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Current Opinion in Structural Biology

Practical approaches to designing novel protein assemblies

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Molecular self-assembly offers a means by which sophisticated materials can be constructed with unparalleled precision. Designing self-assembling protein structures is of particular interest as a result of the unique functional capabilities of proteins. Custom-designed protein materials could lead to new possibilities in therapeutics, bioenergy, and materials science. Although the field was long hampered by the challenges involved in designing such complex molecules, novel approaches and computational tools have recently led to remarkable progress. Here we review recent design studies in the context of three fundamental aspects of self-assembling materials: subunit organization, subunit interactions, and regulation of assembly.

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Current Opinion in Structural Biology 2013, **23**:632–638

This review comes from a themed issue on **Engineering and design**

Edited by **Florian Hollfelder** and **Stefan Lutz**

For a complete overview see the Issue and the Editorial

Available online 1st July 2013

0959-440X/$ – see front matter,

http://dx.doi.org/10.1016/j.sbi.2013.06.002

Introduction

Proteins have immense potential as building blocks for the construction of advanced nanomaterials. Of particular importance is their ability to self-assemble into highly ordered supramolecular complexes [1,2]. This order is manifested in many natural protein assemblies that have evolved as a result of the specialized functions afforded by such structures [3,4]. In addition to being adapted for use in therapeutic, nanotechnology, and materials science applications [5,6], natural assemblies have inspired the development of methods for designing novel self-assembling proteins [7]. The ultimate aim of these studies is to facilitate the design of protein nanomaterials with functions and characteristics that go beyond the capabilities of those assemblies found in nature and that are tailored to specific applications.

Designing protein assemblies with spatial order rivaling that of natural assemblies is challenging due to the complexity of protein structures and interactions. However, new tools and approaches have led to striking progress within the past couple of years [8••,9,10••,11••,12••,13•,14]. Particularly important has been the realization that by making symmetry a central component of design strategies, designing complex architectures such as virus-like cages and even three-dimensional crystals is greatly simplified [15]. The development of increasingly sophisticated computational tools for modeling and designing proteins has also been instrumental, especially methods for modeling symmetric arrangements of proteins [11••,16] and designing new protein–protein interactions [17,18]. Finally, incorporating controllable interactions between proteins and small molecules [19], metal ions [9], or other proteins [12••] has led to approaches capable of producing dynamic assemblies that can be regulated in a manner reminiscent of many natural protein assemblies. Collectively, these developments have resulted in exciting progress in the field.

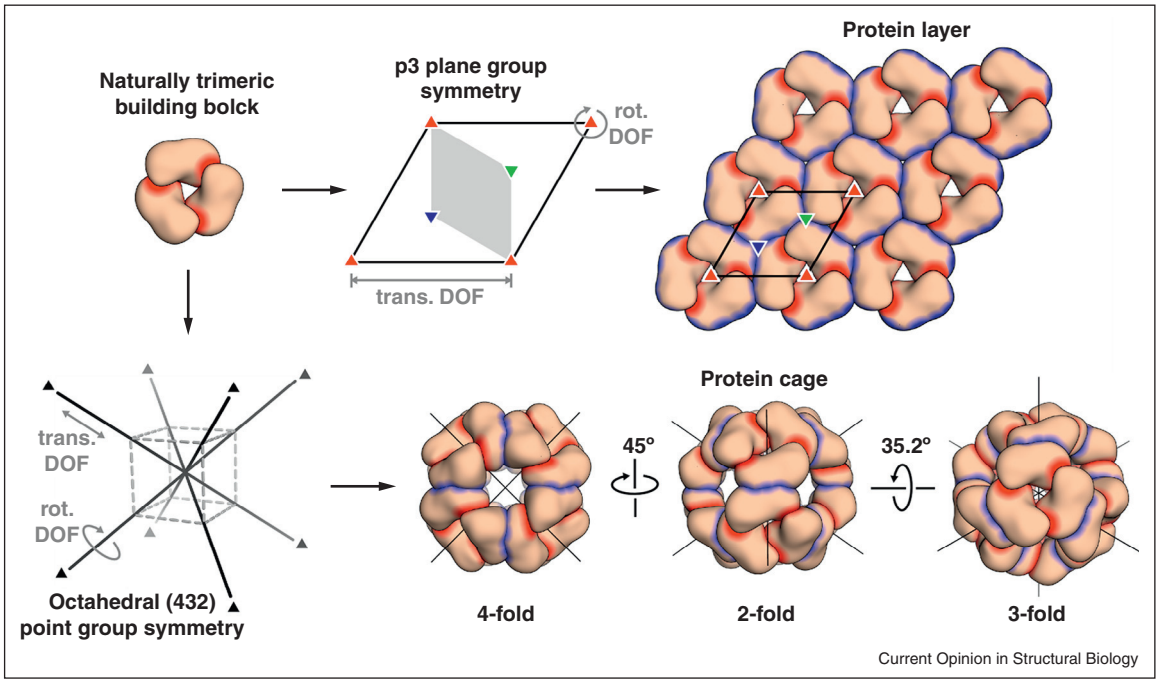
In this review we discuss the principles employed in recent efforts to design complex and geometrically specific protein assemblies, with a focus on practical approaches. The studies reviewed include reports of designed oligomers, large cages, two-dimensional layers, and three-dimensional crystals. We consider in separate sections the strategies used in these studies to address three fundamental aspects of any supramolecular assembly: the spatial organization of subunits, the interactions between subunits, and regulation of assembly. For discussions of progress in the design of filamentous assemblies or the assembly of small peptides, the reader is referred to recent reviews [20–22].

Subunit organization in designed protein assemblies

Many properties of protein assemblies are governed by the spatial organization of the constituent subunits. Beyond the direct relationship to morphology, subunit organization also influences assembly pathways [23,24] and function [3]. During evolution, the subunit organization of natural protein assemblies is not predetermined, but rather arises spontaneously as a consequence of mutations that affect the shapes of the subunits and the interactions between them. In contrast, when one attempts to design an assembly with particular features, the subunit architecture is usually specified beforehand and is an important consideration. In this section we discuss various principles and practical effects related to subunit organization.

The central concept concerning subunit organization in ordered protein assemblies is symmetry. From a design perspective, the greatest utility of symmetry is that it

Figure 1



Principles of subunit organization in designed protein assemblies. The construction of two hypothetical assemblies from the same naturally trimeric building block is used to illustrate several principles related to the spatial organization of subunits. (1) Organizing the building blocks according to p3 symmetry yields an unbounded layer (top), while organizing them according to octahedral symmetry yields a finite cage (bottom), demonstrating the central role of symmetry in determining assembly morphology. In each case, the trimeric building blocks are organized by aligning their threefold symmetry axes to each threefold axis in the target architecture, depicted using the triangle symbol. Differently colored triangles in the p3 symmetry diagram indicate distinct threefold elements of symmetry, and the asymmetric unit is shaded gray. (2) The pre-existing organization of the trimeric subunits within the building block fixes several rotational and translational degrees of freedom (DOFs) within each architecture, leaving, in these cases, only one DOF of each type as indicated in gray. Variation of these DOFs generates assemblies with different detailed structures; only one hypothetical subunit configuration is shown for each architecture. (3) Each combination of building block and symmetry group (symmetric architecture) has a minimum requirement for the number of distinct contact types, referred to as the minimum contact number. For both symmetric architectures shown, this number is two. The interface within the building block, shaded red, provides one of these contacts — a key advantage of using oligomeric building blocks — while the interface that must be designed is shaded blue. As the illustration shows, only a single designed interface is required to generate both architectures. The lack of contacts at the fourfold axis of the octahedral assembly demonstrates that additional contacts, although they could be present, are not required to generate the full assembly. The designed interface could be generated using any of the approaches for designing novel subunit interactions described in the text.

makes possible the construction of large structures through symmetric repetition of a small number of distinct subunit interactions (Figure 1). This is of critical importance given the difficulty of designing precise interactions between proteins; each novel interaction required to generate a desired assembly increases the difficulty of the design problem. DNA nanotechnology [25] provides an illustrative counterpoint. Symmetry is unnecessary for the construction of essentially arbitrarily shaped nanoscale DNA structures [26,27] due to two factors: first, the ease with which a very large number of specific interactions can be encoded and second, stable and reliable structural motifs that allow predictive positioning of the interacting sequences.

The characteristic symmetry group of a symmetric protein assembly defines the mathematical transformations that relate the position of each subunit to every other. In this way, the symmetry group directly determines the gross morphological features of an assembly, the details of which are provided by the shapes of the subunits and their orientations within the symmetry group (Figure 1). This relationship often dictates the choice of symmetric architecture when one sets out to design a novel assembly. Most symmetry groups contain more than one symmetry operator, which enables certain approaches related to subunit organization that can simplify the challenge of designing these complex architectures. First, in many such groups it is not necessary for the subunits to physically interact at all of the symmetry elements in order to generate the full assembly. Rather, contacts are necessary at only a certain subset of elements, the number of which can be defined for each symmetry group and is referred to as the minimum contact number [15,28]. An example using octahedral point group symmetry is depicted in Figure 1. Practically,

this phenomenon allows the designer to minimize the number of novel interactions between subunits that must be designed.

Secondly, naturally oligomeric proteins may be used as building blocks when targeting symmetry groups that contain multiple operators. Oligomeric protein structures are nearly always symmetric, and most exhibit finite point group symmetries [3]. Such proteins can be used as starting points for the design of larger assemblies by aligning their symmetry axis (or axes) with axes of the same order in a target symmetric architecture (Figure 1). This design strategy provides several useful advantages. First, the pre-existing interface(s) within the oligomer can provide, without modification, one or more of the requisite contacts in the target assembly. This strategy therefore yields another method for reducing the number of new subunit interactions that must be designed. As an ancillary benefit, the oligomeric interfaces also serve to define several of the subunit rigid body degrees of freedom. This effect tends to be more important in design approaches where these degrees of freedom are explicitly sampled in order to identify specific subunit configurations as design targets [10••,11••]. Second, in approaches based on designing non-covalent interactions between oligomeric building blocks, the energetic effect of each designed contact is increased in proportion to the symmetry of the building block [29]. The energy required of each designed subunit interaction is therefore lessened, which simplifies the design problem. This last idea assumes that the oligomeric building block is an intermediate in the assembly pathway. This phenomenon occurs frequently in natural protein assemblies and has been suggested to provide a more robust route to fully assembled structures [23,24]. Third, approaches in which multiple oligomeric building blocks are genetically fused together have recently demonstrated that when building blocks with relatively high symmetries are used (e.g., dihedral point groups), the alignment of a common symmetry axis between neighboring building blocks can provide an additional element of geometrical control [12••] (Figure 2c). For these reasons, the organizational strategy of using oligomeric building blocks has been applied in many successful examples of protein assembly design employing various types of subunit interactions. It is noteworthy that the same strategy also appears to have played a role, most likely for the first two reasons, in the evolution of many natural protein assemblies [3,23].

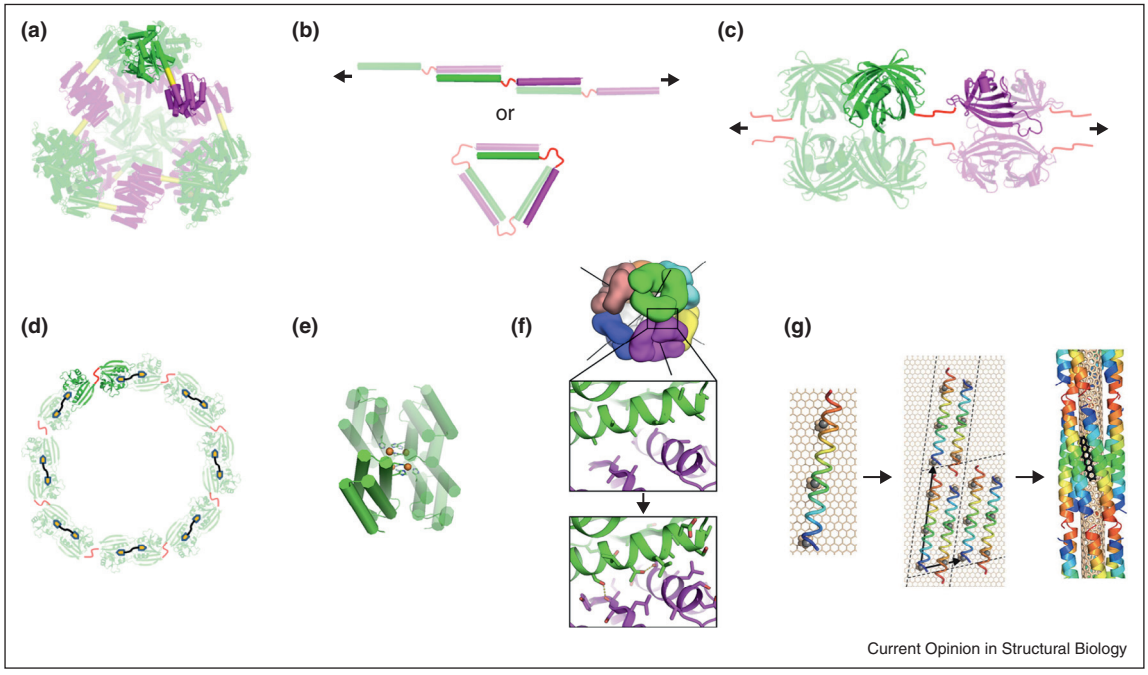
Subunit interactions in designed protein assemblies

The interactions between the subunits of macromolecular assemblies are the central consideration of design approaches. Not only are the subunit interactions the driving force for self-assembly; their geometries also ultimately control the spatial organization of the assembly by defining the relative orientations of the subunits. The hallmarks of stability and precisely defined geometries in natural protein assemblies are generally the result of large protein–protein interfaces that comprise many individually weak interactions [4]. However, methods for designing such interfaces were not available until very recently. For this and other reasons, various approaches have been developed for engineering novel interactions between proteins that lead to self-assembly. In this section we review these approaches, the materials designed by each, and their relative strengths and weaknesses at present.

Fusion strategies are based upon the concept of fusing two protein domains that naturally oligomerize into a single molecule. This endows the molecule with multiple interaction interfaces, which makes possible the construction of a wide variety of symmetric architectures as described above. Among many possibilities, particular architectures are targeted by choosing appropriate oligomerization domains (e.g., dimers or trimers) and linking the two domains such that their interfaces are oriented properly for assembly into the target architecture. In the landmark study that introduced the fusion approach [15], continuous alpha-helical linkers were designed between different pairs of oligomerization domains to control their relative orientation, and designed fibers and tetrahedral cages were obtained (Figure 2a). However, recently reported crystal structures of the tetrahedral assembly revealed significant bending and twisting in the helical linkers [30•,31], suggesting that additional mechanisms for controlling relative orientation may be warranted. Nevertheless, several subsequent studies have attempted to generalize this initial fusion strategy by removing the requirement for rigid helical linkers and using flexible linkers instead (Figure 2b). A variety of small oligomers and fibers have been obtained using this appealingly straightforward method [32–34], though the resulting assemblies are often somewhat polydisperse. Flexible linkers were also used in a recent report in which the oligomerization domains had relatively high symmetries that allowed the alignment of neighboring domains along a common symmetry axis [12••]. This organizational strategy results in assemblies in which multiple linkers connect neighboring oligomerization domains, thereby providing control of the relative orientation of the domains despite the flexibility of the linkers (Figure 2c). Although the strategy can only be applied to unbounded assemblies, it requires minimal design and has been shown to produce very highly ordered fibers and layers, as well as three-dimensional crystal-like solids. In summary, the various fusion methods are simple and powerful approaches for designing novel assemblies. However, controlling the orientation of the fused domains, which is critical for obtaining a specific structure with high accuracy, has not always proven straightforward.

Ligand-mediated assembly is another strategy that does not require the design of new protein–protein interactions if oligomeric building blocks are used. In this approach,

Figure 2



Various approaches to designing novel subunit interactions in protein assemblies. **(a)** Fusion-based approaches use linker segments to genetically fuse two protein interaction domains into a single molecule. While technically the pre-existing interfaces of the domains provide the interactions between subunits, the design approach is focused on the covalent interaction between the domains. The rigid alpha-helical linker used in the original fusion study is shown in yellow, while the two oligomerization domains are magenta and green. **(b)** Flexible linkers, depicted in red, are very straightforward to design but often lead to polydisperse assemblies. **(c)** By using oligomerization domains with relatively high symmetries and aligning neighboring building blocks along a symmetry axis they both have in common, orientational specificity can be encoded even with flexible linkers. **(d)** Multi-functional ligands, depicted here in yellow and black, have been used as a means of introducing novel subunit interactions. In this case, the other interaction is provided by a flexible linker (red) that fuses two copies of a monomer into a single molecule. **(e)** Metal-directed assembly makes use of protein–metal interactions to induce assembly by designing partial metal binding sites on protein surfaces. **(f)** Recently, methods have been developed for the design of novel assemblies through computational protein–protein interface design. **(g)** Designed interactions between alpha-helical peptides and carbon nanotubes led to templated arrays of the peptides on nanotube surfaces.
Panels (f) and (g) adapted from [10,44•] with permission.

small molecules containing multiple functional groups that react with or bind to existing binding sites in proteins are used to provide one of the interactions in a designed assembly [19]. The small molecules themselves may or may not be symmetric. Additional interactions are provided by the interfaces within the oligomeric building blocks (Figure 2d). Together, the symmetry of the oligomers and that of the small molecules define the architecture of the assembly. Fibers, layers, crystals, and cyclical ring-like assemblies have all been designed using ligand-mediated assembly [35–37], and the nature of the interaction affords multiple possibilities for regulation of assembly. However, in a manner similar to the flexible linker fusion approaches, flexibility in the small molecule mediators has often been found to lead to mixtures of various assemblies.

Metal-directed protein assembly takes advantage of the strength and geometrical specificity of protein–metal complexes to introduce novel subunit interactions. Partial metal binding sites are designed on the surfaces of proteins in order to induce assembly through completion of metal coordination by multimerization (Figure 2e). This mode of interaction offers a relatively simple and powerful means of generating novel assemblies, and has been used to design a number of different kinds, including helical bundles [38,39], small oligomers [14,40–43], and extended protein arrays [8••]. In several cases, computational protein design has been used to optimize the residues surrounding the designed metal binding sites [8••,14,38]. In addition, the nature of the metal-mediated interaction readily lends itself to regulation. At present, the major drawback of metal-directed assembly is that it is often, although not always [14], difficult to anticipate the structures of the resulting assemblies [9], which can make the targeted design of specific architectures challenging.

Methods for generating protein assemblies through the design of novel protein–protein interfaces have recently been developed. Although design by inspection was used in early efforts to induce complex formation [29], computational approaches have proven invaluable for

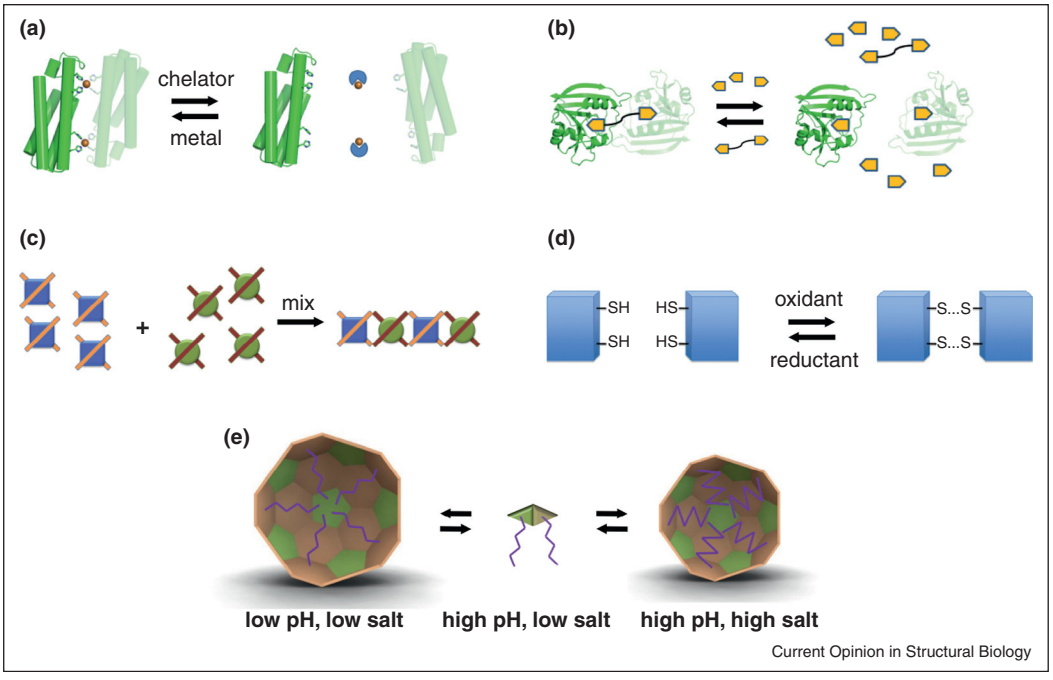
designing more complex interfaces and architectures. The main advantage of the interface design approach is that it allows very specific subunit configurations to be designed with high accuracy by virtue of the remarkable geometric specificity of protein–protein interfaces. However, this capability comes at the expense of simplicity; sophisticated algorithms for modeling and designing extensive interfaces are required. Typically, target configurations are identified by an initial symmetric docking step that combines varying the degrees of freedom in the system with low-resolution or sequence-independent measures of the suitability of a configuration for design. Residues at the interfaces of suitable subunit configurations are then computationally designed in order to generate low-energy, geometrically specific interfaces that drive assembly (Figure 2f). The desired symmetry is typically rigidly enforced throughout the design process. This framework has been used to design novel dimers [13•], tetrahedral and octahedral protein nanocages [10••], and a three-dimensional crystal [11••], all with remarkable accuracy. In addition, a variation in which nonbiological nanostructures were used to template assembly yielded novel carbon nanotube-peptide hybrid nanomaterials [44•] (Figure 2g). To date, strategies for regulating the formation of the designed assemblies have not yet been incorporated into these methods. Combining the high accuracy of the interface design approaches with the opportunities for regulation afforded by the approaches discussed above would represent a significant step toward the ultimate goal of designing functional protein nanomaterials.

Regulation of assembly

Many natural protein assemblies dynamically assemble and disassemble to modulate their functions according to the needs of the cell [2]. These proteins serve as inspiration for the design of 'smart' protein nanomaterials that respond in a desired manner to changes in their environment. Although several approaches to altering the properties of less ordered protein-based materials such as hydrogels have been developed (reviewed in [45]), highly ordered assemblies like those discussed here can present distinct challenges. Various strategies for regulating these assemblies have been demonstrated recently, and the focus on regulation will likely grow in the near future as the field matures beyond the design of static structures.

The metal-directed assembly approach described above offers several means by which assembly can be regulated. Because such a high fraction of the interface energy driving assembly depends on the presence of the appropriate metal ion, the addition or removal of metal provides

Figure 3



Various approaches to regulating the assembly state of designed protein assemblies. **(a)** Metal-directed assembly offers several means of regulating assembly state, such as the addition or chelation of the required metal ions. **(b)** Ligand-mediated assembly is similar in that it requires the multivalent ligand for assembly; disassembly occurs in the presence of monovalent ligand that competes for binding. **(c)** Assembly of materials held together by protein–protein interfaces can be initiated by mixing separately purified building blocks if the materials comprise more than one distinct type. **(d)** Disulfide bonds provide a facile and therapeutically relevant mechanism of regulating assembly. **(e)** In a recent study, genetic fusion of elastin-like peptides, depicted here in purple, to the interior of a virus capsid produced assemblies that formed distinct architectures depending on environmental conditions.

a simple and direct mechanism for triggering assembly or disassembly [9,43] (Figure 3a). In fact, varying the type or concentration of metal constitutes a method of control: a variety of morphologically distinct assemblies can result from the same protein building block depending on the identity of the metal used [42] or the ratio of the added metal to protein [8••,41]. As might be expected, pH has also been shown to influence the designed metal-directed interactions, and, therefore, the resulting assemblies [8••,43].

Ligand-mediated assembly also provides multiple opportunities for regulating the assembly process. Addition of the small molecule mediator to purified protein building blocks can trigger assembly [35,36], while addition of competitive inhibitors can cause disassembly [46] (Figure 3b). Asymmetry in the small molecule can offer additional control through stepwise assembly of successively larger building blocks, which has been found to both improve long-range order and prevent uncontrolled aggregation [36].

Although generally less straightforward than regulating ligand-mediated or metal-mediated assemblies, various methods have been developed to regulate assemblies that have interfaces consisting only of protein–protein interactions. Simple mixing of purified components has been shown to be a successful method of initiating assembly [12••,33]. However, this approach necessitates that the assembly comprise more than one distinct kind of building block, and it does not address controlled disassembly (Figure 3c). Interfaces dependent upon stabilization by disulfide bonds result in assemblies controlled by the redox environment experienced by the protein [47], a facile and potentially therapeutically relevant mechanism of regulation (Figure 3d). A third, recently published approach [48] involves fusing two independent protein domains, one of which is already capable of assembling into several ordered structures, while the other directs assembly toward one of the ordered structures or the other in response to environmental conditions (Figure 3e). This study also highlights the ability of the assembly state of certain naturally occurring complexes (e.g., virus capsids) to be regulated by changes in ionic strength, pH, or temperature [49]. These characteristics are encoded in the noncovalent interactions within the protein subunits and their interfaces, although in ways that are not yet fully understood. As these behaviors are dissected in greater detail, and as methods for designing protein–protein interfaces improve, it is likely that similar characteristics will be incorporated into designed interfaces.

Conclusions and outlook

In the past few years, new approaches and continually improving computational tools have resulted in exciting advances in the design of self-assembling proteins. A variety of strategies are now available for designing novel materials, each with its own focus: ease of design, opportunities for regulation, or high accuracy. Fundamentally, these properties are not mutually exclusive, and in the near future the relative weaknesses of each approach will likely be addressed. Concurrently, demonstrations of functional custom-designed materials are expected. Given the rapid recent progress in the field, the design of completely novel protein-based molecular machines with functions tailored to specific applications seems much closer than it did only a few years ago.

Acknowledgements

The authors thank Jacob Bale and Dr Jeremy Mills for excellent comments and suggestions.

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