Hetero- vs Homogeneous Nucleation of Protein Crystals Discriminated by Supersaturation

ARTICLE *in* CRYSTAL GROWTH & DESIGN · MAY 2011

Impact Factor: 4.89 · DOI: 10.1021/cg101460z

CRYSTAL GROWTH & DESIGN

ARTICLE

pubs.acs.org/crystal

Hetero- vs Homogeneous Nucleation of Protein Crystals Discriminated by Supersaturation

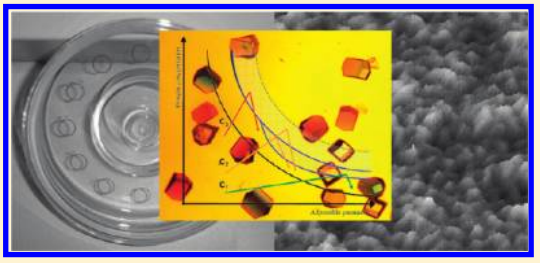
Published as part of the Crystal Growth & Design virtual special issue on the 13th International Conference on the Crystallization of Biological Macromolecules (ICCBM13).

Giovanna Tosi,[†] Simona Fermani,[†] Giuseppe Falini,[*,†] José A. Gavira,[*,‡] and Juan Manuel Garcia Ruiz[‡]

[†]Dipartimento di Chimica "G. Ciamician", Alma Mater Studiorum Università di Bologna, Via Selmi 2, 40126 Bologna, Italy

[‡]Laboratorio de Estudios Crystalográfico, Instituto Andaluz de Ciencias de la Tierra CSIC/Universidad de Granada, Av. Fuentenueva s/n, 18002 Granada, Spain

ABSTRACT: In protein crystallization trials there is always some ambiguity in defining if the nucleation event occurred by a homogeneous or a heterogeneous process. One of the reasons comes from the difficulty to compare experiments with or without a heterogeneous nucleant under the same chemical conditions. The outcome is that it is difficult to figure out the values of supersaturation addressing homo- versus heterogeneous nucleation. Recently it has been shown that the crystallization *mushroom* allows performance of sitting drop vapor diffusion experiments under identical physical−chemical conditions and that functionalized surfaces are favorable heterogeneous substrates for protein crystallization. With the aim to discriminate between nucleation processes, three model proteins, lysozyme, glucose isomerase, and thaumatin, have been crystallized at different starting concentrations, using positively and negatively charged surfaces, in the crystallization *mushroom*. The results show that (i) the heterogenenous nucleation does not crucially affect the nucleation process under conditions of high supersaturation and that (ii) the nucleating action of the functionalized surfaces is mainly related to their superficial charge distribution more than to their absolute charge, according to both classical and colloidal nucleation theories.



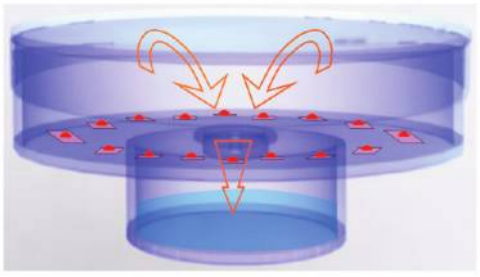
1. INTRODUCTION

The studies conducted on protein crystallization generally focus on how to maximize the probability of nucleation,[1,2] i.e. nucleation frequency, J (see Garcia-Ruíz, 2003, for details),[3] with this being the critical step. There are two main ways to increase nucleation frequency, J: either by changing the relation between the surface and volume energies, according to Gibbs−Thompson expression, or by introducing in the system any material that decreases the energy nucleation barrier, facilitating the crystal formation at lower supersaturation values.[4] In practice, the simple way to change the surface to volume energies ratio is adding preformed seeds into the droplets, from previous experiments or from homologous proteins.[5] Heterogeneous nucleation can be driven by subjecting the drops to materials of various nature that are either supporting the drop or added into it.[4−16]

A number of materials that fits the required criteria, i.e. surface ordered at the molecular level, presence of ionizable groups, local concentration of cavities, nano- and mesoscopic structures, have recently been tested by Georgieva and co-worker.[6] Among them, fragments of human hair allowed them to crystallize not only model proteins but also a polysaccaride-specific Fab fragment and the potato protease inhibitor.[6] Thakur et al. have shown that other materials, such as dried seaweed, horse hair, cellulose, and hydroxyapatite, can be used as nucleating substrates, able to increase the crystallization success of several model proteins, especially when a mix of those materials is used.[7] Two kinds of layer silicate, semisynthetic micromica and natural chlorite, have also been tested as heterogeneous nucleant materials. Added as powder to a hanging drop, they were able to aid protein crystallization. In particular, micromica powder has shown a strong influence even in the absence of precipitants.[8] Also, microporous synthetic zeolite molecular sieves have turned out to be able to selectively facilitate new crystal forms which did not grow spontaneously from solution, acting as a crystallization catalyst.[9] Zeolite-mediated crystallization also improved the crystal quality in almost all of tested proteins (5 of 6).[9] Poly vinyldiene-fluoride membranes with pore sizes of 400 nm have been successfully used as heterogeneous nucleant surfaces: they promoted the growth of perfect lysozyme crystals, allowing to obtain two distinct polymorphic forms.[10] Nucleation of lysozyme crystals has also been promoted by using glass slide surfaces chemically modified with (3-aminopropyl)triethoxysilane, poly(2-hydroxyethylmethacrylate), and poly-L-glutamic acid.[11] Moreover, polymer-induced nucleation has been used for the production of high-quality lysozyme crystals and to induce selective nucleation of multiple macromolecule crystal forms.[12] Surfaces

Received: November 4, 2010
Revised: March 14, 2011

   dx.doi.org/10.1021/cg101460z | *Cryst. Growth Des.* XXXX, XXX, 000–000



SURFACES			PROTEINS		
Silanization of mica sheets		Sulphonation time of polystyrene films		pI	Net charge ($pH_{cryst.}$)
ref.##	100% (N-propyl.)	ref.### (0 min.)	Lysozyme	11.21	+12.0 (4.5)
micaB	70% (N-propyl.)	5 min.			
	30% (3-aminop.)	30 min.	Glucose isomerase	5.14	-15.8 (7.0)
micaC	50% (N-propyl.)	1 h			
	50% (3-aminop.)	8 h	Thaumatin	8.05	+4.7 (6.8)
micaD	70% (N-propyl.)	24 h			
	30% (3-aminop.)	48 h			
micaE	100% (3-aminop.)				

Figure 1. (Top) Schematic representation of the crystallization *mushroom* and experimental setting in the *mushroom*. The arrows indicate the vapor diffusion process. (Bottom) Table summarizing the chemical reactions on surfaces and their effects on the hydrophobicity and density of the chargeable functional groups. For sulfonated polystyrene films, the different times of sulphonation are reported. The letters A−E indicate the different mixtures used in the silanization of mica sheets.

exposing ionizable functional groups, which were prepared by embedding charged polypeptides in gelatin or silk films and by chemical functionalization of mica sheets and polystyrene films, have been used in protein crystallization trials.[13,14] The covering of carbon nanotube films with gelatin has successfully allowed their application as protein crystallizers.[15] Recently, the ratio of heterogeneous versus bulk crystal nucleation has been studied from two sources of commercial lysozyme, pointing out the crucial role of impurities.[16]

Here we have studied the supersaturation effects on the crystallization of three model proteins, lysozyme, glucose isomerase, and thaumatin, on sulfonated polystyrene films and silanized mica sheets.[13,14,17] The experiments were carried out under identical vapor diffusion conditions in the crystallization *mushroom* (Triana Science & Technology).[18] The aim is to experimentally evaluate discriminating values of supersaturation on homo- versus heterogeneous nucleation processes.

2. EXPERIMENTAL SECTION

2.1. Materials. Chicken egg-white lysozyme (E.C. 3.2.1.17), thaumatin from arils of the African shrub *Thaumatococcus daniellii* (T-7638), *N*-propyltriethoxysilane, 3-aminopropyltriethoxysilane, and polystyrene pellets were purchased from Sigma-Aldrich, while glucose isomerase was purified from *Streptomyces rubiginosus* (HR7-100) from Hampton research. Muscovite mica (V-1 quality) samples were purchased from Electron Microscopy Science. The other chemicals were high-grade reagents (Merck). The crystallization *mushrooms* were purchased from Triana Science & Technology.

2.2. Functionalization of Surfaces. Mica surfaces and sulphonated polystyrene films were prepared as previously described.[13,14,17] Mica sheets were functionalized by silanization reaction in the vapor phase using *N*-propyltriethoxysilane, hydrophobic, and 3-aminopropyltriethoxysilane, hydrophilic. Mica was functionalized with only N-propyltriethoxysilane (mica A) and only 3-aminopropyltriethoxysilane (mica E). A mixture of *N*-propyltriethoxysilane and 3-aminopropyltriethoxysilane in the ratio 30% (v/v)−70% (v/v) or 50% (v/v)−50% (v/v) or 70% (v/v)−30% (v/v) was used to functionalize mica sheets and obtain mica B, C, and D, respectively. These functionalized substrates have increasing hydrophilicity and density of exposed ionizable chemical groups going from mica A to mica E. Polystyrene films were obtained by dissolving polystyrene pellets in 1,2-dichloroethane (7% w/w). They were successively sulphonated for increasing times, from 5 min to 48 h, in order to raise the density of exposed sulphonated groups. To generalize their application, the surfaces were not buffered at the crystallization pH before the crystallization experiments, as previously done.[17]

2.3. Crystallization Experiments. The crystallization trials were carried out using the crystallization *mushroom*[18] (Figure 2a and b), a glass tool to perform sitting drop vapor diffusion experiments under identical environmental conditions. It contains 12 marked positions in which sitting drops can be deposited. The equilibration of the drops (6 μL) occurs against a unique reservoir (5 mL). Trials were carried out at 293 K. Three model proteins, lysozyme, glucose isomerase, and thaumatin, were used in this study. The experimental work was done in two consecutive steps:

dx.doi.org/10.1021/cg101460z |*Cryst. Growth Des.* XXXX, XXX, 000–000

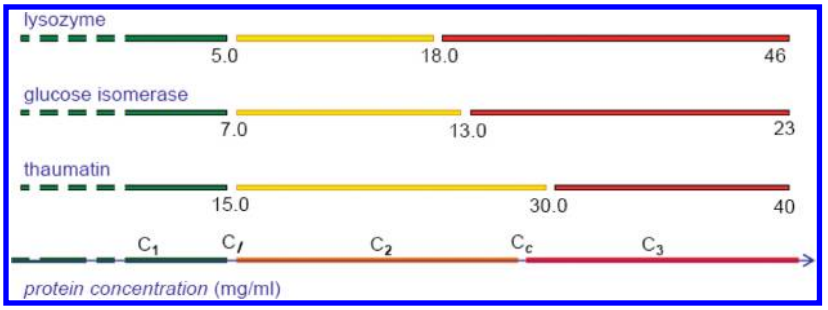


Figure 2. Experimental ranges of protein concentration observed in crystallization on functionalized surfaces for lysozyme, glucose isomerase, and thaumatin. In the range of concentration C_3, the presence of functionalized surfaces did not influence the crystallization parameter with respect to the reference. In the range of concentration C_2, the crystallization of protein was observed both on functionalized surfaces and on the reference, but on the former the waiting time was shorter than that on the latter. At concentrations under the lowest value of the range C_2, here indicated as C_1, the crystallization of protein was observed only in the presence of functionalized surfaces. C_c and C_l indicate the critical and limiting concentrations, respectively.

2.3.1. Determination of the Crystallization Conditions in the Crystallization Mushroom. First, several crystallization *mushrooms* were set up for each model protein. In each one, 12 drops were made by mixing the protein solution (three protein concentrations) and the reservoir solution in four different ratios: 1:1, 1:2, 1:3, and 2:1. In this way, in each experiment a wide area of the model protein phase diagram could be explored. The aim of this work was to identify the crystallization conditions that produce a countable number of single crystals (less than 20) of as large as possible (at least above 150 μm) average size. Lysozyme was optimally crystallized in the crystallization *mushroom* with 46.0 mg/mL protein concentration in 50 mM Na-acetate buffer at pH 4.5 and using 4.0% (*w*/*w*) NaCl in 50 mM Na-acetate pH 4.5 as reservoir solution in a drop settled by mixing 3 μL of protein solution with 3 μL of reservoir. For thaumatin, the best conditions were found by mixing 3 μL of a protein solution at 40 mg/mL in Milli-Q water with 3 μL of a reservoir solution of 0.26 M Na/K tartrate and 0.1 M KH_2PO_4 pH 6.8. The commercial crystalline suspension of glucose isomerase was dialyzed at 277 K first for one day against Milli-Q water and then for two days against 100 mM HEPES pH 7.0. Successively, the protein solution was concentrated using a 50 k Amicon Ultra-4 centrifuge and filter device (from Millipore) to a final concentration of about 300 mg/mL in 100 mM HEPES pH 7.0. Glucose isomerase was optimally crystallized using a drop obtained by mixing 3 μL of a reservoir solution of 10% (w/w) PEG 1000 and 0.20 M $MgCl_2$ with 3 μL of the protein solution at 23 mg/mL concentration.

Model proteins theoretical pI and net charge at the corresponding crystallization pH were calculated using protein calculator v3.3.

2.3.2. Crystallization Experiments with Surfaces in the Crystallization Mushroom. Once the reservoir solution and protein concentration were optimized, experiments in the crystallization *mushroom* were performed using functionalized surfaces. In each crystallization *mushroom,* one of the twelve positions was used as conventional reference, six were used to host polystyrene sulphonated films, and five were used to host silanized mica sheets. The surfaces were anchored to the crystallization *mushroom* with a piece of double-stick tape. In a thirteenth unmarked position, a polystyrene film piece was added and tested as further hydrophobic reference surface (Figure 1 top). The surface influence experiments were carried out by reducing the starting concentration of each protein until it reached a value at which no crystal appeared on the conventional reference. Each experiment was repeated at least five times, and the reported data were the median of the obtained results. The experiments were daily monitored up to one month by optical microscopy, and for each drop the number and the medium size of the crystals were annotated. The crystallization trials were carried out at the temperature 293 K.

3. RESULTS

The aim of this study is to evaluate at which supersaturation values the nucleation events, homogeneous versus heterogeneous, can be discriminated and to investigate the effect of the relative protein/surface charge distribution. The protein crystallization trials were carried out by the vapor diffusion method in the crystallization *mushroom*, inducing the nucleation on the charged surfaces. The starting protein concentration in the drop was gradually reduced until nucleation was not observed in the reference after one month. The waiting time for nucleation on reference surfaces (completely hydrophilic surfaces) and on partially charged (or hydrophobic/-philic) surfaces was compared. Crystallization density and average crystal size were noted as well. Three model proteins—lysozyme, glucose isomerase, and thaumatin—have been crystallized in the presence of two charged functionalized surface families—amino-silanized mica sheets and sulphonated polystyrene films. Three different hydrophobic surfaces—siliconized coverslips, mica sheets functionalized with a hydrophobic silane, and polystyrene films—were used as references (Figure 1). The surfaces have been used to set up multiple vapor diffusion experiments in the sitting drop method using the crystallization *mushroom*. This allowed to monitor the influence of the features of surfaces on crystal nucleation under identical environmental conditions (Figure 1, top).

The above surfaces meet two experimental criteria required to carry out microcrystallization in the vapor diffusion method: a controlled hydrophobicity able to preserve the drop shape and a surface transparency to light. Moreover, they have a low roughness that allows exclusion of the topographic effects.[11] Mica sheets with increasing amounts of exposed positive charges (mica B−E) have been prepared by carrying out silanization reactions in which silane mixtures with increasing relative amounts of 3-amminopropiltrietossisilane with respect to *N*-propyltriethoxysilane have been used. The surface density of sulphonated groups on polystyrene films has been increased with the sulfonation reaction time. The higher the sulfonation time (48 h, polyst. 48 h) was, the higher the charge density and hydrophilicity obtained (Figure 1, bottom).

Crystallization experiments on the two families of functionalized surfaces (mica and polystyrene) were carried out in the crystallization *mushroom* using the optimized precipitant concentrations and varying the protein concentration. A general trend was observed for the three model proteins reducing the protein concentration from the starting values (46.0 mg/mL for lysozyme, 23.0 mg/mL for glucose isomerase, and 40.0 mg/mL for thaumatin) to the minimum one investigated in this study

dx.doi.org/10.1021/cg101460z |*Cryst. Growth Des.* XXXX, XXX, 000–000

Table 1. Median Waiting Time (w.t.), Crystallization Density (c.d.), and Average Crystal Sizes (D) for Lysozyme, Glucose Isomerase, and Thaumatin Grown on Functionalized Surfaces in the Crystallization Mushroom

	lysozyme						glucose isomerase						thaumatin					
	18.0 mg/mL			5.0 mg/mL			13.0 mg/mL			7.0 mg/mL			30.0 mg/mL			15.0 mg/mL		
	w.t. (days)	c.d.[a] (c./mm^2)	D[b] (mm)	w.t. (days)	c.d.[a] (n.c./mm^2)	D[b] (mm)	w.t. (min)	c.d.[a] (n.c./mm^2)	D[b] (mm)	w.t. (days)	c.d.[a] (n.c./mm^2)	D[b] (mm)	w.t. (days)	c.d.[a] (n.c./mm^2)	D[b] (mm)	w.t. (days)	c.d.[a] (n.c./mm^2)	D[b] (mm)
refer.[#]	2	~15	0.20				12	<10	0.20				15	~10	0.38			
refer.[##]	1	~30	0.22	9	~25	0.09	30	mass.[c]	0.20	1	~10	0.11	13	<5	0.30	24	~15	0.11
mica B	1	~30	0.20	8	~25	0.08	30	mass.[c]	0.20	1	mass.[c]	0.11	13	mass.[c]	0.23	21	~15	0.19
mica C	1	~30	0.19	8	~30	0.08	60	>50	0.20	1	~20	0.15	9	mass.[c]	0.38	21	~25	0.30
mica D	1	~30	0.18	8	~25	0.09	30	>100	0.18	1	mass.[c]	0.15	9	mass.[c]	0.38	17	~20	0.30
mica E	1	~30	0.19	8	~20	0.08	30	>200	0.20	1	~10	0.18	9	<5	0.38	17	~20	0.30
refer.[###]	2	~30	0.19	13	~30	0.08	30	>15	0.20	1	~10	0.15	7	~20	0.23			
polyst. 5′	1	~40	0.20	9	~25	0.08	30	mass.[c]	0.20	1	~10	0.15	14	~15	0.38	17	~20	0.34
polyst. 30′	1	~30	0.19	9	~30	0.08	30	>50	0.18	1	~10	0.38	13	~10	0.23	21	~15	0.26
polyst. 1 h	1	~35	0.18	8	~35	0.07	30	>100	0.18	1	~10	0.38	14	~20	0.30	21	~10	0.23
polyst. 8 h	1	~40	0.18	8	~30	0.08	30	mass.[c]	0.20	1	>50	0.38	13	~10	0.23	21	mass.[c]	0.15
polyst. 24 h	1	~35	0.18	7	~20	0.09	30	mass.[c]	0.18	1	~10	0.15	14	~20	0.38	24	<40	0.23
polyst. 48 h	1	~40	0.18	7	~25	0.09	30	>15	0.20	2	~10	0.15	9	~15	0.38	24	~15	0.26

[#] Siliconized glass of the crystallization mushroom. [##] Hydrophobic mica surface. [###] Polystyrene film. [a] Number of crystals observed per surface unit (mm^2). [b] The value refers to the average length of the longest axis of the crystal calculated from a set of several dozen crystals. [c] Massive crystallization.

(5.0 mg/mL for lysozyme, 7.0 mg/mL for glucose isomerase, and 15.0 mg/mL for thaumatin). In the highest range of protein concentrations (46.0−18.0 mg/mL for lysozyme, 23.0−13.0 mg/mL for glucose isomerase, and 40.0−30.0 mg/mL for thaumatin), there was no substantial difference between references and both kinds of charged surfaces. In fact, the median waiting time, crystallization density, and average crystal size were roughly of the same order of magnitude (data not reported). Lowering the protein concentration below the lowest value of the above ranges, hereafter the critical concentration (C_c), the formation of crystals was observed on references and charged functionalized surfaces, but on the latter the median waiting time was significantly reduced (Figure 2, Table 1). The lower the protein concentration was, the more evident was the effect. At the lowest studied protein concentration (concentration limit, C_l) crystal formation was observed only on charged surfaces but not on the conventional reference (Figure 2, Table 1).

The optimal crystallization conditions in the crystallization *mushroom* for lysozyme were found at a concentration of 46.0 mg/mL. However, differences between the crystallization process carried out on the conventional references and the one on the two families of functionalized surfaces were observed only when the protein concentration was reduced to 18 mg/mL. At this concentration lysozyme crystals appeared on the conventional reference and on the polystyrene film after a waiting time of two days, while they showed up after one day on all the other surfaces. The crystallization density of surfaces was at least double with respect to that of the conventional reference, while no differences between them were observed in terms of crystal dimensions. At the concentration of 5.0 mg/mL, crystals formed only on the two families of surfaces, micas and polystyrene, but not on the conventional reference. The waiting time on micas was about 8 days, while on polystyrene it decreased from 13 and 7 days going from refer.[###] to polyst. 48 h. In terms of crystallization density, no substantial differences between the different surfaces were observed. The sizes of crystals grown both on reference and on functionalized surface were of the same order of magnitude (Table 1, Figure 3).

Glucose isomerase was optimally crystallized in the crystallization *mushroom* at concentration of 23 mg/mL, obtaining less than 10 crystals of about 0.20 mm long. The protein crystallization was then progressively reduced in additional experiments. The effect of the functionalized surfaces started to be evident at concentrations below 13.0 mg/mL. In fact, all functionalized surfaces were able to decrease the median waiting time from 120 min (conventional reference) to 30 min, except mica C, for which the median waiting time was 1 h. Also, polystyrene films and N-propyl silanized mica (mica A) decreased by four times the median waiting time. Moreover, at 13.0 mg/mL, the crystallization density on all micas was always higher than that on the conventional reference. Among the five tested micas, the two most hydrophobic ones (mica A and B) showed a massive precipitation of crystals. When the amount of amminosilane used to functionalized mica was higher than 30% (*v*/*v*) (micas C−E), the massive crystallization was arrested; however, it increased upon moving from mica C to E. Also, sulfonated polystyrene films increased the crystallization density with respect to the conventional reference; however, no clear correlation with the waiting time was observed, having the lowest crystal densities on polystyrene films and polystyrene sulphonated, polyst. 48 h. The average size of the crystals was always about 0.20 mm, almost regardless of the initial crystallization density. At a concentration of 7.0 mg/mL, no crystals grew on the conventional reference after one month while formation of crystals was observed after 1 or 2 days on all the other surfaces. At this protein concentration, the crystal density (about 10 crystals/mm^2) was almost the same on the polystyrene films, both sulphonated or not, while the average size of the crystals was maximum (about 0.38 mm) for intermediate sulphonation times. On the functionalized mica surfaces, the glucose isomerase crystals showed smaller dimension (>0.18 mm) than those grown on polystyrene films but with similar nucleation density excepted on micas D and B, on which massive nucleation was observed (Table 1, Figure 3).

Thaumatin set up experiments in the crystallization *mushrooms* were carried out at 40 mg/mL. This starting concentration was

dx.doi.org/10.1021/cg101460z |*Cryst. Growth Des.* XXXX, XXX, 000–000

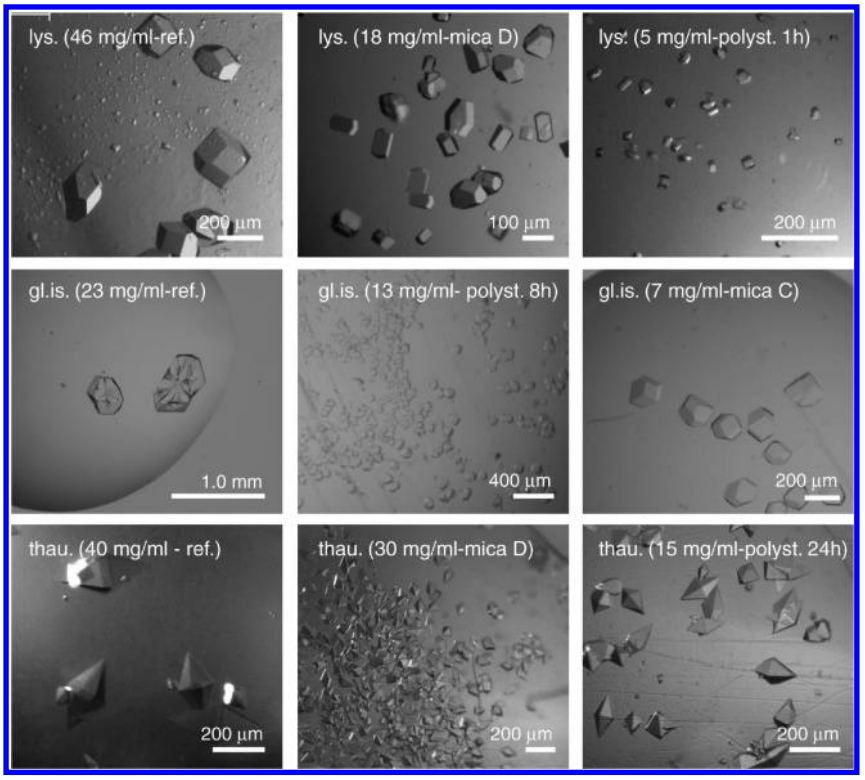


Figure 3. Crystals of lysozyme (lys.), glucose isomeras (gl.is.), or thaumatin (thau.) grown in the crystallization *mushroom* on different surfaces. The starting protein concentration and the surface on which the crystal grew are reported in parentheses in the figure labels. The surface codes are reported in Figure 1.

lowered to 15 mg/mL, in 5 mg/mL steps, at which crystals always appeared on surfaces but never on references. Below a concentration of 30 mg/mL, the first effects of surfaces on the crystallization event began to be evident (Table 1, Figure 2). Micas C−E, polystyrene film, and polyst. 48 h decreased the median waiting time of about 1 week with respect to the conventional reference, while micas A and B and the other sulphonated polystyrene films did not show a clear effect. A massive crystallization was observed on micas B−D, while on hydrophobic mica (ref.[##]) and mica E only a few crystals (<5 crystals/mm^2) grew. On polystyrene films a crystal density of 10−20 crystals/mm^2 was observed. The crystals grown at this concentration (30 mg/mL) show an average size of 0.23−0.38 mm, independently of the surface used. At 15 mg/mL, crystals did not grow on conventional reference and polystyrene film, while they always appeared on functionalized surfaces. The median waiting time decreased progressively, moving from hydrophobic mica (ref.[##]) (24 days) to mica E (17 days). An opposite trend was observed on polystyrene films, for which the waiting time increased gradually from 17 days (polystyrene film, ref.[###]) to 24 days (polyst. 48 h). All micas and sulfonated polystyrene films seemed to influence the crystallization density in the same way, with the exception of polystyrene 8 h and 24 h; the former were more efficient. Under the assayed experimental conditions, with the average size of crystals ranging between 0.11 mm and 0.34 mm, we have not found any correlation with the kind of the surface or functionalization (Table 1, Figure 3).

4. DISCUSSION

In recent years a number of researchers have focused their studies on the crystallization of proteins using heterogeneous additives in the search for a nucleating substrate of wide applicability.[2,19] In this research we have developed a method to study hetero- versus homogeneous nucleation under identical crystallization environments. This comes from the merging of two recent methods for the controlled crystallization: (*i*) the use of charged surfaces as heterogeneous nucleants and (*ii*) the crystallization *mushroom*. The maximum amount of attention was paid in the preparation of all the experimental setup, to avoid the presence of any heterogeneous substrate beside the nucleating surfaces. Three model proteins—lysozyme, glucose isomerase, and thaumatin—have been used in the crystallization trials. The nucleation behavior of these protein is well know. This is an advantage because in this research nucleation mechanisms were studied in depth. With a never crystallized protein, the nucleation behavior is not known, so it is impossible to run fundamental studies, without having a blank. For each protein, two borderline concentrations have been observed. One, that we define as C_{lim}, below which nucleation was observed only on charged surfaces and not on the conventional reference (region C_1 in Figure 2), and another, higher than C_{lim} and referred to as C_{crit}, above which we did not observe any effect of the charged surfaces on the nucleation with respect to the references (region C_3 in Figure 2). In the region of concentrations C_2 ($C_{lim} < C_2 < C_{crit}$) the nucleation was observed on references and charged surfaces, having on the latter a shorter waiting time. These results can be discussed on the basis of the classical theories of protein crystallization; that is, in the heterogeneous nucleation, the presence of a surface (hydrophobic/-philic) decreases the work required to create critical nuclei ($\Delta G^*_{het} = [(2 + \cos\theta)(1 - \cos\theta)^2/4]\Delta G^*$), where θ is the wetting angle of the spherical nucleus, and locally increases the probability of nucleation (J) with respect to other locations in the system. To illustrate this concept, a

dx.doi.org/10.1021/cg101460z |*Cryst. Growth Des.* XXXX, XXX, 000–000

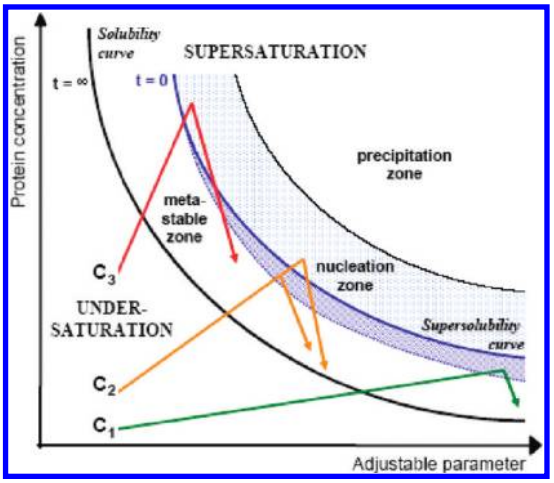


Figure 4. Schematic illustration of the phase diagram for a protein crystallization experiment in the vapor diffusion setup. The solubility curve separates the undersaturated region from the supersaturated one. On it the nucleation time is infinite. The supersaturation region is separated into three different regions: the metastable zone, the nucleation zone, and the precipitation zone. The metastable zone and the nucleation zone are separated by the supersolubility curve. On it the nucleation is instantaneous. The expanded surface of the nucleation zone at lower supersaturations (confined by the dotted supersolubility curve) represents the contribution to the nuclei stabilization due to a heterogeneous nucleation process. Three starting protein concentrations are considered ($C_1 < C_2 < C_3$). At the highest one (C_3), the nucleation occurs without appreciable contribution of the surfaces by a homogeneous mechanism. At the middle concentration (C_2), the nucleation can be either homogeneous or heterogeneous. In this case the heterogeneous process is time favored with respect to the homogeneous one, but both processes can occur. At the lowest concentration (C_1), the nucleation events can be only heterogeneous, driven by the surfaces, since the crystallization curve could never reach the homogeneous nucleation zone in the experimental time.

schematic representation of the phase diagram for an experiment of protein crystallization under vapor diffusion conditions in the absence and presence of a heterogeneous substrate is shown in Figure 4. The presence of a heterogeneous substrate expands the nucleation region toward the lower supersaturation region with respect to the homogeneous one. It is important to note that the location of the metastable limit depends not only on the energetics of nuclei formation but also on how fast the supersaturation evolves.[20] This means that the nucleation density, the final crystal size, and the structural quality of the crystal are a function not only of the supersaturation at which precipitation occurs but also of the rate of supersaturation.[3,21] In the crystallization *mushroom*, the rate at which supersaturation increases only depends on the starting concentration values in the drop, with the geometry of the experimental setup being a constant.[22,23] Indeed, in the crystallization *mushroom* we could compare identical physical and chemical environments—same CO_2 pressure, same number of particles (that could work as plausible nucleation center) per unit of volume, same temperature, and same humidity. The only change was the solid surface on which the crystal nucleates (assuming there is no nucleation on the surface of the drop−air interface). This makes it possible to explore in a rational way the phase diagram evaluating the effects of the functionalized surfaces on the waiting time (nucleation time) as a function only of the starting protein concentration in the drop.

At high concentration of protein, the nucleation takes place mainly by a homogeneous mechanism, as usually occurs in small volumes,[24,25] and thus, the presence of the heterogeneous surface does not influence the stochastic nature of the nucleation phenomenon. This is represented by line C_3 in the phase diagram reported in Figure 4. In the range of starting concentration reported as C_2 (Figure 4), the results show that on the charged surfaces the waiting time is shorter than that on the reference surfaces (Table 1). In this case we can suppose that a heterogeneous nucleation occurs in the presence of a charged surface, while a homogeneous one occurs in the presence of reference surfaces. When the starting concentration is below the limit concentration (C_{lim}), the waiting time on the reference surface is longer than the experimental time (one month in this case), while protein crystallization was observed on the functionalized surfaces, line C_1 in Figure 4. There, the increase of supersaturation lead to the metastable curve in a measurable span of time only in the presence of functionalized surfaces. It is interesting to note that we did not observe a correlation between the average protein charge at the crystallization pH and the charges of the functionalized surfaces (Figure 1). This observation confirms our previous results and pinpoints that the crucial parameter is not the absolute charge of protein or the functionalized surfaces charge distribution.[13,26] These results also confirm that even though the homogeneous surface can play a role as a function of its nature, any treatment producing an heterogeneous distribution of charge/hydrophobicity in islands of a wide range in size can promote nucleation. Therefore, the intrinsic distribution of charge along protein surfaces and the heterogeneity of the functionalized surfaces generate a pull of potential interaction, giving rise to the expected effect from the classical nucleation theory, in agreement with the observations of Thakur and collaborator working surfaces of different nature.[7,21]

The capability of a heterogeneous control of the nucleation rate has been reported for several hydrophobic surfaces and glasses.[27] This followed the original experiments of inducing protein nucleation by the presence of different mineral surfaces with the aim of looking for epitaxial control of nucleation.[28] However, there is an increasing amount of experimental evidence showing that induced-nucleation is rather controlled by weak interactions between crystal nuclei or clusters of protein molecules and heterogeneous substrates.[13,19,26] Under these conditions, protein molecules conserve part of their rotational and translation freedom, which may allow them to reorganize in stable crystal nuclei, eventually stabilized by the heterogeneous substrates. In this view, the classical one step approach to protein nucleation, based on the existence of local density fluctuations and a large structural difference between the degree of order in the solution and the nuclei, loses its power of explanation. On the contrary, the nonclassical colloid nucleation theory is based on a two-step mechanism: (*i*) separation of high protein concentration *regions* from the bulk of the solution at low protein concentration and (*ii*) the formation of crystal nuclei inside these high-concentrated regions,[13,14,18,21] initially proposed for high concentrated protein solutions close to the liquid−liquid boundary[29] and, more recently, in an undersaturated solution of the protein lumazine synthase,[30] could be more realistic.

5. CONCLUSION

This research, by means of crystallization experiments under identical chemical conditions in the crystallization *mushroom*, has allowed exploration in a rational way of the phase diagram and discrimination between homo- and heterogeneous nucleation

dx.doi.org/10.1021/cg101460z |*Cryst. Growth Des.* XXXX, XXX, 000–000

events. Moreover, the systematic study of the protein/surface overall charge indicates that the key parameter, to favor the nucleation, is the heterogeneity of the functionalized surfaces that generates a pull of potential nucleation sites. Both classical and colloid nucleation theories could explain the functionalized surfaces nucleation effect, but the stabilization of the metastable liquid cluster fits better with the experimental observations.

AUTHOR INFORMATION

Corresponding Author

*E-mail: (G.F.) giuseppe.falini@unibo.it. Telephone: +39 051 2099484. Fax: +39 051 2099456. Email: (J.A.G.) gavi@lec.csic.es.

ACKNOWLEDGMENT

We thank Dr. Ernesto Ciabatti for carrying out the lysozyme crystallization trials. The authors are grateful for the financial support by Grant No. 0013890 of bilateral project 2007/2008 CNR-CSIC Italy-Spain. G.T., S.F., and G.F. thank the CIRMSB for financial support. Other support includes the OptiCryst project of the VI European Framework Program and the Project "Factoría Española de Crystalización" Consolider-Ingenio 2010.

REFERENCES

(1) Khurshid, S.; Govada, L.; Chayen, N. E. *Cryst. Growth Des.* **2007**, *7* (11), 2171–2175.
(2) Bolanos-Garcia, V. M.; Chayen, N. E. *Prog. Biophys. Mol. Biol.* **2009**, *77*, 3–12.
(3) Garca-Ruiz, J. M. *J. Struct. Biol.* **2003**, *142*, 22–31.
(4) Nanev, C. N. *Cryst. Res. Technol.* **2007**, *42*, 4–12.
(5) Bergfors, T. *J. Struct. Biol.* **2003**, *142*, 66–76.
(6) Georgieva, D. G.; Kuil, M. E.; Oosterkamp, T. H.; Zandbergen, H. W.; Abrahams, J. P. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63*, 564–570.
(7) Thakur, A. S.; Robin, G.; Guncar, G.; Saunders, N. F. W.; Newman, J.; Martin, J. L.; Kobe, B. *PLoS ONE* **2007**, *2*, 1091.
(8) Takehara, M.; Ino, K.; Takakusagi, Y.; Oshikane, K.; Nureki, O.; Ebina, T.; Mizukami, F.; Sakaguchi, K. *Anal. Biochem.* **2008**, *373*, 322–329.
(9) Sugahara, M.; Asada, Y.; Morikawa, Y.; Kageyama, Y.; Kunishima, N. *Acta Crystallogr., D* **2008**, *64*, 686–695.
(10) Zhanga, X.; Zhanga, P.; Wei, K.; Wanga, Y.; Ma., R. *Desalination* **2008**, *219*, 101–117.
(11) Liu, Y.-X.; Wang, X.-J.; Lu, J.; Ching, B.-C. *J. Phys. Chem. B* **2007**, *111*, 13971–13978.
(12) Grzesiak, A. L.; Matzger, A. J. *Cryst. Growth Des.* **2008**, *8*, 347–350.
(13) Fermani, S.; Falini, G.; Minnucci, M.; Ripamonti, A. *J. Cryst. Growth* **2001**, *224*, 327–334.
(14) Falini, G.; Fermani, S.; Conforti, G.; Ripamonti, A. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2002**, *58*, 1649–1652.
(15) Asanithi, P.; Saridakis, E.; Govada, L.; Jurewicz, I.; Brunner, E. W.; Ponnusamy, R.; Cleaver, J. A. S.; Dalton, A. B.; Chayen, N. E.; Sear, R. P. *ACS Appl. Mater. Interface* **2009**, *1*, 1203–1210.
(16) Hodzhaoglu, F. V.; Nanev, C. N. *Cryst. Res. Technol.* **2010**, *45*, 281–291.
(17) Tosi, G.; Fermani, S.; Falini, G.; Gavira, J. A.; Garcia Ruiz, J. M. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2008**, *64*, 1054–1061.
(18) García-Ruiz, J. M.; Hernández-Hernández, M. A.; Gómez-Morales, J. *Ind. Crystallogr.* **2005**, *1901*, 963–968.
(19) Saridakis, E.; Chayen, N. E. *Trends Biotechnol.* **2009**, *27*, 99–106.
(20) Nyvlt, J. *Collect. Czech. Chem. Commun.* **1983**, *48*, 1977–1983.
(21) Garca-Ruiz, J. M.; Novella, M. L.; Otalora, F. *J. Cryst. Growth* **1999**, *196*, 703–710.
(22) Diller, D. J.; Hol, W. G. J. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *55*, 656–663.
(23) Luft, J. R.; DeTitta, G. T. *Methods Enzymol.* **1997**, *276*, 110–131.
(24) Kuhn, P.; Wilson, K.; Patch, M. G.; Stevens, R. C. *Curr. Opin. Chem. Biol.* **2002**, *6*, 704–710.
(25) Uber, D. C.; Cornell, E. W.; Nordmeyer, R. A.; Kolbe, W. F. *J. Appl. Crystallogr.* **2002**, *35*, 278–281.
(26) Chayen, N. E.; Saridakis, E.; Sear, R. P. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 597–601.
(27) Nanev, C. N.; Tsekova, D. *Cryst. Res. Technol.* **2000**, *35*, 189–195.
(28) McPherson, A.; Shlichta, P. *Science* **1988**, *239*, 385–387.
(29) ten Wolde, P. R.; Frenkel, D. *Science* **1997**, *277*, 1975–1979.
(30) Gliko, O.; Pan, W.; Katsonis, P.; Neumaier, N.; Galkin, O.; Weinkauf, S.; Vekilov, P. G. *J. Phys. Chem. B* **2007**, *111*, 3106–3114.