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Effect of surface chemistry of novel templates on crystallization of proteins

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ABSTRACT

The heterogeneous crystallization of four model proteins (Lysozyme, Thaumatine, Catalase and Ferritin) on novel organic templates is reported. The chemical surface modification of glass substrates is described. The organic layers formed are characterized by contact angles' measurement with their dispersive and polar surface energy components determinated. Crystallization studies were completed using the hanging drop technique. The substrate surface chemistry was found to influence several aspects of protein crystallization: the number of crystals formed, the sizes of crystals and the crystal morphology. Data obtained confirm the theoretical suggestions that templates with specific surface chemistry can be successfully used for the control of nucleation and morphology of protein crystals.

1. Introduction

A major interest in the crystallization of proteins is primarily driven by a need to undertake the X-ray structural determinations of biomolecules. The crystalline state is also a desirable physical form for drug delivery of some therapeutic proteins, as well as for the manufacturing of other bio-active products, such as enzymes. An important inherent property benefit of protein crystals over their commonly used amorphous solid state or solution forms is their improved physical and chemical stability.

Finding optimal conditions for the crystallization of any individual protein is often a prolonged and expensive procedure, as it involves the determination of a wide set of experimental variables necessary for protein crystals formation including specific stabilizers, pH, temperature as well as protein, salt buffer concentrations, etc. (McPherson, 1999; Ducruix and Giege, 1999; Bergfors, 1999). Many proteins are still not crystallized, because the appropriate conditions have not been found. Consequently many researchers have devoted substantial efforts to understanding and controlling the crystallization process.

It is well known that heterogeneous nucleation can be very helpful tool for facilitating crystal nucleation, and that crystal formation at preferential phase boundaries occurs much more often than homogeneous nucleation as it requires less free energy (Eq. (1)):

$$\Delta G_{heterogeneous} = \Delta G_{homogeneous} f(\theta) \quad (1)$$

where $\Delta G_{heterogeneous}$ is the free energy needed for heterogeneous nucleation, $\Delta G_{heterogeneous}$ is the free energy needed for homogeneous nucleation and θ is the wetting contact angle. The contact angle of a new phase condensing on a solid surface is determined by the resultant force balance between the interfacial adhesive (Ψ_{adh}) forces between the substrate surface and a molecule of the new forming phase and interfacial cohesive (Ψ_{coh}) forces between two molecules in this new phase (Eq. (2)) (Mutaftschiev, 1993; Kaischew, 1950). The function included in Eq. (1) has the form

$$f(\theta) = 1/2 - 3/4\cos\theta + 1/4\cos^3\theta = 1 - \frac{\Psi_{adh}}{\Psi_{coh}} \quad (2)$$

Critical nuclei formed on surfaces depend on adhesion free energy between the molecule and the surface. If the surface free energy of adhesion is zero ($\Psi_{adh}=0$), then the critical nucleus is the same size as would be found in the bulk. If the adhesion is higher than the cohesion energy ($\Psi_{adh} > \Psi_{coh}$) then crystallization does not occur as all the surface is covered by a layer of adherent molecules. In case that adhesion energy is lower than cohesion

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($0 < \Psi_{adh} < \Psi_{coj}$), but higher than zero, then the critical nucleus is smaller in size than formed in the bulk and the energy for its formation is smaller (Eq. (1)) and depends on the relation Ψ_{adj}/Ψ_{coh} (Mutaftschiev, 1993; Kaischew, 1950).

The interactions between surfaces and solute molecules depend on the chemical and physical structures of both species. The wetting contact angle, θ, is a measure for these interactions and its relationship to the interfacial energies of the contacting phases is presented by the well known Young's equation (Eq. (3)):

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG}\cos\theta \tag{3}$$

where γ_{SG} is the solid–gas interfacial energy; γ_{SL} the solid–liquid interfacial energy; γ_{LG} the liquid–gas interfacial energy.

The surface free energy is a result of various intermolecular forces at the interface and is commonly subdivided by convention according to the various interactions that occur including dispersive (van der Waals) forces and polar interactions. Polar interactions include a range of short range forces including hydrogen bonding, acid/base interactions, etc. The total surface free energy is the sum of contributions from the different intermolecular forces at the surface (Eq. (4)):

$$\gamma_{LG} = \gamma_{LG}^{d} + \gamma_{LG}^{p}$$
$$\gamma_{SG} = \gamma_{SG}^{d} + \gamma_{SG}^{p} \tag{4}$$

where the superscripts d and p refer to the dispersion and polar surface energy components, respectively.

The surface free energy of a solid–liquid interface can be described as a function of individual force components of the solid and the liquid (Eq. (5)) (Owens and Wendt, 1969):

$$\frac{\gamma_{LG}(1+\cos\theta)}{2} = \sqrt{\gamma_{SG}^{P}}\sqrt{\gamma_{LG}^{P}} + \sqrt{\gamma_{SG}^{D}}\sqrt{\gamma_{LG}^{D}} \tag{5}$$

By measuring the contact angle θ of two different liquids against the solid of interest, simultaneous equations are obtained which can be solved for the dispersive and polar components of the surface energy of the solid surface γ_{SG}^{d} and γ_{SG}^{p} provided the values of γ_{LG}^{d} and γ_{LG}^{p} are known. In the current work water and methylene iodide were used.

There are numerous reports in the literature for surfaces containing ionizable groups found to noticeably influence crystallization of inorganic salts (Mann et al., 1988; Litvin et al., 1997; Falini et al., 1996; Landau et al., 1997). Bearing in mind that salts are highly polar molecules obviously processes of their crystallization can be driven by outer electrostatic forces. Protein molecules contain on their surfaces hydrophobic and hydrophilic patches, which can be responsible for all different types intermolecular interactions. This means the crystallization of macromolecules will depend more on surface properties than small salt compounds. Attempts to find correlations between proteins and surfaces that can improve or just change their crystallization parameters are also recorded in the literature and different substrates have been used for this purpose—mineral surfaces (McPherson and Schilichta, 1988; Kimble et al., 1998), lipid films (Edwards et al., 1994; Kornberg and Darst, 1991; Kubo et al., 2007), polymers (Grzesiak and Matzger, 2008), protein thin film (Pechkova and Nicolini, 2001,2002), molecular imprinted polymer (Saridakis et al., 2011), as well as applying chemical modification of mica (Falini et al., 2002; Simone et al., 2006; Tang et al., 2005; Tosi et al., 2008) or glass surface (Liu et al., 2007; Nanev and Tsekova, 2000; Rong et al., 2002; Sun et al., 2010, Tsekova et al., 2002).

Here we report the preparation of new surface chemical treated crystallization templates, the determination of their dispersive and polar surface energies components and crystallization performance of Lysozyme, Thaumatin, Catalase and Ferritin on them.

2. Materials and methods

2.1. Materials

Four different proteins were used for this work. They were purchased from Sigma-Aldrich: Chicken Egg White Lysozyme (Cat no. L-6876), Thaumatin from *Thaumatococcus daniellii* (T-7638), Catalase (Bovine liver catalase) (Cat. no. C-9322) and Ferritin from equine spleen, solution 56 mg/ml (Cat no. F4503). Salt and buffer components were also Sigma-Aldrich products, namely PIPES, Sodium Potassium Tartrate, Tris, PEG 6K, MPD and $CdSO_4$.

All used solvents were analytical-grade reagents (Sigma, Merck). Microscope glass slides were $18 \times 18\ mm^2$. Silanising reagents were as follows dichlorodimethylsilane (40140), dodecyltriethoxysilane (44237), Trimethoxy(3,3,3-trifluoropropyl) silane (91877), (3-iodopropyl)trimethoxysilane (58035), phenyltrimethoxysilane (79240) and (3-Aminopropyl)trimethoxysilane (281778). Toluene was dried and kept on molecular sieves. Silanisation of the glass coverslips was carried out in an Aldrich® AtmosBag (Cat no. Z530212) in a N_2 atmosphere.

2.2. Glass silanisation

Glass slides were cleaned with a hot mixture of concentrated nitric and sulphuric acids (3:1 concentrated H_2SO_4:HNO_3) at 50–60 °C for about 1 h prior to silanisation. (Caution: This mixture should be handled carefully as it is a very strong oxidant!) Then after cooling, the acidic mixture was removed from the glass slides, and the slides were rinsed in deionized water until pH of water leaving glass is the same as a pH of water going in. Finally the washed slides were dried in an oven at 120 °C. Dried glass slides were immersed in 3–5% (w/w) silanising reagent in anhydrous toluene for 24 h. This procedure was performed in a N_2 atmosphere to avoid side reactions of the silanising reagents with oxygen. After the glass slides had been removed from the solution, they were washed with toluene and ethanol. The glass slides were dried in an oven at 120 °C. Arslan et al. (2006) reported that glass beads treated with 3-Aminopropyl-triethoxysilane (APTES) in a similar way were found to absorb about 1–2 residues/nm^2.

2.3. Surface characterization—contact angle measurement

A Kruss DSA 10 MK2 drop shape analysis system was used to measure the advancing contact angles. This equipment consisted of a digital camera used to view the drop, mechanical moving needle and dosing pump controlled by proprietary software. Measurements started with a small drop of the chosen fluid on the surface of a slide. Then individual drops were added from needle to the drop on the surface, and the contact angle was measured between each drop additions. This was repeated until the advancing contact angle was achieved for a series of water and diiodomethane droplets.

2.4. Solutions used for protein crystallization

2.4.1 Lysozyme was crystallized from solutions of 20 mM citrate buffer, pH=4.8, precipitant 1 M NaCl and protein 5–15 mg/ml.

2.4.2 Catalase crystals were obtained from 100 mM Tris, pH=8.4 in the presence of 5% PEG 6 K (w/v) and 5% MPD (v/v) in concentrations 12.5–20 mg/ml protein.

2.4.3 Thaumatin crystallization solutions were in 50 mM PIPES, pH=6.8 with 0.34 M sodium potassium tartrate and 16–30 mg/ml thaumatin.

2.4.4 Commercial form of Ferritin solution (56 mg/ml) was first diluted by 0.2 M acetate buffer, pH=5 and then mixed with