



Structure-based design of novel polyhedral protein nanomaterials

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Organizing matter at the atomic scale is a central goal of nanotechnology. Bottom-up approaches, in which molecular building blocks are programmed to assemble via supramolecular interactions, are a proven and versatile route to new and useful nanomaterials. Although a wide variety of molecules have been used as building blocks, proteins have several intrinsic features that present unique opportunities for designing nanomaterials with sophisticated functions. There has been tremendous recent progress in designing proteins to fold and assemble to highly ordered structures. Here we review the leading approaches to the design of closed polyhedral protein assemblies, highlight the importance of considering the assembly process itself, and discuss various applications and future directions for the field. We emphasize throughout the exciting opportunities presented by recent advances as well as challenges that remain.

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Introduction

Self-assembling proteins are encoded by organisms across all domains of life as well as viruses, and adopt a variety of architectures ranging from unbounded crystals and filaments to bounded, polyhedron-like assemblies [1]. A subset of these, closed polyhedral protein assemblies, have three distinct genetically defined surfaces arrayed symmetrically with high spatial precision, which engenders modularity to their biological functions (Figure 1a): (i) interiors, which can compartmentalize other molecules; (ii) exteriors, which interact with the surrounding environment; and (iii) inter-subunit surfaces, which define the architectures of the assemblies themselves and tune their assembly/disassembly processes [2].

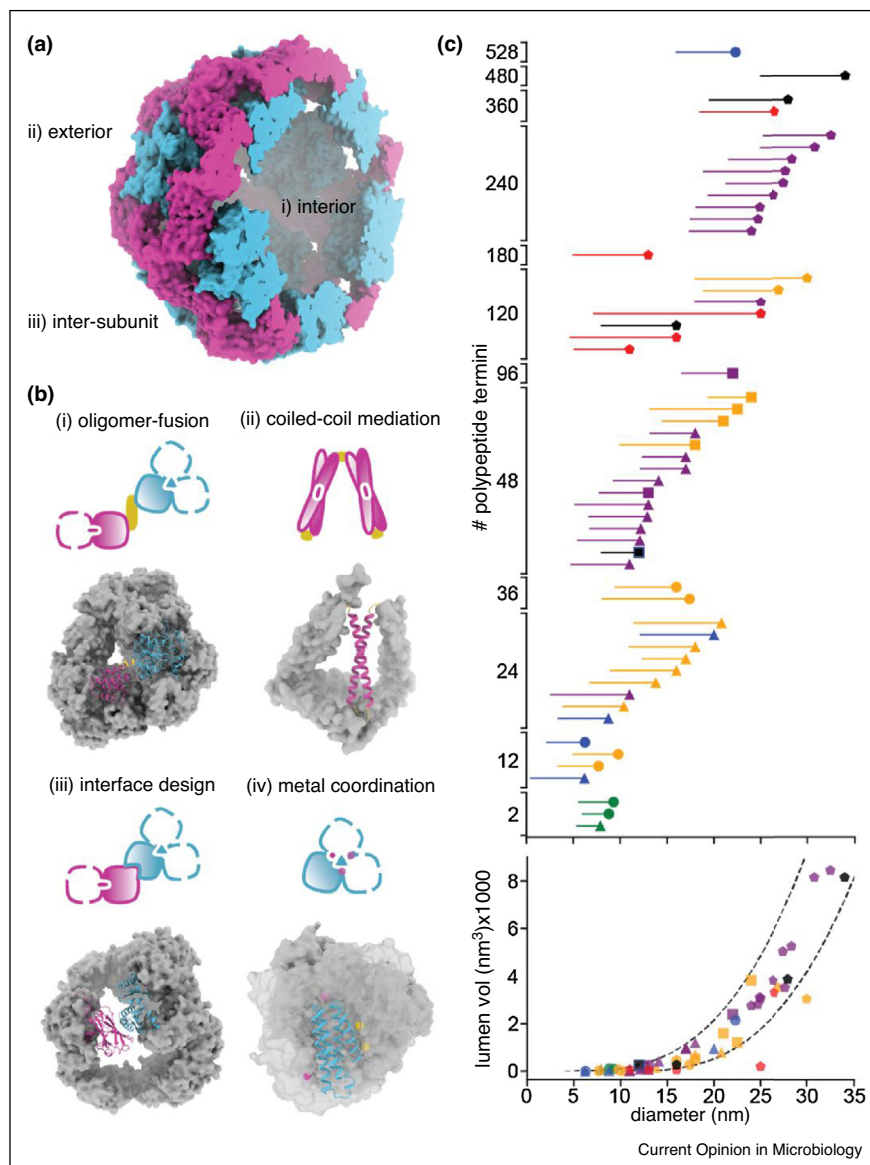
A number of naturally occurring hollow polyhedral protein nanomaterials (e.g., viruses, ferritin, and bacterial microcompartments) have been successfully appropriated for a variety of biomedical applications, usually by displaying or encapsulating molecules of interest [3]. However, these efforts are constrained by the relative paucity of these nanomaterials and their low tolerance to major modifications. By leveraging synthetic as well as natural sequence space, protein design can vastly expand the number and variety of possible protein nanomaterials [4]. Furthermore, new properties may emerge from multimerization of protein building blocks that do not naturally assemble into higher-order architectures. The degree of structural order can also be tuned through design as required by specific applications. While these features are common to all designed protein nanomaterials (e.g., fibers, 2D lattices, crystals), as has been previously reviewed [5–7], here we focus on novel polyhedral protein nanomaterials. More specifically, we discuss the most common strategies for designing these types of container-like structures in the context of three key characteristics of any protein nanomaterial: structure, assembly, and function.

Structures of designed self-assembling proteins

The defining feature of a self-assembling system is that the information determining its structure must be encoded by two highly interdependent properties of its building blocks: their three-dimensional structure(s), and their mode(s) of interaction. Building blocks that exhibit a high degree of order in their structures and interactions produce more highly ordered materials, while flexibility and promiscuity tend to result in materials that are less defined. Essentially all strategies for designing large and complex ordered protein nanomaterials share the common theme that many copies of simpler building blocks must interact in specific orientations [7]. Several design strategies have been developed to produce bounded 3D protein materials (Figure 1b), most commonly obeying point group symmetries such as tetrahedral, octahedral, and icosahedral symmetry. Each strategy is capable of yielding nanomaterials with defined structural features such as the number of termini, shell thickness, and internal volume (Figure 1c).

The structure-based approach that pioneered the explicit use of symmetry as a design element combines multiple distinct self-oligomerizing domains into a single polypeptide building block through *genetic fusion* [8–11] (Figure 1b

Figure 1



Structure-based design of novel protein nanomaterials.

(a) Example of a rationally designed protein nanomaterial (I53_dn5) [39**], highlighting the three distinct surfaces available for modification: (i) interior, (ii) exterior, and (iii) inter-subunit.

(b) Most common strategies for the rational design of 3D bound protein materials. Schematic representation and surface maps of successful examples of tetrahedral assemblies resulting from each design strategy (i. PDB ID 3VDX [9], ii. [24], iii. PDB ID 6OT7 [31**], iv. PDB ID 4NWN [26]).

(c) Survey of structural specifications of well-defined designed assemblies. Outer diameters (top) of the design models are plotted from explicitly stated published values, or values estimated from scale images or experimental data. Inner diameters (left end of colored lines) correspond to published values, or are estimated by subtracting the width of the protein component(s) from the outer diameter. Lumen volumes (bottom) are estimated from inner diameter, and plotted against outer diameter; the black dashed traces plot the relationship between outer diameter and lumen volume for spheres with 2 nm and 5 nm shell thicknesses. Colored polygons and circles in both plots correspond to: black, natural assemblies; purple, *interface design*; blue, *metal-mediated*; orange, *genetic fusion*; red, *coiled-coil*, green, *edge traversal*; pentagons, icosahedral symmetry; squares, octahedral symmetry; triangles, tetrahedral symmetry; circles, other.

(i). Each self-oligomerizing domain is aligned to a rotational symmetry axis in the target architecture, a strategy that can in theory be used to design a very wide variety of architectures comprising combinations of multiple point

group symmetries [7]. In this way, genetic fusion of self-associating domains into a single polypeptide elegantly satisfies the requirement of multiple interfaces to drive assembly and ensures the equimolar stoichiometry

appropriate for many simple architectures. For example, a recent report described the assembly of a genetic fusion of trimeric, pentameric, and dimeric components into a structure with icosahedral symmetry, the only point group that can perfectly accommodate these three rotational symmetry axes [12*].

The simplicity and modularity of *coiled-coils* as assembly modules has facilitated the development of several strategies for designing supramolecular protein assemblies. The genetic fusion approach has been widely used in conjunction with self-assembling peptides [13] of two coiled-coil domains [14,15] or of coiled-coil domains and oligomeric proteins [16–19,20*]. Additional strategies include the use of disulfide bonds between distinct coiled-coil oligomers, which has generated assemblies up to 100 nm in diameter [21,22], and an edge traversal strategy resembling DNA nanotechnology [23] that produces monomolecular polyhedra [24] (Figure 1b(ii)).

Over the last several years, computational methods for *de novo interface design* have been developed that are conceptually generalizable to the production of symmetric assemblies from any well defined protein building block (Figure 1b(iii)). These methods have been used to design—with atomic-level accuracy—protein assemblies ranging from cyclic homooligomers to polyhedral assemblies to unbounded filaments and layers [25–27]. Recently, *de novo* interface design has been combined with genetic fusion to develop a new hierarchical design approach (Hsia *et al.*, bioRxiv doi:<https://doi.org/10.1101/2020.07.27.221333>).

Designing *metal coordination* sites between protein building blocks (Figure 1b(iv)) is another powerful and versatile approach to produce new supramolecular structures [28]. The dependence of assembly on the directionality of protein-metal interactions—and therefore the identities of the metal ions present—can result in specific assemblies and introduce overall chirality [29,30,31**].

Control over assembly, disassembly, and structural homogeneity

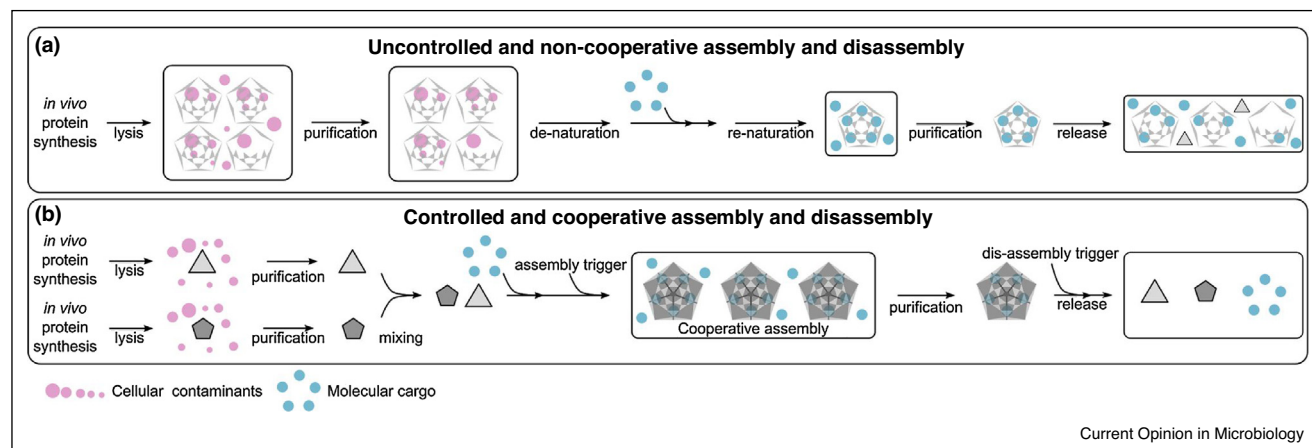
In addition to three-dimensional structure, the dynamics of assembly and disassembly processes are defining characteristics of a given multisubunit complex [32]. Here we consider assembly *dynamics* to encompass mechanisms of subunit flux between the unassembled and higher-order state (s), as well as the potential relationship between assembly conditions and heterogeneity of the resulting structures. Consideration of these processes during design can have substantial functional import. For example, assembly of single-component (i.e., homomeric) architectures is generally constitutive, proceeding *in situ* during protein production in the cell. This may lead to inadvertent encapsulation of cellular contaminants [12*,20*] and render the nanomaterial lumen inaccessible for functionalization (Figure 2a).

In contrast, assembly of multi-component architectures, proteic or otherwise, are inherently controllable, proceeding upon mixing of the independent components [16,17,26,29,31**,33–35] (Figure 2b).

Cooperative assembly is effectively a two-state process, where low concentrations of subunits are sufficient to drive self-organization toward a single low-energy architecture. Cooperativity is often aided by hierarchical organization, where a higher order assembly is preceded by the formation of particular assembly competent substructures. *Non-cooperative* systems in contrast are polymorphic, featuring incomplete versions of the designed structure, disordered aggregates, and other off-target structures. Non-cooperative outcomes often result from a combination of high-affinity interactions between building blocks, as well as flexibility in the orientations between them, such as when two (or more) obligate oligomers are genetically fused in the absence of geometric control [10,11,14,17,20*]. Hence design efforts employing the genetic fusion approach tend to focus on engineering rigid linkers between the oligomerization domains [8–10,12*] (Hsia *et al.*, bioRxiv doi:<https://doi.org/10.1101/2020.07.27.221333>). Selecting components of the highest possible rotational symmetry in the architecture of interest also constrains the diversity of thermodynamically preferred ‘closed’ structures which satisfy all binding interfaces [12*,18]. In *de novo* interface design, both issues can be circumvented by encoding relatively weak protein–protein interactions between rigid protein oligomers. This strategy produces proteins that assemble hierarchically, first into oligomeric building blocks and subsequently (via a cascade of low-order reactions akin to classical nucleation) [36,37] into a milieu of only oligomers and completely closed polyhedral structures, devoid of alternative closed architectures [25,38]. Extension of this method to multi-component architectures yields additional control over the assembly process via component sequestration and mixing *in vitro* [26,34,39**]. A recent study exploited this property of multi-component assemblies to establish that partially assembled structures only arise from *in vitro* assembly in extreme non-stoichiometric regimes [35].

Many studies have demonstrated the versatility of multi-component *in vitro* assembly of metal-protein complexes [29,40,41]. In addition to their integral role as non-proteic components of designed *de novo* assemblies, metals can be introduced into existing natural or synthetic protein nanomaterials (e.g., ferritin) to enable control over their assembly states [20*,33]. The dynamic control mechanisms offered by metal-mediated assembly fundamentally derive from the ‘switchability’ of metal-ligand interactions, which can be enabled and disabled by straightforward manipulation of chemical, oxidoreductive, and thermal triggers [28–30]. Densely packed ionizable residues on coiled-coil components have also been shown to effect more cooperative assembly [24]

Figure 2



Comparison of controlled and uncontrolled assembly and disassembly.

(a) An example of an assembly that is both uncontrolled and non-cooperative, which proceeds simultaneously with protein synthesis, non-specifically encapsulating intracellular contaminants (violet) and resulting in a variety of higher-order structures. A disassembly and purification step is thus required to access the nanomaterial interior and allow for error correction to proceed toward a single target structure that could encapsulate a molecular cargo (blue). Heterogeneity of the assembled product leads to inconsistent cargo protection and release properties.

(b) An ideal multi-component assembly is inherently controllable by intentional sequestration and mixing of individually prepared components. Alternatively (or additionally), assembly can be triggered by a physical or biochemical condition, allowing simultaneous assembly and encapsulation. Cooperative assembly yields a uniform product, which prevents premature cargo release.

and pH-responsive disassembly [22] in materials that do not rely on metal ions, suggesting a general and precise route to genetically encoding dynamic assembly behavior into protein nanomaterials.

Current applications

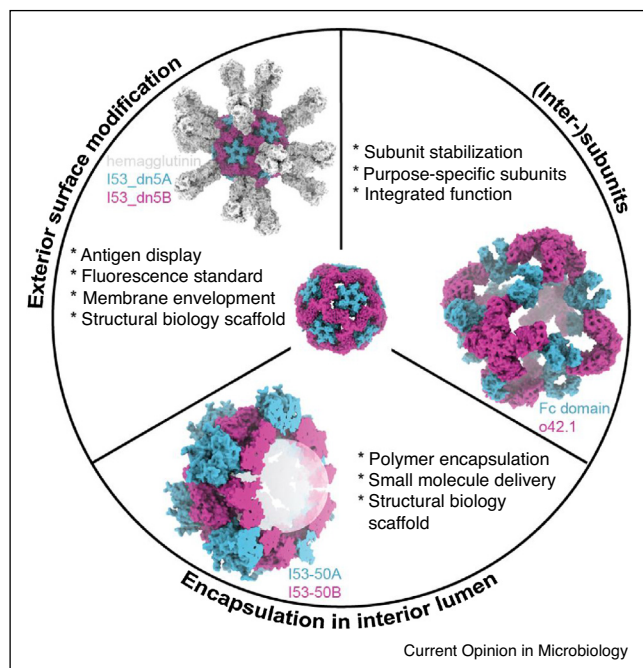
The ultimate aim of designing novel protein nanomaterials is to construct simple, robust, and controllable protein-based technologies tailored to specific applications (Figure 3). The size, shape, and symmetry of protein nanomaterials, as well as the number and location of termini and other functional elements, can all affect functional performance [42], highlighting the importance of design strategies that enable precise control of these properties. Although the approaches described above are still early in their development, designed protein materials are already being applied in various contexts, and reports of truly customized materials are beginning to appear [39^{••}].

Considering first the exterior surface, genetic fusion of functional protein domains is a straightforward way to display a wide variety of molecules, from a defined number of GFP molecules to function as fluorescence standards [38,43] to organized arrays of antigens in nanoparticle vaccines that induce potent or broad immune responses [44,45,46[•],47,48,65]. Fusing an adaptor system such as Spy-Tag/SpyCatcher is a useful variation that allows rapid prototyping of multivalent materials through selective and irreversible conjugation [49–51]. Adaptor-mediated functionalization can also be used to overcome symmetry mismatches between

nanoparticle components and displayed antigens [52]. Designed protein nanomaterials have also been explored as scaffolding platforms that have achieved near-atomic resolution of the structure of a small protein bound to a rigidly fused DARPin adaptor [53,54]. Finally, modification of designed protein assemblies with membrane-binding and ESCRT-recruiting domains led to their release from cells inside cell-derived membrane envelopes [55]. The protein-directed formation of a membrane bilayer can be useful, for example, in protecting cargoes and transporting them across membrane barriers in delivery applications. An alternative approach to increase cargo protection is to modify key physicochemical features of the assemblies themselves, such as the electrostatic charge density lining pores that connect the assembly lumen to bulk solvent [56].

In addition to driving assembly of designed nanomaterials, inter-subunit interactions can directly contribute to nanomaterial function in several ways. Self-assembling proteins often have greatly enhanced thermal and chemical stability compared to monomers, a general physicochemical phenomenon that is shared by natural and designed assemblies alike [20[•],30,35,38,57]. In some cases, enhanced stability has been conferred to molecules displayed on the exterior of the polyhedral assemblies [46[•],47]. Moreover, as any stable protein—natural or designed—can be used as a component, one can incorporate pre-existing functionality into designed protein nanomaterials by choosing purpose-specific components such as enzymes [20[•],35] or building blocks designed

Figure 3



Selection of current applications of rationally designed polyhedral protein assemblies, grouped by the surface addressed for their realization. Examples are provided for each category: a nanoparticle vaccine based on the two-component icosahedral nanoparticle I53_dn5 displaying genetically fused influenza hemagglutinin [65], the two-component octahedral assembly o42.1, which integrates Fc domains as dimeric components (Divine *et al.*, doi:<https://doi.org/10.1101/2020.12.01.406611>), and the two-component icosahedral nanoparticle I53-50-v4 with its mRNA-packaging interior lumen highlighted [56].

specifically for optimal genetic fusion of viral glycoprotein antigens [39**]. An excellent recent example is the use of antibodies as components of designed multi-component assemblies, achieved by co-opting the naturally occurring Fc-Protein A interaction (Divine *et al.*, doi:<https://doi.org/10.1101/2020.12.01.406611>).

Lastly, the interior lumens of such materials can be engineered to encapsulate molecules of interest. For example, the histidine-rich interior surface of a metal-mediated assembly bound and immobilized a flexible microperoxidase, enabling determination of the structure of this challenging target [40]. More commonly, positive charges on the interior surface of protein assemblies have been engineered or evolved to interact with negatively charged molecules such as nucleic acids for delivery applications [22,56,58]. Most recently, this electrostatic mechanism was leveraged to create a hydrophobic interior compartment by trapping anionic surfactants, expanding the portfolio of possible encapsulation targets from polar to hydrophobic molecules [59]. Alternatively, encapsulation can be achieved through a

genetic fusion approach that introduces-specific molecular interactions [55]. Collectively, these studies highlight the modularity and versatility with which protein nanomaterials may be engineered.

Challenges and opportunities

Although there has been tremendous recent progress in designing novel protein nanomaterials and functionalizing them for a variety of applications, even a cursory glance at their natural counterparts make clear that the field has only begun to scratch the surface of what is possible. Innovative approaches that combine high throughput methods for screening novel designed assemblies with structural validation are needed to drive improvements in design methods by enabling rapid iterative feedback from experimental data. The value of such large-scale design-build-test cycles was recently demonstrated for the *de novo* design of small monomeric proteins [60]. Current design strategies yield closed polyhedral protein nanomaterials from a relatively limited range of symmetries, sizes, pore diameters, and lumen volumes (Figure 1c) [61]. Developing methods to introduce local asymmetry, control and exploit structural flexibility, generate ultra-modular off-the-shelf components with stereotyped interactions, and use protein polyhedra themselves as building blocks are clear next steps for the field that will expand the range of assemblies accessible to design and bring us closer to the ultimate goal of constructing arbitrary protein objects ranging from the nano- to the micro- and even macroscales. Current design approaches are mainly based on hydrophobic and metal-mediated interactions, as reliably designing polar protein-protein interactions, including water-mediated interactions, remains a major challenge [62–64]. Robust methods for polar interface design would enable more versatile control of conformational and assembly dynamics, facilitating the design of materials that autonomously sense and respond to environmental cues in the context of *in vitro* and *in vivo* applications, such as changes in small molecule concentrations, pH, or subcellular localization. Incorporating explicit consideration of assembly dynamics into amino acid sequence design and *in vitro* assembly strategies could lead to specific partial assemblies, and hence controlled multiphase assembly, nanomaterials with continually modulable valency, and asymmetric materials. Additional opportunities lay in integrating these design elements to provide specific structural and functional features such as assembly porosity, enzymatic activity and cascades, controlled nucleation of inorganic materials, and enhanced biocompatibility. Finally, a fundamental advantage of designing nanomaterials from protein building blocks is that they are fully genetically encoded, which makes possible their genetic delivery for *in vivo* applications, a clear avenue for future exploration. Unlike complex protein folding landscapes, the principles guiding the symmetric self-organization of folded protein monomers into multivalent polyhedral structures have

proven comprehensible, and demonstrable by bottom-up approaches from both native and *de novo* designed building blocks. Applied without constraint, these principles may facilitate the modular assembly of any extant protein component, including combinations of natural and designed components which could never have emerged from evolutionary processes.

Declaration of interests

N.P.K. is a co-founder, shareholder, paid consultant, and chair of the scientific advisory board of Icosavax, Inc.

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