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Figure 1

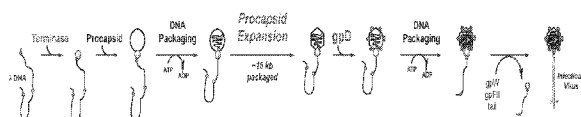
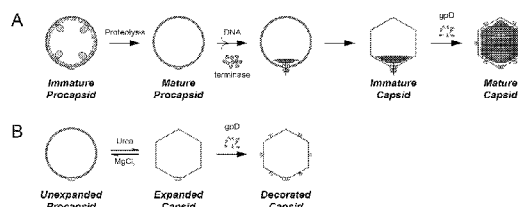


Figure 7



(57) Abstract: The present invention provides viral-based nanoparticles for therapeutic and diagnostic use, and methods for making and using the nanoparticles. Specifically, such nanoparticles comprise decoration-competent viral particles shells such as expanded capsids of phages, stabilized with engineered decoration proteins that have been linked to one or more compounds not naturally occurring on a wild type viral capsid.

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Theragnostic Particles

Related Applications

5 This application claims priority to U.S. Provisional Patent Application Serial Number 61/754,458 filed January 18, 2013, incorporated by reference herein in its entirety.

Background

10 Viral-based particle systems can be modified to present a high density of ligands in a defined symmetric pattern; this type of display can increase the avidity of binding to target biomolecules. For example, bacteriophage lambda has been used for phage display applications through peptide fusions with either the major tail protein (gpV) or the gpD decoration protein (Maruyama et al., 1994; Mikawa et al., 1996). Studies have shown that gpD may be modified at either the N- or C-terminus to present peptides at the capsid surface
15 for phage display applications. However, all current phage based display systems are limited by the required construction of decoration or major capsid protein fusion constructs within the context of an infectious viral particle, while the constructs have been constructed *in vivo*, thus limiting these systems to peptide and protein fusion constructs expressed within infected cells in the context of an infectious virus. As a result, the stoichiometry of
20 the fusion proteins cannot be controlled on the resulting infectious viral particles, the modified constructs are limited to peptide and protein display ligands, and the fusion proteins are limited to fusions at the N- or C-terminus of the decoration protein.

Summary of the Invention

25 In one aspect, the present invention provides theragnostic particles, comprising a plurality of engineered decoration proteins bound to an outer surface of decoration competent viral particle shell, wherein the engineered decoration proteins comprise decoration proteins linked to one or more compound not naturally occurring on a wild type viral capsid, and wherein the one or more compounds have at least one feature selected from the group consisting of:

- 30 (a) the one or more compounds are non-proteinaceous compounds;
 (b) the one or more compounds are present on the theragnostic particle in a defined ratio relative to the engineered decoration protein;

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(c) the one or more compounds comprise two or more different compounds, wherein the two or more different compounds are present on the theragnostic particle in a defined ratio relative to each other; and

(d) the one or more compounds are linked to the engineered decoration protein at a site on the engineered decoration protein other than the N-terminus or the C-terminus.

In another aspect, the invention provides pharmaceutical composition, comprising the theragnostic particles of the invention and a pharmaceutically acceptable carrier.

In a further aspect, the invention provides an isolated recombinant protein comprising the amino acid sequence of SEQ ID NO: 10 (gpD(S42C)), as well as gpD S42C linked to one or more compounds of interest. The invention also provide isolated nucleic acids encoding the isolated protein, recombinant expression vectors comprising the isolated nucleic acids, and isolated host cells comprising the recombinant expression vectors of the invention.

In another aspect, the invention provides an *in vitro* method for preparing a theragnostic particle, comprising decorating a decoration competent viral particle shell *in vitro* with a defined amount of engineered decoration proteins linked to one or more compounds, wherein the engineered decoration proteins stabilize the decoration competent viral particle shell to produce a theragnostic particle.

Summary of the Figures

Figure 1. Phage Lambda Assembly Pathway. A multi-genome concatemer is depicted, which serves as the preferred DNA packaging substrate *in vivo*. Details of the packaging pathway are provided in the Example 1.

Figure 2. Expansion of the Lambda Procapsid *in Vitro*. Panel A. *Magnesium and Urea Stabilize the Procapsid and Expanded Capsid Shells, Respectively.* Procapsids were expanded in 2.5 molar urea, which affords the expanded capsid shell (Urea). The expanded shells were then buffer exchanged into either high magnesium (15 mM, TMB) or low magnesium (1 mM, TMSO) buffer, as indicated. The migration of the procapsid (●) and expanded capsid (●) in the agarose gel is indicated at right of the gel. Panel B. *Urea-Triggered Expansion is Strongly Temperature Dependent.* Procapsids were incubated in 2.5 molar urea for 15 minutes at the indicated temperature and then analyzed by 0.8% agarose gel. Panel C. *Urea-Triggered Expansion is Inhibited by Salt.* Procapsids were expanded as described in Materials and Methods except that NaCl was added to the reaction mixture as

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indicated. *Panel D. Procapsid Expansion is Fully Reversible.* Procapsids were expanded in 2.5 molar urea for 15 minutes at 4°C (Urea) and then buffer-exchanged into TMB buffer. The identical sample was again expanded and buffer exchanged as indicated.

Figure 3. Thermodynamic Characterization of Urea-Triggered Procapsid Expansion.

5 *Panel A.* Procapsids in the absence (○) or in the presence of 3 mM (●) or 10 mM (◇) MgCl₂ were incubated on ice in the presence of increasing concentrations of urea, as indicated, and the fraction of expanded capsids was quantified by gel assay. Each data point represents the average of at least three separate measurements with standard deviations indicated with bars. The solid line represents the best fit of the data to equation 1. *Panel B.*
10 The data presented in Panel A was analyzed according to equation 2 to afford the concentration of urea required to expand half of the procapsid shells ([urea]_{1/2}). *Panel C.* The free energy of capsid expansion (●) and the denaturant *m* values (○) derived from Panel A are plotted as a function of MgCl₂.

Figure 4. Magnesium Drives Contraction of the Expanded Capsid to the Procapsid

15 **State.** MgCl₂ at the indicated concentration was added to expanded capsids in the absence (○) or presence (●) of 2.5 molar urea as described in Materials and Methods. The fraction of contracted capsids was quantified by agarose gel assay. Each data point represents the average of at least three separate measurements with standard deviations indicated with bars. The solid lines represent the best fit of the data according to Equation 3.

Figure 5. Urea-Expanded Capsids are Biologically Functional. *Panel A. Expanded*

Capsids Bind the gpD Decoration Protein. Procapsids and expanded capsids (in the absence of urea) were incubated with gpD as described in Materials and Methods and the products were analyzed by 0.8% agarose gel. Note that the gpD-decorated shell migrates faster than both the procapsid and the expanded capsid shells. *Panel B. The gpD Protein Stabilizes the*

25 *Expanded Capsid Shell.* Micrographs of negatively stained procapsids (i), expanded capsids (ii), expanded capsids that have been re-contracted with 15 mM MgCl₂ (iii), and gpD-decorated expanded shells (iv). Note that the expanded shells are fragile and deteriorate during preparation for EM analysis. Expanded capsids that have been re-contracted with magnesium or stabilized with gpD are structurally sound. *Panel C. Expanded Capsids are*

30 *Biologically Active.* Procapsids and GpD-decorated expanded procapsids (Capsids) were used in an *in vitro* DNA packaging assay. The data represent the average of three separate experiments with standard deviations indicated with bars. *Inset.* DNAase resistant (packaged) viral DNA was analyzed by gel assay. Note that only full-length genomic DNA

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(48.5 kb, arrow) is rendered DNAase resistant. This indicates that both capsid preparations package DNA in a processive manner.

Figure 6. Model for Reversible Procapsid Expansion and gpD Addition. Upper

Pathway. Procapsid expansion is triggered by DNA packaging *in vivo*. Mg^{2+} strongly stabilizes the procapsid conformation. Packaged DNA binds Mg^{2+} at the interior procapsid surface, lowering the free energy required for the expansion transition. GpD trimer spikes assemble at hydrophobic patches exposed at the icosahedral three-fold axes of the expanded shell lattice and stabilize the particle. Lower Pathway. Procapsid expansion is triggered by urea *in vitro*. Mg^{2+} strongly stabilizes the procapsid conformation and higher concentrations of urea are required to trigger the transition. GpD trimer spikes assemble at hydrophobic patches exposed at the icosahedral three-fold axes of the expanded shell lattice. The decorated particles are structurally robust and biologically active.

Figure 7. Bacteriophage lambda capsid *in vivo* (Panel A) and *in vitro* (Panel B) assembly.

At full saturation, 420 copies of gpD decorate the capsid surface, which is composed of 415 copies of the major capsid protein (gpE). Details described in example 2.

Figure 8. The gpD Trimer Spike and gpD Constructs Used in this Study. Panel A. Side view of the gpD-WT trimer. The gpD trimer is shown in cartoon representation with each subunit colored a different shade of blue and with serine 42 depicted as red spheres. Panel B. The gpD constructs used in this study.

Figure 9. GFP-Tagged gpD Adds to the Lambda Capsid *in Vitro*. Expanded capsids were incubated with an increasing ratio of gpD-wt:gpD-GFP as indicated. The decoration mixture was fractionated by agarose gel electrophoresis and the gel was stained with either Coomassie blue (Panel A) or visualized with UV light (Panel B) as described in Materials and Methods. Note that unincorporated H6-gpD-GFP also appears on the agarose gel.

Panel C. Unreacted gpD-WT and gpD-GFP was removed from the decoration reaction mixture using Amicon Ultra-0.5 centrifugation filters and the decorated capsids were analyzed by SDS-PAGE.

Figure 10. Expanded capsids can be decorated with varying ratios of gpD and

gpD(S42C::sMannose) (sMannose). Panel A. Decorated capsids were separated from

excess gpD and sMannose on an agarose gel and stained with Coomassie. Panel B.

Decorated capsids were separated from unincorporated gpD and sMannose by buffer exchange using Amicon Ultra-0.5 centrifugation filters and the protein content of the particles were analyzed by SDS-PAGE.

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Figure 11. *Lambda Capsids Can be Decorated with Multiple Ligands in a Defined Ratio.*

Panel A. Expanded capsids can be decorated with varying ratios of gpD(S42C), sMannose, and gpD-GFP. Decorated capsids were separated from excess gpD and modified gpD on an agarose gel and stained with Coomassie. The migration of the decorated capsids on an agarose gel was unique for each variant. *Panel C.* When illuminated by UV light, GFP-decorated capsids fluoresce. *Panel C.* Decorated capsids were separated from unincorporated gpD, sMannose and gpD-GFP by buffer exchange using Amicon Ultra-0.5 centrifugation filters and the particles were analyzed by SDS-PAGE. *Panel D.* To assess the ability of decorated capsids to bind to Concanavalin A (Con A), a mannose-specific lectin, agglutination assays were performed. Only those decorated with sMannose bound specifically to Con A.

Figure 12. Electron micrographs of decorated capsids. Details described in example 2. Scale bar, 50 nm.

Figure 13. Capsids decorated with modified gpD retain DNA packaging activity. Panel A.

Capsids fully decorated with sMannose are fully competent, but those decorated with 100% gpD-GFP are compromised when full-length lambda is used as the packaging substrate. Panel B. Capsids decorated with up to 50% gpD-GFP are fully competent for packaging..

Detailed Description of the Invention

It must be noted that, as used in the specification, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

Any terms not directly defined herein shall be understood to have the meanings commonly associated with them as understood within the art of the present disclosure. Certain terms are discussed herein to provide additional guidance to the practitioner in describing the compositions, devices, methods, and the like, of embodiments of the present disclosure, and how to make or use them. It will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms can be used for any one or more of the terms discussed herein. No significance is to be placed upon whether or not a term is elaborated or discussed herein. Some synonyms or substitutable methods, materials and the like are provided. Recital of one or a few synonyms or equivalents does not exclude use of other synonyms or equivalents, unless it is explicitly stated. Use of examples, including examples of terms, is for illustrative purposes only and does not limit the scope and meaning of the embodiments of the present

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disclosure herein.

As used herein, "about" means +/- 10% of the recited value.

All embodiments disclosed herein can be used in combination, unless the context clearly indicates otherwise. Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be
5 construed in an inclusive sense, as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to". Words using the singular or plural number also include the plural or singular number, respectively. Additionally, the words "herein," "above" and "below" and words of similar import, when used in this application,
10 shall refer to this application as a whole and not to any particular portions of this application.

The following description provides specific details for a thorough understanding of, and enabling description for, embodiments of the disclosure. However, one skilled in the art will understand that the disclosure may be practiced without these details. In other
15 instances, well-known structures and functions have not been shown or described in detail to avoid unnecessarily obscuring the description of the embodiments of the disclosure. Persons skilled in the relevant art can appreciate that many modifications and variations are possible in light of the embodiment teachings. Accordingly, the disclosure is intended to be illustrative, but not limiting, of the scope of invention.

20 In a first aspect the present invention provides an *in vitro* method for preparing a theragnostic particle, comprising decorating a decoration competent viral particle shell *in vitro* with a defined amount of engineered decoration proteins linked to one or more compounds, wherein the engineered decoration proteins stabilize the decoration competent viral particle shell to produce a theragnostic particle.

25 The present invention overcomes the shortcomings of viral particle systems in the art, which did not permit capsid decoration with a defined amount of engineered decoration proteins linked to compounds of interest. The methods of the present invention permit construction of theragnostic (i.e.: can be used for therapeutic or diagnostic use) particles decorated with defined amounts of engineered decoration protein, and where the engineered
30 decoration proteins are linked to any compound or multiple compounds of interest including non-proteinaceous compounds, which can themselves be added in defined amounts to the resulting viral particle. Further, the engineered decoration proteins of the invention can be modified at any suitable position on the protein that does not interfere with

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binding to the expanded viral particle shell. . As such, the theragnostic particles made by the methods of the invention can be used for any suitable therapeutic or diagnostic purpose, as described in more detail herein. In one embodiment, the expanded viral particle shells are decorated with a stoichiometric ratio of engineered decoration proteins.

5 As used herein, the “viral particle” is a protein shell of a virus (capsid) or virus-like particle. For use in the present invention, the viral particles are based on capsids of double stranded DNA viruses that normally package their genome into preassembled procapsid shells. Packaging in these viruses triggers a major re-orientation and expansion of the proteins assembled into the procapsid shells, yielding the mature capsid shell that is subsequently stabilized with the addition of decoration proteins. Exemplary such double
10 stranded DNA viruses include, but are not limited to bacteriophages lambda, T4, 21, L, P4, herpesvirus and adenoviruses.

The viral particle can be an isolated capsid or may be produced by any suitable means, such as recombinant expression of the structural proteins and subsequent viral
15 particle assembly using the recombinantly expressed structural proteins *in vitro*; in the latter case the particles are known as virus-like particles (VLP). In one embodiment, described in more detail in the examples that follow, vectors can be designed to express all proteins required to assemble a functional capsid in a host cell, such as *E. coli*, and the particle can then be purified. The viral particle may comprise or consist of wild type or mutated capsid
20 structural proteins, so long as the mutated structural proteins can form the engineered viral particle (e.g., functional mutant structural proteins). The viral particles may be isolated and stored or preassembled and stored, or may be isolated or assembled at the time of carrying out the methods of the invention. Assembly or isolation can be carried out by any suitable means, which are well within the level of skill in the art based on the disclosure herein.

25 Exemplary isolation and assembly techniques are provided in the examples that follow.

In one embodiment, a virus-like particle is produced by *in vitro* assembly of isolated capsid structural proteins. The capsid structural proteins to be used will depend on the virus that the viral particle is based on. Capsid structural proteins for each of bacteriophages lambda, T4, L, P22, 21, and P4 and for the eukaryotic adenovirus and herpesviruses are well
30 known to those of skill in the art, as are methods for their recombinant production or isolation. In one non-limiting embodiment, a lambda phage viral particle is used. In this embodiment, the structural proteins may be portal protein gpB (SEQ ID: 1) and major capsid protein gpE (SEQ ID: 2), and the minor capsid protein gpC (SEQ ID: 3), or the

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scaffolding protein contained therein (SEQ ID NO: 36; residues 309 to 439 of SEQ ID NO:3), or functional mutants thereof.

In another embodiment, a bacteriophage T4 viral particle is used; in this embodiment, the structural proteins are the T4 structural proteins, or functional mutants thereof. In a further embodiment, a bacteriophage L viral particle is used; in this embodiment, the structural proteins are the bacteriophage L structural proteins, or functional mutants thereof. In another embodiment, a bacteriophage P22 viral particle is used; in this embodiment, the structural proteins are the bacteriophage p22 structural proteins, or functional mutants thereof. In another embodiment, a bacteriophage 21 viral particle is used; in this embodiment, the structural proteins are the bacteriophage 21 structural proteins, or functional mutants thereof. In a further embodiment, a bacteriophage P4 viral particle is used; in this embodiment, the structural proteins are the bacteriophage P4 structural proteins, or functional mutants thereof. The structural proteins and their amino acid sequences for each of these viral structural proteins known to those of skill in the art.

The viral particle is "decoration competent" in that the particle is either (a) prepared or isolated in a form that can add decoration protein to the shell without further manipulation, (b) contacted with an expansion agent *in vitro* under conditions and for a time suitable to produce an expanded viral particle shell. that will be stabilized in the expanded state by decoration with the decoration protein, or (c) contacted with an agent *in vitro* under conditions and for a time suitable to produce a shell that has undergone a conformational change such that it will be stabilized in that conformation state by decoration with the decoration protein. Packaging in phages lambda, T4, 21, P22, and L, DNA triggers major conformational changes that alter the shell morphology and cause shell expansion. The herpesviruses similarly undergo major conformational changes with DNA packaging that alter the shell morphology but they are not typically described as expanding. Purification affords a mixture of pre-change and post-change species in most cases.

Thus, in one embodiment, the viral particle shell has under extensive conformational change that renders the shell competent for decoration with the decoration protein. All procapsids undergo a major structural re-organization of the shell as a result of DNA packaging during the viral particle maturation process. Decoration of the shell with the decoration protein stabilizes this embodiment of a decoration competent viral particle shell.

In another embodiment, the methods involve expanding the viral particle by contacting it with an expansion agent *in vitro* under conditions and for a time suitable to

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produce an expanded viral particle shell. Any suitable expansion agent that can expand a specific viral particle can be used, including but not limited to chaotropic agents (including but not limited to urea), pH changes, heating, etc. The methods do not require any specific amount of expansion of the viral particle; the expansion may be similar to the expansion
5 seen upon genome packaging during the normal viral life cycle. Any suitable expansion agent can be used, as deemed most appropriate in light of the specific of the engineered viral particle and all other relevant factors. In one embodiment, a chaotropic agent such as urea is used. In one non-limiting example, when bacteriophage lambda viral particles are used, urea can be used as the chaotropic agent; in this embodiment, the resulting viral
10 particle shell can optionally be contracted and expanded repeatedly if desired. In a further embodiment using bacteriophage lambda viral particles, expansion of the cell is done at about 4°C. In other embodiments, the expansion agent can be heat (range of 4°C to 50°C), pH (pH range of 3-9), or other expansion agent. Based on the teachings herein and the specific viral particle being used, those of skill in the art will be able to determine the most
15 appropriate expansion agent for a given use. Similarly, conditions and times suitable to produce the expanded virus shell can be determined by those of skill in the art based on the teachings herein and in light of the specific viral particle being used.

In another embodiment, the methods, agents, and techniques described for particle expansion are applied to driving the conformational change of the shell required to afford a
20 decoration competent particle shell, in those viral capsids that do not undergo particle expansion.

The methods comprise decorating the decoration competent viral particle shells *in vitro* with engineered decoration proteins. As used herein “decorating” means binding a plurality of decoration proteins to the viral particle shells, such that the decoration proteins
25 stabilize the decoration competent viral particle shell. Since the methods are carried out *in vitro*, the decoration proteins can be added stoichiometrically, in any desired amount to the decoration competent viral particle shells. As is known by those of skill in the art, a variety of double stranded DNA viruses possess decoration proteins that stabilize procapsid shells to produce the mature capsid shell. Non-limiting examples of such decoration proteins
30 include, but are not limited to, the lambda gpD decoration protein, T4 head outer capsid (Hoc) protein, T4 small outer capsid (Soc) protein, Shp protein of phage 21, the Dec protein of phage L, and the Psu protein of phage P4, and protein IX of adenoviruses. The plurality of decoration proteins that are added depend on the viral particle used, the modifications

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made to the decoration protein, and the intended use of the viral particles, but include enough decoration proteins to bind to at least 80% of the binding sites for the decoration protein that are present on the expanded viral particle shell (e.g.: on the structural proteins defining the shells. In various further embodiments, the engineered decoration proteins
5 bind to at least 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or to all binding sites for the decoration protein that are present on the expanded viral particle shell.

As will be understood by those of skill in the art, the choice of decoration protein will depend on the viral particle/structural proteins being used. Thus, if the viral particle is lambda phage based, lambda phage structural proteins (or functional mutants thereof) and
10 the engineered lambda decoration protein (engineered gpD protein) will be used. The wild type gpD amino acid sequence is shown in SEQ ID: 4. Similarly, if the viral particle is bacteriophage T4 then T4 structural proteins (or functional mutants thereof) and the engineered T4 decoration protein (engineered Hoc or Soc protein) will be used. The wild type Hoc amino acid sequence is shown in SEQ ID:5; The wild type Soc amino acid
15 sequence is shown in SEQ ID:6. If the viral particle is bacteriophage 21, then bacteriophage 21 structural proteins (or functional mutants thereof) and the engineered bacteriophage 21 decoration protein (Shp protein) will be used. The wild type Shp amino acid sequence is shown in SEQ ID:7. If the viral particle is bacteriophage L, then bacteriophage L structural proteins (or functional mutants thereof) and the engineered
20 bacteriophage L decoration protein (Dec protein) will be used. The wild type Dec amino acid sequence is shown in SEQ ID:8. If the viral particle is bacteriophage P22, then bacteriophage P22 structural proteins (or functional mutants thereof) and the engineered bacteriophage L decoration protein (Dec protein) will be used, since P22 does not make a decoration protein *in vivo*, but can be decorated with Dec from phage L *in vitro*. If the viral
25 particle is bacteriophage P4, then bacteriophage P4 structural proteins (or functional mutants thereof) and the engineered bacteriophage P4 decoration protein (Psu protein) will be used. The wild type Psu amino acid sequence is shown in SEQ ID:9.

The decoration proteins for use in the methods of the invention are “engineered” in that they are not the wild type proteins, but have been modified and are linked to one or
30 more compounds of interest. Since the methods of the present invention can be carried out *in vitro*, the decoration proteins can be modified in any desirable way, providing a unprecedented increase in the functionality that can be displayed on the surface of the resulting particles compared to prior art methods. In one embodiment, the decoration

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proteins have an altered amino acid sequence from wild type, such as an insertion, deletion, one or more substitutions, etc. Non-natural amino acid residues can be incorporated into the engineered decoration proteins as suitable for a given purpose. Any suitable modifications can be made to the decoration proteins so long as the resulting engineered decoration
5 protein can bind to the viral particle shell and stabilize it.

In one non-limiting embodiment, any solvent accessible residue on a shell bound decoration protein can be modified as desired, such as to facilitate linkage to one or more compounds of interest. For example, residues in the shell-bound gpD trimer spike that are exposed to solvent include, but are not limited to Thr1, Ser2, Lys3, Glu4, Thr5, Phe6, Thr7, His8, Tyr9,
10 Gln10, Pro11, Gln12, Gly13, Asn14, Ser15; Gly25, Gly26, Leu27, Ser28, Ala29, Lys30, Ala31; Asp40, Thr41, Ser42, Ser43Arg44, Lys45; Asp49, Gly50, Thr51, Thr52, Asp53; Asp66, Gln67, Thr68, Ser69, Thr70, Thr71; Arg81, Tyr82, Glu83, Asp84; Glu89, Ala90, Ala91, Ser92, Asp93, Glu94, Thr95, Lys96, Lys97, Arg98, Thr99; the N- and C-terminal residues of the protein. In one non-limiting embodiment, one or more residue may be
15 substituted with a Cys residue (including but not limited to an S42C substitution), to facilitate binding of other compounds to the decoration proteins. In another embodiment, a non-natural amino acid, including but not limited to azidohomoalanine (Aha) can be incorporated using genetic manipulation of the gene to allow alternate means for chemical modification of the decoration protein, including “click” chemistry.

Note that the N-terminal methionine shown for gpD in SEQ ID NO 4 is generally excluded from the mature protein by cell degradation. For purposes of this application, the numbering for gpD excludes the (optional) N-terminal methionine. Thus, for example, Ser42 represents the 42 amino acid in the mature gpD sequence (deleted for the N-terminal methionine), but is the 43rd amino acid in the sequence if the N-terminal methionine is
25 counted. The numbering system follows that reported in the crystal structure of gpD (Yang et al, (2000) Nat.Struct.Biol.vol7, pp. 230) which does not included the N-terminal methionine encoded by the gene sequence.

The engineered decoration proteins comprise engineered decoration proteins linked to one or more compound. In another embodiment, the engineered decoration proteins
30 comprise engineered decoration proteins linked to one or more compound selected from the group consisting of nucleic acids, lipids, carbohydrates, polypeptides, polymers, organic molecules, inorganic molecules, or combinations thereof. In a further embodiment, the engineered decoration proteins comprise engineered decoration proteins linked to one or

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more non-proteinaceous compound, such as nucleic acids, lipids, carbohydrates, polymers, organic molecules, inorganic molecules (e.g. magnetic beads and quantum dots, among others), or combinations thereof. In all of these embodiments, the linkage can be at any suitable position on the decoration protein, which is not possible using previously known methods (which are limited to engineered fusion proteins, such that linkage is at the N- of
5 C-terminus of the decoration protein). In various embodiments, the linkage can be at the N- or C- terminus of the decoration protein, or at any suitable internal residue of the decoration protein (e.g., other than the N-terminal or C-terminal residue of the decoration protein). Any suitable techniques can be used to link the one or more compounds to the decoration
10 proteins; such techniques are well within the level of those of skill in the art based on the teachings herein. As will be understood by those of skill in the art, any suitable combination of decoration proteins can be used, as appropriate for a given expanded viral particle.

In further embodiments, the one or more compounds may be any suitable compound
15 for the theragnostic particles of the invention, including but not limited to therapeutic compounds, diagnostic compounds, adjuvants, antigens, antibodies, etc.

The decorating comprises contacting the decoration competent viral particle shells *in vitro* with the engineered decoration proteins for a time and under conditions suitable to bind the decoration proteins to the viral particle shells. The range of viral particle
20 decoration is limited by the initial concentrations of the engineered decoration proteins thereby allowing tunability and precise decorating of the viral particle shell with desired amounts and ratios of the one or more compounds that is not possible using prior methods. Specific conditions will depend on the viral particle-decoration protein used, the nature of the modification to the engineered decoration protein, and all other relevant factors. It is
25 well within the level of those of skill in the art to determine appropriate incubation conditions, based on the teachings herein. Exemplary conditions are provided in the examples that follow. In one exemplary and non-limiting embodiment in which lambda phage viral particles are used with engineered gpD decoration proteins, the contacting is carried out at about room temperature to 37°C.

30 In all of these embodiments, the one or more compounds linked to the decoration proteins may comprise two or more different compounds. In this further embodiment the two or more different compounds can be present on the decoration competent particle shell in a defined ratio relative to each other. For example, the decoration proteins may comprise

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two sets of decoration proteins, a first set linked to compound 1 and a second set linked to compound 2. Since the methods of the invention are carried out *in vitro*, the decorating can comprise contacting the expanded viral particles with a ratio of the first and second sets of decoration proteins that affords the desired stoichiometric ratio of the two different compounds on the theragnostic particle.

In one non-limiting embodiment, the decoration proteins are bacteriophage lambda gpD proteins, or functional mutants thereof. The lambda gpD decoration protein is a monomer in solution but adds to expanded viral particles as a trimer spike to each of the 140 icosahedral three-fold axes (formed by the structural proteins) on the expanded viral particle surface. The N-terminus of each gpD protein interacts with the capsid shell to provide stabilizing contacts required for shell integrity, while the C-termini exit the gpD trimer spike proximate to the shell surface. Thus, prior art methods that are limited to linking compounds *in vivo* to the N- or C-termini of gpD may hinder gpD trimer assembly and interfere with its ability to stabilize the capsid. Further, prior work has been limited to N-terminal or C-terminal peptide and protein fusion proteins that are used to decorate infectious phage *in vivo*. In one embodiment, the engineered gpD proteins comprise a S42C substitution (SEQ ID NO: 10), which can be the sole decoration protein or can be combined with wild type gpD decoration proteins, or other gpD functional mutants.

The resulting theragnostic particles can be devoid of any packaged material ("empty") within the particle, or may include any desired cargo material packaged on the interior of the particle. For example, DNA is efficiently packaged into the decorated capsids and can be modified to carry specific genes of interest.

In a second aspect, the present invention provides theragnostic particles, comprising a plurality of engineered decoration proteins bound to an outer surface of a decoration competent viral particle shell, wherein the engineered decoration proteins comprise decoration proteins linked to one or more compound not naturally occurring on a wild type viral capsid, and wherein the one or more compounds have at least one feature selected from the group consisting of:

- (a) the one or more compounds are non-proteinaceous compounds;
- (b) the one or more compounds are present on the theragnostic particle in a defined ratio relative to the engineered decoration protein;

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(c) the one or more compounds comprise two or more different compounds, wherein the two or more different compounds are present on the theragnostic particle in a defined ratio relative to each other; and

(d) the one or more compounds are linked to the engineered decoration protein
5 at a site on the engineered decoration protein other than the N-terminus or the C-terminus.

All terms used in the first aspect of the invention have the same meaning when referred to in other aspects of the invention. Thus, the decoration competent viral particle shell may be either an expanded viral particle that is stabilized in the expanded state by the decoration proteins, or (b) a viral particle shell that has under extensive conformational
10 change that allows decoration by the decoration protein.

The compositions of the invention overcome the shortcomings of viral particle systems in the art. The theragnostic particles of the invention comprise stoichiometric amounts of engineered decoration protein, and the engineered decoration proteins can be
15 linked to any compound or multiple compounds of interest including non-proteinaceous compounds, which can themselves be added stoichiometrically. Further, the engineered decoration proteins can be modified at any suitable position on the protein that does not interfere with binding to the viral particle shell. As such, the theragnostic particles can be used for any suitable therapeutic or diagnostic purpose, as described in more detail herein.
20 The ability to decorate viral particles with engineered decoration proteins provides an attractive approach to develop "designer" nanoparticles of defined composition and multi-partite, symmetric presentation.

In one embodiment, the theragnostic particle comprises a lambda phage expanded viral particle shell is used. In this embodiment, the structural proteins may be protease
25 gpC/scaffolding protein gpNu3 (SEQ ID NO: 3) or the scaffolding protein (SEQ ID NO: 36), portal protein gpB (SEQ ID NO: 1) and major capsid protein gpE (SEQ ID NO: 2), or functional mutants thereof.

In one non-limiting embodiment, a lambda phage viral particle is used. In this embodiment, the structural proteins may be portal protein gpB (SEQ ID: 1) and major
30 capsid protein gpE (SEQ ID: 2), and the minor capsid protein gpC (SEQ ID: 3), or the scaffolding protein contained therein (SEQ ID NO: 36), or functional mutants thereof.

In another embodiment, a bacteriophage T4 viral particle is used; in this embodiment, the

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structural proteins are the T4 structural proteins, or functional mutants thereof. In a further embodiment, a bacteriophage L viral particle is used; in this embodiment, the structural proteins are the bacteriophage L structural proteins, or functional mutants thereof. In another embodiment, a bacteriophage 21 viral particle is used; in this embodiment, the structural proteins are the bacteriophage 21 structural proteins, or functional mutants thereof. In another embodiment, a bacteriophage 22 viral particle is used; in this embodiment, the structural proteins are the bacteriophage 22 structural proteins functional mutants thereof. In a further embodiment, a bacteriophage P4 viral particle is used; in this embodiment, the structural proteins are the bacteriophage P4 structural proteins, or functional mutants thereof. The structural proteins and their amino acid sequences for each of these viral structural proteins are known to those of skill in the art.

Non-limiting examples of decoration proteins include, but are not limited to, the lambda gpD decoration protein (SEQ ID NO:4), T4 head outer capsid (Hoc) protein (SEQ ID NO:5), T4 small outer capsid (Soc) protein (SEQ ID NO:6), Shp protein of phage 21 (SEQ ID NO:7), the Dec protein of phage L (SEQ ID NO:8), and the Psu protein of phage P4 (SEQ ID NO:9).

The plurality of decoration proteins present on the particle will depend on the viral particle used, the modifications made to the decoration protein, and the intended use of the viral particles, but include enough decoration proteins to bind to at least 80% of the binding sites for the decoration protein that are present on the expanded viral particle shell (e.g.: on the structural proteins defining the shells). In various further embodiments, the engineered decoration proteins bind to at least 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or to all binding sites for the decoration protein that are present on the expanded viral particle shell.

As will be understood by those of skill in the art, the choice of decoration proteins will depend on the viral particle shell/structural proteins being used. Thus, if the viral particle is lambda phage-based, lambda phage structural proteins (or functional mutants thereof) and the engineered lambda decoration protein (engineered gpD protein) will be used. Similarly, if the viral particle is bacteriophage T4 then T4 structural proteins (or functional mutants thereof) and the engineered T4 decoration protein (engineered Hoc or Soc protein) will be used. If the viral particle is bacteriophage 21, then bacteriophage 21 structural proteins (or functional mutants thereof) and the engineered bacteriophage 21 decoration protein (shp protein) will be used. If the viral particle is bacteriophage P22, then

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bacteriophage 22 structural proteins (or functional mutants thereof) and the engineered bacteriophage L decoration protein (dec protein) will be used. If the viral particle is bacteriophage P4, then bacteriophage P4 structural proteins (or functional mutants thereof) and the engineered bacteriophage P4 decoration protein (psu protein) will be used.

5 The decoration proteins for use in the methods of the invention are “engineered” in that they are not the wild type proteins, but have been linked to one or more compound not naturally occurring on a wild type viral capsid to provide added functionality. The modifications comprise one or more of

- (a) the one or more compounds being non-proteinaceous compounds;
- 10 (b) the one or more compounds are present on the theragnostic particle in a defined ratio relative to the engineered decoration protein;
- (c) the one or more compounds comprise two or more different compounds, wherein the two or more different compounds are present on the theragnostic particle in a defined ratio relative to each other; and
- 15 (d) the one or more compounds are linked to the engineered decoration protein at a site on the engineered decoration protein other than the N-terminus or the C-terminus

 The decoration proteins can be modified in any desirable way as disclosed herein, providing a unprecedented increase in the functionality that can be displayed on the surface of the resulting particles compared to prior art methods. In one embodiment, the decoration
20 proteins have an altered amino acid sequence from wild type, such as an insertion, deletion, one or more substitutions, etc. Any suitable modifications can be made to the decoration proteins so long as the resulting engineered decoration protein can bind to the viral particle shell and stabilize it. In one non-limiting embodiment, any solvent accessible residue on a shell bound decoration protein can be modified as desired, such as to facilitate linkage to
25 one or more compounds of interest.

 The engineered decoration proteins comprise engineered decoration proteins linked to one or more compound of interest. In one embodiment, the one or more compounds are selected from the group consisting of nucleic acids, lipids, carbohydrates, polypeptides, polymers, organic molecules, inorganic molecules, or combinations thereof. In a further
30 embodiment, the engineered decoration proteins comprise engineered decoration proteins linked to one or more non-proteinaceous compound, such as nucleic acids, lipids, carbohydrates, polymers, organic molecules, inorganic molecules (e.g. magnetic beads and quantum dots, among others), or combinations thereof. In all of these embodiments, the

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linkage can be at any suitable position on the decoration protein, which is not possible using previously known methods (which are limited to engineered fusion proteins, such that linkage is at the N- or C-terminus of the decoration protein). In various embodiments, the linkage can be at the N- or C- terminus of the decoration protein, or at any suitable internal
5 residue of the decoration protein (e.g., other than the N-terminal or C-terminal residue of the decoration protein). The one or more compounds can be linked to the decoration proteins using any suitable linkage; determining an appropriate linkage based on the specifics of the one or more compounds and the decoration protein are well within the level of those of skill in the art based on the teachings herein. As will be understood by those of
10 skill in the art, any suitable combination of decoration proteins can be used, as appropriate for a given expanded viral particle.

In further embodiments, the one or more compounds may be any suitable compound for the theragnostic particles of the invention, including but not limited to therapeutic compounds, diagnostic compounds, adjuvants, antigens, antibodies, etc.

15 The one or more compounds linked to the decoration proteins may comprise two or more different compounds. In this embodiment the two or more different compounds can be present on the viral particle shell in a defined ratio relative to each other. For example, the decoration proteins may comprise two sets of decoration proteins, a first set linked to compound 1 and a second set linked to compound 2. Since the methods of the invention are
20 carried out *in vitro*, the decorating can comprise contacting the decoration competent viral particles with a ratio of the first and second sets of decoration proteins that reflects the desired stoichiometric ratio of the two different compounds on the theragnostic particle.

In one non-limiting embodiment, the engineered decoration proteins comprise bacteriophage lambda gpD proteins, or functional mutants thereof. The lambda gpD
25 decoration protein is a monomer in solution but adds to the expanded viral particle as a trimer spike to each of the 140 icosahedral three-fold axes (formed by the structural proteins) on the expanded viral particle surface. In one embodiment, the engineered gpD proteins comprise a S42C substitution (SEQ ID NO: 10), which can be the sole decoration protein on the particle, or can be combined with wild type gpD decoration proteins, or other
30 gpD functional mutants, each comprising the one or more linked compounds (which may be the same or different between the same or different gpD forms). For example, residues in the shell-bound gpD trimer spike that are exposed to solvent include, but are not limited to Thr1, Ser2, Lys3, Glu4, Thr5, Phe6, Thr7, His8, Tyr9, Gln10, Pro11, Gln12, Gly13, Asn14,

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Ser15; Gly25, Gly26, Leu27, Ser28, Ala29, Lys30, Ala31; Asp40, Thr41, Ser42, Ser43, Arg44, Lys45; Asp49, Gly50, Thr51, Thr52, Asp53; Asp66, Gln67, Thr68, Ser69, Thr70, Thr71; Arg81, Tyr82, Glu83, Asp84; Glu89, Ala90, Ala91, Ser92, Asp93, Glu94, Thr95, Lys96, Lys97, Arg98, Thr99, and the N- and C-terminal residues of the protein. In one non-limiting embodiment, one or more residue may be substituted with a Cys residue (including but not limited to an S42C substitution), to facilitate binding of other compounds to the decoration proteins. In another non-limiting embodiment, a non-natural amino acid, including but not limited to azidohomoalanine (Aha) can be incorporated using genetic manipulation of the gene to allow alternate means for chemical modification of the decoration protein, including "click" chemistry.

The resulting theragnostic particles can be devoid of any packaged material ("empty") within the particle, or may include any desired cargo material packaged on the interior of the particle. For example, DNA is efficiently packaged into the decorated capsids and can be modified to carry specific genes of interest.

The theragnostic particles of the present invention can be used for any suitable purpose. In one embodiment, the engineered decoration proteins are linked to one or more therapeutic moieties (including but not limited to small molecule drugs, therapeutic antibodies, glycoproteins, carbohydrate polymers, therapeutic nucleic acids (siRNA, antisense RNA, shRNA, gene therapy constructs, etc.), antigens (for use in vaccines), adjuvants (to stimulate an immune response), etc., and the particles can be administered as a therapeutic to a subject in need of treatment. In another embodiment, synthetic polymers including PEG can be used to provide "stealth" characteristics for immune evasion or pH sensitive polymers for escape from cellular endosome compartments.

In various embodiments, the therapeutic is selected from the group consisting of alkylating agents, angiogenesis inhibitors, antibodies, antimetabolites, antimitotics, antiproliferatives, aurora kinase inhibitors, apoptosis promoters (for example, Bcl-xL, Bcl-w and Bfl-1) inhibitors, activators of death receptor pathway, Bcr-Abl kinase inhibitors, BiTE (Bi-Specific T cell Engager) antibodies, biologic response modifiers, cyclin-dependent kinase inhibitors, cell cycle inhibitors, cyclooxygenase-2 inhibitors, growth factor inhibitors, heat shock protein (HSP)-90 inhibitors, demethylating agents, histone deacetylase (HDAC) inhibitors, hormonal therapies, immunologicals, inhibitors of apoptosis proteins (IAPs) intercalating antibiotics, kinase inhibitors, mammalian target of rapamycin inhibitors, microRNA's, mitogen-activated extracellular signal-regulated kinase

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inhibitors, multivalent binding proteins, non-steroidal anti-inflammatory drugs (NSAIDs), poly ADP (adenosine diphosphate)-ribose polymerase (PARP) inhibitors, platinum chemotherapeutics, polo-like kinase (Plk) inhibitors, proteasome inhibitors, purine analogs, pyrimidine analogs, receptor tyrosine kinase inhibitors, retinoids/deltoids plant alkaloids, small inhibitory ribonucleic acids (siRNAs), topoisomerase inhibitors and the like.

In another embodiment, the engineered decoration protein comprises one or more diagnostic, localization, and/or imaging moieties (including but not limited to fluorophores, radioactive tracers, dyes, diagnostic antibodies or other ligands, ligands for surface proteins on cells being targeted, etc.), and can be administered to a subject for diagnostic or imaging purposes. For example, the one or more compound may comprise a diagnostic or imaging agent. Many such imaging agents are known to those of skill in the art. Examples of imaging agents suitable for use in the disclosed particles are radioactive isotopes, fluorescent molecules (including fluorescent proteins such as green fluorescent protein, red fluorescent protein, blue fluorescent protein, etc.), magnetic particles (including nanoparticles), metal particles (including nanoparticles), phosphorescent molecules, enzymes, antibodies, ligands, and combinations thereof, while diagnostic agents may comprise a compound that is a diagnostic marker for a particular disorder bound to such an imaging agent. Methods for detecting and measuring signals generated by imaging agents are also known to those of skill in the art. For example, radioactive isotopes can be detected by scintillation counting or direct visualization; fluorescent molecules can be detected with fluorescent spectrophotometers; phosphorescent molecules can be detected with a spectrophotometer or directly visualized with a camera; enzymes can be detected by detection or visualization of the product of a reaction catalyzed by the enzyme; antibodies can be detected by detecting a secondary detection label coupled to the antibody.

In a further embodiment, the imaging agents can comprise a fluorescent imaging agent, while diagnostic agents may comprise a compound that is a diagnostic marker for a particular disorder bound to the fluorescent imaging agent. A fluorescent imaging agent is any chemical moiety that has a detectable fluorescence signal. This imaging agent can be used alone or in combination with other imaging agents. Examples of suitable fluorescent agents that can be used in the compositions and methods disclosed herein include, but are not limited to, quantum dots, fluorescein (FITC), 5-carboxyfluorescein-N-hydroxysuccinimide ester, 5,6-carboxymethyl fluorescein, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), fluorescamine, OPA, NDA, indocyanine green dye, the cyanine dyes (e.g., Cy3,

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Cy3.5, Cy5, Cy5.5 and Cy7), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid, acridine, acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl]phenylinaphthalimide-3,5 disulfonate, N-(4-anilino-1-naphthyl)maleimide, anthranilamide, BODIPY, Brilliant Yellow, coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcoumarin (Coumaran 151), cyanosine, 4',6'-diaminidino-2-phenylindole (DAPI), 5',5''-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red), 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin diethylenetriamine pentaacetate, 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansylchloride), 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC), eosin, eosin isothiocyanate, erythrosin B, erythrosine, isothiocyanate, ethidium bromide, ethidium, 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein isothiocyanate, IR144, IR1446, Malachite Green isothiocyanate, 4-methylumbelliferone, ortho cresolphthalein, nitrotyrosine, pararosaniline, Phenol Red, B-phycoerythrin, o-phthaldialdehyde, pyrene, pyrene butyrate, succinimidyl 1-pyrene butyrate, Reactive Red 4 (Cibacron[R] Brilliant Red 3B-A), 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodamine (Rhod), 5,6-tetramethyl rhodamine, rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), tetramethyl rhodamine, tetramethyl rhodamine isothiocyanate (TRITC), riboflavin, rosolic acid, coumarin-6, and the like, including combinations thereof. These fluorescent imaging moieties can be obtained from a variety of commercial sources, including Molecular Probes, Eugene, Oreg. and Research Organics, Cleveland, Ohio, or can be synthesized by those of ordinary skill in the art.

In another example, the imaging agents can comprise a Magnetic Resonance Imaging (MRI) agent, while diagnostic agents may comprise a compound that is a diagnostic marker for a particular disorder bound to the MRI agent. A MRI agent is any chemical moiety that has a detectable magnetic resonance signal or that can influence (e.g., increase or shift) the magnetic resonance signal of another agent. This type of imaging agent can be used alone or in combination with other imaging agent. By combining an MRI

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imaging agent and, for example, a fluorescent imaging agent, the resulting agent can be detected, imaged, and followed in real-time via MR I. Other imaging agents include PET agents that can be prepared by incorporating an ^{18}F or a chelator for ^{64}Cu or ^{68}Ga . Also, addition of a radionuclide can be used to facilitate SPECT imaging or delivery of a radiation
5 dose, while diagnostic agents may comprise a compound that is a diagnostic marker for a particular disorder bound to the PET agent.

In some embodiments, the diagnostic agent is a diagnostic imaging agent, including but not limited to position emission tomography (PET) agents, computerized tomography (CT) agents, magnetic resonance imaging (MRI) agents, nuclear magnetic imaging agents
10 (NMI), fluoroscopy agents and ultrasound contrast agents. Such diagnostic agents include radioisotopes of such elements as iodine (I), including ^{123}I , ^{125}I , ^{131}I etc., barium (Ba), gadolinium (Gd), technetium (Tc), including ^{99}Tc , phosphorus (P), including ^{31}P , iron (Fe), manganese (Mn), thallium (Tl), chromium (Cr), including ^{51}Cr , carbon (C), including ^{14}C , or the like, fluorescently labeled compounds, or their complexes, chelates, adducts and
15 conjugates. Any suitable PET agents can be used, including but not limited to carbon-11, nitrogen-13, oxygen-15, fluorine-18, 11C-metomidate, and glucose analogues thereof, including but not limited to fludeoxyglucose (a glucose analog labeled with fluorine-18).

The particles can also be used in viral research, such as to identify protein components required for viral assembly and mechanistic interrogation of the assembly
20 process.

In a further embodiment, the invention provides pharmaceutical compositions, comprising the theragnostic particles of any embodiment or combination of embodiments of the invention and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier is non-toxic, biocompatible and is selected so as not to detrimentally affect the
25 biological activity of the multimers (and any other therapeutic agents combined therewith). Exemplary pharmaceutically acceptable carriers for peptides are described in U.S. Patent No. 5,211,657 to Yamada. The compositions may be formulated into preparations in solid, semi-solid, gel, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, suppositories, inhalants, and injections, allowing for oral, parenteral,
30 or surgical administration. Suitable carriers for parenteral delivery via injectable, infusion, or irrigation and topical delivery include distilled water, physiological phosphate-buffered saline, normal or lactated Ringer's solutions, dextrose solution, Hank's solution, or propanediol. In addition, sterile, fixed oils may be employed as a solvent or suspending

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medium. For this purpose any biocompatible oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids, such as oleic acid, find use in the preparation of injectables. The carrier and agent may be compounded as a liquid, suspension, polymerizable or non-polymerizable gel, paste or salve. The carrier may also
5 comprise a delivery vehicle to sustain (i.e., extend, delay, or regulate) the delivery of the agent(s) or to enhance the delivery, uptake, stability, or pharmacokinetics of the therapeutic agent(s). Such a delivery vehicle may include, by way of non-limiting example, microparticles, microspheres, nanospheres, or nanoparticles composed of proteins, liposomes, carbohydrates, synthetic organic compounds, inorganic compounds, polymeric
10 or copolymeric hydrogels, and polymeric micelles. Suitable hydrogel and micelle delivery systems include the PEO:PHB:PEO copolymers and copolymer/cyclodextrin complexes disclosed in International Publication No. WO 2004/009664 A2, and the PEO and PEO/cyclodextrin complexes disclosed in U.S. Publication No. 2002/0019369 A1. Such hydrogels may be injected locally at the site of intended action, or subcutaneously or
15 intramuscularly to form a sustained release depot.

For intrathecal (IT) or intracerebroventricular (ICV) delivery, appropriately sterile delivery systems (e.g., liquids; gels, suspensions, etc.) can be used to administer the compositions. For oral administration of non-peptidergic agents, the compositions may be carried in an inert filler or diluent such as sucrose, cornstarch, or cellulose.

20 The compositions of the present invention may also include biocompatible excipients, such as dispersing or wetting agents, suspending agents, diluents, buffers, penetration enhancers, emulsifiers, binders, thickeners, flavoring agents (for oral administration). Exemplary formulations can be parenterally administered as injectable dosages of a solution or suspension of the multimer in a physiologically acceptable diluent
25 with a pharmaceutical carrier that can be a sterile liquid such as water, oils, saline, glycerol, or ethanol. Additionally, auxiliary substances such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions comprising modified polypeptides. Additional components of pharmaceutical compositions include petroleum (such as of animal, vegetable, or synthetic origin), for example, soybean oil and
30 mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers for injectable solutions.

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The pharmaceutical composition can also be administered in the form of a depot injection or implant preparation that can be formulated in such a manner as to permit a sustained or pulsatile release of the multimers and other therapeutic (if present).

The pharmaceutical composition may further comprise (a) a lyoprotectant; (b) a surfactant; (c) a bulking agent; (d) a tonicity adjusting agent; (e) a stabilizer; (f) a preservative and/or (g) a buffer. In some embodiments, the buffer in the pharmaceutical composition is a Tris buffer, a histidine buffer, a phosphate buffer, a citrate buffer or an acetate buffer. The pharmaceutical composition may also include a lyoprotectant, e.g. sucrose, sorbitol or trehalose. In certain embodiments, the pharmaceutical composition includes a preservative e.g. benzalkonium chloride, benzethonium, chlorhexidine, phenol, m-cresol, benzyl alcohol, methylparaben, propylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. In other embodiments, the pharmaceutical composition includes a bulking agent, like glycine. In yet other embodiments, the pharmaceutical composition includes a surfactant e.g., polysorbate-20, polysorbate-40, polysorbate-60, polysorbate-65, polysorbate-80, polysorbate-85, poloxamer-188, sorbitan monolaurate, sorbitan monopalmitate, sorbitan monostearate, sorbitan monooleate, sorbitan trilaurate, sorbitan tristearate, sorbitan trioleate, or a combination thereof. The pharmaceutical composition may also include a tonicity adjusting agent, e.g., a compound that renders the formulation substantially isotonic or isoosmotic with human blood. Exemplary tonicity adjusting agents include sucrose, sorbitol, glycine, methionine, mannitol, dextrose, inositol, sodium chloride, arginine and arginine hydrochloride. In other embodiments, the pharmaceutical composition additionally includes a stabilizer, e.g., a molecule which, when combined with a protein of interest substantially prevents or reduces chemical and/or physical instability of the protein of interest in lyophilized or liquid form. Exemplary stabilizers include sucrose, sorbitol, glycine, inositol, sodium chloride, methionine, arginine, and arginine hydrochloride. The pharmaceutical composition can be packaged in any suitable manner.

In a third aspect, the present invention provides isolated recombinant protein comprising or consisting of the amino acid sequence of gpD(S42C) (SEQ ID NO: 10). As demonstrated in the examples that follow, gpD(S42C) is a particularly useful decoration protein. The crystal structure of the gpD trimer spike reveals that Ser42 is positioned at the apex of the spike in all three subunits (Figure 8A). Modification of this residue thus places

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any linked compound projecting away from the capsid surface and into solution for optimal display, and with minimal insult to gpD spike assembly and shell integrity. There are no other cysteine residues in the native protein, and thus the functional gpD mutant provides a unique site for chemical linkage via any suitable means, such as using maleimide-based tags. The use of these and other linking chemistries to link two compounds to each other is well known in the art, and examples are provided below.

In another embodiment, the invention provides isolated recombinant protein comprising or consisting of the amino acid sequence of gpD as modified by an amino acid substitution at one or more residues selected from the group consisting of Thr1, Ser2, Lys3, Glu4, Thr5, Phe6, Thr7, His8, Tyr9, Gln10, Pro11, Gln12, Gly13, Asn14, Ser15; Gly25, Gly26, Leu27, Ser28, Ala29, Lys30, Ala31; Asp40, Thr41, Ser42, Ser43Arg44, Lys45; Asp49, Gly50, Thr51, Thr52, Asp53; Asp66, Gln67, Thr68, Ser69, Thr70, Thr71; Arg81, Tyr82, Glu83, Asp84; Glu89, Ala90, Ala91, Ser92, Asp93, Glu94, Thr95, Lys96, Lys97, Arg98, and Thr99. In one embodiment, the one or more wild type amino acids are substituted with a Cys residue and/or an azidohomoalanine (Aha) residue.

In a further embodiment, a composition is provided comprising (a) isolated recombinant protein comprising or consisting of the amino acid sequence of SEQ ID NO: 10 (gpD(S42C)); and (b) one or more compounds linked to the recombinant protein via the cysteine at position 42. The compositions according to this embodiment are particularly useful for constructing theragnostic particles according to the present invention. The compound may be any suitable compound, including but not limited to compounds selected from the group consisting of nucleic acids, lipids, carbohydrates, polypeptides, polymers, organic molecules, or inorganic molecules, or combinations thereof. In a further embodiment, the compound is a non-proteinaceous compound, such as nucleic acids, lipids, carbohydrates, polymers, organic molecules, inorganic molecules (e.g. magnetic beads and quantum dots, among others), or combinations thereof. In a further embodiment, the composition comprises a plurality of such recombinant proteins with linked compounds, wherein the linked compounds may be the same or different from one recombinant protein to the next in the plurality.

In a further aspect, the invention provides isolated nucleic acids encoding the recombinant gpD(S42C) protein of the invention. The nucleic acids may comprise RNA or DNA, and can be prepared and isolated using standard molecular biological techniques, based on the teachings herein. The nucleic acids may comprise additional domains useful

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for promoting expression and/or purification of the encoded protein, including but not limited to polyA sequences, modified Kozak sequences, and sequences encoding epitope tags, export signals, and secretory signals, nuclear localization signals, and plasma membrane localization signals.

5 In a further aspect, the present invention provides recombinant expression vectors comprising the nucleic acid encoding the recombinant gpD(S42C) protein operatively linked to a promoter. "Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the disclosed nucleic
10 acids in a mammalian system may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The construction of expression vectors for use in transfecting prokaryotic cells is also well known in the art, and thus can be accomplished via standard techniques. (See,
15 for example, Sambrook, Fritsch, and Maniatis, in: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA, and
20 may comprise any other components as deemed appropriate for a given use, including but not limited to selection markers such as an antibiotic-resistance gene.

 In a still further aspect, the present invention provides host cells comprising the recombinant expression vectors disclosed herein, and progeny thereof, wherein the host cells can be either prokaryotic or eukaryotic. The cells can be transiently or stably
25 transfected. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et
30 al., 1989, Cold Spring Harbor Laboratory Press; *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY). Techniques utilizing cultured cells transfected with expression vectors to produce quantities of polypeptides are well known in the art.

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The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various
5 equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize.

All of the references cited herein are incorporated by reference. Aspects of the disclosure can be modified, if necessary, to employ the systems, functions and concepts of the above references and application to provide yet further embodiments of the
10 disclosure. These and other changes can be made to the disclosure in light of the detailed description.

Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these
15 embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure. Accordingly, the disclosure is not limited.

Example 1

20 The assembly of “complex” DNA viruses such as the herpesviruses and many tailed bacteriophages includes a DNA packaging step where the viral genome is inserted into a pre-formed procapsid shell. Packaging triggers a remarkable capsid expansion transition that results in thinning of the shell and an increase in capsid volume to accept the full-length genome. This transition is considered irreversible; however, here we demonstrate that the
25 phage λ procapsid can be expanded with urea *in vitro* and that the transition is fully reversible. This provides an unprecedented opportunity to evaluate the thermodynamic features of this fascinating and essential step in virus assembly. We show that urea-triggered expansion is highly cooperative and strongly temperature dependent. Thermodynamic analysis indicates that the free energy of expansion is influenced by magnesium
30 concentration (3 – 13 kcal/mol in the presence of 0.2 – 10 mM Mg^{2+}) and that significant hydrophobic surface area is exposed in the expanded shell. Conversely, Mg^{2+} drives the expanded shell back to the procapsid conformation in a highly cooperative transition that is also temperature dependent and strongly influenced by urea. We demonstrate that the gpD decoration protein adds to the urea-expanded capsid, presumably at hydrophobic patches

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exposed at the three-fold axes of the expanded capsid lattice. The decorated capsid is biologically active and sponsors packaging of the viral genome *in vitro*. The roles of divalent metal and hydrophobic interactions in controlling packaging-triggered expansion of the procapsid shell are discussed in relation to a general mechanism for DNA-triggered
5 procapsid expansion in the complex dsDNA viruses.

Introduction

The pathways for the assembly of an infectious virus from macromolecular precursors are remarkably similar in all of the complex double stranded DNA (dsDNA)
10 viruses both eukaryotic and prokaryotic^{1,2}. In particular, the DNA replication, procapsid assembly, and genome packaging pathways are strongly conserved in the herpesvirus groups and in many bacteriophages^{3;4;5;6;7}. In these cases, a terminase enzyme specifically recognizes viral DNA and the terminase motor translocates the duplex into the interior of a pre-formed procapsid^{3;4;5;6}. DNA packaging triggers a major reorganization of the proteins
15 assembled into the procapsid shell, which typically results in expansion of the shell into a thinner, more angularized icosahedral structure^{8;9;10}. Bacteriophage lambda (λ) has been extensively characterized genetically, biochemically, and structurally and provides an ideal system in which to define the molecular details of genome packaging^{7;11;12}.

Assembly of the λ procapsid follows an ordered pathway that is generally conserved
20 from phage to the herpesviruses. Briefly, assembly initiates with self-association of the portal protein (gpB) into a dodecameric ring structure^{12;13;14;15}. This nucleates polymerization of the major capsid protein (gpE) into an icosahedral shell, chaperoned by co-polymerization with the scaffolding protein (gpNu3)^{16;17;18;19}. A limited number of viral protease proteins (gpC) are also incorporated into the nascent procapsid interior, which
25 auto digests, degrades the scaffold protein, and removes 20 residues from the N-terminus of roughly half of the portal proteins^{13;14}. The proteolysis products exit the structure to afford the mature procapsid composed of a portal ring situated at a unique vertex of the icosahedral shell; this **portal vertex** provides a hole through which viral DNA can enter during packaging and exit during infection.

30 Genome packaging represents the intersection of the DNA replication and procapsid assembly pathways^{3;7}. The terminase enzyme specifically recognizes viral DNA and then binds to the portal vertex of an empty procapsid (Figure 1). This activates the terminase motor, which translocates DNA into the procapsid interior, fueled by ATP hydrolysis. Upon

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packaging ~15 kb DNA the procapsid undergoes an expansion process, which involves a significant reorganization of the capsid proteins assembled into the shell (Figure 1; discussed further below)^{7; 11; 20; 21; 22; 23}. Conventional wisdom dictates that procapsid expansion represents “a major irreversible change in the assembled capsid proteins of the procapsid shell”^{22; 24; 25}.

In a number of viral systems, this irreversible transition can be artificially triggered *in vitro* using temperature, pH, or denaturants^{26; 27; 28; 29; 30; 31}. In this study we examine urea-triggered expansion of the λ procapsid and report the surprising observation that the transition is fully reversible. The equilibrium is strongly affected by urea concentration, magnesium concentration, salt, and temperature. We present physical, biochemical, and structural studies that characterize this transition and confirm that the urea-expanded structures faithfully recapitulate those generated by DNA packaging *in vivo*. The relevance of these studies with respect to a general mechanism for DNA-triggered procapsid expansion in the complex dsDNA viruses is discussed.

Results

Urea Triggers Expansion of the Lambda Procapsid. Expansion of the λ procapsid *in vivo* is triggered upon packaging of ~ 15 kb duplex DNA (Figure 1)^{20; 21; 23}. We examined a variety of approaches to artificially expand procapsids to study this transition *in vitro*. In contrast to other viral systems, neither pH in the range of 3-9 nor heat in the range of 4°C to 50°C are effective in promoting expansion. Prior work has demonstrated that the λ procapsid can be artificially expanded by incubation with four molar urea for 30 minutes on ice²⁸. Here we recapitulate this result and demonstrate that incubation of our purified procapsids in 2.5 molar urea on ice for 15 minutes triggers procapsid expansion (Figure 2A). Due to the time required for analysis by agarose gel, an accurate quantitation of the reaction rate is not possible using this assay. We note, however, that procapsid expansion is relatively rapid and essentially complete in ~ 1 minute by gel assay (data not shown). Interestingly, urea-triggered procapsid expansion is strongly temperature dependent. While procapsids expand rapidly and completely on ice, the transition is strongly inhibited by elevated temperature (Figure 2B). We considered that this might reflect a kinetic effect; however, urea-triggered expansion was not observed even after 24 hours at 25°C. Urea-triggered expansion is also inhibited by NaCl in a concentration dependent manner (Figure

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2C). Salt inhibition is likely responsible for the observation that the λ procapsid does not expand in the presence of 4 molar guanidinium hydrochloride. For the remainder of this work we will use the term “*procapsid*” to describe the contracted shell (●) and the term “*capsid*” to describe the expanded, angularized structure (⬢; see Figure 2A).

5 *Expansion of the Lambda Procapsid is Reversible.* Previous studies reported that dialysis of urea from the reaction mixture affords a preparation of λ capsids that remain in the expanded state²⁸. In contrast, we observe that the structures contract back to the procapsid state when urea is removed by buffer exchange into TMB buffer (Figure 2A). Close inspection of the published data reveals that the primary difference between our
10 studies and previous work is the buffer used to remove urea from the sample. This was investigated and we show that the capsids remain in the expanded state when exchanged into the TMSO buffer used in the prior studies (10 mM Tris buffer, pH 8, containing 1 mM MgSO_4 and 10 mM NaN_3). In contrast, the expanded shells contract back to the procapsid conformation when exchanged into our standard TMB buffer (50 mM Tris buffer, pH 8,
15 containing 15 mM MgCl_2 and 7 mM β -ME) (Figure 2A). It is generally accepted that procapsid expansion is an irreversible process in virus development and this surprising observation was more fully explored.

Each of the components in the two buffers was individually examined, which reveals that the increased concentration of Mg^{2+} in our TMB buffer is responsible for
20 driving the expanded structure back to the procapsid state; none of the other components has any effect. We next evaluated other metals and the data presented in Table 1 demonstrate that capsid contraction is driven by all of the divalent metals examined. In contrast, none of the monovalent salts have any effect at similar concentrations. Finally, we examined the extent to which the transition is reversible. Procapsids that had been
25 previously expanded with urea and then contracted by buffer exchange into TMB buffer (15 mM Mg^{2+}) were again expanded in urea and again buffer exchanged into TMB buffer. The data presented in Figure 2D demonstrate that the λ procapsid can be repeatedly expanded and contracted in solution by urea and Mg^{2+} , respectively.

30 **Table 1. Di-Valent Metals Drive Contraction to the Procapsid Conformation.**

Procapsids were expanded in 2.5 M urea on ice for 15 minutes and then exchanged into buffer containing 1 mM MgCl_2 and the indicated salt at 15 mM, unless otherwise indicated.

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In all cases, the preparations contained either exclusively expanded capsid or contracted procapsid structures, as indicated.

Salt (15 mM)	Capsid State
MgCl ₂	Contracted
MgSO ₄	Contracted
CaCl ₂	Contracted
BaCl ₂	Contracted
MnCl ₂	Contracted
Ni(NO ₃) ₂	Contracted
ZnCl ₂	Contracted
NaCl	Expanded
Na phosphate	Expanded
KCl	Expanded
K glutamate	Expanded
100 mM NaCl	Expanded
500 mM NaCl	Contracted

5

Thermodynamic Analysis of Urea-Triggered Procapsid Expansion. The data presented above demonstrate that urea-triggered expansion of the λ procapsid is reversible. Moreover, close inspection of the gels fails to reveal any evidence of intermediate states between the contracted procapsid and expanded capsid conformations (see Figure 2). These observations suggest that the expansion reaction may be modeled as a reversible, two-state transition and we adapted analytical tools developed to characterize two-state protein unfolding reactions³². Procapsids were expanded as described in Materials and Methods except that the concentration of MgCl₂ and urea was varied as indicated. In the presence of 0.2 mM MgCl₂, procapsid expansion is urea-concentration dependent and strongly cooperative (Figure 3A). Analysis of the data according to a reversible, two-state transition (Equation 1)³² affords a free energy of expansion, $\Delta G(\text{H}_2\text{O}) \sim 3$ kcal/mol. We note that the expansion transition under these conditions is extremely facile and occurs with essentially

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no pre-transition baseline. The error associated with this analysis is thus rather large; $\Delta G(\text{H}_2\text{O}) = 3.3 \pm 4.4 \text{ kcal/mol}$.

As described above, Mg^{2+} drives the expanded shell back to the contracted procapsid conformation. We interpreted the data to indicate that urea and Mg^{2+} have antagonistic effects on the conformation of the λ capsid and we directly tested this hypothesis. The above urea-expansion study was repeated except that MgCl_2 was included in the reaction mixture at 3 mM or 10 mM. The data clearly indicate that Mg^{2+} antagonizes urea-triggered procapsid expansion in a concentration-dependent manner (Figure 3A) and that the concentration of urea required to expand half of the procapsids ($[\text{urea}]_{1/2}$) is strongly affected by Mg^{2+} (Figure 3B). Analysis of the expansion data according to Equation 1 affords a $\Delta G(\text{H}_2\text{O})$ of $10.0 \pm 3.0 \text{ kcal/mol}$ and $13.4 \pm 2.4 \text{ kcal/mol}$ in the presence of 3 mM and 10 mM Mg^{2+} , respectively (Figure 3C). For comparison, “typical” protein unfolding reactions report $[\text{urea}]_{1/2}$ between 3-6 molar and a $\Delta G(\text{H}_2\text{O})$ that ranges between 5-15 kcal/mol^{32; 33; 34; 35}. Finally, we note that the denaturant “*m*” value for the transition is relatively large and insensitive to the concentration of Mg^{2+} in the reaction mixture (Figure 3C). This is discussed further below.

Mg^{2+} Driven Capsid Contraction. To further explore the antagonist effects of urea and Mg^{2+} , a preparation of expanded capsids in buffer lacking both urea and Mg^{2+} was prepared by buffer exchange. The expanded capsids were then incubated in the presence of increasing concentrations of MgCl_2 and the fraction of structures that had contracted back to the procapsid state was quantified by gel assay. The data presented in Figure 4 demonstrate that Mg^{2+} drives the expanded shell back to the procapsid conformation in a concentration dependent and strongly cooperative manner; analysis of the data according to Equation 3 affords a $[\text{Mg}^{2+}]_{1/2} = 1.1 \pm 0.1 \text{ mM}$. We next repeated the experiment as described above except that 2.5 molar urea was included in the reaction buffer. While Mg^{2+} can still drive the contraction transition, significantly higher concentrations of divalent metal are required; $[\text{Mg}^{2+}]_{1/2} = 6.5 \pm 0.4 \text{ mM}$ (Figure 4).

Biological Activity of the Expanded λ Capsids. We have demonstrated that the λ procapsid can be reversibly expanded and contracted *in vitro*, which has allowed a rigorous thermodynamic characterization of the transition (*vide supra*). Evaluation of these data with specific reference to virus assembly requires that urea-triggered expansion mimic the natural pathway that is triggered by DNA packaging *in vivo*. The three most essential functions required of procapsids during DNA packaging are (i) the ability to bind the

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terminase motor and sponsor DNA packaging, (ii) the ability to bind the gpD decoration protein to the expanded capsid lattice, and (iii) the capacity of the decorated shell to physically withstand the internal forces generated by the packaged λ genome.

Our lab has developed an *in vitro* DNA packaging assay where viral DNA is packaged into purified procapsids in a defined biochemical assay system^{36, 37}. These studies have demonstrated that the packaging reaction is magnesium-dependent and that the optimal MgCl_2 concentration is ~ 5 mM. This presents a problem when trying to package into the expanded capsids since they contract in the presence of > 1 mM Mg^{2+} (Figure 4). Therefore, as a first step towards demonstrating the biological activity of the urea-expanded capsids, we examined gpD binding to the capsid shell. An expanded capsid preparation in 1 mM Mg^{2+} (no urea) was prepared and purified gpD was added to the mixture. Figure 5A clearly shows that the gpD decoration protein efficiently adds to the expanded capsid lattice. Several features of this interaction are noteworthy. First, while gpD binds to the expanded capsid, no interaction is observed with unexpanded procapsid shell (Figure 5A); this feature is similarly observed *in vivo*^{7, 38}. Second, the gpD-decorated shell migrates faster in the gel than does the expanded capsid alone. This indicates that gpD addition affords a more negatively charged capsid shell. Third, gpD stabilizes the expanded shell and the decorated capsids no longer contract in the presence of elevated Mg^{2+} concentrations (Medina, Kruse, and Catalano, unpublished;³⁹). Finally, electron micrographs demonstrate that the gpD decorated structures show thinning of the capsid shell and increased angularization of the icosahedral structure (Figure 5B), as observed *in vivo*²³. We note that the expanded shells are fragile and easily damaged during preparation for EM analysis (see Figure 5B). In contrast, the gpD-decorated shells are robust and intact structures are evident in the micrographs.

To further demonstrate biological activity, we examined the genome packaging activity of the gpD-decorated capsids. Terminase-mediated packaging of the λ genome into a capsid renders the duplex resistant to DNase and the packaging products are visualized on an agarose gel³⁶. Our standard DNA packaging assay was modified by pre-incubating the expanded capsids with gpD and then adding the other reaction components required for genome packaging. The data presented in Figure 5C demonstrate that the expanded, gpD coated capsids are fully competent for DNA packaging. Importantly, the only packaging product is the full-length λ genome (inset). This indicates that packaging into the urea-expanded capsids is highly processive and that DNA-filled particles can withstand the

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tremendous internal forces generated by the tightly packaged, highly pressurized DNA genome^{20; 40; 41}. These features mirror the observations with *bone fide* λ capsids both *in vitro*^{20; 40} and *in vivo*^{7; 14; 38}.

5 Discussion

The packaging of a viral genome into a pre-formed procapsid structure is an essential step in the assembly of complex dsDNA viruses. A universal feature is that DNA packaging triggers maturation of the procapsid, often resulting in expansion and thinning of the shell to afford a larger, more angularized structure. Dogma contends that this step is irreversible so that unidirectional assembly of the viral particle is ensured. This presumption is consistent with a number of *in vitro* studies where procapsid expansion has been artificially triggered by using pH, heat, or denaturants^{26; 27; 28; 30; 42; 43; 44}, however, there has been some indication that strict irreversibility may not necessarily be the case. For instance, pH-induced expansion of the HK97 procapsid proceeds through a number of intermediates and there is evidence that at least one transition may be partially (10%) reversible⁴⁵. That said, the fully expanded capsid structure is stable and does not contract back to the procapsid conformation. Thus, our demonstration that urea-triggered expansion of the λ procapsid is fully reversible and that the expansion-contraction transition can be repeated for multiple cycles is unexpected and quite remarkable.

Energetic Features of Procapsid Expansion. We have demonstrated that Mg^{2+} and urea stabilize the contracted and the expanded conformations of the λ capsid, respectively. We have further shown that inter-conversion between the two structures is highly cooperative and fully reversible. These features allow a rigorous thermodynamic characterization of the expansion reaction, which has not been possible in any other system. Our data indicate that the free energy of urea-triggered procapsid expansion, $\Delta G(H_2O)$, is ~ 13 kcal/mol in the presence of 10 mM $MgCl_2$. This translates directly to ~ 90 piconewtons•nanometer (pN•nm) work done to expand an individual capsid. It is generally presumed that packaging DNA into the procapsid interior generates pressure that ultimately provides the energy, at least in part, required to trigger expansion^{6; 7; 8; 20}. Previous single-molecule studies suggest that expansion is triggered when the terminase motor inserts ~ 15 kb duplex DNA into the procapsid (a magnesium concentration of 10 mM was used in the laser tweezer studies) (see Figure 1)²⁰. The motor generates ~ 5 pN force at this point^{46; 47}, which reflects the energy required to condense the DNA into the confines of the procapsid

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interior. To the first approximation, this force will generate a pressure of 0.06 N/cm^2 on the inside of the shell. Expansion increases the diameter of the particle from 50 nm to 60 nm ^{23, 41}, which results in a volume increase of 4.5×10^{-20} liters. This means that the mechanical work performed by the motor at the point of procapsid expansion is $\sim 70 \text{ pN}\cdot\text{nm}$ per capsid (work = $P\Delta V$), which to the first approximation corresponds quite well to the work required to expand the structure with urea *in vitro* ($\sim 90 \text{ pN}\cdot\text{nm}$, above). While admittedly qualitative in nature, these simple calculations indicate that the free energy of procapsid expansion by urea is commensurate with the work performed by the motor at the point of expansion during DNA packaging. This is discussed further below.

Magnesium Stabilization of the Procapsid ~ Possible Biological Role? Divalent metals play an essential role in the assembly of many viruses. For example, Ca^{2+} plays an important role in the assembly and stabilization of polyoma virus^{48; 49; 50} and herpes virus⁵¹ particles. Similarly, early studies demonstrated an important role for Mg^{2+} in the assembly and stability of an infectious λ virus^{52; 53}. Chelation of Mg^{2+} with EDTA destroys λ infectivity and it has been proposed that Mg^{2+} serves as a counter-ion to maintain DNA in the condensed state within the nucleocapsid⁵⁴. Stabilization of the procapsid conformation by divalent metals as demonstrated here indicates that there is a metal-capsid interaction in addition to the metal-DNA interaction. Indeed, recent structural studies have identified putative metal-binding sites localized at the 3-fold axes on the interior surface of the HK97 procapsid⁵⁵. We suggest that this feature is recapitulated in λ and that this metal-capsid interaction is responsible, at least in part, for stabilizing the procapsid conformation. This model has some interesting predictions.

In addition to the pressure generated by the packaged DNA (*vide supra*), it is presumed that duplex interactions with the inner surface of the procapsid shell trigger the expansion transition; however, mechanistic details of these putative interactions remain obscure. We suggest the following. Condensation of DNA within the capsid interior is energetically unfavorable due, in part, to significant charge-repulsion by the closely packed phosphodiester backbone. Charge neutralization by polyamines and divalent metals is required to ensure efficient condensation and packaging of the genome^{54; 56; 57}. The terminase motor translocates over 600 base pairs/second into the capsid interior^{20; 58} and it is tempting to speculate that the rapidly packaged duplex effectively strips the Mg^{2+} bound at the interior capsid surface. This would serve to decrease the free energy requirement for expansion of the shell, perhaps as much as 10 kcal/mol (Figure 3), and to promote the

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transition. As shown here, expansion is accompanied by addition of gpD to the capsid surface, which stabilizes the expanded shell and prevents contraction back to the procapsid state. In this situation, divalent metals are free to bind to and stabilize the condensed DNA. Work currently underway in our lab seeks to directly measure the duplex length requirement for procapsid expansion in the presence of various divalent metals to directly test this hypothesis.

Procapsid Expansion Exposes Hydrophobic Surface Area. We propose that the procapsid conformation is stabilized not only by divalent metal, but also by hydrophobic interactions between the capsid proteins in the contacted shell (see Figure 6). The expansion transition requires disruption of these interactions and exposure of hydrophobic patches on the expanded capsid surface. This hypothesis is based in part on the observation that urea triggers procapsid expansion; while the mechanism remains controversial, it is generally accepted that urea disrupts hydrophobic interactions and effectively “solvates” hydrophobic residues in water^{34; 59; 60}. Thus, in analogy to protein unfolding, urea stabilizes the expanded capsid conformation and shifts the equilibrium towards the expanded state. The model is also consistent with the observation that procapsid expansion is strongly inhibited by salt and increased temperature, both of which increase the strength of hydrophobic interactions. For instance, expansion occurs efficiently at 4°C but not at 25°C, a temperature range in which the strength of hydrophobic interactions increase to a maximum at ~ 20°C^{59; 61}. In sum, our data are consistent with a transition that requires overcoming hydrophobic interactions.

The model proposed in Figure 6 is further consistent with the large denaturant “*m*” value obtained in our thermodynamic analysis of urea-triggered expansion. This value reflects the dependence of ΔG on denaturant concentration, which is related to the heat capacity change (ΔC_p). This in turn is related to the change in hydrophobic surface area; the larger the *m*, the greater hydrophobic surface area is exposed in the transition³³. The *m* value obtained here (> 4 kcal/mol•molar) is relatively large compared to most protein unfolding reactions (typically 1-3 kcal/mol•molar^{32; 34; 35; 62}). This indicates that a large hydrophobic surface area is exposed upon capsid expansion. Importantly, while the $\Delta G(H_2O)$ for expansion increases with increasing magnesium concentration, the denaturant *m* value remains relatively constant. This indicates that while magnesium stabilizes the procapsid state, it does not affect the change in hydrophobic surface area exposed upon transition to the expanded conformation. In other words, the conformation of the expanded

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state is the same regardless of the Mg^{2+} concentration, as would be expected of a simple two-state transition.

Mechanism for Addition of gpD to the Expanded Capsid Lattice. The terminase motor packages DNA to liquid crystalline density within the capsid, which generates over
5 20 atmospheres of internal pressure^{20; 63; 64; 65}. Viral decoration proteins add to the surface of the expanded capsid structure to stabilize the shell against the tremendous internal forces generated by the tightly packaged DNA^{20; 40; 41}. Specifically, the λ gpD protein assembles as a trimeric spike at the three-fold axes of the expanded capsid lattice^{23; 41}. While gpD is dispensable for packaging of duplexes up to ~ 40 kb, packaging of larger duplexes requires
10 gpD to maintain capsid integrity⁴⁰.

Structural studies indicate that the base of the gpD trimer is hydrophobic and it has been proposed that the spike interacts with hydrophobic patches on the capsid surface^{41; 66; 67}; however, biochemical evidence for this model is lacking. Our data indicate that procapsid expansion is associated with exposure of hydrophobic patches on the capsid
15 surface. We propose that these patches, which are buried in the procapsid conformation, are exposed at the icosahedral three-fold axes of the expanded shell and provide a nucleation site for assembly of the gpD trimer. Importantly, gpD adds to the surface of the expanded capsid efficiently at room temperature but only poorly at 4°C (data not shown; see³⁹); this temperature dependence is consistent with an increase in hydrophobic binding energy
20 within this temperature range (*vide supra*)^{59; 61}.

Although our data show that expansion of the λ procapsid is fully reversible, gpD addition effectively locks the capsid shell into the expanded state. Indeed, large-scale conformational changes accompany DNA packaging in all of the complex dsDNA viruses and this is intrinsically irreversible. *In vivo*, these transitions are further made irreversible
25 by proteolytic cleavage events, capsid protein cross-linking events, or the addition of decoration proteins as is observed in λ . Subsequently, “finishing” proteins add in an ordered, step-wise sequence, interactions that are nucleated by addition of the previous protein. In this manner, the particle progresses to the next step of assembly to minimize “off-pathway” intermediates and ensures fidelity in the assembly process.

30 *Why Do Procapsids Expand?* Procapsid expansion is a common feature in the packaging of viral DNA but it is not clear why this is necessary. One possibility is that the assembly of a procapsid shell provides a mechanism for the terminase packaging motor to select only packaging competent shells. This presumes that pre-expanded capsids represent

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defective, off pathway intermediates that may result from aborted packaging events; however, our data demonstrate that the expanded λ capsid is catalytically competent and packages DNA quite efficiently. Indeed, this has similarly been demonstrated in the bacteriophage T4 system^{68; 69}. Thus, while DNA packaging triggers the transition *in vivo*,
5 expansion *per se* does not influence the capacity of the shell to accept the viral genome.

This begs the question as to why viruses utilize a procapsid structure at all. One possibility proposed by Johnson and co-workers is that the extensive interactions between the protein subunits assembled into the mature capsid shell are unlikely to form in a single assembly step⁵⁵. This model suggests that the procapsid represents a metastable
10 intermediate that is required for fidelity in shell assembly and that expansion is a consequence of the transition to the stable mature shell. Such a transition implies irreversibility; however, our data show that this need not be the case.

Conclusions. We have demonstrated that magnesium and urea stabilize the contracted and expanded conformations of the λ capsid shell, respectively. The λ system is
15 unique in that expansion is fully reversible, which has allowed thermodynamic characterization of the transition. Expansion of the shell is associated with exposed hydrophobic surface area to which the gpD decoration protein adds. This stabilizes the expanded capsid lattice, abrogates contraction, and provides structural integrity so that the genome can be tightly packaged into the capsid interior. This work further provides
20 mechanistic insight into how DNA packaging triggers shell expansion that is generalizable to the complex dsDNA viurses, both prokaryotic and eukaryotic.

Materials and Methods for Example 1

Materials and Methods. Tryptone, yeast extract, and agar were purchased from
25 DIFCO. Molecular biology enzymes and mature λ DNA were purchased from New England Biolabs. Chromatography media was purchased from GE Healthcare and urea was purchased from Fisher Scientific. All other materials were of the highest quality available. Unless otherwise stated, Tris buffers were adjusted to the indicated pH at a temperature of 4°C. Bacterial cultures were grown in shaker flasks utilizing an Innova 4430 incubator-
30 shaker. All protein purifications utilized the Amersham Biosciences ÄKTApurifier™ core 10 System from GE Healthcare. UV-VIS absorbance spectra were recorded on a Hewlett-Packard HP8452A spectrophotometer. Sucrose density gradients were prepared using a Biocomp Model 107 Gradient Master® and gradient centrifugation utilized a Beckman L-

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90K ultracentrifuge with a SW 28 rotor.

Protein Purification. λ terminase and the *Escherichia coli* Integration Host Factor (IHF) were purified as previously described^{70 71}. The gpD decoration protein was purified by our published protocol³⁶, with modification. Briefly, gpD was expressed from the pT7Cap vector and ammonium sulfate was added to the cell lysis supernatant to 50% saturation. The mixture was gently stirred on ice for 50 minutes and insoluble protein was removed by centrifugation (9 K x g, 20 minutes). The supernatant was adjusted to 90% ammonium sulfate, stirred on ice for 50 minutes and insoluble protein was harvested by centrifugation (30 K x g, 30 minutes). The pellet was resuspended in 20 mM Tris buffer, pH 8, containing 1 mM EDTA and 15 mM NaCl and the sample was applied to a DEAE column equilibrated with the same buffer. The column flow-through fraction, which contained gpD, was applied to a S-300 gel filtration column equilibrated and developed with 20 mM Tris buffer, pH 8, containing 1 mM EDTA, and 100 mM NaCl. The gpD containing fractions were pooled, dialyzed into 20 mM Tris buffer, pH 8, containing 1 mM EDTA and concentrated to ~500 μ M using an Amicon[®] centrifugal filter unit. All of our purified protein preparations were homogenous as determined by SDS-PAGE (not shown).

Procapsid Purification. λ procapsids were purified as described previously³⁶, but the purified preparations contained variable amounts of pre-expanded procapsids. To obtain a homogenous preparation of unexpanded procapsids, the sample was fractionated on a 0.8% agarose gel, which readily separates expanded capsids from unexpanded procapsids (see Figure 2). The lower band was excised with a sterile razor blade and the unexpanded procapsids were eluted from the gel slice into Buffer A (25 mM Tris base, 192 mM glycine, and 1 mM MgCl₂) using a Bio-Rad Model 422 Electro-eluter (100 V for 1 hour). The sample was dialyzed into TMB buffer (50 mM Tris buffer, pH 8, containing 15 mM MgCl₂ and 7 mM β -ME) and concentrated using an Amicon[®] centrifugal filter unit.

Procapsid Expansion and Buffer Exchange Protocol. A freshly prepared stock solution of 8 molar urea in water was used for all of the expansion experiments. Unless otherwise specified, equal volumes of purified procapsids and 8 molar urea were mixed to afford a reaction mixture (20 μ l) containing 30 nM procapsids in 10 mM Tris buffer, pH 8, containing 2.5 molar urea and 3 mM MgCl₂. Subsequent modification of the expansion reaction composition was accomplished by a buffer exchange protocol. Briefly, a sufficient volume of buffer was added to adjust the magnesium and urea concentrations as indicated in each experiment and the mixture was then concentrated to its original volume using an

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Amicon® centrifugal filter unit. The expansion reaction mixtures were incubated on ice for 15 minutes and procapsid expansion analyzed by agarose gel assay, as described below.

Analysis of Capsid Expansion and Contraction. The samples were applied to a 0.8 % agarose gel and electrophoresis was performed at 110 v for 100 minutes. The contracted
5 procapsid (lower) and expanded capsid (upper) bands were visualized by staining with Coomassie brilliant blue. Video images of the de-stained gels were captured using an EpiChemi³ darkroom system with a Hammamatsu camera (UVP Bioimaging Systems) and the video images were quantified using either the LabWorks 4.6 (UVP Bioimaging Systems) or the ImageQuant (Molecular Dynamics) software packages.

Thermodynamic Analysis of Urea-Triggered Procapsid Expansion. Procapsid
10 expansion in urea is a two-state, reversible transition and we adapted analytical tools developed to characterize reversible protein unfolding transitions to analyze the data, as follows. The fraction of procapsids in the expanded state was quantified by agarose gel assay (above). The data were fit by nonlinear, least-squares analysis using the linear
15 extrapolation method as outlined by Santoro and Bolen³² according to equation 1:

$$F_E = \frac{(m_P \cdot [U] + b_P) + (m_E \cdot [U] + b_E) \cdot \exp\left[-\left(\frac{\Delta G_{H_2O}}{RT} + \frac{m_G \cdot [U]}{RT}\right)\right]}{1 + \exp\left[-\left(\frac{\Delta G_{H_2O}}{RT} + \frac{m_G \cdot [U]}{RT}\right)\right]} \quad (1)$$

where F_E is the fraction of capsids in the expanded state as a function of the urea concentration ($[U]$). The values m_P and m_E represent the slopes and b_P and b_E represent the y-intercepts of the pre- and post-transition baselines, respectively. R is the ideal gas
20 constant and T is the temperature (kelvin). The free energy of procapsid expansion (ΔG_{H_2O} ; kcal/mol) and the denaturant m value (m_G ; kcal/mol•molar) represent the intercept and slope, respectively, of the linear dependence of the expansion transition energy as a function of denaturant concentration. Each data set was fit to equation 1 with m_P , b_P , m_E , b_E , ΔG_{H_2O} , and m_G as parameters using the IGOR® graphics/analysis package (WaveMetrics, Lake
25 Oswego, OR).

The urea-expansion data were also fit according to equation 2:

$$F_E = Base + \left[\frac{Max}{1 + \exp\left(\frac{[urea]^{1/2} - [urea]}{mr}\right)} \right] \quad (2)$$

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where F_E is the fraction of capsids in the expanded state as a function of the urea concentration ($[urea]$). Base and Max represent the pre- and post-transition baselines, m_T is the transition slope, and $[urea]_{1/2}$ is the concentration of urea required to expand half of the procapsids to the expanded state. Each data set was fit to equation 2 with Base, Max, $C_{1/2}$ and m_T as parameters using the IGOR[®] graphics/analysis package (WaveMetrics, Lake Oswego, OR).

Analysis of Mg^{2+} -Triggered Capsid Contraction. Expanded capsids were prepared as described above in the absence and presence of urea at the indicated concentration. The samples were incubated in the presence of the indicated concentration of $MgCl_2$ at room temperature for five minutes and the fraction of expanded capsid structures quantified by agarose gel and video densitometry as described above. The data were fit by nonlinear, least-squares analysis according to equation 3:

$$F_E = Base + \left[\frac{Max}{1 + \exp\left(\frac{[Mg]_{1/2} - [Mg]}{m_T}\right)} \right] \quad (3)$$

where F_E is the fraction of capsids in the expanded state as a function of the Mg^{2+} concentration, $[Mg]$. Base and Max represent the pre- and post-transition baselines, m_T is the transition slope, and $[Mg]_{1/2}$ is the concentration of Mg^{2+} required to contract half of the expanded shells to the procapsid state. Each data set was fit to equation 2 with Base, Max, $[Mg]_{1/2}$ and m_T as parameters using the IGOR[®] graphics/analysis package (WaveMetrics, Lake Oswego, OR).

DNA Packaging into Expanded Capsids. The *in vitro* DNA packaging reaction was performed as described previously³⁶, with modification. Briefly, purified procapsids (40 nM capsid; 16.6 μ M gpE capsid protein) were expanded as described above and then buffer exchanged into 10 mM Tris buffer, pH 8. Purified gpD was then added to a final concentration of 15 μ M and the mixture was incubated for 30 minutes at room temperature. The gpD-coated, expanded capsids were added to a reaction mixture containing 50 mM Tris buffer, pH 7.4, containing 9 mM NaCl, 5 mM $MgCl_2$, 2 mM spermidine, 1.3 mM β -ME, 1 mM ATP, 100 nM IHF, and 2 nM mature λ DNA. The packaging reaction (20 μ l) was initiated with the addition of terminase holoenzyme to a final concentration of 100 nM and allowed to proceed for 30 minutes at room temperature. DNase (10 μ g/ml) was then added to the reaction mixture and incubated at room temperature for five minutes. The DNase

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reaction was stopped with the addition of phenol:chloroform (21 μ l) and the aqueous layer was removed and loaded onto a 0.8% agarose gel. Packaged (DNase resistant) DNA was quantified by video densitometry as previously described³⁶.

References for Example 1

1. Calendar, R. & Abedon, S. T. (2006). *The Bacteriophages*, Oxford University Press, New York, N.Y.
2. Roizman, B. & Palese, P. (1996). Multiplication of Viruses: An Overview. In *Fields Virology* Third edit. (Fields, B. N., Knipe, D. M. & Howley, P. M., eds.), pp. 101-11. Lippincott-Raven, New York, N.Y.
3. Rao, V. B. & Feiss, M. (2008). The bacteriophage DNA packaging motor. *Annu Rev Genet* **42**, 647-81.
4. Roizman, B., Knipe, D. M. & Whitley, R. J. (2007). Herpes Simplex Viruses. In *Fields Virology* Fifth edit. (Knipe, D. M. & Howley, P. M., eds.), pp. 2501-2602. Lippincott, Williams and Wilkins, Philadelphia, PA.
5. Catalano, C. E. (2005). Viral Genome Packaging Machines: An Overview. In *Viral Genome Packaging Machines: Genetics, Structure, and Mechanism* (Catalano, C. E., ed.), pp. 1-4. Kluwer Academic/Plenum Publishers, New York, N.Y.
6. Baines, J. D. & Weller, S. K. (2005). Cleavage and Packaging of Herpes Simplex Virus 1 DNA. In *Viral Genome Packaging Machines: Genetics, Structure, and Mechanism* (Catalano, C. E., ed.), pp. 135-149. Kluwer Academic/Plenum Publishers, New York, N.Y.
7. Feiss, M. & Catalano, C. E. (2005). Bacteriophage Lambda Terminase and the Mechanism of Viral DNA Packaging. In *Viral Genome Packaging Machines: Genetics, Structure, and Mechanism* (Catalano, C. E., ed.), pp. 5-39. Kluwer Academic/Plenum Publishers, New York, N.Y.
8. Jardine, P. J. & Anderson, D. L. (2006). DNA Packaging in Double-Stranded DNA Phages. In *The Bacteriophages* 2nd edit. (Calendar, R. & Abedon, S. T., eds.), pp. 49-65. Oxford University Press, New York, N.Y.
9. Baines, J. D. & Duffy, C. (2006). Nucleocapsid Assembly and Envelopment of Herpes Simplex Virus. In *Alpha Herpesviruses: Molecular and Cellular Biology* (Sandri-Goldin, R. M., ed.), pp. 175-204. Caister Academic Press, Norfolk, VA.
10. Alonso, J. C., Tavares, P., Lurz, R. & Trautner, T. A. (2006). Bacteriophage SPP1. In *The Bacteriophages* 2nd edit. (Calendar, R. & Abedon, S. T., eds.), pp. 331-349. Oxford University Press, New York, N.Y.

- 43 -

11. Hendrix, R. W. & Casjens, S. (2006). Bacteriophage Lambda and its Genetic Neighborhood. In *The Bacteriophages* 2nd edit. (Calendar, R. & Abedon, S. T., eds.), pp. 409-447. Oxford University Press, New York, N.Y.
12. Hendrix, R. W., Roberts, J. W., Stahl, F. W. & Weisberg, R. A. (1983). *Lamba II*,
5 Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Medina, E., Wieczorek, D. J., Medina, E. M., Yang, Q., Feiss, M. & Catalano, C. E. (2010). Assembly and Maturation of the Bacteriophage Lambda Procapsid: gpC Is the Viral Protease. *J. Mol. Biol.* **401**, 813-830.
14. Georgopoulos, C., Tilly, K. & Casjens, S. (1983). Lambdoid Phage Head Assembly.
10 In *Lambda II* (Hendrix, R. W., Roberts, J. W., Stahl, F. W. & Weisberg, R. A., eds.), pp. 279-304. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Kochan, J. & Murialdo, H. (1983). Early Intermediates in Bacteriophage Lambda Prohead Assembly. II. Identification of Biologically Active Intermediates. *Virology* **131**, 100-115.
- 15 16. Medina, E. M., Andrews, B. T., Nakatani, E. & Catalano, C. E. (2011). The Bacteriophage Lambda gpNu3 Scaffolding Protein is an Intrinsically Disordered and Biologically Functional Procapsid Assembly Catalyst. *Journal of Molecular Biology* **412**, 723-736.
17. Johnson, J. E. (2010). Virus Particle Maturation: Insights into Elegantly
20 Programmed Nanomachines. *Current Opinion in Structural Biology* **20**, 210-216.
18. Fane, B. A. & Prevelige, P. E. (2003). Mechanism of Scaffolding-Assisted Viral Assembly. In *Virus Structure* (Wah, C. & John, E. J., eds.), Vol. Volume 64, pp. 259-299. Academic Press, Oxford, UK.
19. Dokland, T. (1999). Scaffolding Proteins and their Role in Viral Assembly. *Cellular and Molecular Life Sciences* **56**, 580-603.
25
20. Fuller, D. N., Raymer, D. M., Rickgauer, J. P., Robertson, R. M., Catalano, C. E., Anderson, D. L., Grimes, S. & Smith, D. E. (2007). Measurements of single DNA molecule packaging dynamics in bacteriophage lambda reveal high forces, high motor processivity, and capsid transformations. *J Mol Biol* **373**, 1113-22.
- 30 21. Hohn, T., Wurtz, M. & Hohn, B. (1976). Capsid Transformation During Packaging of Bacteriophage Lambda DNA. *Phil. Trans. R. Soc. Lond.* **276**, 51-61.
22. Hohn, T., Morimasa, T. & Tsugita, A. (1976). The Capsid Protein of Bacteriophage Lambda and of its Prehead. *Journal of Molecular Biology* **105**, 337-342.

- 44 -

23. Dokland, T. & Murialdo, H. (1993). Structural Transitions During Maturation of Bacteriophage Lambda Capsids. *J. Mol. Biol.* **233**, 682-694.
24. Steven, A. C., Heymann, J. B., Cheng, N., Trus, B. L. & Conway, J. F. (2005). Virus Maturation: Dynamics and Mechanism of a Stabilizing Structural Transition that Leads to Infectivity. *Current Opinion in Structural Biology* **15**, 227-236.
25. Black, L. W. (1989). DNA Packaging in dsDNA Bacteriophages. *Annual Review of Microbiology* **43**, 267-292.
26. Duda, R. L., Hempel, J., Michel, H., Shabanowitz, J., Hunt, D. & Hendrix, R. W. (1995). Structural Transitions During Bacteriophage HK97 Head Assembly. *Journal of Molecular Biology* **247**, 618-635.
27. Galisteo, M. L. & King, J. (1993). Conformational Transformations in the Protein Lattice of Phage P22 Procapsids. *Biophysical Journal* **65**, 227-235.
28. Kunzler, P. & Hohn, T. (1978). Stages of Bacteriophage Lambda Head Morphogenesis: Physical Analysis of Particles in Solution. *Journal of Molecular Biology* **122**, 191-211.
29. Imber, R., Tsugita, A., Wurtz, M. & Hohn, T. (1980). Outer Surface Protein of Bacteriophage Lambda. *Journal of Molecular Biology* **139**, 277-295.
30. Newcomb, W. W., Homa, F. L., Thomsen, D. R., Booy, F. P., Trus, B. L., Steven, A. C., Spencer, J. V. & Brown, J. C. (1996). Assembly of the Herpes Simplex Virus Capsid: Characterization of Intermediates Observed During Cell-free Capsid Formation. *Journal of Molecular Biology* **263**, 432-446.
31. Conway, J. F., Duda, R. L., Cheng, N., Hendrix, R. W. & Steven, A. C. (1995). Proteolytic and Conformational Control of Virus Capsid Maturation: The Bacteriophage HK97 System. *Journal of Molecular Biology* **253**, 86-99.
32. Santoro, M. M. & Bolen, D. W. (1988). Unfolding Free Energy Changes Determined by the Linear Extrapolation Method. 1. Unfolding of Phenylmethanesulfonyl Alpha-Chymotrypsin Using Different Denaturants. *Biochemistry* **18**, 8063-8068.
33. Pace, C. N. & Shaw, K. L. (2000). Linear Extrapolation Method of Analyzing Solvent Denaturation Curves. *Proteins Suppl.* **4**, 1-7.
34. Santoro, M. M. & Bolen, D. W. (1992). A Test of the Linear Extrapolation of Unfolding Free Energy Changes over an Extended Denaturant Concentration Range. *Biochemistry* **31**, 4901-4907.

- 45 -

35. Bolen, D. W. & Santoro, M. M. (1988). Unfolding free energy changes determined by the linear extrapolation method. 2. Incorporation of delta G degrees N-U values in a thermodynamic cycle. *Biochemistry* **18**, 8069-8074.
36. Yang, Q. & Catalano, C. E. (2003). Biochemical characterization of bacteriophage
5 lambda genome packaging in vitro. *Virology* **305**, 276-87.
37. Gaussier, H., Yang, Q. & Catalano, C. E. (2006). Building a virus from scratch: assembly of an infectious virus using purified components in a rigorously defined biochemical assay system. *J Mol Biol* **357**, 1154-66.
38. Murialdo, H. & Becker, A. (1978). Head Morphogenesis of Complex Double-
10 Stranded Deoxyribonucleic Acid Bacteriophages. *Microbiological Reviews* **42**, 529-576.
39. Medina, E. (2010). Growing Pains of Bacteriophage Lambda: Examination of the Maturation of Procapsids into Capsids. Ph.D., University of Washington.
40. Yang, Q., Maluf, N. K. & Catalano, C. E. (2008). Packaging of a Unit-Length Viral
15 Genome: The Role of Nucleotides and the gpD Decoration Protein in Stable Nucleocapsid Assembly in Bacteriophage Lambda. *Journal of Molecular Biology* **383**, 1037-1048.
41. Lander, G. C., Evilevitch, A., Jeembaeva, M., Potter, C. S., Carragher, B. & Johnson, J. E. (2008). Bacteriophage Lambda Stabilization by Auxiliary Protein
20 gpD: Timing, Location, and Mechanism of Attachment Determined by Cryo-EM. *Structure* **16**, 1399-1406.
42. Conway, J. F., Cheng, N., Ross, P. D., Hendrix, R. W., Duda, R. L. & Steven, A. C. (2007). A Thermally Induced Phase Transition in a Viral Capsid Transforms the Hexamers, Leaving the Pentamers Unchanged. *Journal of Structural Biology* **158**,
25 224-232.
43. Jardine, P. J. & Coombs, D. H. (1998). Capsid Expansion Follows the Initiation of DNA Packaging in Bacteriophage T4. *Journal of Molecular Biology* **284**, 661-672.
44. Lee, K. K., Tsuruta, H., Hendrix, R. W., Duda, R. L. & Johnson, J. E. (2005). Cooperative Reorganization of a 420 Subunit Virus Capsid. *Journal of Molecular
30 Biology* **352**, 723-735.
45. Lata, R., Conway, J. F., Cheng, N., Duda, R. L., Hendrix, R. W., Wikoff, W. R., Johnson, J. E., Tsuruta, H. & Steven, A. C. (2000). Maturation Dynamics of a Viral Capsid: Visualization of Transitional Intermediate States. *Cell* **100**, 253-263.

- 46 -

46. Fuller, D. N., Raymer, D. M., Kottadiel, V. I., Rao, V. B. & Smith, D. E. (2007). Single phage T4 DNA packaging motors exhibit large force generation, high velocity, and dynamic variability. *Proc Natl Acad Sci U S A* **104**, 16868-73.
47. Tsay, J. M., Sippy, J., Feiss, M. & Smith, D. E. (2009). The Q motif of a viral
5 packaging motor governs its force generation and communicates ATP recognition to DNA interaction. *Proc Natl Acad Sci U S A* **106**, 14355-60.
48. Salunke, D. M., Caspar, D. L. & Garcea, R. L. (1989). Polymorphism in the Assembly of Polyomavirus Capsid Protein VP1. *Biophysical Journal* **56**, 887-900.
49. Salunke, D. M., Caspar, D. L. D. & Garcea, R. L. (1986). Self-assembly of purified
10 polyomavirus capsid protein VP1. *Cell* **46**, 895-904.
50. Brady, J. N., Winston, V. D. & Consigli, R. A. (1977). Dissociation of Polyoma Virus by the Chelation of Calcium Ions Found Associated with Purified Virions. *The Journal of Virology* **23**, 717-724.
51. Yanagi, K. & Harada, S. (1989). Destabilization of Herpes Simplex Virus Type 1
15 Virions by Local Anesthetics, Alkaline pH, and Calcium Depletion. *Archives Virology* **108**, 151-159.
52. Sternberg, N. & Weisberg, R. (1977). Packaging of Coliphage Lambda DNA : II. The Role of the Gene D Protein. *Journal of Molecular Biology* **117**, 733-759.
53. Sternberg, N. & Hoess, R. H. (1995). Display of peptides and proteins on the surface
20 of bacteriophage lambda. *Proceedings of the National Academy of Sciences* **92**, 1609-1613.
54. Bode, V. C. & Harrison, D. P. (1973). Distinct Effects of Diamines, Polyamines, and Magnesium ions on the Stability of Lambda Phage Heads. *Biochemistry* **12**, 3193-3196.
- 25 55. Gertsman, I., Gan, L., Guttman, M., Lee, K., Speir, J. A., Duda, R. L., Hendrix, R. W., Komives, E. A. & Johnson, J. E. (2009). An Unexpected Twist in Viral Capsid Maturation. *Nature* **458**, 646-650.
56. Nurmammedov, E., Castelnovo, M., Medina, E., Catalano, C. E. & Evilevitch, A. (2012). Challenging Packaging Limits and Infectivity of Phage Lambda. *Journal of*
30 *Molecular Biology* **415**, 263-273.
57. Fuller, D. N., Rickgauer, J. P., Jardine, P. J., Grimes, S., Anderson, D. L. & Smith, D. E. (2007). Ionic effects on Viral DNA Packaging and Portal Motor Function in

- 47 -

Bacteriophage phi29. *Proceedings of the National Academy of Sciences* **104**, 11245-11250.

58. Yang, Q., Catalano, C. E. & Maluf, N. K. (2009). Kinetic Analysis of the Genome Packaging Reaction in Bacteriophage Lambda. *Biochemistry* **48**, 10705-10715.
- 5 59. Baldwin, R. L. (1986). Temperature Dependence of the Hydrophobic Interaction in Protein Folding. *Proc Natl Acad Sci U S A* **83**, 8069-8072.
60. Makhatadze, G. I. & Privalov, P. L. (1992). Protein Interactions with Urea and Guanidinium Chloride : A Calorimetric Dstudy. *Journal of Molecular Biology* **226**, 491-505.
- 10 61. Schellman, J. A. (1997). Temperature, Stability, and the Hydrophobic Intereaction. *Biophysical J.* **73**, 2960-2964.
62. Myers, J. K., Pace, C. N. & Scholtz, J. M. (1995). Denaturant m values and Heat Capacity Changes: Relation to Changes in Accessible Surface Areas of Protein Unfolding. *Protein Science* **4**, 2138-2148.
- 15 63. Tzlil, S., Kindt, J. T., Gelbart, W. M. & Ben-Shaul, A. (2003). Forces and Pressures in DNA Packaging and Release from Viral Capsids. *Biophysical Journal* **84**, 1616-1627.
64. Evilevitch, A., Lavelle, L., Knobler, C. M., Raspaud, E. & Gelbart, W. M. (2003). Osmotic Pressure Inhibition of DNA Ejection from Phage. *Proceedings of the*
- 20 *National Academy of Sciences* **100**, 9292-9295.
65. Nurmammedov, E., Castelnovo, M., Catalano, C. E. & Evilevitch, A. (2007). Biophysics of viral infectivity: matching genome length with capsid size. *Q Rev Biophys* **40**, 327-56.
66. Yang, F., Forrer, P., Dauter, Z., Conway, J. F., Cheng, N., Cerritelli, M. E., Steven,
- 25 A. C., Pluckthun, A. & Wlodawer, A. (2000). Novel Fold and Capsid-Binding Properties of the Lambda Phage Display Platform Protein gpD. *Nature Structural Biology* **7**, 230-237.
67. Iwai, H., Forrer, P., Pluckthun, A. & Guntert, P. (2005). NMR Solution Structure of the Monomeric Form of the Bacteriophage Lambda Capsid Stabilizing Protein gpD.
- 30 *Journal of Biomolecular NMR* **31**, 351-356.
68. Rao, V. B. & Black, L. W. (1985). DNA packaging of bacteriophage T4 proheads in vitro. Evidence that prohead expansion is not coupled to DNA packaging. *J Mol Biol* **185**, 565-78.

- 48 -

69. Zhang, Z., Kottadiel, V. I., Vafabakhsh, R., Dai, L., Chemla, Y. R., Ha, T. & Rao, V. B. (2011). A Promiscuous DNA Packaging Machine from Bacteriophage T4. *PLoS Biol* **9**, e1000592.
70. Tomka, M. A. & Catalano, C. E. (1993). Physical and kinetic characterization of the DNA packaging enzyme from bacteriophage lambda. *J Biol Chem* **268**, 3056-65.
71. Filutowicz, M., Grimek, H. & Appelt, K. (1996). Purification of the Escherichia coli Integration Host Factor (IHF) in One Chromatographic Step. *Gene* **147**, 149-150.

Example 2

With the development of phage display as a platform for vaccines, viral nanoparticles are transitioning from research tools to therapeutic delivery systems. The popularity of lambda in phage display applications has been due in part to the ease in fusing functional peptides and proteins to the capsid decoration protein gpD. The full potential of the lambda capsid as a therapeutic nanoparticle has yet to be exploited and we are developing bacteriophage lambda for use as a theragnostic nanoparticle system.

To date, all gpD fusion constructs that have been used for phage display applications have been constructed *in vivo*. These systems have thus been limited to peptide and protein fusion constructs expressed within *Escherichia coli* cells in the context of an infectious virus. For instance, a gpD-yellow fluorescence protein fusion construct (gpD-EYFP) expressed from a lambda lysogen *in vivo* affords infectious virus particles decorated with EYFP {Alvarez, 2007 #680}. We have previously demonstrated that purified gpD can be efficiently added to expanded lambda capsids in a defined biochemical reaction mixture *in vitro*. Here we demonstrate that purified gpD-GFP can similarly be used to decorate the lambda capsid *in vitro*. We have further constructed several single-cysteine gpD proteins that allow site-specific modification of the decoration protein with non-proteinaceous ligands. We show that one such construct, gpD(S42C), can be selectively modified with mannose. The resulting synthetic gpD “glycoprotein” is functional in the capsid decoration reaction. Finally, we demonstrate that capsids can be modified with gpD-wild type, gpD-GFP, and gpD-mannose decoration proteins in a defined and tunable surface density. Importantly, the poly-display shell is competent in the DNA packaging reaction *in vitro*, indicating that the capsid can be filled with functional genes as desired. This work sets the stage for the construction of “designer” nanoparticles that can be manufactured to display

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peptides, proteins, carbohydrates, synthetic polymers, and small molecules in a defined composition, symmetrically displayed on the shell surface, and that can carry specific genes for targeted delivery.

We have developed a hybrid theragnostic nanoparticle assembly that harnesses the bacteriophage lambda system for the targeted delivery of drugs and molecular probes. This invention is based on the assembly of engineered viral capsid shells *in vitro* using purified scaffold and major capsid proteins. For delivery applications, DNA is efficiently packaged into the decorated capsids and can be modified to carry specific genes of interest. In addition, the surface of the particle is symmetrically decorated with an external capsid protein that assembles as trimer spikes at the 140 three-fold icosahedral axes. The decoration protein can be specifically conjugated with protein and/or synthetic moieties in defined ratios to enhance cellular targeting/uptake of the particle, to avoid immune surveillance, or alternatively, to enhance immune response to the capsid as a defined antigenic particle. These engineered viral nanoparticles can be tailored in specific ways to afford a delivery vehicle with defined surface characteristics for both diagnostic and therapeutic applications.

Introduction

A brief background of lambda replication relevant to developing a lambda nanoparticle is presented in Figure 7A. The capsid assembly pathway *in vivo* initiates with the assembly of the viral portal protein (gpB) into a dodecameric ring-like structure. The gpNu3 scaffolding protein mediates ring assembly and then chaparones self-assembly of the the major capsid protein (gpE) into an icosahedral shell. This “immature procapsid” contains 420 copies of gpE, a single portal ring situated at a unique vertex, and ~200 copies of gpNu3 inside the shell. A viral protease (gpC) is also incorporated into the immature capsid, which trims 12 residues from the N-terminus of gpB, digests gpNu3 and auto-proteolyzes so that the internal proteins can exit the shell. This affords the “mature procapsid” into which viral DNA is packaged.

Genome packaging is catalyzed by a terminase enzyme. Upon packaging ca. 15 kb duplex DNA, the shell undergoes a remarkable expansion process which results in thinning and increased angularization of the shell and a 2-fold increase in capsid volume. The gpD “decoration” protein is expressed in high-concentration in the phage-infected cell and is a monomer in solution; however, the protein self-assembles as trimer spikes at the 140 three-fold icosahedral axes of the expanded shell (420 copies per shell). GpD stabilizes the shell

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such that it can withstand the tremendous force generated upon packaging the 48.5 kb genome (> 25 atmospheres). Subsequent addition of finishing proteins and a viral tail afford an infectious lambda particle (Figure 7A).

The *in vitro* assembly system described in Example 1 provides an opportunity to
5 decorate the lambda capsid with gpD-modified constructs under defined reaction conditions. Here we describe procedures that allow the assembly of hybrid theragnostic nanoparticles that harnesses the bacteriophage lambda system.

Lambda capsid assembly *in vivo* initiates with the formation of the portal complex, a dodecameric ring through which DNA enters the capsid during packaging and exits during
10 infection of the host. Co-polymerization of the scaffolding protein (gpNu3) and the major capsid protein (gpE) nucleates at the portal, forming an immature procapsid shell composed of 415 copies of gpE (Figure 7A). Within the procapsid interior are ~70-200 copies of gpNu3 and 10 copies of the protease (gpC). Residing at one of the 5-fold vertices of the icosahedral shell is the portal. The immature icoahedral shell is rounded in appearance with
15 a diameter of 50-55 nm. Proteolysis by gpC causes maturation of the procapsid with the removal of gpC and gpNu3 from procapsid interior. Partial cleavage of the portal structure where approximately half of the portal proteins (gpB) is degraded to B* with the cleavage of the 20 N-terminal residues also occurs during procapsid maturation. The terminase complex packages lambda DNA into the capsid interior, triggering expansion of the
20 procapsid to the more angular capsid when approximately 30% is packaged. During expansion gpE undergoes conformational changes, which forms openings in the icosahedral capsid at its 3-fold vertices through which DNA can escape. To stabilize the expanded capsid, 420 copies of the head protein (gpD) add as trimers to the 3-fold vertices of the icosahedral capsid.

Lambda capsid expansion is strictly required for gpD addition. *In vivo* expansion is
25 triggered during DNA packaging. *In vitro* expansion can be triggered by DNA packaging; however, it can also be artificially triggered by the denaturant urea, as described in example 1. The expanded capsids are then decorated with gpD *in vitro*, rendering them stable and biologically functional. Furthermore, tails can be attached to mature capsids forming
30 biologically viable lambda viruses.

Phage display exploits gpD two-fold: its symmetric display and high copy number, at the surface of the icosahedral capsid. However, it is currently limited to proteinaceous ligands decorating an infectious virus since N- and C-terminal gpD fusion proteins are

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expressed from lysogens *in vivo*. Because gpD was be expressed and purified at high concentration and purity for structural determination, we constructed gpD expression vectors to show that gpD can be modified *in vivo* and *in vitro* and that the modified gpD can decorate the capsid surface in combination at defined ratios.

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Materials and Methods

Materials. Terrific broth (Difco), tryptone, yeast extract, ampicillin, urea, and ThermoScientific "Halt"[®] EDTA-free protease inhibitor cocktail (100X) were purchased from Fisher Scientific. Mature λ DNA (*c1857ind 1 Sam 7*) was purchased from Invitrogen. Restriction enzymes were purchased from New England BioLabs. Chromatography media was purchased from GE Healthcare Life Sciences. All other materials were of the highest quality available. Unless otherwise stated, the pH of all buffers was adjusted at 4°C. Cell lysis utilized a Thermo Scientific IEC "French" laboratory press. All protein purifications utilized the Amersham Biosciences ÄKTApurifier™ core 10 System from GE Healthcare and sucrose gradients were prepared on a BioComp 107ip Gradient Master. Protein concentrations were determined spectrally using a ThermoScientific NanoDrop 2000c spectrophotometer.

Construction of pT₇Cap Dam7am43. The plasmid pT₇capDam7am43 expresses the lambda major capsid protein (gpE), the scaffolding protein (gpNu3), the portal protein (gpB) and the capsid protease (gpC), which spontaneously assemble functional procapsids that can be purified in high yield {Yang, 2003 #16}. This vector was constructed by modification of pT₇cap {Yang, 2003 #16} to obviate the expression of gpD; codons 7 (UUU) and 43 (UCC) were mutated to amber stop codons (UAG) using the QuikChange II site-directed mutagenesis kit (Agilent) according to the manufacturers instructions.

Expression and Purification of Lambda Procapsids. Expression of procapsid proteins from pT₇capDam7am43 was performed as previously described for pT₇cap {Yang, 2003 #16} except that Terrific broth was used in place of 2xYT media. Procapsid were purified as described, with minor modification, and unless otherwise indicated all procedures were conducted at 4°C and with ice-cold buffers. Briefly, the cell pellet was resuspended in 50 ml Buffer A (50 mM Tris, pH 8, containing 10 mM MgCl₂, 100 mM NaCl) containing 2 µg/ml DNase I and the cells were lysed by French press (2-3 passages, 650 psi). The lysate was clarified by centrifugation (7,650 x g x 25 minutes) and the procapsids were then harvested by centrifugation (131,453 x g x 3 hours). The procapsids

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were resuspended by overlaying 5 mL Buffer A onto the pellet overnight at 4 °C. The supernatant was aspirated and then diluted with 15 mL TM buffer (50 mM Tris, pH 8, containing 20 mM MgCl₂), loaded onto a HiTrap Q (5 mL) column equilibrated with TM Buffer, and then eluted with a linear gradient to 1 molar NaCl. The procapsid containing
5 fractions (eluting at ~ 100 mM NaCl) were pooled, dialyzed against Buffer A, concentrated in Amicon Ultra-15 Filters (Millipore), applied to a 10-40% sucrose gradient in the same buffer, and centrifuged for 3 hours at 27,000 rpm (SW28 rotor). The procapsid band was visualized in ambient light, harvested by aspiration, and dialyzed against Buffer A. The dialyzed procapsids were then concentrated in Amicon Ultra-15 Filters between 100 and
10 300 nM prior to storage at 4 °C.

Construction of Variant gpD Expression Plasmids. A summary of the proteins described in this work is presented in Figure 2B, each of which was expressed from plasmids constructed as follows. First, vectors that express wild-type gpD protein, without and with a N-terminal hexaHistidine tag (gpD-WT and H6-gpD-WT, respectively) were
15 constructed by PCR amplification of the *D* gene using genomic λ DNA as the template. The primers are described in Table 2 and the plasmids in Table 3. The expected PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega), digested with *NdeI* and *BamHI*, and cloned into similarly digested pET21a (Novagen) to afford plasmids p(D) and p(H6D), both of which served as templates for mutagenesis of serine 42 to
20 cysteine 42 via the QuikChange II site-directed mutagenesis kit (Agilent). The subsequent plasmids expressed mutated gpD proteins (gpD(S42C) and H6-gpD(S42C); Figure 8B).

To generate gpD proteins containing N- and C-terminal linkers terminating with a unique cysteine residue (cys-gpD-H6 and H6-gpD-cys, respectively; Figure 8B), the *D* gene was amplified by PCR using genomic λ DNA as a template and the primers presented in
25 Table 2. The plasmids were constructed in the same manner as those described above.

To construct the plasmid expressing the N-terminal 6-histidine-tagged gpD, the *D* gene with a linker region encoded following its 3'-end was inserted in-frame into the vector pRSET_EmGFP (Invitrogen) between the restriction sites *NdeI* and *NcoI*. The PCR primers are described in Table 2. The plasmid was constructed in a similar manner as those
30 described above.

Multiple cloning sites were added to the 5'- and 3'-end of the *D* gene separately to generate plasmids that will express proteins of choice fused to gpD via linkers at the N- and

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C-termini, respectively. The plasmid was constructed in a similar manner as those described above. The PCR primers are described in **Table 2**.

Table 2. Oligonucleotide primers. Original DNA sequences are underlined. Restriction sites are indicated by italics. Stop codons are in bold.

Primer	Sequence
<i>Mutagenesis</i>	
Dam7 mut for	5'-CGAGCAAAGAAACCTGAACCCATTACC-3' (SEQ ID NO: 11)
Dam7 mut rev	3'-GGTAATGGGTTCAGGTTTCTTTGCTCG-5' (SEQ ID NO: 12)
Dam43 mut for	5'-GCTGATGCTGGACACCTGAAGCCGTAAGCTGGTTGC-3' (SEQ ID NO: 13)
Dam43 mut rev	3'-GCAACCAGCTTACGGCTTCAGGTGTCCAGCATCAGC-5' (SEQ ID NO: 14)
D(S42C) mut for	5'-CCGCTGATGCTGGACACCTGCAGCCGTAAGCTGGTTGC-3' (SEQ ID NO: 15)
D(S42C) mut rev	3'-GCAACCAGCTTACGGCTGCAGGTGTCCAGCATCAGCGG-5' (SEQ ID NO: 16)
<i>Recombination</i>	
D forward	5'-GTGTAAGGGATGCATATGACGAGC-3' (SEQ ID NO: 17)
D-NdeI-BamHI for	5'-CACACCAGTGTAACATATGGGATCCACGAGCAAAGAAACC-3' (SEQ ID NO: 18)
D rev	3'-GTGATGAAGGGGATCCTTAAACGATGC-5' (SEQ ID NO: 19)
H6-D for	5'-CGATTTGCTGAAACATATGCACCATCACCACCAACACAGAGCAAAGAAACC-3' (SEQ ID NO: 20)
D-H6 rev	3'-GCCGCACAGGGATCCTTTAGTGATGATGGTGATGAACGATGCTG-5' (SEQ ID NO: 21)
cys-D for	5'-GCCGTTAACGATCATATGTGCGGATCAGGGTCAGGGAGTGGTAGCACGAGCAAAGAAACC-3' (SEQ ID NO: 22)
D-cys rev	3'-CCCGTAAAAA AAGCCTCGAGTTAGCAACTTCCTGATCCAGAGCCAGATCCAACGATGCTGATTGC-5' (SEQ ID NO: 23)
MCS-D for	5'-GCTGAACACACAGCTAGCGGGGGGACTGCGACGAGCAAAGAAACC-3' (SEQ ID NO: 24)
D-MCS rev	3'-GGCGGCCCTTTAGGCTAGCACTCCAAAGTCCAACGATGCTGATTGC-5' (SEQ ID NO: 25)
D-GFP rev	3'-CCCGTAAAAACCATGCGAGTGCCGCCCTTCCTCCTCCAGAGCCAAGTCCAACGATGCTGATTGCC-5' (SEQ ID NO: 26)

Table 3.

Plasmid	Parental vector	Forward primer	Reverse primer
<i>For gpD expression</i>			
pD	pET21a	D for	D rev
pD(S42C)	pET21a	D(S42C) mut for	D(S42C) mut rev
pcys-D	pET21a	cys-D forw	D rev
pD-cys	pET21a	D-NdeI-BamHI forw	D-cys rev
pMCS-D	pET21a	MCS-D forw	D rev
pD-MCS	pET21a	D forw	D-MCS rev
pH6-gpD	pET21a	H6-D forw	D rev
pH6-gpD(S42C)	pET21a	D(S42C) mut forw	D(S42C) mut rev
pcys-gpD-H6	pET21a	cys-D forw	D-H6 rev
pH6-gpD-cys	pET21a	H6-D forw	D-cys rev
pH6-gpD-DGFP	pRSET_EmGFP	H6-D forw	D-GFP rev
<i>For procapsid expression</i>			
pT7capDam7	pKKT7E	Dam7 mut forw	Dam7 mut rev
pT7capDam7am43	pKKT7E	Dam43 mut forw	Dam43 mut rev

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Purification of the Modified gpD Proteins. Expression of the gpD protein constructs was performed as described for procapsid expression above. For all constructs except gpD-GFP (see below), the induced cells were harvested by centrifugation (6,430 x g x 30 minutes) and the cell pellet was resuspended in Buffer B (20 mM Tris, pH 8, 20 mM NaCl, 0.1 mM EDTA, 1 mM DTT) containing 2 µg DNase I. The cells were lysed by French press (2-3 passages, 650 psi) and the lysate was clarified by centrifugation (7,650 x g x 25 minutes). The supernatant was heated to 50°C for 15 minutes and immediately chilled on ice for 15 minutes; insoluble protein was removed by centrifugation (10,000 x g x 45 minutes). The supernatant was dialyzed against Buffer B at 4 °C overnight, clarified by centrifugation (10,000 x g x 10 min) and then loaded onto a HiTrap Q HP (5 mL) column equilibrated with Buffer B; gpD is not retained by the column, and the flow-through was collected and concentrated using Amicon Ultra-15 Filters. The concentrated protein was dialyzed overnight against Buffer B, clarified by centrifugation (10,000 x g x 10 min), and then loaded onto a Superose 6 10/300 (24 mL) column equilibrated with Buffer B. The gpD-containing fractions were collected, dialyzed overnight against Buffer B, and then stored at 4°C until use.

Storage of gpD(S42C) in buffer containing β-ME resulted in adduction of the reducing agent to the cysteine residue to afford a mixed disulfide that precluded chemical modification with maleimide reagents (data not shown). Therefore, gpD(S42C)-containing fractions eluting from the Superose 6 column above were pooled, dialyzed against Buffer B in the absence of any reducing agent, and TCEP was added to a final concentration of 1 mM prior to storage at 4 °C.

Finally, H₆-gpD-GFP was expressed as described above, except that 20 mM glucose was added to the growth media. The cell pellet was resuspended in Buffer H (20 mM Tris, pH 8, 150 mM NaCl, 0.1 mM EDTA, 7 mM β-ME, 25 mM imidazole) and the cells were disrupted by sonication (two 10 sec bursts separated by a 10-sec break). DNase I (2 µg/ml) and Thermo Scientific "Halt" EDTA-free protease inhibitor cocktail (1X) was added, and the resuspension was incubated on ice for 30 minutes. The mixture was again sonicated (10 x 10 sec pulses separated by 10 sec breaks in-between), and insoluble material was removed by centrifugation (8,000 x g x 10 minutes). The clarified lysate was loaded onto a HisTrap FF (5 mL) column equilibrated with Buffer H, and the proteins were eluted with a 10-column volume gradient to 500 mM imidazole. The H₆-gpD-GFP-containing fractions

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(bright green in color) were dialyzed overnight against Buffer H (minus imidazole), concentrated using an Amicon Ultra-0.5 filter and stored at 4 °C until use.

Synthesis of 6'-Maleimidohexanamido-polyethyleneglycol mannoside. A solution of 2-(2-(2-(Amido)ethoxy-ethoxy)ethyl-O- α -D-mannoside (20 mg, 6.4×10^{-2} mmol) in methanol (1 ml) was added to a solution of 6-maleimidohexanoic acid N-hydroxysuccinimide ester (25 mg, 7.7×10^{-2} mmol) in methanol (0.5 ml). The reaction mixture was stirred at RT for 2 hrs. The desired product was obtained after removal of solvent under reduced pressure following purification by silica column chromatography. The identity of product was confirmed by mass spectrometry and by ¹H and ¹³C NMR.

Conjugation and purification of Mannose-conjugated gpD(S42C). The H6-gpD(S42C) stock solution was dialyzed into PBS buffer (1 ml) to which a solution of maleimide-activated mannose in 50 mM PBS buffer, pH 6.6 (800 μ L), was added. The reaction mixture was stirred at RT for 48 hours and then loaded onto a HiTrap Con A (1 ml) column equilibrated with 20 mM Tris buffer, pH 7.4 (4 °C), containing 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂. The protein was eluted over a 0-100% gradient to Buffer X (20 mM Tris buffer, pH 6.4 (4 °C), containing 0.2 M methyl- α -D-mannopyranoside and 0.5 M NaCl). The gpD(S42C::sMannose)-containing fractions were pooled and dialyzed against 50 mM Tris buffer, pH 8, containing 150 mM NaCl, concentrated using an Amicon Ultra-0.5 filter and stored at 4 °C until further use. The expected product was confirmed by mass spectrometry.

Procapsid Expansion. Expanded capsid shells were prepared as previously described in example 1, with modification. Briefly, purified procapsids and a freshly prepared stock of 6 M urea were mixed to afford a reaction mixture (20 μ L) containing 40 nM procapsids in 10 mM Tris buffer, pH 8, containing 2.5 M urea and 1 mM MgCl₂. The reaction was allowed to proceed for 60 min on ice, and the expanded capsids were then buffer exchanged into TMN buffer (10 mM Tris buffer, pH 8, 1 mM MgCl₂) using an Amicon Ultra-0.5 Filter (Millipore).

Decoration of Expanded Capsids with gpD Proteins. Unless otherwise indicated, purified decoration proteins were added to expanded capsids to afford a reaction mixture containing 40 nM capsids and 50 μ M decoration protein in TMN buffer. The reaction mixture was incubated at room temperature for 60 min and the capsids analyzed by agarose gel assay (below). When required, unincorporated decoration proteins were removed prior

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to analysis either by Superose 6 column chromatography or by buffer exchange using an Amicon Ultra0-0.5 Filter (Millipore).

Capsid Decoration Assay. Reaction mixtures were applied to a 1.2 % agarose gel, and the capsids fractionated by electrophoresis at 100 V for 180 minutes. The expanded
5 capsid and gpD-decorated capsid bands were visualized by Coomassie brilliant blue stain. Video images of the destained gels were captured using an EpiChemi darkroom system with a Hamamatsu camera (UVP Bioimaging Systems), and quantitation of the bands was performed using the ImageQuant[®] data analysis package (Molecular Dynamics)

Electron microscopy. Samples were applied to 300 mesh carbon coated copper grids
10 (Electron Microscopy Sciences) treated by negative glow discharge prior to sample application. Staining was achieved using Nano-W (Nanoprobes) according to manufacturer protocols. Bright field transmission electron microscopy was performed at the NanoTech User Facility at the University of Washington on a FEI Tecnai G2 F20 S-Twin TEM.

Genome Packaging Assay. The genome packaging assay was performed as
15 described in example 1, with modification. Briefly, decorated and expanded capsids at a final concentration of 15 nM were added to a reaction mixture composed of 50 mM Tris buffer, pH 8, containing 1.5 mM NaCl, 10 mM MgCl₂, 2 mM spermidine, 0.42 mM β-ME, 1 mM ATP, 50 nM IHF, and 2 nM full-length λ DNA. The packaging reaction (20 μL) was initiated with the addition of terminase to a final concentration of 100 nM and allowed to
20 incubate for 30 minutes at room temperature. DNase I was then added to a final concentration of 10 μg/mL and incubated for 5 minutes before the reaction was stopped with the addition of phenol:chloroform (21 μL). The aqueous layer was removed and loaded onto a 0.8% agarose gel. The DNase-resistant (packaged) DNA was quantified using video densitometry and ImageQuant (Molecular Dynamics) software package.

Agglutination Assay. Decorated capsids (1 μM gpD equivalent) were added to 10 mM HEPES buffered saline solution containing 150 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂. Concanavalin A (ConA, 1 μM) was then added to initiate the reaction and agglutination was detected by monitoring the increase in absorbance (350 nm) at 1- minute intervals. α-D-mannose was added to a final concentration of 5 mM after 20- minutes
30 incubation time to competitively displace the sMannose.

Results

The ability to decorate lambda capsids with modified gpD constructs provides an attractive approach to develop “designer” nanoparticles of defined composition and multi-partite, symmetric presentation. Towards this end, we first constructed a plasmid that expresses a HIS-tagged protein composed of an N-terminal gpD domain fused to a C-terminal green fluorescent protein domain (gpD-GFP; Figure 8B). This protein is analogous to the previously described gpD-EYFP fusion protein used to decorate phage particles *in vivo* (Alvarez et al., 2006). GpD-GFP can be purified to homogeneity and in high yield.

Decoration of Lambda Capsids with gpD-Green Fluorescent Protein in vitro. The decoration of expanded lambda capsids with wild-type gpD (gpD-WT) can be conveniently monitored *in vitro* using an agarose gel assay as described in example 1. Here we utilize this assay to determine whether gpD-GFP can similarly be added to the expanded shell *in vitro*. In this case, the decorated capsids are visualized in two ways; first by Coomassie blue staining for total protein content (Figure 9A) and second by fluorescence imaging for the presence of GFP (Figure 9B). As previously demonstrated, capsids decorated with gpD-WT migrate as a distinct, higher mobility band in the agarose gel. In the presence of increasing amounts of gpD-GFP, progressively slower migrating species appear, consistent with decoration with increasing density of gpD-GFP (Figures 9A, 9B). At maximal gpD-GFP densities, two distinct bands are apparent in the gel indicating that two populations are present. Exactly what these two species represent is unclear, but we presume that the upper and lower bands are partially vs. completely decorated capsid shells, respectively.

To confirm that the gpD proteins have in fact added to the capsid shell, the capsids were separated from unreacted material using centrifugal filters and the protein content of the decorated shells was examined by SDS-PAGE; the data clearly indicate that both proteins stably add to the capsid (Figure 9C). Importantly, the surface density of both gpD-WT and gpD-GFP can be tuned by adjusting the ratio of these two proteins included in the reaction mixture. In the presence of gpD-WT alone, the ratio of major capsid protein (gpE) to gpD-WT in the decorated particles is ~ 1:1, as anticipated for a fully decorated shell capsid (Figure 7, Table 4). As the amount of gpD-GFP is increased in the reaction mixture, the density of the modified protein on the decorated particles increases to an average surface density of ~ 77% (Figure 8C; Table 4). Particle decoration does not appear to be linear, suggesting that gpD-WT outcompetes gpD-GFP for addition to the capsid surface (data not shown). This is not surprising given that the GFP domain (26.9 kDa) adds significant bulk

to the small gpD polypeptide (11.4 kDa), which likely interferes with the assembly of the trimeric spikes at the capsid surface (see Figure 8A).

Table 4. Decoration of Lambda Capsids by gpD Variants

Variant	Surface Density (% relative to gpD)
gpD-WT	100
gpD(S42C)	90 ± 16
gpD(S42C::sMannose)	82 ± 12
H ₆ -gpD-GFP	77 ± 14

5

Construction of gpD-Vectors for Diverse Phage Display Applications. The lambda gpD protein has been used in a variety of phage display applications {Beghetto, 2011 #688}. To date, the gpD fusion constructs have appended peptides to either the N-terminus or C-terminus of the protein and decorated phages have been assembled *in vivo* from the induction of the modified lambda lysogens. While useful, this approach has several limitations. First, display *in vivo* restricts potential ligands to those that do not affect the development of viable viral particles. It is quite likely that many desired ligands will in fact adversely affect the yield of infectious virus. Second, the current phage display systems require construction of modified lambda genomes and induction of lambda lysogens *in vivo* to afford the modified particle. This approach is laborious and is ultimately controlled by the whims of the *E. coli* cell, which does not allow facile manipulation shell decoration density. We reasoned that the *in vitro* gpD-decoration procedure described above could be adapted for the display of any peptide/protein on the capsid surface and at defined surface densities. Towards this end we constructed a vector that possesses a multi-cloning site immediately downstream from the *D* gene (gpD-MCS, Figure 8B), which allows facile expression of C-terminal tagged gpD-fusion proteins that can be used to decorate the lambda capsid *in vitro*, as described for gpD-GFP above. We also constructed an analogous vector that allows facile generation of N-terminal tagged fusion proteins (MCS-gpD). These vectors allow rapid and efficient expression of gpD-fusion proteins that can be used to decorate the lambda capsid *in vitro* with total control of surface display density.

A third major limitation to all current phage display platforms including lambda is that the systems are limited to proteinaceous tags. To address this limitation, we next constructed vectors that express gpD containing a unique cysteine residue either at the N-terminus (Cys-gpD-H₆) or the C-terminus (H₆-gpD-Cys) of the protein (Figure 8B). The

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purified proteins provide scaffolds that can be site-specifically modified with a variety of ligands, not limited to peptides, using simple maleimide chemistry. This is discussed further below.

A final limitation of current phage display systems centers on the location of the N- and C-termini of gpD bound to the capsid surface. GpD is a monomer in solution but adds to the 140 three-fold axes of the icosahedral shell as a trimeric spike (Figures 7, 8A). The N-terminus of each subunit directly interacts with the capsid shell to provide stabilizing contacts required for shell integrity {Lander, 2008 #351}, while the C-termini exit the gpD trimer spike proximate to the shell surface (Figure 8A) {Lander, 2008 #351}. In both cases, appending bulky ligands could easily hinder gpD trimer assembly and interfere with its ability to stabilize the DNA-filled shell. Indeed, this was observed with phage decorated with various GFP variants *in vivo* {Alvarez, 2007 #680; Zeng, 2011 #681; Nicastro et al., 2013} and is consistent with the incomplete decoration observed with gpD-GFP *in vitro* (see Figure 9A).

The crystal structure of the gpD trimer spike reveals that Ser42 is positioned at the apex of the spike in all three subunits (Figure 8A). Modification of this residue would place the desired tag projecting away from the capsid surface and into solution for optimal display, and with minimal insult to gpD spike assembly and shell integrity. We therefore constructed vectors (with and without N-terminal histidine tags) that express gpD in which Ser42 has been changed to Cys42 (H₆-gpD(S42C) and gpD(S42C), respectively; Figure 8B). Importantly, there are no other cysteine residues in the native protein and this provides a unique site for reaction with maleimide-based tags.

Chemical Synthesis of gpD(S42C::sMannose). Cysteine-modified gpD constructs provide proteins that can be efficiently and specifically modified using maleimide chemistry. To demonstrate the utility of this approach, we synthesized 6'-maleimido-hexan-amido-polyethyleneglycol mannoside (sMannose) as described in Materials and Methods. This was used to chemically modify gpD(S42C) to afford the gpD(S42C::sMannose) glycoprotein, which was purified to homogeneity.

We next examined capsid decoration by gpD(S42C::sMannose). As anticipated, gpD-WT adds to the expanded capsids to afford a unique band of higher mobility in the agarose gel compared to unexpanded (undecorated) procapsids (Figure 10A). Increasing the fraction of gpD(S42C::sMannose) included in the reaction mixture results in gradual retardation of capsid migration in the gel, consistent with progressive decoration by the gpD

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glycoprotein. To confirm this presumption, the capsids were separated from unreacted material and the protein content of the decorated capsids analyzed by SDS-PAGE. Unlike gpD-GFP, the density of gpD(S42C::sMannose) on the decorated particles increases in a linear fashion (data not shown) and to a higher average surface density of ~ 82% (Figure 10B, Table 4).

The Lambda Capsid Can Be Decorated with Multiple Tags in a Defined

Composition. As demonstrated above, modified gpD constructs can be used to decorate the lambda capsid with a large protein (GFP) and with a mannose glycoprotein. Here we demonstrate that the shell can be decorated with multiple ligands simultaneously. Lambda capsids were incubated with gpD-WT, gpD(S42C::sMannose), and gpD-GFP alone or in combination. The reaction mixture was analyzed by agarose gel assay, which clearly demonstrates that the proteins, alone and in combination, add to the capsid shell to afford decorated particles with a unique mobility in the gel (Figures 11A, 11B). Analysis of the isolated, decorated shells by SDS-PAGE demonstrates that (i) each construct efficiently adds to the shell and that (ii) all three proteins can add to the capsid when simultaneously present in the reaction mixture (Figure 11C). Within this context, we note that gpD-WT and the gpD(S42C::sMannose) glycoprotein migrate closely in SDS-PAGE. In order to confirm the presence of both proteins on the decorated capsid surface, we utilized an agglutination assay. In this assay, the presence of mannose is detected by agglutination of the particles by with Concanavalin (ConA). As expected, neither gpD-WT nor gpD-S42C decorated capsids exhibit a positive agglutination response, while gpD-GFP decorated capsids exhibit a weak (non-specific) agglutination response (Figure 5D). In contrast, capsids decorated with gpD(S42C::sMannose) show a strong response indicating (i) mannose is present on the capsid surface and (ii) it is efficiently displayed for interaction with ConA.

Structural Characterization of the Decorated Lambda Capsids. A major advantage of utilizing viral capsids as display platforms is that under ideal conditions they can display the tags in a symmetric and defined manner. In the case of the gpD constructs discussed here, tags of known composition can be appended to the display protein in a site-specific manner, which can then be presented as trimers at the three-fold icosahedral axes of the capsid shell. While it is clear that our modified gpD constructs efficiently bind to the lambda capsid, confirmation that the shells remain intact for optimal display of the tags is important. We therefore utilized electron microscopy to examine the decorated shells as described in Materials and Methods. Capsids decorated with gpD-WT are composed of an

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intact icosahedral shell with thinned walls and significant angular facets (Figure 12), as observed with an infectious lambda virus {Dokland, 1993 #414; Lander, 2008 #351}. The gpD(S42C) decorated capsids are similar in morphology, consistent with the conservative mutation in the protein and their near-native gel migration in the agarose gel (Figure 11A).

5 Capsids decorated with the gpD(S42C::sMannose) glycoprotein similarly display a thin and angularized shell, though perhaps not as faceted as wild-type decorated shells (Figure 12). The gpD-GFP decorated shells also possess the thin wall phenotype and similarly appear less faceted than wild type. In addition, the shells have an extra density on the exterior shell surface, which we presume reflects the presence of the large GFP domain appended to the capsid surface (Figure 12). Decorated capsids isolated from reaction mixtures containing
10 gpD-WT, gpD(S42C::sMannose), and gpD-GFP in a 40:40:20 ratio also possess extra staining density at the capsid shell, though not as prominent as those fully-decorated with gpD-GFP; this is consistent with the lower surface density of gpD-GFP on these particles (Figure 11A).

15 *Functional Characterization of the Decorated Lambda Capsids.* Procapsids serve as receptacles for the viral genome, and gpD is required to provide structural integrity to the DNA-filled shell (Figure 7) {Yang, 2008 #}. In the absence of gpD, the capsid cannot withstand the intense pressures generated by the packaged DNA (> 50 atmospheres) and the shell fractures, rendering the DNA accessible to DNase digestion {Yang, 2008 #1}. Here
20 we utilize an *in vitro* DNA packaging assay to determine if capsids decorated with modified gpD proteins are functionally active in capsid shell stabilization.

The genome packaging reaction was performed as described in Materials and Methods using capsids decorated with either gpD-WT, gpD(S42C), gpD(S42C::sMannose), or gpD-GFP. As shown in Figure 7A, gpD(S42C) and the gpD(S42C::sMannose)
25 glycoprotein are as efficient as wild-type gpD in the DNA packaging reaction. In contrast, capsids decorated with H₆-gpD-GFP are seriously compromised when the surface density of the protein exceeds 50% (Figure 7B). Thus, while the GFP-modified protein can fully decorate the expanded shell (Table 4), it does not necessarily impart the requisite structural integrity required to retain a fully-packaged genome. This is consistent with the observation
30 that infectious lambda virus decorated with gpD-GFP variants are less stable compared to wild-type virus *in vivo* (Alvarez et al., 2006, Nicastro et al., 2013).

Discussion

Decoration of the lambda capsid is a powerful tool in the development of nanoparticles for theragnostic applications. Its relative ease aids in the modification of the capsid, allowing the display of tags in a defined and tunable manner. We have developed an *in vitro* decoration system in which lambda capsids are decorated with non-proteinaceous ligand conjugated to gpD. Validation of the system was first confirmed using the modified gpD fusion protein, gpD-GFP.

Lambda capsids can be decorated *in vitro* with gpD-GFP. Characterization of the gpD-GFP *in vitro* decorated capsids is consistent with the gpD-EYFP and gpD-GFP *in vivo* decorated viruses (Alvarez et al., 2008; Nicastro et al., 2013). The added bulk of GFP affects the stability of both the *in vivo* and *in vitro* decorated capsids. Because the C-terminus of gpD lies near the capsid surface (Chang et al., 2004; Lander et al., 2008), gpD-GFP may interfere with the addition of the gpD trimers to the capsid surface, hence, the two distinct populations of gpD-GFP decorated capsids visualized by agarose gel analysis when only gpD-GFP is present. Representation of the gpD-GFP decorated capsids on SDS-PAGE is an average of the two populations. The exact coverage for each population is unknown, but we surmise that one population has a higher surface density than the other. Despite this, gpD-GFP is ineffective in stabilizing capsids to the same extent as the other gpD variants. The added bulk of GFP to the C-terminus of gpD most likely interferes with the ability of gpD to stabilize the capsid. The apical gpD residue 42 is an ideal candidate for conjugation.

The advent of gpD(S42C) (and similar gpD functional mutants) and the capacity of the system to be tunable circumvents the issues of capsid stability. Modification of the apical residue S42C does not decrease capsid stability to the same extent as modifications to either the N- or C-termini of gpD. Addition of gpD(S42C::sMannose) to the capsid, although not as complete as gpD-WT, does provide sufficient capsid stability. This suggests that full coverage of the capsid shell by gpD may not be a strict requirement for packing competency. About 80% coverage appears to be the minimum required assuming that the modification tag does not interfere with structural stability of the capsid, as the case with gpD-GFP but not gpD(S42C::sMannose). Added bulk to the structurally compact gpD-WT at the apical residue 42 may lower efficiency of trimer formation depending on the size of the conjugant. This may be avoided with the initial addition of gpD-WT at a partial complement. Although how the gpD variants are participating in trimer formation is

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unclear, gpD-WT stabilizes the capsid structure by limiting the saturation of the modified gpD variant and its potential hazard of capsid instability either within in each trimer or between trimers.

The range of capsid decoration is limited by the initial concentrations of the gpD variants thereby allowing tunability. Capsids decorated with gpD-WT and gpD-GFP appear as diffuse bands on agarose gels compared to capsids decorated with a single variant (Figures 9A, 11A), suggesting that the gpD-WT is masking the partiality of gpD-GFP. Capsids decorated with gpD-WT and gpD(S42C::sMannose) appear as discrete bands with increasing concentrations of gpD(S42C::sMannose) (Figure 10A). Although the size disparity between the two variants is minimal by comparison, the structural difference between gpD-WT and gpD(S42C::sMannose) is at an apical residue, not at a region involved in capsid stability like gpD-GFP. Decoration of the capsid by gpD(S42C::sMannose) is most likely more homogenous compared to that by gpD-GFP. Capsids decorated with all three gpD variants appear as a diffuse band on agarose gel (Figure 11A), implicating gpD-GFP as the factor causing heterogeneity in capsid decoration. Because gpD-GFP is initially limited, its role in decoration is depressed but not suppressed. Our system's tunability is advantageous, allowing the inclusion of proteinaceous and non-proteinaceous ligands that would otherwise be disadvantageous to capsid stability.

One potential downstream application of the *in vitro* decorated capsids would be their involvement *in vitro* assembled viruses. We have previously developed an assay for assembling via lambda viruses *in vitro* (Gaussier et al., 2006). Genetically modified DNA can be packaged into *in vitro* decorated capsids. The attachment of tails to the mature capsid prevents exit of the packaged DNA as well as produces a carrier for DNA which can be targeted to a specific cell type, for example, via a non-proteinaceous modification conjugated to gpD. We have utilized our *in vitro* capsid assembly system to decorate lambda capsids with proteinaceous and non-proteinaceous modification. The ability to tune the decoration of lambda capsids *in vitro* obviates possible capsid instability of bulky modifications but not limiting what can be added to the capsid surface. This expands the utility of the capsid display system.

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References for Example 2

1. Alvarez, L. J., Thomen, P., Makushok, T., and Chatenay, D. (2007) Propagation of Fluorescent Viruses in Growing Plaques., *Biotechnol. Bioeng.* 96, 615-621.
2. Nicastro, J., Sheldon, K., El-zarkout, F. A., Sokolenko, S., Aucoin, M. G., and Slavcev, R. (2013) Construction and Analysis of a Genetically Tuneable Lytic Phage Display System, *App. Microbiol. Biotechnol.*
3. Yang, Q., and Catalano, C. E. (2003) Biochemical characterization of bacteriophage lambda genome packaging in vitro, *Virology* 305, 276-287.
4. Gaussier, H., Yang, Q., and Catalano, C. E. (2006) Building a virus from scratch: assembly of an infectious virus using purified components in a rigorously defined biochemical assay system, *J Mol Biol* 357, 1154-1166.
5. Medina, E., Nakatani, E., Kruse, S., and Catalano, C. E. (2012) Thermodynamic Characterization of Viral Procapsid Expansion into a Functional Capsid Shell, *J. Mol. Biol.* 418, 167-180.
6. Beghetto, E., and Gargano, N. (2011) Lambda Display: A Powerful Tool for Antigen Discovery, *Molecules* 16, 3089-3105.
7. Lander, G. C., Evilevitch, A., Jeembaeva, M., Potter, C. S., Carragher, B., and Johnson, J. E. (2008) Bacteriophage Lambda Stabilization by Auxiliary Protein gpD: Timing, Location, and Mechanism of Attachment Determined by Cryo-EM, *Structure* 16, 1399-1406.
8. Zeng, L., and Golding, I. (2011) Following Cell-fate in E. coli After Infection by Phage Lambda, *Jornal of Visualized Experiments* 56, 3363.
9. Dokland, T., and Murialdo, H. (1993) Structural Transitions During Maturation of Bacteriophage Lambda Capsids, *J. Mol. Biol.* 233, 682-694.
10. Yang, Q., Maluf, N. K., and Catalano, C. E. (2008) Packaging of a Unit-Length Viral Genome: The Role of Nucleotides and the gpD Decoration Protein in Stable Nucleocapsid Assembly in Bacteriophage Lambda, *Journal of Molecular Biology* 383, 1037-1048.

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We claim

1. A theragnostic particle, comprising a plurality of engineered decoration proteins bound to an outer surface of decoration competent viral particle shell, wherein the engineered decoration proteins comprise decoration proteins linked to one or more
5 compound not naturally occurring on a wild type viral capsid, and wherein the one or more compounds have at least one feature selected from the group consisting of:
 - (a) the one or more compounds are non-proteinaceous compounds;
 - (b) the one or more compounds are present on the theragnostic particle in a defined ratio relative to the engineered decoration protein;
 - 10 (c) the one or more compounds comprise two or more different compounds, wherein the two or more different compounds are present on the theragnostic particle in a defined ratio relative to each other; and
 - (d) the one or more compounds are linked to the engineered decoration protein at a site on the engineered decoration protein other than the N-terminus or the C-terminus.
- 15 2. The theragnostic particle of claim 1, wherein the one or more compounds are non-proteinaceous compounds.
3. The theragnostic particle of claim 1 or 2 wherein the one or more compounds are present on the theragnostic particle in a defined ratio relative to the engineered decoration protein.
- 20 4. The theragnostic particle of any one of claims 1-3, wherein the one or more compounds comprise two or more different compounds, wherein the two or more different compounds are present on the theragnostic particle in a defined ratio relative to each other.
5. The theragnostic particle of any one of claims 1-4, wherein the one or more compounds are linked to the engineered decoration protein at a site on the engineered
25 decoration protein other than the N-terminus or the C-terminus.
6. The theragnostic particle of any one of claims 1 and 3-5, wherein the engineered decoration proteins comprise engineered decoration proteins linked to one or more compound selected from the group consisting of nucleic acids, lipids, carbohydrates, polypeptides, polymers, organic molecules, inorganic molecules, and combinations thereof.
- 30 7. The theragnostic particle of any one of claims 1-5, wherein the engineered decoration proteins comprise engineered decoration proteins linked to one or more non-proteinaceous compound selected from the group consisting of nucleic acids, lipids, carbohydrates, polymers, organic molecules, inorganic, and combinations thereof.

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8. The theragnostic particle of any one of claims 1-7, wherein the one or more compounds are selected from the group consisting of therapeutic compounds, diagnostic compounds, adjuvants, antigens, antibodies, and combinations thereof.

9. The theragnostic particle of any one of claims 1-8, wherein the one or more
5 compounds linked to the decoration proteins comprise two or more different compounds.

10. The theragnostic particle of any one of claims 1-9, wherein the decoration competent viral particle shell is an expanded viral particle shell.

11. The theragnostic particle of any one of claims 1-10 wherein the decoration
10 competent viral particle shell is a bacteriophage lambda, bacteriophage T4, bacteriophage L, bacteriophage P22, bacteriophage 21, bacteriophage P4, herpesvirus, or adenovirus decoration competent viral particle shell.

12. The theragnostic particle of claim 10, wherein the expanded viral particle shell is an expanded bacteriophage lambda particle shell, and where the engineered decoration proteins are gpD proteins, functional mutants thereof, or combinations thereof.

15 13. The theragnostic particle of claim 12, wherein the gpD proteins, functional mutants thereof, or combinations thereof comprise gpD S42C proteins.

14. The theragnostic particle of claim 12, wherein the gpD proteins, functional mutants thereof, or combinations thereof comprise gpD functional mutations with an amino acid substitution at one or more of residues Thr1 Ser2, Lys3, Glu4, Thr5, Phe6, Thr7, His8,
20 Tyr9, Gln10, Pro11, Gln12, Gly13, Asn14, Ser15; Gly25, Gly26, Leu27, Ser28, Ala29, Lys30, Ala31; Asp40, Thr41, Ser42, Ser43Arg44, Lys45; Asp49, Gly50, Thr51, Thr52, Asp53; Asp66, Gln67, Thr68, Ser69, Thr70, Thr71; Arg81, Tyr82, Glu83, Asp84; Glu89, Ala90, Ala91, Ser92, Asp93, Glu94, Thr95, Lys96, Lys97, Arg98, and Thr99.

15. The theragonistic particle of claim 14, wherein the amino acid substitution
25 comprises substituting a cysteine or azidohomoalanine residue for the wild type residue.

16. A pharmaceutical composition, comprising:

- (a) the theragnostic particle of any one of claims 1-15; and
- (b) a pharmaceutically acceptable carrier.

17. An isolated recombinant protein comprising the amino acid sequence of SEQ ID
30 NO: 10 (gpD(S42C)).

18. The isolated recombinant protein of claim 17, further comprising one or more compounds linked to the recombinant protein via the cysteine at position 42.

19. An isolated nucleic acid encoding the recombinant protein of claim 17.

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20. A recombinant expression vector comprising the isolated nucleic acid of claim 19.
21. An isolated host cell, comprising the recombinant expression vector of claim 20.
22. An *in vitro* method for preparing a theragnostic particle, comprising
decorating a decoration competent viral particle shell *in vitro* with a defined amount of
5 engineered decoration proteins linked to one or more compounds, wherein the engineered
decoration proteins stabilize the decoration competent viral particle shell to produce a
theragnostic particle.
23. The method of claim 22, wherein the decorating comprises decorating the viral
particle shells *in vitro* with a stoichiometric ratio of engineered decoration proteins.
- 10 24. The method of claim 22 or 23, wherein the decoration competent viral particle shell
is an expanded viral particle shell, and wherein the method comprises expanding the viral
particle by contacting it with an expansion agent to produce the expanded viral particle
shell.
25. The method of claim 24, wherein the expansion agent is selected from the group
15 consisting of chaotropic agents, pH, heat, and combinations thereof.
26. The method of claim 25, wherein the expansion agent is urea.
27. The method of any one of claims 22-26, wherein the one or more compounds are
selected from the group consisting of nucleic acids, lipids, carbohydrates, polypeptides,
polymers, organic molecules, inorganic molecules, and combinations thereof.
- 20 28. The method of any one of claims 22-27, wherein the one or more compounds are
one or more non-proteinaceous compound.
29. The method of claim 28, wherein the one or more non-proteinaceous compound is
selected from the group consisting of nucleic acids, lipids, carbohydrates, polymers, organic
molecules, inorganic molecules, and combinations thereof.
- 25 30. The method of any one of claims 22-29, wherein the one or more compounds
comprise two or more different compounds, wherein the two or more different compounds
are present on the theragnostic particle in a defined ratio relative to each other.
31. The method of any one of claims 22-30, wherein the one or more compounds are
selected from the group consisting of therapeutic compounds, diagnostic compounds,
30 adjuvants, antigens, antibodies, and combinations thereof.
32. The method of any one of claims 22-31, wherein the decoration competent viral
particle shell is an decoration competent bacteriophage lambda, bacteriophage T4,

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bacteriophage T4, bacteriophage P22, bacteriophage 21, bacteriophage L, bacteriophage P4, herpesvirus, or adenovirus viral particle shell.

33. The method of any one of claims 22-32, wherein the decoration competent viral particle shell is an expanded bacteriophage lambda particle shell, and where the engineered
5 decoration proteins are gpD proteins, functional mutants thereof, or combinations thereof.

34. The method of claim 33, wherein the gpD proteins, functional mutants thereof, or combinations thereof comprise gpD S42C proteins.

35. The method of claim 33, wherein the gpD proteins, functional mutants thereof, or combinations thereof comprise gpD functional mutations with an amino acid substitution at
10 one or more of residues Thr1, Ser2, Lys3, Glu4, Thr5, Phe6, Thr7, His8, Tyr9, Gln10, Pro11, Gln12, Gly13, Asn14, Ser15; Gly25, Gly26, Leu27, Ser28, Ala29, Lys30, Ala31; Asp40, Thr41, Ser42, Ser43Arg44, Lys45; Asp49, Gly50, Thr51, Thr52, Asp53; Asp66, Gln67, Thr68, Ser69, Thr70, Thr71; Arg81, Tyr82, Glu83, Asp84; Glu89, Ala90, Ala91, Ser92, Asp93, Glu94, Thr95, Lys96, Lys97, Arg98, and Thr99.

15 36. The method of claim 35, wherein the amino acid substitution comprises substituting a cysteine or azidohomoalanine residue for the wild type residue.

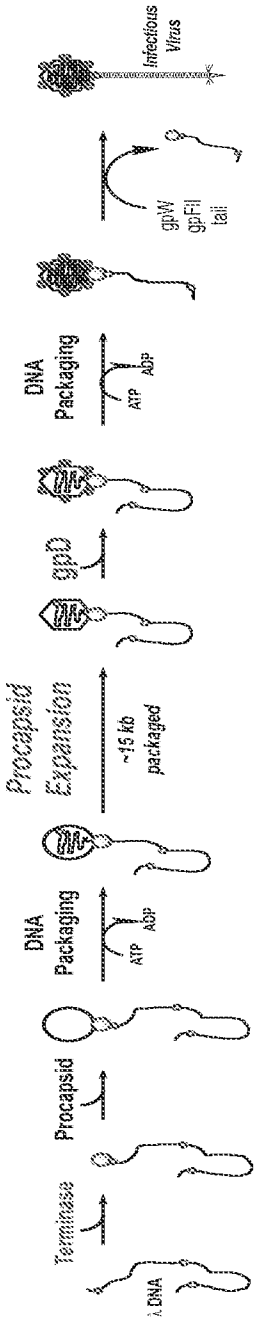
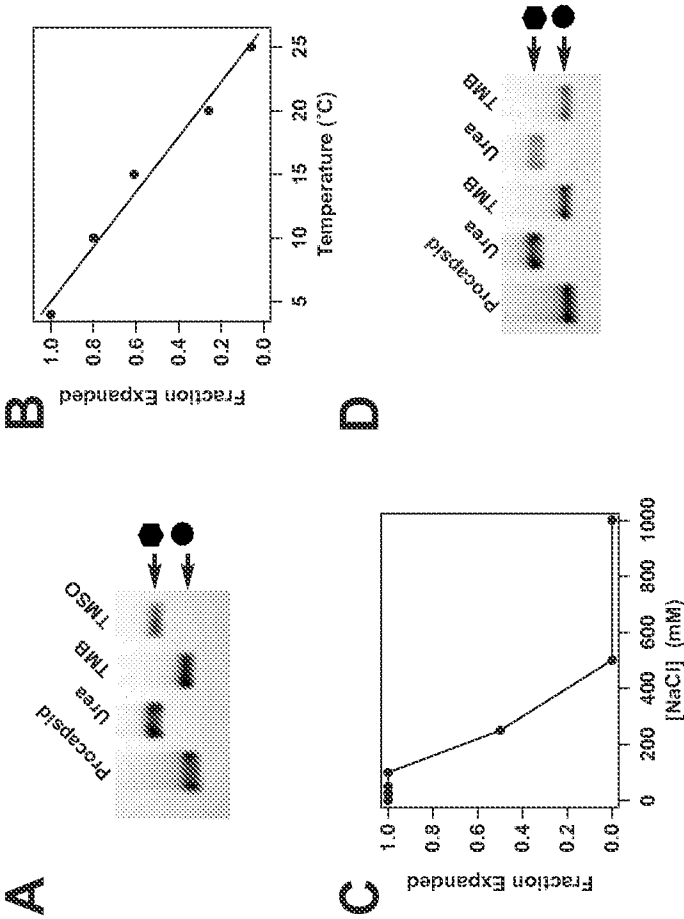


Figure 1

Figure 2



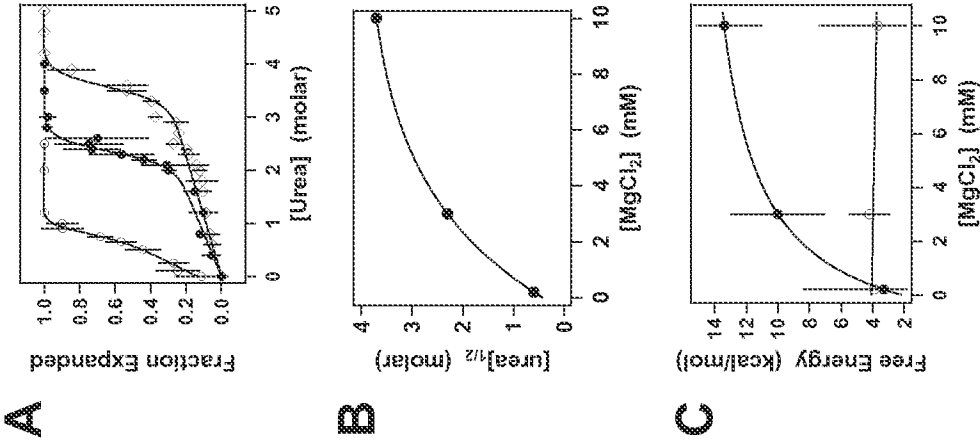


Figure 3

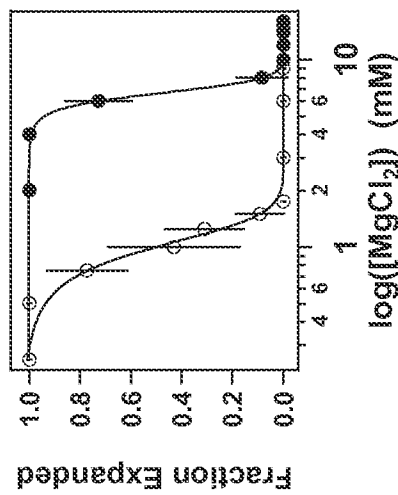


Figure 4

Figure 5

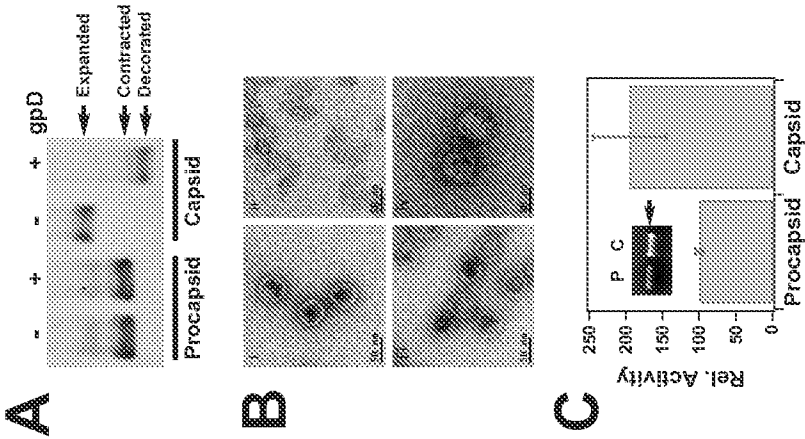


Figure 6

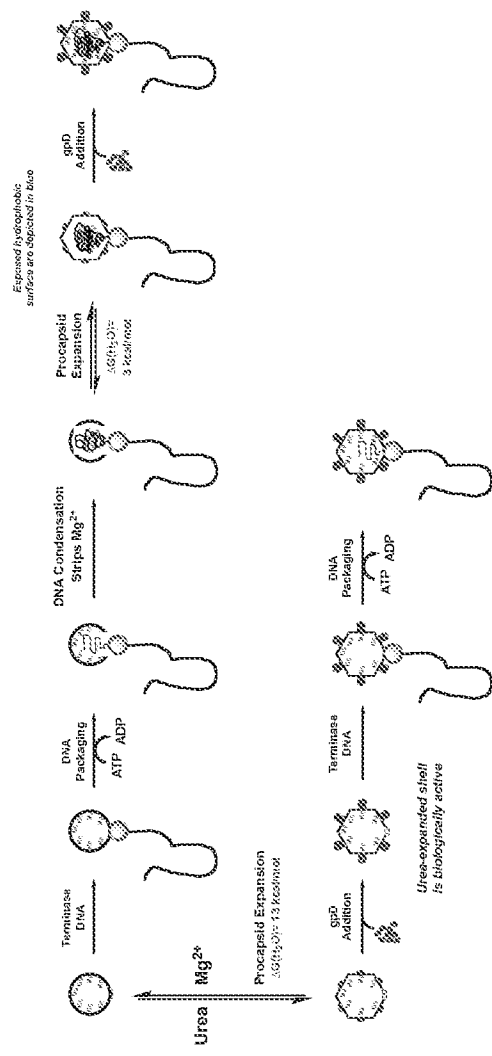
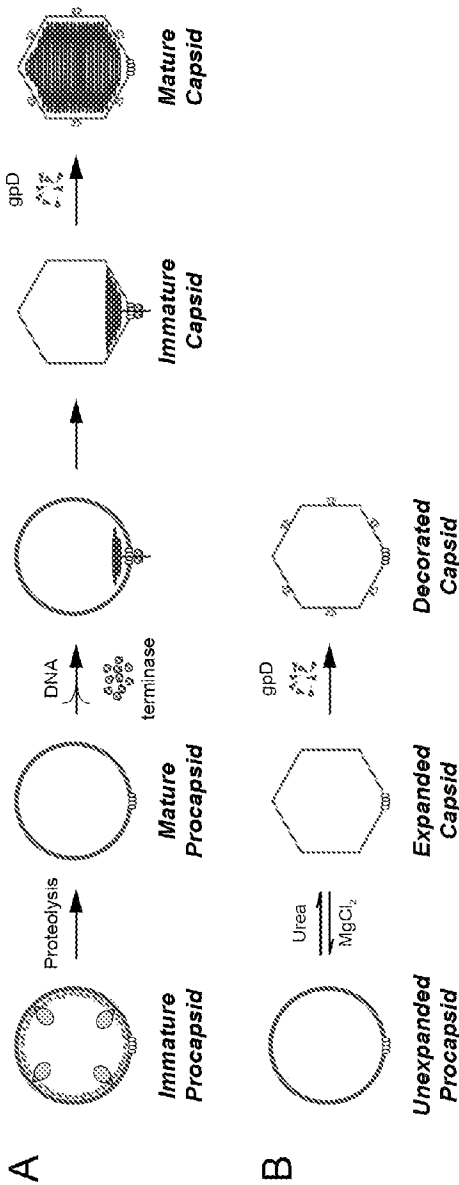


Figure 7



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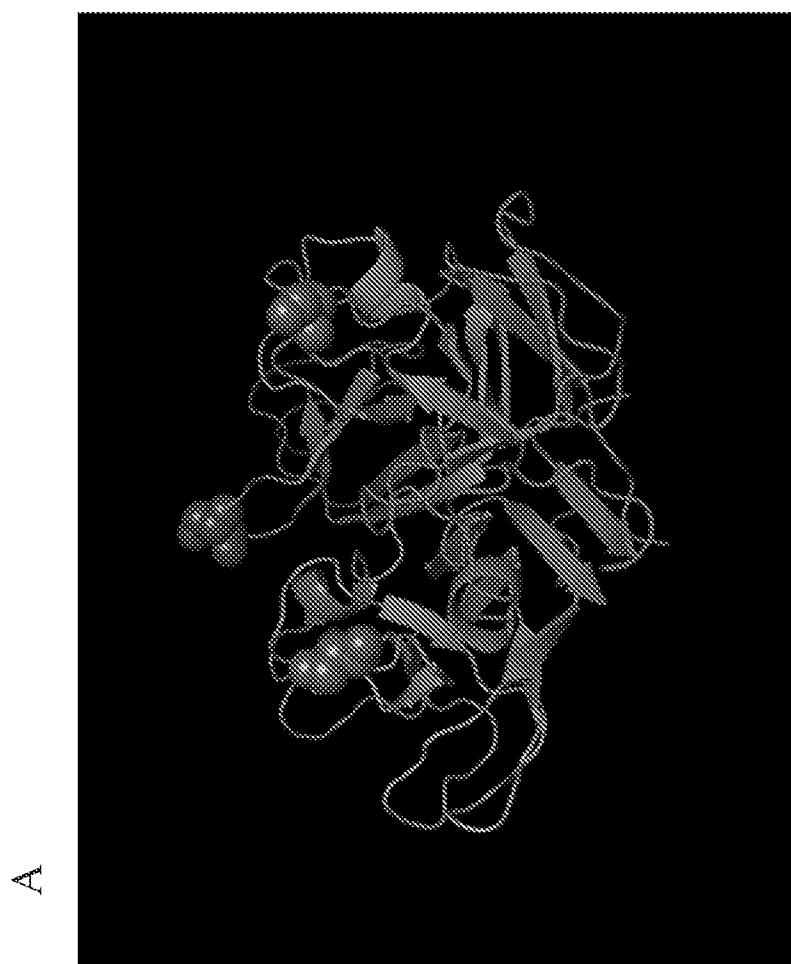


Figure 8

Figure 8

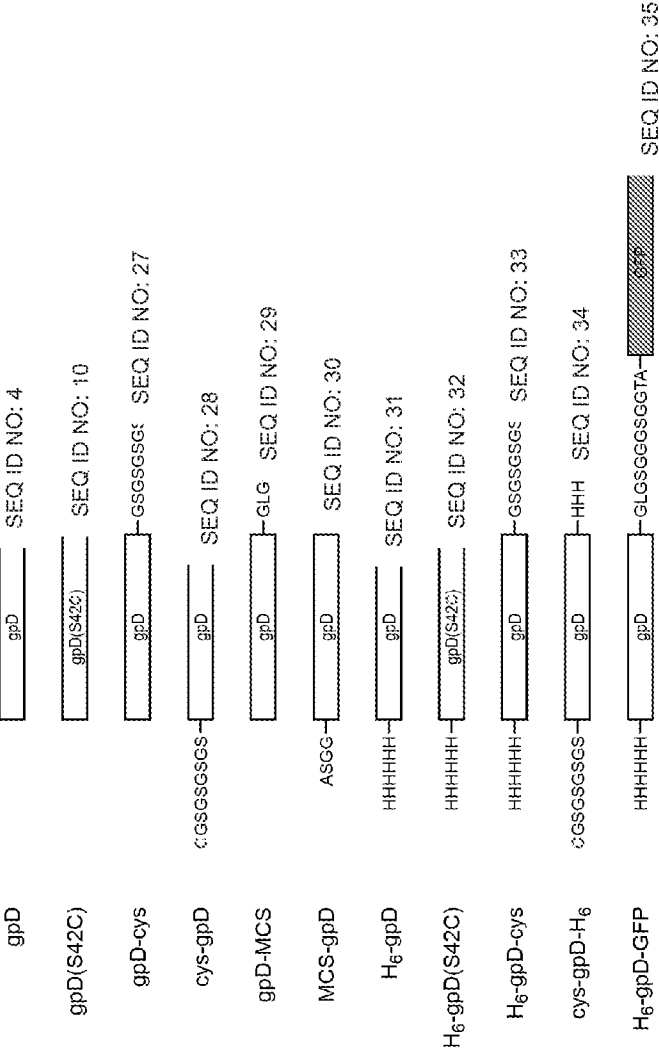


Figure 6

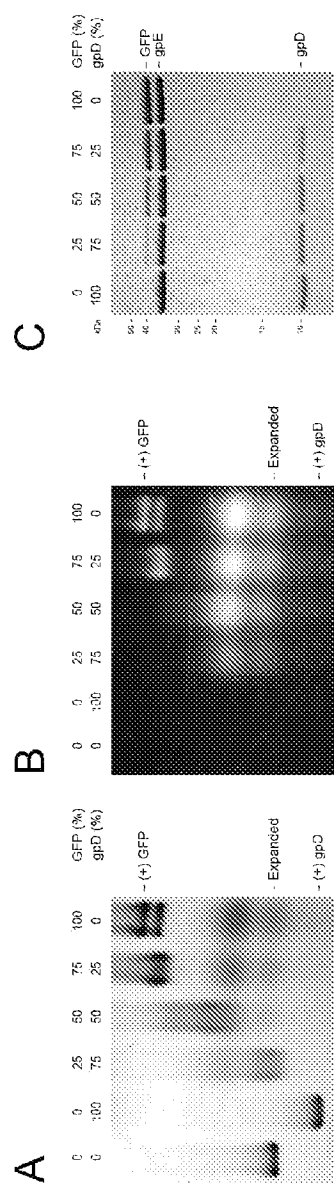


Figure 10

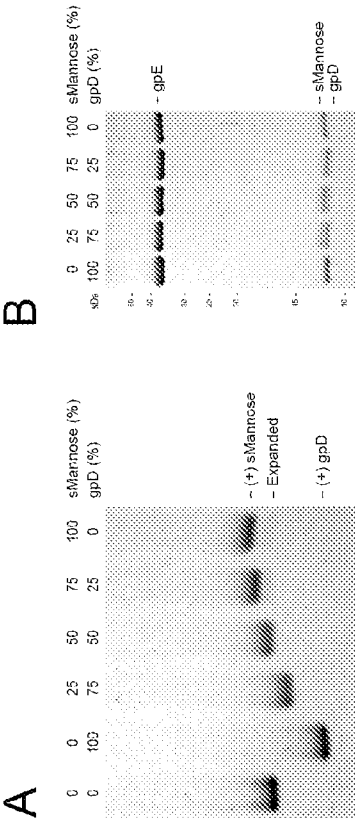
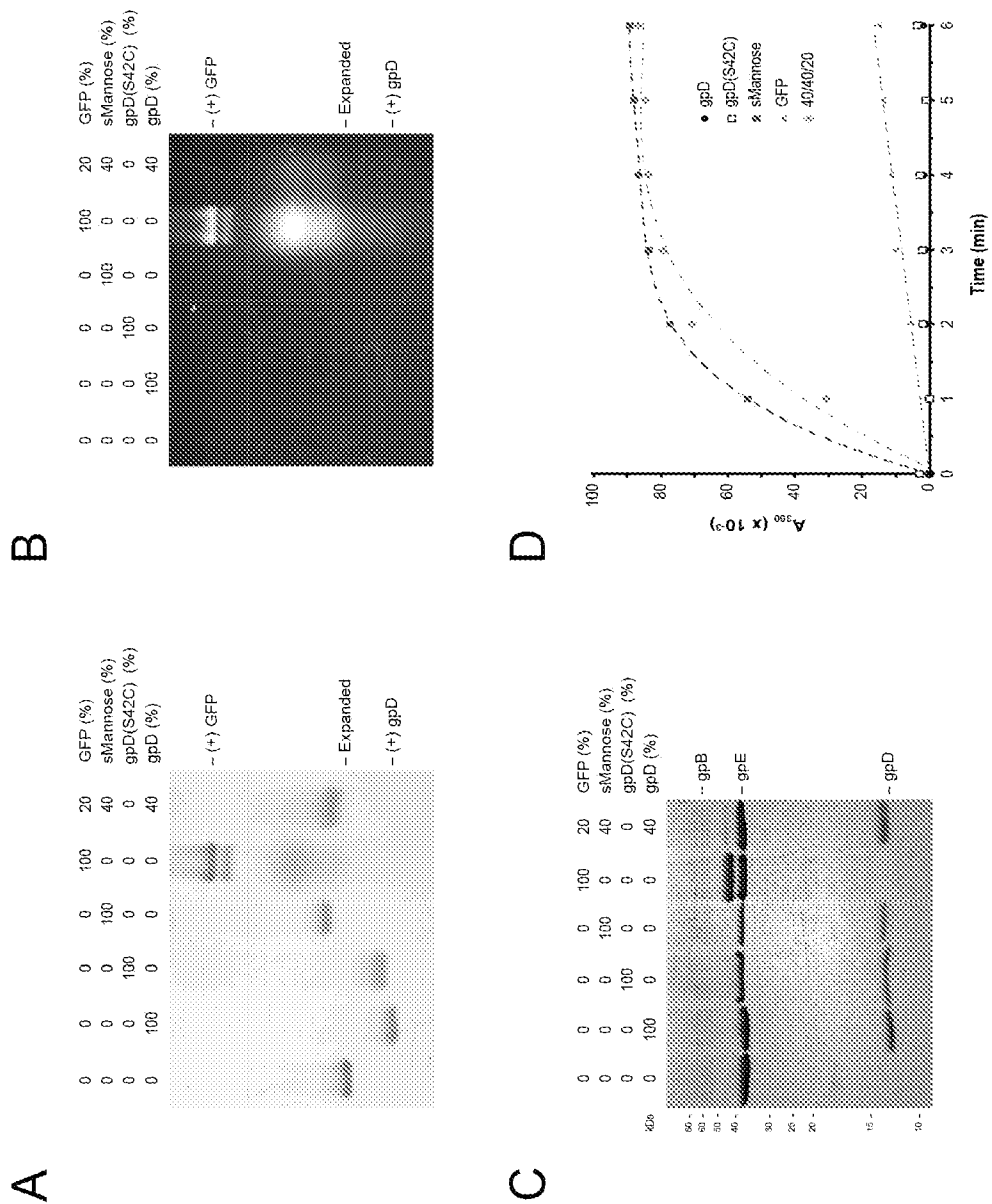


Figure 11



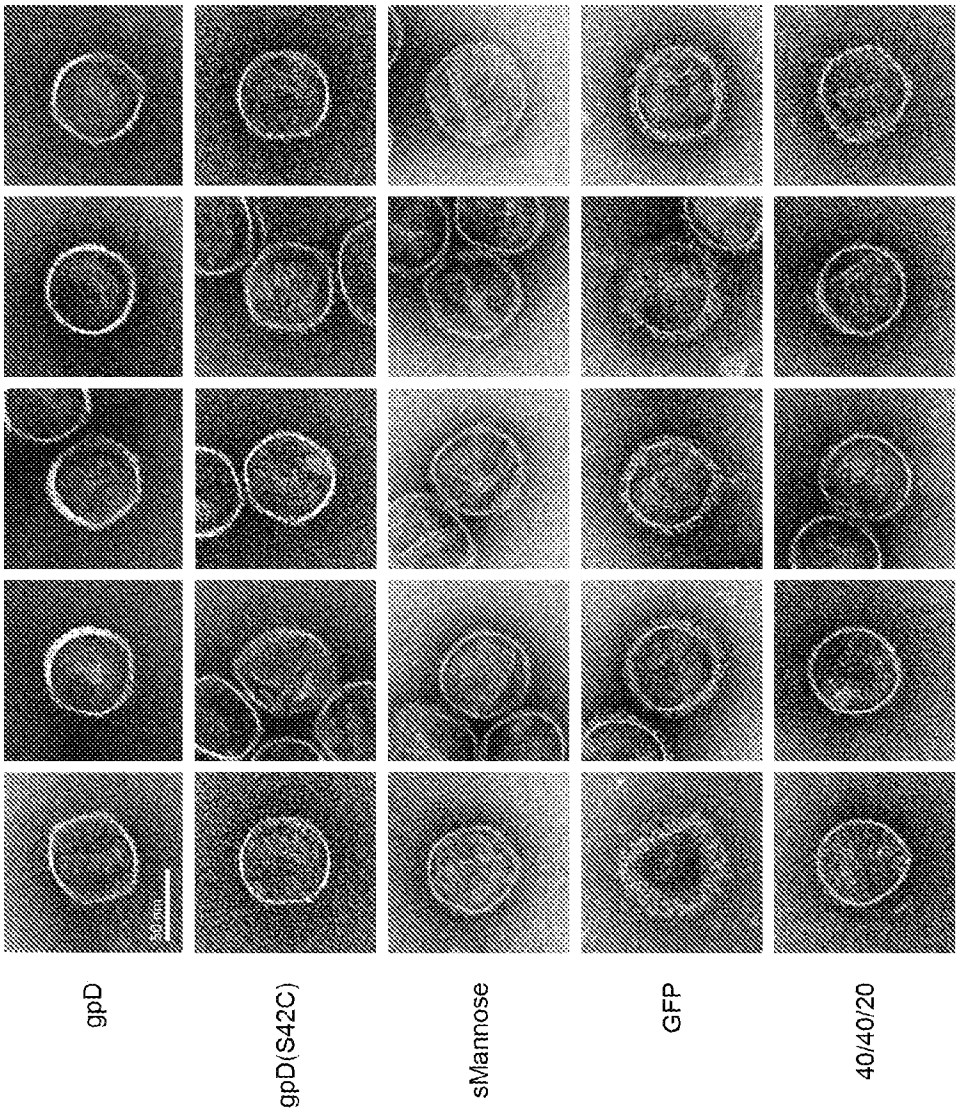
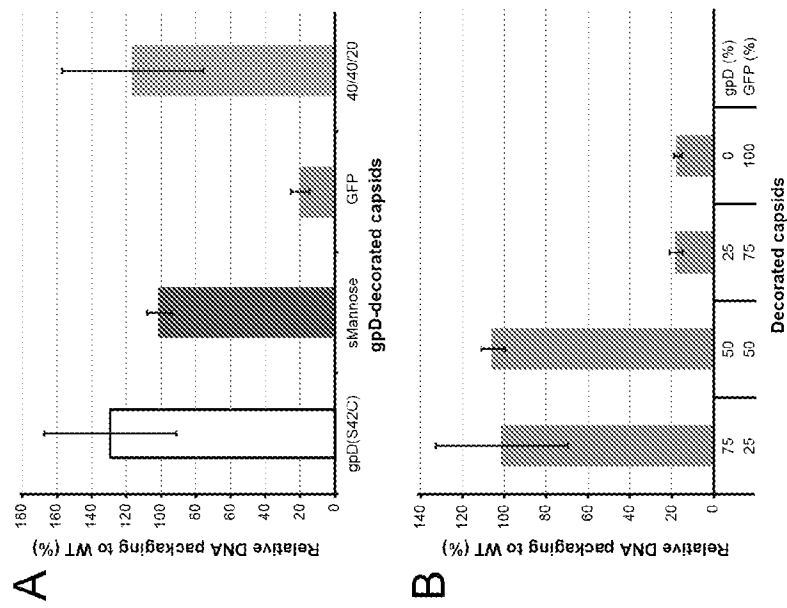


Figure 13



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/012206

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N7/00 C07K14/001
 ADD. C12N15/1037

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	WO 2008/115296 A2 (UNIV ROCHESTER [US]; DEWHURST STEPHEN [US]; TREANOR JOHN J [US]) 25 September 2008 (2008-09-25)	1-12,16, 22,23, 27-33 14,35 13,15, 17-21, 34,36
X Y	----- PARENT K.N. ET AL.: "Stepwise molecular display utilizing icosahedral and helical complexes of phage coat and decoration proteins in the development of robust nanoscale display vehicles.", BIOMATERIALS, vol. 33, 8 May 2012 (2012-05-08), pages 5628-5637, XP002722060, figure 1 ----- -/--	1-3,6-8, 10,11, 16, 22-25, 27-29, 31,32 26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 March 2014

Date of mailing of the international search report

17/04/2014

Name and mailing address of the ISA/

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Authorized officer

Galli, Ivo

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/012206

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE UniProt [Online] 11 July 2012 (2012-07-11), "SubName: Full=Head decoration protein;", XP002722061, retrieved from EBI accession no. UNIPROT:I2PRL2 Database accession no. I2PRL2 sequence	14,35
Y	----- MEDINA E. ET AL.: "Thermodynamic characterization of viral procapsid expansion into a functional capsid shell.", J. MOL. BIO., vol. 418, 23 February 2012 (2012-02-23), pages 167-180, XP002722062, the whole document	26
A	----- NURMEMMEDOV E. ET AL.: "Challenging packaging limits and infectivity of phage lambda", J. MOL. BIO., vol. 415, 15 November 2011 (2011-11-15), pages 263-273, XP002722063, the whole document	1-36
A	----- YANG Q., MALUF N.K. & CATALANO C.E.: "Packaging of a unit-length viral genome: The role of nucleotides and the gpD decoration protein in stable nucleocapsid assembly in bacteriophage Lambda", J. MOL. BIO., vol. 383, 3 September 2008 (2008-09-03), pages 1037-1048, XP002722064,	1-36
A	----- US 2012/214867 A1 (LIU JINNY LIN [US] ET AL) 23 August 2012 (2012-08-23) figure 1 -----	1-36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2014/012206

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008115296 A2	25-09-2008	US 2010284967 A1 WO 2008115296 A2	11-11-2010 25-09-2008
US 2012214867 A1	23-08-2012	NONE	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/012206

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. (means)
- ☐ on paper
- ☒ in electronic form
- b. (time)
- ☒ in the international application as filed
- ☐ together with the international application in electronic form
- ☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: