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Crystallization of proteins on functionalized surfaces

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Functionalized mica sheets and polystyrene films exposing ionisable groups have been used as heterogeneous nucleating surfaces for model proteins. Surfaces with different densities of amino or sulfonated groups have been prepared. Crystallization trials were carried out using the hanging-drop vapour-diffusion method. The results show that using these surfaces the starting protein concentration necessary to form crystals is reduced. The effect of these surfaces on the crystallization process may be the consequence of electrostatic interactions between charged residues of proteins and ionisable groups on surfaces. These interactions can be attractive or repulsive, depending on the relative charge of the protein and the surface at the crystallization pH. Both phenomena can induce an increase of the local protein concentration on the surface or in its proximity, favouring nucleation. Moreover, a reduction of the waiting time (an estimation of the nucleation time) was also observed for some proteins, suggesting a surface-stabilization effect on crystal nuclei.

1. Introduction

The study of the three-dimensional crystallographic structures of proteins is a starting point to understanding their structure–function relationships. This usually requires single crystals of high diffraction quality. Since there is no well defined law that allows the determination of which chemical and physical experimental conditions can lead a protein to crystallize, a 'trial-and-error' approach is usually applied. Currently, numerous (thousands) crystallization conditions can be tested using completely automated systems. However, this approach usually involves large financial investments and requires a substantial amount of protein sample. Moreover, it is a common experience that the chance of success in the crystallization of a protein is not directly correlated to the number of conditions tested (Kimber *et al.*, 2003). Knowledge of the protein chemistry and the use of nucleating agents can help in the search for crystallization conditions (Benvenuti & Mangani, 2007).

Most reported protein-crystallization experiments occur through homogenous nucleation. However, the presence of foreign solids, such as the container surface or dust particles, can favour heterogeneous nucleation in many cases (McPherson, 1999; Sear, 2003). Surfaces which are specifically employed as heterogeneous nucleants can be classified into three main groups depending on their structural and morpho-

Table 1
Representation of surface functionalization carried out by silanization of mica sheets with mixtures of silanes (mica A–E) or sulfonation of polystyrene films for different times (polyst. 1–5).

Surface-contact angles and roughnesses are reported and their standard deviations are given in parentheses. The codes A–E and 1–5 indicate an increasing superficial density of charged functional groups.

	Surface functionalization	Roughness (nm)	Contact angle (°)
Reference†		0.50 (0.06)	104.4 (1.5)
Mica A	0%(*v*/*v*) 3-apes‡	0.25 (0.09)	98.7 (2.1)
Mica B	30%(*v*/*v*) 3-apes	0.38 (0.17)	86.9 (1.9)
Mica C	50%(*v*/*v*) 3-apes	0.29 (0.07)	83.0 (2.3)
Mica D	70%(*v*/*v*) 3-apes	0.30 (0.12)	81.7 (2.5)
Mica E	100%(*v*/*v*) 3-apes	0.29 (0.07)	81.3 (2.7)
Polyst. 1	5 min§	0.40 (0.09)	81.5 (2.0)
Polyst. 2	30 min	0.42 (0.15)	78.1 (2.2)
Polyst. 3	1 h	0.38 (0.17)	76.4 (2.4)
Polyst. 4	8 h	0.68 (0.18)	53.3 (2.5)
Polyst. 5	48 h	1.5 (0.5)	35.1 (3.5)

† Siliconized cover slip, on which chlorinated organopolysiloxanes are the exposed groups. ‡ 3-Aminopropyltriethoxysilane. The values indicate the percentage of 3-aminopropyltriethoxysilane in the 3-aminopropyltriethoxysilane/*N*-propyltriethoxysilane binary mixture. § Sulfonation time of polystyrene surfaces in sulfuric acid.

logical characteristics. McPherson & Shlichta (1988) were the first to introduce the idea of using the crystalline structure of flat mineral surfaces as a template for protein nucleation. Since then, several patterned surfaces have been tested as nucleant agents, such as structured membranes, peptide monolayers and Langmuir monolayers (Edwards *et al.*, 1994; Pack *et al.*, 1997; Rong *et al.*, 2002; Tsekova *et al.*, 2002; Krafft & Goldmann, 2003; Curcio *et al.*, 2006). A second group of nucleating surfaces is characterized by the presence of pores and/or charged randomly distributed functional groups. Porous glass materials have been successfully used to decrease the induction time of nucleation and to obtain crystals at concentrations at which crystals were not observed on silanized cover slips (Chayen *et al.*, 2001; Rong *et al.*, 2004). It has also been proved that materials with pores can promote protein nucleation more effectively than smooth surfaces (Page & Sear, 2006) and Chayen *et al.* (2006) have proposed a theory for nucleation on disordered porous bioactive gel-glasses. Fermani *et al.* (2001) have introduced the use of polystyrene films exposing sulfonate groups and the use of biopolymeric matrices with entrapped charged polypeptides. Mica sheets functionalized with silanes containing different functional groups have been also used (Falini *et al.*, 2002; Tang *et al.*, 2005). Biopolymers and biominerals represent a third class of nucleation materials having crystalline surfaces and controlled roughness and/or pore sizes. For example, hairs have been used to induce protein nucleation at low starting concentrations (D'Arcy *et al.*, 2003; Georgieva *et al.*, 2007).

The use of nucleating surfaces has been also proposed to guide the crystallization towards a selected polymorph, a subject that is particularly important in drug production (Simone *et al.*, 2006). Moreover, it has been reported that crystals formed in the presence of heterogeneous substrates diffracted better than those obtained using conventional methods (Yoshizaki *et al.*, 2001; Sugahara *et al.*, 2008).

Here, we present work on the influence of surfaces exposing ionisable groups (sulfonated polystyrene films and aminosilanized mica sheets) on the crystallization of insulin and ribonuclease A. These results have been compared with previously reported work on the crystallization of lysozyme, concanavalin A and thaumatin in the presence of the same type of surfaces (Fermani *et al.*, 2001; Falini *et al.*, 2002). These findings are discussed in order to propose mechanisms by which the functionalized surfaces could promote and shorten protein crystallization and minimize protein consumption in this process.

2. Materials and methods

2.1. Materials

Bovine pancreatic insulin (EC No. 234-291-2), ribonuclease A from bovine pancreas (type I-A; EC 3.1.27.5), polystyrene, *N*-propyltriethoxysilane, 3-aminopropyltriethoxysilane and Sigmacote (chlorinated organopolysiloxane in heptane) were purchased from Sigma–Aldrich. Muscovite mica (V-1 quality) samples were purchased from Electron Microscopy Science. The other chemicals were high-grade reagents (Merk or Sigma–Aldrich). Ultrapure water (0.22 µS, 298 K) was used in all experiments.

2.2. Preparation of surfaces

Reference silanized glass cover slips were prepared as follows: glass cover slips were dipped in Sigmacote solution, air-dried and extensively washed with water. The preparation of silanized mica sheets and sulfonated polystyrene films was optimized with respect to that previously described (Fermani *et al.*, 2001; Falini *et al.*, 2002).

The mica sheets were immersed in 0.5 *M* HCl solution for 2 h (Fang & Knobler, 1995) and then left to dry overnight in a nitrogen-gas atmosphere in a desiccator. The silanization reaction was carried out in the vapour phase for 18 h in a desiccator containing 100 µl of a silane mixture. The density of the ionisable groups was varied using mixture of two silanes, *N*-propyltriethoxysilane (A) and 3-aminopropyltriethoxysilane (B), at five different percentage ratios: 100%(*v*/*v*) A, 70%(*v*/*v*) A, 50%(*v*/*v*) A, 30%(*v*/*v*) A and 100%(*v*/*v*) B. The mica samples were immersed in the same buffer used for crystallization trials for at least one night.

In order to improve the quality of the polystyrene films, the solvent and the concentration of the starting polystyrene solution were changed with respect to those previously reported (Fermani *et al.*, 2001): polystyrene pellets were dissolved in 7.0%(*w*/*w*) 1,2-dichloroethane and 9.0 ml of the obtained solution was poured into a glass Petri dish (5.5 cm diameter) and left overnight under a chemical hood. The films were formed by solvent evaporation at room temperature, which was completed by incubating the films at 333 K for about 12 h. The remaining procedures were performed as previously described.

2.3. Characterization of surfaces

Contact angles were determined using the sessile-drop method at room temperature. 5 µl pure water was dropped onto the surface of the functionalized samples and onto the surface of the silanized glass cover slip, respectively. The drop was left undisturbed for about 1 min and its shape was then recorded with a digital camera. The contact-angle values reported in Table 1 were the average of at least three measurements.

The surface roughness was evaluated by means of atomic force microscopy. A Digital Instruments Nanoscope III atomic force microscope (AFM) was used to observe the topography of sulfonated polystyrene films, chemically modified mica sheets and silanized glass cover slips. All images were obtained in tapping mode using micro-fabricated silicon nitride cantilevers (Digital Instruments). The mean roughness, defined as the arithmetic average of the absolute values of the surface-height deviation measured from the mean plane surface, was calculated over a square of 1 µm using the Nanoscope software. The values reported in Table 1 were the average of at least four measurements.

2.4. Crystallization experiments

The crystallization trials were carried out by the vapour-diffusion method using the hanging-drop technique at 293 K. The final volume of each drop was 5 µl, containing the protein and reservoir solutions in an equal ratio. 750 µl reservoir solution was used. Insulin was crystallized (Dodson *et al.*, 1978) in the presence of 0.01 *M* EDTA, 0.30 *M* Na_2HPO_4 pH 9.5 and 0.50%(*v*/*v*) xylene and ribonuclease A was crystallized (King *et al.*, 1956) in the presence of 55%(*v*/*v*) 2-methyl-2,4-pentanediol, 0.10 *M* sodium cacodylate pH 6.5 and 3.7 m*M* nickel chloride. The starting concentration of both proteins, insulin (20.0 mg ml^{-1} in 5 m*M* EDTA, 0.15 *M* Na_2HPO_4 pH 9.5) and ribonuclease A [25.0 mg ml^{-1} in water, 0.25%(*v*/*v*) xylene], was lowered until no crystal growth was observed on the reference surface. All the experiments were repeated at least five times. In order to estimate the median waiting time (defined as the time spanning from the settling of the experiment to the observation of the first crystals using an optical microscope with crossed polarizers), all the experiments were monitored at least twice per day using an optical microscope. In each drop, the number and average size of the crystals were noted.

3. Results and discussion

3.1. Functionalized surface characterization

Crystallization trials with insulin and ribonuclease A were carried out in the presence of negatively charged sulfonated polystyrene films or positively charged silanized sheets of mica. The number of charged functional groups on surfaces was controlled by chemical reaction. These surfaces have low superficial roughness and are transparent to light, which are two important requirements for protein-crystallization trials using the hanging-drop technique. Moreover, inexpensive reagents and simple chemical reactions were used in their preparation (Fermani *et al.*, 2001; Falini *et al.*, 2002). Table 1 summarizes the preparation of the functionalized surfaces and reports measurements of their roughness and contact angle. The conventional chlorinated organopolysiloxane-coated glass cover slip was used as a reference. Mica surfaces showed a roughness that was always lower than the reference surface, irrespective of the silane mixture used in the functionalization reaction. Polystyrene surfaces showed a roughness of the same

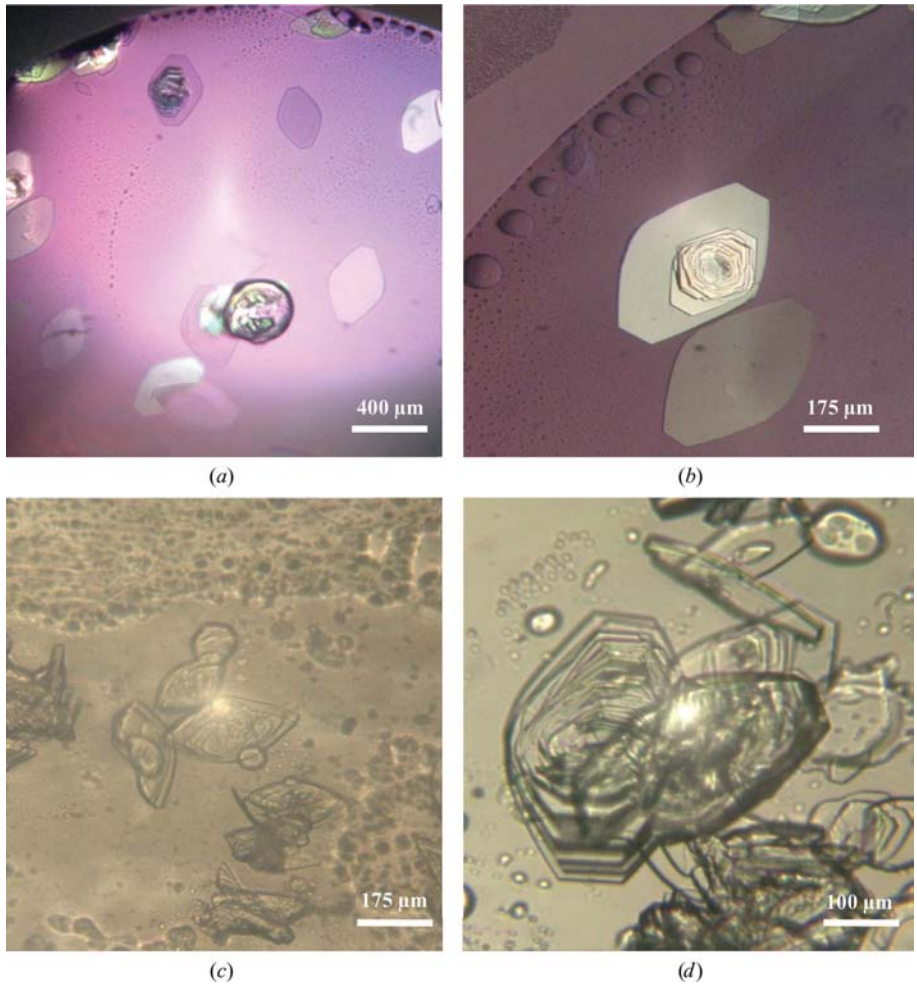

Figure 1
Optical micrographs of ribonuclease A crystals grown on the reference surface (*a* and *b*) and the polystyrene surface with the highest content of sulfonated groups (*c* and *d*). The crystals were grown using a starting protein concentration of 20.0 mg ml^{-1}.

order of magnitude as the reference surface, apart from that prepared with the highest sulfonation time (polyst. 5, 48 h). The low roughness of these two types of surfaces gives confidence that topographical factors should be almost absent in the crystallization process. The measurement of surface-contact angles gives an evaluation of surface hydrophilicity (or hydrophobility). As expected, surface hydrophilicity increases with the relative amounts of the aminosilanes used in the silane mixture for mica-sheet functionalization and with the sulfonation time of polystyrene films. The distribution of charged functional groups on these surfaces has been studied. It has been reported that a mixture of silanes forms a monolayer on the mica surface in which silanes cluster in islands of different sizes (Lyubchenko *et al.*, 1993; Crampton *et al.*, 2005). In contrast, sulfonate groups are homogeneously distributed on polystyrene-film surfaces (Addadi *et al.*, 1987).

3.2. Crystallization of insulin and ribonuclease A on functionalized surfaces

Insulin and ribonuclease A were crystallized using the experimental conditions reported in the literature but slightly adapted to the experimental setup used. The capability of the surface to affect protein crystallization was evaluated by comparing parameters such as the median waiting time, the density of crystallization and the average crystal size. Crystallization trials were carried out using different starting protein concentrations. The results are illustrated in Figs. 1 and 2 and summarized in Tables 2 and 3.

Ribonuclease A was crystallized using a starting concentration of between 25.0 and 2.5 mg ml^{-1} on mica or polystyrene-functionalized surfaces. Crystal formation was not influenced by the presence of functionalized mica surfaces until the starting concentration was reduced to values equal or below 10.0 mg ml^{-1} (Table 2). At this concentration, using mica E functionalized only with hydrophilic silane, the waiting time was about 2 d, in contrast to waiting times that were at least doubled using the other silanized surfaces (Table 2). At a protein concentration of 7.5 mg ml^{-1}, the waiting time was about 4 d for crystals grown in the presence of mica A or the reference surface, both of which have a hydrophobic surface, about two weeks in the presence of mica surfaces functionalized with silane mixtures (micas B–D) and about 6 d in the presence of mica E. When the starting concentration was reduced to 2.5 mg ml^{-1}, crystals only grew on mica surfaces with a high content of amino (hydrophilic) groups (micas C–E). The density of crystallization did not appear to be strongly influenced by the silanized mica surfaces.

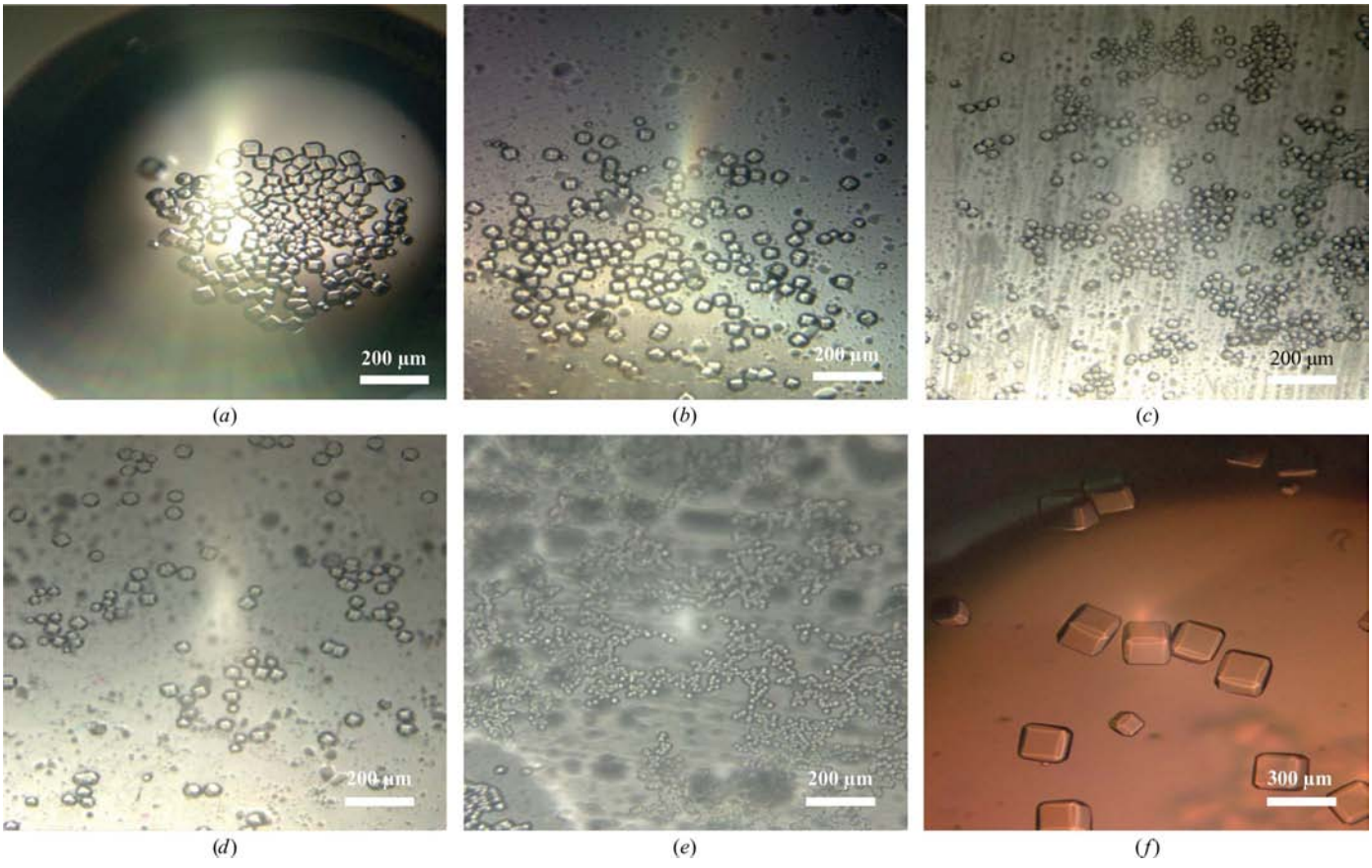

Figure 2
Optical micrographs of insulin crystals grown on sulfonated polystyrene surfaces (*a*–*e*) and the reference syrface (*f*). (*a*) polyst. 1, (*b*) polyst. 2, (*c*) polyst. 3, (*d*) polyst. 4, (*e*) polyst. 5. The crystals were grown using a starting protein concentration of 10.0 mg ml^{-1}.

Table 2
Median waiting time (w.t.), crystallization density (c.d.) and average crystal size (*d*) for ribonuclease A crystals grown on functionalized surfaces.

	10.0 mg ml^{-1}			7.5 mg ml^{-1}			2.5 mg ml^{-1}		
	w.t. (d)	c.d.†	*d*‡ (mm)	w.t. (d)	c.d.†	*d*‡ (mm)	w.t. (d)	c.d.†	*d*‡ (mm)
Reference§	5	3	0.33	5	3	0.29	—	—	—
Mica A	5	3	0.43	4	2	0.26	—	—	—
Mica B	5	6	0.36	11	3	0.30	—	—	—
Mica C	4	6	0.39	11	3	0.27	11¶	0.5	0.17
Mica D	4	5	0.26	12	2	0.16	9¶	0.5	0.16
Mica E	2	6	0.43	6	4	0.21	35¶	1	0.17
Polyst. 1	5	3	0.40	4	3	0.26	—	—	—
Polyst. 2	5	3	0.30	4	6	0.24	—	—	—
Polyst. 3	5	4	0.29	6	5	0.32	4¶	1	0.13
polyst. 4	5	6	0.21	6	6	0.14	4¶	1	0.14
Polyst. 5	2	9	0.34	2	10	0.14	4¶	1.5	0.11

† Number of crystals observed per surface unit (mm^2). ‡ The value refers to the average length of the longest axis of the crystal calculated from a set of several dozen crystals. § Reference: silanized glass cover slip. ¶ Crystal formation was only observed in a few experiments (about 25%).

Table 3
Median waiting time (w.t.), crystallization density (c.d.) and average crystal size (*d*) for insulin crystals grown on functionalized surfaces.

	2.0 mg ml^{-1}			1.0 mg ml^{-1}			0.75 mg ml^{-1}		
	w.t. (d)	c.d.†	*d*‡ (mm)	w.t. (d)	c.d.†	*d*‡(mm)	w.t. (d)	c.d.†	*d*‡ (mm)
Reference§	1	12	0.02	2	9	0.02	7¶	6	0.03
Mica A	1	34	0.02	0.5	10	0.03	3	8	0.02
Mica B	1	35	0.03	1	20	0.02	2	10	0.03
Mica C	1	34	0.06	1	30	0.01	2	15	0.03
Mica D	1	46	0.01	1	35	0.02	1	35	0.02
Mica E	1	48	0.01	1	35	0.01	1	50	0.02
Polyst. 1	1	45	0.01	1	12	0.01	6	6	0.02
Polyst. 2	1	50	0.09	2	20	>0.01	6	9	0.02
Polyst. 3	1	55	0.01	3	35	0.01	4	9	0.01
Polyst. 4	1	††	††	3	50	>0.01	3	12	0.01
Polyst. 5	1	††	††	2	††	††	2	30	>0.01

† Number of crystals observed per surface unit (mm^2). ‡ The value refers to the average length of the longest axis of the crystal calculated from a set of several dozen crystals. § Reference: silanized glass cover slip. ¶ Crystal formation was only observed in a few experiments (about 25%). †† Massive crystallization.

However, at each concentration the highest value of crystallization density was observed in the presence of mica E. The average size of the ribonuclease A crystals was mainly controlled by the starting concentration and not by the type of mica surface used (Table 2).

In the presence of sulfonated surfaces, ribonuclease A crystallized as large aggregates when starting concentrations above 10.0 mg ml^{-1} were used. Fig. 1 shows crystals grown at a concentration of 20.0 mg ml^{-1} using polystyrene film sulfonated for 48 h (polyst. 5) and the reference surface. The formation of these aggregates made it difficult to evaluate the average crystal size and the crystallization density. The waiting time was affected by the presence of sulfonated surfaces only when starting protein concentrations equal or lower than 10.0 mg ml^{-1} were used. At this concentration and for polyst. 5 the waiting time was shorter (2 d) than for other polystyrene surfaces (5 d). A similar influence of the surface was observed at a protein concentration of 7.5 mg ml^{-1}. When the protein concentration was reduced to 2.5 mg ml^{-1} a waiting time of about 4 d was observed and, more importantly, crystals formed only in the presence of polystyrene films that had been sulfonated for more than 1 h (polyst. 3–5). The average crystal size at the concentration of 10.0 mg ml^{-1} varied between 0.2 and 0.4 mm and was independent of the type of polystyrene film used. At a protein concentration of 7.5 mg ml^{-1} the average crystal size was around 0.25 mm using polyst. 1–3 and about 0.14 mm for polyst. 4 and 5. At the lowest starting protein concentration (2.5 mg ml^{-1}) an average crystal size of about 0.14 mm was observed. In the presence of these surfaces, the crystallization density increased proportionally to the degree of sulfonation.

It is important to note that at a starting ribonuclease A concentration of 2.5 mg ml^{-1} crystal formation was observed only in about 25% of trials for both surface families (more than ten on each surface). However, this observation does undermine the nucleating role of the functionalized surfaces as crystal formation was not observed on the reference surface. The ribonuclease A waiting time using functionalized surfaces showed a significant variability and in some experiments was longer than that observed on the reference surface (silanized surface with high hydrophobicity). A possible reason for this variability could be the presence of trace amounts of impurities in solution. Moreover, minimal protein degradation cannot be excluded in lengthy crystallization experiments.

Insulin was crystallized on functionalized surfaces using a range of starting concentrations from 20.0 to 0.75 mg ml^{-1}. This protein has a high tendency to crystallize using starting concentrations above 2.0 mg ml^{-1}. Under these conditions, the influence of surfaces on crystallization parameters can only be evaluated qualitatively. In Fig. 2(*a*) a view of crystals grown on sulfonated polystyrene surfaces using a starting concentration of 10.0 mg ml^{-1} is shown. A large number of crystals formed on functionalized surfaces (Figs. 2*a*–2*e*) with respect to the reference surface (Fig. 2*f*). Moreover, the crystallization density increased proportionally to the density of sulfonate groups on the surfaces (Figs. 2*a*–2*e*), with a concomitant reduction of the average crystal size. Similar behaviour was observed using silanized mica sheets. The waiting times, average sizes and crystallization densities of insulin crystals grown on mica and polystyrene functionalized surfaces using starting protein concentrations of 2.0, 1.0 and 0.75 mg ml^{-1} are reported in Table 3. When a starting insulin

concentration of 2.0 mg ml^{-1} was used, the waiting time was about 1 d in the presence of the functionalized surfaces or the reference surface. At a concentration of 1.0 mg ml^{-1} the waiting time on the reference was almost double that on all mica functionalized surfaces, while the waiting time on sulfonated polystyrene surfaces was unrelated to the density of sulfonation. Using a starting protein concentration of 0.75 mg ml^{-1} on the reference surface, crystals formed after about one week and only in a few experiments (less than 25%). They always appeared after a waiting time of about 1 d and not longer than 5 d using amino-silanized mica sheets and sulfonated polystyrene films, respectively. Moreover, in the presence of functionalized polystyrene surfaces the waiting time decreased with the increase of the amount of sulfonate groups from about 6 d on polyst. 2 to about 2 d using polyst. 5. The crystallization density on the reference surface decreased as the protein concentration was reduced (Table 3). In the presence of surfaces with an increasing number of ionisable functional groups, an increase in crystallization density was observed at each protein concentration. Interestingly, the crystallization densities observed on mica and polystyrene functionalized surfaces were always higher than those observed on the reference surface. This effect was also present for mica A, which has a reference-like hydrophobic surface but differs in roughness and contact angle (Table 1). At a protein starting concentration of 2.0 mg ml^{-1} the crystallization densities of insulin using silanized mica surfaces increased to a value of about 48 crystals mm^{-2}. This value is close to the lowest crystallization density observed in the presence of polyst. 1 (45 crystals mm^{-2}) at the same protein concentration. Under the same conditions the crystallization density increased to 55 crystals mm^{-2} in the presence of polyst. 3 and appeared as a massive precipitation using polyst. 4 and polyst. 5. When the starting protein concentration was reduced to 1.0 or 0.75 mg ml^{-1} a progressive decrease in crystallization density was observed. This influence was more marked using sulfonated polystyrene films than with silanized mica sheets. Insulin precipitated in all the experiments, forming small crystals (around 10 µm along the main axis). As the density of charged functional groups on the surfaces increased, the average crystal size slightly decreased while the crystallization density increased.

Table 4
Summary of the results of the model protein crystallization experiments using the sulfonated polystyrene films and the silanized mica sheets that gave the most evident effects with respect to the reference.

The lowest starting protein concentration at which crystals formed on functionalized surfaces and the reference surface are also reported. Waiting times (w.t.), crystallization densities (c.d.) and average crystal size (*d*) are reported as relative values, equal (=), higher (+) or lower (−) in comparison to those for the reference.

				Sulfonated polystyrene films			Silanized mica sheets		
Protein	Concentration† (mg ml^{-1})	Crystallization pH	Protein charge‡	w.t.§	c.d.§	*d*§	w.t.§	c.d.§	*d*§
Ribonuclease A	2.5/7.5	6.5	+8	−	+	−	=	+	−
Insulin	0.75/1.0	9.5	−9	=	+	−	−	+	−
Lysozyme¶	5.0/10.0	4.5	+2	=	=	=	−	=	=
Concanavalin A¶	10.0/10.0	9.0	∼0	−	+	−	††	††	††
Concanavalin A¶	3.0/10.0	6.0	−9	††	††	††	−	+	−
Thaumatin¶	2.0/2.0	6.8	+5	††	††	††	−	+	−

† The first and second values indicate the lowest starting protein concentration at which crystals formed in the presence of functionalized surfaces and the reference surface, respectively. ‡ The protein charge at the crystallization pH was calculated using the pI, the MW and the titration curve tool from the *ExPASy* server. § These observations refer to the lowest starting protein concentration at which crystals were observed on both functionalized surfaces and the reference surface. The surfaces are those with the most evident effect on crystallization processes, usually those with the highest density of ionizable functional groups. ¶ Data reported in Fermani *et al.* (2001) and Falini *et al.* (2002). †† Crystallization trials were not carried out under these conditions.

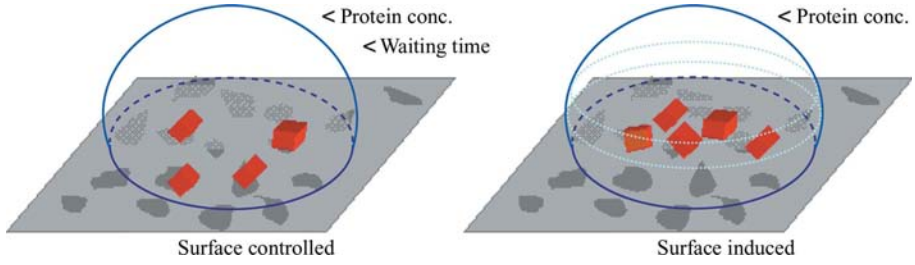

Figure 3
Schematic representation of surface effects on protein crystallization. Crystallization can be controlled (left) or induced (right). In the first case protein nucleation occurs on the surface, which stabilizes the nuclei. In the second case repulsive forces are present between the surface and the protein. These move the proteins out from the surface and increase their concentration in a thin layer in its proximity (dashed circles). In the scheme, drops and crystals are not shown on the same scale.

3.3. Effects of functionalized surfaces on protein crystallization

The results for the crystallization of insulin and ribonuclease A in the presence of functionalized surfaces have been compared with findings obtained using lysozyme, thaumatin and concanavalin A on the same surfaces (Fermani *et al.*, 2001; Falini *et al.*, 2002). A summary of the variation of crystallization parameters using functionalized surfaces with respect to the reference surface is reported in Table 4. The lowest starting protein concentration at which crystals formed on the functionalized surfaces or reference surface and the protein charge at the crystallization pH are also reported. Insulin and ribonuclease A have a charge of about −9 and +8, respectively, at the pH values of the crystallization conditions. Under the same pH conditions amino-silanized mica surfaces (micas B–E) are positively charged, whereas sulfonated polystyrene surfaces are negatively charged. Thus, electro-

static attractions or repulsions may be present as a function of the relative charges of the surface and the protein. Similar considerations can be performed for the other model proteins reported. It is possible to note that with the exception of lysozyme, charged surfaces always increased the crystallization density and reduced the nucleation time (measured here as the median waiting time) with respect to the reference surface. It has been demonstrated experimentally and theoretically that the interaction between proteins and surfaces promoting nucleation requires weak forces that concentrate proteins in the proximity of the surface (Chayen *et al.*, 2006; Sear, 2007). While the physics and chemistry which govern homogeneous nucleation of proteins have been accurately investigated (García-Ruiz, 2003), research on the processes that control their heterogeneous nucleation is still in progress. Recent studies suggest that the heterogeneous nucleation of protein crystals cannot be described in the same way as the heterogeneous nucleation of ionic solids, in which an epitaxial mechanism of nucleation is commonly involved (Galkin & Vekilov, 2000; Sear, 2007). Protein crystals are stabilized by weak lattice energies (McPherson, 1999). Thus, the interaction between proteins and heterogeneous surface should be weak enough to let protein molecules be free to reorganize themselves in rotation and translation to associate in stable crystal nuclei.

The ionizable functional groups present on the surfaces should allow protein–surface interactions by electrostatic forces. Since the superficial charge density can be modulated, the force of the electrostatic interaction can be also varied. When attractive interactions are present, protein molecules tend to concentrate close to the surface, locally increasing the supersaturation that favours crystal nucleation and growth. In addition to this effect, the surface can also stabilize already formed nuclei by interaction with a specific crystal face or it can favour the formation of crystal nuclei by clustering ordered motifs of protein molecules. It can be supposed (similarly to the concept used to explain the nucleation properties of glass substrates with pores of a wide range of sizes; Chayen *et al.*, 2006) that surfaces with a random distribution of functional groups offer many different potential patterns of interaction with crystal nuclei. This can be described as a controlled mechanism with the surface playing an active role in the nucleation process.

In the presence of repulsive forces, the protein molecule does not settle in the thin layer close to the surface. As a consequence, it can be supposed that the protein concentrates in the upper layer and its crystallization can be achieved using a lower starting protein concentration. As the surface does not play a direct role in affecting the crystallization process in this case, this mechanism is described as surface-induced. A schematic representation of these two mechanisms is shown in Fig. 3.

In conclusion, it has been shown that functionalized surfaces are able to induce protein crystallization at concentrations lower than those required by the reference surface. The random distribution of the functional groups on the surfaces results in a reduction of the waiting time occurring in some cases, which may suggest surface stabilization of the crystal nuclei. Thus, it is conceivable to design surfaces suitable to control nucleation kinetics in order to resolve the conflict between the necessity of nucleation at low supersaturation and the need for a protein concentration sufficient to sustain crystal growth.

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