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Research review paper

The biomedical and bioengineering potential of protein nanocompartments

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ABSTRACT

Protein nanocompartments (PNCs) are self-assembling biological nanocages that can be harnessed as platforms for a wide range of nanobiotechnology applications. The most widely studied examples of PNCs include virus-like particles, bacterial microcompartments, encapsulin nanocompartments, enzyme-derived nanocages (such as lumazine synthase and the E2 component of the pyruvate dehydrogenase complex), ferritins and ferritin homologues, small heat shock proteins, and vault ribonucleoproteins. Structural PNC shell proteins are stable, biocompatible, and tolerant of both interior and exterior chemical or genetic functionalization for use as vaccines, therapeutic delivery vehicles, medical imaging aids, bioreactors, biological control agents, emulsion stabilizers, or scaffolds for biomimetic materials synthesis. This review provides an overview of the recent biomedical and bioengineering advances achieved with PNCs with a particular focus on recombinant PNC derivatives.

1. Introduction

In living organisms, subcellular compartmentalization serves various physiological roles including the housing of nucleic acids, cell signalling, sequestration of metabolic processes, and biomineralization. To accommodate such diverse functions, biological nanocompartments are highly variable in size, structure, and composition. For example, many are lipid-based, as in the case of eukaryotic exosomes and membrane-bound organelles, whereas others are protein-based, including viral capsids, **b**acterial **m**icro**c**ompartments (BMCs), and eukaryotic vault ribonucleoproteins. Inspired by these natural nanocage architectures, recombinant or entirely synthetic nanocompartments are now being engineered as novel platforms for a wide range of biomedical and nanobiotechnology applications.

Of these various biological nanoparticles, **p**rotein **n**ano**c**ompartments (PNCs) have many distinct advantages over competing chemically synthesized, polymer, or lipid nanoparticles (Cho et al., 2008; Faraji and Wipf, 2009; Molino and Wang, 2014; Yan et al., 2015). Among the broad range of PNCs that will be discussed in this review, each with uniform size, structure, and composition, most are remarkably monodisperse and the protein subunits can often self-assemble *in vivo* or *in vitro* with high cargo encapsulation efficiency (Aniagyei et al., 2008; Künzle et al., 2018). Assembled PNCs or their component proteins can be produced in a variety of expression systems including bacterial, plant, insect, yeast, and mammalian cells, or even cell-free expression systems, each having their own advantages and disadvantages depending on the intended application (Bundy et al., 2008; Diaz et al., 2018; Fuenmayor et al., 2017; Huang et al., 2017b; Sheng et al., 2017). Specific to their potential as *in vivo* delivery vehicles, PNCs are biocompatible, protect their cargo from degradation (Peng et al., 2010), and can be selectively targeted to defined cell types *via* exterior conjugation to various targeting ligands (Ashley et al., 2011; Li et al., 2013; Stephanopoulos et al., 2010) or by exploiting the native tropism of a viral capsid (Lee et al., 2019). In fact, multiple PNC surfaces are amenable to genetic or chemical functionalization, including the exterior and interior surfaces and the subunit interfaces, which can be further used to modify cargo type, enhance cargo encapsulation, or facilitate cargo release (Buehler et al., 2014; Diaz et al., 2018; Rohovie et al., 2017). Finally, PNCs are highly repetitive structures and the appropriate size to both induce potent immune responses

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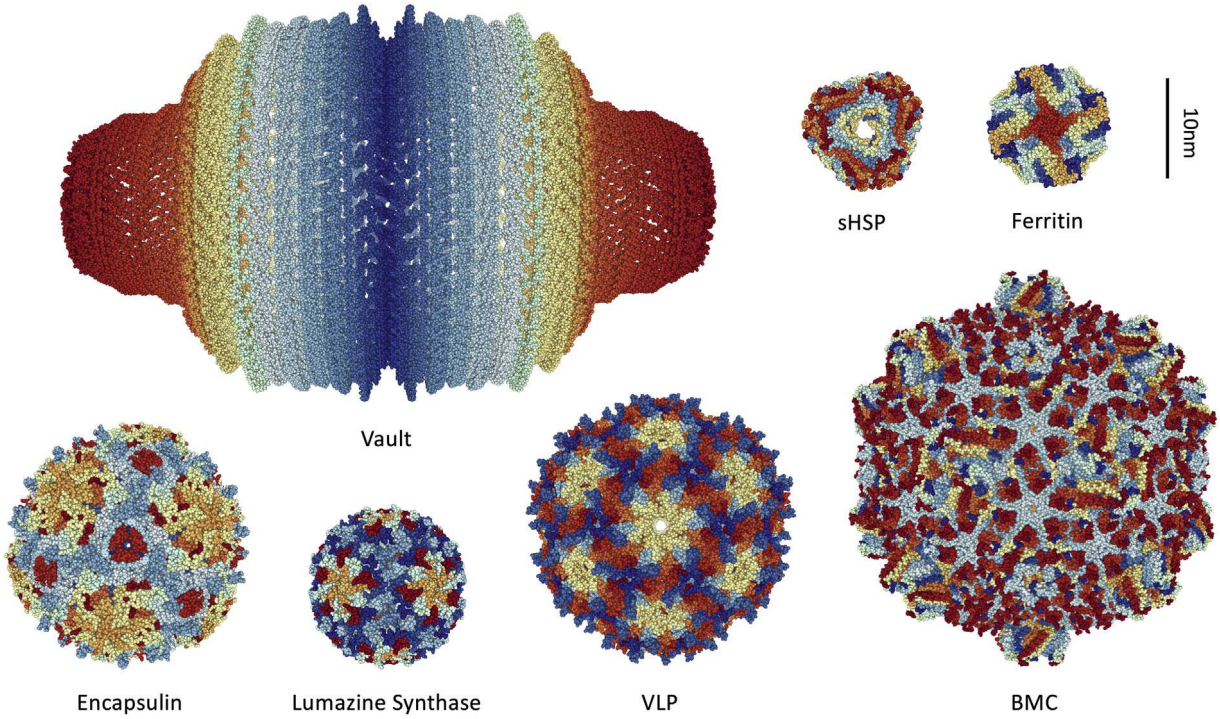

Fig. 1. Size and structural comparison of typical examples of biological protein nanocompartments. From top left to bottom right, the *Rattus norvegicus* major vault protein shell (PDB ID: 2QZV; (Anderson et al., 2007)); *Methanococcus jannaschii* sHSP (PDB ID: 1SHS; (Kim et al., 1998a)); *Escherichia coli* ferritin, EcFtnA (PDB ID: 1EUM; (Stillman et al., 2001)); *Thermotoga maritima* encapsulin nanocompartment (PDB ID: 3DKT; (Sutter et al., 2008)); *Aquifex aeolicus* lumazine synthase (PDB ID: 1HQK; (Zhang et al., 2001)); bacteriophage MS2 VLP (PDB ID: 7MSF; (Rowsell et al., 1998)); and the *Haliangium ochraceum* BMC shell (PDB ID: 5V74; (Sutter et al., 2017)). Space filling models (with a rainbow spectrum indicating subunits from blue N-terminus to red C-terminus) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) using the NGL Viewer (available online at www.rcsb.org) (Berman et al., 2003; Berman et al., 2000; Rose et al., 2018; Rose and Hildebrand, 2015) (Table 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(for use as vaccines and immunotherapies (Bachmann and Jennings, 2010; Mohsen et al., 2017; Slutter and Jiskoot, 2016) and to be taken up via the endocytotic pathway (Albanese et al., 2012), notably enabling transcytosis of the blood-brain barrier and overcoming this significant obstacle to efficient drug delivery to the brain (Anand et al., 2015). Altogether, PNCs are highly versatile, modular platforms that can enhance the efficacy and specificity of a variety of applications, while also often being relatively simple to bioengineer compared to competing nanoparticle platforms.

The PNC platforms that are most commonly adapted for non-native functions include **v**irus-like **p**articles (VLPs)(Rohovie et al., 2017; Schwarz et al., 2017), BMCs (Kerfeld et al., 2018), encapsulins (Giessen, 2016), enzyme-derived nanocages, specifically lumazine synthase and the E2 component of pyruvate dehydrogenase (Azuma et al., 2018; Dalmau et al., 2008), ferritins (Jutz et al., 2015), **s**mall **h**eat **s**hock **p**roteins (sHSPs)(Han et al., 2008), and vault ribonucleoproteins (Rome and Kickhoefer, 2013). Each with their own size, composition, cargo-loading mechanisms, environmental tolerances, and chemical properties, the natural diversity of biological PNCs supports an extensive range of potential applications (summarized in Fig. 1 and Table 1). This review presents a brief overview of biological PNCs and compiles the recent advances in the biomedical and bioengineering applications of recombinant PNCs including vaccine development, delivery of therapeutics, diagnostic imaging, administration of biological controls, catalysis, nanomaterials synthesis, and beyond.

2. Types of Protein Nanocompartments (PNCs)

2.1. Virus-like particles (VLPs)

Viral coat (capsid) protein subunits self-assemble to produce hollow icosahedral nanocages that lack infectious nucleic acids. There are many examples of animal virus-derived, bacteriophage-based, and plant virus-based VLPs that are being actively studied and developed for various applications (Rohovie et al., 2017). One of the most well-characterized animal virus VLPs is the **h**epatitis **B** **v**irus (HBV), which is

Table 1
Comparative summary of size, symmetry, and biological function of various protein nanocompartments.

Protein nanocompartment	Diameter (nm)	Subunits	Symmetry	Biological function
Virus-like particle (non-enveloped)	27–64	Variable	Icosahedral	Transfer of viral nucleic acids
Bacterial microcompartment	40–200	Variable	Polyhedral or icosahedral	Sequestration of metabolic processes
Encapsulin nanocompartment	24–32	60 or 180	Icosahedral	Variable (metabolism, iron storage, oxidative stress response)
Lumazine synthase	15	60	Icosahedral	Riboflavin synthesis
E2	25	60	Icosahedral	Subunit of the pyruvate dehydrogenase complex
Ferritin	12	24	Octahedral	Iron storage
DNA-binding protein from starved cells	8	12	Tetrahedral	Iron storage
Small heat shock protein	12	24	Octahedral	Molecular chaperone
Vault ribonucleoprotein	35–42 × 65–75	78	Dihedral	Implicated in immunity, nucleocytoplasmic transport, & drug resistance

composed of 240 coat proteins that produce an icosahedral (triangulation number (T) = 4) nanocage measuring 35 nm in external diameter (Wynne et al., 1999). The bacteriophage MS2 and Qβ VLPs, as well as the plant-derived **c**ow**p**ea **m**osaic **v**irus (CPMV) and **c**owpea **c**hlorotic **m**ottle **v**irus (CCMV) VLPs, are slightly smaller icosahedrons (T = 3), consisting of 120–180 subunits with external diameters of 27–31 nm (Golmohammadi et al., 1996; Golmohammadi et al., 1993; Lin et al., 1999; Speir et al., 1995). The P22 bacteriophage VLP, on the other hand, is one of the largest known icosahedral (T = 7) VLPs and is composed of approximately 420 coat proteins and 100–300 removable scaffold proteins with an external diameter of 58–64 nm (Parent et al., 2010).

Depending on the complexity of the virus from which they are derived, VLPs are produced from one or more structural proteins that mimic the size, morphology, and immunogenicity of native virions. The component capsid proteins of VLPs can also tolerate various internal and external surface modifications to alter the particle size, antigenicity, cell-type specificity, and cargo for a wide range of applications. Many excellent reviews discussing these modifications, as well as the stability, expression, and purification of the major classes of VLPs are available (Glasgow and Tullman-Ercek, 2014; Lee et al., 2016b; Rohovie et al., 2017; Schoonen and van Hest, 2014; Schwarz et al., 2017; Smith et al., 2013; Zeltins, 2013).

VLPs are especially suitable for use as vaccine platforms because they display antigenic epitopes in a multimeric, repetitive, and highly spatially organized conformation that efficiently induces both humoral (B-cell) and cellular (T-cell) immune responses (Bachmann and Jennings, 2010; Gamvrellis et al., 2004; Slutter and Jiskoot, 2016; Zhao et al., 2013). In addition, because native viruses may be membrane-enveloped or non-enveloped, so can VLPs, which affords additional opportunities for displaying glycoprotein antigens in a lipid bilayer to effectively induce host immunity (Dai et al., 2018; Metz et al., 2013). However, beyond vaccines, the utility of enveloped VLPs is greatly limited by the decreased range of applicable expression systems, poor particle self-assembly *in vitro,* reduced particle uniformity, complicated purification requirements, and the additional viral impurities that may be present in the lipid membrane (Dai et al., 2018; Lua et al., 2014; Pitoiset et al., 2015). Thus, this review focuses primarily on non-enveloped VLPs as they have been more widely adopted for the broad range of biomedical and bioengineering applications discussed herein.

2.2. Bacterial microcompartments (BMCs)

Lacking membrane-bound organelles, prokaryotes instead produce self-assembling, proteinaceous organelles called BMCs. These selectively permeable complexes encapsulate and co-localize enzymes to enhance the efficiency of segregated metabolic processes. Ultimately, the BMC acts as a barrier, confining and protecting enzymes and substrates while conversely shielding the surrounding cell from volatile and cytotoxic intermediates. Some BMCs are similar in size and shape to viral capsids (the *Haliangium ochraceum* BMC, for example, is 40 nm in diameter)(Sutter et al., 2017) whereas others can be much larger, up to 200 nm in diameter. BMCs consist of multiple conserved non-homologous structural proteins, BMC-H, BMC-T, and BMC-P, which are analogous to the major and minor capsid proteins of viruses (Krupovic and Koonin, 2017; Yeates et al., 2010).

While many different metabolic pathways have been identified in BMCs, the three best studied are carbon dioxide fixation in anabolic carboxysomes, 1,2-propanediol utilization (Pdu), and ethanolamine utilization (Eut) (Kerfeld and Erbilgin, 2015; Yeates et al., 2010). Other reviews elaborate further on the structure, native function, and enzyme targeting mechanisms of the various carboxysomes and metabolosomes that have been identified to date (Bobik et al., 2015; Kerfeld et al., 2018; Kerfeld and Erbilgin, 2015). Generally, BMC enzymes contain N- and/or C-terminus encapsulation peptide sequences that form amphipathic helices, which have been demonstrated to be sufficient for targeting of novel enzymes to recombinant BMCs (Aussignargues et al., 2015; Fan et al., 2010; Lawrence et al., 2014). The introduction of non-native catalytic pathways to BMCs is of particular interest for use as industrial bioreactors, but re-engineered carboxysomes also have notable potential for synthetically enhancing plant carbon fixation and improving agricultural productivity.

2.3. Encapsulin nanocompartments

Only relatively recently discovered, bacteria and archaea also produce a minimal icosahedral PNC consisting of self-assembling encapsulin proteins. Encapsulin nanocompartments are comprised of a single structural protein that has no sequence or structural similarities to BMC proteins but appears to share an evolutionary origin with the capsid proteins of tailed bacteriophages (McHugh et al., 2014; Nichols et al., 2017). Like viral capsids, the shell size of encapsulins, as well as the size and chemical nature of their pores, varies between species. For example, the *Thermotoga maritima* encapsulin is composed of 60 subunits, forming a T = 1 icosahedron that is 24 nm in external diameter, whereas the *Pyrococcus furiosus* and *Myxococcus xanthus* encapsulins are comprised of 180 protomers, forming T = 3 icosahedrons that are 30–32 nm in diameter (Akita et al., 2007; McHugh et al., 2014; Sutter et al., 2008).

The *in vivo* functions of encapsulins are diverse and include iron sequestration in *M. xanthus,* lignin degradation in *Rhodococcus jostii* RHA1, and the oxidative stress response in *T. maritima* (McHugh et al., 2014; Rahmanpour and Bugg, 2013; Sutter et al., 2008). The endogenous enzymes involved in these processes are typically internally anchored by conserved C-terminal extensions to the interior surface of the particle and so non-native enzymes or other protein cargo can be encapsulated in these PNCs via a similar mechanism (Cassidy-Amstutz et al., 2016; Putri et al., 2017; Rurup et al., 2014; Sutter et al., 2008). Others describe alternative cargo-loading mechanisms, methods of functionalization, as well as the structure, stability, and expression of various encapsulins in more detail (Giessen, 2016; Nichols et al., 2017; Sigmund et al., 2018).

2.4. Enzyme-derived nanocages

2.4.1. Lumazine synthase

An enzyme complex, consisting of an outer shell of lumazine synthase subunits and a core of riboflavin synthase subunits, catalyzes the penultimate step of riboflavin (vitamin B2) synthesis in plants, fungi, archaea, and bacteria (Azuma et al., 2018; Ladenstein et al., 2013). In the absence of riboflavin synthase, the lumazine synthase of some prokaryotes, including *Bacillus subtilis, Aquafex aeolicus,* and others, forms hollow icosahedral (T = 1) assemblies of sixty identical subunits with approximately 15 nm exterior and 9 nm interior diameters (Bacher et al., 1986; Kumar et al., 2011; Morgunova et al., 2010; Zhang et al., 2001). The morphology and functionalization of spherical lumazine synthase nanocages, with particular focus on that of *A. aeolicus*, was recently reviewed in detail (Azuma et al., 2018).

2.4.2. E2 component of the pyruvate dehydrogenase complex

In the glycolysis metabolic pathway of eukaryotes and Gram-positive bacteria, the pyruvate dehydrogenase multienzyme complex catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, which may then be utilized in the citric acid cycle. This complex consists of a structural core of dihydrolipoyl acyltransferase (E2) subunits to which peripheral subunits of pyruvate decarboxylase (E1) and dihydrolipoyl acyltransferase (E3) are non-covalently bound (Domingo et al., 2001). Alone, the E2 component self-assembles to produce a homogenous 60-subunit dodecahedral nanocage structure with icosahedral symmetry (T = 1). The E2 scaffold has external and internal diameters of 24 m and 18 nm, respectively, and twelve 5 nm pores (Izard et al., 1999). E2 subunits, like those of other PNCs, are amenable to functionalization

with foreign peptides and engineered variants of the *Geobacillus stearothermophilus* E2 scaffold have been shown to be unusually thermostable, such that unfolding begins only at temperatures upwards of 80 °C (Dalmau et al., 2008; Domingo et al., 2003; Domingo et al., 2001).

2.5. Ferritins and ferritin homologues

Highly conserved across eukaryotes and prokaryotes, ferritins are spherical PNCs that act as iron storage proteins to regulate iron availability and limit oxidative stress. Ferritins are composed of 24 subunits with octahedral symmetry and have exterior and interior diameters of 12 nm and 8 nm, respectively (Crichton and Declercq, 2010; Watt, 2011). Though they typically house approximately 2000–2500 iron atoms within their mineral core, ferritins have a theoretical maximum storage capacity of approximately 4500 iron atoms (Chasteen and Harrison, 1999; Jutz et al., 2015). Devoid of iron oxide cargo, the empty ferritin shell is often referred to as apoferritin.

Due to their biological iron storage role, these PNCs are especially suitable for the biomimetic synthesis of compact inorganic nanomaterials and targeted delivery of contrast agents for diagnostic imaging (Jutz et al., 2015). In addition, ferritins contain 14 pores for ion exchange, of which eight are hydrophilic threefold channels (for entry of iron ions) and six are hydrophobic fourfold channels. This diversity in pore selectivity affords the additional potential to facilitate transport and delivery of a wide variety of therapeutic molecules (Takahashi and Kuyucak, 2003). Human ferritin is of particular relevance to cancer therapeutics as it natively interacts with cells through the Transferrin Receptor 1, which is overexpressed by many types of tumor cells (Fan et al., 2012; Li et al., 2010).

Prokaryotes and archaea also produce a smaller ferritin homologue called Dps (**D**NA-binding **p**rotein from **s**tarved cells), which is similarly important for the sequestration and rapid oxidation of iron for protection of DNA against oxidative damage (Su et al., 2005). Like ferritins, Dps proteins form four-helix bundles that assemble into spherical nanocages. However, in contrast to typical ferritins, Dps proteins are dodecamers with tetrahedral symmetry and an external diameter of approximately 8 nm (Grant et al., 1998; Ilari et al., 2000). Consequently, the diameter of the empty internal cavity is only approximately 4–5 nm, which lessens their cargo-loading capacity compared to other biological PNCs.

2.6. Small heat shock proteins (sHSPs)

As a family of molecular chaperones, **s**mall **h**eat **s**hock **p**roteins (sHSPs) normally function to protect newly translated proteins from misfolding and endow thermotolerance to cells *in vivo* (Han et al., 2008; Kim et al., 1998a; Kim et al., 1998b). While the oligomeric state and diameter of sHSPs can be highly variable (Chang et al., 1996; Ehrnsperger et al., 1999; Haley et al., 2000; Lee et al., 1995), many form spherical complexes with a central cavity including *Methanococcus jannaschii* Hsp16.5, *Saccharomyces cerevisiae* Hsp26, and human Hsp27 (Haley et al., 2000; Haslbeck et al., 1999; Kim et al., 1998a). The well-characterized *M. jannaschii* Hsp16.5 assembles into a hollow 24 subunit cage with octahedral symmetry, exterior and interior diameters of 12 nm and 6.5 nm, respectively, and a relatively large 3 nm diameter pore for solution exchange (Kim et al., 1998a, 1998b). This small PNC is stable in temperatures up to 70 °C and within a pH range of ~5–11, making it an attractive platform for applications in harsh environments or industrial-scale manufacturing (Bova et al., 2002; Flenniken et al., 2005; Flenniken et al., 2003).

2.7. Vault ribonucleoproteins

Vaults are barrel-shaped cytoplasmic ribonucleoprotein complexes that are abundant and highly conserved among eukaryotes (Kedersha et al., 1990; Kedersha and Rome, 1986). Measuring approximately 40 nm in diameter and 70 nm in length, vault shells form the largest known non-icosahedral PNCs (Kedersha and Rome, 1986; Mikyas et al., 2004; Tanaka and Tsukihara, 2012). While their precise function remains elusive, they have been linked to innate immunity, multidrug resistance, cell signalling pathways, and nucleocytoplasmic transport (Berger et al., 2009; Chugani et al., 1993; Hamill and Suprenant, 1997; Kolli et al., 2004; Kowalski et al., 2007; Scheffer et al., 1995; Steiner et al., 2006; Yu et al., 2002).

The outer vault shell consists primarily of **m**ajor **v**ault **p**rotein (MVP) subunits, which are sufficient to form vault nanocompartments alone. *In vivo*, MVPs interact with two additional proteins, **te**lomerase-associated **p**rotein **1** (TEP1) and **v**ault **p**oly-**A**DP-**r**ibose **p**olymerase (VPARP), as well as one or more untranslated vault RNAs (Buehler et al., 2011). Besides genetic fusion to MVP, peptide cargo can be packaged into vault-derived PNCs via the MVP **int**eraction (INT) domain derived from VPARP (Kickhoefer et al., 2005; Wang et al., 2018). Similar to other PNCs, chemical and genetic modification of vaults has been previously shown to improve targeting, cellular uptake, and conjugation to various agents, but these PNCs show particular promise for drug delivery as they have low immunogenicity (Benner et al., 2017; Buehler et al., 2011; Kickhoefer et al., 2009; Rome and Kickhoefer, 2013). A review by Rome and Kickhoefer (2013) describes the engineering of vaults as a platform technology in further detail (Fig. 1).

3. Biomedical applications

3.1. Vaccines

3.1.1. Unmodified VLP vaccines

The most widely studied and successful biomedical application of VLPs is antigen presentation for vaccines. Though novel PNC derivatives are the primary focus of this review, it is worth briefly examining the clinical success of employing unmodified VLPs as anti-viral vaccines. VLPs retain the morphology and antigenicity of the virion from which they were derived and so can be used to induce strong immune responses without synthetic modifications or added adjuvants (Bachmann and Jennings, 2010; Zhao et al., 2013). Furthermore, as VLPs do not contain viral genetic material and thus are not infectious, they are safer than inactivated or live attenuated vaccines. The first commercially available VLP-based vaccines were the HBV vaccines, Recombivax HB® (Merck) and Engerix-B® (GlaxoSmithKline; GSK). Both are composed of the HBV surface antigen and have proven effective in preventing HBV infection worldwide (Andre, 1990; Andre and Safary, 1987; McAleer et al., 1984). The exterior L1 major capsid protein of **h**uman **p**apilloma**v**irus (HPV) has also been successfully used to develop the commercial prophylactic HPV vaccines, Gardasil® (Merck) and Cervarix® (GSK), which have led to a prominent reduction of HPV infection incidence in young adults and the consequent reduction of associated cervical cancers and precancerous lesions (Harper and DeMars, 2017; Ma et al., 2011). Two additional *Escherichia coli* derived VLP vaccines against HPV are also now in clinical trials and an HPV6b VLP vaccine shows promise for treatment of genital warts (Huang et al., 2017a; Zhang et al., 2000). Hecolin® (Innovax), a vaccine against **h**epatitis **E** **v**irus (HEV) composed of HEV 239 capsid proteins, was the first VLP-based vaccine to be derived from *E. coli* and approved for use in humans (though only approved in China) (Li et al., 2015; Zhang et al., 2009; Zhu et al., 2010). Various other enveloped and non-enveloped VLP-derived vaccines have reached preclinical or clinical trials for protection against human enterovirus 71 (causing hand, foot and mouth disease); (Chung et al., 2008; Zhao et al., 2017), **h**uman **i**mmunodeficiency **v**irus (HIV; (Pankrac et al., 2018)), norovirus (Atmar et al., 2011), chikungunya virus (Metz et al., 2013; Metz and Pijlman, 2016), Zika virus (Boigard et al., 2017), influenza (Kang et al., 2015; Landry et al., 2010; Lopez-Macias et al., 2011), and many other viral pathogens (Yan et al., 2015).

3.1.2. Chimeric PNC-based viral vaccines

Despite the commercial success of many unmodified VLPs as vaccines, there are a number of important advantages to chemically or genetically pseudotyping PNCs for vaccine development. First, modification and assembly of complex multicomponent VLPs can be replaced by modular antigen presentation on simpler homogenous PNCs to reduce vaccine manufacturing complexity and cost. Even very large antigens, such as the 18kDa rotavirus VP8* antigen, can be conjugated via flexible linkers to single VLP subunits (Lua et al., 2015; Tekewe et al., 2017).

PNCs can also be used to alleviate the steric hindrance from particle glycosylation to enhance the activation of host immune responses. The extensive glycosylation of many viruses, such as the Epstein-Barr virus, is thought to enable antibody evasion and reduce the effective immunity induced by inactivated or live-attenuated vaccines. Moreover, transmembrane protein variants that have been truncated to remove the glycan-rich domains are often misfolded or destabilized in their soluble form. However, when truncated major envelope protein variants from the Epstein-Barr virus were fused to the surface of ferritins or encapsulins, they were conformationally stabilized, symmetrically distributed, and elicited potent neutralizing activity (Kanekiyo et al., 2015).

The highly repetitive, modular, and symmetrical PNC architecture supports the presentation of an increased density of antigens in their natural conformation and allows efficient uptake by antigen-presenting cells to stimulate both humoral and cellular immune responses (Domingo et al., 2003; Gamvrellis et al., 2004; Slutter and Jiskoot, 2016). As an example, it has been postulated that the unusual ability of HIV to evade host neutralization responses is partially due to the low density and high spatial separation of HIV envelope glycoprotein (Env) spikes, which impedes bivalent binding by immunoglobulin G (Klein and Bjorkman, 2010). Indeed, HIV-1 VLPs with a 10-fold increase in the density of Env spikes (obtained through selection of producer cells) show superior activation of Env-specific B-cells (Stano et al., 2017). Other key HIV-1 immunogens, such as the Tat and Gag(p17) proteins, have also been fused to the surface of ferritin and the *G. stearothermophilus* pyruvate dehydrogenase E2 subunit, which were successful in eliciting potent immune responses in mice (Caivano et al., 2010; Li et al., 2006). Besides the challenges associated with HIV vaccines, there are also limitations in the efficacy of influenza VLP-based vaccines, which may be partially due to their polymorphous, asymmetrical structure (Chroboczek et al., 2014). To overcome this obstacle, the cytotoxic T lymphocyte epitope from influenza A was presented on the symmetrical **s**imian **v**irus **40** (SV40) VLP and protection against influenza A virus was imparted without the use of adjuvants (Kawano et al., 2014). Furthermore, when highly conserved influenza haemagglutinin structures were fused to *Helicobacter pylori* ferritin particles, this PNC vaccine-elicited potent H1N1 antibody titers more than ten-fold higher than those from a licensed inactivated vaccine with no autoimmune reaction (Kanekiyo et al., 2013). The display of hemagglutinin spikes on the much smaller ferritin nanocage may serve to increase the angle of display and exposure of conserved regions of the protein, increasing the accessibility to broad-spectrum neutralizing antibodies and thereby increasing the effectiveness of the vaccine against multiple influenza serotypes (Schwarz and Douglas, 2015).

Finally, whereas surface display of antigens can generate neutralizing antibodies to prevent infection, encapsulation of antigens within PNCs can also be used to preferentially stimulate T-cell responses, aiding in the clearance of infected cells. In model antigen delivery systems, the genetic fusion of ovalbumin peptides (OT-1 and OT-2) to the internal cavity of ferritin and *A. aeolicus* lumazine synthase efficiently induced antigen-specific T-cell proliferation, differentiation, and selective killing of target cells in mice (Han et al., 2014; Ra et al., 2014). Schwarz et al. (2016) demonstrated successful co-encapsidation of two **r**espiratory **s**yncytial **v**irus (RSV) proteins (M and M2) within P22 bacteriophage VLPs, which elicited T-cell memory responses against both antigens after intranasal administration in mice. Others have successfully sequestered the large, conserved nucleoprotein from influenza in P22 VLPs, which enabled broad protection against both H1N1 and H3N2 influenza in mice (Patterson et al., 2013). This method of vaccine development may ultimately help overcome many of the challenges associated with the rapid mutation rate and genomic reassortment of influenza virus, the diversity of hemagglutinin and neuraminidase surface proteins, and the resulting limited efficacy of conventional vaccines.

3.1.3. Veterinary VLP vaccines

Just as native and chimeric VLPs have shown promise for the development of human vaccines, they can similarly be employed for veterinary medicine. There are currently only two veterinary VLP vaccines commercially available, Porcilis PCV® (Intervet International) and Ingelvac® CircoFLEX® (Boehringer Ingelheim), for use against porcine circovirus infection in domestic pigs (Kekarainen et al., 2008; Wu et al., 2012). Other candidate VLP vaccines are in development for protection against porcine encephalomyocarditis virus (Jeoung et al., 2011), bovine papillomavirus type 1 (Love et al., 2012), infectious bursal disease virus (Wang et al., 2012); bluetongue virus (Mokoena et al., 2019; Roy, 2004; Thuenemann et al., 2013), feline calicivirus (Di Martino et al., 2007), rabies virus (Fontana et al., 2015), African horse sickness virus (Dennis et al., 2018), and many others (Crisci et al., 2012; Liu et al., 2012).

3.1.4. Bacterial or parasitic disease vaccines

In addition to viral antigens, modified PNC vaccines can be utilized to present epitopes or encapsulate immunogenic proteins from bacterial pathogens and parasites. MalariVax™ and Mosquirix™, for example, are chimeric HBV-derived malaria vaccines that display the *Plasmodium falciparum* circumsporozoite epitopes on their surface and have shown promise in clinical trials (Moorthy et al., 2003; Nardin et al., 2004). The E2 scaffold and chikungunya virus VLPs have also been used to present *Plasmodium* spp. circumsporozoite epitopes on their surface, the latter of which induced significantly higher titers of anti-malarial antibodies than HBV and *Helicobacter pylori* ferritin-derived PNCs in recent preclinical studies (Domingo et al., 2001; Urakami et al., 2017). In addition, intranasal application of vaults engineered to bind IgG and encapsulating either the major outer membrane protein or the polymorphic membrane protein G-1 of *Chlamydia muridarum* have effectively induced anti-chlamydial immune responses in mice. Notably, these engineered vaults avoided both induction of auto-antibody production against the major vault protein and the destructive inflammation usually associated with adjuvants (Champion et al., 2009; Jiang et al., 2017a).

3.1.5. DNA vaccines

DNA vaccination involves the administration of a DNA plasmid containing the gene for a protein antigen, expression of which induces both humoral and cytotoxic T-cell-mediated immune responses in the host (Schalk et al., 2006). Numerous human DNA vaccines have entered clinical trials for various cancers (Eriksson et al., 2013; Tiriveedhi et al., 2014; Yuan et al., 2013) and infectious diseases including HIV (Kalams et al., 2013; MacGregor et al., 1998; Mulligan et al., 2006; Mwau et al., 2004), malaria (Moorthy et al., 2003; Richie et al., 2012), Zika virus (Gaudinski et al., 2018), influenza virus (Houser et al., 2018; Jones et al., 2009), and HBV (Godon et al., 2014; Rottinghaus et al., 2003; Yang et al., 2017a). Currently, plasmids are administered to human tissues via intradermal or intramuscular injection, electroporation, or particle-mediated “gene gun” delivery. However, naked nucleic acids have a very short half-life and a low efficiency of cellular uptake, limiting the effectiveness of DNA vaccines relative to inactivated, live attenuated, or VLP vaccines.

Encapsulation in PNCs may help overcome many of the major challenges that currently restrict the success of DNA-based vaccines (as

well as gene therapies; see Section 3.3) by shielding the DNA vector from degradation and enhancing infection of target cells. In mice, hepatitis E VLPs loaded with HIV *env* cDNA notably enhanced the efficacy of oral vaccination and elicited cellular and humoral immune responses both locally and systemically (Takamura et al., 2004). HPV VLPs have also been used to package and deliver DNA vaccines against the model antigen, ovalbumin, and an antigen derived from the RSV M and M2 proteins in mice (Graham et al., 2010; Peng et al., 2010). In the latter case, T-cell and antibody immune responses in recipient mice were comparable to an approximately 10,000-fold higher dose of naked DNA. Nevertheless, while many DNA vaccines have been administered in combination with an attenuated virus or VLPs as adjuvants to boost T-cell immunogenicity (Gangadhara et al., 2017; Houser et al., 2018; Moorthy et al., 2003; Mwau et al., 2004; Ye et al., 2010), the use of VLPs (or other PNCs) as DNA vaccine delivery vehicles has yet to gain momentum.

3.1.6. Contraceptive vaccines

Contraceptive vaccines that generate a humoral or cell-mediated immune response against proteins critical for the production of gametes, fertilization, or pregnancy recognition have been proposed as an alternative to conventional methods of contraception (Lekhwani et al., 2014). A contraceptive vaccine targeting **h**uman **c**horionic **g**onadotropic (hCG) hormone, a hormone required for implantation and produced only after fertilization, has been engineered with RNA bacteriophage PP7 VLPs and was capable of eliciting antibodies in mice that efficiently inhibited hCG activity (Caldeira et al., 2015). Other potential antigens, including zona pellucida, gonadotropin releasing hormone, and sperm surface antigens, have been considered for use in contraceptive vaccines but have been rejected for use in humans due to associated side-effects, cross-reactivity, and irreversible infertility (Caldeira et al., 2015; Naz et al., 2005). Regardless, contraceptive PNC vaccines displaying these latter antigens have alternative potential as biological control agents (see Section 4.5.1).

3.1.7. Vaccines against cancer and other human diseases

Cancer immunotherapy has emerged as a less invasive alternative to conventional cancer treatments such as surgery, chemotherapy, and radiotherapy and can confer long-term remission or immunity (Dimberu and Leonhardt, 2011). Cancer vaccines can be classified as preventative (in the case of prophylactic vaccines against oncoviruses such as HPV) or therapeutic, stimulating the host immune system to fight pre-existing cancers (Liu, 2014; Ong et al., 2017). In the latter case, tumor-specific or tumor-associated antigens can be displayed with high spatial density on the surface of PNCs, which mimic the morphology of viral pathogens and thus efficiently stimulate innate immune responses. In mice, directional display of **h**uman **e**pidermal growth factor **r**eceptor-**2** (HER2), an overexpressed marker of invasive mammary carcinomas and many other cancers, induces potent autoantibody responses (Palladini et al., 2018). A single-chain dimer version of the MS2 coat protein has also been used to develop an immunotherapy against the human cysteine-glutamate antiporter protein xCT, which is highly expressed in tumorspheres, and this vaccine effectively reduced pulmonary metastases in mouse models (Bolli et al., 2018). Besides VLPs, encapsulin subunits have been fused to the OT-1 peptide of ovalbumin protein for suppression of melanoma tumor masses, E2 PNCs have been simultaneously conjugated to melanoma-associated gp100 epitopes and anti-tumor immunostimulatory CpG oligodeoxynucleotides for prophylactic immunization of aggressive murine melanoma tumors, and human ferritin-based PNCs have similarly shown promise for dense presentation of tumor antigens in proof-of-concept targeting to lymph nodes in mouse models (Choi et al., 2016; Lee et al., 2016a; Molino et al., 2016).

As an alternative to antigen presentation on the surface of PNCs, immunostimulatory molecules can be encapsulated within PNCs and targeted to dendritic cells to enhance anti-tumor immunity without eliciting systemic inflammation. For instance, vaults packaged with anti-tumor CCL21 lymphoid chemokines inhibit the growth of established lung cancers *in vivo* (Kar et al., 2011). Furthermore, co-delivery of short immunostimulatory CG DNA motifs (CpGs) as vaccine adjuvants within E2 nanocages and various VLPs has been demonstrated to enhance dendritic cell activation and cross-presentation (Molino et al., 2013; Storni et al., 2004). Of particular note, a promising melanoma vaccine composed of Qβ bacteriophage VLPs coupled to the melanocyte differentiation antigen, Melan A (or MART-1), and loaded with CpGs has had measurable success in Phase II clinical trials (Goldinger et al., 2012; Goldinger et al., 2010).

Finally, the multivalent presentation of various peptides on PNCs can be used to overcome the B-cell tolerance for self-antigens or other small peptides in the treatment of non-cancer human diseases. For the treatment of hypertension, a Qβ bacteriophage VLP has been used to present a peptide derived from the angiotensin II receptor type 1, which effectively decreased the blood pressure of hypertensive mice and may also be effective for prevention of diabetic nephropathy (Chen et al., 2013; Ding et al., 2016). Similar Qβ bacteriophage VLP-based strategies displaying nerve growth factor for treatment of chronic pain (Rohn et al., 2011), nicotine haptens to prevent relapses after tobacco smoking cessation (Maurer et al., 2005), and tumor necrosis factor-α for treatment of inflammatory disorders have also been implemented (Spohn et al., 2007). In addition, bluetongue virus VLPs have been used to display ghrelin as a potential obesity therapy. Administration of these VLPs in mice resulted in increased anti-ghrelin antibody titers, significantly reduced cumulative food intake, and increased energy expenditure (though no changes in body weight were observed); (Andrade et al., 2013). Finally, perhaps the most promising non-cancer PNC immunotherapy is a VLP-derived immunotherapy against aggregated amyloid-beta (Aβ) plaques within the brain for the treatment of Alzheimer's disease. Immunization of mice with murine leukemia virus, HPV, or Qβ VLPs displaying Aβ peptides elicited anti-Aβ antibody responses at low doses and without the use of adjuvants. Compared to unvaccinated controls, mice vaccinated with Aβ-VLPs demonstrated a significant 40–70% reduction in Aβ plaque load (Bach et al., 2009; Chackerian, 2010; Chackerian et al., 2006). Furthermore, the multivalent display of short, N-terminal Aβ-derived peptides (rather than full-length Aβ) enabled B-cell responses in the absence of unwanted T-cell responses (Chackerian, 2010; Chackerian et al., 2006; Fig. 2). Clinical trials with a short Aβ-conjugated Qβ VLP vaccine (CAD106) are currently ongoing and vaccination appears generally well-tolerated by patients (Vandenberghe et al., 2017; Wiessner et al., 2011; Winblad et al., 2012).

3.2. Cancer ablation and chemotherapeutic drug delivery

3.2.1. Drug delivery

Besides cancer immunotherapy, PNCs can also be used to specifically target conventional chemotherapeutics to tumors (Lee et al., 2016b; Molino and Wang, 2014). The vast majority of drugs are small hydrophobic molecules that, when not encapsulated in a delivery vehicle, have reduced pharmacokinetic properties, poor solubility, and can cause adverse non-specific systemic cytotoxicity in the absence of appropriate targeting mechanisms. In addition to enhancing the bioavailability and targeted distribution of encapsulated drugs, the disassembly of most PNCs in acidic environments also provides a pH-controlled mechanism of drug release that coincides with the physiology of the endolysosomal compartment encountered during cellular uptake (Flenniken et al., 2005; Moon et al., 2014; Ren et al., 2011). Of the various PNCs described in this review, human ferritin may be of particular utility in the delivery of cancer therapeutics as it natively interacts with the transferrin receptor 1, a highly expressed marker of human cancer cells, without additional targeting ligand functionalization (Fan et al., 2012; Li et al., 2010; Liang et al., 2014).

Various VLPs have been successfully used to encapsidate or display

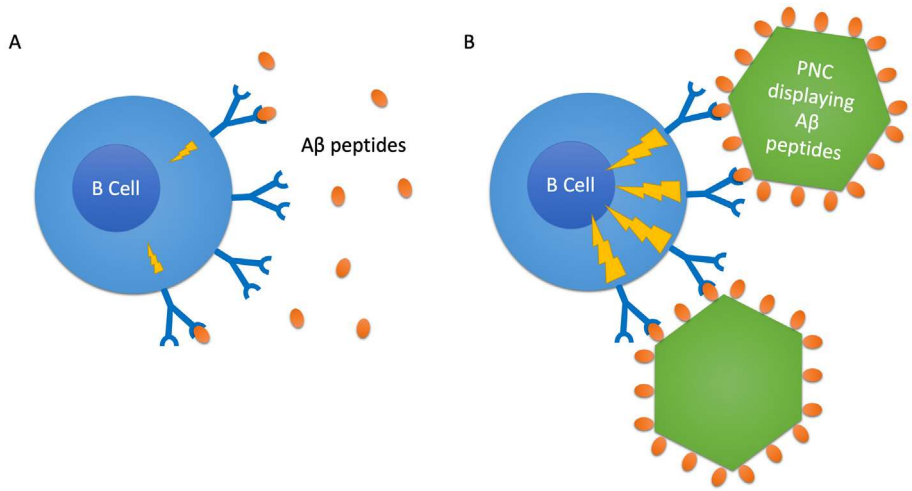

Fig. 2. PNC presentation of Aβ peptides enhances B-cell activation in Alzheimer's disease immunotherapy. Unlike free, unorganized antigens (A), highly multivalent antigens displayed on the surface of VLPs (B) can mediate extensive cross-linking of B-cell receptors, resulting in stronger signaling to the naïve B-cell. Consequently, this increased signaling also enhances downstream interactions between Aβ-specific B-cells and helper T-cells (Chackerian, 2010).

the anti-cancer drug doxorubicin, which demonstrates enhanced cytotoxicity when conjugated to VLPs compared to free drug (Aljabali et al., 2013; Ashley et al., 2011; Barwal et al., 2016; Ren et al., 2007; Zhao et al., 2011). Other PNCs, including H-ferritin, the E2 component of pyruvate dehydrogenase, the *M. jannaschii* sHSP, and a heat-stable encapsulin variant, have also been used to target and deliver doxorubicin to various types of cancer cells (Flenniken et al., 2005; Flenniken et al., 2006; Liang et al., 2014; Moon et al., 2014; Ren et al., 2012; Ren et al., 2011). Besides doxorubicin, human ferritin-packaged olaparib, a poly (ADP-ribose) polymerase inhibitor that impairs DNA repair, exhibits 1000-fold higher anti-cancer activity than free olaparib in breast cancer cells (Mazzucchelli et al., 2017). Ferritins and MS2 VLPs have also been loaded with the platinum anticancer drugs cisplatin and carboplatin (Falvo et al., 2013; Ji et al., 2012; Xing et al., 2009; Yang et al., 2007), and MS2 VLPs have also been used to deliver taxol and ricin toxin A-chain to specifically kill targeted cancer cells (Ashley et al., 2011; Wu et al., 2009). Finally, encapsulation of proflavine (an intercalating agent) in CPMV VLPs demonstrates cytotoxicity similar to free proflavine when applied to cervical, breast, and colon cancer cell cultures but with a vastly decreased risk of off-target mutagenic side-effects (Yildiz et al., 2013).

The small size of many PNCs also affords the additional potential for targeting pharmaceutical peptides, antibiotics, and chemotherapeutic drugs specifically to the brain. While the blood-brain barrier has historically been an enormous obstacle in the delivery of brain therapeutics, many PNCs are readily transcytosed and can act as a shuttle for encapsulated drugs. Anand et al. (2015), for example, genetically incorporated the analgesic marine snail peptide, ziconotide, within the interior cavity of the bacteriophage P22 capsid and chemically conjugated the cell-penetrating HIV Tat peptide to the exterior of the VLP. The ziconotide cargo was successfully translocated via a recyclable noncytotoxic endocytic pathway in both an *in vitro* human microvascular endothelial cell model of the blood-brain barrier and an *in vivo* rat brain microvascular endothelial cell model. While these experiments did not demonstrate transport into the central nervous system nor confirm retention of peptide activity for the regulation of neuropathic pain, they did confirm biocompatibility, stability in the blood, and appropriate trafficking of the PNCs.

Vaults show particular promise as drug carriers as they exhibit a large internal volume (approximately 50×10^6 nm^3) for encapsulation of cargo, are abundant among eukaryotes and so have minimal cytotoxicity and immunogenicity, and dissociate into halves in low pH environments, enabling more precise control over cargo release (Buehler et al., 2011; Goldsmith et al., 2007). Thus, vaults have been uniquely bioengineered to enhance the sequestration of small lipophilic drug compounds through the addition of interior lipophilic modifications. Discoidal lipid bilayer fragments derived from truncated apolipoprotein-AI, called nanodisks, have been used to create nanodisk-INT complexes designed to enhance encapsulation and efficacy of insoluble, hydrophobic drug cargos such as **A**ll-***trans*** **R**etinoic **A**cid (ATRA), a vitamin A derivative useful for treatment of cancer and many other illnesses (Buehler et al., 2011). This technology was later refined and simplified by instead attaching the amphipathic α-helix from the hepatitis C virus phosphoprotein, NS5A, to the N-terminus of MVP to form an internalized protein-based lipophilic ring. These lipophilic recombinant vaults were shown to reversibly incorporate hundreds to thousands of ATRA, amphotericin B, or bryostatin 1 molecules, the latter of which is of particular therapeutic interest for the treatment of Alzheimer's disease, cancer, and HIV eradication (Buehler et al., 2014; Fig. 3).

Finally, as PNCs are amenable to multiple interior and exterior surface modifications, there is significant potential for highly specific combination cancer therapies that integrate targeted delivery of encapsulated chemotherapeutic cargo, anti-tumor surface peptides, and presentation of tumor-specific or tumor-associated antigens for immunotherapy. Hepatitis C core VLPs have been used to display both interferon, which inhibits tumor cell growth, and arginine-glycine-aspartic (RGD) peptides, which target and bind highly expressed integrins on the cell surface of many tumors (Li et al., 2013). In proof-of-concept experiments, these chimeric VLPs specifically targeted tumor cells, significantly inhibited migration and invasion of cancer cells, and suppressed tumor growth in a xenograft model of human breast cancer. Though the specific combination of chemotherapeutic drug delivery and cancer immunotherapy remains untested, PNCs have been successfully modified for both loading of non-native cargo and antigen presentation (Lagoutte et al., 2018). Furthermore, a chimeric VLP has shown promise as a dual-purpose influenza vaccine and vehicle for targeted delivery of doxorubicin to colon carcinomas, suggesting that combined cancer immunotherapy and chemotherapy is plausible (Deo et al., 2015).

3.2.2. *Photodynamic therapies*

PNCs have also been demonstrated to enhance photodynamic chemotherapy, which uses low-intensity light energy to activate a non-toxic chemical dye or photosensitizer. The resulting reactive oxygen species induce cell damage and ultimately apoptosis of targeted tumor cells. Without a delivery vehicle, however, photosensitizers often have poor water solubility, lack tumor selectivity, and may exhibit toxicity. To remedy these challenges, a wide range of photosensitizers, including hypocrellin B, phthalocyanine dyes, buckyballs (C_{60}), and various porphyrins, have been encapsulated in or displayed on the surface of modified ferritins and VLPs (Huang et al., 2017a; Jiang et al., 2017b;

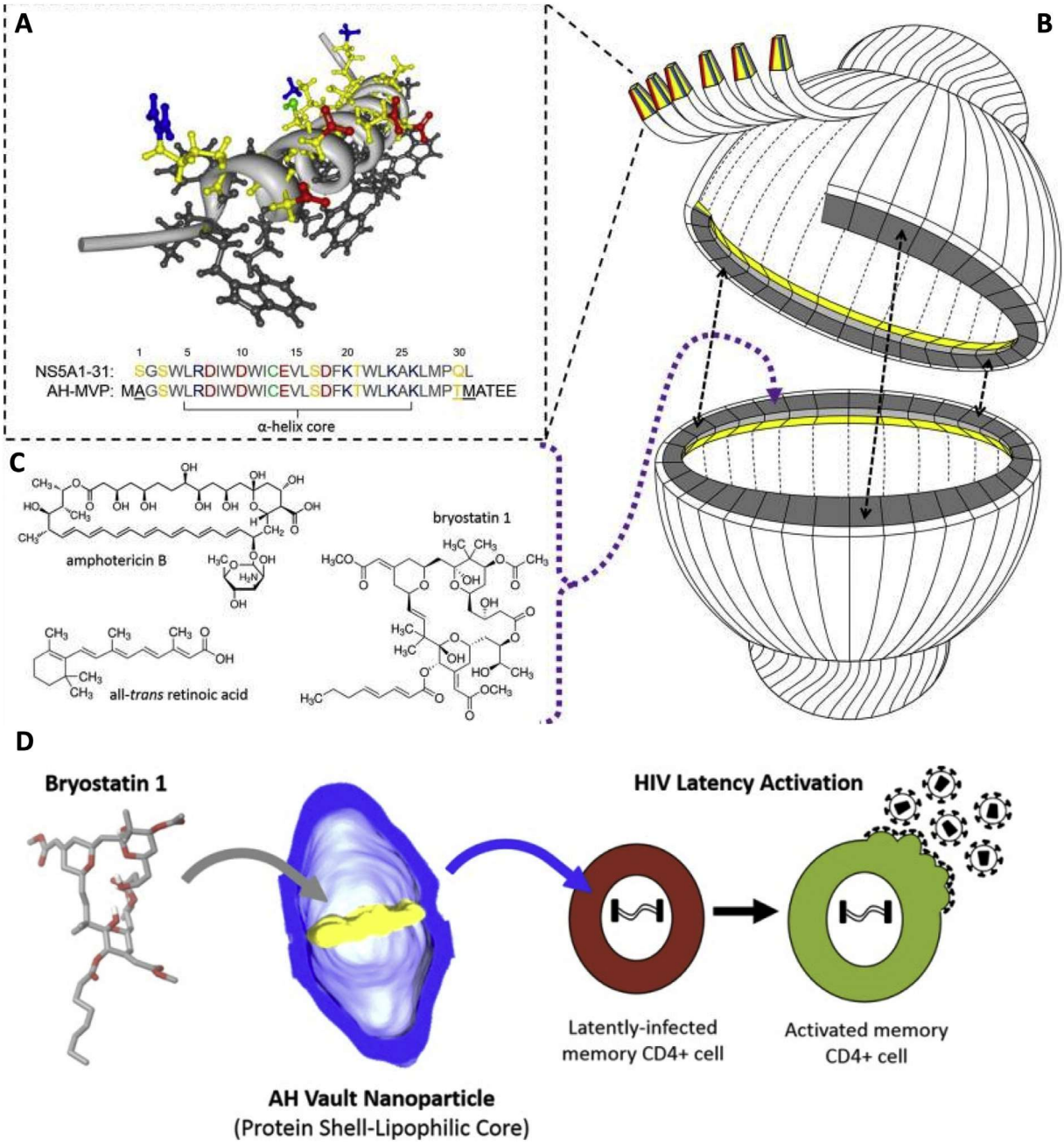

Fig. 3. A recombinant vault with a lipophilic interior shows promise for the delivery of hydrophobic drugs. (A) Three-dimensional structure of the hepatitis C NS5A1-31 **a**mphipathic α-**h**elix (AH) domain highlighting its asymmetric charge distribution along the polar face (PDB ID: 1R7E). Hydrophobic residues are in black, while polar residues are in yellow with acidic (Glu/Asp) and basic (Arg/Lys) functional groups in red and blue, respectively. The cysteine residue SH is in green. (B) Simplified cutaway schematic of an AH-containing vault depicting the attachment of AH to MVP with subsequent vault self-assembly and self-association of adjacent AH domains to form an internalized lipophilic ring for sequestering small therapeutic compounds. (C) Three sample therapeutic compounds that become reversibly associated with AH vaults. (D) Bryostatin 1 is of particular therapeutic interest because of its ability to potently induce expression of latent HIV. Adapted and reprinted with permission from Buehler et al. (2014). Copyright 2014 American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Luque et al., 2014; Setaro et al., 2015; Stephanopoulos et al., 2010; Wen et al., 2016; Zhen et al., 2013). Dual functionalization with a wide variety of targeting ligands notably enhances the specific delivery and localized concentration of photosensitizers in tumors. Stephanopoulos et al. (2010) used MS2 VLPs displaying DNA aptamers to target porphyrins to protein tyrosine kinase 7 receptors on Jurkat leukemia cells whereas others have used RGD-targeted ferritins to deliver zinc hexadecafluorophthalocyanine or sinoporphyrin sodium to subcutaneous tumors and 4T1 cancer cells, respectively (Huang et al., 2017a; Zhen et al., 2013). In all cases, these PNCs exhibited high tumor-specific uptake and accumulation, minimal non-specific toxicity, pronounced photodynamic activity, and good rates of tumor inhibition.

3.2.3. *Thermal therapies*

Similar to photodynamic chemotherapy, photothermal therapy involves laser irradiation of photothermal materials to produce cytotoxic temperatures within targeted cells. Copper sulfide-loaded ferritin nanocages, for instance, have been used to achieve complete photothermal ablation of tumors in mice without any long-term toxic effects from treatment (Wang et al., 2016b). In addition, RGD-targeted ferritin

packaged with sinoporphyrin sodium not only produced reactive oxygen species (photodynamic therapy) upon light stimulation, but also laser-triggered local hypothermia, enhancing cancer cell cytotoxicity in comparison to photothermal or photodynamic therapy alone (Huang et al., 2017a). Finally, besides laser irradiation, thermal therapies can also be triggered by other methods. Fantechi et al. (2014), for example, mineralized iron oxide within cobalt-doped human ferritin (modified to display α-melanocyte-stimulating hormone peptides for targeting to melanoma cells) and used an alternating magnetic field to effectively induce hyperthermia *in vitro*.

3.2.4. Radiotherapies

Radiation therapy is a well-established method of eradicating localized tumors by inflicting DNA damage (either directly or indirectly via reactive oxygen species). Encapsulation of radioactive particles within PNCs may serve to both enhance targeting specifically to tumor cells and reduce cytotoxicity near the primary treatment site. Hainfeld (1992) was the first to demonstrate loading of radioactive uranium atoms within ferritin particles. Later, Wu et al. (2008a, 2008b) similarly used ferritin as a template for precipitation of non-radioactive yttrium and lutetium phosphate nanoparticles, ultimately providing an efficient method for generating the counterpart (and clinically relevant) radioactive yttrium and lutetium nanoparticles for use in both cancer radiotherapy and diagnostic radioimaging. Interestingly, radiotherapy is also known to impart therapeutic benefits against tumors distant from the treatment site and evidence suggests this "abscopal effect" may be facilitated by an immune response elicited by antigen release from dying tumor cells (Grass et al., 2016). Though not yet tested, it is plausible that radiotherapy-loaded PNCs may be further functionalized to simultaneously act as an adjuvant to enhance these immunotherapeutic effects.

3.3. Gene therapies

Even in the absence of native nucleic acid binding domains, many VLPs readily package foreign nucleic acids *in vitro* or *in vivo* (Chen et al., 2010; El Mehdaoui et al., 2000; Kimchi-Sarfaty et al., 2006; Ou et al., 1999; Touze and Coursaget, 1998). Besides DNA vaccines (refer to Section 3.1.5), the targeted delivery of DNA or RNA in PNCs has many diverse therapeutic applications including gene knockdown via **RNA i**nterference (RNAi), selective killing of tumor cells, augmentation of mutations that cause chronic disease, or complete replacement of defective genes (Seow and Wood, 2009). Encapsulation of nucleic acid therapies in PNCs may increase bioavailability, prevent the rapid degradation of the nucleic acids, enhance tissue-specific targeting and expression, and reduce the cost and complexity of manufacturing.

To date, the most commonly used pseudovirions for PNC-based gene delivery have been derived from SV40 or **JC** polyoma**v**irus (JCV) as they package non-native DNA without viral genetic material or packaging signal sequences, can accommodate large plasmids up to 17.7 kb (with SV40; 9.4 kb with JCV), and have high transduction efficiency (Chen et al., 2010; Fang et al., 2012; Kimchi-Sarfaty et al., 2006). In an early demonstration by Kimchi-Sarfaty et al. (2006), both intratumoral and intraperitoneal administration of SV40 capsids loaded with plasmids encoding the truncated *Pseudomonas exotoxin* gene (PE38) effectively reduced the size of human adenocarcinomas growing in mice. Furthermore, when administered in combination with doxorubicin, the SV40 pseudovirions reduced the weight loss side effects observed with chemotherapy alone. Since, JCV VLPs have been used to encapsidate and deliver plasmid DNA encoding a "suicide gene", the herpes simplex virus thymidine kinase, to human colon carcinoma cells, glioblastomas, lung adenocarcinomas, and diffuse B-cell lymphomas in mouse models (Chao et al., 2015; Chao et al., 2016; Chao et al., 2018; Chen et al., 2010). In all cases, these JCV pseudovirons, when combined with ganciclovir treatment, resulted in targeted cytotoxicity in infected cells.

Owing largely to their low immunogenicity and broad tissue tropism, recombinant **a**deno-**a**ssociated **v**irus (AAV) particles are also particularly promising PNC vectors for the delivery of gene therapies despite their reduced DNA packaging capacity (~5 kb) (Naso et al., 2017). Though the majority of current AAV expression systems require helper viruses and/or the co-transfection of viral genes for capsid assembly and packaging, complicating their classification as PNCs as defined in this review, there has been recent progress in refining more conventional AAV VLP production strategies in bacteria to improve the scalability and uniformity of therapeutic AAV preparations (Le et al., 2019). In addition to the numerous AAV gene replacement therapies currently in development (Goswami et al., 2019), several AAV vectors have been approved for clinical use including Glybera (Alipogene tiparvovec) for the treatment of lipoprotein lipase deficiency (Yla-Herttuala, 2012), Luxturna (Voretigene neparvovec) for a rare type of retinal dystrophy (Russell et al., 2017), and Zolgensma (Onasemnogene abeparvovec) for treatment of pediatric patients with spinal muscular atrophy (Mahajan, 2019). However, the exorbitant cost of approved AAV gene therapies has limited their clinical practicality and it is expected that continued improvements to PNC manufacturing methods will enhance the accessibility of AAV (and other viral vector) gene therapies in the future.

Besides DNA transfer, VLPs have also shown potential for delivery of various RNAs including **s**hort **i**nterfering **RNAs** (siRNAs) for RNAi. In early *in vitro* proof-of-concept RNAi experiments, SV40 pseudovirions and chimeric HBV capsid proteins fused to RNA binding domains were used to encapsidate siRNA molecules, which efficiently silenced fluorescent protein gene expression in target cells (Choi et al., 2011a; Kimchi-Sarfaty et al., 2005). MS2 capsids have since been used as a delivery vehicle for siRNA against *bcl* oncogene transcripts (conjugated to the capsid assembly RNA signal sequence), which caused Bcl2 knockdown and apoptosis in HeLa cells as effectively as commercial lipid transfection agents but with enhanced target specificity (Galaway and Stockley, 2013). As a possible treatment for osteoporosis caused by excessive **r**eceptor **a**ctivator for **n**uclear factor-**k**appaB ligand (RANKL; which has a role in the regulation of bone metabolism), JCV VLPs have been utilized to deliver synthetic RANKL siRNA molecules to rat osteoblasts both *in vitro* and *in vivo* (Hoffmann et al., 2016). JCV VLPs have also been used to package **i**nter**l**eukin-**10** (IL-10) interfering RNA for targeted silencing of this proinflammatory cytokine, which has potential applications in the treatment of systemic lupus erythematosus and other autoimmune diseases where IL-10 is upregulated (Chou et al., 2010).

In addition to the delivery of siRNAs to downregulate aberrant gene expression, VLPs have also been loaded with various dysregulated **l**ong **n**on-**c**oding **RNAs** (lncRNAs) and **mi**cro**RNAs** (miRNAs). For instance, as another potential therapy for systemic lupus erythematosus, MS2 VLPs (conjugated to the HIV-1 Tat cell-penetrating peptide) have been used to package miRNA-146a, which effectively reduced both the expression of autoantibodies and levels of proinflammatory cytokines when administered to lupus-prone mice (Pan et al., 2012a; Pan et al., 2012b). The same system was subsequently used to deliver miRNA-146a to human peripheral blood mononuclear cells where they suppressed the differentiation and function of osteoclasts, the major cells responsible for bone resorption, demonstrating further utility as a method of preventing excessive bone loss in osteoporosis (Yao et al., 2015). To accommodate various novel target cells and cargos, development of this modified MS2-based system is ongoing, with the most recent versions being used to deliver miRNA-122 and the lncRNAs RNA *MEG3* for the treatment of hepatocellular carcinoma (Chang et al., 2016; Wang et al., 2016a).

Synthetic **p**eptide **n**ucleic **a**cids (PNAs), which are DNA analogs synthesized on a polyamide backbone, have also emerged as potent regulators of gene expression. However, while their artificial nature affords PNAs the ability to bind both DNA and RNA sequences with high affinity and circumvent degradation by cellular enzymes, it also

greatly impairs cellular uptake (Koppelhus and Nielsen, 2003). To mitigate this challenge, Macadangdang et al. (2011) exploited SV40 VLPs to package and deliver PNA molecules against the *MDR1* gene, which is frequently overexpressed in drug-resistant cancers. Notably, antigene PNA delivered with the SV40 system not only increased the sensitivity of cultured cancer cells to chemotherapy, but also drastically reduced transduction times (from 48 to 96 h to only 2.5 h) compared to naked PNA delivery or lipid-based transfection methods.

Finally, genome editing with CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) technologies is anticipated to revolutionize the treatment of human genetic diseases (Cai et al., 2016). However, highly specific targeted delivery of the CRISPR-associated protein-9 (Cas9) DNA endonuclease and guide RNA remains a significant challenge. By fusing Cas9 to the bacteriophage P22 scaffolding protein, Cas9 and a single-guide RNA complex have been successfully encapsidated within P22 VLPs through association with truncated P22 coat proteins as they self-assemble (Qazi et al., 2016). Lentivirus VLPs, consisting of a fusion of the HIV-1 Gag domain to Cas9, have also been used to demonstrate delivery of the CRISPR-Cas9 system *in vivo* (Montagna et al., 2018). In both cases, the encapsidated Cas9 retained its site-specific endonuclease activity and thus these VLPs show potential in human biomedicine for enhancing the delivery of CRISPR/Cas9 systems and reducing detrimental off-target effects.

3.4. Medical imaging

Contrast agents are targeted to specific tissues within the human body to enhance the visibility of structures for diagnostic imaging. Common agents include quantum dots for fluorescent particle tracking, gadolinium chelates or iron oxide for **m**agnetic **r**esonance **i**maging (MRI), and radioactive tracers for **p**ositron **e**mission **t**omography (PET). The pharmacokinetics, relaxivity, biocompatibility, and signal-to-noise ratio of many contrast agents can be improved by encapsulation in PNCs (Schwarz and Douglas, 2015). Furthermore, as PNCs are amenable to multivalent incorporation of both targeting peptides and imaging agents, the specificity of biodistribution can also be greatly enhanced.

Due to their biological capacity to nucleate iron oxide, ferritins are particularly suitable for iron oxide nanoparticle synthesis for use as a targeted MRI contrast agent (Kitagawa et al., 2017; Uchida et al., 2006) but encapsulins co-expressed with ferritin-like native cargo also efficiently sequester iron (Sigmund et al., 2018). Ferritins loaded with various contrast agents, including iron oxide or magnetite nanoparticles, are even more specifically well-suited as a platform for imaging vascular inflammation as they have been demonstrated to accumulate in macrophages in human atherosclerotic plaques, even in the absence of additional targeting modifications (Terashima et al., 2011; Uchida et al., 2008). In addition to iron oxide, ferritins, lumazine synthase, and various VLPs have alternatively been loaded with paramagnetic manganese(III) porphyrins or gadolinium complexes for high-field MRI (Aime et al., 2002; Allen et al., 2005; Liepold et al., 2007; Millan et al., 2014; Prasuhn et al., 2007; Qazi et al., 2013; Qazi et al., 2014; Song et al., 2015; Usselman et al., 2015). Notably, covalent sequestration of chelated gadolinium ions on the lengthy (300 nm) interior or exterior surfaces of the rod-shaped **t**obacco **m**osaic **v**irus (TMV) VLP, or the thermally-transitioned spherical TMV nanoparticle (170 nm), produced the highest relaxivities measured for a VLP-derived contrast agent (Bruckman et al., 2013). Besides use in delivering MRI contrast agents, bacteriophage MS2 VLPs have also been loaded with quantum dot 585 for particle tracking in human hepatocellular carcinomas, and internally conjugated to [^{18}F] fluorobenzaldehyde for use in PET scans (Ashley et al., 2011; Hooker et al., 2008).

In addition to diagnostic imaging alone, PNC-guided precision delivery of contrast agents has also enabled theranostics such as PET, fluorescence, or PAI-guided photothermal and photodynamic therapies (Huang et al., 2017b; Wang et al., 2016b); MRI monitoring and alternating magnetic field or radio frequency ablation of tumors with iron oxide-loaded PNCs (Fantechi et al., 2014; Nguyen et al., 2016; Rohovie et al., 2017); and PET monitoring of targeted anticancer treatments (Choi et al., 2011b).

3.5. Antimicrobials

The growing resistance of human microbial pathogens to conventional antibiotics is now a global public health problem and novel antibacterials are urgently needed (World Health Organization, 2014). The diverse utility of PNCs for inorganic materials synthesis (see Section 4.1), as bioreactors (see Section 4.2), and for targeted phototherapy (refer to Section 3.2.2), may also support a wide variety of new antimicrobial strategies.

One such strategy that can be enhanced by PNCs is not new: metal-based antimicrobials. A wide variety of metal ions have antibacterial properties, and many can be readily mineralized within PNC structures. Silver, for example, is commonly used to coat various hard surfaces, including flooring, appliances, and food storage containers, and is readily found in numerous consumer products such as athletic clothing, wound dressings, and deodorant (Turner, 2017). When silver is synthesized within *T. maritima* encapsulins, these nanoparticles are imparted with unique antimicrobial properties that are superior to commercially available citrate-capped silver nanoparticles (Giessen and Silver, 2016b; Fig. 4).

Bacteriolytic enzymes, such as lysozymes, offer a promising alternative to standard antibiotic drugs. However, in the absence of a protective delivery vehicle, the clinical efficacy of the cationic lysozyme is hampered by aggregation with negatively charged biopolymers and interactions with specific lysozyme inhibitors, which both undermine its antibacterial properties (Callewaert et al., 2012; Dostal et al., 2015; Gill et al., 2011). Thus, CCMV VLPs have been engineered to selectively, efficiently, and stably encapsidate T4 lysozyme molecules, which remained active and effectively degraded the cell walls of target *Micrococcus luteus* populations (Schoonen et al., 2017).

Similarly, PNCs can also be re-engineered as catalytic nanoreactors for the *production* of antimicrobial materials. The P22 bacteriophage VLP has been used for encapsulation of a hydrogen peroxide-producing enzyme, the NADH oxidase from *P. furiosus*, which was fused to the P22 scaffolding protein to encourage encapsulation. The enzyme remained catalytically active, inhibiting the growth of bacterial populations, and the VLP itself was resistant to the oxidizing effects of hydrogen peroxide (Patterson et al., 2014). CCMV VLPs have also been loaded with glucose oxidase, which catalyzes the oxidation of glucose into gluconolactone and produces hydrogen peroxide as a side product, using an alternative non-covalent encapsulation mechanism mediated by DNA tags (Brasch et al., 2017).

As another alternative to conventional antibiotics for the treatment of localized infections, both antibiotic-resistant bacteria and biofilms are susceptible to photodynamic therapy (Wainwright, 2009; Wainwright and Crossley, 2004). Similar to photodynamic chemotherapy for tumor ablation, bacterial cell death is induced by cytotoxic reactive oxygen species, which are produced by chemical photosensitizers when stimulated by light of the appropriate wavelength. The selectivity and efficacy of antimicrobial photodynamic therapy can be enhanced by dual functionalization of a PNC with both a photosensitizer and microbe-specific targeting mechanisms. Suci et al. (2007) modified CCMV VLPs to encapsidate a photosensitizer (a ruthenium complex). These PNCs were then targeted to *Staphylococcus aureus* populations by two mechanisms: electrostatic conjugation to cationic poly-L-lysine polymers, which enhance the activity of photosensitizers and have antimicrobial properties but are relatively non-specific, or biotin-mediated conjugation to antibodies selective for *S. aureus*. Both targeting methods significantly enhanced photodynamic inactivation of microbial populations. For future biomedical applications, attachment of multiple targeting ligands to the relatively large surface area of PNCs

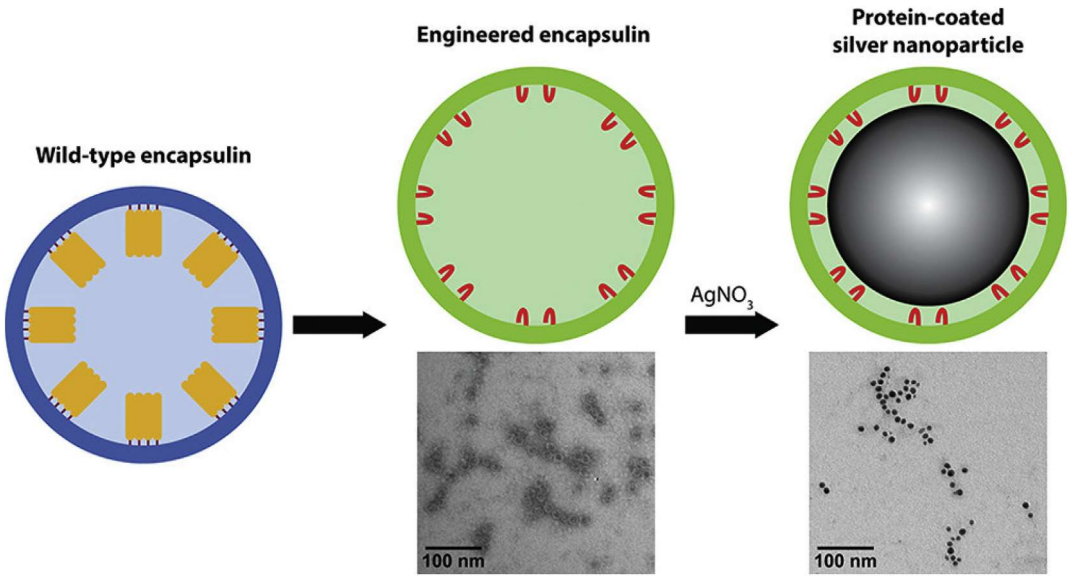

Fig. 4. Synthesis of antimicrobial silver nanoparticles using engineered encapsulin scaffolds. Schematic of a *T. maritima* encapsulin PNC engineered to contain internal silver precipitation sites. TEM images of empty modified encapsulins (bottom left) and protein-coated silver nanoparticles (bottom right) are also shown. Reprinted with permission from Giessen and Silver (2016b). Copyright 2016 American Chemical Society.

may offer even more finely tuned species selectivity.

4. Bioengineering applications

4.1. Materials synthesis

Metallic nanoparticles exhibit unique electronic, magnetic, chemical, and mechanical properties that are of growing interest for various applications including data storage, battery microelectrodes, contrast imaging (refer to Section 3.4), theranostics (refer to Sections 3.2.2–3.2.4), and more (Hosein et al., 2004; Jutz et al., 2015). With predictable size, surface charge, and symmetry, PNC architecture can be harnessed as a template for spatially controlled, size-constrained biomimetic assembly of a wide range of inorganic nanomaterials. For many applications, the use of PNCs as templates for materials synthesis offers some advantages over existing fabrication methods (such as lithography and gas- or liquid-phase processes). PNCs can reduce material costs, improve the environmental sustainability of production, offer greater consistency and control over both length and diameter, increase monodispersity, and enhance biocompatibility and targeting for biomedical purposes (Schwarz and Douglas, 2015; Schwarz et al., 2017).

In the first demonstration of an entirely synthetic repurposing of CCMV capsids, Douglas and Young (1998) exploited the positively-charged interior surface as an interface for controlled inorganic crystal mineralization. Since, various other PNCs have been adapted to encourage mineralization of a wide range of oxides, metals, and alloys. For example, CCMV, CPMV, and many other VLP scaffolds have been used for the biomineralization of titanium dioxide, nickel, iron, platinum, cobalt, and other nanoparticles (Aljabali et al., 2010; Klem et al., 2008). Ferritins, as well as the smaller *L. innocua* ferritin-like Dps nanocage, are inherently suitable to synthesize iron oxides (including maghemite, magnetite, and hematite), but have also been used to mineralize metallic iron, iron-platinum, cobalt oxide, copper sulfide, cadmium selenide, palladium, silver, and cobalt, and many other nanomaterials (Allen et al., 2003; Hosein et al., 2004; Kang et al., 2011; Kasyutich et al., 2010; Kramer et al., 2004; Okuda et al., 2010; Parker et al., 2008; Uchida et al., 2006; Ueno et al., 2004; Usselman et al., 2010; Wang et al., 2016b). In addition, modified *M. jannaschii* sHSPs have served as reaction vessels for the mineralization of both iron oxide and cobalt platinum alloy nanoparticles (Flenniken et al., 2003; Klem et al., 2005) and *T. maritima* encapsulins have been used for synthesis of gold and silver nanoparticles (Giessen and Silver, 2016a; Künzle et al., 2018).

For assembly of nanowires, nanotubes, or high surface area composites, TMV and tomato mosaic virus-derived VLPs have been demonstrated to be versatile scaffolds for mineralization at a longer length scale. These VLPs are helical rods (300 nm × 18 nm) with very high stability under a wide range of pH values and reaction conditions. By exploiting natural electrostatic interactions or by modifying peptide composition, the external surfaces of these VLPs have been harnessed as elongated substrates for mineralization of PbS, CdS, iron oxide, nickel, platinum, gold, tin, silicon, and other nanotubes (Altintoprak et al., 2015; Bromley et al., 2008; Chen et al., 2011; Chen et al., 2012; Gerasopoulos et al., 2008; Gorzny et al., 2008; Lee et al., 2006; Royston et al., 2008; Shenton et al., 1999; Fig. 5). Moreover, by preventing deposition on the exterior surface, the 4 nm diameter core can facilitate the specific formation of encapsulated 3 nm nanowires. To date, nanowires of silver, gold, copper, nickel, cobalt, platinum, palladium, and various alloy compositions have been produced in this manner (Balci et al., 2006; Balci et al., 2012; Dujardin et al., 2003; Knez et al., 2003; Kobayashi et al., 2010; Tsukamoto et al., 2007; Yang et al., 2013). The longer (900 nm) helical bacteriophage M13 VLP has similarly enabled the synthesis of gold, cobalt oxide, cobalt-platinum, iron-platinum, and other hybrid nanowires (Huang et al., 2005; Mao et al., 2004; Nam et al., 2006; Reiss et al., 2004). Ultimately, these high surface area inorganic-organic composites and metallic nanowires have significant potential in many downstream applications such as contrast imaging, catalysis, sensors, batteries, transistors, photoelectrochemical solar cells, and high density data storage (Atanasova et al., 2011; Bruckman et al., 2013; Chen et al., 2011; Chiang et al., 2012; Gerasopoulos et al., 2008; Górzny et al., 2010; Rong et al., 2009; Royston et al., 2008; Yang et al., 2013).

4.2. Catalysis

Many PNCs are suitable for use as *in vivo* or *in vitro* nanoreactors, especially BMCs and encapsulins for which the targeting peptide sequences for internal enzyme sequestration have been identified and validated. However, alternative mechanisms for targeting and immobilizing enzymes within PNCs, such as component fusion to SpyTag and SpyCatcher peptides (Zakeri et al., 2012), have also been used to enable multi-enzyme encapsulation within VLPs (Giessen and Silver,

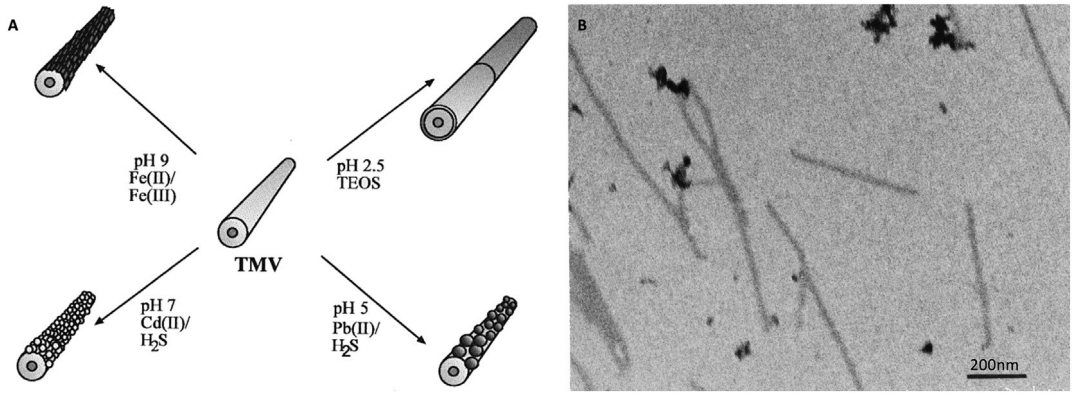

Fig. 5. Synthesis of inorganic-organic composite nanotube materials using TMV templates. (A) Schematic of nanotube synthesis. Clockwise from top left: oxidative hydrolysis (iron oxide); sol-gel condensation (silica); coprecipitation (PbS and CdS nanocrystals). (B) The TEM image of ultra-thin iron oxide nanotubes formed by aerial oxidation of TMV suspensions in Fe^2 solution. Adapted and reprinted with permission from Shenton et al. (1999). Copyright 1999 John Wiley and Sons.

2016a). In any case, the encapsulation of one or more enzymes within a PNC serves to enhance enzyme kinetics and enable spatially controlled multi-step catalytic pathways. The potential use of encapsulated hydrogen peroxide-producing enzymes as antimicrobial agents, as an example, as previously described (refer to Section 3.5). The confinement of alternative enzymes, such as alcohol dehydrogenase, in PNCs may serve to enhance many industrial reactions relevant to chemical, food, and pharmaceutical production. Furthermore, such enzyme-containing PNCs have been demonstrated to remain catalytically active when immobilized on surfaces or within larger complexes, making them ideal for use as *in vitro* protein-based sensors, flow cells, or in higher-order superlattices (Patterson et al., 2014; Patterson et al., 2012; Putri et al., 2017; Uchida et al., 2018).

4.2.1. Lignin degradation and bioethanol production

One potential application of PNC bioreactors is in the industrial manufacturing of cellulose-based products and bioethanol production. Lignin is a substantial component of plant biomass and, as such, more effective methods of lignocellulosic degradation are in high demand. Recombinant *R. jostii* RHA1 peroxidase DypB has been assembled into encapsulin nanocompartments *in vitro* and showed enhanced lignin degradation activity compared to native DypB, suggesting that PNCs have significant potential in biomass deconstruction (Rahmanpour and Bugg, 2013). A simple ethanol bioreactor has also been assembled by tagging both a pyruvate decarboxylase and an alcohol dehydrogenase with BMC-targeting peptides to direct them into empty Pdu-derived BMCs (Lawrence et al., 2014). Not only were the purified BMCs capable of ethanol production from pyruvate *in vitro*, but they produced 56% more ethanol than free enzymes and the modified *E. coli* strains also demonstrated elevated levels of *in vivo* ethanol production. In the future, the fabrication of multi-enzyme PNC bioreactors or PNC superlattice structures for coupled multi-step reactions may soon enable sequential, controlled enzymatic processes for both lignin degradation and subsequent bioethanol production from plant biomass.

4.2.2. Bioremediation

Biological remediation of contaminated environments is currently limited by the highly constrained biochemical conditions required for microbe growth (such as oxygen levels, pH, nutrient availability, and others) and the instability of free enzymes (Wang et al., 2015). PNC bioreactors may provide an alternative mechanism for immobilizing and stabilizing enzymes while maintaining catalytic efficiency. O'Neil et al. (2013) sequestered multiple copies of the *Brevundimonas diminuta* **p**hospho**t**ri**e**sterase (PTE) enzyme within the bacteriophage P22 VLP, which has broad utility as a bioremediation tool against harmful insecticides and chemical nerve agents such as soman and sarin. Furthermore, Wang et al. (2015) successfully sequestered INT-fused **m**anganese **p**eroxidase (MnP), a fungal enzyme previously employed in the biodegradation of organic contaminants, in vault PNCs. Vault-packaged MnP exhibited significantly higher stability and three times higher phenol biodegradation in 24 h than free MnP-INT, reinforcing that PNCs have significant potential in the development of efficient, cost-effective, and environmentally sustainable bioremediation methods.

4.3. Water treatment

The mineral iron core of ferritins acts as a sorbent for oxoanions including orthophosphate, arsenate, and vanadate and, as such, can be exploited for water treatment and purification applications (Honarmand Ebrahimi et al., 2010). Jacobs et al. (2010) first demonstrated the potential utility of the thermostable *P. furiosus* ferritin for industrial-scale control of water biofouling, achieving almost complete phosphate removal at ambient temperature and pH with high efficiency. Sevcenco et al. (2015) later adopted a similar approach for both phosphate and arsenate adsorption, effectively reducing these pollutants to residual levels well below ecological and health-based guidelines, respectively. Though it remains to be seen if PNCs will be further developed and broadly adopted for water purification processes, these studies strongly support that ferritins are an exceptionally stable, cost-effective, and recyclable alternative to existing chemical and microbiological treatment methods.

4.4. Pickering emulsions

Emulsions are widely used in the food, cosmetic, and pharmaceutical industries. Pickering emulsions utilize solid particles as stabilizers, which accumulate at the interface between two immiscible phases and reduce the probability of coalescence. While various inorganic particles have been used as stabilizers, novel biocompatible and biodegradable stabilizers are sought to reduce toxicity and improve safety for *in vivo* usage (Yang et al., 2017b). PNCs have emerged as potential organic stabilizers with TMV, CPMV, and turnip yellow mosaic virus VLPs all having been successfully employed as emulsifiers at oil/water interfaces (He et al., 2009; Kaur et al., 2009; Russell et al., 2005; Wang et al., 2017). However, the surface activity of many biomolecules is often readily affected by pH, ionic concentration, temperature, and other environmental conditions. Sarker et al. (2017) recently developed surface-active *G. stearothermophilus* E2 PNCs that stabilize Pickering emulsions in neutral to basic pH's, ionic concentrations up to 250 mM, and storage temperatures up to 50 °C (Fig. 6). Furthermore, the optimized E2 emulsion was pH-switchable such that the emulsion could be reversibly separated into emulsion and serum phases at lower pH values.

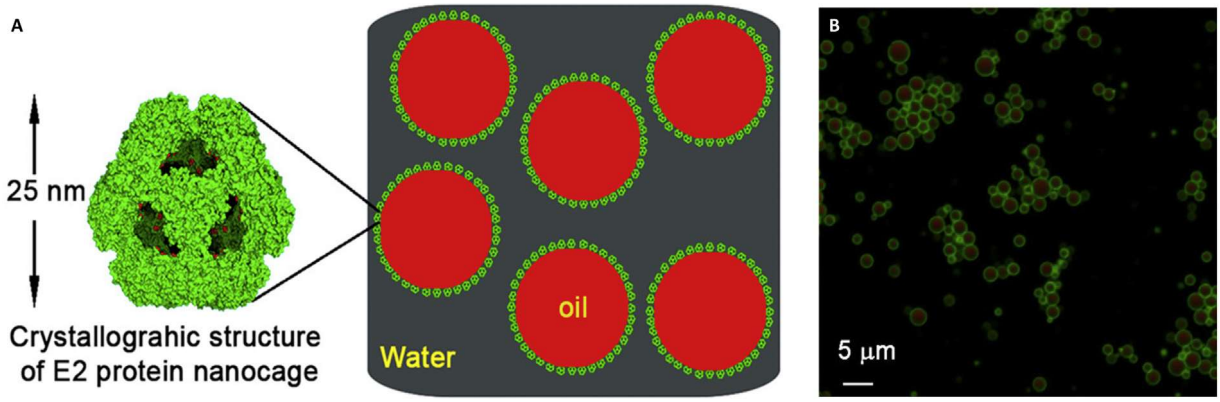

Fig. 6. E2 protein nanocages as Pickering emulsion stabilizers. (A) Schematic diagrams of the crystallographic structure of an E2 protein nanocage and an E2-stabilized Pickering emulsion. (B) Confocal microscopy fluorescence image of a coarse Pickering emulsion. The oil phase has been stained with Nile red and the modified E2 protein cages were conjugated with AF488 (green). Adapted and reprinted with permission from Sarker et al. (2017). Copyright 2017 American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.5. Biological controls

4.5.1. Contraceptive vaccines for the control of wild animal populations

Due to the irreversible infertility associated with many of the contraceptive vaccines in development (refer to Section 3.1.6), these vaccines may have more significant potential for the humane control of wild animal populations such as stray canines, brushtail possums, rodents, and other pests. The rod-shaped Johnson grass mosaic virus, for example, has been used to present the mouse zona pellucida glycoprotein-3 and spermatozoa-specific YLP_{12} peptides, which successfully induced subfertility in immunized animals (Choudhury et al., 2009). Gonadotrophin-releasing hormone and zona pellucida proteins have also been chemically conjugated to rabbit haemorrhagic virus VLPs for potential use in the control of brushtail possum populations in New Zealand (Cross et al., 2011). However, the use of VLPs may actually reduce the contraceptive efficacy as antibodies would also be generated against the viral coat protein (Gupta and Minhas, 2017). In the future development of this particular application, a non-immunogenic PNC platform (such as vaults) may be preferable to VLPs.

4.5.2. Control of algal blooms

Targeted PNC delivery of cytotoxic compounds may provide an economical and environmentally-sustainable method of controlling harmful algal blooms in aquatic ecosystems and human water sources, especially in areas where aeration or chemical treatments are not feasible. The capsid protein of HcRNAV34, a virus that infects the toxic dinoflagellate, *Heterocapsa circularisquama*, has been used to encapsidate and specifically target an algicidal compound, thiazolidinedione 49 (TD49), to harmful *H. circularisquama* populations. Encapsidated TD49 demonstrated precise host selectivity and a more potent cytotoxic effect than TD49 alone (Kang et al., 2015). Similar VLP-based approaches for control of brown tide blooms (*Aureococcus anophagefferens*), red tide alga (*Heterosigma akashiwo*), *Emiliana huxleyi* blooms, and many other harmful phytoplankton populations are plausible (Bratbak et al., 1996; Gastrich et al., 2004; Nagasaki et al., 1994).

4.5.3. Pest control

Just as PNCs are being adopted to encapsulate algal cytotoxins, they are also being harnessed to improve the bioavailability and reduce the required dosage of pesticides used in agricultural applications. For instance, Cao et al. (2015) packaged abamectin, a biological pesticide against parasitic nematodes, within red clover necrotic mosaic virus VLPs to improve root protection against disease. Relative to free abamectin, encapsulation in VLPs increased dispersion of abamectin in the soil and enabled a controlled release strategy, effectively enlarging the limited zone of protection around growing root systems without compromising pesticide efficacy. An alternative anthelmintic nematicide, crystal violet, has also been encapsidated within chemically-modified tobacco mild green mosaic virus VLPs, which similarly improved soil motility but offered poorer treatment efficacy than free drug (Chariou and Steinmetz, 2017). For plant protection against aphids, Bonning et al. (2014) fused a spider-derived insect neurotoxin to a luteovirus coat protein, which, even without assembly of the component coat proteins into a complete VLP, was sufficient for targeted delivery into the insect hemocoel after consumption and caused significant mortality in four tested aphid pest species. In the future, considering the demonstrated utility of PNCs for nucleic acid delivery (refer to Section 3.3), there is also considerable potential for PNC delivery of various RNAi-based pest controls that are currently in development (Baum and Roberts, 2014; Kolliopoulou et al., 2017; Palli, 2014).

4.6. Agricultural productivity

Population growth and loss of arable land is currently outpacing agricultural productivity. To address projected demands for major food crops, engineering carbon fixation has been identified as a promising method of improving plant photosynthetic efficiency and increasing biomass output. Plant photosynthesis is limited by the carbon-fixing enzyme D-**ri**b**u**lose-1,5-**bis**phosphate **c**arboxylase/**o**xygenase (RuBisCO), which exhibits a slow turnover and wasteful oxygenase activity. In cyanobacteria, the carboxysome BMC acts as a carbon dioxide concentrating mechanism and the cyanobacterial RuBisCO has higher rates of CO_2 fixation than that of photosynthetic plants (Fig. 7). Theoretically, the bioengineering of carboxysomes and implementation into plant chloroplasts could enhance photosynthetic carbon fixation and reduce photorespiration, ultimately increasing crop yield by up to 60% (Lin et al., 2014; McGrath and Long, 2014; Price et al., 2013; Zarzycki et al., 2013). Thus, efforts have been made to introduce the β-cyanobacteria carboxysome operon into plant chloroplasts and the structural components have been shown to appropriately assemble into microcompartments (Giessen and Silver, 2017; Hanson et al., 2016; Lin et al., 2014; McGrath and Long, 2014). To reduce the complexity of carboxysome transfer and assembly in non-native systems, a chimeric *Synechococcus elongatus* BMC protein has been developed that structurally and functionally replaces four carboxysome genes while retaining the photosynthetic capabilities of the assembled PNC (Gonzalez-Esquer et al., 2015). A smaller synthetic CO_2-concentrating PNC has also been derived from encapsulin nanocompartments by implementing a minimal carboxysome operon, and co-packaging of RuBisCO and carbonic anhydrase has been demonstrated in *A. aeolicus* lumazine synthase, though a significant kinetic effect was not observed in the latter PNC (Frey et al., 2016; Giessen and Silver, 2017). In any case,

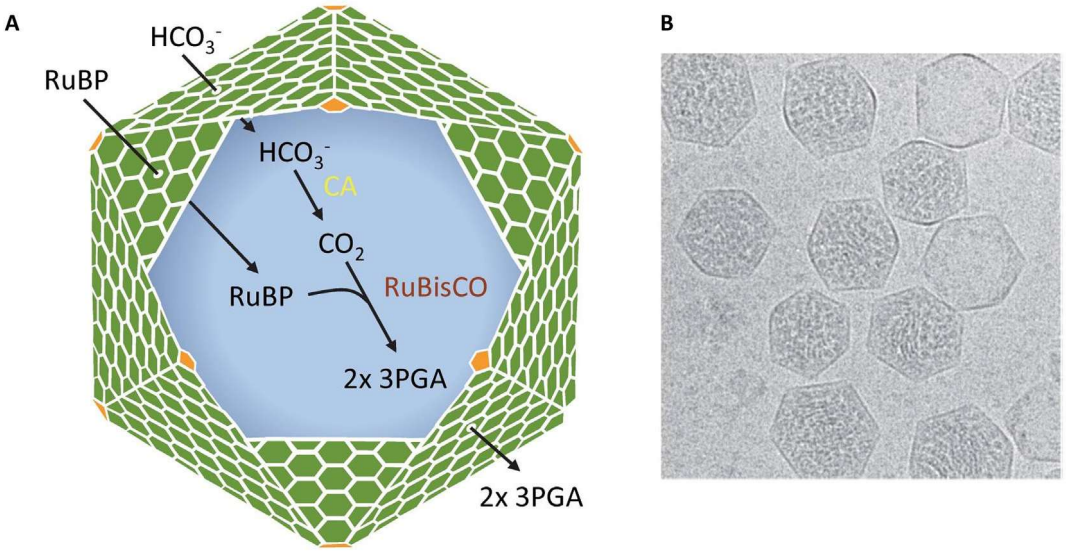

Fig. 7. The carboxysome. (A) A model of carboxysome function. The protein carboxysome shell is proposed to sequester CO_2 to enhance the activity of RuBisCO. (B) Electron micrograph of purified carboxysomes from *Halothiobacillus neapolitanus*. Adapted and reprinted with permission from Bobik et al., 2015. Copyright 2015 John Wiley and Sons.

carboxysomes and other carbon-concentrating PNCs require significant further engineering (specifically the addition of a bicarbonate ion transporter and inhibition of stromal carbonic anhydrase) to be completely functional in plants (Hanson et al., 2016).

Alternatively, there is additional potential to optimize photorespiration rather than attempting to minimize it (Zarzycki et al., 2013). Using genetic modification of *Arabidopsis thaliana*, the cyanobacteria dicarboxylic acid cycle and the *E. coli* glycerate pathway have each been introduced into chloroplasts, resulting in improved biomass production by up to 30% (Kebeish et al., 2007; Maier et al., 2012). While such pathways have yet to be implemented using PNCs, there is obvious potential for integration of multiple catabolic pathways within carboxysomes or other PNCs to circumvent the release and refixation of photorespiratory by-products, concentrate carbon dioxide in close proximity to RuBisCO, and ultimately enhance carbon fixation for improved biomass output.

5. Summary

Genetic engineering of proteins has been widely used to redesign biological nanocage architectures to produce recombinant PNCs with precise structure, size, and function. For biomedical and bioengineering purposes, these novel PNCs offer distinct advantages over other nanoparticle platforms and yet can also support further functionalization with chemical, polymer, or lipid motifs for an increased spectrum of non-native applications. In addition, increasing knowledge and fundamental understanding of PNC structure, assembly, cargo-loading, and targeting mechanisms will further enable the rational *de novo* design of entirely synthetic PNCs for highly specific applications (Bale et al., 2015; Grossi et al., 2017; King et al., 2012; Lai et al., 2012; Lai et al., 2014; Padilla et al., 2001). As such, self-assembling PNCs can provide diverse, versatile platforms for a variety of potential applications including vaccines, targeted cargo delivery, enhancement of industrial reactions, and synthesis of biomimetic materials.

While the commercial translation of these biotechnologies is largely restricted to vaccines to date, the extensive clinical, preclinical, and proof-of-concept success of various recombinant PNCs suggests considerable future potential for implementation of PNC platforms in agriculture, industrial processes, and medicine. Currently, the most significant challenge impeding the widespread adoption of PNCs is industrial scalability. While some recombinant PNCs greatly improve the cost-effectiveness of manufacturing relative to existing or proposed alternatives (for example, Tekewe et al., 2017), others are limited by the poor yield of many cell-based expression systems and cumbersome purification methodologies (for elimination of contaminating cellular debris and, in some cases, empty or incompletely assembled PNCs). It is probable that the continued advancement of cell-free protein expression systems will encourage adoption of more PNC-based products and processes, as these methods increase yield, reduce costs, eliminate viral or host cell contamination, and reduce the complexity of required quality control measures (Bundy et al., 2008; Sheng et al., 2017). Ultimately, in parallel with innovations in rational design and cell-free protein expression technologies, we anticipate that minimalist recombinant PNC compositions will revolutionize nanobiotechnology and the prevention and treatment of human disease.

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