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Design and biosynthesis of functional protein nanostructures

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Proteins are one of the major classes of biomolecules that execute biological functions for maintenance of life. Various kinds of nanostructures self-assembled from proteins have been created in nature over millions of years of evolution, including protein nanowires, layers and nanocages. These protein nanostructures can be reconstructed and equipped with desired new functions. Learning from and manipulating the self-assembly of protein nanostructures not only help to deepen our understanding of the nature of life but also offer new routes to fabricate novel nanomaterials for diverse applications. This review summarizes the recent research progress in this field, focusing on the characteristics, functionalization strategies, and applications of protein nanostructures.

protein nanostructures, protein nanowires, S-layer, protein nanocages, self-assembly, biosynthesis, functionalization

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Introduction

In Feynman's famous talk entitled "There's Plenty of Room at the Bottom", at Caltech in 1959, he said "a biological system can be exceedingly small…they do all kinds of marvelous things—all on a very small scale". Now, we have been able to reconstruct many of these elegant things *in vitro*, accordingly allowing us to learn from the nature of life and to design novel functional nanostructures. Just giving our own examples, during the last decade, we constructed a series of nanostructures using natural proteins. They can be sorted into one-dimensional, two-dimensional, three-dimensional and hierarchical nanostructures with a variety of novel functions (Figure 1). This review will summarize the major research progress in this field.

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One-dimensional biological nanostructures—protein nanowires

Self-assembling protein nanowires

One-dimensional biological nanostructures usually refer to a kind of nanomaterials whose primary structures are composed of biomacromolecules. These nanostructures generally have a confined diameter at the nanoscale (less than 100 nm) and a high aspect ratio (more than 1,000). Unlike one-dimensional inorganic nanomaterials, one-dimensional biological nanostructures have typical characteristics: (i) inherent biocompatibility and bioactivity derived from biomacromolecules, making them perfectly qualified for biological applications; (ii) high specific surface area and highly ordered molecular arrangement, making them ideal platforms for the construction of combinatorial materials or nanodevices, and (iii) flexible adjustment of biomolecular building blocks by genetic engineering, biological interactions or chemical

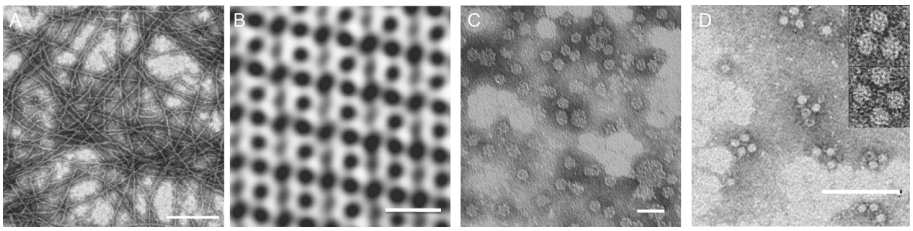

Figure 1 Protein nanostructures of different dimensions. A, Transmission electron microscopy (TEM) image of nanowires assembled from the yeast prion protein Sup35. Scale bar: 500 nm. B, Real map of self-assembled EA1 obtained by the Fourier transform of the TEM raw data. Scale bar: 10 nm. Figure adapted from Wang et al., 2015 (Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim). C, TEM image of polymorphic protein nanocages assembled from the major capsid protein of simian virus 40 (SV40). Scale bar: 50 nm. D, TEM image of hierarchical nanostructures of ferritin. Scale bar: 100 nm. Figure adapted from Men et al., 2016 (Copyright 2015 American Chemical Society).

modification, allowing the production of programmable assemblies with desirable functionalities. Due to these advantages, one-dimensional biological nanostructures are receiving increasing attention in recent years. With the development of interdisciplinary research, a large number of one-dimensional biological nanostructures have been proposed for various purposes in biological, chemical, biomedical, electric, and food-related fields (Cao and Mezzenga, 2019; Moore and Hartgerink, 2017).

Typically, three strategies are used to prepare one-dimensional biological nanostructures. One is based on the natural aggregation of proteins *in vivo*. In general, amyloid fibrils associated with various human diseases (such as Alzheimer's disease, Type II diabetes, and Huntington disease) are widely observed *in vivo* due to the linear self-assembly of misfolded proteins (so-called amyloid proteins). To date, a variety of amyloid proteins (such as yeast prion Sup35, Ure2, collagen, and fibroin) have been discovered to form one-dimensional amyloid fibrils. The second approach depends on mimicking the natural self-assembly process *in vitro*. Researchers have proposed a seeding-induced self-assembly strategy that first breaks amyloid fibrils into small fragments. Following the introduction of monomeric proteins, those small fragments can serve as seeds to facilitate the assembly of monomers into nanowires (Figure 2A) (Men et al., 2009). Superior to naturally formed one-dimensional biological nanostructures, nanowires of different lengths can be obtained based on the seeding-induced self-assembly method by adjusting the molar ratios between seeds and monomers. In addition, such a method enables the introduction of functional ligands into the nanowires through genetic engineering. The third method is artificial design by virtue of indirect interactions to arrange biomolecules in periodic linear structures (Figure 2B–F) (Luo et al., 2016). Rationally designed biomacromolecules with metal-binding motifs (such as histidine, cysteine, and glutathione) can be interconnected in the presence of metal ions (such as Ni^{2+}, Zn^{2+}, and Mg^{2+}), owing to the metal-ligand coordination (Kuan et al., 2018; Li et al., 2019a). Besides, electrostatic self-assembly (Sun et al., 2016a), host-guest interactions (Hou et al., 2013), receptor-ligand interactions (Luo et al., 2016), and protein-nucleic acid interactions (Chandrasekaran, 2016) have all been used to construct one-dimensional biological nanostructures. Over the past decade, although great efforts have been made to fabricate one-dimensional biological nanostructures with designed modules, precise control of their size distributions and functionalities remains a huge challenge. In virtue of the richness of biomolecules, a variety of one-dimensional biological nanostructures have been fabricated using peptides, proteins, cellulose, nucleic acids, and viruses as building blocks. In this review, we will focus on protein-based one-dimensional nanostructures, also known as protein nanowires.

Functionalization of protein nanowires

Functionalization is a prerequisite for the achievement of protein nanowires with desired features. Functional ligands can be introduced before or after the formation of protein nanowires (Figure 3). The former generally involves modifying the self-assembly domains with functional ligands by genetic engineering to produce fusion proteins as building blocks, which subsequently assemble into functional protein nanowires based on the intrinsic self-assembly ability or indirect interactions between building blocks. Using this strategy, a series of functional biomolecules (such as enzymes, fluorescent proteins, and antigens) have been genetically fused with self-assembling domains to obtain functional protein nanowires (Zhou et al., 2014a). Interestingly, Liu and colleagues used an electrostatic self-assembly method to construct dual-enzyme functionalized nanowires (Sun et al., 2015). A glutathione peroxidase catalytic center was introduced to the outside of the stable protein one (SP1) to form a cricoid structure, and a superoxide dismutase catalytic center was introduced into the polycationic PAMAM dendrimer bearing manganese porphyrin. Each PAMAM dendrimer allowed electrostatic interaction with two cricoid proteins in the opposite orientation to form a sandwich structure, thereby promoting the nanowire assembly.

The latter is to conjugate functional ligands through chemical modifications by harnessing active groups (such as amino, carboxyl, and sulfhydryl) on protein nanowires

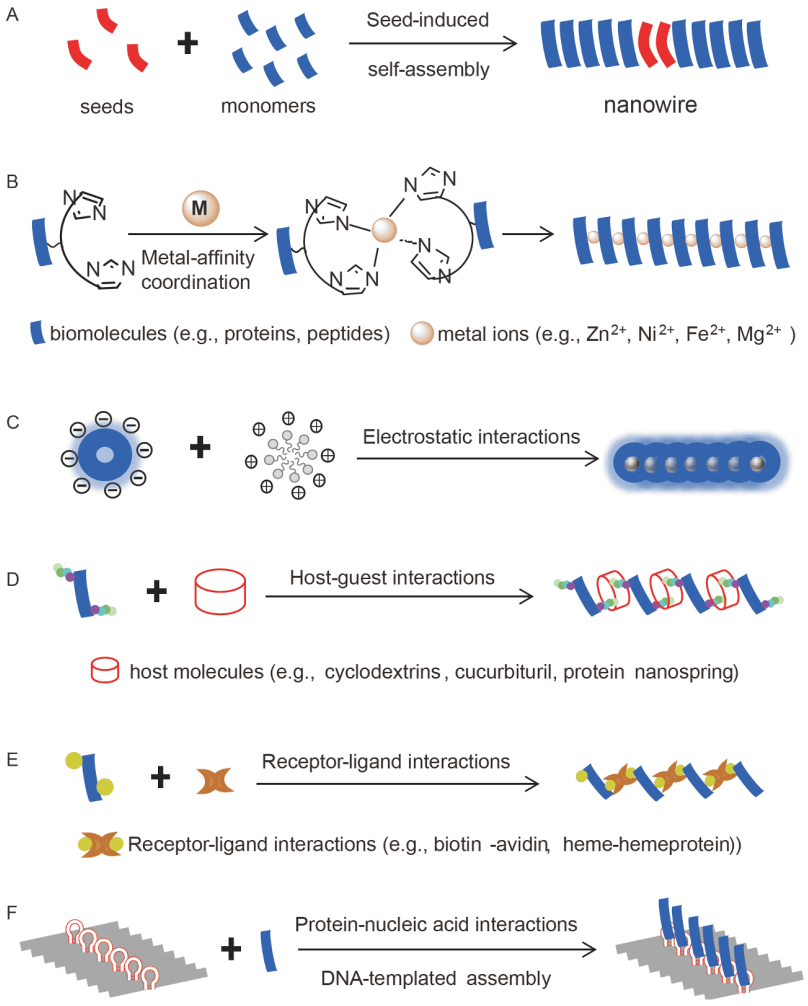

Figure 2 Common strategies to prepare one-dimensional biological nanostructures based on (A) seeding-induced self-assembly, (B) metal-affinity coordination, (C) electrostatic interactions, (D) host-guest interactions, (E) receptor-ligand interactions, and (F) DNA-templated aptamer-protein interactions.

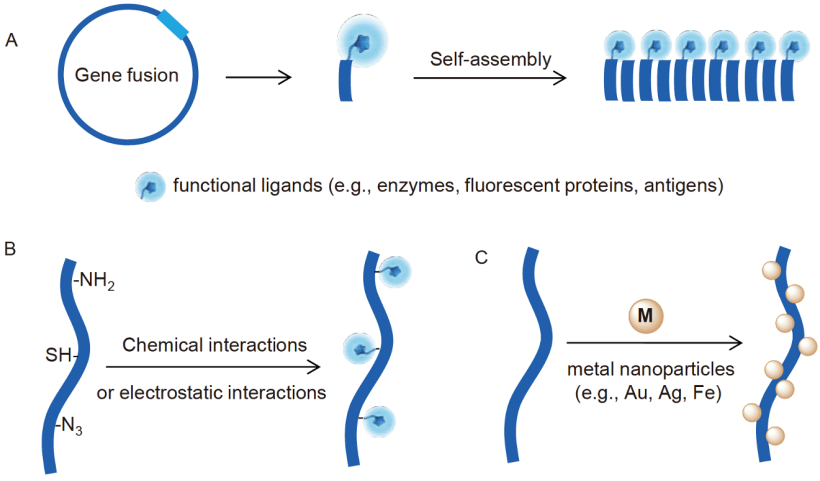

Figure 3 Modification of protein nanowires with functional ligands by (A) genetic engineering, (B) chemical or electrostatic interactions, or (C) *in situ* synthesis of metal nanoparticles.

(Buell et al., 2010). For example, Seki and colleagues produced a biotinylated protein nanowire using condensation reactions between amino groups on human serum albumin nanowires and carboxyl groups on biotins, resulting in stable

covalent modification. Furthermore, this biotinylated protein nanowire was readily conjugated to streptavidin-modified functional molecules due to the high affinity between biotin and streptavidin (Omichi et al., 2014). Therefore, biotinylated protein nanowires may provide a flexible platform for constructing more functional protein nanowires. Mezzenga and colleagues prepared graphene-functionalized amyloid fibrils through electrostatic interactions between negatively charged graphene and positively charged amyloid fibrils (Li et al., 2012a). Considering the unique advantage that the electronegativity of protein nanowires can be adjusted by changing the pH, electrostatic interactions may offer a general route to conjugate protein nanowires with functional groups bearing opposite charges.

In addition, owing to the existence of metal-reducing or metal-binding domains, protein nanowires can be employed as templates to promote the *in situ* synthesis of metal nanoparticles to introduce novel functionalities. Zhang and colleagues produced a ZrO_2-decorated amyloid fibril membrane with defluorinating ability using β-lactoglobulin-assembled amyloid fibrils as a template. The electrostatic interactions between Zr ions and charged amino acids on the amyloid fibrils were responsible for the recruitment of Zr ions and *in situ* formation of ZrO_2 nanoparticles (Zhang et al., 2019). Mezzenga and colleagues found that amino acids including cysteine, tyrosine, and tryptophan in the β-lactoglobulin amyloid fibrils can be used as reducing agents and stabilizers for the preparation of bioavailable iron nanoparticles (Shen et al., 2017b). Using a similar strategy, several inorganic nanoparticles (such as gold, silver, and calcium carbonate nanoparticles) have been anchored on protein nanowires (Nyström et al., 2018; Shen et al., 2017a). Furthermore, silicified protein nanowires can also be achieved by the hydrolysis-condensation of a silica precursor (e.g., tetraethoxysilane) in the presence of β-lactoglobulin amyloid fibrils (Rima and Lattuada, 2018).

Applications of protein nanowires

Protein nanowires with highly-ordered structure and large specific surface area can display a variety of functional moieties through the aforementioned strategies. In return, these functional ligands aligned with orientated arrays typically exhibit improved performance over their individuals or macroscopic materials. In addition to the prominent biological properties of protein nanowire itself, the introduction of functional moieties may also create synergistic effects or collaborative functions. Taking both advantages, functional protein nanowires have been widely used in biotechnology, biomedicine, and materials science (Wei et al., 2017). In particular, we will highlight the recent progress on the applications of protein nanowires in biosensors, catalysis, and biomedical materials.

Protein nanowire-based biosensors

A biosensor is a typical interdisciplinary product that includes specific probe fabrication, target recognition, and signal transduction. Since it is fairly easy to introduce functional ligands with specific binding ability (such as peptides, protein G, and antigens) or signal-generating ability (such as enzymes, fluorescent proteins, and fluorescent dyes) into protein nanowires by genetic engineering or surface modification, protein nanowires as an emerging bio-nanotechnology platform provide a powerful tool for constructing biosensors. Zhang and colleagues have conducted systematic investigations that make full use of self-assembling protein nanowires to achieve highly sensitive biosensing. They constructed bifunctional protein nanowires, which significantly increased the sensitivity of pathogen detection (Men et al., 2009; Men et al., 2010). Protein G (SPG) with the antibody-binding ability and methyl parathion hydrolase (MPH) with the signal-generating ability were genetically fused with the self-assembly domain of Sup35, respectively. The Sup35-SPG can be used as seeds to induce the self-assembly of Sup35-MPH, and the resultant protein nanowires integrated with a large number of signal molecules achieved a 100-fold enhancement of detection sensitivity over conventional methods. In addition to enzymes, fluorescent proteins and dyes can also be introduced into the nanowires as signal outputs for highly sensitive biosensing. Besides, Gerrard and colleagues displayed glucose oxidase on protein nanowires through the interaction between avidin and biotin and then anchored the sulfhydryl-bearing protein nanowires on the surface of gold nanoparticles to achieve sensitive glucose detection by an electrochemical method (Sasso et al., 2014).

When combined with high-throughput protein microarray techniques, protein nanowires enable multiplexed pathogen detection (Men et al., 2016). Inspired by the fact that periodic biological nanostructures often exhibit synergistic polyvalent interactions to enhance binding affinity to target molecules, Zhang and colleagues have recently developed a new strategy using the self-assembly of antigen proteins into nanowires to enhance the binding affinity for high-efficiency target capture. The use of magnetic beads to support antigen nanowires enabled rapid and sensitive immunoassays, with both strong binding affinity and rapid magnetic separation and enrichment (Figure 4A) (Men et al., 2018). Therefore, the protein nanowire can serve as a universal platform for specific antibody detection by altering the antigen on its surface (Lee et al., 2013).

Protein nanowire-based catalysis

The use of protein nanowires in catalysis is usually based on two ideas. One is that immobilizing enzymes on protein nanowires can endow them with distinct catalytic behaviors. Through rational design and genetic modification, these

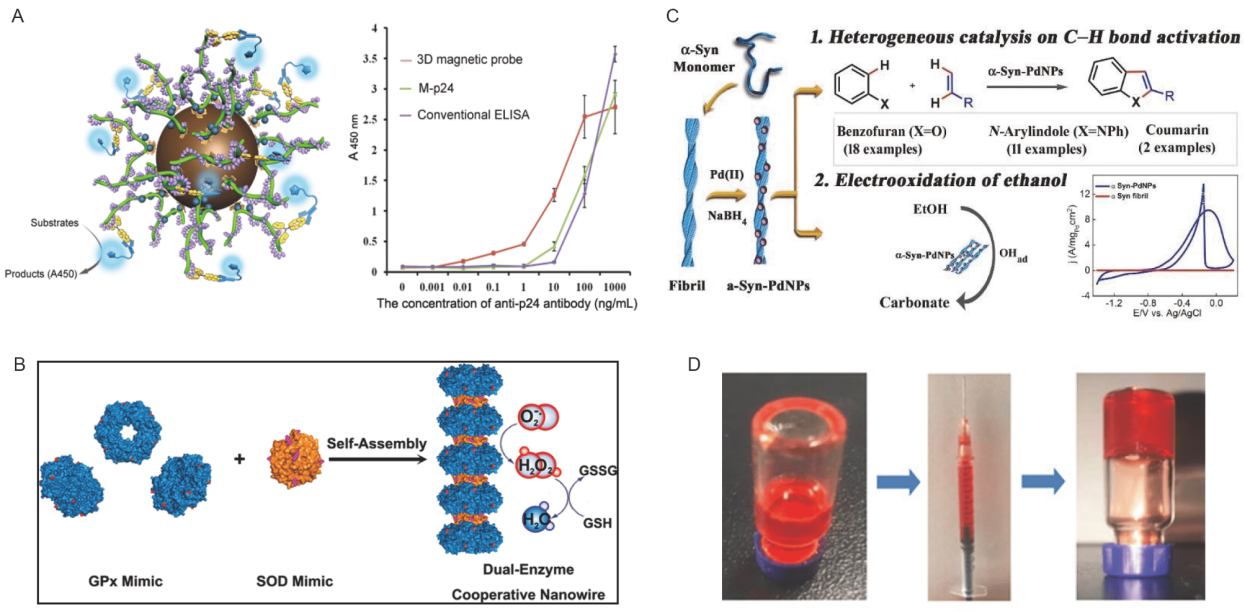

Figure 4 Applications of protein nanowires. A, Biosensing. Figure reprinted from Men et al., 2018 (Copyright 2018 American Chemical Society). B and C, Catalysis. Figures reprinted from Sun et al., 2015 (Copyright 2015 American Chemical Society) and Jayarajan et al., 2019 (Copyright 2019 Royal Society of Chemistry). D, Injectable hydrogel. Figure reprinted from Yang et al., 2019 (Copyright 2019 American Chemical Society).

catalytic nanowires with high density and ordered molecular arrays can reduce activity damage and even improve enzyme activity. The other is that protein nanowires can be good scaffolds for supporting metal nanoparticles to produce metallic protein nanowires with catalytic activities.

In early 2002, Wickner and colleagues constructed a variety of catalytic nanowires based on the self-assembly of the yeast prion protein, and studied the effects of side-chain groups on the prion self-assembly based on the measurement of catalytic activity (Baxa et al., 2002). Perrett and colleagues found that display of different enzymes, alkaline phosphatase, and horseradish peroxidase on the prion domain of Ure2 can lead to different enzymatic activities (Zhou et al., 2014a). As the fusion of alkaline phosphatase provided an effective catalyst for the phosphatase reaction comparable to commercial agents, they then assembled the alkaline phosphatase-Ure2 fibrils into microgel particles in combination with a microfluidic technique, further extending their applications in microflow chemistry (Zhou et al., 2015a). Recently, Liu and colleagues made a great contribution to the construction of artificial nanoenzymes based on protein nanowires. The active site of glutathione peroxidase was introduced into the capsid protein of the tobacco mosaic virus. With the self-assembly of capsid proteins, catalytic nanowires were obtained, which showed dramatically improved catalytic activity (Hou et al., 2012). Furthermore, glutathione peroxidase and superoxide dismutase were introduced into the nanowires to construct a multi-enzyme catalytic system for cooperative antioxidative applications (Figure 4B) (Sun et al., 2015).

On the other hand, Liu and colleagues constructed an Ag nanoparticle-nanowire composite through biomineralization (Qiao et al., 2018). Notably, in the process of catalytic reduction from p-nitrophenol to p-aminophenol, the catalytic activity of the Ag nanoparticle-nanowire composite was 4.19 times that of the hydrothermally synthesized Ag nanoparticles, resulting from the higher affinity between the composite and the substrate. Similarly, a variety of metal nanoparticles (such as Au, Pd, Cu, and Pt) with catalytic activities can be equipped on protein nanowires to develop new catalytic properties (Jayarajan et al., 2019). For example, using α-Synuclein fibrils as a template, Pd nanoparticle-nanowire composite was formed, which acted as a heterogeneous catalyst for dual applications in C–H bond activation and ethanol electrooxidation (Figure 4C).

Protein nanowire-based biomedical materials

Benefitting from the unique features of low-cytotoxicity and excellent stability in the physiological environment, amyloid fibrils have stimulated active studies in the construction of biomedical materials. Amyloid fibril-based hydrogels/aerogels have shown great potential in injectable reagents, cell culture and tissue engineering, and antibacterial response (Nyström et al., 2018).

Mezzenga and colleagues prepared pH-responsive hydrogels based on β-lactoglobulin fibrils and $CaCO_3$ nanoparticles. The $CaCO_3$ nanoparticle was employed as a crosslinker to promote the formation and stabilization of the gel network. The hybrid gel system exhibited a self-healing property by changing the pH value from neutral to acidic, making it feasible for injectable materials (Shen et al., 2017a). In a recent study, Ma and colleagues used fragments

of hen egg-white lysozyme to form amyloid fibrils, which were then enhanced by magnesium ions to produce an injectable hydrogel (Figure 4D). The hydrogel with low cytotoxicity and excellent thixotropic property was capable of drug delivery with the pre-loaded small-molecule drug doxorubicin (Yang et al., 2019).

Owing to their natural cell attachment properties, amyloid fibril-based hydrogel/aerogels can be used as matrixes to support cell growth and promote tissue regeneration (Reynolds, 2019). By immobilizing cell adhesion molecules or cytokines on the surface of protein nanowires, cells can grow and differentiate along the axis of protein nanowires. Furthermore, by modulating inducers at specific sites, directed cell growth and differentiation can be achieved with spatial selectivity, and eventually, a regenerative tissue skeleton is formed. Maji and colleagues also demonstrated that the thixotropic feature of hydrogels is beneficial for cell culture (Jacob et al., 2015). They used amyloid fibril-based hydrogels to grow a range of cell types (such as SH-SY5Y neuronal cells and L929 fibroblasts) in 2D and 3D cell culture, and then employed growth factors wrapped in the hydrogels to drive differentiation of human mesenchymal stem cells (Das et al., 2017). Through freeze-drying and cross-linking, amyloid fibril-based porous aerogels can be obtained, allowing cell penetration and proliferation (Nyström et al., 2017).

Besides, the deposition of silver onto the amyloid fibrils endows the resultant silver-nanowire aerogels with a good antibacterial response, originating from the well-known antibacterial activity of silver (Nyström et al., 2018). Furthermore, with the introduction of natural polyphenols (e.g., epigallocatechin gallate) into amyloid fibrils, reversible and thermal-resistant hybrid hydrogels can be achieved, exhibiting antibacterial activity against Gram-negative and Gram-positive bacteria (Hu et al., 2018). During the gelation of amyloid fibrils, antibacterial drugs such as tetracycline and penicillin can also be loaded into the gel for antimicrobial purposes (Cao and Mezzenga, 2019).

In summary, great efforts have been made in the preparation, functionalization, and application of protein nanowires, but several issues remain to be resolved in the future, including mechanism revelation, controllable uniformity, and high-qualified functionalities. The apparent trend of interdisciplinary study may greatly contribute to the development of protein nanowires. For instance, protein nanowires of controllable length can be prepared over a narrow range by virtue of stoichiometric regulation and highly efficient purification techniques. In addition to the aforementioned applications, the employment of protein nanowires in other disciplines such as electronics can uncover unprecedented functions, such as conductive protein nanowires (Sun et al., 2018; Tan et al., 2016).

Two-dimensional nanostructures—S-layer

Self-assembling S-layer

The surface layer (S-layer) is a single molecular layer with the crystal lattice, widely existing on the surface of archaea and bacteria. S-layer connects to the cell membrane or cell wall in a non-covalent form, completely enveloping the bacteria and forming a natural barrier between the organism and the external environment. Most S-layers consist of a single protein, forming a symmetric structure of P1, P2, P3, P4 or P6, with the number of subunits as 1, 2, 3, 4 or 6, respectively. The arrangement of subunits can be oblique (P1, P2), square (P4) or hexagonal (P3, P6) symmetry (Figure 5) (Sleytr et al., 2011). The S-layer thickness of bacteria is about 5–20 nm, the central spacing of each morphological unit is 3–35 nm, and the diameter of the hole occupying 70% of the surface of the S-layer is 2–8 nm (Sleytr and Sára, 1997; Sleytr et al., 1994). In organisms with an S-layer, proteins used to form the S-layer usually account for 10%–20% of total cell proteins. For a medium-sized rod-shaped bacterium, it takes approximately 5×10^5 monomers to form the entire S-layer (Schuster et al., 2013). During about 20–30 min of bacterial growth, the formation of the S-layer reflects a dynamic process of perfect supramolecular assembly.

Applications of bacterial S-layer

Over the past 30 years, the richness of the S-layer in terms of structure, chemistry, genetics, morphogenesis, and function has fully reflected its potential applications in many nano-related fields. S-layer proteins can be reassembled into two-dimensional nanolattice structures *in vitro*, which have the same or similar natural S-layer structure at various interfaces or surfaces of suspension, solid-phase support, lipid film, etc. (Ferner-Ortner-Bleckmann et al., 2013; Lopez et al., 2010; Pum and Sleytr, 2014; Toca-Herrera et al., 2005; Ucisik et al., 2013). Importantly, studies on the structure and function of S-Layer indicate that the fusion protein can still self-assemble into crystal lattice after recombination and expression of the S-layer gene with foreign fragments. Therefore, to obtain more ideal biological nanomaterials, the natural properties of the S-layer can be changed by gene manipulation. There have been reports of S-layer protein SbsB fused with streptavidin, SgsE or glucose-1-phosphate thymidyltransferase (RmlA), which can self-assemble into artificial S-layer *in vitro* (Moll et al., 2002; Schäffer et al., 2007). Using S-layer protein SbpA, the researchers also successfully constructed S-layers with the green fluorescent protein, camel antibody, and IgG-binding domain (Ilk et al., 2004; Pleschberger et al., 2004; Völlenkle et al., 2004). The *in vitro* self-assembly system lays a foundation for the application of bacterial S-layer in nanotechnology and inspires

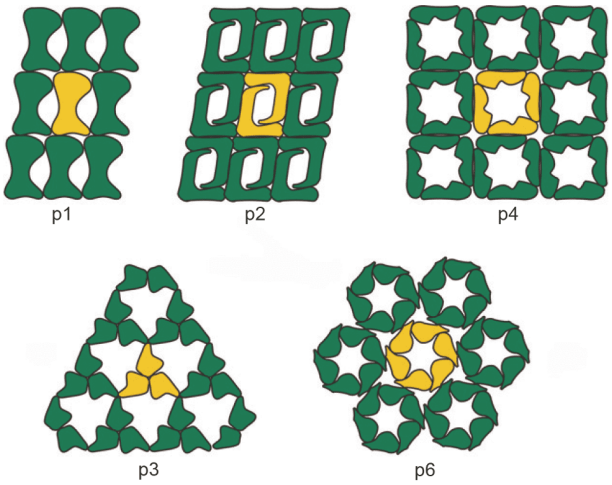

Figure 5 Schematic drawing of oblique (P1, P2), square (P4), and hexagonal lattice symmetry (P3, P6) of S-layer crystallin lattice. Figure adapted from Sleytr et al., 2011 (Copyright 2011 Elsevier).

development of two-dimensional biological nanodevices. At present, either a natural S-layer or a modified S-layer can be adopted in the following ways.

S-layer as ultrafiltration membrane and molecular sieve

As mentioned above, the S-layer is a regular, ordered two-dimensional lattice structure composed of a single protein. The surface of the S-layer has holes of the same size and shape, with each molecular unit down to the sub-nanometer level. Using this property, the researchers fixed the S-layer with glutaraldehyde and reduced the pressure under the condition of sodium borohydride to prepare ultrafiltration membranes with uniform pore size and various specifications, which can be used to screen nanoparticles of different sizes (Sára and Sleytr, 1987; Schuster et al., 2001; Weigert and Sára, 1995).

Controlled immobilization of functional molecules on S-layer

In the fields related to nanotechnology, such as biosensor, molecular electronics, and non-linear optics, it is very important to achieve the directional immobilization of functional molecules. The S-layer surface is arranged orderly and densely with a variety of functional groups, including carboxyl, amino, and hydroxyl. For example, there are approximately 60 carboxyl groups on each S-layer subunit of *Bacillus sphaericus*, corresponding to a charge density of 1.6 carboxyl groups per nm^2 (Sleytr et al., 1996). Therefore, S-layer can bind functional molecules by covalent/non-covalent bonds. For the covalent binding, it is mainly achieved by chemical crosslinking between the carboxyl group and the amino group. Non-covalent bonds depend on the interactions between charges. After immobilization of functional molecules on the S-layer, these molecules remain active, and the S-layer can recrystallize on the surface of various solid-phase supports. To date, many functional molecules of interest, such as enzymes, antibodies, and antigens, have been successfully immobilized on the surface of the S-layer and used as highly sensitive sensors for the detection of blood sugar and other analytes (Picher et al., 2013; Rothbauer et al., 2013; Scheicher et al., 2013).

S-layer-supported lipid membranes

S-layer proteins not only self-assemble into the S-layer on artificial lipid membranes such as Langmuir-Blodgett films, planar lipid membranes, and liposomes (Schuster and Sleytr, 2000), but also stabilize the lipid membranes and significantly increase their life expectancies (Ucisik et al., 2013). Compared to solid-supported lipid membranes, S-layer-coated functional lipid membranes offer additional advantages in physiochemistry, biological stability, handleability, and multifunctionalization. Therefore, S-layer-supported lipid membranes are promising nanocarriers that can be used in highly sensitive and specific biosensors, drug delivery, and other biomedical applications. Up to now, the safety of S-layer-coated lipid membranes has been extensively evaluated. For example, it was reported that the emulsomes coated by the S-layer with a characteristic crystal lattice can be taken up by human liver carcinoma cells without significant cytotoxicity (Schuster et al., 2001). Besides, Hollmann et al. achieved the safe and efficient delivery of calcein to human colon adenocarcinoma cells using S-layer-coated liposomes, suggesting the potential of S-layer-coated liposomes as oral delivery carriers (Hollmann et al., 2007; Hollmann et al., 2018).

S-layer as a scaffold for fabrication of metallic nanoparticle arrays

Orderly metallic nanoparticles have unique photoelectric and magnetic properties that can be adjusted by changing the density of nanoparticles and the distance between nanoparticles (Li et al., 2016; Sheng et al., 2014; Xie and Yan, 2017). Fabrication of two-dimensional regular arrays of metal nanoparticles for flexible nanodevices is a rapidly developing research field. Some advanced techniques such as electron beam lithography, chemical vapor deposition, and metal etch can be used to fabricate well-defined nanoparticle arrays, but these techniques are costly and difficult to acquire nanoarrays with super-resolution (<10 nm spacing). Considering that S-Layers have various crystal lattices at the nanoscale, they are natural biotemplates for the manufacture of metallic nanoparticles. Similar to the bacterial mineralization in the natural environment, when the S-layer is placed in a solution containing metal ions, metal ions can be reduced and precipitated into holes on the surface of the S-layer by reducing agents. The spatial distribution of metal nanoparticles depends on the crystal lattice of the S-layer itself. In principle, the S-layer can be used as a template to

synthesize metal nanoparticle superlattices with a wide particle size distribution, different particle spacings, and various symmetries. Using this strategy, researchers have successfully fabricated high-quality nanoparticle arrays such as CdS, Au, Pt, and Pd (Liu et al., 2008; Mark et al., 2006; Shenton et al., 1997; Tang et al., 2008; Wahl et al., 2001). Compared with physical and chemical methods, the S-layer provides a mild, low-cost and easy-operate template for the preparation of orderly nanoparticle arrays with high resolution.

S-layer-based biosensors

Given the ability of S-layer proteins to self-assemble on the surface of Si, gold, and other electrodes, the S-layer lattice is often used to link bioreceptor and transducer interface for bioreceptor immobilization, signal amplification, low detection limit, etc. (Schuster, 2018). Glucose oxidase (GOx) biosensors are the most widely used. To date, the S-layer has been successfully used in a variety of GOx biosensors, including electrochemical biosensors and fiber-optic biosensors, enabling the rapid, sensitive and specific detection of glucose (Neubauer et al., 1993; Neubauer et al., 1996; Picher et al., 2013). Besides, S-layers were also employed on cholesterol sensors and oxygen sensors for excellent performance (Ferraz et al., 2011; Guimarães et al., 2014; Scheicher et al., 2009). In recent studies, S-layer-based sensors were reported to detect folate receptors in breast cancer cells and cellular markers in hepatic oval cells and cancer cells (Damiati et al., 2017; Damiati et al., 2018a; Damiati et al., 2018b). As can be seen, more and more S-layer-based biosensors are being applied for biomedical purposes.

Although it has been confirmed that more than 400 kinds of bacteria and archaea in nature have an S-layer structure, researchers have shown great interest in the S-layer of only a small number of bacteria, such as *Bacillus sphaericus* and *Bacillus stearothermophilus*. In addition, they mainly studied the *in vitro* self-assembly ability of S-layer proteins and related physicochemical properties, but the intrinsic biological functions of S-layer proteins were usually ignored. In fact, S-layers from different bacteria or archaea not only represent a great variety of crystal structures but also have a diversity of biological functions. Therefore, more S-layers need to be explored for their wide application in biosensors.

EA1 (non-virulent protein) as the main protein of the S-layer of *Bacillus anthracis* is widely distributed on the surface of vegetative cells and spores, enabling it a potential protein marker of *Bacillus anthracis* (Wang et al., 2009). Besides, other researchers have also confirmed that the protein, as the main antigen of *Bacillus anthracis*, can induce serological reaction and produce a large number of specific antibodies, which can be used as a basis for the serological diagnosis of anthrax (Makam et al., 2014; Shlyakhov et al., 2004). Based on the above characteristics, we constructed a bifunctional two-dimensional enzyme array by *in vitro* self-assembly of EA1, and applied EA1-based nanoarrays and magnetic beads to the serological diagnosis of anthrax (Figure 6) (Wang et al., 2015). In this study, EA1 was genetically fused with methyl parathion hydrolase (MPH) to degrade methyl parathion and provide a label for signal amplification; EA1 served not only as a nanocarrier but also as a binding molecule to capture anthrax-specific antibodies; the EA1-MPH complex formed a highly ordered monolayer with the same P1 symmetry and similar lattice parameters as the natural S-layer of *Bacillus anthracis*; the chimeric nanocatalyst greatly improved the enzymatic stability of MPH and achieved highly sensitive detection of anthrax-specific antibodies in serum samples. Taken together, this research not only provided a novel nanobiosensor but also proposed a new model for the development of multifunctional nanostructures with biomedical value using the bacterial S-layer.

Three-dimensional biological nanostructures—protein nanocages

Self-assembling protein nanocages

There are various kinds of protein nanocages self-assembled from one or several protein subunits in organisms, which generally have the symmetry of tetrahedron, octahedron, and icosahedron. Protein nanocages have been recognized as an important class of scaffolds for biological nanodevices because of their appropriate nanoscale sizes (10–200 nm), high homogeneity, large-scale preparation by biosynthesis, convenient modification by chemical and genetic engineering, good biocompatibility, biodegradability, availability of inner and outer surfaces, and controllable switch between assembly and disassembly. Functional nanostructures and nanodevices derived from protein nanocages have been successfully applied in cancer thermotherapy, bioimaging, biosensing, drug delivery, tissue engineering, catalysis, and other fields (Flenniken et al., 2009).

Typical protein nanocages include virus-based nanoparticles (VNPs), ferritins, DNA-binding proteins from starved cells (Dps), heat shock proteins (Hsp), etc. VNPs are formed by viral capsid proteins through self-assembly. They do not contain the viral genome and cannot replicate independently, and therefore they are non-infectious. VNPs usually have icosahedral symmetry, similar to the capsid of natural viruses, and range in size from tens to hundreds of nanometers (Pokorski and Steinmetz, 2011; Salunke et al., 1989; Sun et al., 2007). Many viral capsid proteins can self-assemble *in vitro* to form VNPs, including plant viruses (e.g., brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), cowpea mosaic virus (CPMV), red clover necrosis mosaic virus (RCNMV), and turnip yellow mosaic virus (TYMV)), animal and human viruses (e.g., simian virus 40

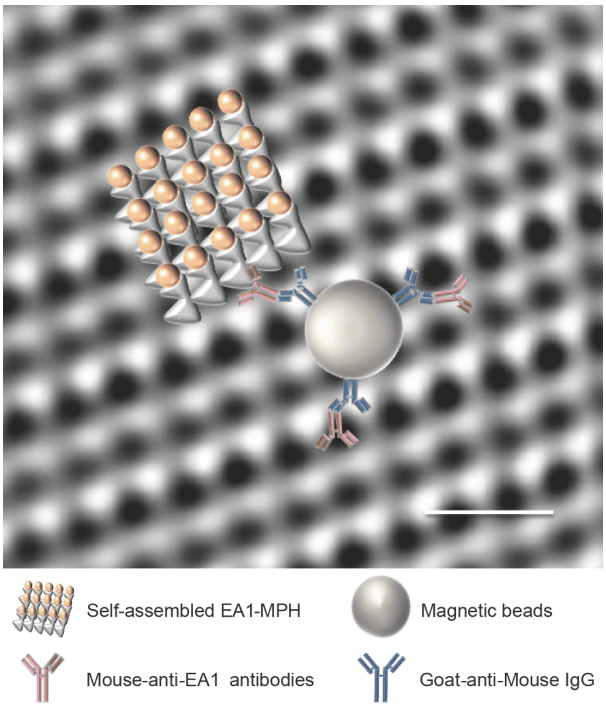

Figure 6 Illustration of an S-layer-based biosensor for detection of anthrax antibodies. The background picture reveals the altered regular structure of the S-layer sheet of EA1-MPH. The foreground picture illustrates the detection of anthrax antibodies with self-assembled EA1-MPH. Scale bar: 10 nm. Figure adapted from Wang et al., 2015 (Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim).

(SV40), human papillomavirus (HPV), and hepatitis B virus (HBV)), and bacteriophages (e.g., P22, MS2, and Qbeta) (Li and Wang, 2014). Ferritin has an octahedral cage structure self-assembled from 24 identical or homologous subunits with an outer diameter of 12 nm and an inner diameter of 8 nm (Arosio and Levi, 2002). Dps belongs to the ferritin superfamily, also known as mini-ferritin, which has an octahedral cage structure like ferritin. The difference is that Dps is assembled from 12 monomers with an outer diameter of about 9 nm and an inner diameter of about 4.5 nm (Almirón et al., 1992). Hsp is widely found in a variety of cells and is produced by cells under stress conditions (especially high temperatures). The Hsp from *Methanococcus jannaschii* consists of 24 identical subunits arranged in octahedral symmetry with a 12 nm outer diameter and a 6.5 nm inner diameter; the Hsp cage has eight 3 nm-sized triangular windows and six 1.7 nm-sized square windows (Kim et al., 2003). Diverse protein nanocages in living organisms constitute a pool of protein-based nanomaterials and have inspired *de novo* design of self-assembling proteins with desired properties (Malay et al., 2019), which is enriching the pool.

Functionalization of protein nanocages

Due to well-defined structural features, protein nanocages are often used as templates or scaffolds for the construction of multifunctional nanostructures. Functional components can be loaded into the inner cavity or onto the outer surface, and more complex multifunctional nanostructures can be obtained using both the inner cavity and the outer surface (Douglas and Young, 2006). There are several ways to load functional components onto/in protein nanocages (Figure 7): (i) fusing peptides or proteins to the N- or C-terminus of nanocage subunits through genetic engineering; (ii) connecting functional elements to the inner or outer surfaces of protein nanocages by covalent or non-covalent interactions; (iii) packaging functional elements in the inner cavity through controllable assembly of protein nanocages; (iv) *in situ* synthesis of materials at the sites of interest by using affinity tags on protein cages, which can be introduced by genetic engineering or chemical modification.

In 1998, Douglas et al. used the pH-dependent gating mechanism of CCMV VNPs to load precursor materials and synthesized paratungstate and decavanadate nanoparticles through mineralization in VNPs. In this way, they pioneered a method of constrained synthesis of inorganic nanomaterials within protein nanocages (Douglas and Young, 1998). Subsequently, various inorganic nanomaterials such as β-titanium dioxide, Fe_2O_3, CdS, CuS, and Au were successfully synthesized in protein nanocages (Klem et al., 2008; Reichhardt et al., 2011; Zhou et al., 2014b). Besides, the packaging of pre-synthesized nanoparticles has become another way to load nanomaterials in protein nanocages and has been implemented in VNP self-assembly systems of various viruses, including BMV, CCMV, alphavirus, RCNMV, MS2, SV40, and HBV. The nanomaterials that have been packaged include AuNPs, CdSe quantum dots (QDs), Fe_3O_4, and $CoFe_2O_4$ (Aniagyei et al., 2009; Chen et al., 2006; Daniel et al., 2010; Dixit et al., 2006; Dragnea et al., 2003; Goicochea et al., 2007; Huang et al., 2007; Loo et al., 2006; Loo et al., 2007; Sun et al., 2007; Tsvetkova et al., 2012). In most cases, guest materials are encapsulated into protein cages by dialyzing the mixture of protein cage building blocks and cargoes against assembly buffers. Differently, we have recently reported a simple concentrating method for cargo encapsulation in light of the apparent critical assembly concentration of protein cages, which has the advantage of being more friendly to the cargoes to package (Li et al., 2019b).

Since 2005, our team has developed a self-assembly system of SV40 VNPs (composed of VP1 pentamer, the major capsid protein of SV40) and carried out a series of studies on packaging nanoparticles with different particle sizes and surface modifications. The natural capsid of SV40 is an icosahedron (T=7) with a diameter of about 45 nm, but VP1 tends to form a regular icosahedron VNP with T=1 icosahedral symmetry in the presence of QDs (Li et al., 2011b; Li et al., 2009; Salunke et al., 1989). SV40 VNPs can package CdSe@ZnS QDs with different surface coatings (such as

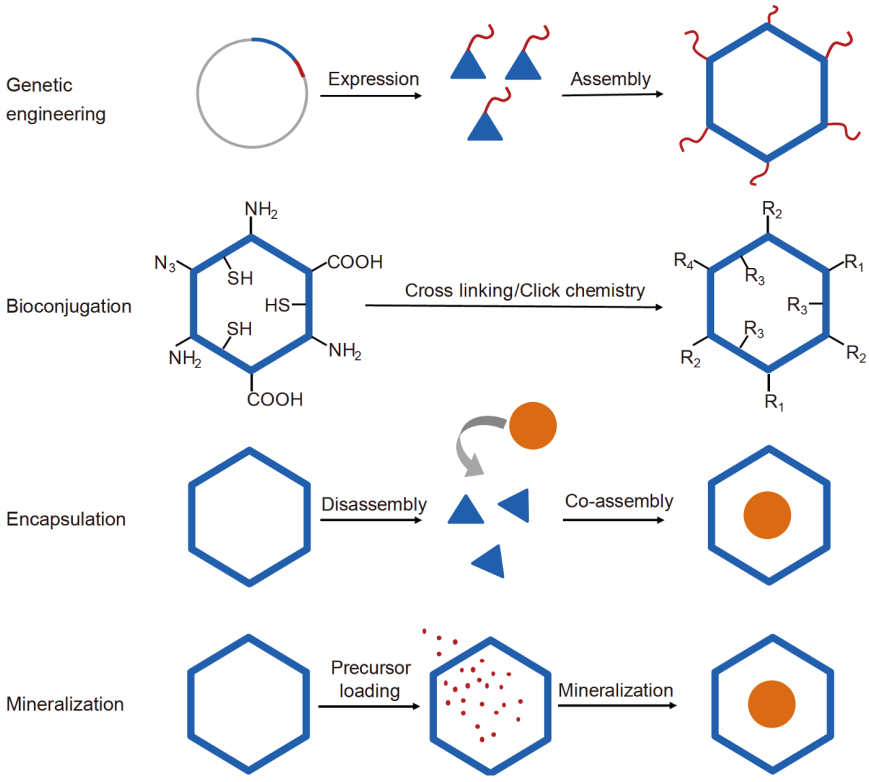

Figure 7 Routes to functionalizing protein nanocages.

mercaptopropionic acid, DNA, PEG with methoxyl terminus, and PEG with amino terminus) (Li et al., 2010), Ag_2S QDs (Li et al., 2015; Li et al., 2012b), and AuNPs (Wang et al., 2011). We also found that QDs can induce VNP assembly (Gao et al., 2013) and that cysteines at positions 9 and 104 of VP1 play a key role in stabilizing the VNP-nanoparticle heterogeneous structures. In addition to chemically synthesized nanomaterials, foreign proteins and DNA origami can also be packaged into protein nanocages by rational design (Glasgow et al., 2012; Minten et al., 2009). Similarly, the controllable synthesis of nanomaterials and assembly of presynthesized nanomaterials can also be performed on the outer surface of protein nanocages. For example, Co, Ni, Fe, Pt, Co-Pt, and Ni-Fe nanomaterials have been successfully mineralized on the surface of CPMV VNPs (Aljabali et al., 2010; Aljabali et al., 2011). Besides, Johnson and Finn et al. arranged AuNPs orderly on the CPMV surface and proposed the concept of VNPs as an addressable template for the ordered assembly of nanomaterials (Blum et al., 2005; Blum et al., 2004; Chatterji et al., 2005; Wang et al., 2002).

Meanwhile, more complex nanostructures can be constructed by utilizing the inner and outer surfaces of protein nanocages, which provide more space for functional coupling at the nanoscale. Since 2011, we have reported a series of studies on the orderly assembly of nanoparticles by using SV40 VNPs as a template and synergistically utilizing their internal and external spaces. The protein-nanoparticle interface can be rationally regulated via genetic engineering and chemical modification. Besides, precise control of particle composition, number, spacing, and other parameters has been achieved, providing an instructive model for nanophotonics research (Li et al., 2012b; Li et al., 2011a; Li et al., 2011b; Zhou et al., 2015b).

Applications of protein nanocages

By virtue of the aforementioned strategies, protein nanocages can be functionalized to fulfill various goals and have thus received extensive attention in basic research and nanobiotechnology innovation, from targeted delivery to nanophotonics.

Protein nanocages as carriers of bioactive molecules

Protein nanocages are widely used as delivery carriers of bioactive molecules such as vaccines, nucleic acids, polypeptides, and small-molecule drugs. For example, virus-like particles formed by self-assembly of L1, the major capsid protein of HPV, have been successfully developed and translated into the HPV vaccine (Paavonen et al., 2007). Lim et al. encapsulated polyacid and anti-cancer drug doxorubicin in the VNPs of Hibiscus chlorotic ringspot virus (HCRSV), which increased the loading efficiency of doxorubicin. Folic acid on the outer surface of the VNP imparted cancer-targeting capacity. This nano-sized system greatly

enhanced the doxorubicin uptake capacity of ovarian cancer cells and the cancer-killing capacity of doxorubicin (Ren et al., 2007). Kimchi-Sarfaty et al. used SV40 VNPs to package plasmid as a gene therapy vector, showing a high transfection efficiency (Kimchi-Sarfaty et al., 2002). Recently, we have efficiently loaded multiple anti-tumor peptide drugs into P22 VNPs via genetic fusion for intracellular delivery, in which a tumor-specific cleavable linker was present between the peptide drug and the capsid protein for controlled release (Figure 8A) (Wang et al., 2018). Curiel and Everts used adenoviral vectors to deliver AuNPs into cells, where AuNPs were loaded on the adenoviral vectors by covalent bonds or van der Waals force. The ability of adenoviral vectors to infect and target tumor-related antigens was maintained, which can be used in hyperthermia of tumor sites (Everts et al., 2006; Saini et al., 2008). Taken together, bioactive molecule delivery is a promising application of protein nanocages, which has attracted considerable attention. A more detailed introduction can be found in several elegant reviews or perspectives (Edwardson and Hilvert, 2019; He et al., 2019; Liu et al., 2019; Molino and Wang, 2014; Rohovie et al., 2017).

Protein nanocage-based bioimaging and biosensing

The co-assembly of protein nanocage and fluorescent probe can be used to develop novel fluorescence imaging strategies. In 2009, we reported a study to dynamically monitor SV40 VNP infection in living cells by encapsulating fluorescent QDs in VNPs. QD-VNPs were found to mimic early steps of the SV40 infection. This packaging strategy fundamentally avoided the interference of QDs on VNP-host cell interactions (Li et al., 2009). In 2015, real-time dynamic fluorescence imaging of SV40 VNPs *in vivo* was achieved for the first time by packaging Ag_2S QDs, which emit fluorescence in the second near-infrared window (NIR-II), with the advantages of deep penetration and high spatio-temporal resolution (Figure 8B) (Li et al., 2015). Recently, we have constructed a multi-functional VNP nanodevice that enabled fluorescence imaging and targeted drug delivery for theranostic of atherosclerotic plaques at different stages *in vivo* (Sun et al., 2016b). Besides, Wang et al. demonstrated a fluorescent labeling method for enveloped viruses. By modifying a sequence complementary to the HIV pseudoviral genomic RNA on QD, QD was packaged into the viral capsid, and the infection behavior of the enveloped pseudoviruses with the VSV-G envelope and the HIV capsid was tracked (Zhang et al., 2013). Furthermore, high-density organic fluorescent molecules loaded in CPMV VNPs and near-infrared fluorescent dyes packaged in the BMV VNPs have also been demonstrated for *in vivo* fluorescence imaging (Gupta et al., 2013; Lewis et al., 2006).

Recently, magnetic resonance imaging (MRI) contrast agents based on Gd^{3+} have attracted much research interest. Macromolecules such as polymers, dendrimers, and liposomes are applied in the loading of Gd^{3+}, as it is expected that many Gd^{3+} loaded in a single molecule can increase the imaging relaxation rate (Hooker et al., 2007). In this regard, protein nanocages are ideal carriers for loading Gd^{3+} because of their controllable self-assembly, large inner space, and addressability of modification sites. In 2005, Douglas et al. utilized CCMV VNPs to load Gd^{3+} via the Ca^{2+}-binding sites inherent in CCMV VNPs (Allen et al., 2005). Later, they loaded Gd^{3+} using P22 "Wiffleball" (WB) particles. The pore size of P22 WB VNPs was about 10 nm, which facilitated material exchange inside and outside VNPs. The particle diameter was 64 nm, and each P22 VNP can carry up to 9,100 Gd^{3+}. At 298 K 0.65 T, the relaxation rate reached 41,300 $(mmol\ L^{-1})^{-1}\ s^{-1}$ (Qazi et al., 2013). In addition, they initiated atom transfer radical polymerization in the P22 VNPs, restricted polymer synthesis, and loaded high-density Gd^{3+} on polymers for imaging vascular inflammation (Kosuge et al., 2013; Lucon et al., 2012). On the other hand, VNPs containing superparamagnetic iron oxide nanoparticles (SPIONs) have also been assembled for MRI. SPIONs can produce a negative contrast effect by reducing the transverse relaxation time (T_2). In 2007, Huang et al. reported the first example of SPION@VNPs for MRI using the VNPs of BMV (Huang et al., 2007). Then the magnetic BMV VNPs were demonstrated to be able to penetrate the plant cell wall into the cytoplasm and cell junctions, holding potential in the investigation of plant growth and development (Huang et al., 2011). Besides, MRI applications have also been explored with VNPs derived from other viruses such as HBV (Shen et al., 2015) and SV40 (Enomoto et al., 2013).

Protein cage-based nanophotonics

Many nanomaterials have unique optical properties. When they are assembled in a controlled manner, new properties or functions may be produced because of their interactions. Protein nanocages are ideal templates for the directional assembly of optical nanostructures due to their appropriate mesoscopic scale and addressable structure. In 2007, Wang and Charbonniere et al. established a prototype of biological nanoparticles for time-resolved immunofluorescence analysis using TYMV VNPs (Barnhill et al., 2007). Based on the principle of fluorescence resonance energy transfer (FRET), a sensor derived from the VNPs with multivalent modification can be developed. In 2011, we and collaborators constructed a series of QD-AuNP hybrid nanostructures with SV40 VNPs as the template to quantitatively study the energy transfer between QDs and AuNPs. The experimental data, consistent with theoretical predictions, showed that the surface plasmon resonance (SPR) coupling effect between the 4 nm AuNPs on the SV40 VNP surface was weak, while AuNPs had a strong cumulative quenching effect on QD

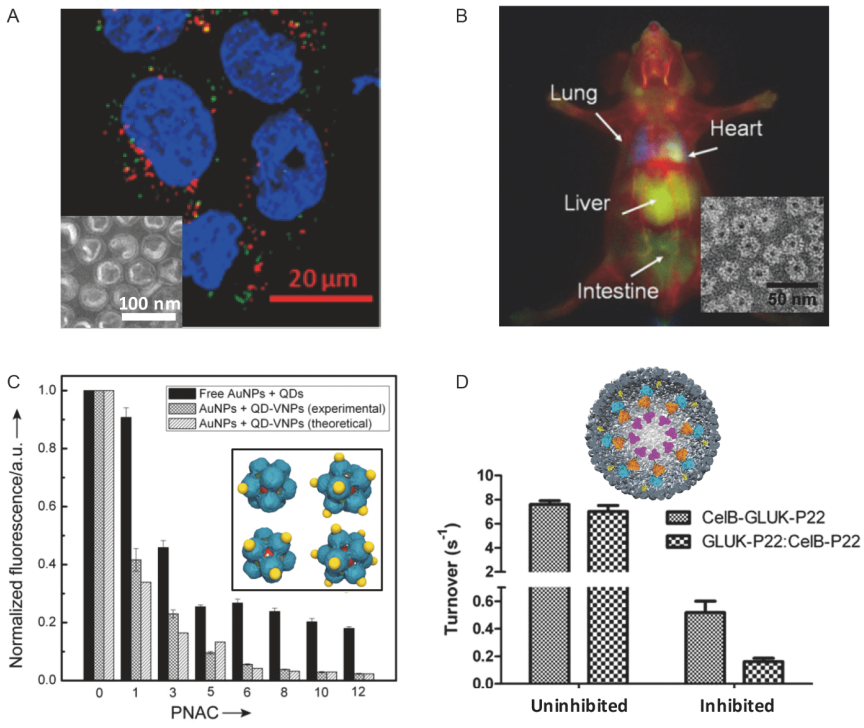

Figure 8 Diverse applications of nanostructures derived from protein nanocages. A, Controlled release of peptide drugs delivered by a P22 VNP-based system. Figure adapted from Wang et al., 2018 (Copyright 2018 Royal Society of Chemistry). B, NIR-II real-time monitoring of *in vivo* distribution of VNPs by virtue of encapsulated Ag_2S QDs. Figure adapted from Li et al., 2015 (Copyright 2015 American Chemical Society). C, Photonic interactions in VNP-templated hybrid AuNP-QD nanoarchitectures with well-controlled parameters. Figure adapted from Li et al., 2011b (Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim). D, Formation of a synthetic metabolon by co-encapsulating 2 or 3 enzymes within a P22 VNP. Figure adapted from Patterson et al., 2014 (Copyright 2014 American Chemical Society). Insets show the TEM images (A and B) or models (C and D) of functionalized structures derived from protein nanocages.

fluorescence (Figure 8C) (Li et al., 2011b). Besides, in 2013, Francis et al. packed AuNPs inside the MS2 VNPs and attached fluorescent dyes to the outer surface of VNPs for fluorescence enhancement study. By using DNA of different lengths to tune the distance between dyes and the AuNP in the protein cage, they observed a distance-dependent fluorescence enhancement effect (Capehart et al., 2013). A more detailed review of this topic can be found in a recent article (Petrescu and Blum, 2018).

Protein cages as nanoreactors

When enzymes are encapsulated in protein nanocages, confined reaction space can be formed to control the transfer of substrates and products, achieving the regulation of the catalytic reactions. Carboxysomes are natural examples of using protein cages as nanoreactors, and their selective permeability to CO_2 and O_2 is critical for their efficient carbon sequestration in living organisms (Yeates et al., 2008). Artificial protein nanoreactors have also been designed and constructed, generating a platform for study of enzymatic catalysis and development of novel biomaterials. Cornelissen et al. packaged a single horseradish peroxidase and a precisely controlled amount of lipase B in CCMV VNPs respectively. Their results showed that the activities of the packed enzymes significantly increased, independent of the number of enzymes per VNP (Comellas-Aragonès et al., 2007; Minten et al., 2011). Douglas et al. expressed different enzymes that catalyzed related reactions as fusions with the P22 scaffold protein, all of which were encapsulated in the inner cavity of the P22 VNPs and formed a cascade (Figure 8D) (Patterson et al., 2014). In addition, P22 VNPs containing β-glucosidase could be further arranged into a protein macromolecular framework through templated assembly, resulting in condensed-phase materials with high local concentration of enzymes (McCoy et al., 2018). It is envisioned that these bio-inspired protein nanostructures will, in return, enrich the toolbox of synthetic biology.

Conclusion and outlook

Biological nanostructures are formed by self-assembly guided by physical and chemical laws and biological principles. Some of them form the cellular structures and some constitute biological devices or molecular machines functioning in cells. Synthesis of these nanostructures *in vitro* helps to understand how life systems form ordered structures and deepen our understanding of life mechanisms. Furthermore,

these biological nanostructures have many marvelous characteristics and are easy to redesign using gene manipulation tools to achieve desired functions.

There are countless natural nanostructures in cells. Current research has only touched a very limited number of them. Even so, their identity in structures and functions already shows the beauty of nature and the great application potential. The extended and in-depth study of biological nanostructures will strongly push forward the development of nanobiology and bionics.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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