or two Nanogold clusters. We note that this behaviour has been observed before for a similar particle (Cheng et al., 1999).

Given the approximately 80 Å (8 nm) diameter of the dodecahedral cavity (Fuschiotti et al., 2006) and the approximately 27 Å (2.7 nm) diameter of the entire Nanogold moiety including its organic shell, we do not expect to see encapsidation of large and highly visible aggregates, but rather of single or double Nanogold particles. Co-projection with surrounding dodecahedral density reduces their visibility further. We also expect only a limited number of successful encapsidations, unless there is an unlooked-for affinity between Nanogold and the penton base subunit. Nonetheless, evidently promising particles were selected for analysis – several are shown in Fig. 2(b) – and may be compared with control particles prepared in the absence of Nanogold (Fig. 2f). To enhance signal from any encapsidated moieties, the overlaid density due to the dodecahedron was removed by projecting equivalently oriented images from the reconstruction of the empty dodecahedra (Fuschiotti et al., 2006) and subtracting these from the EM images (Fig. 2c–e). Small cores of dark density that are significantly stronger than the noisy background remain after subtraction (Fig. 2e), indicating that gold clusters were encapsidated successfully in these particles. Control images subjected to the same procedure resulted only in background (Fig. 2f–i).

In conclusion, our demonstration of controlled assembly is an important basis for the development of Ad3 dodecahedra towards therapeutic application. Furthermore, successful encapsidation of a marker molecule indicates the feasibility of our approach. The following considerations remain for development of the Ad3 dodecahedron as a drug-delivery vector. Packaging of small molecules must avoid their escape by diffusion through the perforations in the dodecahedral shell, either by polymerization to increase the diameter of the moiety or by modifying the interior surface of the cavity to retain the cargo, such as at the truncated amino terminus (Fuschiotti et al., 2006). Additionally, a sufficient quantity of cargo molecules will need to be delivered into each target cell, which will depend both on packing density within the cavity and on the efficiency of dodecahedral entry (Fender et al., 1997; Garcel et al., 2006). These steps remain to be evaluated fully, but will build on the results presented here.

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Development of adenovirus 3 dodecahedron for therapy

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