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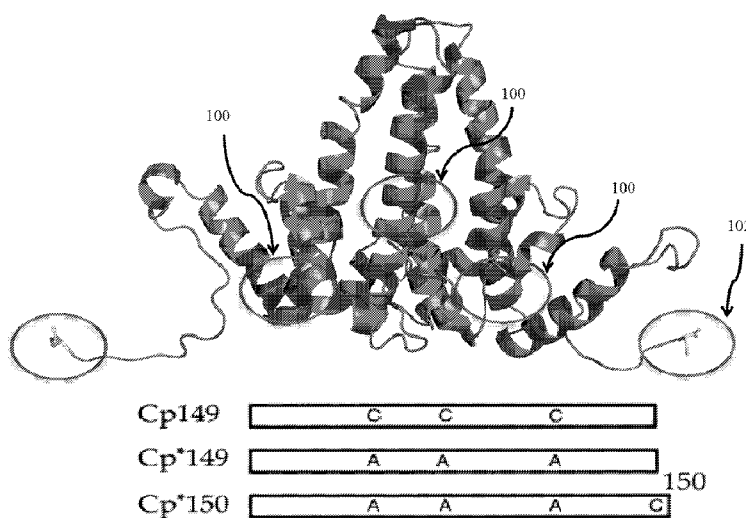
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(54) Title: HOLEY CAPSID PLATFORM



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FIG. 1A

(57) Abstract: A self-assembling protein capsid including removable sub-units is disclosed along with a method for modifying a capsid to create a holey capsid.

HOLEY CAPSID PLATFORM

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 62/019,719, filed July 1, 2015, the entire disclosure of which is hereby expressly incorporated by reference.

GOVERNMENT LICENSE RIGHTS

[0002] This invention was made with government support under GM100071 and AI077688 awarded by the National Institutes of Health. The Government has certain rights in the invention.

FIELD

[0003] The present disclosure relates generally to virus-like particles (“VLP’s”) referred to as holey capsids, which are capsid platforms to which subunits can be removed and added as needed, at specific amounts, and at specific sites. More particularly, the present disclosure relates to methods for adding to and removing from virus capsids specific subunits, in one embodiment a Hepatitis B Virus (“HBV”) capsid.

BACKGROUND

[0004] The assembly of hundreds of identical proteins into an icosahedral virus capsid is a remarkable feat of molecular engineering. How this occurs is poorly understood. Virus capsids containing hundreds of subunits have evolved to assemble with high fidelity in a short period of time. In many cases, capsid formation is sufficiently robust that, given the right conditions, purified capsid proteins spontaneously assemble into icosahedral virus-like particles. Despite a large effort, the underlying principles of capsid assembly are far from fully understood.

[0005] Capsid assembly reactions necessarily have a single starting point of bulk subunits and a single ending point of complete capsids. The assembly of HBV is of particular interest because it is a devastating pathogen, and because it is an attractive target for the development of new assembly-directed antiviral molecules.

[0006] Thus, there is a need to create a nano-scaffolding to which components can be added to and subtracted from at will. Therefore, discussed herein is a holey capsid, illustratively formed from HBV core protein, to which components can be added and subtracted.

SUMMARY

[0007] VLP's are useful tools as cargo nanocarriers for toxins, proteins, drugs, and metals. They provide specificity to targeted cells during delivery and minimize unwanted contact with non-targeted cells. Although other types of nanoparticles such as lipids can serve as nanocarriers, they lack cell-specificity. In the case of human VLP's, the HBV core protein is an excellent choice to create the VLP's, primarily because it has the ability to carry other foreign cargo besides its genome.

[0008] One of the issues with the synthesis of cargo-filled VLP's is that the cargo needs to be assembled into nanoparticles during encapsulation. Encapsulation of these pre-formed cargo particles also presents a challenge where the capsid would have to assemble around the cargo. The present disclosure overcomes these problems by creating a hybrid capsid assembled with a desired ratio of "passivated" subunits (being passivated through addition of N-ethyl maleimide (NEM)) and non-passivated subunits. Once a hybrid capsid forms, the passivated dimer subunits can then be easily removed by chemical treatment of the fully assembled capsid resulting in a capsid, bearing holes referred to herein as a "holey capsid."

[0009] These holes, or pores, (roughly equivalent to the size of a dimeric unit) can then serve as a channel for the introduction of cargo. This can allow for the synthesis of bulky polymers within the capsid due to the ability of large polymer building blocks to

pass through the capsid's holes and into the interior of the holey capsid where they may polymerize. It has been shown that the nature of the functional groups on the nanoparticles may affect the assembly of the virus core protein or the encapsulation efficiency. Some embodiments of the present disclosure provide a more versatile nanocarrier to be used with a range of cargo.

[0010] During the replication life cycle of HBV, the nucleocapsid functions as a carrier for relaxed circular dsDNA and pregenomic ssRNA. In vitro, the capsid adaptation to these different cargo compositions in the cell makes the HBV nucleocapsid an attractive VLP for non-genomic cargo, such as non-genomic therapeutic agents. In vitro, the nucleocapsid is capable of encapsidating RNA as well as other nanoparticles. Some embodiments of the present disclosure include methods to control capsid assembly and assembly of capsid multimers. In other embodiments, new cargos are incorporated in assembling capsids and/or in preformed capsids. Still in other embodiments, preassembled capsids are used to form crystal-like solids.

[0011] Addition of cargo attached to the Cp149 (where Cp stands for "Core protein") dimer and reincorporation into the holey capsid can be an effective method for making nanocarriers. In one embodiment, green fluorescent protein ("GFP") is chosen as the cargo attached to Cp149 dimer (GFP is a characterized protein and its fusion to other proteins are documented).

[0012] In some embodiments, HBV VLP's can be used to carry foreign cargo such as silver or gold nanoparticles and quantum dots that are suitable for plasmonic application. A bimetal lattice of nanoparticles with novel optical properties, in some embodiments, consists of alternating layers of metal (i.e. gold, silver) filled VLP's. This arrangement of layered VLP's, in some embodiments, gives rise to novel optical properties that may be neither the individual nor the sum of the cargo's optical properties. In other embodiments, these VLP's may exhibit synergy.

[0013] Holey capsids are platforms to which subunits can be added as needed, at specific amounts, and at specific sites. Data from high-performance liquid chromatography (“HPLC”), fluorescence, and charge detection mass spectrometry (“CDMS”) show that holey capsids are receptive to re-incorporation of subunits.

[0014] As noted, there is a need for nano-scaffolding components that can be added to at will. Synthesized holey capsids have been formed and have demonstrated the ability to reincorporate Cp*150 Bo-labeled dimer subunits back into the holey capsids. In one embodiment, producing holey capsids utilizes Cp150 mutants (Cp*150), in which cysteines at positions 48, 61, and 107 have been replaced with alanines, and an additional cysteine has been added to the C-terminus of the protein. Cp*150 allows crosslinking of dimers to form very stable capsids. This also allows for the synthesis of a new hybrid capsid formed by the assembly of two different types of subunits (Cp*150 blocked with N-Ethylmaleimide (NEM) – “passivated” Cp*150.NEM – and “non-passivated” Cp*150 subunits).

[0015] In some embodiments, the ratio of passivated to non-passivated subunits is 1:3. Once the hybrid nucleocapsid is formed, the “passivated” dimers can be removed to create a “holey” capsid. In some embodiments, the holey capsid has an average of 19 missing dimers. At least some of the holes in the holey capsid can be back-filled with fluorescently labeled dimer. Total Internal Reflection Fluorescence (“TIRF”) microscopy in combination with resistive pulse sensing equipment can help characterize the back-filled capsids.

[0016] In some embodiments, cargo other than Cp*150 Bo-labeled dimer can be attached to the dimer subunits. Such cargo may include, but is not limited to, green fluorescent proteins (“GFP”) fused to Cp149 dimer (GFP-Cp 149 dimer). The GFP protein (27kDa protein) is approximately the same size as an HBV monomer, and its incorporation into the holey capsid may be impeded compared to Cp*150 Bo-labeled dimer. Therefore, the speed of such incorporation is determined in some embodiments. A time course for the reincorporation of modified dimer onto the holey capsid by

chromatography and fluorescence anisotropy experiments can be performed. The reincorporated GFP-Cp149 dimer in the holey capsid can be analyzed by mass spectrometry to see how many GFP molecules are present in the interior of the holey capsid. Testing GFP-Cp149 dimer reincorporation in a nanofluidic device can also provide important information on single molecule studies using resistive pulse sensing and fluorescence.

[0017] The manipulation of the assembly and disassembly of the capsid allows the creation of a holey capsid that include channels which are large enough to deliver cargo, e.g. about core protein dimer size, that cannot otherwise be packaged into the naturally occurring wild type capsid. Theoretically, the cargo that can be packaged inside the capsid is limited only by the size of the pores in the holey capsid.

[0018] Without a prior art holey capsid, there is a need for the ability to attach cargo to dimer subunits and reincorporate them into the capsid. This form of cargo encapsulation allows control over the number of cargo attached per capsid. This method also depends less on a diffusion mechanism for encapsulation of small molecules into the HBV capsid. The GFP proteins fused to the CP149 tethered dimer allow only one GFP per dimer, increasing the probability of successful incorporation into holey capsid. In some embodiments, the added subunits may be bound to organic and inorganic catalysts, other proteins (e.g. receptors, receptor-binding signaling molecules, enzymes, toxins), specific dyes, metals, and therapeutic molecules. Holey particles may also serve as low density carriers for immunogenic polypeptides as for vaccines or immune assays.

[0019] Manipulating the assembly and disassembly reaction of the HBV core protein capsid allows synthesis of a holey capsid. Stability requires a mutant protein that covalently crosslinks the structure. The holes can be back-filled with labeled protein. In other embodiments, other modified molecules can be added or subtracted, controlling what subunits are encapsulated and what features are present on the capsid surface and interior.

[0020] Thus, herein disclosed is, in one embodiment, a self-assembling protein capsid comprising: non-passivated sub-units that form a stable complex and passivated, removable sub-units, wherein said non-passivated sub-units form a holey capsid when one or more of said passivated sub-units are removed from the capsid.

[0021] In some embodiments, the capsid is a hepatitis B virus core protein capsid. In other embodiments, the capsid comprises at least one mutant of the hepatitis B virus core protein, and the mutant is capable of crosslinking. In other embodiments, the capsid further includes at least one mutant of the hepatitis B virus core protein capable of covalently crosslinking via disulfide bonds or other chemistry. Still in other embodiments, the capsid further comprises at least one mutant of the hepatitis B virus core protein capable of covalently crosslinking via disulfide bonds or other chemistry, and said capability to covalently crosslink can be passivated. In yet other embodiments, the capsid comprises at least one mutant of the hepatitis B virus core protein, and said at least one mutant contains at least one insertion.

[0022] The non-removable sub-units can be selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5**. In other embodiments, the non-removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion. Still in other embodiments, the removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

[0023] In yet still other embodiments, the non-removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion. In some embodiments, the self-assembling protein capsid further includes incorporated sub-units, wherein said incorporated sub-

units back fill the holey capsid. In other embodiments, the incorporated sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity or 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

[0024] In still other embodiments, the incorporated sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity or 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion, and wherein the one or more insertions are one or more polypeptides selected from the group consisting of: receptors, receptor-binding molecules, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

[0025] In yet other embodiments, the incorporated sub-units are bound to moieties selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins. Still in other embodiments, the removable subunits are passivated with N-Ethylmaleimide. The removable subunits can be removed from the capsid using a chaotrope.

[0026] Also disclosed is a method for modifying a capsid comprising the steps of: combining passivated and non-passivated sub-units, wherein said sub-units self-assemble to form the capsid; crosslinking the non-passivated sub-units; and removing at least one sub-unit from the capsid to form a holey capsid. In some embodiments of the method, the capsid is a hepatitis B virus core protein capsid. In other embodiments, the removed sub-units are selected from the group consisting of: sequences that contain at least one component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5**.

[0027] Still in other embodiments, the removed sub-units are selected from the group consisting of: sequences of about at least 80% similarity to **SEQ. ID. NOS. 2, 3,**

4, or 5. In some embodiments, the method further comprises the step of incorporating sub-units, wherein said sub-units back fill the holey capsid. In other embodiments, the sub-units are bound to a moiety selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins.

[0028] In some embodiments, the sub-units are selected from the group consisting of: subunits with inserts that encode receptors, receptor-binding peptides, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

[0029] Additionally disclosed herein is a method for identifying holey capsid incorporatable sub-units comprising the steps of: providing a holey capsid; providing a sub-unit, wherein said subunit comprises an identifiable marker; combining the sub-unit with the holey capsid; identifying the amount of sub-unit incorporated into the holey capsid; and comparing to a holey capsid control. In some embodiments, the capsid is a hepatitis B virus core protein capsid. In other embodiments, the sub-units are bound to a moiety selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins.

[0030] And in other embodiments, the sub-units are selected from the group consisting of: subunits with insertions that encode receptors, receptor-binding peptides, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The features of this disclosure, and the manner of attaining them, will become more apparent, and the disclosure itself will be better understood by reference to the following description of embodiments of the disclosure taken in conjunction with the accompanying drawings.

[0032] FIG. 1A shows a graphical representation of Cp*150 mutant dimer.

[0033] FIG. 1B is a graphical representation showing Cp*150 termini are concentrated at fivefold and quasi-sixfold vertices to facilitate crosslinking.

[0034] FIG. 2 is a diagram showing holey capsid assembly from mutant Cp*150 dimer and mutant passivated Cp*150 dimer. NEM and removal of mutant passivated Cp*150 by Urea.

[0035] FIG. 3A shows smaller intermediates (1-16 dimers) as detected on the CDMS.

[0036] FIG. 3B shows CDMS of a holey capsid before reincorporation of subunits or back-filling.

[0037] FIG. 3C shows CDMS analysis after backfilling of a holey capsid and a lack of capsid intermediates.

[0038] FIG. 4A shows a schematic representation of the labeling process of Cp*150 mutants with BoDIPY-FL that has absorbance at 504nm.

[0039] FIG. 4B shows the calculated percent incorporation of Cp*150 Bo labeled dimer in a holey capsid.

[0040] FIG. 5A shows the construct for making a tethered Cp149 dimer by linking the C terminus of one monomer to the N terminus of the second monomer by a GGS linker of five repeats.

[0041] FIG. 5B shows a construct with ligation of eGFP gene extracted from a pEGFP-N1 Vector that can be commercially purchase.

[0042] FIG. 6A shows size-exclusion chromatography ("SEC") analysis of fluorescently labeled Cp*150 dimer for optimum conditions that result in no self-assembly reaction at 280, 504nm.

[0043] FIG. 6B shows SEC of holey capsid back-filled with fluorescently labeled Cp*150 dimer.

[0044] FIG. 7 shows a model for a holey capsid and a model for a back-filled capsid.

[0045] Corresponding reference characters indicate corresponding parts throughout the several views. Although the drawings represent embodiments of the present disclosure, the drawings are not necessarily to scale and certain features may be exaggerated in order to better illustrate and explain the present disclosure. The exemplifications set out herein illustrate an exemplary embodiment of the disclosure, in one form, and such exemplifications are not to be construed as limiting the scope of the disclosure in any manner.

SEQUENCE LISTING

[0046] SEQ. ID. NO. 1: 1 mdidpykefg atvellsflp sdffpsvrdl ldtaaalyrd alespehcsp
hhtalrqail 61 cwgdImtlat wvgtnledpa srdlvvsyvn tnvglkfrql lwfhiscItf gretvleylv 121
sfgvwirtpp ayrppnapil stlpettvvr rrgsprrrt psprrrrsqs prrrrsqsre 181 sqc. Cp183.

[0047] SEQ. ID. NO. 2: 1 mdidpykefg atvellsflp sdffpsvrdl ldtaaalyrd alespehcsp
hhtalrqail 61 cwgdImtlat wvgtnledpa srdlvvsyvn tnvglkfrql lwfhiscItf gretvleylv 121
sfgvwirtpp ayrppnapil stlpettvv. Cp149.

[0048] SEQ. ID. NO. 3: 1 mdidpykefg atvellsflp sdffpsvrdl ldtaaalyrd alespehcsp
hhtalrqail 61 cwgdImtlat wvgtnledpa srdlvvsyvn tnvglkfrql lwfhiscItf gretvleylv 121
sfgvwirtpp ayrppnapil stlpettvvc. Cp150.

[0049] SEQ. ID. NO. 4: 1 mdidpykefg atvellsflp sdffpsvrdl ldtaaalyrd alespehasp
hhtalrqail 61 awgdImtlat wvgtnledpa srdlvvsyvn tnvglkfrql lwfhisaltf gretvleylv 121
sfgvwirtpp ayrppnapil stlpettvvc. Cp*150, mutant.

[0050] SEQ. ID. NO. 5: 1 mdidpykefg atvellsflp sdffpsvrdl ldtaaalyrd alespehcsp
hhtalrqail 61 cwgdImtlat wvgtnledpa srdlvvsyvn tnvglkfrql lwfhiscItf gretvleylv 121

sfgvwirtpp ayrppnapil stlpettvvg gsggsggsgg sggs 165 mdidpykefg atvellsflp sdffpsvrdl
ldtaaalyrd alespehcsp hhtalrqail 225 cwgdltlat wvgtnledpa srdlvvsyvn tnvglkfrql
lwfhiscltf gretvlelv 285 sfgvwirtpp ayrppnapil stlpettv. Tandem-Cp149 (see **FIGS. 5A**
and B).

DETAILED DESCRIPTION OF THE DRAWINGS

[0051] The embodiments disclosed herein are not intended to be exhaustive or limit the disclosure to the precise form disclosed in the following detailed description. Rather, the embodiments are chosen and described so that others skilled in the art may utilize their teachings.

[0052] Referring first to **FIG. 1A**, Cp*150 mutant dimer is shown. Sites 100 indicate cysteines at positions 48, 61, and 107 were mutated to alanines. (See **SEQ. ID. NOS. 3, 4**). Site 102 indicates a new cysteine added to the C-terminal position. The HBV core protein comprises of 183 residues ("Cp183," **SEQ. ID. NO. 1**) with an N-terminal assembly domain (the first 149 residues) and a C-terminal RNA-binding domain (positively charged and acts as a chaperone for the encapsulation of pgRNA).

[0053] Truncated mutant Cp*150 contains 149 residues from the N-terminus. Cp*150 is a mutant that has three cysteines (residues 48, 61 and 107) mutated to alanine and one additional cysteine added to the Cp149 C-terminal end. This mutant allows crosslinking to occur between the C terminal ends of adjacent dimers (imparting higher structural stability) but does not allow the carrying of pgRNA (due to lack of RNA binding domain) and thus is favorable to be used as a nanocarrier.

[0054] Referring to **FIG. 1B**, Cp*150 C termini are concentrated at fivefold and quasi-six fold vertices to facilitate crosslinking. In free dimer, the C-terminal cysteine is available for attaching of fluorescent dyes or NEM.

[0055] Referring now to **FIG. 2**, a diagram for holey capsid assembly from Cp*150 dimer and passivated Cp*150 dimer.NEM, followed by removal of passivated Cp*150 by

Urea, a chaotropic agent, is shown. In other embodiments, other chaotropic agents such as butanol, ethanol, guanidinium chloride, lithium perchlorate, lithium acetate, magnesium chloride, phenol, propanol, sodium dodecyl sulphate, or thiourea may be used. Varying portions of Cp*150 dimers are 100% blocked with NEM to create Cp*150dimer.NEM. These dimer subunits are then coassembled with Cp*150 unblocked dimer to synthesize the hybrid capsid. The hybrid capsids are allowed to crosslink at room temperature for 24h. The oxidized hybrid capsids then undergo urea (3M) treatment to remove the passivated subunits to form a holey capsid. These holey capsids are optionally analyzed in CDMS and HPLC.

[0056] Still referring to **FIG. 2**, two pools of subunits to generate the holey capsids were used; the non-passivated Cp*150 mutant and Cp*150 passivated mutant fully labeled with N-ethyl Maleimide ("NEM") (blocking agent for free cysteine ends). Mixing of these two types of subunits randomly resulted in different degrees of crosslinking in the oxidized hybrid capsid.

[0057] Upon exposure to urea, dimers blocked with NEM fall out of the hybrid capsid resulting in the formation a holey capsid with an average of 19 missing dimer subunits. These intermediates (lacking 1-19 dimer subunits) have been trapped and analyzed by CDMS (See **FIG. 3B**). The lack of intermediates between 0.5-3 MDa can be observed by CDMS (See **FIG. 3A-B**). Without limiting the mechanism to any one specific theory, one possibility is that intermediates above 3 MDa are able to form intact holey capsids that have 3 or more interdimer contacts, whereas intermediates below this mass may become linear chains that have only two or less interdimer contacts per dimer. With the addition of urea, these linear chain intermediates may break apart and fall into the smaller intermediate range detected in the CDMS. Another possibility is that the intermediates in this range have low abundance and therefore cannot be detected in the CDMS or HPLC experiments.

[0058] Referring now to **FIGS. 3A-C**, CDMS graphs are shown. **FIGS. 3B** and **3C** show CDMS comparison of a holey capsid and a back-filled capsid. In **FIG. 3C**, the

absence of intermediates' peaks, compared to **FIG. 3B**, indicates that holey capsids are now complete and back-filled. There is a lack of intermediates, and a higher peak intensity is seen for T=4 as compared to T=3 capsids.

[0059] **FIG. 3A** shows smaller intermediates (1-16 dimers) as detected on the CDMS. The measured masses were overestimated by 5 percent and were calibrated to the T=3 and T=4 peaks of HBV to increase their accuracy. Those holey capsids that had an average of 19 missing dimers were very stable structures and could be further analyzed. Upon exposure to urea, dimers blocked with NEM fell out of the hybrid capsid resulting in the formation of a holey capsid with an average of 19 missing dimer subunits. These intermediates (lacking 1-19 dimer subunits) have been trapped and analyzed by CDMS as shown in **FIG. 3B**. The lack of intermediates between 0.5-3 MDa can be observed by CDMS.

[0060] Referring now to **FIGS. 4A** and **4B**, a schematic diagram and a graph showing the results of the assembly reaction of holey capsid and Cp*150 Bo labeled dimer are shown. **FIG. 4A** is a schematic representation of the labeling process of Cp*150 mutants with BoDIPY-FL that has absorbance at 504nm. **FIG. 4B** shows the percent incorporation of Cp*150 Bo labeled dimer in a holey capsid. An average of 19 missing dimer (16%) subunits was determined. The average number of dimers backfilled into each capsid was derived from the SEC analysis.

[0061] Referring now to **FIGS. 5A** and **5B**, schematic diagrams are shown for a method of creating GFP-Cp149 dimers and reincorporating them into a holey capsid. **FIG. 5A** shows the construct for making a tethered Cp149 dimer by linking the C terminus of one monomer to the N terminus of the second monomer by a GGS linker of five repeats. **FIG. 5B** shows the construct from Shepherd et al. with the ligation of eGFP gene extracted from a pEGFP-N1 Vector that can be commercially purchase.

[0062] Referring to **FIGS. 6A** and **6B**, a holey capsid was backfilled with fluorescently labelled CP*150 dimer to quantify the average number of holes per capsid.

FIG. 6A is a graph showing SEC of fluorescently labeled Cp*150 dimer for optimum conditions that result in no-self assembly reaction at 280, 504nm. **FIG. 6B** is a graph showing holey capsid back-filled with fluorescently labeled Cp*150 dimer. 504nm detections shows the amount of labeled dimer in capsid and dimer fractions.

[0063] Referring now to **FIG. 7**, a model for a holey capsid and a model for a back-filled capsid is shown.

[0064] While this invention has been described as having an exemplary design, the present invention may be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains.

EXAMPLES

[0065] *Example 1:* The ratio for non-passivated: passivated dimer was 3:1 (See **FIG. 2**). Other ratios have been tested including 3:1, 2:1, 5:2, and 1:1, but resulted in a very similar average number of holes in the holey capsid (19-23 dimers respectively). Even with a 3:1 ratio, expecting to get 30 missing dimers, only 19 missing dimers were observed.

[0066] Possible factors include but are not limited to competition between passivated and non-passivated dimer for the assembly reaction. Non-passivated dimer may prefer to assemble with other non-passivated dimer when a low amount of Cp*150-NEM is added (3:1). Such a ratio may cause a smaller fraction of Cp*150 holey capsid compared to a T=4 capsid (the fraction of capsids that has 19 passivated dimers compared to the whole sample is ~10-20% based on sucrose gradient).

[0067] When a higher amount of Cp*150-NEM is added (1:1), non-passivated dimer may be forced to assemble with passivated dimer when non-passivated dimers are

used up. This forms unstable capsids with 30 passivated well-distributed dimers that may result in a breakdown into smaller oligomers during urea treatment (1-16 dimers as shown in **FIG. 3A**). A lesser amount of Cp*150.NEM dimer can be added to create a smaller number of holes in the event that it is desired to restrict the number of GFP-Cp 149 dimer incorporation to lesser than 19 dimers due to steric crowding within the capsid.

[0068] *Example 2: FIGS. 3C, 4A, and 4B* illustrate the ability of capsid holes to be filled up for utilization as nanocarriers. A back-fill reaction was performed by adding 30mM NaCl (>30mM NaCl cause self-assembly of the labeled dimer) to a fixed 3 μ M holey capsid and 0-7 μ M Cp*150-Bo DIPY-FL (Bo) labeled dimer. To monitor its incorporation into the holey capsid, UV/Vis spectrometry at 280 and 504nm excitation wavelength was performed.

[0069] The controls included 0-7 μ M Cp*150 Bo-labeled dimer alone in the presence of 30mM NaCl that show a lack of self-assembly below 7 μ M Cp*150 Bo-labeled dimer (protein and salt concentration that cause self-assembly of the labeled dimer induce competition to the incorporation of these labeled dimers in the holey capsid). The back-fill reaction was tested on a Shimadzu HPLC at absorbance of 280nm and 504nm. The absorbance at 504nm corresponds to the free-labeled dimer absorbance alone while the absorbance at 280nm corresponds to the holey capsid and incorporated labeled dimer absorbance.

[0070] By monitoring the absorbance at 280nm and 504nm, the number of Cp*150 Bo-labeled dimers that are incorporated into the holey capsid were determined. An average of 19 dimers (16%), as shown in **FIG. 3C**, was found to be incorporated. In order to confirm that this was maximal incorporation, CDMS was performed on the co-assembled holey capsids. The CDMS results showed that the back-fill reaction was able to complete formation of the T=4 capsid. Holey capsids can serve as platforms for the addition of cargoes as well as reincorporation of the missing subunits following encapsidation of the desired cargo.

[0071] *Example 3:* Total Internal Reflection Fluorescence ("TIRF") characterization of the holey capsids is a tool to analyze the holey capsid and the subsequently reincorporated capsid. A comparison between the signal differences in currents involving Cp*150 holey capsids, the reincorporated capsids, and wild type T=3 and T=4 capsids, allows a determination whether the current of the reincorporated capsid is similar to a T=4 capsid. TIRF microscopy in combination with resistive pulse sensing also allows determination of the average fluorescence per capsid. It is advantageous to determine the individual fluorescence per capsid instead of just an average value.

[0072] Shepherd et al., **FIGS. 5A-B**, has synthesized the tethered Cp149 dimer construct and shown that its expression and purification steps are the same as Cp149 wt. In one embodiment of the present disclosure, eGFP gene is extracted from a pEGFP-N1 vector and fused to the C terminal end of the tethered Cp149 dimer construct from Shepherd et al. In some embodiments, an attachment of 1 GFP per tethered dimer (instead of two GFP per dimer if one Cp149 monomer is fused with one eGFP gene) is generated that does not provide bulk steric hindrance that inhibit reincorporated into holey capsid. Followed by expression and purification of the GFP-dimer protein similar to wild-type Cp149 protein.

[0073] In other embodiments, the kinetic rate of the reincorporation of the GFP-Cp 149 dimers into the holey capsid is measured. A simple time course experiment can be performed using fluorescence anisotropy (fluorescence polarization). The tumbling rates would differ when fluorophore is attached to a virus capsid. These different tumbling rates affect the anisotropy value that can be measured directly from the plate reader. The GFP protein in the interior of the capsid due to its size and quantity (19 GFP-Cp149 dimers can theoretically be incorporated) may exhibit a slower tumbling rate due to local interaction in the interior of the capsid. The GFP-Cp149 dimers reincorporation in the holey capsid would also be tested by CDMS and resistive pulse sensing equipment + TIRF (nanofluidic device).

[0074] In some embodiments, the fusion of GFP protein directly to the tethered Cp149 dimer may prevent the proper folding of the protein. In an alternative embodiment, a glycine linker of 6-8 repeats is inserted between these two genes in the construct. The GFP protein attachment on the Cp149 dimer may not allow incorporation into the holey capsid. Other alternative cargo may be considered such as a tetracysteine tag that is shown to work on the insertion into the immunodominant c/e1 site of HBV core protein. The fluorescence anisotropy experiment may not work as GFP is attached to the C-terminal end of Cp149 dimer, even if it is incorporated into the capsid; its localized interaction is minimal. An alternative approach might be to perform a time course HPLC back-fill experiment. In one alternative embodiment, this allows a determination of the number of GFP-Cp 149 dimers attached and compared it to previous Cp*150Bo labeled dimer.

WHAT IS CLAIMED IS:

1. A self-assembling protein capsid comprising:
non-passivated sub-units that form a stable complex and passivated, removable sub-units, wherein said non-passivated sub-units form a holey capsid when one or more of said passivated sub-units are removed from the capsid.
2. The self-assembling protein capsid according to claim 1, wherein the capsid is a hepatitis B virus core protein capsid.
3. The self-assembling protein capsid according to claim 1, wherein the capsid comprises at least one mutant of the hepatitis B virus core protein, and wherein said mutant is capable of crosslinking.
4. The self-assembling protein capsid according to claim 3, wherein the capsid further comprises at least one mutant of the hepatitis B virus core protein capable of covalently crosslinking via disulfide bonds or other chemistry.
5. The self-assembling protein capsid according to claim 3, wherein the capsid further comprises at least one mutant of the hepatitis B virus core protein capable of covalently crosslinking via disulfide bonds or other chemistry, and wherein said capability to covalently crosslink can be passivated.
6. The self-assembling protein capsid according to claim 1, wherein the capsid comprises at least one mutant of the hepatitis B virus core protein, and wherein said at least one mutant contains at least one insertion.
7. The self-assembling protein capsid according to claim 1, wherein the non-removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5**.

8. The self-assembling protein capsid according to claim 1, wherein the non-removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

9. The self-assembling protein capsid according to claim 1, wherein the removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

10. The self-assembling protein capsid according to claim 1, wherein the non-removable sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

11. The self-assembling protein capsid according to claim 1, further comprising incorporated sub-units, wherein said incorporated sub-units back fill the holey capsid.

12. The self-assembling protein capsid according to claim 11, wherein the incorporated sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity or 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion.

13. The self-assembling protein according to claim 11, wherein the incorporated sub-units are selected from the group consisting of: sequences that include a self-assembling component of about at least 60% identity or 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5**, wherein the sequences optionally include at least one insertion, and wherein the one or more insertions are one or more polypeptides selected from the group consisting of: receptors, receptor-binding molecules, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

14. The self-assembling protein according to claim 11, wherein the incorporated sub-units are bound to moieties selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins.

15. The self-assembling protein according to claim 1, wherein the removable subunits are passivated with N-Ethylmaleimide.

16. The self-assembling protein according to claim 15, wherein the removable subunits are removed from the capsid using a chaotrope.

17. A method for modifying a capsid comprising the steps of:

combining passivated and non-passivated sub-units, wherein said sub-units self-assemble to form the capsid;

crosslinking the non-passivated sub-units; and

removing at least one sub-unit from the capsid to form a holey capsid.

18. The method according to claim 17, wherein the capsid is a hepatitis B virus core protein capsid.

19. The method according to claim 17, wherein the removed sub-units are selected from the group consisting of: sequences that contain at least one component of about at least 60% identity to **SEQ. ID. NOS. 2, 3, 4, or 5.**

20. The method according to claim 17, wherein the removed sub-units are selected from the group consisting of: sequences of about at least 80% similarity to **SEQ. ID. NOS. 2, 3, 4, or 5.**

21. The method according to claim 17, further comprising the step of incorporating sub-units, wherein said sub-units back fill the holey capsid.

22. The method according to claim 21, wherein the sub-units are bound to a moiety selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins.

23. The method according to claim 21, wherein the sub-units are selected from the group consisting of: subunits with inserts that encode receptors, receptor-binding peptides, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

24. A method for identifying holey capsid incorporatable sub-units comprising the steps of:

- providing a holey capsid;
- providing a sub-unit, wherein said subunit comprises an identifiable marker;
- combining the sub-unit with the holey capsid;
- identifying the amount of sub-unit incorporated into the holey capsid; and
- comparing to a holey capsid control.

25. The method according to claim 24, wherein the capsid is a hepatitis B virus core protein capsid.

26. The method according to claim 24, wherein the sub-units are bound to a moiety selected from the group consisting of: organic and inorganic catalysts, receptors, receptor-binding signaling molecules, enzymes, toxins, dyes, metals, therapeutic molecules, immunogenic polypeptides, and fluorescent proteins.

27. The method according to claim 24, wherein the sub-units are selected from the group consisting of: subunits with insertions that encode receptors, receptor-binding peptides, enzymes, toxins, immunogenic polypeptides, and fluorescent proteins.

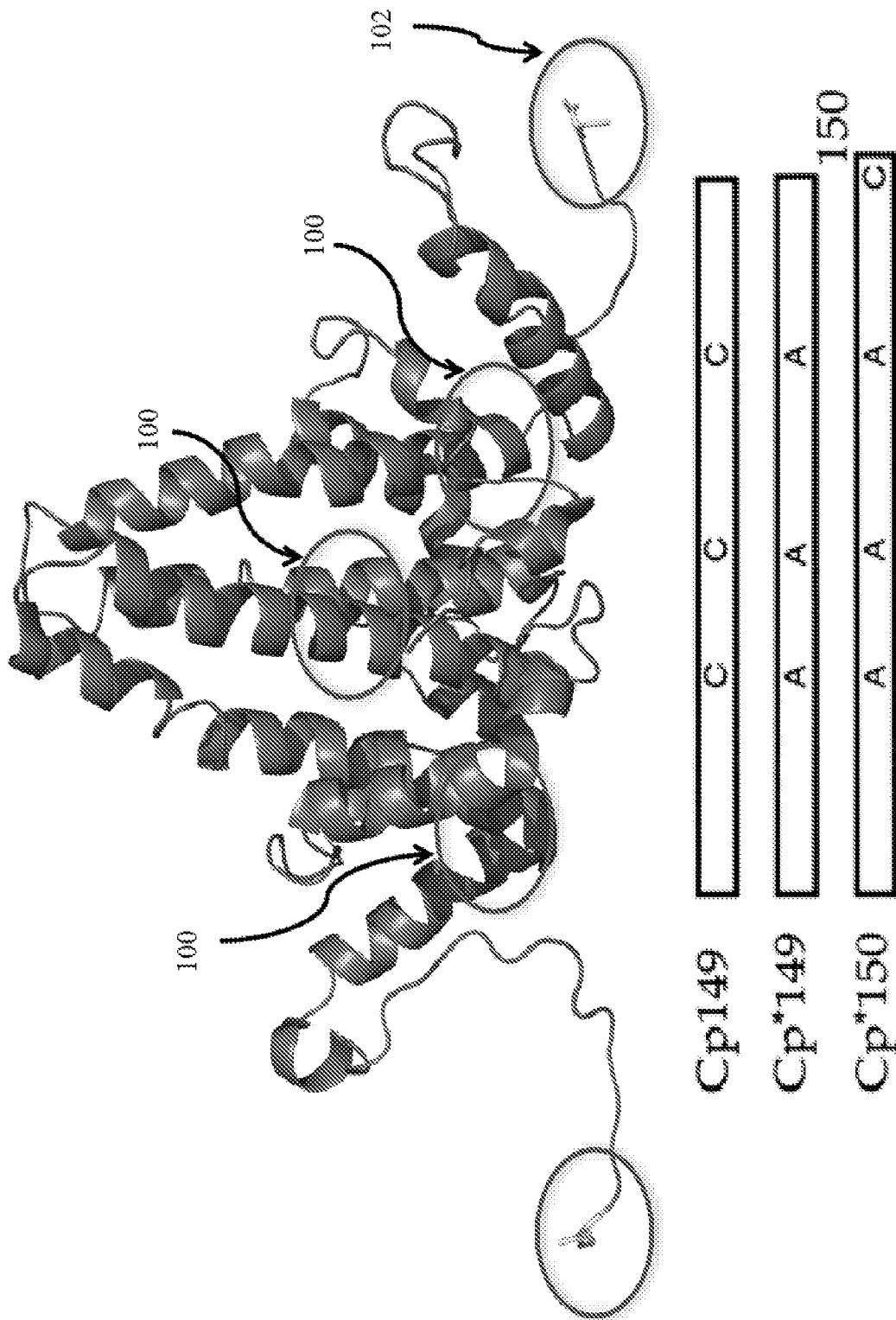


FIG. 1A

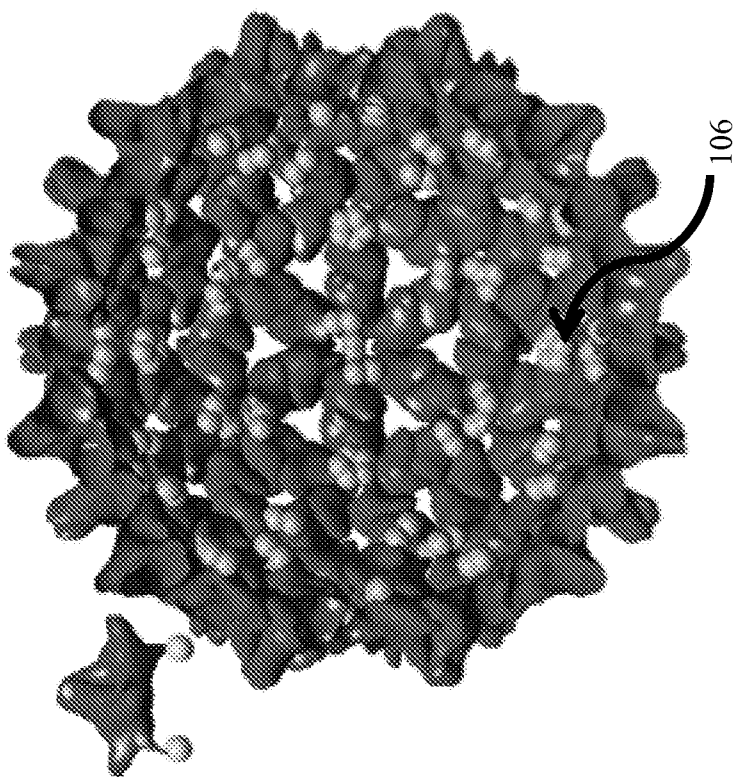


FIG. 1B

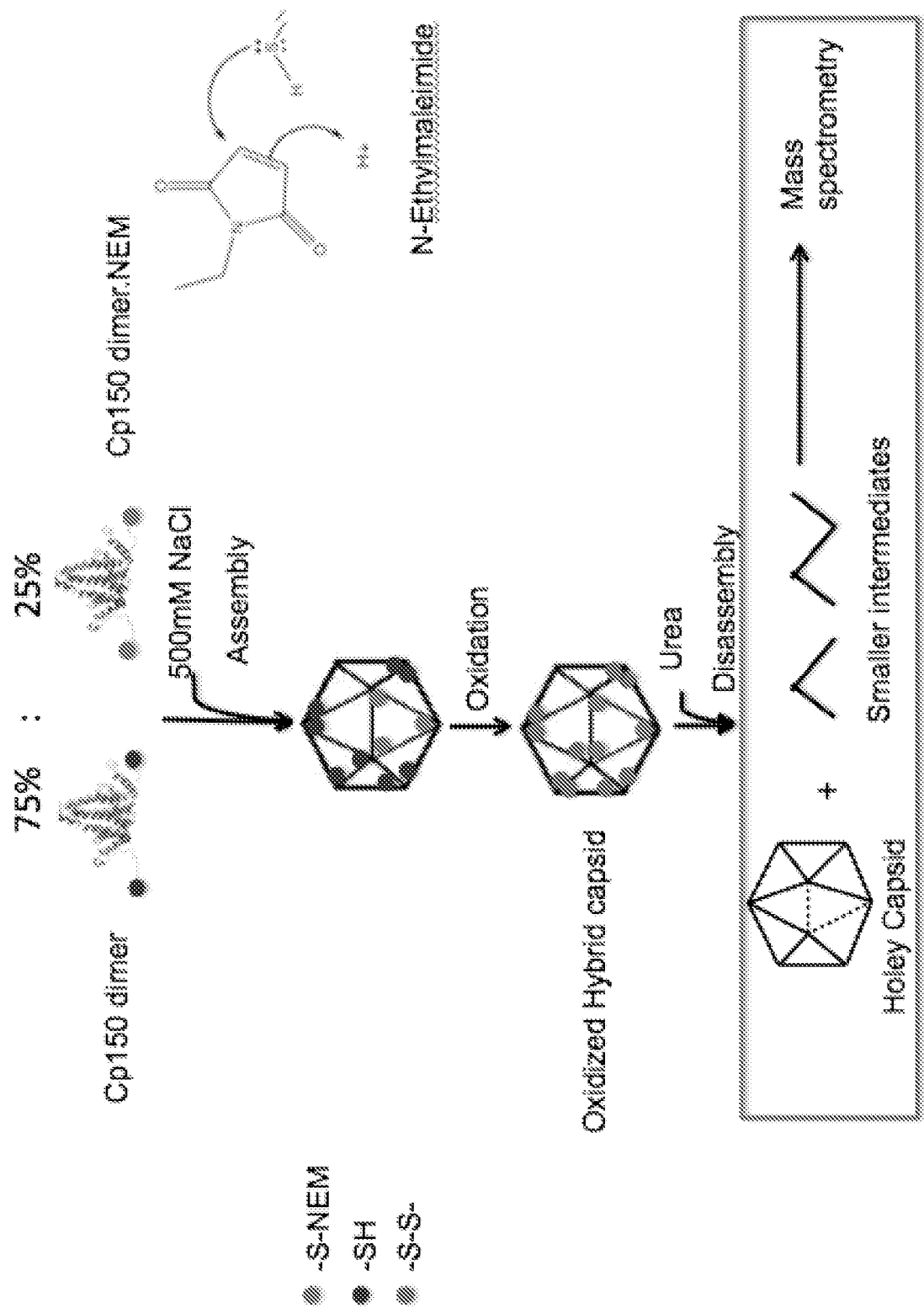


FIG. 2

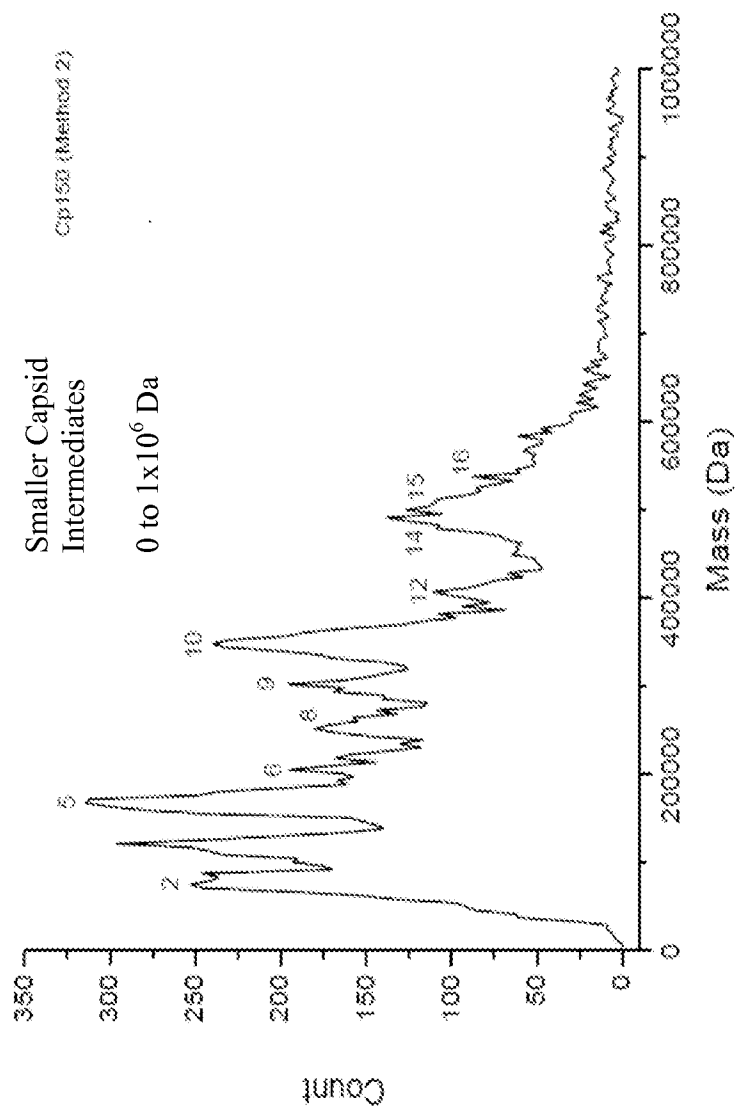


FIG. 3A

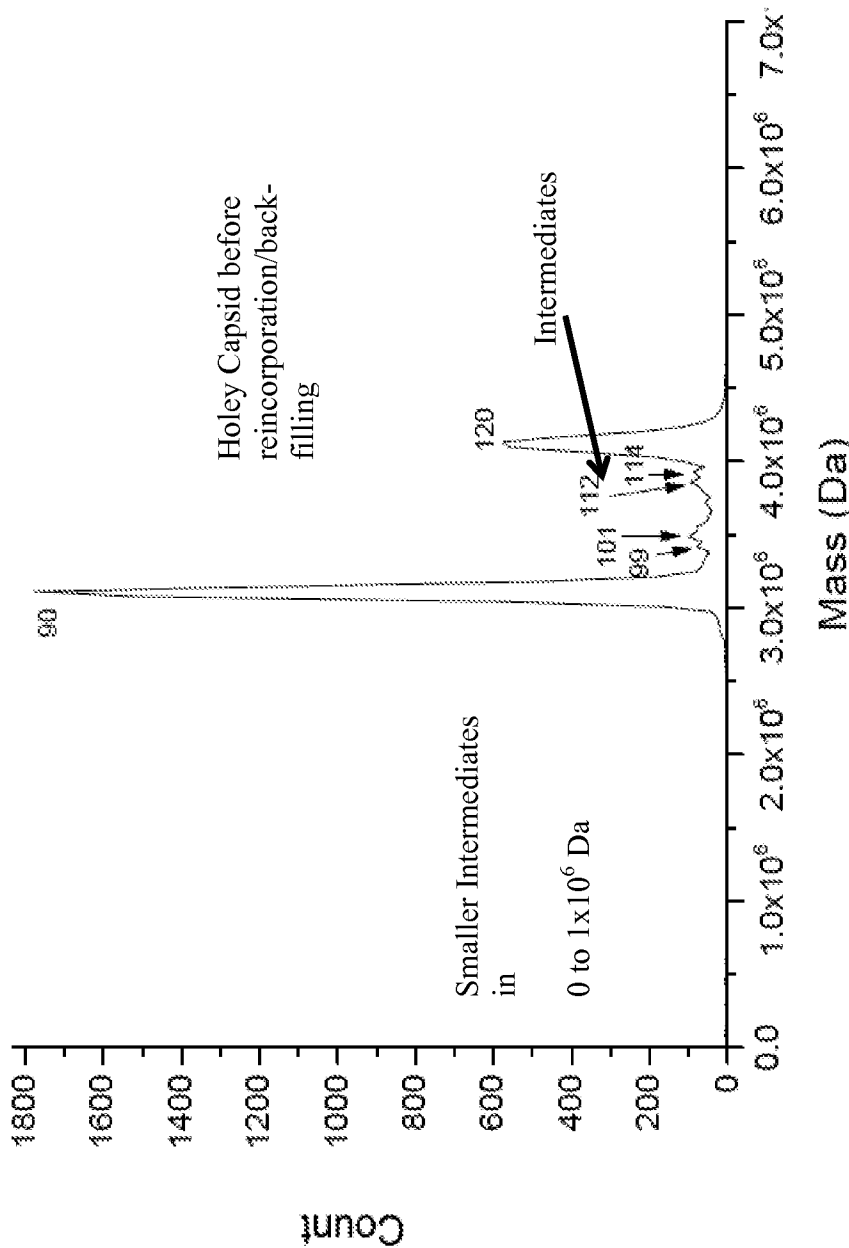


FIG. 3B

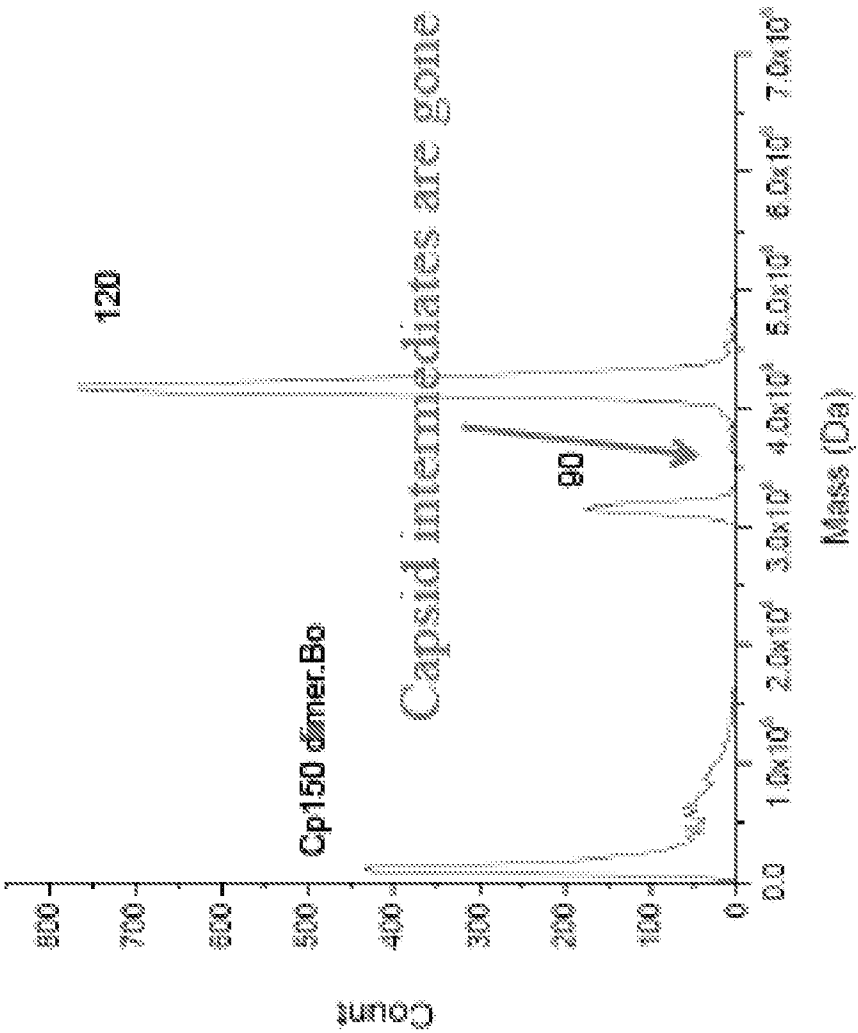


FIG. 3C

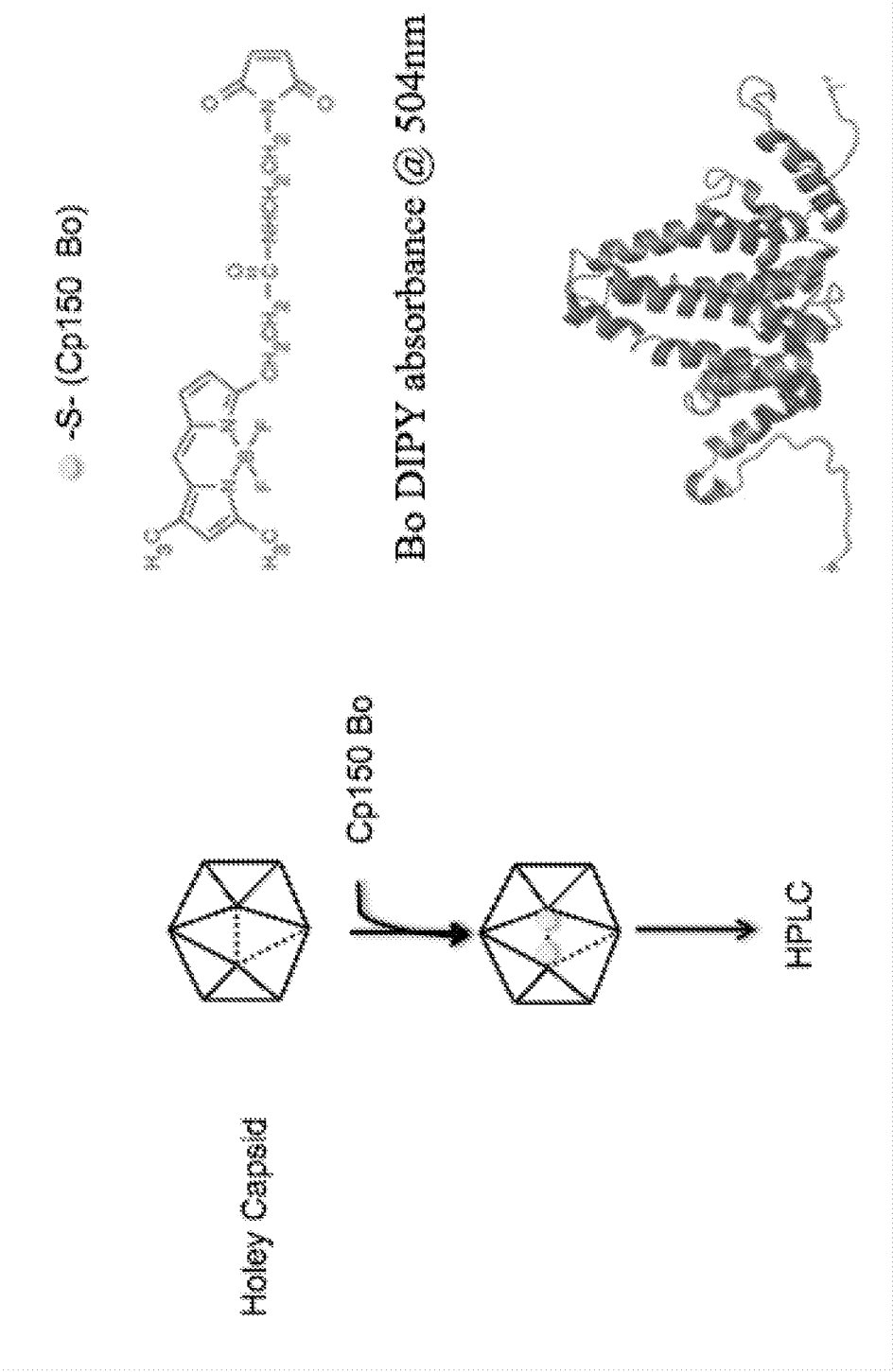


FIG. 4A

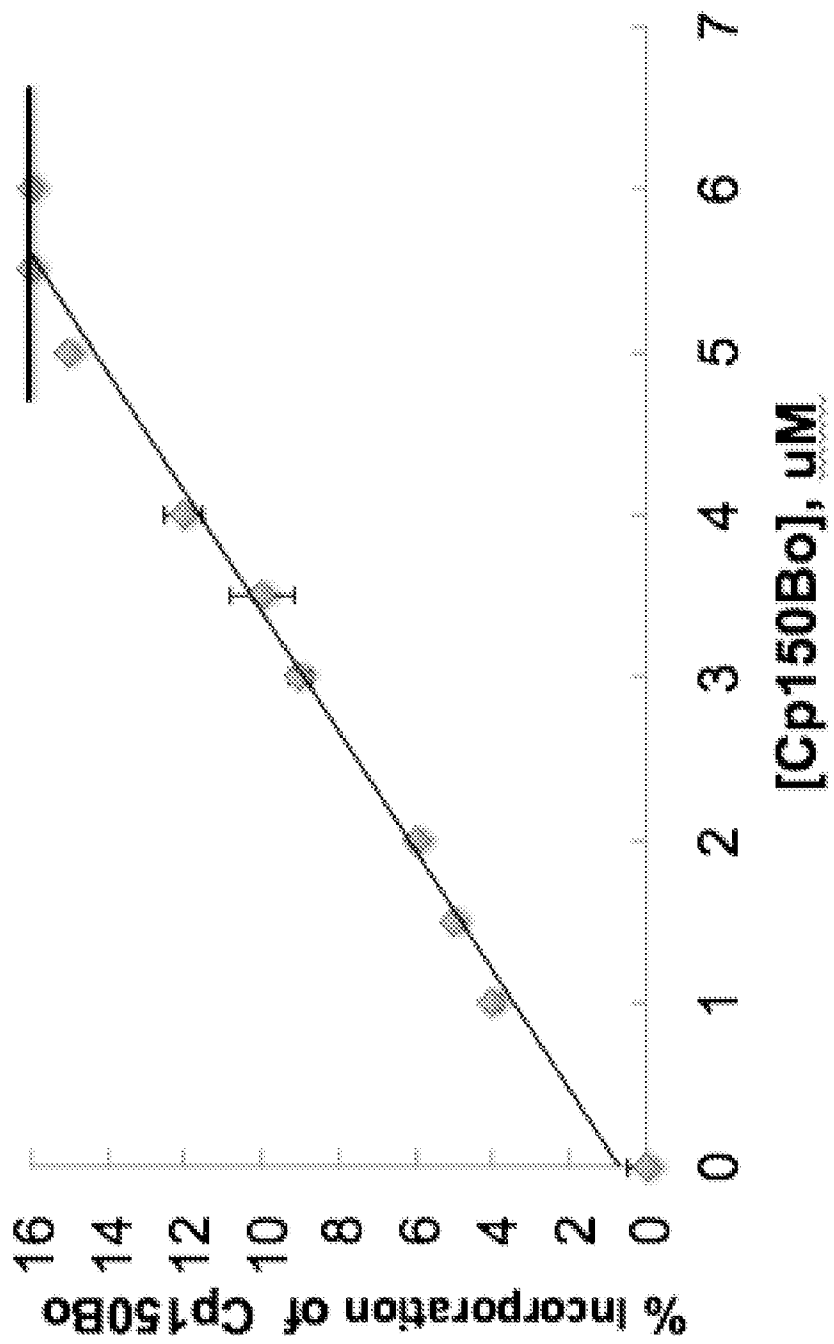


FIG. 4B

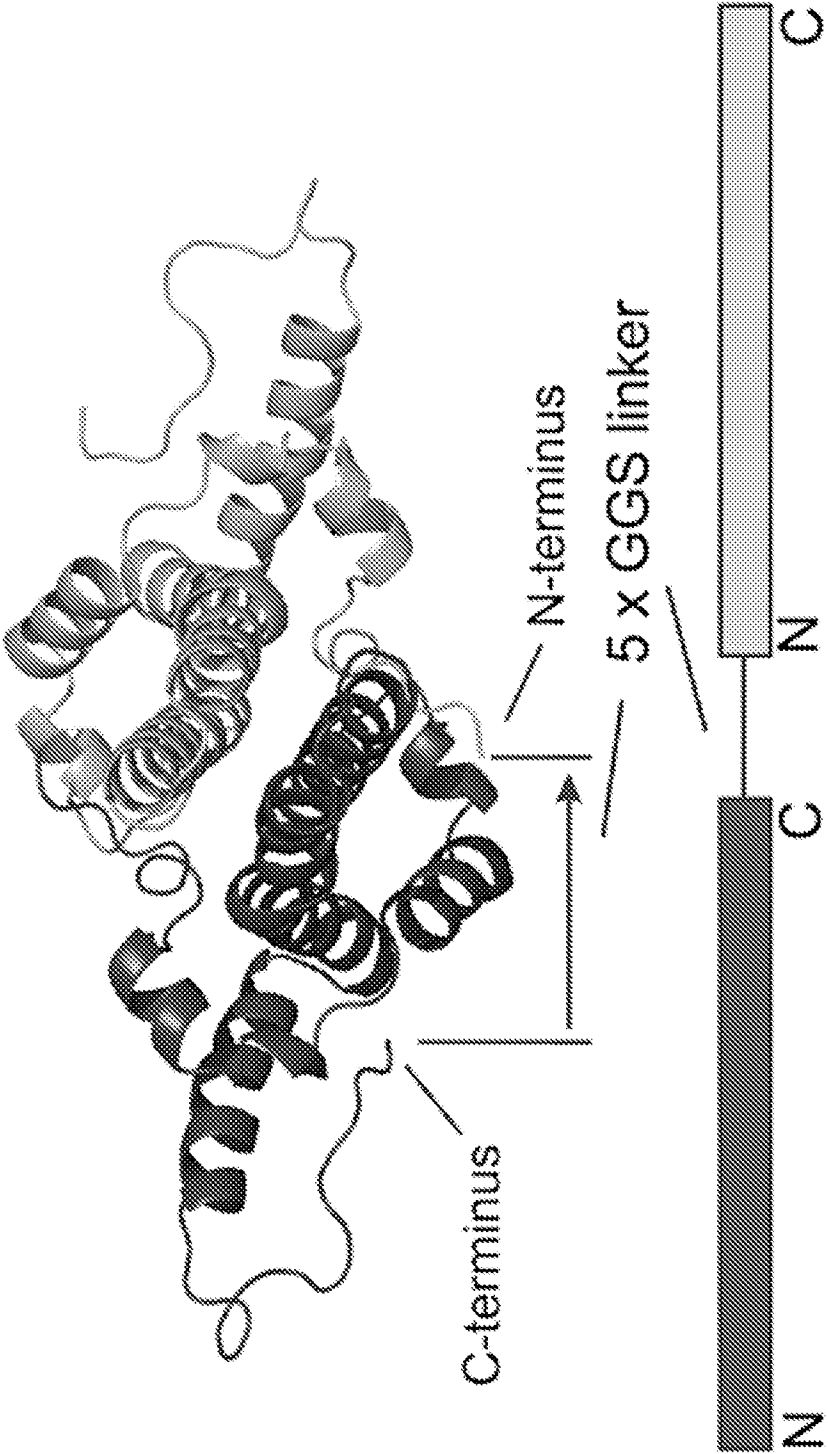


FIG. 5A

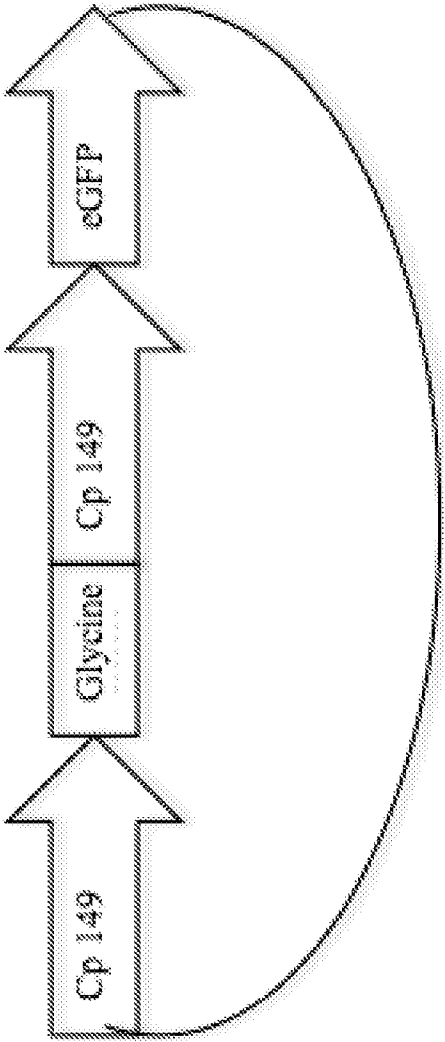
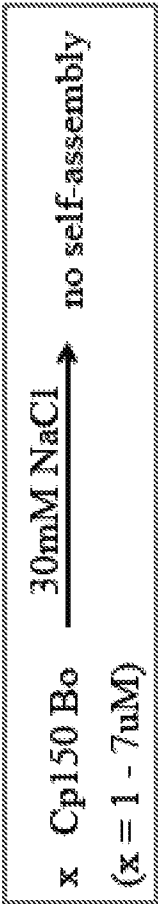


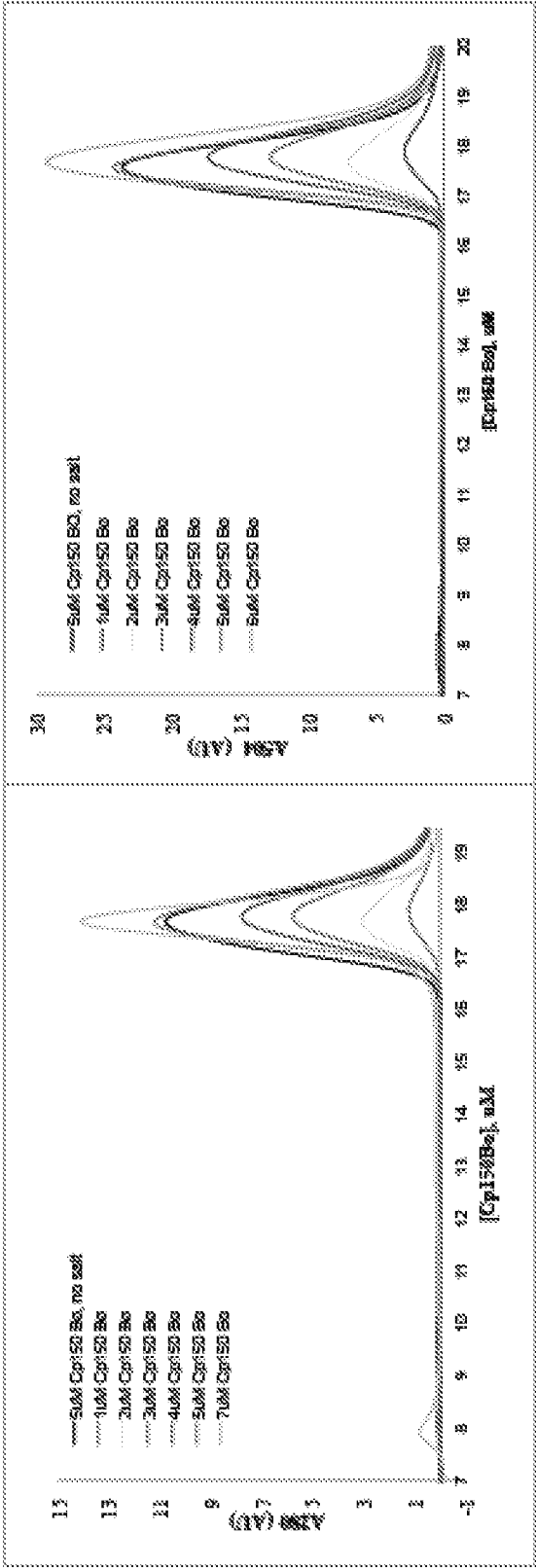
FIG. 5B

HBV assembly depends on protein concentration, ionic strength and temperature.



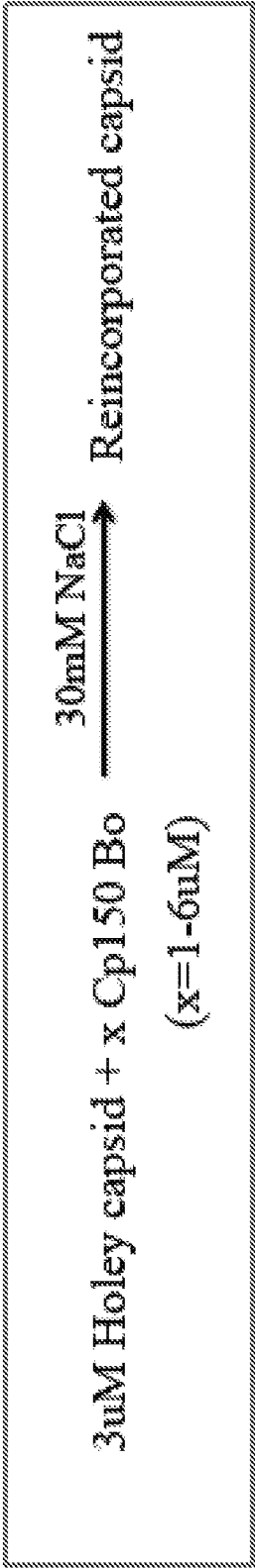
280nm

504nm



<7μM Cp150 Bo + 30mM NaCl show no self assembly reaction

FIG. 6A



280nm

504 nm

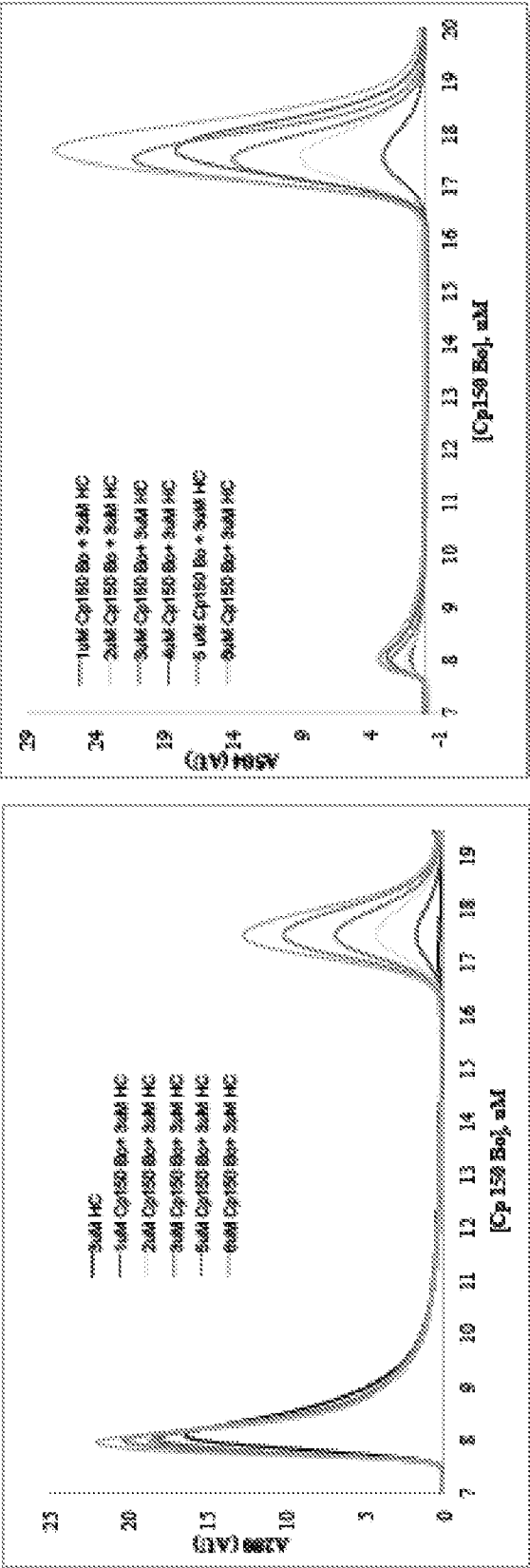


FIG. 6B

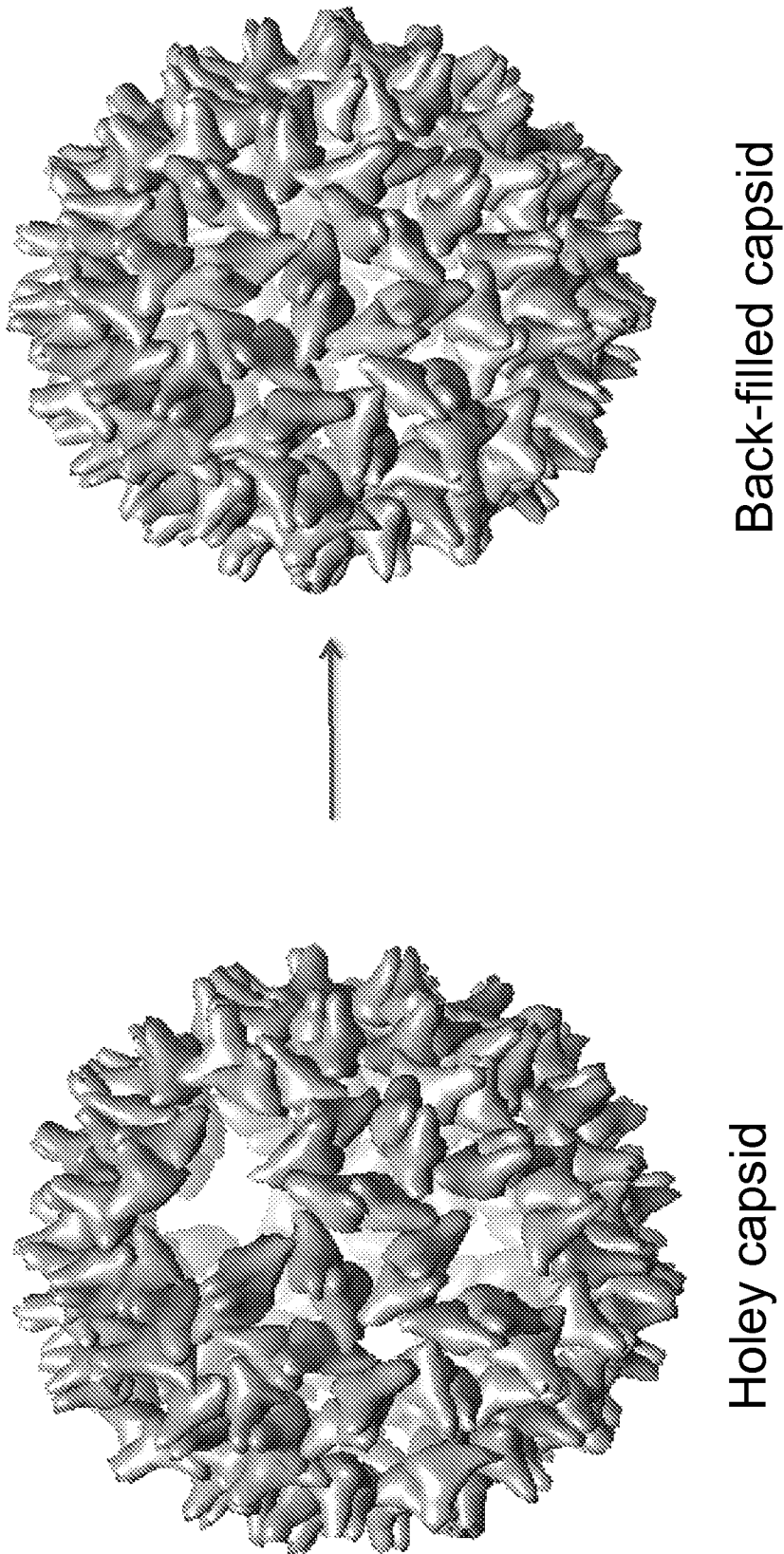


FIG. 7