ELSEVIER

Journal of Crystal Growth 218 (2000) 390–398

JOURNAL OF CRYSTAL GROWTH

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Improved protein crystallization by vapor diffusion from drops in contact with transparent, self-assembled monolayers on gold-coated glass coverslips

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Received 7 July 1998; accepted 23 April 2000
Communicated by A. McPherson

Abstract

The surfaces of glass coverslips of the type typically used for protein crystallization were modified with four types of transparent, chemically distinct self-assembled monolayers (SAMs). The SAM-functionalized surfaces exhibit a much higher degree of order and chemical uniformity than silanized glass, as judged by contact angle measurements. These characteristics lead to a marked increase in the range of solution conditions under which large crystals of lysozyme, α-lactalbumin, ribonuclease, hemoglobin, thaumatin, and catalase are observed to form. The results are rationalized in terms of a marked reduction in the rate of non-productive nucleation relative to the rate of crystal growth.

Keywords: Protein; Crystallization; SAMS; Gold

1. Introduction

The vapor diffusion method, in the guise of the hanging or sitting drop methods, remains one of more the widely used techniques to screen for protein crystallization conditions. We describe herein a new, easily implemented variation on this procedure that broadens the array of currently available conditions. In the examples presented, the new procedure also significantly broadens the range of successful crystallization conditions that lead to large well-formed macromolecular protein crystals.

Protein crystals must be sufficiently large and well ordered for structural studies. Although the narrow beam dispersion of synchrotron radiation can afford the successful analysis of small protein crystals (smallest dimensions $\geqslant 10\,\mu m$), typical home sources of X-rays require protein crystals with substantially larger sizes (smallest dimensions $\geqslant 50$–$250\,\mu m$) to acquire reliable X-ray diffraction data. Some improvement in the growth of ordered crystals has been achieved through crystallization under microgravity conditions, where convective

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0022-0248/00/$ - see front matter
PII: S0022-0248(00)00541-8

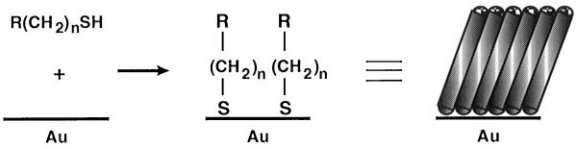
mixing is absent [1]. The absence of convective mixing encourages uniform approach of the solute in solution to the growing crystal. However, the size and shape of the crystal obtained can also depend upon a number of other controllable factors.

The size, shape, and crystalline order of protein crystals are limited by a combination of thermodynamic and kinetic phenomena. The process of crystallization can be divided into three subprocesses: (1) nucleation (or initiation); (2) propagation (growth from nuclei); and (3) termination [2]. If nucleation is faster than propagation, one obtains a showering of inadequately small microcrystals. If, however, nucleation is too slow, crystal growth might fail to proceed on an experimentally accessible time scale, even though the crystalline state might constitute one of the more thermodynamically favorable states of the closed system. Furthermore, some kinetically competitive nucleation processes can lead to amorphous, aggregated states from which the ordered crystalline state is kinetically or thermodynamically inaccessible. Experimental time limitations are particularly significant for crystals grown at low gravity, when experimental duration is limited by the duration of a space flight or by the availability of payload space. Thus, the development of new methods to improve control over the relative rates of nucleation, growth, and termination will undoubtedly enhance efforts to generate high-quality crystals for structural studies.

Variations in both the concentration and the identity of the molecular components of the protein drop solution are often effective at achieving variation in the relative rates of nucleation and growth, but in practice, both of these rates are influenced in unpredictable ways. Protein crystal *nucleation* might plausibly occur either at (1) the interior of the protein solution drop, (2) the liquid–air interface, or (3) the liquid–solid interface where the drop contacts the underlying crystallization apparatus. Secondary nucleation may also occur in the drop at the surfaces of initially formed crystals. Crystal *growth* on the other hand, always occurs at the crystal–solution interface. Thus, by selectively varying the nature of the liquid–solid interface (i.e., the interface between the protein solution and the crystallization apparatus), it should be possible to influence favorably the rate of crystal nucleation (productive nucleation) or to decrease the rate of non-productive nucleation (irreversible formation of an amorphous aggregate) while maintaining relatively constant rates of crystal growth and termination.

This general approach has been employed by others. McPherson and Schlichta, for example, have grown protein crystals epitaxially from the surfaces of mineral crystals [3]. While this work represents a pioneering effort in the area, application of this method to large-scale screening of crystallization conditions is limited by the technical difficulty of introducing insoluble, mineral crystals reproducibly into many small-scale crystallization wells. Furthermore, the chemical nature of the solid at the solid–liquid interface is dictated by the composition and morphology of the mineral crystal, thus providing limited and poorly controllable variabilities. More recently, improved crystallization of streptavidin and RNA polymerase has been observed by Edwards and coworkers using glass coverslips with deposited lipid bilayers as the modified liquid–solid interface [4]. Crystal growth on a lipid bilayer proved to be effective at improving crystal size and morphology. For practical applications, however, use of the lipid-coated glass coverslips is unattractive for at least four reasons: (1) deposition of the bilayer requires a highly specialized apparatus, (2) interfaces modified in this fashion are typically riddled with defects, (3) the films are unstable to long-term storage, and (4) chemical variation of the lipid components of the bilayer system is greatly restricted.

To expand and improve upon these approaches, our research targets the controlled nucleation and growth of protein crystals using the well-defined interfaces formed by self-assembled monolayers (SAMs) on gold [5]. SAMs form spontaneously by the adsorption of organic thiols or disulfides onto the surface of gold, generating lipid-like thin films (Scheme 1). These films are highly ordered and semicrystalline as judged by infrared spectroscopy [6]. They can be stored under ambient conditions for extended periods of time without degradation. A further advantage of using SAMs as a coating for protein crystallization is that simple synthetic



Scheme 1.

organic chemistry can be used to control the local structure and composition of the SAM–liquid interface *at the Ångstrom level*. Indeed, a variety of functional groups can be employed (e.g., OH, NH_2, COOH, etc.). These functional groups can be directed toward the solid–liquid interface (i.e., toward the protein drop) by using alkanethiols having appropriately functionalized ω termini.

Recent methods enable the deposition of structurally uniform, transparent layers of polycrystalline gold onto glass [7,8]. In this paper, we use this methodology in combination with SAM technology to functionalize specifically the surfaces of glass coverslips of the type commonly employed in protein crystallization procedures. We explore the utility of this approach by monitoring the process of crystallization of hen egg lysozyme, bovine α-lactalbumin, porcine pancreatic ribonuclease, horse hemoglobin, thaumatin, and beef liver catalase proteins on a variety of SAMs for which the charge and polarity of the functional groups at the liquid–solid interface are specifically chosen. Thus, we have formed a variety of transparent SAMs on glass coverslips, and have examined the degree to which the structural uniformity and the chemical composition of these SAMs permits control over the relative rates of protein crystal nucleation and growth.

2. Materials and methods

2.1. Reagents

11-Bromoundecanoic acid and 11-bromo-1-undecanol were purchased from Aldrich Chemical Co. Potassium thioacetate was purchased from Lancaster Synthesis. Lithium sulfate was obtained from Sigma Chemical Co. Ammonium sulfate (ultrapure grade) was purchased from Life Technologies, Inc. Gold shot was purchased from Americana Precious Metals. Polyethyleneglycol-4000 (PEG-4000) was purchased from Fluka Chemical Co. Glass coverslips were obtained from Fischer Scientific or Thomas Scientific. Hen egg lysozyme ($3\times$ recrystallized from Sigma or $10\times$ recrystallized from Seikagaku America) was dissolved and dialyzed $3\times$ each against aqueous buffer solution (sodium acetate, 100 mM, pH 4.2) prior to concentration by ultrafiltration and crystallization. Likewise, the other protein solutions (bovine α-lactalbumin, porcine pancreatic ribonuclease, horse hemoglobin, thaumatin, and beef liver catalase) were prepared from commercially available purified protein according to procedures from the literature [9].

2.2. Alkanethiols

Undecanethiol and dodecanethiol were purchased from Aldrich Chemical Co. 11-Thioundecanol, 11-thioundecanoic acid, 12-thioundecanol, and 2-(10-mercaptodecyl)-imidazole were prepared by published procedures [10,11]. The imidazole-substituted compound was stored as the protected thioacetate derivative and was deprotected within one day of deposition onto the gold-coated glass coverslips.

2.3. Evaporation of gold onto glass coverslips

Untreated glass coverslips were washed in a sonicator 3 times each with soap (Alconox) solution, distilled deionized water, and absolute ethanol. In an adaptation of an earlier procedure [7], gold substrates were prepared by thermally evaporating about 25 Å of chromium metal onto the cleaned glass coverslips, followed by evaporating about 150 Å of gold. The freshly prepared gold surfaces were then immersed in ethanolic solutions of the respective thiols and were allowed to equilibrate for 24 h. The resultant SAMs were thoroughly rinsed with ethanol and blown dry with ultrapure nitrogen prior to use. The uniformity of the SAM surfaces was evaluated by liquid contact angle goniometry using a Ramé–Hart Model 100 contact angle goniometer.

2.4. Crystallization experiments

Crystallization trials using the transparent SAM-coated glass coverslips were performed at 22°C by vapor diffusion (hanging drop method) in standard Linbro trays having 24 wells. The initial drop size was held constant at 2 μL for all trials. The coverslips were inverted and sealed against the mouth of the well using Dow high-vacuum silicone grease.

3. Results

Several transparent SAM-coated glass coverslips having a variety of exposed organic functional groups (methyl, imidazole, carboxylic acid, or hydroxyl) were evaluated as substrates for the crystallization of hen egg lysozyme and other proteins. The SAMs were reproducibly uniform as determined by spot checks of the values of the liquid contact angle of hexadecane on the methyl-terminated SAMs, which were derived from the adsorption of undecanethiol (C_{11}) and dodecanethiol (C_{12}). Unlike water, hexadecane is highly sensitive to the degree of order and packing of hydrocarbon surfaces; its use as a characterization tool in contact angle studies is ubiquitous in interfacial science [12,13]. The average values of the hexadecane contact angle on the gold-coated coverslips treated with C_{11} and C_{12} were 37 and 45°, respectively. These values are comparable with literature values for the contact angles of these liquids on the corresponding SAMs on microcrystalline gold [14]. Furthermore, the measured hexadecane contact angle values are significantly higher than that obtained for the surfaces of the commercially available silanized glass coverslips that are available from Hampton Research (13°), indicating that our lipid-like surfaces are substantially more uniform, densely packed, and highly ordered [12,13].

3.1. Lysozyme

In our studies of lysozyme, the crystallization behavior was found to be independent of the commercial source of recrystallized protein, provided that the sample of lysozyme was properly dialyzed against fresh buffer before use. In the crystallization trials, the lysozyme concentrations were varied in the range from 10 to 80 mg/ml. Crystallizations on the SAM-coated coverslips were compared to those on silanized glass coverslips under identical conditions. On the whole, the use of the densely packed and highly ordered SAM surfaces with lysozyme led to a marked enhancement in the rate and extent of crystal nucleation *relative* to precipitate formation (Fig. 1). For proteins other than lysozyme, some of the SAMs led to an increase in the extent of precipitate formation (data not shown).

For crystallization trials at lower lysozyme concentrations (10–40 mg/mL), we observed reproducible differences in crystal nucleation for each of the unique SAM surfaces as well as for the silanized glass surface (see Fig. 1). In particular, at a crystallization time of 1 h, less precipitate was observed on the methyl-terminated (C_{11}) SAMs than on all other surfaces when the salt concentration was low (10–40 mg/mL). At higher salt concentrations (40–60 mg/mL), however, crystals and/or powdery precipitate were observed on all of the surfaces except for the carboxylic acid-terminated SAM, which yielded only powdery precipitate. At 6 h and at intermediate salt concentration (30 mg/mL), crystals formed on the methyl-, imidazole-, and hydroxyl-terminated SAMs but not on the silanized glass surface nor the carboxylic acid-terminated SAM. At 12 h and at low salt concentration (10–20 mg/mL), crystals were observed on all of the SAM surfaces but not on the silanized glass surface.

In crystallization trials at higher lysozyme concentrations (50–80 mg/mL), we also observed reproducible differences in the nucleation of crystals and precipitate on the various surfaces (see Fig. 1). At 1 h, we observed crystals only on the methyl-terminated SAM. Correspondingly, at 6 h the methyl-terminated SAM afforded crystals under the widest variety of conditions. At 12 h, crystals formed on all SAMs over a wider range of conditions than on silanized glass. Fig. 2 shows that at under a chosen set of identical conditions (i.e., low salt and high lysozyme concentration), large crystals were observed on the imidazole-, carboxylic acid-, and methyl-terminated SAMs, while smaller crystals were observed on the hydroxyl-terminated SAM

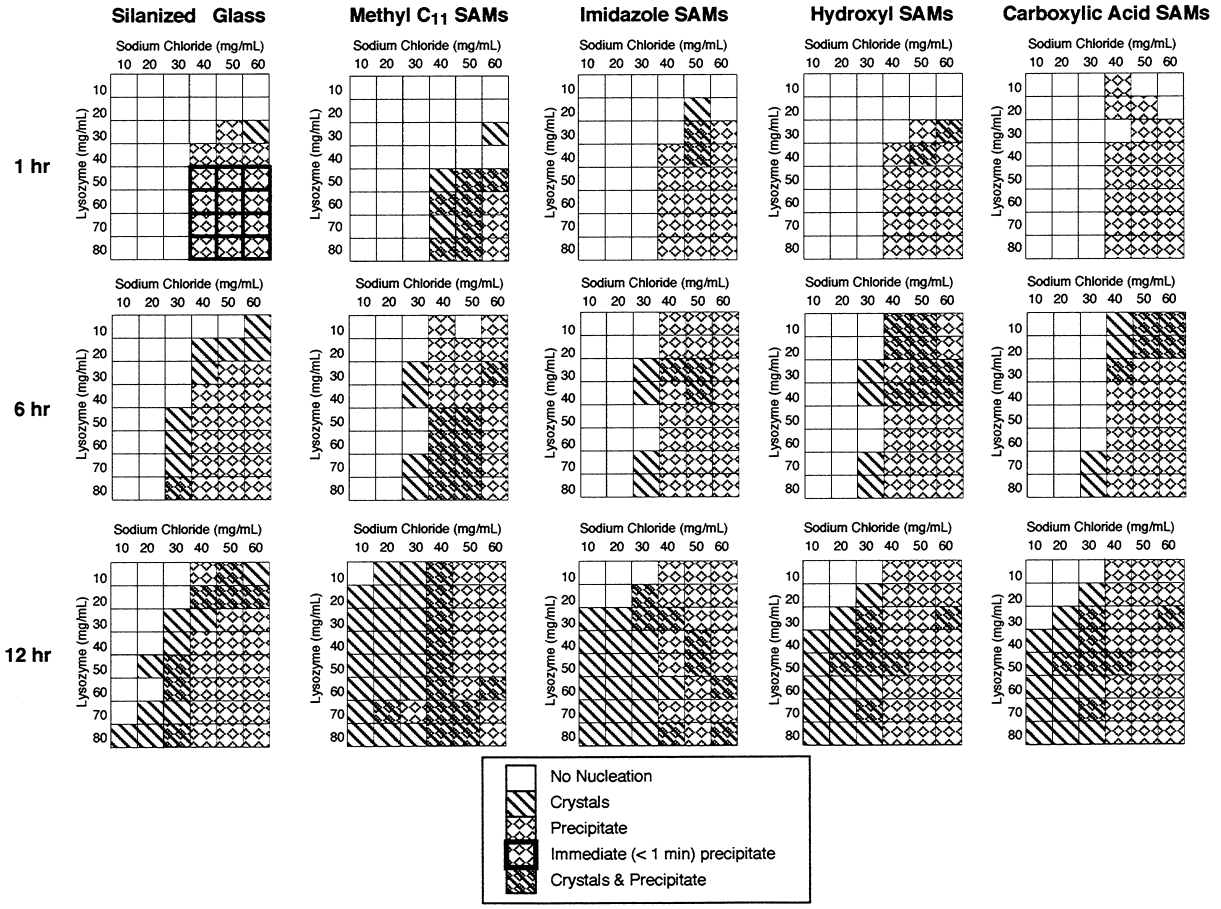


Fig. 1. Crystallization profiles for hen egg lysozyme on five types of chemically modified glass coverslips. In these experiments, the initial drop size of the crystallization mixture was 2 μL.

and on silanized glass. No substantial changes in lysozyme crystallization were observed at times after 24 h for all lysozyme and salt concentrations employed.

We note that high concentrations of both lysozyme and salt eventually led to showers of microcrystals on the SAMs and on silanized glass. However, when the SAM surfaces were used, this showering was delayed until after the appearance of well-formed crystals. Under certain conditions of high lysozyme concentration (Fig. 1), precipitate formed immediately upon contact with silanized glass, but protein remained dissolved with SAMs and eventually yielded large crystals. Qualitatively, we observed that the delayed formation of precipitate at high lysozyme concentration coincided with the formation of several large crystals on the SAMs, but only amorphous precipitate on the silanized glass surfaces.

3.2. Other proteins

In addition to lysozyme, SAMs were observed to influence the crystallization behavior of five other proteins: thaumatin, hemoglobin, α-lactalbumin, ribonuclease, and catalase. No other proteins were examined in this study. Improved crystallization with SAM surfaces occurred reproducibly under a variety of conditions, which are summarized in Table 1 and depicted in Fig. 3. *These crystallization*

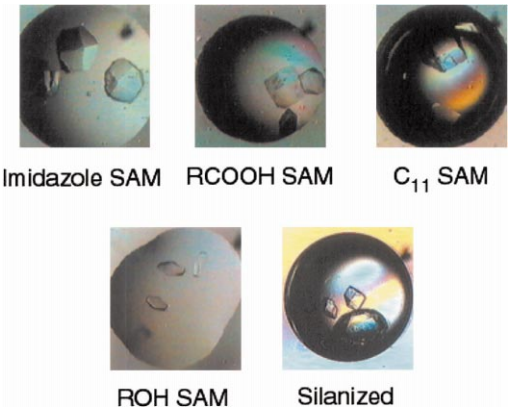


Fig. 2. Illustration of the crystallization behavior typically observed for lysozyme with each of a variety of surface-modified glass coverslips under a chosen set of conditions ([NaCl] = 20 mg/mL; [lysozyme] = 80 mg/mL; time = 12 h). In these experiments, the initial drop size of the crystallization mixture was 2 μL. Under these conditions and others that led to crystallization, the SAM surfaces afforded crystals that were typically larger than those observed with silanized glass. Over a wide range of conditions, the hydroxyl-terminated SAMs exhibited the greatest variation in the relative size of crystals.

studies demonstrate that the highly uniform SAM surfaces significantly and reproducibly broaden the range of protein, salt, and precipitant concentrations (Δc) *that lead to the formation of large protein crystals. In general, the C_{11}- and C_{12}-SAM surfaces afford protein crystals of larger size than can be obtained using silanized glass.* The use of the C_{11}- and C_{12}-SAM surfaces with several of the proteins also enhanced the formation of amorphous precipitate at lower precipitant concentrations than observed with silanized glass (Table 1). However, the initial formation of precipitate occurred at higher precipitant concentrations than did the initial formation of crystals. Although no differences were observed in the limiting concentrations of precipitant for the protein thaumatin, the size of octahedral (i.e., tetragonal bipyramidal) thaumatin crystals was considerably larger (>0.5 mm in length) for crystals that formed with C_{11}- and C_{12}-coverslips at thaumatin concentrations of 20–38 mg/mL after 12 h of incubation (see Fig. 3). Furthermore, for the protein catalase, the amount of precipitate was observed to be higher on the SAM surfaces even though the formation of precipitate occurred at the same salt concentrations for both the SAMs and silanized glass.

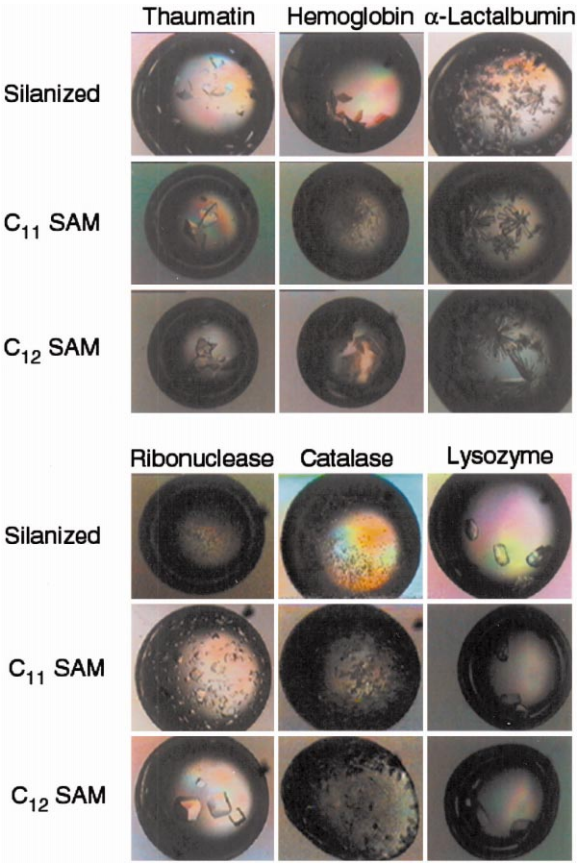


Fig. 3. The crystallization behavior of several different proteins varies with the nature of the chemically modified coverslip surface (i.e., silanized vs. C_{11} SAM vs. C_{12} SAM). Shown here are crystallizations of thaumatin (38 mg/mL, 1.6 M Na–K tartrate, 12 h); horse hemoglobin (65 mg/mL, 24% w/v PEG-4000, 10 d); bovine α-lactalbumin (35 mg/mL, 30% w/v PEG-4000, 22 h); porcine pancreatic ribonuclease (60 mg/mL, 1.8 M CsCl, 0.45 M ammonium sulfate, 36 d); beef liver catalase (35 mg/mL, 10% w/v PEG-4000, 10 h); and hen egg lysozyme (35 mg/mL, 4.0% w/v sodium chloride, 4 d). For each protein, the initial crystallization conditions were the same for all three types of surfaces, and the initial drop size of the crystallization mixture was 4 μL. The buffers used are described in Table 1.

4. Discussion

The enhancements afforded by using SAM-coated coverslips for the crystallization of lysozyme

Table 1
Crystallization of selected proteins on silanized glass and on SAM-coated surfaces[a]

Fixed protein and surfaces	Crystal[b]		Precipitate[c] Lowest PPT	Crystalline morphology and relative size ()[d]
	Lowest PPT	Highest PPT		
α-*Lactalbumin* 35 mg/mL		(PEG-4000)		
Silanized glass	28% w/v	36% w/v	40% w/v	Needles
C11-coated surface	24% w/v	36% w/v	36% w/v	Elongated slabs (L)
C12-coated surface	24% w/v	36% w/v	36% w/v	Elongated slabs (L)
α-*Lactalbumin* 55 mg/mL				
Silanized glass	24% w/v	36% w/v	36% w/v	Needles
C11-coated surface	20% w/v	36% w/v	36% w/v	Elongated slabs (L)
C12-coated surface	20% w/v	36% w/v	32% w/v	Elongated slabs (L)
Horse-Hemoglobin 65 mg/mL		(PEG-4000)		
Silanized glass	16% w/v	32% w/v	28% w/v	Monohedra
C11-coated surface	12% w/v	32% w/v	24% w/v	Monohedra (L)
C12-coated surface	12% w/v	32% w/v	24% w/v	Monohedra (L)
Thaumatin 20–38 mg/mL		(Na–K tartrate)		
Silanized glass	1.0 M	1.6 M	None	Tetragonal bipyramids
C11-coated surface	1.0 M	1.6 M	None	Tetragonal bipyramids (L)
C12-coated surface	1.0 M	1.6 M	None	Tetragonal bipyramids (L)
Ribonuclease 60 mg/mL[e]		(Ammonium sulfate)		
Silanized glass	0.45 M	0.45 M	0.65 M	Trapezohedra
C11-coated surface	0.35 M	0.45 M	0.55 M	Trapezohedra (SL)
C12-coated surface	0.35 M	0.45 M	0.55 M	Trapezohedra (L)
Catalase 20–35 mg/mL		(PEG-4000)		
Silanized glass	11% w/v	13% w/v	10% w/v	Needles
C11-coated surface	10% w/v	13% w/v	10% w/v	Rectangular prisms (L)
C12-coated surface	10% w/v	13 % w/v	10% w/v	Rectangular prisms (L)
Lysozyme 35 mg/mL		(Sodium chloride)		
Silanized glass	4.0% w/v	5.0% w/v	None	Tetragonal pyramids
C11-coated surface	3.0% w/v	5.0% w/v	None	Tetragonal pyramids (L)
C12-coated surface	2.0% w/v	5.0% w/v	None	Tetragonal pyramids (L)

[a]Initial drop size of the crystallization mixture was 4 μL.
[b]Limiting concentrations of precipitant at which protein crystals were observed.
[c]Lowest concentration of precipitant at which amorphous precipitate was observed.
[d]The notation in parentheses indicate the qualitative size of the crystals relative to that observed on silianized glass, where SL = slightly larger and L = larger.
[e]The additive CsCl was used at concentrations of 1.4–2.6 M (i.e., 4 × [ammonium sulfate]). Before use, all proteins were dialyzed against their starting buffer. The buffer solutions for each protein crystallization are (α-lactalbumin) 0.1 M Tris-HCl, pH 8.5; (hemoglobin) 0.1 M sodium phosphate, pH 6.5; (thaumatin) 0.02–0.1 M MOPS, pH 6.8; (ribonuclease) 0.1 M acetate, pH 6.1 containing 4.0 M CsCl; (catalase) 0.02–0.03 M sodium phosphate, pH 6.1; (lysozyme) 0.1 M acetate, pH 4.8. For lysozyme, any discrepencies between these data and those in Fig. 1 probably arise from the different drop sizes used in the two sets of experiments (i.e., 4 vs. 2 μL, respectively).

and the other proteins probably arises from a reduction in the rate of non-productive, amorphous precipitate formation with respect to that of productive crystal nucleation. For lysozyme, all of the functionally diverse SAMs that were analyzed (i.e., those terminated with methyl, imidazole, carboxylic acid, and hydroxyl groups) exhibited a broadening of the useful range of conditions for crystallization for one or more combinations of protein, precipitant, and additives. Observations

with atomic force microscopy (AFM) [15] on mica suggest that nucleation of non-productive aggregates in the crystallization of lysozyme might occur by means of amorphous protein aggregation on exposed silica surfaces. Relative to the well-packed and highly ordered SAM-coated coverslips, the incomplete coverage of silanized glass can possibly promote amorphous protein aggregation at the solution–silicate interface. We note that immediate selective precipitation of some concentrated lysozyme solutions with silanized glass surfaces is consistent with such protein–silica interactions. The selective absence of crystals at low precipitant concentrations on silanized coverslips might likewise result from the formation of soluble non-productive aggregates that are undetectable by standard light microscopy. Thus, at low precipitant concentrations, the formation of soluble, non-productive aggregates might account for the surface-dependent inhibition/absence of crystallization that is observed in the presence of silanized glass coverslips.

We anticipate that there might be instances for which a prudent choice of the functional group that is exposed at a SAM surface will increase the rate of productive crystal nucleation. However, it is significant that variation in the apparatus surface (i.e., the use of SAM-coated coverslips) can decrease the rate of non-productive nucleation relative to productive nucleation for the variety of proteins examined here. Indeed, our results highlight the fact that the important variable to control is the *ratio* of the rates of productive to non-productive nucleation. If non-productive nucleation is suppressed, and if the nucleation of crystals and amorphous precipitate exhibit similar concentration dependences, the rate of crystal formation and/or the ultimate crystal size can be enhanced by increasing the concentration of protein.

5. Conclusions

The use of SAMs at relatively high protein concentrations represents a general method for enhancing the growth of protein crystals. SAMs reproducibly widen the range of solution conditions that yield large crystals for the six proteins examined in this study. In general, the surface of the apparatus can influence the relative rates of crystal nucleation and growth. Our observations suggest that both silanized glass surfaces and SAMs markedly affect nucleation rates – either those for the formation of crystals or those for the formation of non-productive, amorphous aggregates. Silanized glass, for example, appears to promote the nucleation of non-productive aggregates (i.e., those that are kinetically restrained from redissolving to form ordered three-dimensional crystals) for lysozyme at low concentrations of precipitant. The kinetic inhibition of non-productive aggregate formation, which allows faster crystal growth and/or larger crystal size, should prove highly useful to those interested in the determination of three-dimensional macromolecular structures.

Acknowledgements

We thank the National Aeronautics and Space Administration (NRA-96-OLMSA-03) and the Robert A. Welch Foundation for generous financial support.

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