

Constructing arrays of proteins

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The construction of crystalline arrays allows proteins to be presented in a dense, oriented and functional way that also facilitates determination of their structure. Rational design of these supramolecular structures is becoming increasingly tractable with recent successes exploiting both innate protein symmetry and advances in protein–protein interface design. Pre-existing symmetry minimizes the number of non-native interfaces that must be produced, and the use of symmetric interfaces facilitates protein alignment. Arrays in which metal coordination or peptide binding are responsible for the inter-particle associations show particular promise due to the malleable and reversible nature of these interactions. Cross-pollination of the principles that underlie successful strategies is likely to produce rapid advances in this field and consequent benefits to both nanotechnology and structural biology.

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Introduction

Protein arrays, in the broadest sense, are not uncommon. Each protein structure determined by X-ray diffraction requires a crystal that comprises millions of ordered particles. These crystals are produced when both an appropriate form of the protein and appropriate environmental conditions have been empirically established. Their lattices are held together by serendipitous, non-covalent interactions between component particles and, significantly, it is impossible to determine in advance either whether a lattice will form or its exact topology. This presents a bottleneck in the advancement of structural biology and, in particular, structure-based drug discovery.

While protein crystallography is a field many decades old, production of protein arrays by design began in earnest only in the early part of this century and few strategies have borne tangible fruit. This review examines the progress made towards producing designed ‘arrays’, rather than traditional macromolecular crystals that arise from

unpredictable interactions. In particular it considers the most successful systems, the elements that constitute them, and recent developments in related fields that may have applications in this one.

The benefit of crystalline lattices over more standard solution states derives from the coordinated action of identical particles that are densely packed in an identical orientation. The ability to preserve aqueous solvation in arrays and the presence of significant solvent channels frequently allow binding and enzymatic processes to occur in a manner indistinguishable from the native process in bulk solvent [1]. Although protein arrays are not easily adapted to produce structural complexity at the level possible in DNA self-assembly [2], they do provide immediate access to an immense library of protein functionality.

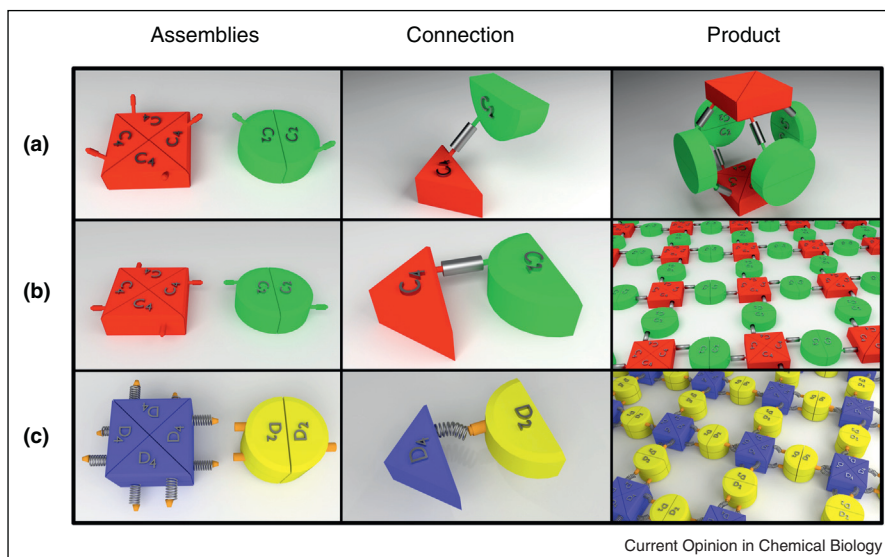
Until protein arrays are more routinely produced, the practical applications of these features will remain largely unexplored. Much, however, can be inferred from uses of traditional protein crystals. Such crystals, where necessary stabilized by cross-linking [3], have been proposed as microporous materials for separation processes [4], pharmaceutical delivery agents [5] and scaffolds for inorganic templating [6–8]. However, perhaps the most outstanding potential of protein arrays remains as a tool for macromolecular structure determination in X-ray crystallography. In this context, key therapeutic targets that do not easily form crystals using conventional methods might either be directly arrayed or hosted within the large solvent channels that are a feature of many array designs [9••].

Approaches to protein array design

In one of the earliest examples of *ab initio* array design, a three-dimensional array of a lectin was produced when exposed to a synthetic, bivalent form of its natural ligand [10]. These arrays diffracted X-rays to only 6 Å resolution and were not in the predicted cubic crystallographic space group, but they remain one of the best examples of an ordered three-dimensional protein array. A related strategy, again exploiting the innate affinity of a protein for its small molecule ligand, allowed production of very limited two-dimensional arrays of aldolase with streptavidin spacers [11].

Two more recent approaches have exploited genetic protein fusions in place of small molecule binding. One very versatile strategy [12] has been used to produce well-defined oligomeric particles, or ‘nanohedra’ [13,14••]. Nanohedra are formed through oriented fusion of symmetric protein oligomers, or ‘assemblies’, such that

Figure 1



The application of symmetry in protein arrays. **(a)** and **(b)** Genetic fusion of protein assemblies that contain a single rotational symmetry axis (nanohedra). Termini in the different protein subunits are joined by genetic fusion to produce rigid links. This defines the relative disposition of the symmetry axes in each assembly. In the examples shown, assemblies with fourfold and twofold rotational symmetry axes (symmetry groups C_4 and C_2 respectively) are fused to produce cages (a) or 2D arrays (b) according to the angle of the joint between subunits. **(c)** Genetic fusion of assemblies that contain multiple symmetry axes (crysallins). Flexible connections are possible due to the connections being formed along a shared symmetry axis. Shown are assemblies with principal rotational symmetry axes of order 4 and 2 that also possess orthogonal twofold symmetry axes (symmetry groups D_4 and D_2 respectively). These are joined using flexible, spring-like connections to produce a 2D array in which the assemblies are aligned along symmetry axes.

their rotational symmetry axes are aligned at a specific angle (Figure 1a,b). The requirement for a specific orientation demands that the linker between assemblies is relatively robust, and this has been accomplished by extension of a C-terminal helix in one assembly into a contiguous α -helix at the N-terminus of a second assembly [13]. The approach places stringent demands upon the geometry and secondary structure of the N-termini and C-termini of the constituent assemblies. However, when a pair of assemblies with appropriate termini has been determined, subsequent design is not difficult and very few empirical sequence adjustments were required in order to exemplify a protein cage. While the strategy has potential for extension to 2D and 3D arrays, to date only discrete particles and filaments have been demonstrated.

Successful protein array production has, however, been shown in a related approach that exploits higher symmetry proteins [9•]. These arrays, or ‘crysallins’, are formed when high-symmetry assemblies are forced by genetic fusion to align along rotational symmetry axes of equal order (Figure 1c). The additional symmetry limits its versatility relative to the nanohedra approach in that it is suitable just for production of extended arrays and not discrete assemblies. However, the design process demands only a flexible fusion linker of appropriate length and position. It also permits a very broad library

of potential components since the geometry of the termini is less stringently defined. Moreover, the lattices produced typically have extremely large voids making them suitable for hosting other large macromolecular biological or inorganic species.

Finally, in a strategy that falls somewhere between these broad design principles and the sequence specific methods used for the optimization of traditional crystallization, metal-coordination has been used to produce 2D and 3D arrays of a modified cytochrome cb_{562} [15•]. The arrays could be directed to stack into a third dimension by means of additional rhodamine-mediated contacts. This approach, exploiting a monomeric base unit, requires more detailed molecular design than those exploiting higher symmetry particles. However, recent developments in this area have made such designs significantly more tractable [16–18].

In each approach described above, two main parameters have been selected: the nature of the protein building blocks, and the means of connecting these blocks. The strategy behind selection of these parameters and their implications will now be examined.

Building blocks – symmetry and number

Ordered protein arrays possess space group symmetry — a combination of rotation and translation symmetry

elements that are present in the extended lattice. Consequently, in the production of arrays, probably the most significant asset of any building block is pre-existing symmetry. Symmetry in a particle minimizes the number of interactions that must be designed *ab initio* in order for blocks to associate into an extended lattice. For example, a naturally oligomeric protein particle that assembles with the symmetry of a cube may be stacked into an extended array using a single designed interface (associating face to face, corner to corner, or edge to edge). In contrast, a monomeric protein with no intrinsic symmetry requires different interfaces to be designed and implemented for extension along each lattice axis in an array.

Successful strategies have typically used naturally occurring multi-subunit protein assemblies whose innate point group symmetry becomes a component of the array space group symmetry. The exception to this is the metal-directed approach [15^{••}] but, even here, a novel, symmetric dimer was produced in the first step of the design process. The potential to increase the native symmetry of proteins artificially is further underlined by recent work that has generated high symmetry octahedral assemblies from lower symmetry natural trimers [19^{••}]. Designed assemblies of this nature can provide building blocks that possess symmetry ideally suited to arrays. However, their production shares many of the same challenges, in particular the synthesis of geometrically defined protein–protein interfaces of an appropriate strength.

Peptides, similarly, may be used to create discrete particles [20[•],21,22] with the potential for subsequent array formation. Moreover, it may be easier to optimize the symmetry and surface properties of peptide assemblies than is the case for natural protein oligomers with significantly more complex folds. When incorporated into arrays as fusion partners for larger proteins, peptides can also provide access to symmetric folds that have not been observed in naturally occurring protein structures [23].

The number of different types of block in an array has significant practical implications. Recombinant proteins are typically expressed in cellular systems in which folding and association events are difficult or impossible to control. For systems that produce a discrete assembly from a single component this is not an issue [14^{••},19^{••}]. However, single components that self-assemble into arrays will typically require disassociation and reconstitution following cell lysis in order to produce a macroscopic product. Depending on the type of inter-particle interactions employed, chaotropes, metal-chelation agents, or specific inhibitors may be used to recover monomeric species for subsequent *in vitro* reconstitution into an array [15^{••}]. However, in many circumstances it is more convenient to construct interacting pairs of blocks that are

expressed and purified independently before mixing to induce self-assembly [9^{••}].

Particle interactions

Alongside these geometric considerations, the principal design choice in construction of protein arrays is that of the inter-particle ‘glue’. The novel protein–protein interactions required have historically been considered more complex to design than either the well-defined base pairing responsible for interactions in their DNA-based equivalents [24–26] or the peptide bonding patterns (in particular coiled-coils and beta-sheets) that have been exemplified in the design of self-assembling structures [27].

The established fields of nucleic acid self-assembly and protein crystallization provide guidance on the character of the forces required for production of protein arrays. These principles include reversibility, modularity and specificity. Self-assembling arrays of peptides, protein, and DNA all share a requirement for reversible, geometrically defined associations [28]. Reversible interactions are enormously beneficial in self-assembly since they permit error-correction and gradual exploration of a variety of states whilst progressing towards that with a global minimum energy. Given inter-particle forces of appropriate geometry, this state may be an extended two or three-dimensional array, or a single symmetric assembly, such as a virus particle. If the geometric constraints are sufficiently strong, then tight, poorly reversible associations may produce a stochastic population of correctly ordered small assemblies, but for extended systems such as arrays, the outcome will at best be glass-like and only locally ordered [29,30].

Recently, experimental [19^{••}] and computational advances [17] have begun to facilitate a rational design approach in the production of novel protein–protein interfaces that exhibit the characteristics required for successful self-assembly and that exploit structural motifs exhibited at native protein–protein interfaces. The binding of an α -helix in a concave, hydrophobic groove is one such example of interface design: the shape complementarity helps control the relative orientations and is more reliable than designing complementary polar contacts [31]. Strand pairing with side-chain interdigitation is another such strategy for designing geometrically controlled protein–protein interactions. Exposed beta-strands on the interacting proteins are combined with redesigned side chains modified to form complementary polar and/or hydrophobic pairings [32,33].

Metal coordination motifs have proved a particularly tractable paradigm [16,18,34[•]]. The use of metal-coordination sites is attractive for a number of reasons. In particular, there are a large number of well-characterized structural motifs that lend themselves

to *in silico* analysis and binding is reversible in a way that can be easily modulated by variation of environmental parameters [15^{••},35,36].

Specific peptide binding has also been shown to be a viable 'glue' for arrays [9^{••}] and one which can be achieved through genetic fusion and optimized through variation in sequence. In many instances, environmental parameters such as ionic strength, pH and temperature can be dynamically modified to modulate the binding strength between a protein and cognate peptide. In this context, it is notable that temperature elevation was critical to the production of designed macroscopic 3D DNA arrays [26].

Coiled-coil interactions are amongst the better understood peptide motifs that have been used for inter-particle association [9^{••}]. The coiled-coil motif is well represented in both natural and synthetic structural libraries [37,38]. Selection can be made on the basis of their symmetry, polarity (parallel/anti-parallel) and/or length, thereby allowing precise control of lattice geometry. However, strength of the interaction in longer coils is likely to limit the reversibility of association [39], and arrays produced in this way have been of limited size.

Another promising approach utilizes small molecule binding. This was extremely effective when used to cross-link lectins [10]. However, association through the exceptionally strong biotin–streptavidin interaction resulted in only small and relatively poorly ordered arrays [11]. This issue may have been exacerbated by geometric inconsistencies that were unmitigated by either significant flexibility or symmetric association.

Whatever the nature of the interactions that bind an array, the design of effective self-assembling arrays is made much easier if it is possible to explore local energy minima before reaching a global minimum energy. Hence, weakly defined local geometry may be averaged over neighbouring units of the array to find a global minimum. In this way, metal-coordination (with a comparatively broad, smooth energy profile in respect of geometry) and flexible fusion links (when married to high symmetry, Figure 1c) offer the potential to make tentative initial associations followed by reorganization to a geometry compatible with an extended lattice. Where the interface symmetry is lower and the imposition of geometry more stringent (Figure 1b), a balance between interaction strength, flexibility and error correction can be more difficult to establish.

Finally, while the design of arrays is very different from the traditional approach to crystallization, it is worth bearing in mind that many aspects of the physical process involved are identical. A considerable body of literature already exists in areas pertinent to both processes such as

crystal nucleation [40–42], and practical aspects of experimental setup and sample preparation [43]. Factors that have been found beneficial in the generation of novel interfaces in crystallization such as surface entropy reduction (SER) [44,45] and surface lysine methylation (SLM) [46] are likewise worthy of consideration in array design.

Conclusions

The construction of self-assembling protein arrays is a young field in which significant areas remain unconquered. In particular, complete *ab initio* design of a self-assembling protein array in three-dimensions with sufficient order to permit atomic resolution diffraction of X-rays is yet to be demonstrated. However, serendipitous stacking of 2D sheets has already produced high quality 3D crystals and similar stacking has been directed by design (although with lower apparent order) [15^{••}]. Self-assembly technology for DNA and peptide arrays has made impressive strides in the last ten years despite facing similar challenges. Increasingly accomplished methodology in both protein–protein interface and array design suggests that the road from 2D to 3D for protein arrays may be even more rapid. If so, it should provide a platform for important advances in the fields of both nanotechnology in general and crystallography in particular.

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