

Two-dimensional crystals of proteins on lipid layers

Roger D. Kornberg and Seth A. Darst

Stanford University School of Medicine, Stanford, California, USA

Proteins bound to lipid layers form single-layer (two-dimensional) crystals amenable to structure determination by electron microscopy and image processing. Recent studies have extended the range of the lipid-layer crystallization approach. In some cases, a simple electrostatic interaction between a protein and a charged lipid will suffice to direct binding, obviating the requirement for a specific lipid-binding ligand for two-dimensional crystal growth. Alternatively, streptavidin can be used to couple a biotinylated protein to a biotinylated lipid layer. Streptavidin itself forms large two-dimensional crystals that diffract to 2.8 Å resolution, showing that ordering on lipid layers can be comparable to that in three dimensions.

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Introduction

Two-dimensional crystals of proteins occur naturally in numerous bacterial and algal cell envelopes, can be formed from purified proteins using methods designed for growing three-dimensional crystals [1] and, in some cases, form spontaneously in solution. Only with the development of a general method of forming two-dimensional crystals of soluble proteins, however, have systematic studies of the crystallization process and a wide range of applications become possible. In this review, the status of this new science is assessed, with the emphasis placed on some recent examples.

Crystallization on lipid layers

The general method of forming two-dimensional crystals depends on constraining proteins in two dimensions without loss of mobility. For this purpose, proteins are adsorbed from an aqueous solution onto a monolayer of lipids, whose lateral diffusion affords the required mobility (Fig. 1). Adsorption may be accomplished in two ways: the proteins are bound specifically to ligands attached to the polar head groups of lipids, which allows the proteins to adopt a unique orientation at the lipid–water interface; or, the proteins are bound non-specifically through electrostatic interactions with a charged lipid layer. In either case, a high affinity of the proteins for the lipid layer assures a high protein concentration at the lipid–water interface. The concentration of protein localized on the lipid surface can be on the order of 500–1000 mg ml⁻¹. This high concentration drives the crystallization process. In all cases studied so far, no further driving force, such as a precipitant in the aqueous solution, is needed; two-dimensional crystals may be formed under physiologic conditions or under other relevant conditions.

The approach has been successfully applied to a dozen different protein molecules, including antibodies [2], enzymes [3,4,5•–7•,8], polypeptide toxins [9,10] and other proteins [11,12•,13]. It seems that with a homogeneous preparation of a protein that binds tightly and specifically to a lipid-based ligand, two-dimensional crystals will invariably be obtained. Crystallization is monitored by electron microscopy or, in favorable instances, by fluorescence microscopy [11,12•].

The most useful application of two-dimensional crystals of proteins so far has been in structure determinations by electron microscopy and image processing [14]. This combined approach is applicable to many proteins that are not amenable to X-ray diffraction analysis. It requires only small (microgram) quantities of material and small crystals (areas containing a few thousand unit cells). It is particularly valuable for large assemblies of polypeptides, which pose the greatest difficulties for X-ray crystallography. Indeed, the best hope of revealing very large molecules at high resolution lies in solving the structures of component parts by X-ray diffraction and fitting the X-ray structures together with the aid of a lower-resolution map from electron crystallography. The two-dimensional crystallographic approach is also advantageous for studies of proteins that interact with membranes. For example, an analysis of cholera toxin crystallized in two dimensions on a lipid monolayer has revealed the structure of the toxin in the act of penetrating a membrane [9,15].

Ultimately, the value of adsorbing proteins through specific interactions onto lipid monolayers may extend beyond electron crystallography. It should be possible to explore in a systematic way the chemistry of interactions in two dimensions (especially at the lipid–water interface) and to elucidate the principles of two-dimensional crystallization. It should be possible to assemble a huge variety of new materials through the synthesis of lipids

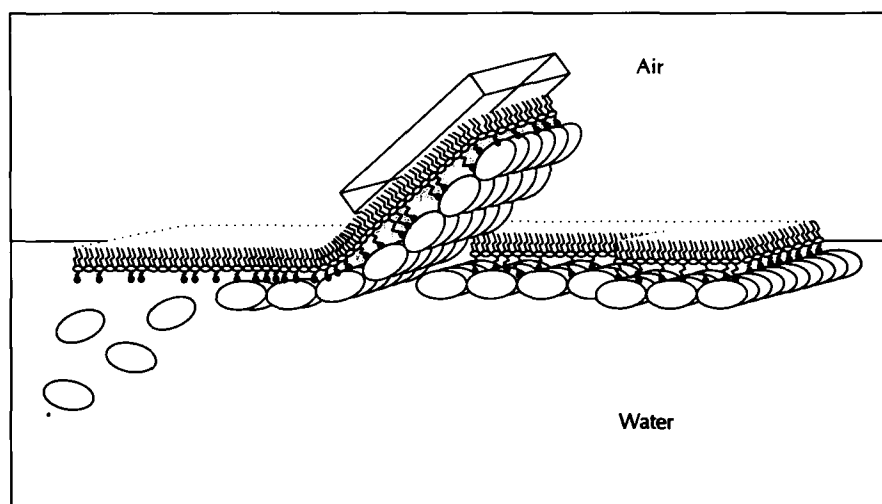


Fig. 1. Diagram of two-dimensional crystallization of protein on a lipid layer and transfer to an electron microscope grid.

with head groups for binding practically any protein, and hydrocarbon chains having different physical properties.

Importance of structure and composition of the lipid layer

An unexpected finding in initial studies of two-dimensional crystallization of antibodies was that no variation in the aqueous solution conditions was required [16]. The crystals formed under a wide range of solution conditions, including physiological conditions. By contrast, the physical state of the lipids was crucial. This dichotomy has proved to be generally applicable in all studies reported. It is therefore possible to form two-dimensional crystals under practically any solution conditions.

The physical state of lipids is readily varied by changing the hydrocarbon-chain composition and the temperature, and by adjusting the density of monolayers spread at the air-water interface. For antibody crystallization, fluid-state lipids are required, yet the monolayer must not be too diffuse [16]. The optimal density is the maximum that can be achieved without collapse of the monolayer. A likely explanation for this behavior is that lipids compete with denatured protein for sites on the air-water interface. A high lipid density prevents extensive coverage by protein, favoring the integrity of the monolayer. The requirement for a high-density lipid layer is probably general, although streptavidin has proved to be a notable exception by forming two-dimensional crystals on monolayers of biotinylated lipid in greatly expanded states [11,12•]. Streptavidin may be less prone than other proteins to interfacial denaturation. Alternatively, streptavidin may possess an unusual propensity for two-dimensional crystallization.

A wide range of lipid-based ligands have been synthesized for two-dimensional crystal growth. Most are derivatives of phosphatidylethanolamine, with ligands attached to the amino group of the ethanolamine moiety. The ligands include haptens for antibody binding (dinitrophenyl) [2], nucleotides for enzyme binding (ATP and dATP) [3,8], steroids for receptor binding (novobiocin)

[17] and biotin for streptavidin binding [11]. Despite this variety and the comparative ease of synthesis, a single lipid-based ligand that could bind a wide range of proteins, obviating the need for new synthesis, is very desirable. The use of a biotinylated lipid in conjunction with streptavidin may serve this purpose [12•], as described in more detail below.

Crystal transfer

Perhaps the most significant technical difficulty in the use of two-dimensional crystals grown on lipid layers for structure determination by electron microscopy is the problem of transferring the crystals from the air-water interface to the surface of an electron microscope grid. The well known Langmuir-Blodgett technique [18] has been used successfully [12•]; this involves transferring an amphiphilic layer at the air-water interface to a solid support by passing the support vertically through the interface. Unfortunately, this technique requires a relatively large aqueous volume (several milliliters) beneath the lipid layer and thus a large amount of protein in solution, therefore defeating one advantage of the two-dimensional crystallization method. Transfer on a small scale (10 μ l of protein solution) has been accomplished by placing an electron microscope grid, coated with a thin film of carbon, directly on the surface of protein droplets coated with lipid and then withdrawing the grid. Some of the lipid layer and the associated protein adhere to the grid as a result of hydrophobic interactions between the hydrocarbon chains of the lipid and the carbon film. Studies of two-dimensional crystallization of the protein streptavidin on monolayers containing biotinylated lipids have revealed severe shortcomings of transfer by adhesion to a carbon film [12•]. Only a small fraction of the crystals present at the air-water interface were transferred to the carbon film, and those crystals that were successfully transferred were often broken apart and deformed by the stresses involved in the transfer process. Improved transfer procedures have recently been developed [19•] (see below) and further improvements are needed.

Crystals or hexagonally close-packed arrays

Two-dimensional crystals have been obtained in a variety of space groups, including crystals with true hexagonal ($p6$ plane group) symmetry [16]. Hexagonal symmetry may, however, be misleading. Experience with cholera toxin crystallized on lipid layers containing its natural membrane receptor, the ganglioside GM1, illustrates the point. Two types of ordered arrays of cholera toxin were obtained, a rectangular lattice and a hexagonal lattice [9]. Noise-filtered images computed from the diffraction patterns revealed pentagonal or hexagonal rings of protein density in the square and hexagonal lattices, respectively. The diameters of the two types of ring were the same, thus they encompassed similar molecular volumes, and in view of the pentameric nature of the B subunit oligomer, the pentagonal structure was clearly correct, whereas the hexagonal rings were an artifact of averaging the pentagonal structure about the six-fold axes of the hexagonal lattice. There was evidently some static or dynamic rotational disorder of the B subunit oligomer about the molecular fivefold axis in the hexagonal lattice, which was not a true crystal, but rather a hexagonally close-packed array.

A similar sort of hexagonal close-packing probably explains the ordering of ferritin in work done long ago, before the idea of crystallization on lipid layers was introduced [20]. Ferritin was adsorbed onto positively charged lipid layers, and hexagonal arrays were observed by electron microscopy. The possible crystalline nature of the arrays was never investigated, and a rather refined analysis would be required to rule out the artifact of close-packing.

Two-dimensional crystals by non-specific binding

The first protein to be crystallized on lipid surfaces without specific ligands for binding was *Escherichia coli* RNA polymerase holoenzyme [4]. Electrostatic interactions between the acidic protein and positively charged lipid surfaces were sufficient for two-dimensional crystallization. This result has now been extended to eukaryotic RNA polymerases [5–7], and three-dimensional structures of these giant protein assemblies have been derived [21,22].

Whereas two-dimensional crystals of yeast RNA polymerase II were poorly ordered compared with those of *E. coli* RNA polymerase [5], an altered form of the enzyme purified from a mutant strain of yeast crystallized with significantly better order than any RNA polymerase crystals studied previously (diffracting to 12–15 Å resolution when preserved in negative stain) [7]. The mutation in the yeast strain eliminated a major source of heterogeneity in subunit composition of the enzyme, indicating the importance of protein purity and homogeneity in the growth of well ordered crystals under conditions in which impurities or multiple forms of a protein compete for binding to the lipids. By contrast, crystallization

of cholera toxin on lipid layers containing its cell-surface receptor was insensitive to the addition of contaminating proteins that did not compete for the receptor [23].

Streptavidin as a general adaptor molecule

Although the lipid-layer crystallization approach has been applied successfully with a variety of ligands for specific protein-binding, appropriate lipid-based ligands are sometimes difficult to obtain. The two-dimensional crystallization of RNA polymerases on charged lipid layers described above demonstrates one way of circumventing this difficulty. Although the modification of lipid-surface properties such as electrostatic charge may prove useful in other applications, this strategy may depend on special properties of the protein and thus may not be widely applicable. An alternative strategy is to obtain or devise a general adaptor molecule that will link a wide variety of macromolecules to lipid layers through specific binding to a common lipid-based ligand. The bacterial protein streptavidin may serve this purpose. Streptavidin is tetrameric, with four high-affinity binding sites for the small molecule biotin [24]. It has been used extensively in the past for the specific adsorption, localization and other analysis of biotin conjugates. When streptavidin was introduced beneath layers of biotinylated lipids, the result was striking: large protein domains up to 200 μm in diameter were observed by epi-fluorescence microscopy [11,12]. Diffraction from electron micrographs showed that these domains were two-dimensional crystals with an unusually large size and a high degree of order [12]. Three-dimensional reconstruction from electron micrographs of crystals in negative stain revealed that the surface topography of the streptavidin molecule agreed very well with the structure determined at atomic resolution by X-ray crystallography [25,26]. From comparison of the electron and X-ray structures, it was apparent that two of the four biotin-binding sites of the streptavidin molecule must be exposed on the surface of the crystal facing the aqueous solution. This ease of access to biotin-binding sites allows interaction with additional biotinylated molecules in solution, as shown by the binding of biotinylated ferritin to streptavidin crystals. The binding was specific, as it was not observed in the presence of excess free biotin, which saturated the sites on the crystals, and ferritin without attached biotin did not bind at all [12].

The free biotin-binding sites on streptavidin bound to lipid layers may be exploited in two ways in studies of additional biotinylated molecules. Firstly, streptavidin may be used to direct the binding of additional biotinylated molecules to lipids in order to crystallize them. This strategy should be effective so long as the additional biotinylated molecules are a good deal larger than streptavidin, so that their interactions, and not biotin–streptavidin interactions, dominate the crystallization process. Secondly, some biotinylated molecules smaller than streptavidin may become well ordered simply through binding to a preformed streptavidin crystal.

A key question about single-layer crystals of proteins formed on lipid layers is whether or not they can be ordered as well as three-dimensional crystals, despite the lack of protein-protein interactions above and below the single layer. To investigate this question, two-dimensional crystals of streptavidin were examined in the unstained state (negative stain, which allows observation by electron microscopy, imposes a resolution limit of about 10 Å because of the granularity of the stain [19•]). To preserve the quality of the unstained crystals and facilitate their analysis by electron microscopy, new procedures had to be developed for transferring the crystals to specimen support grids (see discussion above). The properties of carbon films perforated with holes have proved advantageous in this regard [19•]. When grids coated with holed carbon films were placed on the surface of droplets of streptavidin solution coated with biotinylated lipids, then withdrawn and preserved in a negative stain, two-dimensional streptavidin crystals were observed covering virtually all the holes. Crystals transferred over the holes did not appear to be fragmented like those transferred onto carbon. Crystals suspended over the holes also appeared to be better ordered, diffracting to 10 Å resolution (the limit for specimens in a negative stain), compared with 14 Å resolution for crystals on carbon [19•]. Finally, two-dimensional streptavidin crystals transferred over holes and preserved without staining [27] gave an electron diffraction extending to 2.8 Å resolution [19•]. This result leaves no doubt as to the degree of crystalline order, and raises the possibility of structure determinations at the atomic level, using current methods of cryo-electron microscopy and image processing [28], for proteins crystallized by the lipid-layer approach.

Two-dimensional crystals as seeds for three-dimensional crystal growth

For some proteins, two-dimensional crystallization is accompanied by the formation of ordered arrays several layers thick. These multiple layers sometimes exhibit coherent diffraction, indicative of ordering in the third dimension [7•]. The ordering occurs despite the comparatively low concentration of protein in the aqueous solution (0.1–0.25 mg ml⁻¹) and the lack of precipitants normally used to induce three-dimensional crystallization of proteins, suggesting that two-dimensional crystals may be highly effective as seeds for three-dimensional crystal growth. The prospect of being able to form three-dimensional crystals in difficult cases, possibly even in a physiological solution, from lipid layers merits further investigation.

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RD Kornberg and SA Darst, Beckman Laboratories, Fairchild Center, Department of Cell Biology, Stanford University School of Medicine, Stanford, California 94305-5400, USA.