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Engineering protein nanoparticles for drug delivery☆

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Protein nanoparticles offer a highly tunable platform for engineering multifunctional drug delivery vehicles that can improve drug efficacy and reduce off-target effects. While many protein nanoparticles have demonstrated the ability to tolerate genetic and posttranslational modifications for drug delivery applications, this review will focus on three protein nanoparticles of increasing size. Each protein nanoparticle possesses distinct properties such as highly tunable stability, capacity for splitting or fusing subunits for modular surface decoration, and well-characterized conformational changes with impressive capacity for large protein cargos. While many of the genetic and posttranslational modifications leverage these protein nanoparticle's properties, the shared techniques highlight engineering approaches that have been generalized across many protein nanoparticle platforms.

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Introduction

The use of nanoparticles as a platform for drug delivery has exploded since the first cancer nanomedicine (Doxil) was approved by the FDA in 1995 [1]. Nanoparticles can improve therapeutic efficacy by increasing bioavailability, increasing circulation times, reducing nonspecific toxicity, and enabling cell-specific targeting [2]. Protein-based nanoparticles are of particular interest in drug delivery because they can tolerate a wide range of genetic or posttranslational modifications while maintaining the ability to self-assemble into highly uniform structures [3]. These modifications include the external display of proteins, the internal loading of proteins, and the alteration of subunit–subunit interactions to modulate nanoparticle stability, properties, or behavior.

Many drug delivery platforms require the multifunctionalization of nanoparticles for targeting, loading, and/or controlled release, as shown in Figure 1. From this standpoint, protein nanoparticles are advantageous because genetic and posttranslational modifications enable the controlled surface display of proteins for cell targeting, significant improvements in drug loading [4,5], and stimuli-responsive conformational changes and disassembly for cargo release [6,7]. These properties, along with the multitude of unique structures, sizes, and surface properties, allow protein nanoparticles to be selected and further tuned for specific applications.

There are a multitude of promising protein nanoparticles that are amenable to direct functionalization via genetic or posttranslational modification. These platforms vary in size and origin, and many possess properties such as inherent stability, or thermal responsiveness that can be leveraged when engineering a multifunctional drug delivery platform. In this review, we will highlight engineering strategies used on three differently sized protein-based nanoparticles from viral, bacterial, and phage origin, each offering unique properties for drug delivery applications (Figure 2). While these protein-based nanoparticles are phylogenetically distinct, a toolbox of related genetic and posttranslational modification tools is available for their multifunctionalization.

E2 nanocage

A promising protein nanoparticle of bacterial origin is the E2 component of the pyruvate dehydrogenase multienzyme complex found in *Geobacillus stearothermophilus*. It is composed of 60 subunits that self-assemble into an icosahedral hollow nanoparticle (d=25 nm) with 12 pores (d=5 nm). Owing to its thermophilic origin, it has remarkable thermal stability at temperatures up to 85 °C [7].

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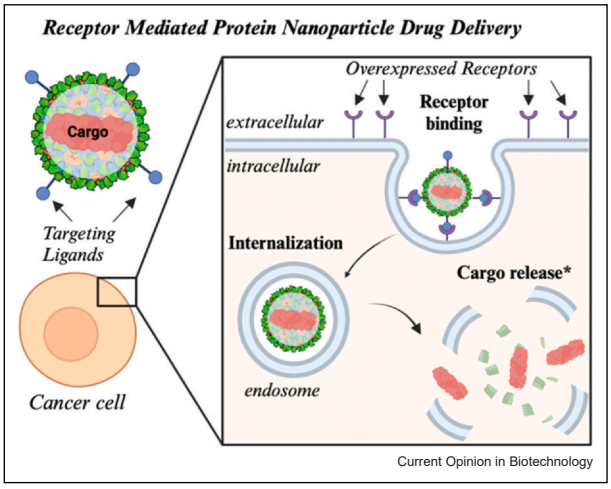
Figure 1



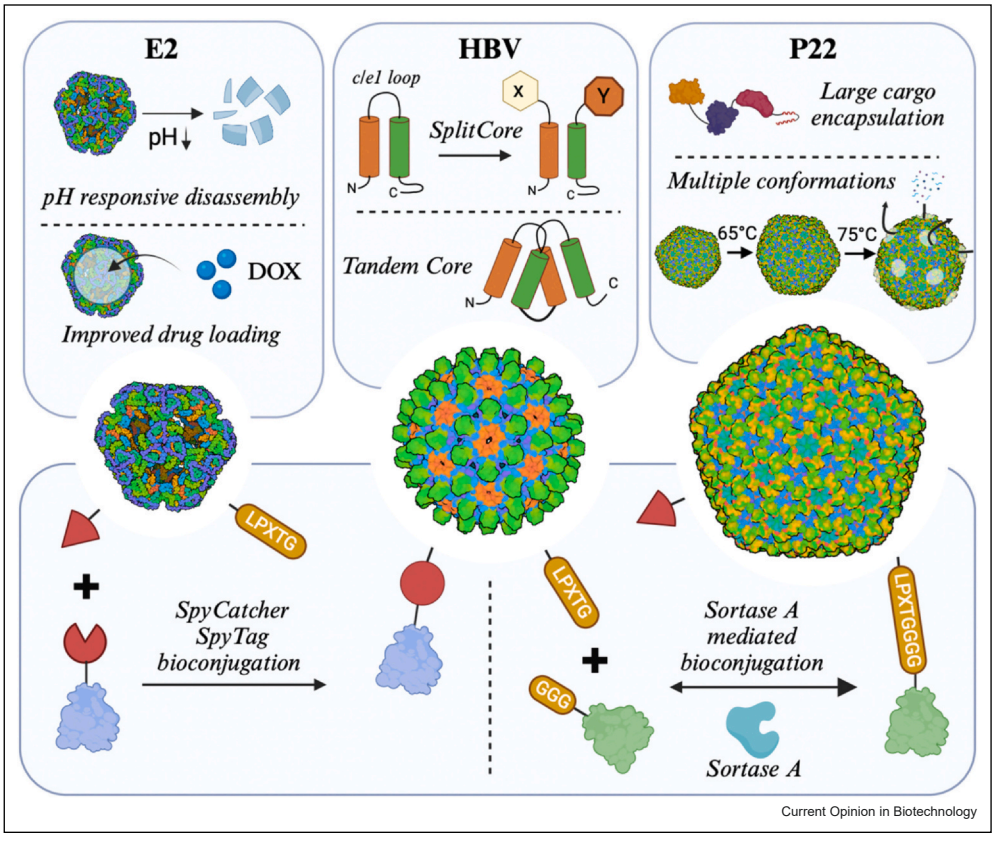
The exterior of protein nanoparticles decorated with targeting ligands that bind to overexpressed receptors on a target cell. Upon uptake, protein nanoparticles experience a pH change that either releases cargo via nanoparticle disassembly* or via another mechanism. Created with Biorender.com.

External modification

While direct genetic fusion of proteins such as green fluorescent protein (GFP) to the exposed N-terminus of E2 has been reported, all fusion proteins are insoluble and refolding is needed to recover the intact E2 structure [10]. To bypass this limitation, external modification of E2 was explored using sortase A (SrtA), which catalyzes the condensation reaction between a C-terminal LPXTG motif and an N-terminal triglycine tag, resulting in the formation of an amide bond [11]. A wide range of C-terminally tagged LPETG proteins were successfully decorated onto the surface of GGG-E2 nanocages, including the large tetrameric β-galactosidase protein, without impacting E2 stability and enzyme activity [11]. This strategy is not limited to proteins; chlorohexane-modified DNA aptamers and fluorescent dyes also have been decorated onto a surface-ligated HaloTag [12]. While robust, SrtA efficiency is limited by the enzyme's reversibility, resulting in limitations on the maximum decoration possible. In the case of LPETG-E2, only about 60% of the subunits can be decorated.

To improve decoration density, the N-terminus of E2 can be fused with SpyTag (ST) to allow external display

Figure 2



E2 has been engineered to disassemble at a pH of 5 for cargo release and has been engineered for improved small-molecule drug loading [4,7], The HBV subunit has been split to allow for dual- fusion display, and has also been fused for larger cargo loading [5,8], and P22 has demonstrated impressive loading potential and well-characterized conformational changes [9]. All have been engineered to enable SC/ST- and SrtA-mediated bioconjugation. Created with Biorender.com.

of SpyCatcher (SC)-fusion proteins with up to 100% efficiency [13,14]. Using SC/ST conjugation, a system capable of recruiting and displaying antibodies on E2's surface was developed by conjugating 60 copies of a fusion protein composed of the protein-A- derived Z-domain and 80 repeats of elastin-like polypeptide (ELP) to ST-E2 [13,14]. This system was used to quantify and purify mAbs; however, the same system could be used to target overexpressed receptors on cancer cells with receptor-specific antibodies [14]. ST-E2 was also decorated with GE11 peptides to target epidermal growth factor receptor (EGFR)-overexpressing cancer cells, resulting in enhanced and cell-specific intracellular uptake in IBC-SUM-149 breast cancer cells [15].

Currently, the SC/ST system is the most effective strategy for E2 surface decoration. Ramirez et al. (2023) [16] compared three approaches for displaying CBU190 antigen on E2, two of which resulted in unusable nanoparticles: direct fusion to the E2 subunit resulted in no E2 assembly, and Ni-NTA His chemistry to link CBU190 to the E2 subunit exhibited poor reaction efficiency and produced CBU190-E2 with low solubility. In contrast, SC/ST modification resulted in 100% E2 subunit decoration, highlighting SC/ST as a powerful posttranslational modification system [16].

Drug release and loading

Most small-molecule therapeutics are hydrophobic, a property that has been exploited to improve the loading capacity of E2 by introducing interior phenylalanine residues to increase the hydrophobicity of the inner E2 surface by up to 118% [4,15]. The increased hydrophobicity allowed up to 30-fold higher doxorubicin (DOX) loading compared with the wild-type E2, and 6-fold higher loading compared with traditional chemical conjugation of DOX to the subunit [4]. This E2 variant was capable of loading 390 DOX per nanocage (~13.4 wt %), outperforming most nanomedicines that typically achieve < 10 wt% loading [4,17]. Encapsulated DOX was released upon protonation in the low-pH environment of lysosomes, resulting in MDA-MB-231 breast cancer cell death [4].

Another promising approach to release interior cargo is to modulate E2's stability such that the nanocage disassembles in acidic environments. This behavior can be desirable for therapeutic delivery because nanoparticles typically experience a shift from pH 7.4 to pH ~5 during the cellular uptake and intracellular trafficking process [18]. Truncating the N-terminus of the monomer subunit weakens the intersubunit interactions, resulting in assembly at pH 7 and disassembly at pH 5 [18]. The disassembled subunits, however, form insoluble aggregates that could be problematic in a drug delivery setting [7]. An improved approach is to substitute key residues with clusters of histidine, which repulse each other when protonated [7]. E2 variants with histidine substitutions in the N-terminal region of the subunit assemble at pH 7 and disassemble at pH 5 without forming insoluble aggregates, offering a potential strategy for pH-inducible cargo release [7].

Multifunctionalization for selective drug delivery

Common goals for drug carriers are high cargo loading, a targeting ligand for uptake, and a mechanism for drug release. This has been achieved with both traditional chemical ligations and with the protein engineering toolbox outlined above. Traditional ligation approaches have been used to chemically conjugate DOX to an interior cysteine in E2, and simultaneously, to chemically ligate PEG–folic acid onto an exterior lysine, resulting in a bifunctional E2 nanocage that carries DOX into EGFR-overexpressing cells [19]. In other examples, four phenylalanine residues were introduced in the E2 interior to improve DOX loading, while ST was added for external conjugation. This approach yielded 30% higher DOX loading compared with traditional chemical ligation and offered selective breast cancer cell uptake when conjugated with SC-linked protein conjugates targeting EGFR (SC-mCherry-$(GE11)_4$) [15]. This fusion protein simultaneously added multiple functionalities, enabling straightforward analysis of cell uptake by measuring mCherry, multivalent EGFR targeting by engaging the high-density clusters of GE11 EGFR-targeting peptide.

Hepatitis-B viral capsid

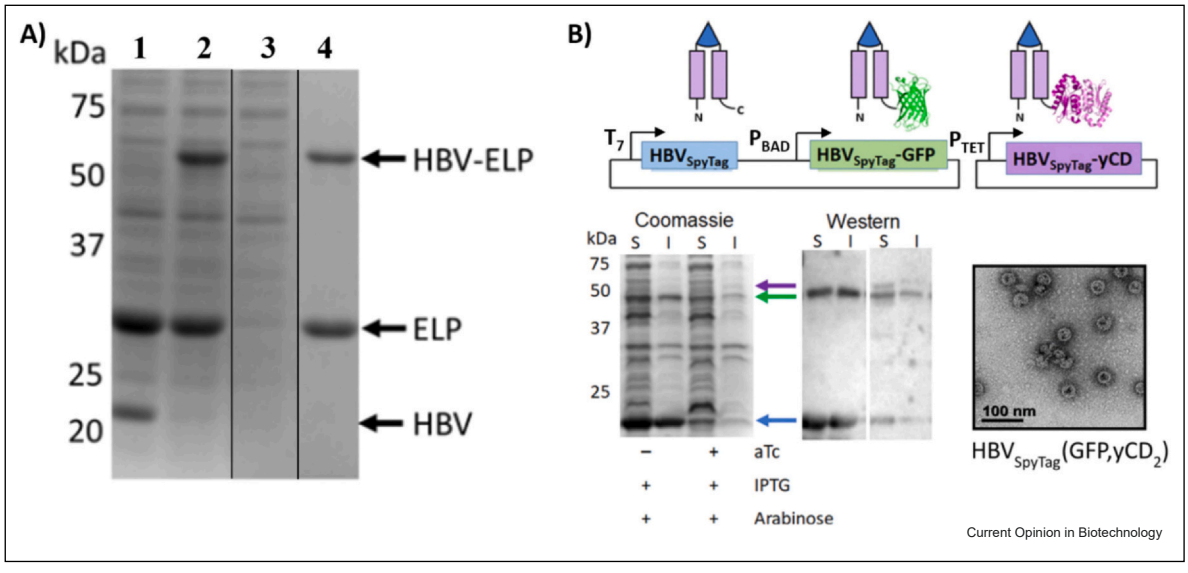
The native hepatitis-B virus (HBV) capsid is made up of a 183-amino acid viral core antigen (HBcAg) comprising a capsid assembly domain, a hinge linker region, and an unstructured arginine-rich protamine domain. Removal of the latter domain abolishes both nonspecific RNA encapsulation during recombinant expression [20], and nonspecific interactions with heparin sulfates, which are critical for cell-type-independent endocytosis [21]; this domain removal gives rise to the more commonly used 149-residue-truncated HBcAg (tHBCAg). tHBcAg and HBcAg capsids have a predominant conformation of T4 capsid of 240 subunits with an exterior diameter of about 36 nm, and a lower proportion T3 capsid of 180 subunits with an exterior diameter of 32 nm [22]. Both capsid conformations have identical intradimer interactions forming a four-helix bundle across dimeric alpha-helical hairpins resulting in surface-exposed exterior spike domains as a characteristic feature of HBV capsids. Each spike domain contains the immunodominant c/e1 B-cell epitope overlapping with the connecting loop of the alpha-helical hairpins. While this domain is primarily responsible for the antigenicity of HBV, it also used as the primary site for surface display through association with weak peptide-binding domains [23–25] or through genetic fusion and posttranslational ligation into the c/e1 loop.

Surface display

Monomeric proteins with spatially close termini have been directly genetically fused into the surface-exposed c/e1 loop [26], while proteins with distant termini can be fused by flanking them with flexible linkers often resulting in heterogeneous and irregular HBV capsids [27]. Proper capsid assembly can be recovered by cleaving one of the peptide bonds connecting an unfavorable c/e1 loop insert to the HBcAg backbone [28]. This observation inspired the development of the SplitCore surface display platform where the tHBcAg or HBcAg backbone is split after the 79th residue at the center of the c/e1 loop and expressed bicistronically as coreN and coreC fragments [8]. This allows for one or two proteins to be displayed as a genetic fusion to the C-terminus of coreN and/or the N-terminus of coreC. SplitCore capsids favor the smaller T3 symmetry particles compared with full-length capsids and have a significant depreciation in particle yield and stability. These effects can be attributed to differences in the relative expression between the two split fragments, which are predominantly dependent on the fusion partner and the partial read-through seen in expression of the two fragments that can result in their linkage together [8].

To decouple the effect of the inserted protein on particle yield, efforts have been made to minimize the size of the fusion partner in expression by moving to a posttranslational modification display approach. A biotin-decorated HBV capsid using a coreN-fusion to a biotin-acceptor peptide and co-expression of a BirA ligase [8] was used to display streptavidin fusion proteins with monomeric biotin-binding sites [29]. Additionally, sortase-mediated ligation and intein-mediated trans-splicing ligation platforms have been developed using a coreN-LPETGG fusion [30] and coreN-int^{N} fusion with a gb1 solubility tag [31], respectively. However, both ligation techniques only achieve 60–70% efficiency or require significant excess of the ligation partner to approach 90% decoration density. More recently, Hartzell et al. and Yur et al. developed SC and ST c/e1 loop genetic fusion tHBcAg capsids, respectively [32,33]. Both were able to achieve up to 100% decoration using the irreversible SC–ST ligation without using the SplitCore system or a large excess of reactant. They also demonstrated that with just a small surface density of ELP, the ligated capsids could be completely purified using inverse-transition cycling (ITC), providing a simple purification scheme to isolate capsids prior or proceeding further surface functionalization, as shown in Figure 3a [32,33]. While the decoration efficiencies of all methods are protein-dependent, the SC and ST c/e1 loop genetic fusion tHBcAg capsids are the most effective platforms for high-density posttranslational surface decoration to date, and provide a good complement to direct c/e1 loop genetic fusions and SplitCore display platforms. Currently, these three surface decoration methods and the peptide-binding HBV c/e1 wild-type loop surface display method have been utilized to display RGD peptides [34–36], EGFR and HER2 affibodies [37,38], folic acid [39], an EGFR-targeting DARPin [33], and different cellular uptake peptides

Figure 3



Exterior and interior modification of ST-decorated HBV. **(a)** ITC purification of ELP-ligated HBV capsids. Lane 1: Ligation of ELP–SC and HBV–ST capsids at t = 0. Lane 2: Overnight ligation of ELP–SC and HBV–ST capsids. Lane 3: Soluble fraction following hot-spin of the first inverse-transition cycle. Lane 4: Purified ELP-conjugated HBV. **(b)** SDS-PAGE with corresponding western blot and transmission electron microscopy of HBV tri-expression system for tunable interior loading of both GFP and yeast cytosine deaminase.
Reprinted with permission from Ref. [33].

[32,40] and cell-penetrating peptides [24,41] to facilitate cellular uptake for protein and drug delivery.

Protein loading

Protein loading into the HBV capsid is primarily done with genetic fusion to the C-terminus of a tHBcAg monomer, which has been demonstrated for both a small 19-kDa siRNA-binding p19 monomer [34] and a 17-kDa nuclease [42]. Loading of the former allows encapsulation of siRNAs with greatly improved stability compared with free siRNA, and the pH-dependent binding interaction allows cytosolic release of siRNA molecules when delivered to B16F10 melanoma cells with RGD-decorated HBV capsids [34,35]. However, more space is required to overcome steric limitations when fusing larger proteins inside the capsid. To create more space by decreasing the number of termini per capsid, a TandemCore tHBcAg platform was developed by linking the C-terminus of one monomer to the N-terminus of another via a flexible linker — halving the number of termini and effectively doubling the space available for each loaded protein [5]. This also allowed heterogeneous surface display through different genetic fusions at the two c/e1 loops per fused monomer; moreover, larger genetic fusions are possible by fusing at only one of the two c/e1 loops. However, the TandemCore platform does show a significant reduction in particle yield compared with other HBV platforms [5]. Further efforts to tune the percentage of functionalized C-termini have focused on creating mosaic particles through *in vitro* co-assembly of functionalized and nonfunctionalized subunits [43]. To better control the composition of the particle while avoiding disassembly and reassembly, Yur et al. implemented a three-promoter expression system that allowed tunable loading of two different proteins in each capsid [33]. This permitted simultaneous and tunable incorporation of both GFP and a prodrug-converting yeast cytosine deaminase (Figure 3b), which enabled cell-selective cytotoxicity in EGFR-overexpressing MDA-MB-468 triple-negative breast cancer cells using a surface-displayed EGFR-targeting designed ankyrin repeat protein (DARPin) [33].

P22 nanocage

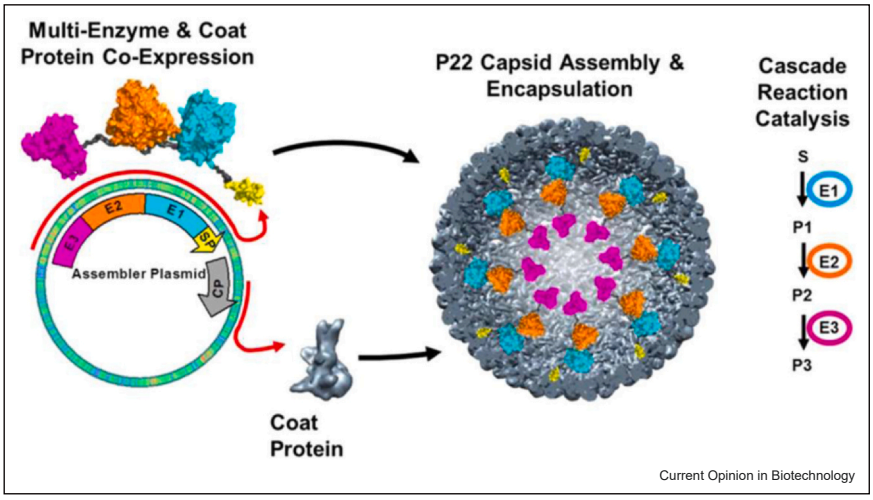
The bacteriophage P22 is a promising virus like nanoparticle (VLP) with impressive cargo loading capabilities. This nanoparticle stands out from others due to two specific properties: an internal scaffold subunit capable of loading large cargo, and the ability to undergo a series of well-defined conformational changes. The P22 nanoparticle consists of 420 capsid proteins (CP) and 100–300 scaffold proteins (SP), which noncovalently aid in assembly [44]. The nanoparticle initially assembles as a 56–58-nm procapsid (PC) that contains 12 pores (d=5 nm), but heating to 65 °C results in the PC expanding into an expanded (EX) conformation that is 62 nm in diameter and maintains the 12 pores. Further heating EX to 75 °C results in 12 pentameric units of the CP falling out, leaving behind the final stable conformation known as wiffle ball (WB), a structure that has a diameter of 62 nm and contains 12 pores (d=10 nm) [45]. With two separate modifiable subunits and three distinct conformations with varying properties, P22 is an exciting platform for an array of nanomedicine applications.

Therapeutic loading and release

While both CP and SP subunits are amenable to modification, the SP subunit stands out in terms of biochemical tunability. Both the N- and C-terminus can be truncated and genetically fused to a protein of interest. When co-expressed with CP, such fusion proteins are loaded into the capsid interior during P22 assembly, or less commonly, CP- and SP-fusion proteins may be expressed separately and assembled using an *in vitro* assembly approach in the cell lysate [46]. A benefit of using the SP-fusion strategy for P22 loading is its ability to rescue the solubility of proteins that are prone to aggregation, such as hemagglutinin (HA) head and α-galactosidase [47].

The loading efficiency of SP-fused cargos varies greatly depending on the cargo; however, high levels of loading can generally be achieved due to the large size of P22. Some notable fusions to SP include Cas9 proteins that maintained functionality [48], a variety of therapeutic peptides including the nonopioid analgesic ziconotide peptide MVIIA [49], and large antigens that typically would have poor nanoparticle loading due to their size or multimeric structure, such as respiratory syncytial virus M/M2 fusion protein that forms a large quaternary structure (70 kDa per M/M2-SP protein, up to 157 M/M2-SP/P22) [50]. The large internal cavity also has attracted much interest as a nanoreactor platform; while most of this work is outside the scope of this review, there are some therapeutic applications. For example, the prodrug-activating enzyme CYP_{BM3} was fused to SP resulting in a nanoparticle (109 CYP-SP/P22) that could be co-delivered to breast cancer cells along with tamoxifen for activation upon cellular uptake [51]. Enzymatic activity in P22 can uniquely be modulated by co-loading wild-type SP along with the enzyme SP, providing a direct lever to tune up or down enzymatic activity [46]. Multiple enzymes can simultaneously be loaded into P22 allowing for the confinement of enzymatic pathways, for example, 15 copies of a 166-kDa fusion protein composed of galactokinase, glucokinase, and B-glucosidase fused to SP were loaded within P22 shown in Figure 4. While the activity of these enzymes lacks a therapeutic application, they demonstrate that the loading capacity of P22 may be the largest cargo capacity demonstrated in a VLP to date [9].

Figure 4



A three-enzyme cascade was loaded into P22, by fusing galacotokinase, glucokinase, and B-glucosidase to the N-terminus of SP. Co-expressing the fusion SP with CP resulted in a self-assembled nanobioreactor containing 15 copies of the 166-kDa cargo.
Reprinted with permission from Ref. [51].

Capsid protein fusion

Once assembled, the C-terminus of CP is externally displayed, while the N-terminus is internally displayed — both are amenable for genetic fusion. A benefit of CP fusion is it results in 420 copies per P22 unlike the variable loading with SP-fusion proteins; however, there are more significant size and charge limitations to consider. The N-terminus of CP has been fused with synergistic therapeutic peptides KLAK and NuBC, which were cleaved off by intracellular cathepsin upon RGD-mediated uptake in breast cancer cells [44]. The positively charged tandem peptides hindered CP assembly, but by fusing a negatively charged protein (enhanced GFP) to SP, assembly was rescued and the 420 tandem peptides were successfully loaded into P22, demonstrating the platform's flexibility [44]. The C-terminus of CP has been modified with recognition motifs ST and LPETG for SC and sortase-mediated conjugation, respectively.

Sortase-mediated conjugation to CP-LPETG also is a viable approach. In one example, 140 GG-GFP and 183 GG-HA head antigen were decorated on the surface of P22 PCs [52]. As previously discussed, the reversibility and sterics of the SrtA system prevents high-efficiency conjugation. Alternatively, CP-ST has also been explored for external decoration of P22. Unlike ST-E2 and ST-HBV, 100% conjugation has not been achieved on ST-P22; however, due to the large number of ST subunits, P22 can display a comparable number of SC proteins as compared with HBV and up to 3.8-fold more than E2. Using ST-P22, 230 copies of the 39-kDa HA head influenza antigen were conjugated onto the PC [53]. Additionally, the EGFR and HER2 affibodies (Afb) have both been conjugated onto the WB conformation; when solubility issues arose when more than 150 Afb/P22 were conjugated, it was hypothesized that excess Afb leads to clusters on the surface that destabilize the capsid [45]. This HER2Afb/EGFRAfb-P22 was loaded with a DOX prodrug that contained a pH-cleavable hydrazine bond resulting in targeted uptake and cargo release in HER2- overexpressing SK-BR-3 cancer cells and EGFR-overexpressing MDA-MB-468 cancer cells [45].

Last, P22 can utilize a trimeric decoration protein (DEC) derived from bacteriophage L to display proteins on its exterior. DEC noncovalently binds up to 240 sites on P22 with as strong as 0.225 nM affinity, resulting in nanoparticle stabilization; the C-terminus of DEC also is available for displaying functional fusion partners [54]. DEC has been used to display the 21-residue SELF peptide, which blocks macrophage recognition and increases P22 circulation time; the 17-kDa soluble region of CD40L, which acts as an adjuvant and recruits B lymphocytes; and a His6 tag, which enables GFPthe conjugation of gold nanoparticles with Ni-NTA chemistry [54,55].

Conclusions and future outlook

Protein nanoparticles remain a burgeoning drug delivery platform due to their amenability to multifunctionalization, resulting in a diverse class of highly specialized carriers for targeted delivery. While there are an abundance of protein nanoparticles being engineered, such as ferritin and encapsulin, E2, HBV, and P22 encompass a broad range of sizes, distinct properties, and demonstrated engineering approaches that are translatable to other protein nanoparticles. It is particularly interesting to see what new functionalities could be created using protein nanoparticles

generated from de novo computation design, as they present geometries and sizes not seen in native systems. With the FDA continuing to approve new protein nanoparticles, it is clear they are an increasingly powerful platform to address many of the current shortcomings in modern drug delivery [2].

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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