

Chapter 1

Two-dimensional Crystallization on Lipid Monolayers

1.1. Overview

In 1971 it was first shown by Fromherz (1971) that an ordered arrangement of protein can be generated underneath a lipid monolayer. Ferritin molecules were observed in a regular arrangement after adsorption to a lipid film. Limited lateral mobility and heterogeneity in the lipid monolayer resulted in only moderately ordered protein in these early experiments. About 10 years later, for the first time Kornberg's group was capable of obtaining 2D crystals of an antibody using a monolayer of lipid hapten (Uzgiris and Kornberg, 1983). Since then, this 2D crystallization technique has been successfully employed for a variety of different proteins (see Appendix). These are mainly water-soluble proteins, but recent studies have shown that this technique is also applicable to membrane proteins. This may provide a much needed extension in the repertoire of membrane protein crystallization, thus improving our structural knowledge of this protein class.

The crystallization on lipid layers is an elegant method because it is possible to work with very dilute protein solutions and still generate a locally high concentration of protein constrained in 2D. Nonetheless, the proteins retain sufficient mobility to allow for organization into crystalline 2D arrays by lateral diffusion. Lipid monolayers can be spread (driven by surface tension) over the whole air/water interface of a drop to form a flat, one molecule thick film. This provides a substrate for protein binding, leading to a layer of closely packed proteins at the interface which can be organized into a 2D crystal suitable for structure determination by electron crystallography.

2 Strategies for Two-dimensional Crystallization of Proteins

The first step in the crystallization process is the adsorption of the protein to a lipid monolayer (Fig. 1.1). This limits the protein to a few orientations relative to the lipid plane which facilitates crystallization. The hydrophilic headgroup of the lipid is responsible for this function and operates as a recognition element for the protein in one of two ways. The first involves electrostatic interactions of a charged lipid layer with the protein allowing non-specific binding to a surface layer. The second occurs by specific binding of protein to a surface monolayer. This is achieved by interaction of the protein with a ligand attached to the polar headgroup of the lipid. Both types of interactions lead to a densely packed protein layer at the lipid/water interface. It is possible to obtain a locally high concentration of protein in the order of 500–1,000mg/mL from a very dilute solution (10–100 μ g/mL) (Kornberg and Darst, 1991).

Lipids provide a substrate for protein binding and therefore a functional basis of the crystallization technique. In general, lipids consist firstly of a hydrophilic headgroup which can carry a charge or a functionalized ligand group, for example a Ni²⁺-NTA group, that can be utilized for protein binding via a His-tag. The second part of a lipid is a long hydrophobic tail which usually consists of two acyl chains. It is necessary for the

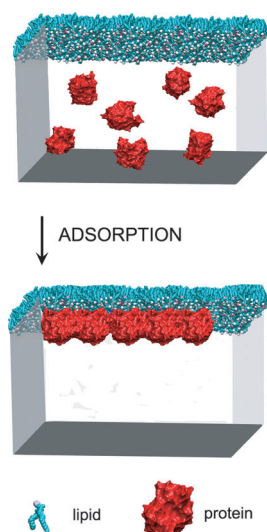


Fig. 1.1 The method of surface crystallization for soluble proteins.

molecule to accommodate its hydrophobic and hydrophilic part in a suitable environment. Therefore, amphiphilic molecules like lipids have a tendency to self-organize. In solution, they form lipid bilayers in the form of vesicles or tubes; at the air/water interface they form lipid monolayers. The hydrophobic tails are sheltered from the water by burying within the bilayer or by pointing into the apolar air. In this way interaction of each part with the opposite phase is minimized. This is favorable because the acyl chains are not able to form hydrogen bonds with water. The organization of the monolayer is driven by the hydrophobic effect associated with the lipid tail groups of the lipid molecules which also determines the stability and fluidity of the monolayer. The structure of this water insoluble moiety is responsible for the physical properties of the lipid layer, because the long hydrophobic chains interact extensively with each other. The length and saturation of the acyl chain as well as branches in the chain influence the fluidity of the lipid at a given temperature. Fluidity of a lipid monolayer is given by intramolecular contacts between the hydrophobic lipid chains. This interaction is dependent on the shape of the hydrophobic tails of the lipids. A *cis*-double-bond introduces a kink in the acyl chain which sterically hinders packing of the hydrocarbon chains in the same fashion as a branched chain and both result in an increased fluidity of the lipid (see Sec. 3.1). It is clear from the literature that the lipid monolayer being in the fluid state is the most favorable condition for the growth of 2D crystals. Although closely packed protein molecules have been found on monolayers in the solid state, 2D crystals were not observed under these conditions (Darst *et al.*, 1991a; Mosser and Brisson, 1991).

The physical properties of the monolayer system are determined mainly by the chemical composition of the lipids, the temperature and the composition of the underlying buffer. In order to allow protein crystallization these parameters have to permit the lateral diffusion of the protein molecules attached to the monolayer. To achieve a favorable physical state of the lipid layer it has often proven useful or even essential to use mixtures of different lipids. As these additional lipids usually do not carry a functional group, they are generally referred to as diluting lipids. A reason for the dependence on diluting lipids might be the difference in surface covered by proteins and the much smaller lipids. A phospholipid occupies about 50 times less area than a 100kDa globular protein.

Therefore, one protein molecule can interact with many lipid molecules. Thus the composition of the lipid layer is of high importance for crystallization, especially in the case of non-specific interactions, as it determines the local electrostatic milieu. Furthermore, it is possible to adjust the fluidity properties for a given monolayer by mixing functionalized lipids with diluting lipids of different structure.

Application of the surface crystallization technique for the creation of 2D crystals always bears the possibility of producing hexagonally closely packed protein, which might be mistaken for protein crystals at low resolution. Some proteins crystals like cholera toxin show a true hexagonal space group (Kornberg and Ribi, 1987), other denser areas of the same protein on the grid were found to exhibit a hexagonal diffraction pattern (Ludwig *et al.*, 1986). Further investigation indicated that the diffraction pattern was due to the presence of hexagonally closely packed protein molecules, and not due to a crystal. Surface crystallization trials generally lead to a closely packed layer of protein and are therefore prone to produce this kind of artifact, which rarely lead to 2D crystals. Figure 1.2 shows different organizations of protein which are common for surface crystallization trials.

The observation of growing 2D crystals on lipid monolayers can also give insight into the fundamental processes during crystallization (Ku *et al.*, 1993). Due to reduced dimensionality, aspects of the transition between disordered and ordered states can be studied more easily. 2D crystals have also been used to promote epitaxial growth of 3D crystals from 2D crystals from lipid layers. In this case, 2D crystals serve as nuclei for the formation of 3D crystals. Furthermore, it has been established that the lipid layers alone can trigger epitaxial crystal growth (Hemming *et al.*, 1995).

In conclusion, there are some general requirements for 2D crystallization of proteins:

- limiting the protein diffusion to a plane;
- a high concentration of the protein in the plane;
- orientation of the protein;
- providing mobility of the protein within the plane to allow sampling of various interaction arrangements.

The physical properties of the lipids spread at the air/water interface, the protein adsorption and binding capacity at the interface, the

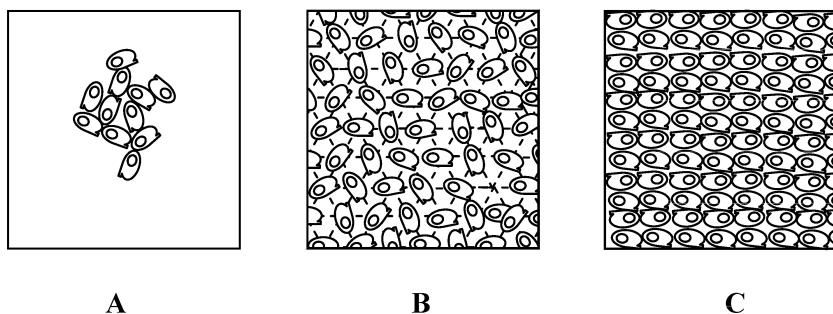


Fig. 1.2 Different stages of protein organization on lipid films: (A) aggregates without order, (B) closely packed hexagonal arrays, (C) 2D crystal.

lipid reorganization induced by protein binding and the crystallization process of the proteins have been extensively studied using numerous biophysical techniques. Some of these techniques include film balance measurements, ellipsometry, light scattering microscopy, epifluorescence microscopy, fluorescence spectroscopy, fluorescence microscopy, phase contrast microscopy, Brewster angle microscopy (BAM), electron microscopy (EM), atomic force microscopy (AFM). More recently, other methods have been developed such as X-ray reflectivity, X-ray grazing incidence diffraction, neutron reflectivity, shear modulus measurements and scanning near field optical microscopy (SNOM). Some of these techniques will be presented in chapter 3.

1.2. Non-specific Adsorption through Electrostatic Interactions

Lipids can contain headgroups with positive, negative, or neutral charges (Fig. 1.3). The attraction of opposite electrical charges provides the basis for electrostatic interactions. Charged lipids can be used to create a charged surface that can interact with the surface potential of a protein. This type of interaction can be compared to processes involved in ion-exchange chromatography:

Proteins carry positive and negative charges, according to the acidic or basic side chains of single amino acids. At acidic pH values the side chain lysine, arginine and histidine are

6 Strategies for Two-dimensional Crystallization of Proteins

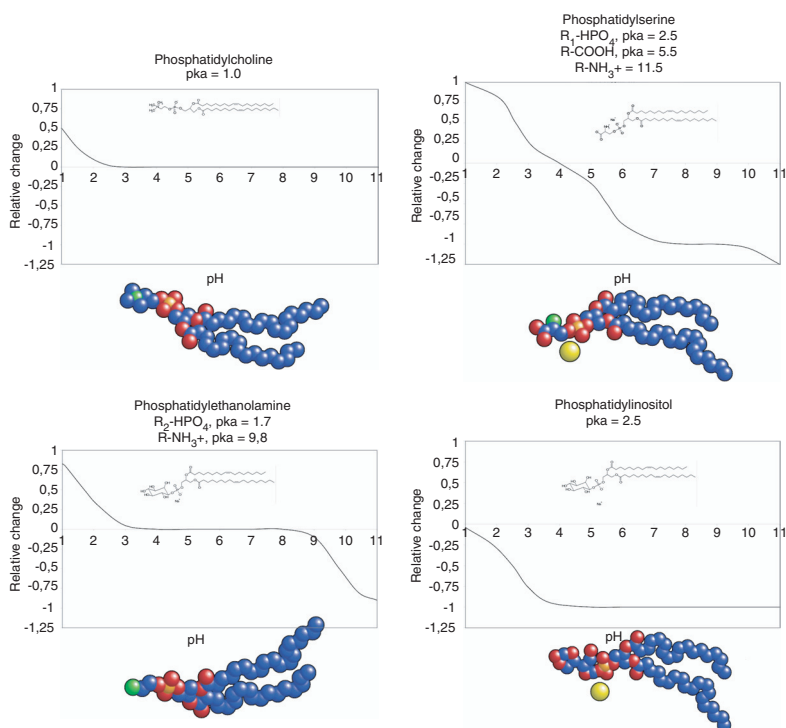


Fig. 1.3 Chemical structure, surface representation and relative charge as a function of pH of some common phospholipids.

protonated and a protein shows a cationic behavior. In contrast, at basic pH the negative charges of aspartic acid and glutamic acid render the protein anionic. Therefore, the net charge of a protein is dependent on the pH of the surrounding buffer and the number of exposed charged amino acids on the surface of the protein. The overall charge of a protein is described by the pI value. If the pH of the buffer is at the pI, the net charge of the protein is zero and therefore the capacity of the protein for electrostatic interactions is low, unless there is an unequal distribution of charged amino acids on the surface of the protein, which leads to regions with a positive or negative charge.

This seems to be the case for α -actinin which forms 2D crystals on a positively charged lipid layer at the isoelectric point of the protein

(Taylor and Taylor, 1993). The properties of the surface potential of the protein and the charge of the lipid layer can be controlled by varying the pH. For lipid layers, mixtures of differently charged lipids can provide different charge patterns on the surface facing the crystallization solution. If these conditions are chosen correctly, electrostatic attraction is sufficient to establish a stable interaction between a protein and the lipid layer. The extent of electrostatic interaction can be mediated not only by changing the pH, but also by altering the ionic strength of the aqueous phase. Increasing ionic strength weakens the electrostatic interaction and ultimately prevents protein binding, usually at a salt concentration of above 400mM.

The first protein that was crystallized on lipid monolayers exploiting electrostatic interactions was the RNA polymerase from *Escherichia coli* (Darst *et al.*, 1988). The attraction between the acidic surface of the protein and the positively charged lipid layer was sufficient to generate 2D crystals at the interface. RNA polymerase II is a very good example for the potential of surface crystallization when it comes to large complexes. The catalytically competent core consists of five different subunits and has a molecular weight of about 400kDa. The complex binds to positively charged lipid layers via electrostatic interaction and forms 2D crystals useful for structure determination (Darst *et al.*, 1988). Another large complex, the 50S ribosomal subunit, was crystallized on negatively charged lipids (Avila-Sakar *et al.*, 1994).

A group of proteins that shows a natural affinity for negatively charged lipids are annexins. The self-assembly capability of some annexins on cell membranes (Kaetzel *et al.*, 2001) seems to be important for membrane-related processes including exocytosis, endocytosis and vesicle trafficking (Gerke and Moss, 1997; Konig *et al.*, 1998; Raynal and Pollard, 1994). The tendency of annexin to naturally form 2D arrays has been used to grow crystals on negatively charged lipid membranes (Mosser *et al.*, 1991; Newman *et al.*, 1989; Newman *et al.*, 1991). 2D crystals of annexin reproducibly form on lipid monolayers containing at least 5% of the negatively charged phosphatidylserine and are strictly Ca^{2+} -dependent (Andree *et al.*, 1990). Annexin V (consisting of four 40kDa subunits) exhibits a high affinity to negatively charged lipids, such as phosphatidylserine ($K_D < 0.1\text{nM}$). The structure of lipid bound annexin V

was presented in 1991 at 20Å (Brisson *et al.*, 1991). The atomic structure of annexin V had simultaneously been solved by R. Huber (Huber *et al.*, 1990). The protein in membrane bound form and its soluble equivalent from X-ray crystallographic work was then compared and the precise orientation of the protein with respect to the membrane was determined (Brisson *et al.*, 1991). Later, a 3D model at 17Å (Voges *et al.*, 1994) and a projection map at 8Å resolution (Olofsson *et al.*, 1994), and then at 6.5Å resolution (Oling *et al.*, 2000) allowed an analysis of the conformational change from soluble to membrane bound form.

Another protein with a natural affinity for negatively charged lipids is brush border myosin-I. As with the annexins, this property is linked to the suggested function of mediating vesicle transport (Celia *et al.*, 1996). To date, three different human blood coagulation factors have been crystallized, utilizing their natural affinity to negatively charged lipids (Stoylova *et al.*, 1994; Stoylova *et al.*, 1998; Stoylova *et al.*, 1999). This affinity has a functional importance as the assembly of different factors into active complexes of the blood clotting cascade requires the preceding binding of these factors to negatively charged phospholipids. Using these data, a hypothetical model of membrane-bound factor VIII associated with the classic hemophilia was proposed.

A pore-forming protein, α -toxin from *Staphylococcus aureus*, is known to interact with lipids. The interaction leads to the oligomerization of the protein, insertion into the membrane and pore formation. A negatively charged lipid monolayer was used to crystallize the protein (Ellis *et al.*, 1997; Olofsson *et al.*, 1990). Reconstruction of a 3D model of an oligomeric pore provided the first glimpse of the process by which the toxin inserts into the membrane.

As the electrostatic interactions are non-specific, the purity of the protein preparation is extremely important to prevent the accumulation of contaminants at the surface which could inhibit the crystallization process. Furthermore, the non-specific nature of the interaction limits the probability that a protein is pre-oriented at the interface which is responsible in facilitating crystallization. Nevertheless, Lebeau *et al.* (1996) observed that the RNA polymerase, which has an acidic surface, can bind in an orientated way upon interaction with a positively charged lipid. In the same study, the RNA polymerase was observed to adsorb (to a lower extent) to polar but uncharged lipids, implying that

hydrogen bonds are also involved in the protein-lipid interaction. On the other hand, if only charged lipids were used, without the addition of neutral dilution lipids, no crystals were obtained, probably due to interference of the protein-protein interactions necessary for crystallization by the strongly charged surface.

It was shown for the chaperonin TF55 that differently charged lipids resulted in crystals with the protein in different orientations (Ellis *et al.*, 1998). Neutral lipids lead to an interaction of the protein in a side-on manner, whereas negatively charged lipids resulted in an end-on attachment to the lipid monolayer. Although only the latter crystals were ordered well enough for structure determination, it is interesting to note that a lipid-dependent orientation of a protein could be helpful for structure determination by electron crystallography. The missing information due to a limited tilt range (referred to as the missing cone problem) could be filled in by using crystals with a different orientation towards the crystal plane (Bischler *et al.*, 1998).

It is possible to form 2D protein crystals at a phase interface other than the lipid/water interface of a monolayer. Aoyama *et al.* (1995) conducted trials using a thin layer of the organic liquid dehydroabietylamine (DHAA) which carries a positive charge on its amino group (Fig. 1.4). Since it is insoluble in water, it will not denature protein. It forms a sharp interface with the aqueous protein solution. Its positive charge attracted negatively charged proteins to the interface and 2D crystals of various proteins, namely ferritin, catalase, chaperonin and the 50S ribosome were obtained at resolutions of 20–28Å (Aoyama *et al.*, 1995). Crystal sizes for the first three proteins were between 5µm and 0.5µm whereas the 50S ribosome yielded only very small crystals which could still be used to calculate a 3D map to approximately 28Å. A drawback of using a thin 3D liquid is an elevated noise level due to the greater thickness of

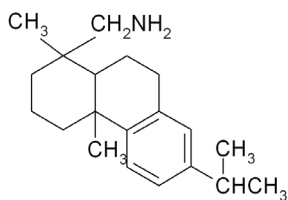


Fig. 1.4 DHAA (dehydroabietylamine).

the layer compared to a lipid monolayer. A similar organic compound carrying a negative charge has not yet been found.

Another alternative to lipid monolayers is the use of a synthetic polypeptide poly(1-benzyl-L-histidine). This polypeptide was spread at the air/water interface and its positively charged imidazole groups were able to attract catalase, ferritin or streptavidin from which 2D crystals grew (Furuno and Sasabe, 1993; Sato *et al.*, 1993). A higher temperature was required for crystallization (up to 40°C for 30min) to compensate for the lower fluidity compared to using unsaturated phospholipids.

Other proteins have been crystallized using electrostatic interaction, but it seems not to be a general method for every protein. Although we know from ion chromatography that it is theoretically possible to find conditions for an electrostatic interaction for every protein dependent on its surface charge pattern, the lack of specificity in binding and pre-orientation may cause difficulties in succeeding to find crystallization conditions for a particular protein. A more straight forward way is the use of specific adsorption of proteins via ligands, which is described in the next section.

1.3 Specific Adsorption via Ligands

The crystallization of protein on lipid monolayer using natural lipid headgroups will be presented first. This approach was used for a small number of proteins. The ability to synthesize lipids carrying various synthetic groups on lipid headgroups has been exploited and was a major breakthrough for the development of the 2D crystallization on lipid monolayers. Functionalized lipids carrying small molecules or metal ions will be subsequently described.

1.3.1. Molecular recognition between protein and lipid headgroup

Cholera toxin B/monosialoganglioside

Cholera toxin is one of the first proteins that have been crystallized by specific anchoring to planar lipid films (Ludwig *et al.*, 1986). This protein

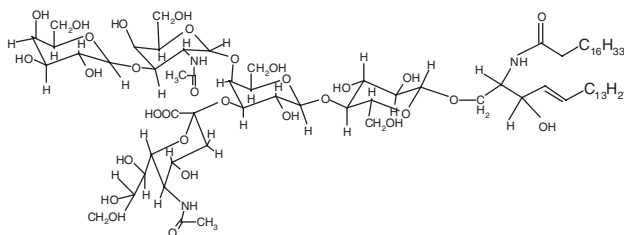


Fig. 1.5 GM₁, monosialoganglioside.

is secreted by the bacterium *Vibrio cholerae* and is composed of two different subunits A ($M_W = 27\text{kDa}$) and B ($M_W = 11.6\text{kDa}$) assembled with the stoichiometry AB₅. The B₅ assembly is responsible for binding of the protein to its cellular receptor, monosialoganglioside GM₁ (Fig. 1.5). The A₁ fragment of the A subunit catalyzes the ADP-ribosylation of the Gs α regulatory component of the adenylate cyclase complex (van Heyningen, 1983). The ultimate result of cholera toxin action is an extensive fluid loss characteristic of the disease.

A 3D model of cholera toxin B subunit has been calculated at medium resolution (Ribi *et al.*, 1988). The 2D crystals of cholera toxin-GM₁ complexes are extremely well ordered once transferred onto an electron microscope grid. An electron diffractogram exhibiting electron diffraction peaks extending to a resolution better than 0.35nm has been produced (Mosser *et al.*, 1992) and demonstrates the potential for high resolution electron crystallographic analysis of this type of monolayer crystals.

1.3.2. Functionalized lipids

Various synthetic lipids made of a protein ligand coupled to a lipid molecule have been synthesized. These protein ligands are often small molecules: biotin, ATP or ions: Ni²⁺, Cu²⁺; but they can also be larger such as steroid-derivative lipids. Lipids can also exhibit multifunctional groups. Firstly, moiety-carrying lipids (where molecules are attached to lipids) will be described (Sec. 1.3.2.1), then metal chelating lipids and multi functionalized lipids will be presented (Secs. 1.3.2.2 and 1.3.2.3).

Imagination and creativity helped to originate numerous functionalized lipids. This approach was not only motivated by the hope of producing a general method for 2D crystallization, but also by the need to create biofunctionalized interfaces. The lipid monolayer spread at the air/water interface was then used as a model system to investigate protein functions such as molecular and cellular recognition processes at the lipid interfaces. Biosensing technologies using biofunctionalized interfaces are another intensively studied field of research. It is important to know about the existence of these specially synthesized lipids as they can also be used for setting up 2D crystals. A short presentation of these designed lipids initially developed to create biofunctionalized surfaces will be given in Sec. 1.3.2.4.

1.3.2.1. Moiety-carrying lipids

Biotin-lipid streptavidin system

The high affinity of streptavidin for biotin ($K_D = 10^{-15}$ M) which is only one order of magnitude lower than for a covalent bond (Green, 1975), is at the origin of many biotechnological applications. The complex has been used for anchoring streptavidin onto biotinylated planar lipid surfaces. Streptavidin is tetrameric with four high affinity binding sites for the small molecule biotin (vitamin H). It has been used extensively in the past for specific adsorption, localization and other analysis of biotin conjugates.

When streptavidin, introduced into the subphase under the layer of biotinylated lipids (Fig. 1.6), was studied for the first time by epifluorescence microscopy, the result was striking. Large protein domains up to $200\mu\text{m}$ in diameter were visualized (Blankenburg *et al.*, 1989). This structural study was pursued with image analysis of electron micrographs and led to a 3D model of the streptavidin tetramer at 14\AA resolution

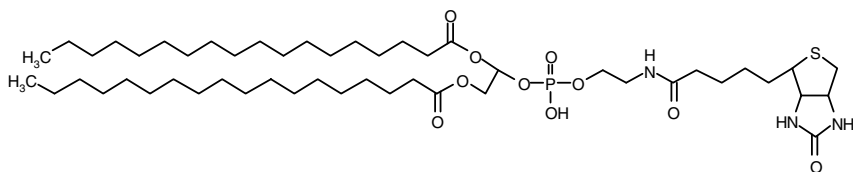


Fig. 1.6 Biotinylated-lipid: biotin-LC-DPPE.

(Darst *et al.*, 1991a) which was compared to the known X-ray structure (Hendrickson *et al.*, 1989; Weber *et al.*, 1989). Extension of the investigation and development of cryo-specimen preparation techniques allowed the first high resolution projection map (3Å) for an interfacially crystallized soluble protein to be calculated (Avila-Sakar and Chiu, 1996). A comparison of this projection map with the X-ray structure of streptavidin allowed assignment of β -sheet structures in the projection map. The excellent agreement of the projected density with the atomic model provides strong evidence that this type of monolayer crystal has the potential for high resolution electron crystallographic analysis. Comparison of the electron and X-ray structures confirmed that biotin-binding sites of streptavidin are available and appropriately orientated to bind additional biotinylated macromolecules in solution. Streptavidin has four binding sites for the biotin, two biotin binding sites are interacting with the biotinylated lipids and the two other binding sites are on the opposite side, facing the solution. This first layer made of a 2D crystal of streptavidin could provide a scaffold for the formation of another layer of protein molecules. Specific binding of biotinylated ferritin to lipid-bound streptavidin was demonstrated by electron microscopy (Darst *et al.*, 1991a). Nonetheless crystallization of streptavidin bound protein using these preformed 2D crystals have not been reported so far despite various attempts.

Recently, immobilization of biotinylated DNA on 2D streptavidin crystals has been reported by Crucifix *et al.* (2004). DNA was end-labeled with a biotin moiety in order to decorate 2D crystals of streptavidin which formed in contact with a biotinylated lipid layer. RNA polymerase was then able to interact with the DNA, and was immobilized by that strategy in various orientations. The immobilized nucleoprotein complexes were then suitable for 3D reconstruction from isolated molecular views, a method which requires usually a homogeneous angular distribution. The use of immobilized DNA while keeping it accessible for interacting partners opens the possibility to drive the macromolecular assemblies into a different functional state by simply changing the incubation buffer.

The streptavidin/biotin system provides a convenient model for studying the molecular mechanisms underlying protein crystallization since the crystallization conditions are well known. The relationship

between lipid-protein molecular recognition, crystallization solution conditions, and the crystal properties such as coherence, space group and morphology is an important issue. A better understanding of these relationships will aid in the design of rational strategies for promoting high-quality protein crystallization and for controlling protein assembly at interfaces in the biomaterial and nanotechnology fields.

The combined macroscopic and microscopic observations of the 2D crystals of streptavidin in various conditions shed light on the intermolecular interactions taking place between neighboring streptavidin proteins during the process of 2D crystallization. Depending on the conditions of crystallization, streptavidin crystals can form a variety of macroscopic morphologies. These macroscopic patterns can be observed using a Brewster angle microscope (BAM) (Frey *et al.*, 1996) or by epifluorescence microscopy and were compared to the associated microscopic crystal structures observed using electron microscopy (Gast *et al.*, 1999). For instance, lattices with P_1 symmetry appear for $1.5 < \text{pH} < 5$, P_1 and P_2 for $5 < \text{pH} < 6$, and C_{222} symmetry for $7 < \text{pH} < 11$. P_1 crystals nucleate rapidly and form a particular pattern of thin needle-shaped crystals. C_{222} crystals grow more isotropically and exhibit H- and X-shapes (or butterfly-like shapes) as observed with BAM (Fig. 1.7). The transition from C_{222} to P_1 or P_2 crystals can be accomplished in minutes by lowering the pH. Dynamics of 2D protein crystallization growth have been investigated, introducing avidin in the subphase as a non crystallization protein contaminant. The macro-pattern of the 2D crystals changed according to the concentration of avidin in the solution and shifted from dendritic growth to amorphous finger and needle like structure (Ku *et al.*, 1992).

The molecular mechanisms underlying asymmetrical growth in 2D streptavidin crystals have been investigated in more detail. Comparison



Fig. 1.7 H- and X-shaped streptavidin crystals observed with a Brewster angle microscope.

of the macroscopic morphology of 2D crystals of mutated streptavidin with 2D crystals of wild type streptavidin has been presented by Edwards *et al.* (2002): At neutral pH, streptavidin forms crystals with C_{222} symmetry and X-shaped morphology that arises from asymmetric growth rates. The molecular mechanism coupling biotin binding and growth asymmetry has been clearly shown using a mutated streptavidin (threonine 20 replaced by alanine). It appears that Tyr22-Thr20 hydrogen bond interaction across the protein-protein contact interface is the crystallization contact that is altered by biotin binding to give growth asymmetry in wild-type crystals. Using the same strategy, it has been shown that the mutant protein crystallizes with a different morphology than the wild type protein when lysine 132 is replaced by leucine (Edwards *et al.*, 1998). Lysine 132 seems also essential for the crystal morphology. The side chains of lysine 132 interact with each other across the dyad-related crystal contacts. These studies and others (Ratanabanankoon and Gast, 2003; Wang *et al.*, 1999; Yaticilla *et al.*, 1998) help to understand the complicated effects of intermolecular interactions on 2D protein crystal growth. This understanding is essential for the production of large and well ordered crystals.

Lipid-hapten

Early experiments using a lipid monolayer for 2D crystallization of proteins utilized a lipid hapten (DNP-PE) monolayer that had an affinity to a monoclonal antibody (anti-DNP IgG) (Uzgiris and Kornberg, 1983). A monolayer of this lipid hapten was used to form an ordered array of antibodies. After that, the C1q component of the complement was bound to the Fc tail of the antibody. The decorated antibodies also formed an ordered array and allowed the calculation of a projection map of the entire antigen-antibody-complement complex.

ATP-lipid

The universality of the ATP ligand in nature makes the use of ATP-lipid very attractive: any ATP-binding protein should bind to the ATP/lipid interface, the first step of the 2D protein crystallization process.

The use of an effector nucleotide linked to lipids allowed the formation of 2D crystals of the B₁ subunit of ribonucleotide reductase.

The projection map revealed structural features of the subunit up to 18Å resolution (Ribi *et al.*, 1987). The effector-lipid consisted of dATP coupled through the γ -phosphatyl group and a ϵ -aminocaproyl linker to phosphatidylethanolamine (Fig. 1.8A). Another class of hydrolyzable and non-hydrolyzable ATP-lipids where the nucleotides are covalently attached via the C₈- or N₆-position of the adenine ring to a synthetic lipid has been synthesized (Fig. 1.8B and Fig. 1.8C) (Schmitt and Tampe, 1996). These ATP-lipids can be applied not only to anchor, orient and crystallize ATP-binding proteins at a lipid interface, but they also have the ability to act as an energy source in the 2D plane. These ATP-lipids were characterized by various enzyme assays in

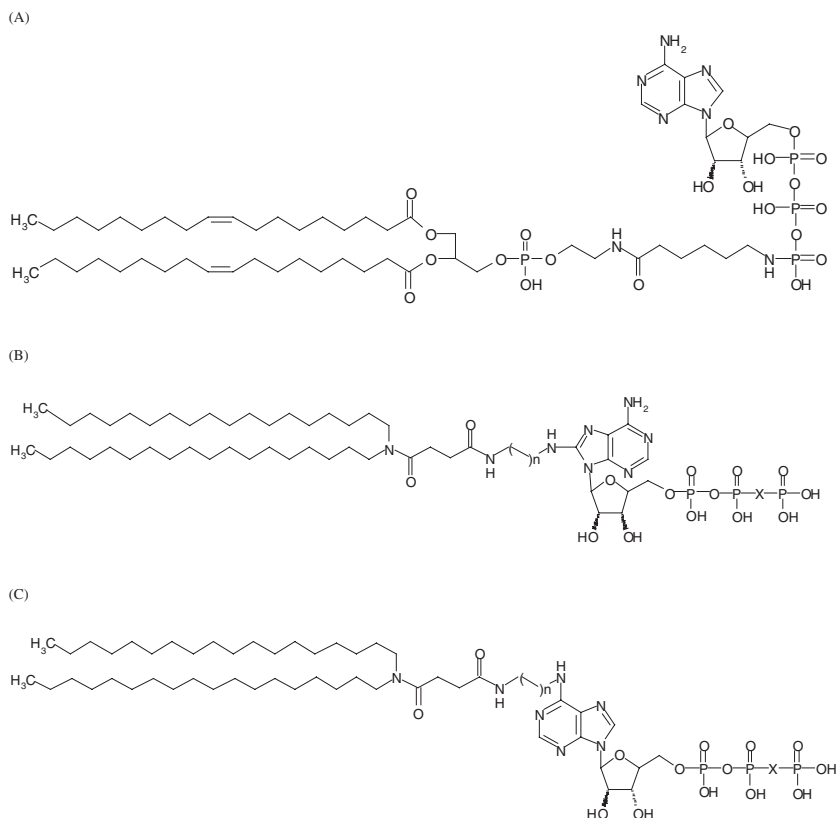


Fig. 1.8 ATP-lipids: (A) dATP-aminocaproyl-PE. (B) DODA-HM-C8-AMPPCP; (C) DODAHM-N6-AMPPCP; nonhydrolyzable (X = CH₂) and hydrolyzable (X = O) lipid. The spacer length can vary from ethylene (n = 1) to hexamethylene (n = 3).

micellar solution, resulting in ATPase and competition activities that are comparable to their free counterparts. Actin was used as a model for an ATP-binding protein attaching to an ATP-lipid. Following binding to ATP-lipid, drastic changes in the viscoelastic properties and shape transitions of vesicles were observed by phase contrast microscopy (Schmitt and Tampe, 1996).

Novobiocin-lipid

2D crystals of the *Escherichia coli* DNA gyrase B subunit were obtained upon specific interaction with novobiocin linked phospholipid films. DNA gyrase is a heterotetramer with an A_2B_2 stoichiometry. The B subunit has a M_W of 90kDa and was shown to bind coumarins such as the antibiotic novobiocin in the nanomolar concentration range. Therefore, novobiocin was an obvious natural ligand to attach to lipids in order to initiate structural studies of the B subunit. The specially designed lipid consists of the antibiotic group, novobiocin, modified and anchored to the lipid at the coumarin region, which preserved its affinity for the protein (Fig. 1.9). A polyethylene oxide hydrophilic spacer was placed between the dioleoylphosphatidic acid and the antibiotic in order to improve accessibility (Lebeau *et al.*, 1996). A projection map (Lebeau *et al.*, 1990) and later a 3D model (Celia *et al.*, 1994) of the gyrase B subunit were produced.

Dichlorophenyl-lipids

In the pioneering work of Hirata and Miyaka (1994), functionalized lipids (quinonylphospholipid) were designed to bind the bacterial photosynthetic reaction center onto monolayers. Later, new functionalized

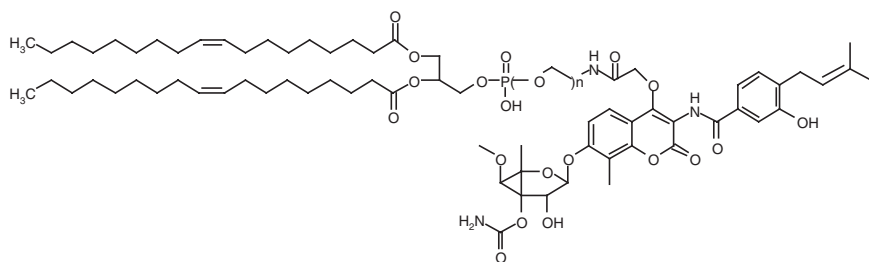


Fig. 1.9 Novobiocin-lipid. The spacer length can vary from $n = 1$ to $n = 4$.

lipids were synthesized (Trudel *et al.*, 2001). These dichlorophenyl-lipids (DCPU-lipids, Fig. 1.10) bound very efficiently to the Q_b site of photo-system II core complex, but no crystals were reported.

Other specific lipid ligands

Phospholipids linked to steroid-hormones derivatives (Fig. 1.11) have been synthesized for the study of the crystallization of progesterone and estradiol receptors (Lebeau *et al.*, 1991).

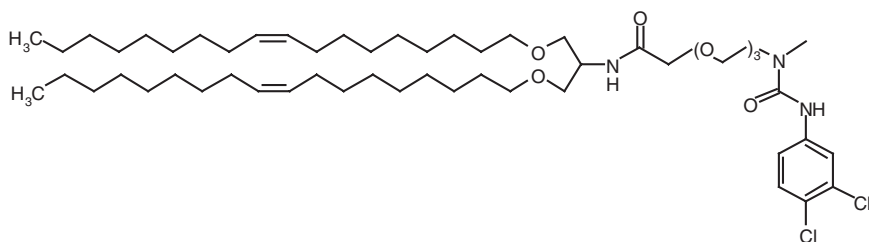


Fig. 1.10 DCPU lipid.

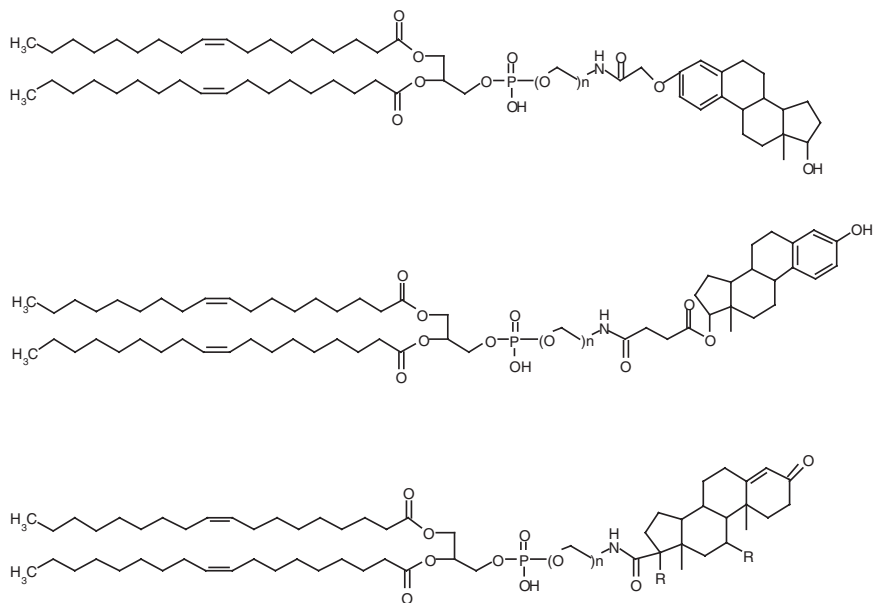


Fig. 1.11 Steroid-hormone derivatives lipids designed for two-dimensional crystallization experiments with hormone-binding receptors $R = OH$ or $R = H$, $n = 1-4$.

Glycerolipids linked to hydroxamate derivatives were designed for crystallization of mammalian aminopeptidase M (Altenburger *et al.*, 1992).

1.3.2.2. Metal-chelating lipids (Histidine-tag/ Ni^{2+} lipid headgroup)

Although the lipid-layer crystallization approach has been applied successfully to a variety of ligands for specific protein-binding, appropriate lipid-based ligands are sometimes difficult to obtain. An alternative strategy is to 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. In this strategy, strong interactions between a Ni^{2+} ion carried by specially synthesized lipids and a His-tag from expressed protein have been successfully used.

It was shown initially by electron spin resonance (ESR) that native proteins are targeted to Cu^{2+} -IDA lipid assemblies through coordination by surface histidine amino acids (Shnek *et al.*, 1994). The copper lipids have now been almost entirely replaced by the nickel chelates for routine protein binding. A short historical reference to the copper lipid is given at the end of the Sec. 1.3.2.4.

NTA-Nickel lipid headgroup design

An optimal design was found to be the quadridentate nitrilotriacetic acid (NTA, also called *N,N*bis[carboxymethyl]glycine (Fig. 1.12A), which binds to Ni^{2+} with three carboxyl groups and one nitrogen (Hochuli *et al.*, 1987). The remaining two ligand positions in the octahedral coordination sphere of Ni^{2+} are available for tight but reversible selective protein interactions. A string of six histidines was found to produce a strong and specific bonding to Ni^{2+} when it was held by NTA, replacing the two water molecules on the Ni^{2+} with histidine side chains. NTA represents an optimal compromise between the tridentate iminodiacetic acid (IDA, Fig. 1.12B) derivatives which bind Ni^{2+} weakly through three coordination positions and the pentadentate tris(carboxymethyl) ethylenediamine, which occupies five coordination positions on Ni^{2+} , leaving only one free for protein binding.

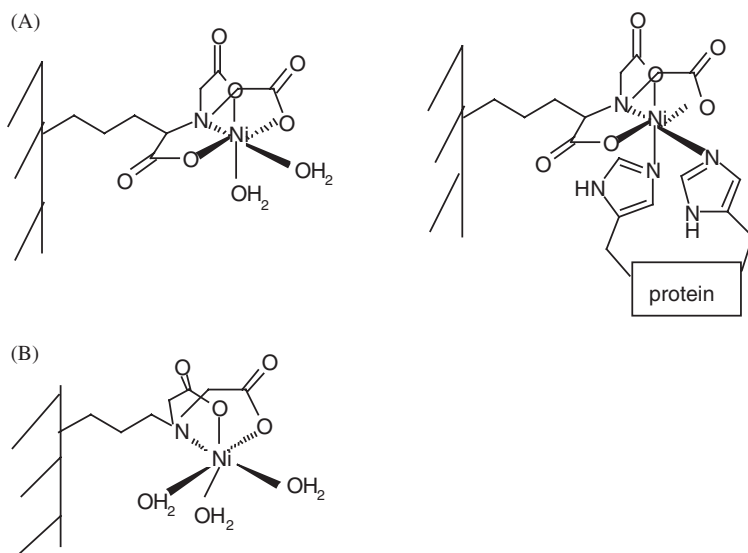


Fig. 1.12 Ni²⁺-NTA (nitrilotriacetic acid) and Ni²⁺-IDA (iminodiacetic acid) headgroup design. (A) Ni²⁺-NTA; (B) Ni²⁺-IDA.

Histidine-tag

Histidine is a relatively rare amino acid, accounting for only 2% of the amino acids in globular proteins. Only half are exposed on the protein surface. Therefore, on average, a 100-residue protein will only have one surface histidine. Consequently design of a protein expression system with His-tag became very attractive. If the high affinity for chelated metals is conferred on a specific protein, it becomes unique and thereby the adsorption of the protein on the lipid monolayer will be unidirectional, a prerequisite for the formation of 2D crystals.

Binding properties of Ni²⁺-Histidine

The dissociation constant (K_D) of 6 His-tag proteins to Ni²⁺-NTA has been measured to be 10^{-13} M at pH 8 (Schmitt *et al.*, 1993). This binding affinity is stronger than those of most antibodies which typically range from 10^{-6} to 10^{-12} M (Harlow and Lane, 1988). Only the avidin/biotin system shows a stronger binding with a K_D of 10^{-15} M (Green, 1975). A segment made of 10 histidines binds even tighter

(Hoffmann and Roeder, 1991) but seems to be unnecessarily long, so six histidines are commonly used.

The pKa of the histidine is close to 7.0. The proper interaction between histidine and a Ni^{2+} -complex occurs in the pH range 7.5–8.5. The interaction between Ni^{2+} and nitrogen in the imidazole ring is ionic and can be abolished by protonating these nitrogens, lowering the pH below the pKa value. The typical elution conditions for stripping a His-tag protein off a Ni^{2+} column is to lower the pH to a value < 5 (Schmitt *et al.*, 1993; Schmitt *et al.*, 1994), to use a pH gradient from 6.0 to 4.0, or to add a chelating agent (up to 100mM EDTA) (Paborsky *et al.*, 1996). Milder conditions are nevertheless required to avoid denaturation of proteins at low pH (Hoffmann and Roeder, 1991). It was found that imidazole could be used instead of change of pH, competing with the imidazole groups of the histidine residues for the Ni^{2+} . Typically, with a standard buffer and pH adjusted to 8, 50 to 200mM imidazole is necessary for a purification or elution step.

Effect of imidazole

In the crystallization trough, no adsorption of His-tag recombinant HIV-1 reverse transcriptase on a Ni^{2+} -lipid monolayer was observed when imidazole (100mM) or EDTA (1mM) was present in the protein buffer (Kubalek *et al.*, 1994). Imidazole displaces the protein by competing for the Ni^{2+} -NTA group.

In the case of the 2D crystallization of yeast RNA polymerase I on Nickel-chelating lipids, a minimum imidazole concentration was required to form crystalline areas (40–90mM) (Bischler *et al.*, 1998) whereas at a concentration higher than 200mM imidazole, the protein no longer bound. The low concentration of imidazole required for the enzyme to crystallize on these lipids reflects the behavior of His-tag proteins in immobilized metal ion affinity chromatography. Small amounts of imidazole are believed to prevent non-specific binding (mediated, for instance, by surface histidines) and favor the specific interaction with the engineered 6 His-tag.

Interestingly, the injection of imidazole (200mM final) into the crystallization trough, after crystals had formed, did not provoke any major desorption of the 2D crystals of His-HupR (a transcription factor from

Rhodobacter capsulatus) as shown using ellipsometric measurements (Courty *et al.*, 2002). However, the observation of the electron microscope grids after transfer of the monolayer showed that the crystals were smaller and contained multiple defects when compared with crystals before the addition of imidazole. A partial complexation of the Ni^{2+} ion with imidazole occurs as the interaction with imidazole is weaker than that with hexa-histidine ($K_D = 10^{-8}\text{M}$ compared with 10^{-13}M at pH 8.0 (Schmitt *et al.*, 1993)). In addition, the cohesive force within a crystal resulting from protein-protein interactions contributes to prevent a massive desorption of the protein. In a separate experiment, 200mM imidazole initially present in the protein solution prevented any adsorption of proteins to the monolayer.

Effect of EDTA, DTT and various ions

A concentration of 10mM EDTA prevented crystal formation of RNA polymerase I (Bischler *et al.*, 1998), EDTA disrupts binding of protein by fully complexing the Ni^{2+} ions. The addition of divalent or trivalent cations at a final concentration of 5mM had different effects depending on the nature of the ion. Poor transfer of the RNA polymerase I crystals onto the electron microscope grid was observed in the presence of Fe^{3+} , Zn^{2+} , or Cu^{2+} , whereas crystals could be observed in the presence of Ca^{2+} and Mg^{2+} . Although all multivalent cations should be able to out-compete the Ni^{2+} ions, this distinct behavior probably reflects the variable affinity of the divalent cations for the NTA moiety and the 6 His-tag.

The effect of additional molecules interfering with the 2D crystallization of His-MoCa — a viral capsid protein — has been reported by Barklis *et al.* (1997). A solution containing 5mM NiCl_2 , 10mM EDTA, 5mM DTT, and 5mM β -mercaptoethanol prevented the adsorption of the His-protein to the Ni^{2+} -lipid monolayer.

In the case of a membrane protein, the proton-ATPase from plant plasma membrane, the addition of 1mM EDTA and 0.25mM DTT did not interfere with the formation of crystalline monolayers. These conditions were similar to those used routinely for column chromatography of the His-tag ATPase on Ni^{2+} -chelating columns. Control experiments in the presence of 10mM EDTA, 150 μM NiSO_4 or 250mM imidazole which would release the His-tag protein from the NTA column did not yield any protein crystalline sheets (Lebeau *et al.*, 2001).

Addition of EDTA in the trough after crystallization of streptavidin on Cu^{2+} lipid monolayers (adsorption through a naturally exposed histidine) lead to vacancies on the layer observed with light scattering microscopy (Schief *et al.*, 2000).

Effect of pH

For the crystallization of His-tag proteins on Ni-lipid monolayers, a pH range of 7.5–8.5 is usually used for the crystallization buffer. Upon injection of concentrated HCl into the subphase to lower pH to 6.5, a rapid and complete desorption of 2D crystals of His-HupR from the lipid layer is observed using ellipsometric measurements (Courty *et al.*, 2002).

Effect of high salt, detergent, glycerol and other buffer conditions

Another interesting property of the interaction of 6 His-tag with Ni^{2+} -NTA is that it is virtually unaffected by high salt (up to 1M), or by various detergents such as 10mM Triton X-100 or up to 1% Tween 20, 10mM dodecyl-maltopyranoside (Lévy *et al.*, 1999), 0.1% (w/w) dodecyl maltoside, (Lebeau *et al.*, 2001), organic solvents, ethanol or glycerol up to 30%, 8M urea or 6M guanidine HCl. A variety of buffer conditions are thus possible making this method especially suitable for membrane protein crystallization.

Crystallization of His-tag proteins

In this paragraph, examples of various proteins crystallized into 2D crystals on a Ni-lipid monolayer will be presented. A small number of Ni-lipids are available. They differ by the structure of the headgroup (ethanolamine, stearylamine), and the aliphatic chains (the length, saturated or unsaturated), they can be phospholipids or just lipids. The lipid monolayer is usually made of a mixture of lipids: the binding lipid and diluting lipids which will give the necessary fluidity of the lipid bilayer. The diluting lipids are often DOPC, DOPE or DOPS. It is useful to try various diluting lipids such as those cited above and others such as *E. coli* lipids. The molar ratio binding lipid:diluting lipid conducive to 2D crystals is usually between 1:1 and 1:15.

The use of the metal chelating phospholipid Ni-NTA-DOPE (Fig. 1.13A) forming the monolayer was successful for the crystallization

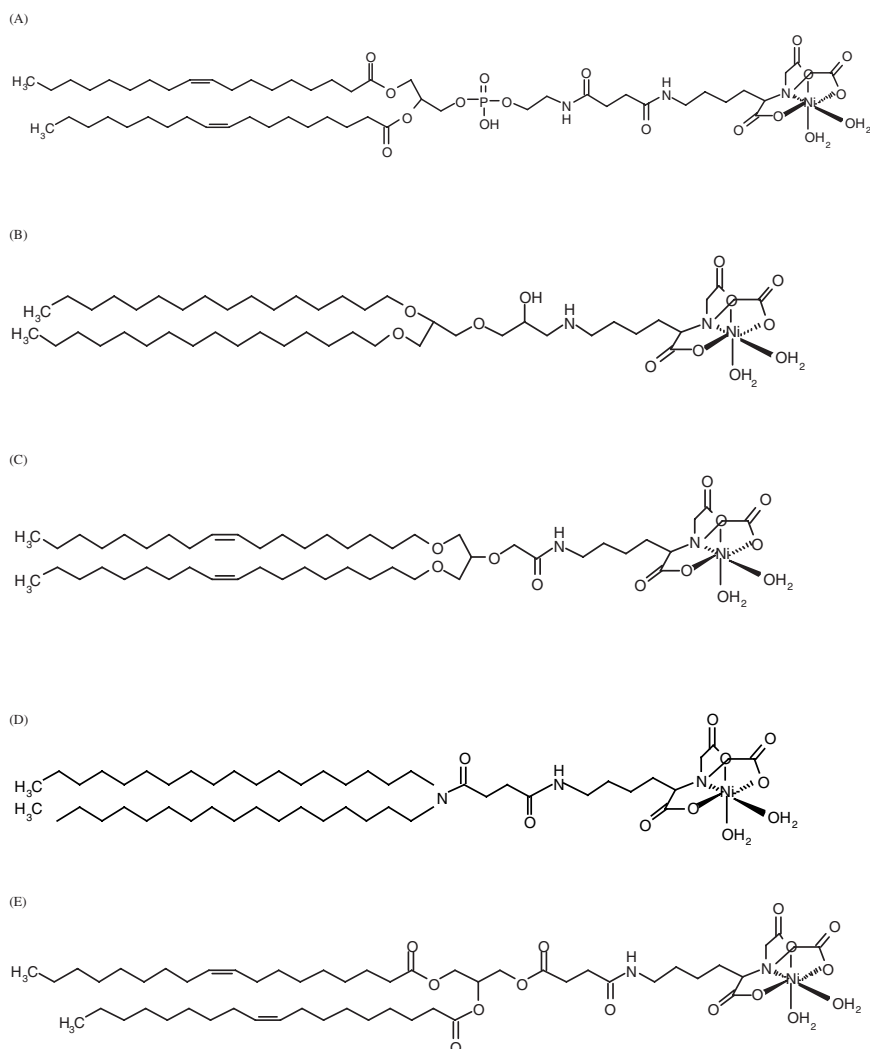


Fig. 1.13 Ni-lipids used for 2D crystallization of protein. (A) Ni-NTA-DOPE; (B) Ni-DHGN; (C) Ni-NTA-DOGA; (D) Ni-NTA-DODA; (E) Ni-NTA-DOGS.

of the His-tag recombinant HIV-1 reverse transcriptase (Kubalek *et al.*, 1994). 2D crystals preserved in negative stain diffracted strongly to $\sim 21\text{\AA}$.

Other successful crystallizations were achieved using Ni-DHGN, a saturated metal chelating lipid (Fig. 1.13B). His-tag Moloney murine leukaemia virus capsid protein (His-MoCa) formed extensive 2D protein crystals with reflections out to 9.5\AA resolution (Barklis *et al.*, 1997).

The His-tag MoCa arrays show some of the retrovirus core (Gag) protein interactions which occur in assembling virus particles. These 2D crystals were of good quality and a 3D structure of this tagged protein has been determined (McDermott *et al.*, 2000).

Another functionalized lipid Ni-NTA-DOGA (Fig. 1.13C) was successfully used to grow 2D crystals of His-tag yeast RNA polymerase I (Bischler *et al.*, 1998). More interestingly, RNA polymerase I which was 6 His-tagged on two different subunits yielded two different crystal forms, the orientation of the enzyme in both crystal forms being different. This is a significant advantage of the technique of 2D crystallization on lipid monolayer as specific His-tags, (or any other peptide tag) can be inserted into the protein of interest at multiple sites using recombinant DNA technology. This may allow the protein to be bound to a lipid monolayer in a variety of different orientations, thus overcoming the “missing cone” problem in electron crystallographic analysis. Combining data from protein crystals in which the molecules are oriented at different angles with respect to the crystal plane, would allow structural data to be collected effectively at all angles. A variation of the Ni-NTA-DOGA, the saturated Ni-NTA-DSA was also tested with the RNA polymerase I but was not conducive to crystallization.

A transcriptional factor from the photosynthetic bacterium *Rhodobacter capsulatus*, His-tag HupR has also been crystallized in 2D on a monolayer of Ni-NTA-DOGA, the crystals diffracted up to 9Å resolution in ice (Vénien-Bryan *et al.*, 1997; Vénien-Bryan *et al.*, 2000). HupR belongs to the nitrogen regulatory protein (NtrC) subfamily of transcriptional regulator. To date, none of the proteins belonging to the NtrC family have been crystallized in 3D form. These proteins are difficult to crystallize due to their propensity to aggregate and also the presence of large flexible domains inside the proteins, which does prevent crystallization in 3D but accommodate in the 2D geometry.

An elegant illustration of the potential of a protein-engineered His-tag to immobilize macromolecules in a predictable orientation at metal-chelating lipid interfaces is found in the work of Thess *et al.* (2002). The recombinant 20S proteasome was His-tagged in various positions, the lipid monolayer was made of the chelating lipid: Ni-NTA-DODA (Fig. 1.13D). Proteasomes His-tagged at their sides displayed exclusively side-on views, as seen on the electron micrographs, while

proteasomes His-tag at their ends displayed exclusively end-views. This oriented immobilization of His-tag proteins at lipid interfaces can assist structural studies, not only 2D crystal but also single molecule analysis. Isotopic structural information is achievable when viewing of the proteins in all possible orientation is possible. Often single particles have a preferred orientation on the electron microscope grid. The use of a lipid monolayer can help to force the protein in another orientation on the grid and gives access to structural information hidden otherwise.

Ni-NTA-DOGS (Fig. 1.13E) was essential for the crystallization of soluble proteins and membrane proteins. The structure of a membrane-bound murine molecule (MHC) and its relationship with the membrane was studied after crystallization on a Ni-NTA-DOGS lipid monolayer (Celia *et al.*, 1999). This molecule is expressed at the surface of cells and plays the role of presenting peptides to T cells.

The helper component proteinase (HC-Pro), a key protein encoded by plant viruses of the genus *Potyvirus* was crystallized with the same Ni-lipid, image analysis of cryo micrographs gave some structural information up to 9Å resolution (Plisson *et al.*, 2003).

2D arrays of a recombinant fragment of human vascular endothelium (VE) cadherin were produced on a lipid monolayer of Ni²⁺-NTA-DOGS (Al-Kurdi *et al.*, 2004). The 2D crystals preserved in uranyl acetate diffracted to 17Å. VE cadherin crystallized in 2D forms compact dimers in contact with the lipid monolayer. It was suggested that this compact *cis*-dimeric state may occur on isolated cells and that this compact form may serve to protect the molecule from degradation. The system of crystallization on a monolayer of lipid is able, as illustrated in this example, to mimic the behavior of biological molecules at the cell surface.

Ni-NTA-DOGS were used for the crystallization of two membrane proteins, FhuA, a high-affinity receptor from the outer membrane of *E. coli*, and F₀F₁-ATP-synthase from thermophilic *Bacillus* PS3 (Lévy *et al.*, 1999). Conditions were found to avoid solubilization of the lipid layer. Image analysis of these negatively stained crystals showed structural information up to 15Å for FhuA and 30Å for F₀F₁-ATP-synthase.

Partially fluorinated Ni²⁺-chelating lipids (Fig. 1.14) that formed stable monolayers at the air/water interface even in the

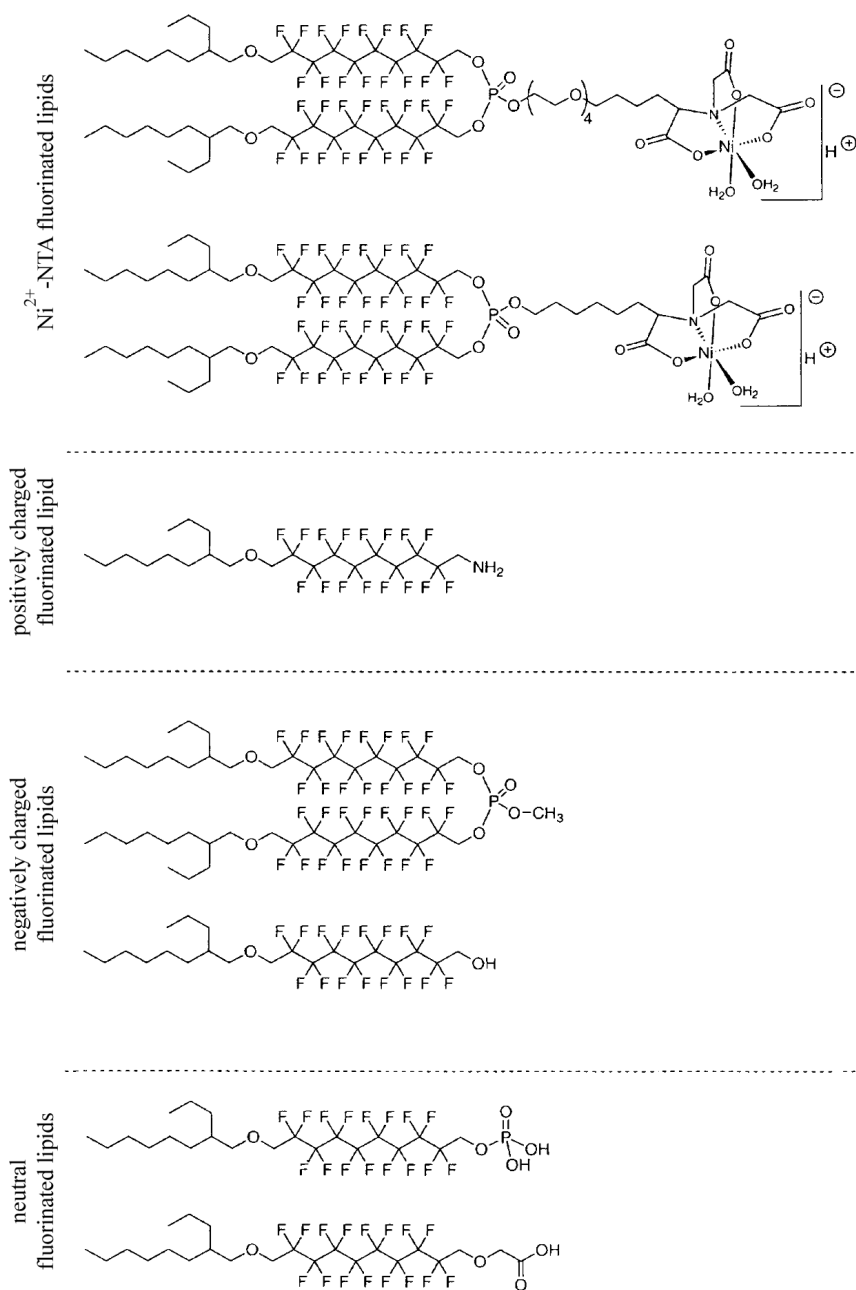


Fig. 1.14 Chemical structure of different fluorinated lipids.

presence of detergents were used to prepare 2D crystals of the plasma membrane proton-ATPase from *Arabidopsis thaliana* which diffracted up to 8Å resolution (Lebeau *et al.*, 2001), details are given in Sec. 2.4.

1.3.2.3. Multifunctionalized lipids

The structures of lipids are very versatile and allow not only the design of monofunctional groups, but also multifunctional groups. A headgroup containing both biotin and NTA chelator moieties have been synthesized in order to bind streptavidin- and polyhistidine-tagged proteins (Drakopoulou *et al.*, 2000). This bifunctionalized lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, (Fig. 1.15) is believed to form a helical tube rather than a monolayer structure. This work was based on previous results where it was shown that biotinylated dioctadecylamine (DODA) molecules were capable of forming tubular structures. Addition of streptavidin to the tubes produced ordered helical streptavidin arrays. Although it is difficult to predict whether the addition of an NTA-moiety close to the biotin group could interfere with the formation of tubes, it has been shown in the case of galactosylceramide tubes that the addition of NTA-lipids does not prevent their formation (Wilson-Kubalek *et al.*, 1998; Wilson-Kubalek, 2000). See also Sec. 6.2.

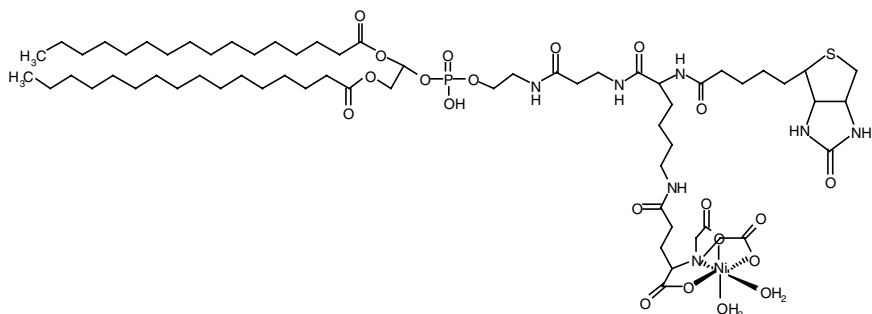


Fig. 1.15 Bifunctionalized lipid containing both biotin and NTA chelator moieties.

1.3.2.4. Immobilization of proteins on biofunctionalized surfaces using synthetic lipids

A variety of lipids and biophysical methods have been developed along with the study of immobilization of proteins on biofunctionalized surfaces. These designed lipids and techniques have greatly benefited the field of 2D crystallization.

Functionalized lipids which have been used for coating surfaces or forming monolayers at the air/water interface in order to immobilize the biomolecules in a defined orientation at the interface is presented in this chapter. Biophysical experiments for the observation of the binding of proteins are briefly described.

Moiety-carrying lipids

Lipid hapten synthesis has been described (Balakrishnan *et al.*, 1982) and these lipids have been used to form a monolayer, coating quartz microscope slides to serve as specific antibody-dependent targets for rat basophil leukaemia cells (Weis *et al.*, 1982). It also has been shown that the use of lipid-hapten and specific anti-hapten IgG antibody was required for the binding of guinea pig peritoneal macrophages to planar lipid monolayers on alkylated glass (Hafeman *et al.*, 1981). Proteins such as antibody fragments have been used as recognition moieties and extensively developed for biotechnological application. Immuno-liposomes have been exploited as vehicles for targeted drug delivery and for gene therapy (Bendas, 2001; Maruyama, 2000). Liposomes endowed with specific binding functions have been employed as simplified model systems to study ligand-membrane receptors interactions (Egger *et al.*, 1992, Lee *et al.*, 1993). The progress in the bacterial expression of functional antibodies such as F_{ab} fragments and as single-chain molecules motivated the use of genetic engineering to convert antibodies into membrane-bound molecules for immuno-liposomes applications (Laukkanen *et al.*, 1994; Martin *et al.*, 1981).

Lipids have also been modified using sugars (Haensler and Schuber, 1988; Spevak *et al.*, 1993), and NAD^+ (Salord *et al.*, 1986), these synthetic lipids are good candidates for 2D crystallization providing an anchor for protein interacting with these groups.

Metal-chelating lipid

Coordination of Cu^{2+} -IDA lipid by surface histidine amino acid has been first described using ESR (Shnek *et al.*, 1994). The interest for Cu^{2+} -lipid then developed as it became a tool for understanding the process of binding and immobilization of proteins on a metal-chelated monolayer of lipids. Among various proteins studied, streptavidin has been chosen most often because the adsorption and crystallization conditions for this test protein are well characterized.

The organization of streptavidin was monitored using fluorescence microscopy and Brewster angle microscopy (Frey *et al.*, 1996; Pack *et al.*, 1997a; Schief *et al.*, 2000; Vogel *et al.*, 1997). Binding of native streptavidin to the Cu-DOIDA lipids (Fig. 1.16A) spread at the air/water interface occurred via a His-87 located on the protein surface near the biotin binding pocket. BAM reveals 2D streptavidin crystals with a microscopic shape that differed from crystals that formed beneath biotinylated lipids.

DOIDA was modified with a fluorescent pyrene moiety to produce Cu-PSIDA (Fig. 1.16B). These chelating lipids served as reporters of membrane reorganization induced by binding of ligands as shown using fluorescence spectroscopy and fluorescence microscopy (Ng *et al.*, 1995; Pack *et al.*, 1997b).

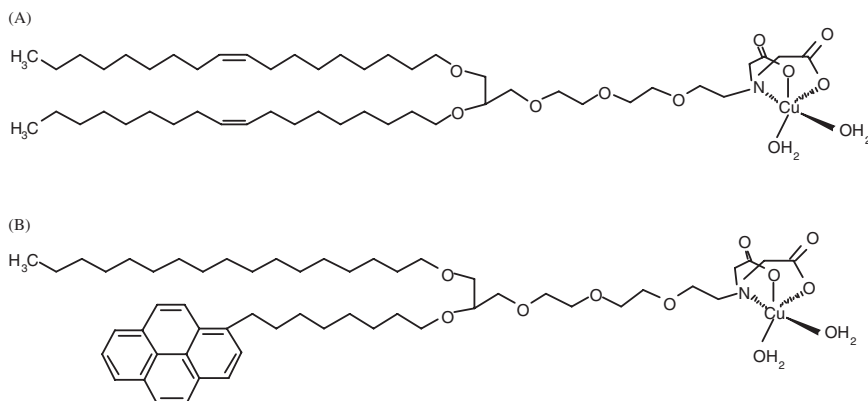


Fig. 1.16 Cu-lipids used for adsorption of proteins on solid surface (A) Cu-DOIDA; (B) Cu-PSIDA.

Ni-NTA-DODA and Ni-NTA-DPPE were also designed to achieve a defined deposition and immobilization of His-tag biomolecules (heat shock factor HSF24 and peptides containing oligohistidine residues) (Dietrich *et al.*, 1995; Dietrich *et al.*, 1996; Schmitt *et al.*, 1994). The NTA chelator was coupled either to a phospholipid, DPPE, or to a synthetic lipid, DODA. Epifluorescence microscopy was used to follow the binding process.

