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Protein nanocage architectures for the delivery of therapeutic proteins

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Abstract

Protein assemblies with cage-like structures are found widely in Nature with a large diversity of structural properties and functionalities. These architectures provide both inspiration for biomimetic design and templates for bioengineering. Inspired by the native utility of protein nanocage (PNC) architectures for cargo loading, transport, and protection, significant effort has been put into the development of PNC-based biomedical applications, including therapeutic delivery. This review summarizes the designs of PNC architectures for the delivery of therapeutic proteins (categorized by the type of therapeutics) and highlights the achieved or potential advantages of the PNCs as delivery systems for these proteins.

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Introduction

Proteins are biomacromolecules that play a major role in almost all the life activities with a variety of functions that derive from their diverse structures. Proteins can self-assemble from multiple subunits into quaternary structures with symmetries that aid functionality. One unique group of self-assembled protein architectures is cage-like complexes. From an evolutionary point of view, the emergence of most protein cages results in compartmentalization, which provides an isolated space for sequestration of materials and biochemical reactions (i.e. protein cages as carriers and reactors) [1]. Viral capsids, for instance, segregate their encapsidated viral genomes from the environment, packaging and releasing the genetic materials in a controlled manner to complete the life cycles [2]. Other prominent examples can be found in the structures classified as bacterial microcompartments, which usually separate metabolic pathways from the bulk cellular environment to enhance the metabolic flux of the cells, such as carboxysomes, or to prevent the leakage of metabolites that are toxic to cells, such as Pdu (1,2-propanediol utilization) microcompartments [1]. Protein cages are also found in eukaryotes, with examples including proteins in the ferritin superfamily, heat shock proteins, and vault proteins, which are involved in stress response and cellular signaling [3]. Apart from naturally occurring examples, the diversity of protein cages is further broadened by biotechnological methods such as rational engineering, directed evolution, and *de novo* design to create new functional protein cage architectures [3,4]. The available library of protein cages covers a broad range of physical properties that are not limited to size (diverse but mostly at nanoscale), shape (e.g. sphere, rod, ring), and surface properties (e.g. electrostatics), which provides architectures and templates for a broad range of applications [5].

The evolution of protein nanocages (PNCs) with diverse functionalities inspires us to redesign and engineer them for different goals. A focus of catalysis is one major theme of the natural utilization of the PNCs [1,3]. Artificial nanoreactors, built from these structures, have been designed to perform selective catalytic activities, and efficient catalytic biomaterials based on these nanoreactors have been developed at multiple length scales [3,5,6]. Natural PNCs often have native guest molecules encapsulated inside their interiors for storage, sequestration, and protection, and repurposing the cages as nanocarriers has been a successful approach to incorporate a wide variety of non-native cargos [3,5]. The finite space inside the cavity of the cages acts as a significant constraint to determine the packing capacity and the size of packaged cargo (e.g. 'head-full' genome packaging mechanism of some bacteriophages, such as P22 [7]), and this property has been used to confine the growth of synthetic materials (e.g. polymers [5,6] and nanoparticles [6,8]). Furthermore, protein cages are self-assembled to form a repetitive pattern of a limited number of building block(s) that is important in their biological recognition [9]. Using this pattern, together with re-engineering protein subunits, has allowed these architectures to be used for surface display (e.g. targeting peptides and immunogenic epitopes) [4,6,9,10]. The use of PNC architectures has become a

powerful platform for biomimetic design and engineering, with great values for applications some of which have already been accomplished by genetic, chemical, and physical manipulation [3,5].

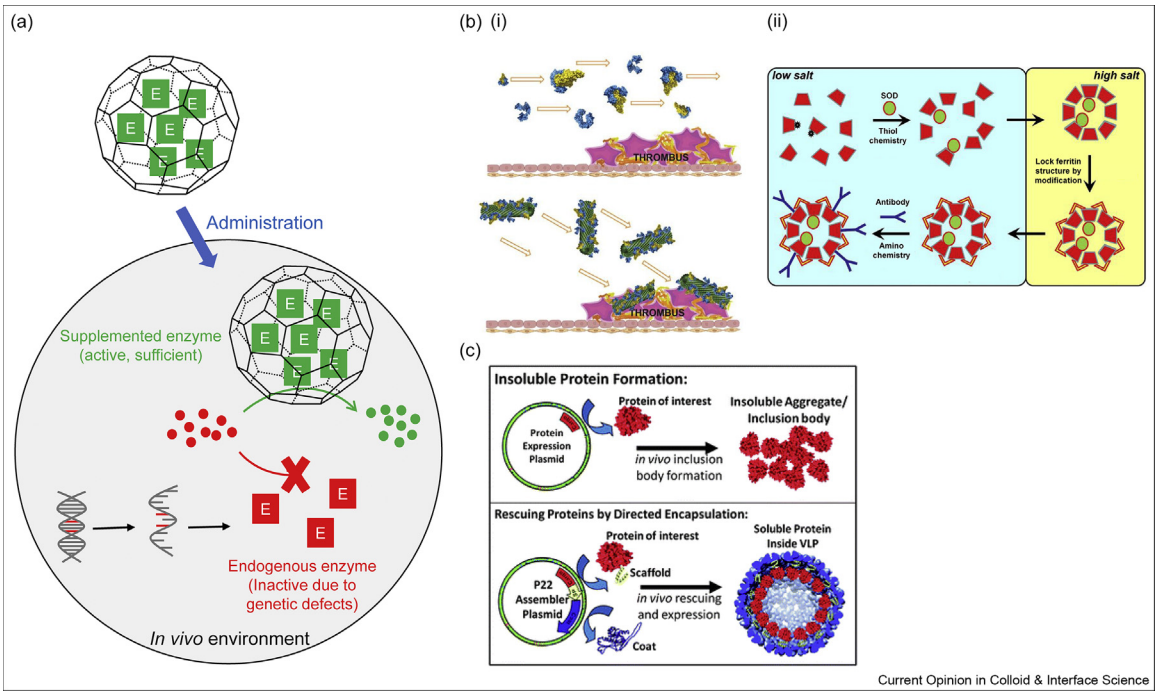
Biomedical utility has been an emphasis of protein cage architecture engineering. Besides those cages that have endogenous therapeutic activity (e.g. proteins in the ferritin superfamily [8] that are discussed later in detail and bacteriophages that could be used in treatment of pathogenic bacterial infections [11]), PNCs are ideal candidates and have been extensively exploited as drug delivery systems [10]. Their advantages are demonstrated by their intrinsic biophysical structures and plasticity for modifications, which have been successfully exploited. First, because drug carriers interact directly with the *in vivo* environment of organisms (including blood, various tissues, and organs), good biocompatibility is required to minimize adverse effects (such as immune responses and toxicity), as well as good structural uniformity and stability to maintain consistency in delivery. The proteinaceous nature of PNCs as drug carriers endows them with high biocompatibility and biodegradability. The cage architecture of PNCs is assembled with relatively strict geometric constraints, of one or a few components, resulting in a highly homogenous population of particles [4,10]. Second, the delivery systems should be able to load drugs readily. The exterior and the hollow interior of PNCs offer large room, and they have been shown as mature and powerful systems to incorporate guest cargos via genetic engineering, bioconjugation, and strong noncovalent interactions [3]. Third, targeting is the main objective of drug delivery, but recent studies have suggested that targeting is not straightforward [12]. However, the vast library of PNCs is with a diverse range of natural tropism, and site-specific delivery at multiple levels (such as organ, cell type, and cellular compartment level) can be realized using passive targeting [4]. Meanwhile, the modifications to the exterior surface with functional moieties can alter the biodistribution for different locations [6]. Fourth, rational modifications on PNCs with molecular precision have also been shown to reduce the interactions with immune systems, prolong circulation time, and change the responses to environment, all of which are beneficial for biosafety, pharmacokinetics, and drug release [4,6,13]. In addition, the economical manufacturing process of PNCs using standard biotechniques is a strength given the ultimate goal of industrial production for applications [4]. As delivery systems, PNCs have been used to load cargos ranging from small molecule compounds to biomacromolecules and nanoparticles for biomedicine including therapeutics (e.g. cancer treatment), prophylactics (e.g. vaccine development), and diagnostics (e.g. magnetic resonance imaging) [14].

This review covers the biomedical use of PNC architectures for the delivery of therapeutic proteins. Therapeutic proteins refer to the proteins displaying activities that directly work with or for therapeutic effects, of which majority have catalytic activities or binding activities. Therefore, delivery of immunogenic proteins, for example, is outside the scope but has been comprehensively reviewed elsewhere [4,6,9,14]. Functional proteins have been shown to be effective as therapeutics for the treatment of genetic disorders of essential endogenous proteins, to effectively supplement the insufficient bioactivities, and to circumvent the regulation and the lag time of transcription and translation processes [15]. PNCs are able to deliver therapeutic proteins with multiple advantages, such as disease site targeting, cargo protection, extension of biological half-life, and enhancement of biocompatibility, which are discussed in detail in each section. In addition, the delivery of enzyme-like activities, such as intrinsic catalytic activities of PNCs and nanozymes, is also included in this review.

Therapeutic enzymes

As biological catalysts, enzymes play a pivotal role in almost all the processes of metabolism to sustain the normal life activities of organisms. Diseases might be induced when an essential enzyme, for processing vital biochemical reactions and regulating cell cycles, is missing, deficient, or inactive, which could be caused by either genetic abnormalities (such as lysosomal storage diseases [16]) or failure to upregulate the enzyme level (such as exocrine pancreatic insufficiency [17] and breast cancer associated with a downregulation and deficiency of caspase 3 [18]). Accordingly, enzyme replacement therapy has been developed as a treatment by direct compensation of the deficient biocatalytic activities with exogenous enzymes. This therapy caters exactly to the origins of the diseases and circumvents the process of transcription and translation, which is advantageous to the efficacy of treatment. However, as exotic biomacromolecules, the supplemented enzymes may show low biocompatibility and cause immune responses such as anaphylaxis [19]. The cells that have no direct contact with bloodstream (e.g. skeletal muscle and bone) or are protected by biological barriers (especially blood–brain barrier) cannot easily access bare enzymes, and targeting to specific tissues or cells usually cannot be realized with bare enzymes [16,19], which might lead to higher required dosage for efficacy resulting in nonspecific toxicity. In addition, frequent dosing may be required due to a short circulation time of exogenous proteins that are likely subject to degradation and inactivation *in vivo*, which could result in a higher expense and lower efficacy of this therapy [16,19]. PNCs, architectures that function naturally to sequester, transport, and protect macromolecular cargos, can be redesigned to incorporate functional enzymes, showing great promise as nanocarriers of therapeutic enzymes that might solve some of the limitations of enzyme replacement therapy (Figure 1A).

Figure 1



PNC architectures for delivery of therapeutic enzymes. **(a)** Schematic representation showing the PNC architecture as a delivery system for enzyme replacement therapy. The essential endogenous enzyme is inactive due to genetic defects (shown in the figure) or deficient due to upregulation failure (not shown in the figure), which results in accumulation of toxic or waste molecules that could cause diseases. PNCs can deliver sufficient amount of the essential enzymes with high catalytic activity to the disease sites in the *in vivo* environment, metabolizing toxic or degrading waste molecules. **(b)** Targeted delivery of therapeutic enzymes is realized by (i) natural or (ii) engineered tropism of PNCs. (i) Delivery of thrombolytic enzyme, streptokinase (STK), was enhanced by TMV's natural tropism to thrombi [21]. Top, free STK displays low interaction with thrombi, resulting in low efficiency of the thrombolytic therapy. Bottom, attached to the exterior surface of TMV, STK is more liable to localize at thrombi for efficacy. Reprinted with permission from the study by Pitek et al [21]. Copyright (2017) American Chemical Society. (ii) The targeted delivery of SOD was achieved by encapsulation inside a ferritin cage with engineered tropism to endosomes in endothelial cells, which is realized by attaching antibodies against an endothelial glycoprotein, called plasmalemmal vesicle-associated protein, on the ferritin surface. Reprinted from the study by Shuvaev et al [24] with minimal alterations, Copyright (2018), with permission from Elsevier. **(c)** The stability of therapeutic enzymes could be stabilized by PNCs. P22 VLP system was shown to rescue α-galactosidase from insoluble aggregates/inclusion body by encapsulation inside the capsids. Reproduced from the study by Patterson et al [27], with permission from The Royal Society of Chemistry. SOD, superoxide dismutase; TMV, tobacco mosaic virus; PNC, protein nanocage; VLP, virus-like particle.

Targeting

The exploitation of natural tropism of PNCs has been successful in targeted delivery of therapeutic enzymes. Wen et al [20] found that the elongated rod shape determines tobacco mosaic virus (TMV), with a favorable flow and margination behavior in blood vessels, contributed to the natural characteristic of thrombus targeting (Figure 1B i). By bioconjugation of thrombolytic enzymes such as streptokinase [21] and tissue plasminogen activator (a serine protease) [22] to the exterior of TMV, new formulations for treating thrombotic diseases have been developed. Compared with free enzymes, these TMV-thrombotic enzyme-conjugated nanoparticles were examined in mouse models with a better or similar efficacy in clot localization and a reduced risk of bleeding side effects resulting from the high clearance rate of the particles. We are designing a system to deliver therapeutic enzymes with virus-like particles (VLPs) derived from bacteriophage P22, taking advantage of their natural biodistribution to the liver [23]. Given that low levels of glutathione (GSH) are associated with multiple hepatic diseases, supplementation of the GSH biosynthetic activity may act as a potential treatment. GSH nanoreactors have been developed by encapsulating essential enzymes of the GSH biosynthetic pathway inside P22 VLPs, and their therapeutic utility has been shown by successfully protecting cells against oxidative stress in an *in vitro* model (Y Wang et al., unpublished). Based on the organ tropism observed for the P22 protein cage architectures, it would be promising to use these nanoreactors as a treatment for GSH-deficient hepatic diseases.

By modifying the exterior surface, PNCs can also be rationally engineered for tissue- and cell-specific targeting. For example, through conjugation of

antibodies against an endothelial cell-specific glycoprotein called plasmalemmal vesicle-associated protein, a thermophilic ferritin was able to specifically deliver enzymes, such as superoxide dismutase, to endosomes of endothelial cells with subcellular precision (Figure 1B ii) [24]. The delivered superoxide dismutase was shown in preclinical experiments to eliminate the increase of superoxide levels in endosomes triggered by lipopolysaccharide, effectively protecting mice from lipopolysaccharide-induced inflammation.

Enzyme stabilization

Once incorporated inside the protein cage architecture and immobilized in that microenvironment, enzymes often show an enhanced stability or activity, compared with free proteins. Lysozyme, a key endogenous host enzyme for protection against bacterial infections, easily aggregates in the presence of negative macromolecules because of its intrinsic electrostatic property, which impedes its utility as an antibiotic. By encapsulation inside cowpea chlorotic mottle virus VLPs, Schoonen et al. [25] showed that T4 lysozyme was enzymatically active within the capsid and stabilized probably due to the charge neutralization from the capsid shell, which paves the way for potential antibiotic applications of lysozyme. In another example, α-glucosidase was decorated on the exterior of VLPs derived from parvovirus B19 and showed enhanced activity compared with free enzyme [26]. In addition, by encapsulation within P22 VLPs, α-galactosidase enzymes can be rescued in their fully catalytically form after being recombinantly produced rather than forming insoluble aggregates in inclusion bodies (Figure 1C) [27]. These systems have therapeutic potentials in treating some types of lysosomal storage disorders, such as Pompe disease and Fabry disease (which are caused by genetic defects of endogenous α-glucosidase and α-galactosidase, respectively) [16]. However, the therapeutic efficacy of these systems has not yet been confirmed, and some potential challenges (such as prolonged biological half-life, and, in some cases, enzyme release) need to be addressed before biomedical applications can be realized.

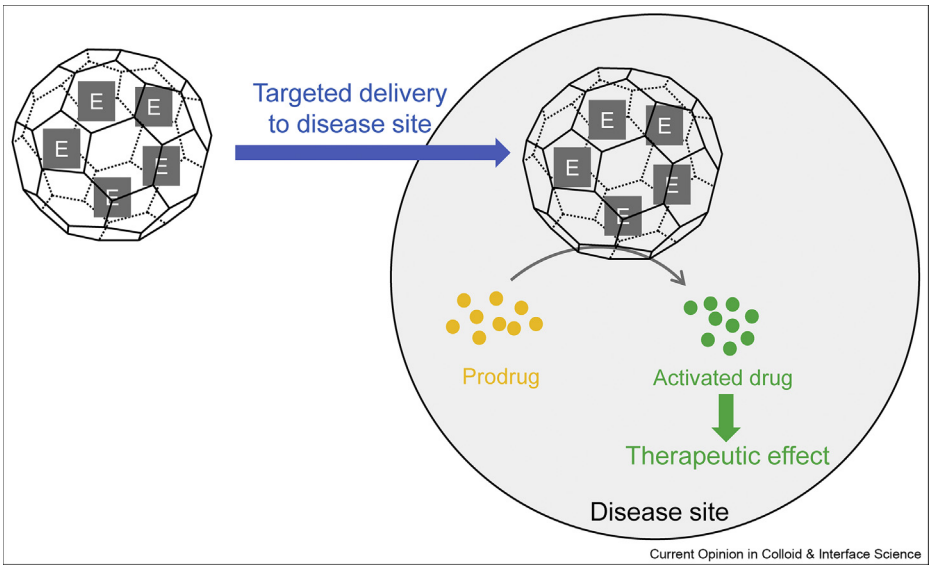
PNCs are composed of multiple protein subunits arranged in a repetitive pattern, and this structural property has been used to restore enzyme function from split protein components. For instance, caspase 8 is a protease that recognizes and degrades endogenous proteins as substrates and induces cell apoptosis. The caspase 8 activity has the potential to be developed into a therapeutic for cancer treatment, but among the challenges for large scale, recombinant production is susceptibility of the expression hosts during protein production. To solve the problem, human caspase 8 was split into two nonfunctional domains, both genetically fused to the Gag protein of avian sarcoma leukosis virus (an enveloped virus) [28]. When simultaneously expressed, the two fusion proteins assembled as chimeric capsids. These capsids were produced in combination with a foreign viral envelop protein, that is, pseudotyped (coproduced) with vesicular stomatitis virus envelope protein (VSV-G), resulting in enveloped VLPs that were replication deficient but able to enter cells due to endocytosis mediated by VSV-G. This strategy protected the recombinant expression host from apoptosis probably by concealing caspase inside the viral envelop, simultaneously maintained the caspase activity and efficiently induced apoptosis of the cells that were administrated with the VLPs. In contrast, the caspase activity could not be restored when the two caspase domains were expressed separately and assembled in different capsids. This suggests that the caspase function was recovered by dimerization of the two split components, which might be facilitated by the Gag–Gag proximity in the self-assembled cage-like structure.

Enzymes for prodrug activation

Prodrugs need to be metabolized to their pharmacologically active forms for therapeutic effect. If this conversion process could be designed to occur when catalyzed by a specific enzyme, the activated drug could be uniquely generated only at the site where the enzyme is found [29]. This enzyme prodrug therapy shifts the design from site-specific delivery of small-molecule drugs to the delivery of macromolecular catalysts. Different strategies have been applied to direct prodrug activation enzymes to the desired sites, including fusion of antibody, lectin, polymers, and bacteria (such as Clostridia for targeting solid tumor), as well as directed delivery of enzyme genes [29,30]. PNCs are good candidates as nanocarriers for targeted delivery of prodrug activation enzymes (Figure 2). They have been identified as having diverse physical properties (size, charge, and shape), as well as chemical and genetic plasticity that can be used for modification and display of surface-exposed ligands to alter the tissue tropism in addition to their natural biodistribution, which can be taken advantage of. Most of these nanocages have little to no barrier for the diffusion of small molecules across the capsid; therefore, the enzyme-incorporated nanocages can directly work as nanoreactors without the need to release the enzymes at the targeted site for prodrug activation [13]. This might be beneficial for prolonging the biological half-life of the enzymes due to the protein stabilization effect and protection afforded by PNCs.

An early example of PNC-based nanoreactors for prodrug activation is the encapsulation of cytosine deaminase within simian vacuolating virus 40 VLPs [31]. Simian vacuolating virus 40 VLPs were shown to have good cell attachment and internalization properties. Pretreatment of the nanoreactors enabled CV-1 cells to almost completely convert the prodrug 5-

Figure 2



Schematic representation showing the PNC architecture as a delivery system for enzyme prodrug therapy. The enzyme for specific activation of a prodrug is encapsulated inside the PNC and targeted to the disease site. The prodrug is converted into its active form catalyzed by the PNC–enzyme nanoreactor (shown in the figure) or the enzyme released from the PNC (not shown in the figure), only showing therapeutic effects at the disease sites. PNC, protein nanocage.

fluorocytosine to 5-fluorouracil (5FU), a cytotoxic molecule used in chemotherapy, which induced cell death with a similar efficiency as direct treatment with 5FU. Cytochrome P450 (CYP), an important enzyme associated with drug metabolism, was also used for prodrug activation. Nanoreactors have been designed for enzyme prodrug therapy by encapsulation of an activity-enhanced variant of CYP inside P22 [32] and cowpea chlorotic mottle virus [33] VLPs. Delivery of P22–CYP nanoreactors into the cytoplasm of Hela cells was realized by lipid-mediated transfection, and the delivered CYP was found to be catalytically active inside the cells.

Nanoreactors for prodrug activation were also developed by displaying enzymes on the exterior of PNCs. An engineered M13 bacteriophage was reported to possess targeting and cell-penetrating properties toward prostate cells, which was achieved by genetic fusion of a peptide called Ypep to the phage exterior [34]. By further presenting horseradish peroxidase, the engineered phage could oxidize indole-3-acetic acid, a prodrug, to produce peroxyl radicals in human PC3 prostate cancer cells, which effectively led to cytotoxicity and cell death.

Prodrug activation enzymes were also delivered using protein capsids originated from enveloped viruses. It was reported that enzymes (Fcy and Fur) that can activate 5FU were genetically fused to avian sarcoma leukosis virus Gag protein, which assembled into capsids that were then pseudotyped (i.e. co-produced) with VSV-G to form infectious enveloped VLPs lacking replication function [28]. The VLPs delivered the enzyme-incorporated capsid into PC3 cells, which then converted 5FU to cytotoxic compounds and significantly decreased cell viability. VLPs derived from human immunodeficiency virus (HIV)-1 have also been successfully developed for delivery of prodrug activation enzymes, which are incorporated by genetic fusion with native protein components inside the viral capsid. For example, thymidine kinase (which activates ganciclovir) was encapsulated inside the protein capsid via fusion to a mutant of Nef (negative regulatory factor, a regulatory protein located inside the capsid) [35], while linamarase (which converts linamarin to cyanide) was encapsulated via fusion to Vpr (viral protein R, located inside the capsid) [36], and both methods realized high enzyme incorporation levels within the VLPs. These two enzymes were both delivered by the VLPs into cancer cells *in vitro* and efficiently activated prodrugs to show therapeutic efficacy.

Self-activities and enzyme-like activities

Self-activities

PNCs have evolved to accomplish many different biological functions (such as cargo transport, storage, stress responses, and cellular metabolism), some of them achieved by biocatalytic activities. For instance, PNCs from the ferritin superfamily act as nanocontainers for iron oxide and maintain iron homeostasis in a controlled fashion. Their intrinsic ferroxidase-like activity catalyzes the formation of a particle of hydrated iron oxide,

by which iron ions are stored inside the PNC cavity [37], an activity that is important in cells for antioxidation and detoxification purposes [8]. Ferritin itself also displays weak peroxidase-like activity that is involved in the redox regulation [8]. These endogenous activities of ferritin play an important role in cellular metabolism, and ferritin, therefore, has biomedical potential against intracellular redox challenges.

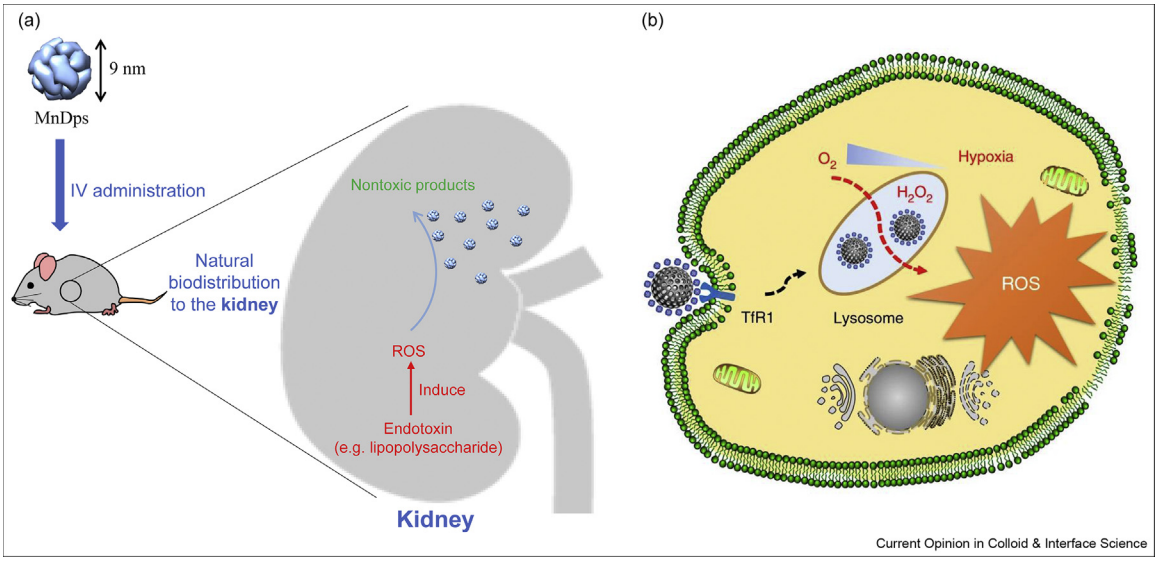
A recent demonstration of self-activity of PNCs for therapeutic use is the DNA-binding protein from starved cells (Dps), a member of ferritin superfamily, for treatment of sepsis-induced kidney injury (Figure 3A) [23]. Dps is a small hollow dodecameric PNC with a native antioxidative function. When loaded with Mn(II) ions at the ferroxidase active sites, Dps shows a stabilized catalase activity, which can be site-specifically delivered to the kidney *in vivo* based on the natural biodistribution of the cage for the removal of damaging H_2O_2 generated from endotoxin-induced oxidative stress. This example is an elegant application of PNCs as therapeutics using both the intrinsic catalytic activities and the natural targeting behavior of the architecture.

Enzyme-like activities

Ferritins can also act as containers for the encapsulation of nanomaterials having enzyme-like activities (nanozymes), which extends the biomedical utility of ferritin protein cages. Many different strategies have been used to synthesize nanozymes within the limited space of the ferritin cavity to constrain the growth of the metal nanoparticles, which is beneficial for the increase in surface area and consequently the size-dependent catalytic activity of the nanozymes [8]. The synthesized ferritin–nanozyme complexes are nanoreactors possessing selective enzyme-like activities. The human ferritin heavy chain subunit is recognized specifically by CD71 (transferrin receptor protein 1), making it useful for cell targeting and internalization, while the exterior surface of the cage can also be modified to affect delivery of ferritin and nanozymes [8]. Ferritin PNCs have demonstrated utility as templates for size-controllable nanozyme synthesis, as nanoreactors for incorporating enzyme-like activities, and as nanocarriers for targeted delivery, which clearly demonstrates advantages for delivery of enzyme-like activities for therapeutic purposes.

The theranostic applications of ferritin nanozymes with designed catalytic activities have been summarized comprehensively in a recent review [8]. Owing to the natural targeting propensity for CD71 (which is upregulated in tumor cells), a series of ferritin nanozymes have been synthesized and investigated for tumor diagnosis and cancer therapy. For example, several ferritins, with encapsulated peroxidase-like nanozymes, have been designed to catalyze chromogenic reactions,

Figure 3



Biomedical utility of self-activities of PNCs and delivery of enzyme-like activities as therapeutics using PNCs. **(a)** Dps loaded with Mn(II) ions (MnDps) is with enhanced catalase-like activity. Dictated by the natural biodistribution property of Dps, MnDps localizes in the kidney in mice after intravenous (IV) administration and shows effective protection against endotoxin-induced kidney injury [23]. **(b)** Human ferritin-conjugated nitrogen-doped porous carbon nanospheres (HFn-N-PCNSs ferritin nanozymes) have a tumor-targeting behavior due to the recognition of CD71 receptor by HFn, as well as the activity from the encapsulated nanozyme of producing reactive oxygen species (ROS) at acidic environment (such as lysosomes). HFn-N-PCNSs were shown to generate ROS in lysosomes of tumor cells and effectively induce tumor regression *in vivo*. Reprinted from the study by Fan et al [39] under Creative Commons license (http://creativecommons.org/licenses/by/4.0/). PNC, protein nanocage.

tested with high accuracy and sensitivity for tumor tissue visualization [8,38]. By encapsulating enzyme-like activities that induce reactive oxygen species, ferritins can be delivered *in vivo* to tumor cells to repress tumor growth (Figure 3B) [8,39]. In contrast, cellular protection against oxidative stress has also been reported through the delivery of ferritin cages with enzyme-like activities [8,40].

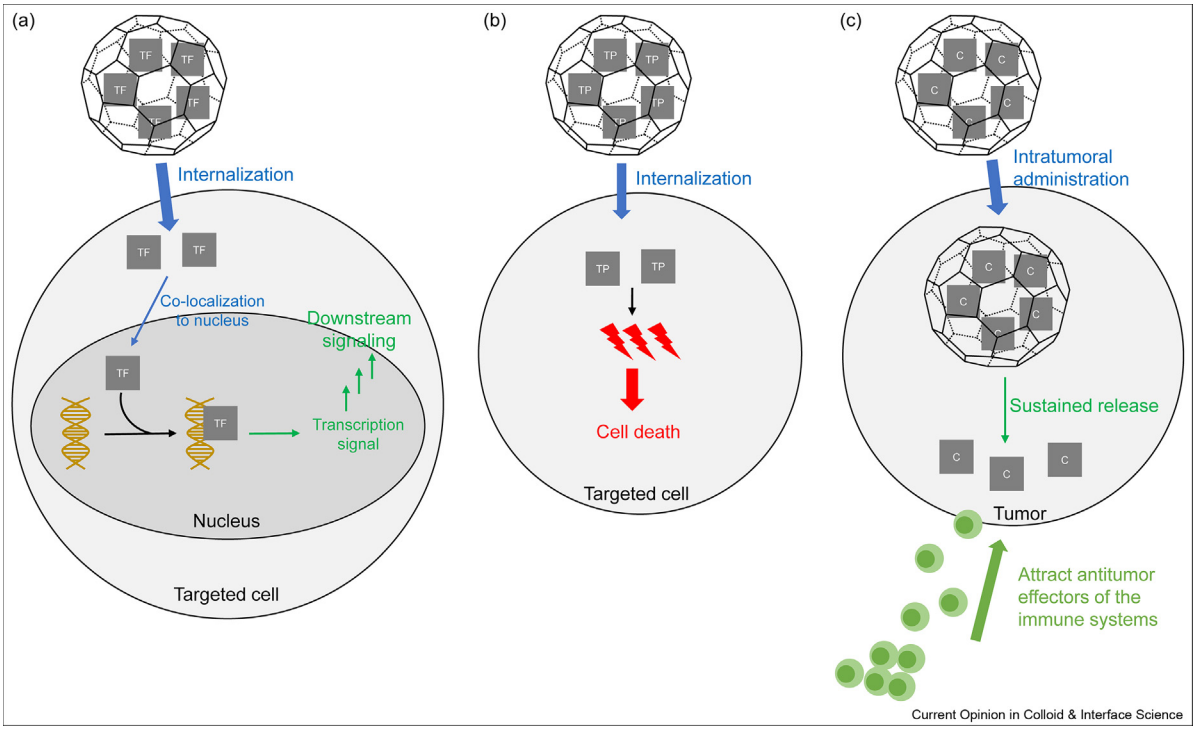
Other therapeutic proteins

Besides biocatalytic activities, proteins that are directly involved in gene regulations, cell apoptosis, and immune responses are also potential therapeutic targets for disease mitigation. Transcription factors, toxic proteins, and cytokines have been tested, pre-clinically and clinically, as treatment options and most of them function by forming complexes with other biomacromolecules to participate in, or influence, cell signaling or regulation of cell cycles. As the activities of these therapeutic proteins are dependent on direct intermolecular binding, there are two main challenges for using them as therapeutics with maximum efficacy and minimum off-target effects. First, the proteins need to be delivered site-specifically, as well as enclosed and protected during delivery, to prevent potential degradation and off-target activities. Second, the delivered proteins need to be released in their fully functional form at the disease sites. PNCs have been used as delivery systems to transport and release these proteins in a safe and efficient manner.

Transcription factors

Transcription factors (TFs) regulate the rate of gene expression, as part of the control of normal cellular and organismal activity including growth, development, and stress responses [41]. A lack of certain TFs for tumor suppressors can result in dysregulation of cell cycles and oncogenesis. Targeted delivery of TFs to interfere with the cell signaling of cancer cells could be effective for the induction of apoptosis and suppression of cancer growth [41]. However, the TFs need to be released inside the cells without protein degradation and transported to the nucleus to be therapeutically effective. Attempts have been made to take advantage of enveloped cage-like protein complexes to direct the internalization of TFs inside cells, with the incorporation of

Figure 4



Schematic representations showing PNC architectures as delivery systems for transcriptional factors, toxic proteins, and proteins with innate immune functions: **(a)** Internalization of PNC–TF complex releases TFs in the cellular cytoplasm, and the nuclear localization signal attached to the TFs directs the transportation into the nucleus for DNA binding and subsequent downstream therapeutic effect. **(b)** After internalization of PNC–toxic protein (TP), TPs are released inside cells, which induces downstream signaling and eventually cell death. **(c)** Administration of PNCs incorporated with proteins with innate immune functions such as cytokines at the location of the tumor could result in a sustained release of the therapeutic protein, which attracts antitumor effectors of the immune system to inhibit tumor growth. PNC, protein nanocage; TF, transcription factor.

appropriate peptide fusions (e.g. nuclear localization/export signal sequence) to direct transport to the nucleus (Figure 4A).

Using murine leukemia virus (MLV, a retrovirus) VLPs, it was shown that TFs could be delivered into cells and induce efficient transcription signals [42]. In this work, a fusion of TFs (such as SOX2) and nuclear export signal (NES) sequences were inserted into the MLV Gag gene, between the matrix and p2 proteins, linked by an MLV protease cleavage site. This strategy not only incorporates the TFs as a part of the immature capsid inside the enveloped VLPs but also allows NES -TF fusion proteins to be liberated as a free protein from the Gag polyprotein after cleavage by MLV protease (i.e. the native protease of MLV that processes Gag-Pol polyprotein into individual functional viral proteins for viral maturation) and subsequently packaged inside the mature capsid. After the cell entry of VLPs and disassembly of the protein capsids, TFs can be released in the cells to target the nucleus and bind DNA. This example demonstrates the release mechanism of the therapeutic proteins via the native disassembly pathway of the PNC (in contrast to the example of an engineered chimeric ferritin cage described in Toxic protein**s**, where the therapeutic protein was released via an active response of the PNC to ionic strength). In the process, the envelope structure outside the protein cage facilitates uptake into the cell, which circumvents endosomal–lysosomal pathway, reducing the risk of protein degradation. Potential engineering of the envelope was also proposed to change the tropism toward different cell types. A similar design has also been successfully developed using HIV-1 VLPs for TF delivery, where the TF-incorporated HIV-1 VLPs showed high resistance to freeze–thaw cycles without significant changes in morphology or TF delivery function, highlighting the stability of the material that is advantageous to these applications [43].

Toxic proteins

Toxic proteins from a broad range of natural sources act as a defense system for the native organisms against herbivores and pathogens [44]. Engineered toxic proteins are good candidates for the treatment of some diseases including cancer and bacterial infections. To be developed as therapeutics, these proteins need to be modified or concealed, to reduce immune responses, and delivered in a site-specific manner. By encapsulation inside PNCs, toxic proteins are hidden during transportation so that toxicity and immunogenicity are suppressed until they are released from the nanocarriers at the desired site of action (Figure 4B).

The MLV VLP system, mentioned previously, has been used to deliver toxic proteins as a potential treatment for cancer [42]. Because the toxic protein is damaging to the host in recombinant expression systems (which was demonstrated in a failed study using HIV-1 VLPs [36]), a bacterial 'toxin–antitoxin' system, MazEF, was used. The toxicity of the therapeutic protein is concealed during material preparation and protein delivery but takes effect after the internalization of the VLPs by the targeted cells. This system showed a good efficacy in the killing of chemotherapy-resistant cancer cells.

A chimeric ferritin nanocage was recently reported to deliver an inducer of apoptosis [45]. This PNC consists of both human ferritin heavy chain subunits, which offer targeting behavior toward CD71 receptors, and engineered archaeal ferritin subunits, which offer an ionic strength-mediated self-assembly/disassembly property and the consequent opportunity for cargo encapsulation and liberation (different from the MLV delivery system mentioned previously, which releases encapsulated proteins via native disassembly of the PNC). In this work, cytochrome c was used as a toxic protein model, which is able to bind apoptotic protease activating factor-1 in the cytoplasm and trigger a series of cell signaling events for apoptosis. Using this chimeric ferritin cage, cytochrome c was successfully delivered and released in myeloid leukemia cells which have a high-level expression of CD71, inducing significant cell death.

PNCs as nanocarriers can also deliver therapeutic proteins to cross biological barriers. The conjugation of the protein transduction domain of HIV-1 Tat peptide to the exterior of P22 VLP resulted in enhanced translocation of the PNC in blood–brain barrier models [46]. In this work, the modified P22 VLPs offer an alternative administration of therapeutic toxic proteins to intrathecal injection for targeting the central nervous system, as demonstrated by ziconotide, an analgesic peptide from the venomous marine snail.

Proteins with innate immune functions

Proteins with innate immune functions are also potential therapeutics. For example, cytokines aid in modulating immune responses, which are important in the defense against both exotic invasion and endogenous stress. Chemokines, a type of cytokine, have been shown to effectively attract antitumor effectors of the immune system, including dendritic cells, lymphocytes, natural killer cells, and natural killer T cells, and could be a treatment for the prevention of tumor growth [47]. The chemokine CCL21, delivered by vault protein cages, induced an enhanced T-cell migration *in vitro*, resulting from a steep chemotactic gradient established by the sustained release of CCL21 (Figure 4C) [47]. This sustained release property is probably due to the occasional escape of non-covalently encapsulated CCL21 from the vault nanoparticle when the protein cage 'breathes' and transiently switches from whole-

vault to half-vault structure. Antigen-presenting capacity of dendritic cells was also boosted *in vitro* by using vault-CCL21 compared with free CCL21. Further *in vivo* experiments showed that a single intratumoral administration of the vault-CCL21 formulation effectively inhibited lung tumor growth, with a similar efficacy to the frequent dosing of large quantity of free CCL21. The properties of slow release, low-immunogenicity, and plasticity of the platform toward modification for targeted delivery make vault proteins a promising delivery system for immune proteins as antitumor therapies.

Conclusion

The use of proteins as therapeutics is an emerging field in biomedicine. However, direct administration of bare proteins has multiple limitations, including lack of targeting, short biological half-life, and induction of unwanted immune responses. The architectures offered by PNCs as nanocarriers of biomacromolecules have been developed as delivery systems for therapeutic proteins, which solves some of the limitations of the protein therapies and extends their utility. PNC architectures have many advantages such as a highly homogeneity population and plasticity toward modifications, and efforts have been made to incorporate therapeutic proteins with different PNCs to achieve enhanced biocompatibility, stability, targeting, and controlled release. The future in-depth understanding on how PNCs behave *in vivo* (e.g. biodistribution properties, cellular internalization and degradation pathways, and interactions with the immune system), along with further diverse engineering on PNCs (e.g. targeted delivery and extension of circulation time), will move this approach toward the clinical utilization of such architecture for the delivery of therapeutic proteins.

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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