**Review**

**Two-dimensional (2D) crystallization of engineered proteins: A review**

Jie Lu*, Sa-Sa Shen, Rong-Rong Li, Qing Lin and Meng-Jie Zheng

National Engineering Laboratory for Cereal Fermentation Technology, School of Chemical and Material Engineering, Jiangnan University, 214122 Wuxi, China.

Accepted 21 October, 2011

Protein’ two-dimensional (2D) crystallization denotes the supreme degree of its ordering, thus 2D protein crystals can be applied in diverse fields ranging from structural biology to materials science with unique performances. To date, 2D crystallization of proteins, especially engineered proteins, is emerging as a powerful tool for bottom-up approaches to the nanofabrication of functional structures. However, till now there is still lack of general method of forming 2D crystals of proteins due to limited understanding of the 2D crystallization process, and the self-assembly of 2D protein crystals often suffers from point defects and grain boundaries resulting from homogeneous nucleation and uncontrollable crystal growth. In this review, we will introduce some aspects of 2D protein crystallization, including key approaches to growing 2D protein crystals and their potential applications in various fields.

**Key words:*** Protein, two-dimensional (2D) crystallization, self-assembly, applications.

**INTRODUCTION**

Self-assembly, different with disorganized aggregation, random assembly, precipitation or deposition, can be defined as the spontaneous association of molecules into organized structures under thermodynamic equilibrium conditions, and currently represents one of the most promising bottom-up strategies for developing the next generation of functional materials (Smith et al., 2011). In general, self-assembling systems can be divided into five key parts: the subunits, a driving force, a repulsive force, a binding force and an environment. The subunits (for example, peptides or proteins) are the building-blocks of the assembled material and may possess both functional and structural roles. The driving force consists of interactions such as electrostatic or hydrophobic/hydrophilic repulsions, and the binding forces including hydrogen bonding and van der Waals, ionic, dipole–dipole and hydrophobic forces which are non-covalent interactions that impart stability to the assembled structure and determine the final architecture(Pelesko, 2007).

Many proteins have shown an inherent ability to form self-assembly structures in suspensions or at interfaces (Sleytr et al., 1993). Through self-assembly, proteins or peptides can build-up a large variety of structures including monolayers, tapes, belts, fibrils, tubes, vesicles, or bilayer membranes (Hosseinkhani et al., 2006a). Comparing with other structures, the crystalline two-dimensional (2D) structures of proteins have more advantages in their applications. For example, because free amino, carboxyl and hydroxyl groups are located on their surfaces in well-defined positions and orientations, 2D protein crystals have the capability of binding functional molecules in a regular fashion and with a maximum dense packing order through electrostatic, hydrophobic, or covalent bonds (Liang and Chen, 2011; Chen et al., 2011). In this paper we introduce the state-of-the-art on the applications and controlled growth of 2D protein crystals.

**TWO-DIMENSIONAL CRYSTALLIZATION OF PROTEINS**

2D crystallization of proteins requires an appropriate interface that will direct the proteins into a preferred
Thus the key he -ed that, as shown in Figure 1, these clusters under agnostic kits, tests such as (3) via ed s. u -d lipid bilayers -ariants of streptavidin -ed. -he general practicality went -2+ structural form -h. -h resulting protein arrays, which in turn affects overall of surface ligand influences the molecular packing of the phospholipids. Their studies reveal that the concentration assembly of streptavidin on mica (1994) pH, ion composition and ionic strength -several environmental parameters such as temperature, and size of the self -proteins -transcriptase, RNA polymerases I and II, and S Cholera toxin subunit B, DNA gyrase B, HIV reverse -2D at interfaces, such as Annexin IV, Anti -surfaces -denaturing proteins. So far, the most frequently used routine to grow 2D protein crystals is to adsorb proteins from an aqueous solution onto the layer of lipids, whose lateral diffusion affords the required mobility. This type of 2D crystallization includes (1) via binding proteins to natural lipid ligands at lipid interfaces (Pum and Sleytr, 1995a); (2) via metal chelation which utilizes lipid head-groups that have been designed to chelate divalent metal ions such as Cu$^{2+}$ and Ni$^{2+}$ (Frey et al., 1996a); (3) via electrostatic interactions, for example, with charged lipid molecules, with polyelectrolytes, etc. (Gergely et al., 2004).

Several proteins have been crystallized in 2D without a lipid layer support. Solid substrates such as silica, mica, metals, carbon films or synthetic polymers have been utilized to facilitate the formation of 2D protein crystals. Although this method has been used successfully to crystallize proteins, many proteins are known to denature at such surfaces (Pum and Sleytr, 1995b).

To date, a number of proteins have been crystallized in 2D at interfaces, such as Annexin IV, Anti-DNP IgG, Cholera toxin subunit B, DNA gyrase B, HIV reverse transcriptase, RNA polymerases I and II, and S-layer proteins (Pum et al., 1993). In general, structural form and size of the self-assembly 2D crystals depend on several environmental parameters such as temperature, pH, ion composition and ionic strength (Pum and Sleytr, 1994). Lou et al. (2007) have investigated the self-assembly of streptavidin on mica-supported phospholipids. Their studies reveal that the concentration of surface ligand influences the molecular packing of the resulting protein arrays, which in turn affects overall crystal morphology. Besides, two variants of streptavidin affect both crystalline lattice and domain morphology (Lou et al., 2008). Fukuto et al. (2010) have studied the 2D assembly of streptavidin on a biotin-bearing lipid monolayer by means of in situ X-ray scattering and optical Brewster angle microscopy measurements at the liquid-vapor interface. The minimum biotin density required for the 2D crystallization of streptavidin is found to be remarkably close to the density of the ligand-binding sites in the protein crystal.

Nevertheless, the general practicability of 2D protein crystallization has been limited by a lack of understanding of how protein ordering is promoted at various interfaces. Using streptavidin as a model system, Hemming et al. (1995) have found that crystal growth of 3D crystals from lipid layers does not require pre-formed 2D crystals, and that lipid layers are effective at promoting epitaxial crystal growth. Chung et al. (2010) have used in situ atomic force microscopy to follow 2D assembly of S-layer proteins on supported lipid bilayers. The obtained molecular-scale picture of multistage protein crystallization revealed that, as shown in Figure 1, monomers with an extended conformation first formed a mobile adsorbed phase, followed by a condensation into amorphous clusters. Then these clusters underwent a phase transition into crystalline clusters (nuclei). Finally growth proceeded by formation of new oligomers exclusively at cluster edges (Dufrène et al., 2001; Pum et al., 2000).

**APPLICATIONS OF 2D PROTEIN CRYSTALS**

2D crystallization of soluble proteins at various interfaces denotes the ultimate degree of 2D ordering of protein molecules, which results in a broad spectrum of applications, ranging from structure determination to ultrafiltration membranes, biosensors, diagnostic kits, vaccine applications, affinity matrices, templates for mineral formation, carrier systems for the functionalization
of inorganic surfaces, and many other aspects of molecular nanotechnology, nanobiotechnology and biomimetics (Sleytr et al., 1999, 2001).

Structure determination

2D protein crystals were initially used for the determination of the three-dimensional (3D) structures of difficult-to-crystallize proteins using electron microscopy and image processing. In particular, as to membrane proteins, the presence of hydrophobic domains often precludes 3D crystallization by traditional methods (van Bruggen et al., 1986), the characterization of 2D crystals by electron diffraction techniques can significantly broaden the knowledge of the molecular function of membrane proteins. The use of 2D crystals for structure determination can reduce the amounts of material and allows the protein to be examined in its native lipid environment, thus 2D crystals have become a principle target for protein crystallographers using electron diffraction techniques (Vonck et al., 1990).

In addition, 2D crystals can serve as “catalysts” for the 3D crystal growth of difficult-to-crystallize proteins. In some cases, 3D crystals can grow under conditions where nucleation would not occur, and thus the 2D crystals will act as nuclei for their growth (Frey et al., 1996b).

Biofilms

One of the applications of 2D protein crystals in biofilms is the development of functional protein coatings that can be engineered at the molecular level to display controlled wettability, porosity, elasticity, or bioadhesiveness (Bayley, 1994). Besides, protein assembly at surfaces could provide new routes to preventing blood coagulation or bacterial adsorption, promote cell adhesion, and/or direct a healing response (Mrksich et al., 1996). The advances in controlling protein order and thus the properties of biofilm surfaces can be expected to play an important future role in designing biomaterials that communicate with living tissue (Kuhl and Griffith-Cima, 1996; Hosseinkhani et al., 2006b, 2007).

The periodicity of pores in some 2D protein lattices has been exploited in ultrafiltration membrane technology. Sara et al. (1992) have studied the permeability of 2D crystals of S-layer from therophilic Bacillaceae. The 2D crystals were found to possess an extremely sharp molecular weight cutoff. This characteristic feature led to attempts for exploiting S-layers as active molecular sieves in ultrafiltration membranes.

The 2D protein crystals have also been exploited as supporting film for functional lipid membranes. Biological lipid membranes are highly selective filters, which have been optimized for specific transport functions and as sensors for external signals. However, their applications are often hampered by their short life time and their low stability. Pum and Sleytr (1994) have shown that synthetic and biological lipid membranes maintained their structural and functional integrity for a much longer period of time when S-layers were used as supporting substrates.

Immobilization matrix/carriers

The procedures for immobilizing functional molecules can be categorized into cross-linking, entrapment, and carrier-binding (Hosseinkhani et al., 2006c; Kalhor et al., 2011). 2D protein crystals can be considered as ideal matrix because functional molecules can be not only aligned in high density on the surface but also show identical positions and orientations on each protomer, resulting from appropriate interactions between protein molecules and functional molecules (Sara et al., 1993).

Self-assembly methods for controlling protein order can be expected to be applicable to a host of targeted drug delivery systems, as they can be used effectively on a wide variety of polymeric supports. Recently 2D protein crystals have been developed in the application of targeted drug delivery systems. For instances, biotinylated antibodies attached to biotinylated liposomes via a streptavidin linker may be used to target liposome-encapsulated drug to tumor-specific receptors (Loughrey et al., 1990). S-layer proteins have been crystallized and cross-linked on the surface of liposomes to stabilize the lipid membrane by modulating the lateral diffusion of the free lipid molecules, thus changing their fluidity and increasing their mechanical stability (Paul et al., 1992). And the suitability of S-layers as carrier/adjuvants for the production of conjugate vaccines has been described by Malcolm et al. (1993).

Biotemplates

2D protein crystals on various types of supports can exhibit different lattice symmetries (for example, oblique, square or hexagonal) with adjustable lattice constants. Taking advantage of their spatially defined physical and chemical surface properties, 2D protein crystals offer an attractive approach for the fabrication of nanoparticle (NP) templates, and have been used successfully as biotemplates for the in situ nucleation of inorganic particles and for binding of NPs via electrostatic interaction. McMillan et al. (2005) have patterned nanoparticle arrays using a genetically engineered crystalline protein template to direct constrained chemical synthesis. In their work, the heat-shock protein TF55β spontaneously assembles into an octadecameric double-ring cage structure called a chaperonin, which in turn readily assembles into 2D crystalline arrays. They
genetically removed a loop on TF55β that occludes the central pore of the assembled chaperonin and added a polyhistidine (His10) sequence to its amino terminus. With these modifications, the solvent-accessible cores of assembled chaperonins possess 180 additional His residues, creating a region with enhanced affinity for metal ions that is spatially constrained by the interior dimensions of the chaperonin. When incubated with Pd (II), the chaperonin cores become sites for selectively initiating the chemical reduction of magnetic transition metal (TM) ions (either Ni²⁺ or Co²⁺) from precursor salts. This procedure yields arrays of bimetallic (here Ni-Pd or Co-Pd) nanoparticles with dimensions defined by the chaperonin. Furthermore, the nanoparticles are patterned into arrays because the 2D crystalline template imparts longer-range order that extends across the engineered protein crystal. Target applications of these patterned NP arrays range from high-density data storage media to directing the catalytic growth of additional materials such as nanotubes on substrates.

Not long after that, Mark et al. (2007) explored the bionanofabrication of silicon nanopillar structures using ordered gold nanoparticle arrays generated using S-layers from the Gram-positive bacterium Deinococcus radiodurans as templates. S-layers preimmobilized onto chemically modified silicon substrates were initially used to template the fabrication of a nanolithographic hard mask pattern comprised of a hexagonally ordered array of 5-nm gold nanoparticles (lattice constant = 18 nm). Significantly, the use of the biotemplated gold nanoparticle mask patterns in an inductively coupled plasma (ICP) etching process successfully yielded silicon nanopillar structures. However, the resultant nanopillars (8-13 nm wide at the tip, 15-20 nm wide at half-height, 20-30 nm wide at the base, and 60-90 nm tall) appeared to lack any significant degree of translational ordering.

**Electronic and photonic devices**

In recent years, much attention has been paid to developing and investigating the use of 2D protein crystals in the construction of electronic and photonic devices such as protein chips, nanobiosensors, new hybrid electronic devices, etc. (Rinaldi et al., 2004). However, the development of protein-based devices requires detailed understanding of the crystallization processes and eventual conformational changes of the biomolecule. The investigation on the intrinsic stability of proteins provides a better understanding of the mechanisms affecting shelf life and operational stability upon crystallization on to a solid surface.

One of the most widely studied applications of 2D protein crystals in molecular electronic devices is the fabrication of biosensors, though prototypes of such novel biosensors have not been developed yet. In this perspective, the use of electron-transfer proteins appears to be particularly attractive due to their natural charge transfer activity in biological systems. Molecular biosensors for the detection of glucose have been constructed using glucose oxidase that was immobilized on protein crystals. These biosensors have been demonstrated to possess a high degree of enzymatic activity, high packing density, short diffusion distance, and a fast response time (Neubauer et al., 1993). Spinke et al. (1993) have successfully constructed a prototypical multilayer system incorporating human chorionic gonadotrophin for use as a pregnancy test, which may be used in a variety of medical diagnostic systems. In addition, highly specific sensors utilizing antibody-antigen recognition have also been constructed. As shown in Figure 2 (Sleytr et al., 2007), the S-layer fusion protein rSbpA31-1068/cAb-prostatespecific antigen (PSA)-N7 carrying the camel antibody (cAb) sequence recognizing the PSA, was recrystallized on gold chips precoated with thiolated secondary cell wall polymer (SCWP), and exploited as nanopatterned sensing layer in surface plasmon resonance (SPR) to detect PSA.

The pioneer work on the electronic structure of 2D protein crystals by Vyaliikh et al. (2004) has opened up new possibilities for low-dimensional hybrid semiconductor devices. In their work, the 2D crystals of...
S-layers have been demonstrated a semiconductorlike behavior with a gap value of ~3.0 eV and the Fermi energy close to the bottom of the lowest unoccupied molecular orbital. Figure 3 schematically illustrates the construction process of a thin film biotransistor.

Mcmillan et al. (2002) have fabricated nanoscale ordered arrays of quantum dots by binding preformed nanoparticles onto 2D protein crystals. Quantum dots can be manipulated and organized into arrays which can be used in next-generation photonic devices (Astier et al., 2005), as shown in Figure 4.

IMPROVING PERFORMANCES OF 2D CRYSTALS

Macromolecular crystals normally exhibit a variety of imperfections or disorder that limit the accuracy of molecular structure determinations and their performances (Malkin and Thorne, 2004). Figure 5 shows examples of crystal disorder, such as molecular positional, orientational or conformational disorder, grain boundaries, dislocation, etc.

Field-induced 2D crystallization

Capillarity-induced 2D assembly, popularly conducted at an air-liquid interface, has several limitations: it applies to relatively large particles only, the clustering is usually non-defect-free and lacks long-range order, and the lattice spacing cannot be adjusted (Mbamala and von Grünberg, 2003). Recently external field-induced 2D assembly has been demonstrated success of produce 2D crystals with well-defined orientations and less defects. In general, electric fields interact with particles in crystallizing system through two mechanisms. As for dielectric particles, they give rise to induced dipole (polarization) effects, which have been used to guide the structure formation of self-assembly, and can produce the orientation of particles. As for particles with pre-existing charge, application of electric fields gives rise to electrokinetic effects, which typically scale linearly with the applied field strength and the free charge (that is, electrophoresis and electroosmosis) (Ku et al., 2007). Xie and Liu (2009) reported an excellent work of 2D colloidal crystallization controlled by an alternating electric field (AEF), and found that the lattice spacing of 2D colloidal crystals could be adjusted statically or dynamically. In our laboratory, AEF has been also applied into the 2D crystallization of proteins, as shown in Figure 6.

Protein engineering

Native proteins usually show unsatisfying performance in terms of activity and stability for a given application (Lu et al., 2010). To overcome this limitation, such protein engineering techniques as directed evolution and rational protein design are often used to tailor the native proteins (Bornscheuer and Pohl, 2001). Rational design (site-directed mutagenesis, SDM, as shown in Figure 7) normally requires both the availability of the structure of the protein and knowledge about the relationships between sequence, structure and mechanism/function, and is therefore very information-intensive. In sharp contrast, as shown in Figure 8, directed evolution involves either a random mutagenesis of the gene encoding the catalyst (for example, by error-prone PCR) or recombination of gene fragments (for example, by DNA shuffling). Libraries thus created are then usually assayed using high-throughput technologies to identify improved variants. For both approaches to protein engineering, the gene(s) encoding the protein(s) of interest, a suitable (usually microbial) expression system,
and a sensitive detection system are prerequisites (Tobin et al., 2000; Sutherland, 2000).

To date, there are some successful examples of improving performances of 2D crystals through protein engineering. Badelt-Lichtblau et al. (2009) have utilized genetic engineering to engineer the S-layer protein for the generation of functionalized nanoarrays. Three C-terminal amino acids were deleted firstly, and then the sequence encoding the short affinity tag Strep-tag II as well as a single cysteine residue was fused to their C-terminal end. Functionalized two-dimensional S-layer lattices formed by rSbpA31-1178/STII/Cys exhibiting highly accessible cysteine residues in a well-defined arrangement on the surface were utilized for the template-assisted patterning of gold nanoparticles. Tang et al. (2008) ordered the arrays of 5 nm gold NPs by...
using the repetitive pattern of a mutated S-layer protein as a binding template.

Although there are sporadic successes in improving proteins’ functions through protein engineering approaches, further development of more-efficient methods to explore protein sequence space will be an ongoing endeavor. Molecular modelling and molecular dynamic calculations will help to uncover the structural basis of functions as well.

CONCLUSIONS AND PERSPECTIVES

2D protein crystals represent unique biomaterials which can be used to generate various structures or devices through a bottom-up approach. However, to date, the most frequently used routine to grow 2D protein crystals is to adsorb proteins from an aqueous solution onto the layer of lipids, whose lateral diffusion affords the required mobility. Accordingly there are three key problems to be resolved as soon as possible in order to expand the applications of 2D protein crystals with well-defined orientations and less defects. The first one is that, is there a general way to assembly proteins into 2D crystals on the solid surfaces directly instead of using lipid layers? It is obviously inconvenient to use 2D crystals grown on lipid layers in many applications (for example, biosensor,
The problem of transferring the crystals from the air-water interface to the target surface. Although some works on the subject have been done, more studies should be conducted further. The second one is that, what is the exact mechanism of protein 2D crystallization at interfaces, particularly at liquid-solid interface? There still lacks of a general method of forming two-dimensional crystals of soluble proteins due to limited understanding of the 2D crystallization process. The third one is that, how to eliminate various kinds of disorder of protein molecules in 2D crystals resulting from homogeneous nucleation and uncontrollable crystal growth? More attentions should be paid to above questions in future.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 21176102 and 21176215), the Scientific Research Foundation for Returned Chinese Scholars and the Fundamental Research Funds for the Central Universities (No. JUSRP30904).

REFERENCES


Kalhor HR, Shahin F, Fouani MH, Hosseinkhani H (2011). Self-assembly of tissue transglutaminase into amyloid-like fibrils using electric displays, etc.) because of the problem of transferring the crystals from the air-water interface to the target surface. Although some works on the subject have been done, more studies should be conducted further. The second one is that, what is the exact mechanism of protein 2D crystallization at interfaces, particularly at liquid-solid interface? There still lacks of a general method of forming two-dimensional crystals of soluble proteins due to limited understanding of the 2D crystallization process. The third one is that, how to eliminate various kinds of disorder of protein molecules in 2D crystals resulting from homogeneous nucleation and uncontrollable crystal growth? More attentions should be paid to above questions in future.


