Bioconjugate Chemistry

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Design of Heat Shock-Resistant Surfaces to Prevent Protein Aggregation: Enhanced Chaperone Activity of Immobilized α-Crystallin

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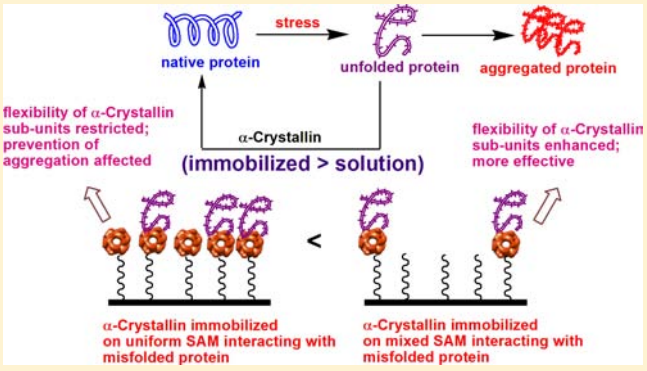
Supporting Information

ABSTRACT: α-Crystallin is a multimeric protein belonging to the family of small heat shock proteins, which function as molecular chaperones by resisting heat and oxidative stress induced aggregation of other proteins. We immobilized α-Crystallin on a self-assembled monolayer on glass surface and studied its activity in terms of the prevention of aggregation of aldolase. We discovered that playing with grafted protein density led to interesting variations in the chaperone activity of immobilized α-Crystallin. This result is in accordance with the hypothesis that dynamicity of subunits plays a vital role in the functioning of α-Crystallin and might be able to throw light on the structure−activity relationship. We showed that the chaperone activity of a certain number of immobilized α-Crystallins was superior compared to a solution containing an equivalent number of the protein and 10 times the number of the protein at temperatures >60 °C. The α-Crystallin grafted surfaces retained activity on reuse. This could also lead to the design of potent heat-shock resistant surfaces that can find wide applications in storage and shipping of protein based biopharmaceuticals.



1. INTRODUCTION

Aggregation of protein occurs due to homotypic association to form oligomers or higher order aggregates. Proteins may undergo aggregation preferably in partially or fully unfolded forms. Aggregation may be dictated by protein unfolding or changes in protein conformation referred to as conformational instability. It may also be driven by protein−protein interactions which lead to colloid formation and is termed as colloidal instability.[1,2] Prevention of aggregation is critical to maintaining complex native structures of proteins. The issue is of utmost importance in the case of engineered proteins which tend to form aggregates more readily. As a consequence, the overexpressed recombinant proteins need to be purified to homogeneity from inclusion bodies.[3,4] The physical stability of monoclonal antibodies must be maintained during all stages of pharmaceutical processing like packaging, shipping, and storage, another area where protein aggregation must be prevented.

Molecular chaperones are the cellular machines which assist polypeptides in folding efficiently in the highly concentrated, complex cellular environment. Heat shock proteins (Hsps) or stress response proteins are a class of molecular chaperones which are generated in cells under mild temperature elevation or other proteotoxic insults, respectively. They resolve misfolded proteins by restoring the normal protein folding environment of cells.[5,6] Hydrophobic interactions are crucial in intra and interprotein interactions, and appropriately placed hydrophobic surfaces appear to play a key role in chaperone activity.

α-Crystallin is the major protein of the mammalian lens in most species. It is expressed at high levels in the lens, where it serves as a refractive element. At the same time, it is a member of the family of small heat shock proteins (sHsps)[7] and has the ability to prevent the precipitation of denatured proteins, increasing cellular tolerance to stress.[8,9] It can prevent heat and

Received: November 6, 2013
Revised: April 1, 2014
Published: April 1, 2014

oxidative stress induced aggregation of proteins. The name "Crystallin" was first coined by Berzelius in 1830 to describe the gelatinous substances that could be isolated from the crystalline lens. It exists as a polydisperse aggregate with an average molecular mass of 800 kDa, assembled from two polypeptide subunits αA and αB at a ratio of 3:1, each having a molecular weight of around 20 kDa.[10,11] Several groups have carried out extensive work to decipher the quaternary structure of this protein. Aquilina et al. have reported a distribution, primarily of oligomers containing 24−33 subunits, with the dominant species composed of 28 subunits.[12] Additionally, lower oligomers, as small as 10-mers and as large as 40-mers, were observed in αB-Crystallin by mass spectrometry. Because of the polydispersity of α-Crystallin, the true molecular mass and size of the isolated α-Crystallin have not yet been determined, and molecular mass values from 300 to 12,000 kDa are variously reported in the literature.[13−15] Both αA and αB are equally effective as molecular chaperones.[16] Unlike other lens proteins (β and γ-Crystallin), the three-dimensional structure of α-Crystallin has not been solved yet by either X-ray crystallography or NMR techniques because of its large aggregative nature. Studies have been attempted to correlate the quaternary structure to chaperone activity of α-Crystallin.[17,18] The exact mechanism of its chaperone activity is yet to be elucidated, but several studies have been carried out to identify the critical residues required for chaperone-like function.[19,20] On the basis of such studies, it is proposed that the hydrophobic region in the α-Crystallin domain is the binding site for denaturing proteins and contributes toward the chaperone-like property.[21] Denatured polypeptides form larger soluble aggregates with α-Crystallin, which suppresses their nonspecific, irreversible aggregation. Not much is known about the mechanistic aspects of recognition of the unfolded state of proteins by α-Crystallin. It can be speculated that the stoichiometry of binding should depend on the extent of exposed hydrophobic surfaces on the target protein, complementarities of the interacting surfaces, and stability of the complex. It has been shown that α-Crystallin can suppress the aggregation of many enzymes when added in stoichiometric amounts.[8,16] This remains a problem as such quantities of α-Crystallin after being used in solution cannot be recovered or reused.

Effects of variations of temperature and high pressure, post-translational modifications, mutagenesis, cross-linking, small molecules, etc. on chaperone activity of αB-Crystallin have been investigated.[22−26] Many of these modifications enhance chaperone activity in vitro and hence might reflect an upregulation of chaperone activity in vivo too. It may be predicted that α-Crystallin exists in a semimobile state in the cellular matrix. Activity studies in solution allow free movement of the protein unlike the restrictive cellular environment. Simulation can be achieved by controlled immobilization. Not only that, at the surface of synthetic material, proteins undergo structural perturbation and that might alter their biological activity. With an aim to explore these aspects, we immobilized α-Crystallin on a self-assembled monolayer (SAM)[27] and studied its chaperone activity. We also changed the concentration of the protein across the surface to address flexibility issues.

α-Crystallin grafted surfaces can be used in storage and transport of various proteins; they can enhance the shelf life of unstable enzymes (viz. restriction enzymes) or other protein based pharmaceuticals like antibody drugs, presence of whose aggregates has the potential for life-threatening immunogenic side effects. Such surfaces can also be useful for the biodetection of unfolded/misfolded proteins and studying protein folding pathways.

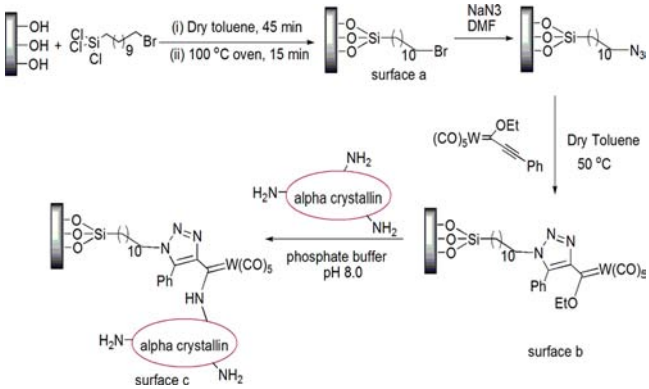
In the present work, we (a) immobilized α-Crystallin (whole protein) on a self-assembled monolayer (SAM) and studied the chaperone activity of the immobilized protein, (b) studied the chaperone activity of immobilized α-Crystallin of variable concentration on mixed SAMs, and (c) compared the chaperone activity of immobilized α-Crystallin with α-Crystallin in solution at different temperatures.

We have shown not only that the specific activity of α-Crystallin remains unaffected by immobilization but also that at higher temperatures, the surface immobilized α-Crystallin is prominently more efficient than an equivalent amount in solution. In addition, these surfaces were shown to be reusable, which increases the scope and applicability of the heat shock resistant surfaces that we have designed. Another significant aspect of our studies was the improvement in activity upon the reduction of the surface density of immobilized α-Crystallin up to a certain limit. We attempted to correlate this result to the structure−function relationship of the protein. We also studied the effect of BSA, immobilzed on uniform and mixed SAMs, on the aggregation of aldolase (as a nonchaperone control) and found a reverse trend compared to that of α-Crystallin.

2. RESULTS

2.1. Surface Analyses. *2.1.1. ATR-IR on Silicon Wafers.* After treating the activated Si surfaces with BUTS (step1 of Scheme 1) ATR-IR showed absorption at 2960 and 2840

Scheme 1. Grafting of α-Crystallin on Fischer Carbene Terminated SAM



cm^{-1}(aliphatic C−H stretching) indicating the presence of a bromo terminated monolayer (surface a). Bromo functionality was substituted by azide and subjected to a "Click" reaction with the Fischer carbene complex (step 3 of Scheme 1). Distinct peaks for CO stretching of the metal pentacarbonyl fragment at ∼2066 cm^{-1} and ∼1942 cm^{-1} were observed (surface b). Aminolysis with the protein (step 4 of Scheme 1) leads to a shift in the peak at 1942 cm^{-1} to ∼1923 cm^{-1} (surface c) (for spectra, see Supporting Information).

2.1.2. AFM and Fluorescence Microscopy on Glass Surfaces. Surface topology of a functionalized glass coverslip was observed with atomic force microscopy, as can be seen in Figure 1. Patches of dense coverage of α-Crystallin was observed on uniform SAM on glass slides. The mean protein covered area on glass chips (10 mm × 10 mm) was calculated

dx.doi.org/10.1021/bc500097q | Bioconjugate Chem. 2014, 25, 888−895

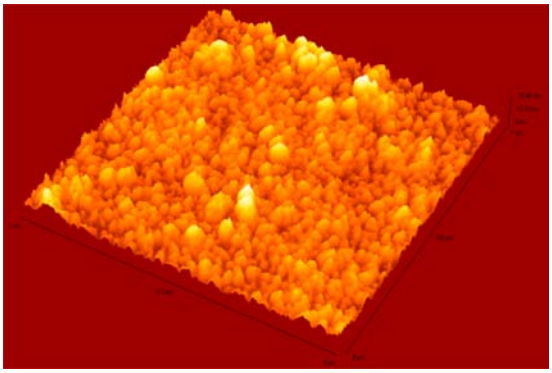


Figure 1. AFM image of uniform protein coverage.

to be 4.31 × 10^{13} nm^2 (from Mat Lab). We know from the literature that α-Crystallin can exist as a spherical aggregate of approximate diameter 14 nm and molecular mass 800 kDa, comprising about 40 subunits.[9] Assuming that the protein we used also exists as a spherical assembly of 40 subunits (of diameter 14 nm), we calculated the number of immobilized proteins to be 2.8 × 10^{11} on a glass chip of 100 sq. mm area (the program for the calculation of the number of proteins is given in the Supporting Information). This many number of proteins correspond to 0.0004 mg of the protein.

A qualitative estimation of the dilution of protein density on the surface on 1:3, 1:5, 1:10, and 1:20 mixed SAMs was done by high resolution scanning electron microscopy and fluorescence microscopy (for images, see Supporting Information). Assuming that on mixed SAMs, the number of proteins would vary accordingly, we calculated the number of proteins on different surfaces. The count was 7 × 10^{10}, 4.7 × 10^{10}, 2.5 × 10^{10}, and 1.3 × 10^{10}, respectively, for 1:3, 1:5, 1:10, and 1:20 mixed SAM bearing glass chips.

2.2. Aggregation Studies. *2.2.1. Chaperone activity of Immobilized α-Crystallin on Uniform SAM on Glass Capillaries.* Aggregation of aldolase (blank) was considered to be 100%. α-Crystallin grafted on uniform SAM on glass capillaries (diameter 0.1 cm) retarded the heat induced aggregation of aldolase (0.1 mg/mL) at 60 °C to ∼54% of the blank. BSA was used as a nonchaperone control for this experiment; it was found to retard the aggregation of aldolase as well but to ∼23% of the blank (Figure 2). We obtained comparable results on reusing the same set of capillaries for two runs, after thorough washing. Percent chaperone activities were calculated from O.D. values at the end point of the aggregation study of each set, following the reported method as mentioned before.

2.2.2. Effect of Surface Protein Dilution. 2.2.2.1. Chaperone Activity of Immobilized α-Crystallin on a Mixed SAM on Glass Capillaries. The comparison of thermal aggregation of aldolase (0.2 mg/mL) in the presence of α-Crystallin coated glass capillaries bearing 1:5 and 1:10 mixed SAMs with α-Crystallin grafted on uniform SAM was interesting. It was found that as protein density was reduced on the surface to 1/10th, the aggregation of aldolase was reduced (∼59%), even though the number of protein molecules was less compared to that of the uniformly coated surface (∼62% aggregation). The 1:5 mixed SAM bearing surface showed lesser effectiveness (∼67% aggregation) compared to that of the uniform surface (Figure 3).

2.2.2.2. Chaperone Activity of Immobilized α-Crystallin on a Flat Glass Surface Bearing a Mixed Monolayer. Aggregation

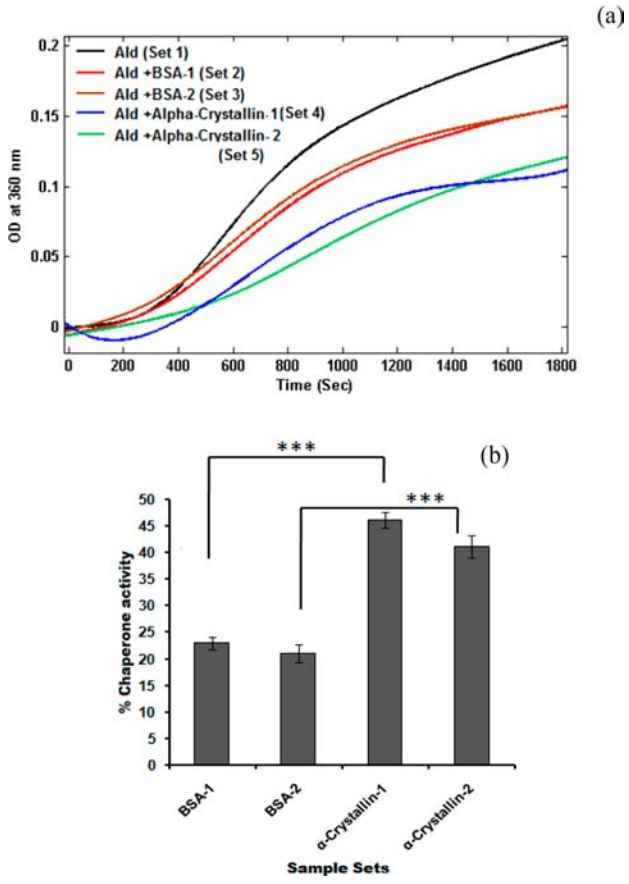


Figure 2. (a) Aggregation profiles (proportional to O.D.) at 60 °C of Set 1-aldolase and Set 2-aldolase in the presence of 10 BSA coated capillaries, Set 3-aldolase when the capillaries of Set 2 were used for a second time after washing, Set 4-aldolase in the presence of 10 α-Crystallin coated capillaries, and Set 5-aldolase when the capillaries of Set 4 were used for a second time after washing. (b) Bar diagram representing the % chaperone activity of BSA and α-Crystallin coated capillaries (at 1800 s) in Sets 2−5. Note: *** = very significant (P < 0.0005), ** = significant (0.0005 < P < 0.05), and x = less significant ($P \geq 0.05$).

of aldolase in the presence of α-Crystallin on uniform SAM (Set 3) was ∼40% of the blank (Set 1), whereas in the presence of the control surface (Set 2), it was merely 13%. On incubation with α-Crystallin coated on 1:3 mixed SAM (Set 4), aggregation of aldolase was suppressed to ∼73% (for O.D., curves see Supporting Information). α-Crystallin coated on 1:5 mixed SAM (Set 5) showed ∼67% aggregation of the target protein. Aggregation of aldolase was retarded to the maximum extent (∼59%) by α-Crystallin on 1:10 mixed SAM (Set 6). Activity of immobilized α-Crystallin on 1:20 mixed SAM was less (∼64% aggregation of aldolase) compared to that on 1:10 SAM (Figure 4). During the heating of aldolase, we measured the average particle size of the protein in Sets 1 and 3. It was found that there was a considerable difference in the mean hydrodynamic diameter of aggregated aldolase, 1106 ± 163 nm and 615.1 ± 90.6 nm, respectively (for figures, see Supporting Information).

2.2.3. Chaperone Activity of Immobilized α-Crystallin on a Flat Glass Surface Compared to That in Solution. We studied the heat induced aggregation of aldolase (0.2 mg/mL) at 60 °C in the presence of immobilized α-Crystallin, obtained from a human eye lens. The activity of immobilized α-Crystallin was

dx.doi.org/10.1021/bc500097q | Bioconjugate Chem. 2014, 25, 888−895

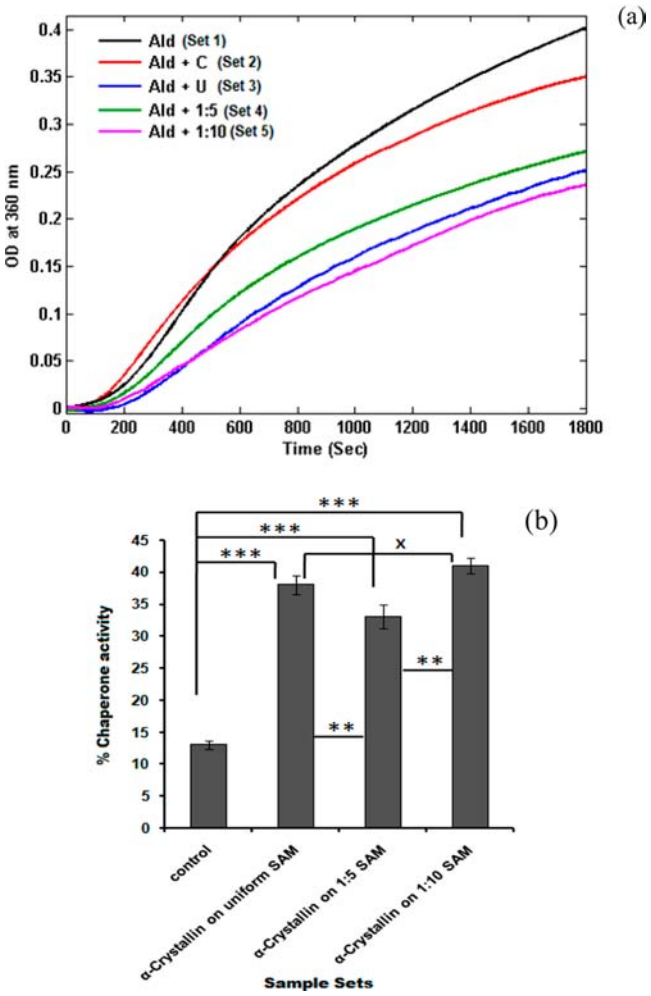


Figure 3. (a) Aggregation profiles at 60 °C of Set 1-aldolase and Set 2-aldolase in the presence of the control surface (coated with amine), Set 3-aldolase in the presence of a glass chip coated with α-Crystallin on uniform SAM, Set 4-aldolase in the presence of a glass chip coated with α-Crystallin on 1:5 mixed SAM, and Set 5-aldolase in the presence of a glass chip coated with α-Crystallin on 1:10 mixed SAM. (b) Bar diagram representing the % chaperone activity of the control and α-Crystallin coated capillaries (at 1800 s) in Sets 2−5. Note: *** = very significant ($P < 0.0005$), ** = significant ($0.0005 < P < 0.05$), and x = less significant ($P \geq 0.05$).

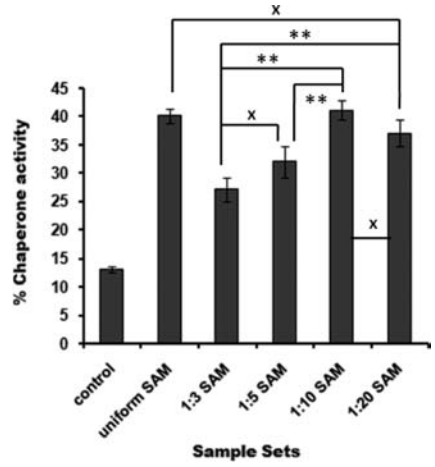


Figure 4. Bar diagram representing the % chaperone activity of the control (Set 2) and α-Crystallin coated capillaries in Sets 3−7. Note: *** = very significant ($P < 0.0005$), ** = significant ($0.0005 < P < 0.05$), and x = less significant ($P \geq 0.05$).

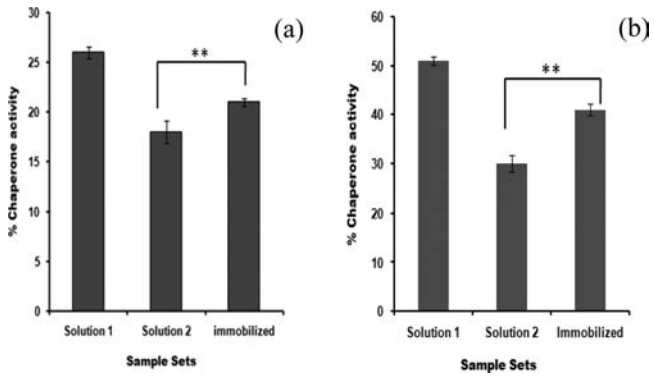


Figure 5. Bar diagram representing the % chaperone activity of (a) α-Crystallin (from human eye lens) and (b) α-Crystallin (from bovine eye lens), in solution and in an immobilized state (at 1800 s) at 60 °C. Note: *** = very significant ($P < 0.0005$), ** = significant ($0.0005 < P < 0.05$), and x = less significant ($P \geq 0.05$).

compared to the activities of two solutions, one containing 10 times as many proteins (solution1, 0.04 mg/mL of α-Crystallin) and the second containing an equivalent number of proteins (solution 2, 0.004 mg/mL of) as the surface. It was found that immobilized proteins showed 21 ± 0.4% chaperone activity compared to 18 ± 1.1% of solution 2 (Figure 5a). Maximum activity was found for solution 1 (26 ± 0.6%).

The same comparison was done for α-Crystallin obtained from a bovine eye lens to ensure that activity variation of immobilized α-Crystallin was because of a different source. The data now showed an activity level at ∼50% (solution 1) and ∼40% (immobilized) in Figure 5b, which is essentially similar to that in Figures 2 and 3. The objective of this experiment was to compare the activity of α-Crystallin in solution with immobilized α-Crystallin. As can be clearly seen from Figure 5a and b, the trend was identical (solution 1> immobilized > solution 2).

2.2.4. Chaperone Activity of Immobilized α-Crystallin on a Flat Glass Surface Compared to the Solution at Higher Temperatures. Similar to the previous experiment, we compared the chaperone activities of solutions 1 and 2 and immobilized α-Crystallin (from human eye lens) at 65 and 70 °C (Figure 6). It was found that as temperature increased the specific activity of α-Crystallin was reduced in solution. Solution 1 though contained 10 times the number of proteins on the surface, but it showed far inferior activity at 65 and 70 °C (1 ± 0.4% and 0.7 ± 0.5% chaperone activity, respectively). Solution 2 showed 1 ± 0.2% and 1.6 ± 0.8% chaperone activity at those temperatures, whereas immobilized α-Crystallin retained detectable chaperone activity even at such high temperatures (26 ± 1.3% and 23 ± 1.4% at 65 and 70 °C, respectively).

2.2.5. Chaperone Activity of Immobilized BSA in Solution and on the Surface. It was found that in solution BSA did not exhibit any marked chaperone activity (6.5 ± 0.5%). BSA coated on uniform SAM showed 16 ± 1.2% activity (for O.D. curves, see Supporting Information). Dilution of immobilized BSA concentration on the surface did not improve the antiaggregation effect of BSA (15 ± 0.9% and 8 ± 0.7% chaperone activity on 1:5 SAM and 1:10 SAM, respectively;

dx.doi.org/10.1021/bc500097q | Bioconjugate Chem. 2014, 25, 888−895

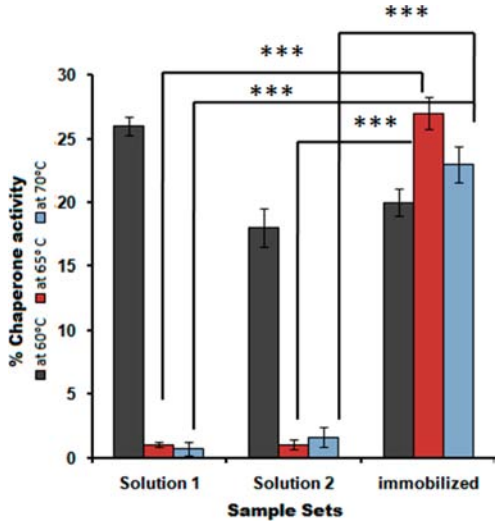


Figure 6. Bar diagram representing the % chaperone activity of α-Crystallin in solution and in an immobilized state (at 1800 s) at 60, 65, and 70 °C. Note: *** = very significant ($P < 0.0005$), ** = significant ($0.0005 < P < 0.05$), and x = less significant ($P \geq 0.05$).

Figure 7), which was the reverse of α-Crystallin (Figures 3 and 4).

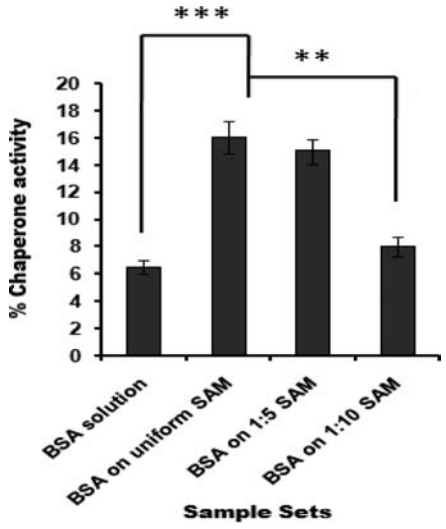


Figure 7. Bar diagram representing the % chaperone activity of BSA in solution and immobilized (at 1800 s) on the surface. Note: *** = very significant ($P < 0.0005$), ** = significant ($0.0005 < P < 0.05$), and x = less significant ($P \geq 0.05$).

3. DISCUSSION

In the present study, immobilization of α-Crystallin on glass and silicon surfaces was achieved following a protocol developed in our group.[28,29] The strategy rests on a facile reaction among an alkoxy Fischer carbene complex (FC), an organometallic functionality, and the amino group of a pendant lysine residue on a protein. Characterization of the functional surface was carried out as reported earlier (see Supporting Information). Silicon wafers allowed us to do spectral studies for the detection of terminal functional groups at different stages of the reaction. Grafting was also done on glass coverslips and slides for AFM, HRSEM, and fluorescence microscopy. Apart from the flat surfaces, we also grafted the protein on outer surfaces of glass capillaries for maximizing the surface area of exposure of grafted protein. The protein concentration on the surface was controlled by varying the concentration of a functional chain on a mixed SAM. The gradual variation of an FC terminated chain with respect to a methyl terminated chain on SAM was monitored by HRSEM and fluorescence microscopy. We have seen earlier that the immobilization protocol and the organometallic functionality involved in the conjugation step do not interfere in the protein recognition or binding events.[29]

Aldolase is a glycolytic enzyme whose catalytic activity is known to be temperature sensitive and has been widely studied.[30−32] It was chosen as the target protein for studying the chaperone activity of α-Crystallin. The idea was to study the thermal aggregation of aldolase in the presence of α-Crystallin. The aggregation profile of aldolase at 60 °C, was monitored in terms of the turbidity/increment of O.D. at 360 nm over a period of 30 min. Aggregation was considered to be complete once a plateau of turbidity was observed and the final O.D. value was taken as 100% aggregation. In presence of capillaries uniformly coated with α-Crystallin, aggregation of aldolase was reduced to ∼54% (Figure 2). In presence of BSA coated capillaries, ∼76% aggregation was observed. It is known that hydrophobic interactions play a key role in the chaperone activity of proteins. Hence, the difference in chaperone activity between the two proteins might be directly related to the availability of their exposed hydrophobic surface to aldolase. It was important to note that the chaperone activities of both α-Crystallin and BSA were practically retained when the same capillaries were used for a second run with a fresh solution of aldolase. Recyclability was a clear advantage of using immobilized chaperone protein compared to the use of the same protein in solution. The former maximizes the use of chaperone protein, while the latter has the disadvantage of waste of material after one use.

Since an element of dynamicity is believed to be important for chaperone activity, varying the concentration of α-Crystallin on SAM was thought to be interesting. A mixed SAM was therefore prepared where the ratio of functional and nonfunctional chains was 1:5 and 1:10. Interestingly, it was found that chaperone activity was marginally reduced in the case of the former, while an improvement of similar order was observed for the latter (Figure 3). This aspect was explored in detail in the study of the denaturation of aldolase in the presence of α-Crystallin immobilized on flat glass surfaces. It can be clearly seen in Figure 4 that there was an initial decrease of chaperone activity when the number of immobilized α-Crystallin was reduced to approximately 1/3rd from the uniformly coated SAM. It showed an increase in chaperone activity as the concentration of the protein was reduced to 1/5th.When the concentration was further reduced to 1/10th of the original, chaperone activity improved and was comparable to that of the uniformly coated SAM. Further reduction of protein concentration on the surface by 2-fold, reduced the chaperone activity of the surface but less significantly (compare % chaperone activities for 1:5 SAM with 1:20 SAM, Figure 4). Reduction in the packing density of immobilized α-Crystallin may allow reorganization of the subunits within the aggregate or small change in the packing domain of the subunits themselves. This, in turn can influence surface exposed hydrophobicity. There is an also an argument that the flexibility of subunits of α-Crystallin might facilitate it to locate denatured polypeptides

dx.doi.org/10.1021/bc500097q | Bioconjugate Chem. 2014, 25, 888−895

and bind with its hydrophobic residues. Our result definitely was in accordance with the thought that dynamicity could enhance chaperone action. This was quite expected as 1:3 mixed SAM would not space out the proteins to an extent that it would improve the dynamicity of the subunits. On going to 1:5 mixed SAM, improvement was observed compared to that in the last set. For protein grafted on a 1:10 mixed monolayer, there was maximum enhancement in the chaperone activity of immobilized α-Crystallin, slightly better than the one in which there was dense coverage of protein. We may interpret this observation as the decrease in number of protein molecules immobilized on a surface in the case of 1:10 mixed SAM compensated by an increase in dynamicity of the protein and flexibility of the subunits. This, allows more space for suitable orientation of the hydrophobic patches and enhances chaperone activity. We were further sure that we were on the right track when we found that the trend started to reverse for 1:20 mixed SAM. This could be because in this set, the number of proteins grafted on the surface was so low that the dynamicity factor could not overcome the deficient number. Dynamicity plays a significant role in making α-Crystallin immobilized on 1:20 mixed SAM more effective in chaperone activity than 1:3 and 1:5 mixed SAMs, where protein concentrations were much higher.

A significant advantage of the immobilization of α-Crystallin was evident when we compared the chaperone activities of the immobilized protein (on flat surface) with protein solutions. With reference to aldolase as before, we found that the chaperone activity of immobilized α-Crystallin, from human eye lenses, (∼21%) was better than the solution (∼18%) containing the same number of protein molecules at 60 °C. The same trend of activities was observed when the same experiment was repeated with bovine α-Crystallin (∼30% for solution and ∼40% for the immobilized protein). These results were consistent with the study reported earlier.[33] In that study, αB-Crystallin was grafted on a random, polymeric, aldehyde terminated chain, where the orientation of grafted proteins was less predictable. In comparison, the present study was conducted with α-Crystallin immobilized on a SAM, where the proteins were properly oriented and more accessible. When the concentration of α-Crystallin in solution was increased 10-fold, the chaperone activity was only improved by 5% and 10% compared to that in the immobilized state (Figure 5a and b). It was not significantly higher than that of the immobilized α-Crystallin. The situation changed dramatically when the temperature was raised to 65 or 70 °C (Figure 6). Temperature variations have been proved to alter the structure of α-Crystallin.[17] It is known that a perturbation in the quaternary structure above 30 °C enhances the protective ability of α-Crystallin perhaps by increasing or reorganizing the hydrophobic surfaces.[18] In that study, the plot of protection vs temperature showed two transitions, one at 30 °C and another at 55 °C. Temperature dependent activity studies of α-Crystallin in solution have been carried out in the past, but comparison with surface activity at temperatures above 60 °C is not known. While the chaperone activity of immobilized α-Crystallin remained practically unaffected at 65 or 70 °C, solutions of α-Crystallin lost most of their chaperone activities and led to almost 100% aggregation of aldolase. This has been observed earlier and happens mostly because of saturation of the chaperone protein leading to coprecipitation with the target protein. Additionally, in solution the protein would be more susceptible to further structural perturbations at such high temperatures, which might have adversely affected its chaperone function. When the protein is immobilized, structural alteration would be restricted, and in turn, interaction with target protein would be less affected. Herein lays another advantage of immobilizing α-Crystallin. Although the temperature regime is not relevant physiologically, it emphasizes the potential of using immobilized α-Crystallin for other practical applications like protein storage, shipment, etc. An experiment on high temperature aggregation of protein is presenting the enhanced form of the storage problem in a short time window. Such high temperature studies (in solution) have been done previously by other groups.[30,31] So, if a chaperone system is capable of preventing the thermal degradation of proteins at ≥60 °C, it would be capable of preventing the thermal denaturation of proteins in the case of long-term storage. Not only that, quite high temperatures are encountered in tropical countries, and preservation of vaccines and other protein based drugs is an issue there. This could inspire one to make storage ampules whose inner walls are coated with α-Crystallin. Such an option would, in principle, reduce the energy consumption needed for deep freezing proteins and avoid repeated freeze–thaw procedures. So, it has immense potential in the real-life biological domain.

In comparison, BSA in solution showed far less chaperone activity than immobilized BSA. Reduction of BSA molecules on the surface (5-fold or 10-fold) revealed a different trend in corresponding chaperone activity (Figure 7). Effect of the reduction of BSA concentration on the surface was the reverse of α-Crystallin. This reflects the difference between the nature of these two proteins (BSA, which is monomeric, and α-Crystallin, which is a polymeric aggregate) and their chaperone activities.

Activity measurement of an enzyme, in the presence of an α-Crystallin coated surface, would be an interesting study. For this purpose, we chose citrate synthase as the model enzyme instead of aldolase for operational simplicity (the results are included in the Supporting Information). Aggregation profile of citrate synthase was studied, and the O.D. profile was similar to that of aldolase aggregation. Then, the activity of citrate synthase was measured in the presence and absence of the α-Crystallin coated surface. Citrate synthase was found to retain 83% of the native state activity due to the chaperone activity of immobilized α-Crystallin. We believe that aldolase activity would also show a similar trend.

4. CONCLUSIONS

In summary, we have developed a method of immobilizing α-Crystallin on glass and silicon surfaces via SAM following a simple protocol. Protein concentration on the surface was controlled on mixed SAM, and chaperone activity was monitored. Improvement of activity up to a certain dilution, supports the idea that chaperone activity of α-Crystallin is dependent on the dynamicity and mobility of its subunits and thereby on its quaternary structure. In addition, we have shown not only that the specific activity of the α-Crystallin is retained on immobilization but also that its chaperone activity in the immobilized state exceeds its activity in solution at high temperatures (≥60 °C). Reusability of these grafted surfaces further confirms that the chaperone activity of α-Crystallin is preserved on immobilization.

Success of the designed heat shock-resistant surfaces described herein can be extended toward the storage and

transport of sensitive proteins, enzymes, and biopharmaceuticals.

5. EXPERIMENTAL PROCEDURES

ATR-IR spectra were recorded on a Nicolet 380 spectrometer. Fluorescence images were acquired using a Leica DM 3000 Upright Trinocular Research Microscope with a Leica DFC 425 Scientific digital camera and LAS software. The topography of the protein coated surface was investigated by atomic force microscopy (diCP-II) with DI Company SPMlab Analysis software. All images were obtained in noncontact mode. High resolution scanning electron microscopy (HRSEM) pictures were taken with a JEOL (JSM-6700F) field emission scanning electron microscope. DLS measurements were done on Nano-ZS (Malvern Instruments). The optical density of aldolase solution was measured on a Thermo Scientific spectrophotometer (Model: Evolution 300). α-Crystallin [lyophilized powder; from bovine eye lens (for experiments (I) and (II)) and from human eye lens (for experiments (III), (IV))] and aldolase from rabbit muscle (powder form) were purchased from Sigma-Aldrich. BSA was obtained from SRL. Solvents like ethanol, DMF, and toluene were purchased from SRL and dried using standard procedures. Reagents were purchased from Sigma-Aldrich and were used as received.

5.1. Immobilization of α-Crystallin on the Surface. Immobilization of α-Crystallin on Fischer carbene terminated SAM on silicon and glass surfaces was achieved following our reported protocol (Scheme 1).[28,29] 0.1 mg/mL of the protein in phosphate buffer (pH 8.0) was used for immobilization.

Mixed SAMs were formed to reduce the density of grafted proteins on the surface. We made a mixed monolayer of 11-bromoundecyltrichlorosilane (BUTS) and decyltricholorosilane (DTS) in desired ratios (1:3, 1:5, 1:10, and 1:20). Glass capillaries (curved surface) and chips (flat surface) were used for aggregation studies.

5.2. Chaperone Activity Assays. The chaperone activity of immobilized α-Crystallin was measured against the aggregation of aldolase as the target protein. All protein solutions for the assay were prepared in 50 mM phosphate buffer at pH 7.22. Aldolase with or without α-Crystallin immobilized on glass surfaces and α-Crystallin in solution (100 μL) or control surfaces (butyl amine coated, with no protein immobilized) were incubated at 60, 65, and 70 °C inside the spectrophotometer, and the thermal aggregation of aldolase (0.2 mg/mL) was measured in terms of increments of optical density (O.D.) at 360 nm at 60 °C for 30 min. All assays were performed in a cuvette of path length 1 cm using a sample volume of 1 mL. Each assay included a control surface to asses any spectroscopic or chaperone contributions from the chemically modified surfaces. The protein coated surfaces were washed thoroughly with water and phosphate buffer before reuse. The chaperone activity was calculated as a percentage according to a reported procedure.[33,34] For comparing the effect of surface concentration on the chaperone activity of α-Crystallin, the protein was grafted on 1:3, 1:5, 1:10, and 1:20 mixed SAMs. We also studied the aggregation profile of aldolase at 60 °C in the presence of BSA as a nonchaperone control. To determine the mean particle size of aggregated aldolase, DLS measurements were done (see Supporting Information). Data for all experiments were analyzed by a paired *t*-test, and the difference of activities under various conditions were found to be significant mostly (the *P* values are tabulated in the Supporting Information and indicated in the figures as well).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

ATR-IR spectra, AFM, HRSEM, and fluorescence images, dynamic light scattering measurements, optical density profiles, and measurement of enzyme activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work has been supported by CSIR (grant number 09/080(0673)/2009/EMR-1) and IFCPAR (project number 4105-1). We thank Professor Dr. K. P. Das (Bose Institute, Kolkata) for his encouragement and to the reviewers for their constructive suggestions.

■ DEDICATION

This work is dedicated to Dr. S. Rajappa on the occasion of his 80th birth anniversary.

■ ABBREVIATIONS

SAM, self-assembled monolayer; Hsp, heat-shock protein; BSA, bovine serum albumin; AFM, atomic force microscopy; ATR-IR, attenuated total reflection infrared; DLS, dynamic light scattering; O.D., optical density

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