# Size Evolution of Protein-Protected Gold Clusters in Solution: A Combined SAXS–MS Investigation

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Supporting Information

ABSTRACT: We report a combined small-angle X-ray scattering (SAXS) and mass spectrometric (MS) study of the growth of gold clusters within proteins, in the solution state. Two different proteins, namely, lysozyme (Lyz) and bovine serum albumin (BSA), were used for this study. SAXS study of clusters grown in Lyz shows the presence of a 0.8 nm gold core, which is in agreement with the Au<sub>10</sub> cluster observed in MS. Dynamic light scattering suggests the size of the cluster core to be 1.2 nm. For



BSA, however, a bigger core size was observed, comparable to the Au<sub>33</sub> core obtained in MS. Concentration- and time-dependent data do not show much change in the core size in both SAXS and MS investigations. When metal-protein adducts were incubated for longer time in solution, nanoparticles were formed and protein size decreased, possibly due to the fragmentation of the latter during nanoparticle formation. The data are in agreement with dynamic light scattering studies. This work helps to directly visualize cluster growth within protein templates in solution.

#### 1. INTRODUCTION

Noble metal nanoclusters belong to one of the rapidly evolving fields in nanoscience today.<sup>1–10</sup> Subnanometer sized clusters comprising a few to a hundred atom cores are of specific interest due to their unusual physical and chemical properties. Investigations of such clusters started with their gas phase analogues, which were studied extensively with mass spectrometry.<sup>1,11-13</sup> Although clusters are many, only very few of the gold and silver clusters have been crystallized so far.14-19 Macromolecular templating is one way to create clusters. DNA, dendrimers, and most recently proteins have been introduced to the cluster community.<sup>3,10,20-23</sup> Many proteins, namely, bovine serum albumin (BSA), human serum albumin (HSA), lactoferrin (Lf), human serum transferrin, pepsin, horseradish peroxidase, ovalbumin, chymotrypsin, insulin, trypsin, lysozyme (Lyz), etc. have been used so far for synthesizing clusters.<sup>24-37</sup> Protein protected clusters exhibit intense luminescence, which has been exploited mostly for sensing of specific molecules and bioimaging.<sup>26,29–31,38–43</sup> Besides, retention of biological activity of insulin after cluster formation has also been shown recently.<sup>3</sup> Usually proteins are characterized by crystallography, cryoelectron microscopy, nuclear magnetic resonance (NMR), etc. Although crystal structure determination is the best option for analyzing the protein structure,<sup>44,45</sup> it has not been possible so far to get crystals of protein protected clusters due to the inherent difficulty to form crystals at experimental conditions of cluster

synthesis. Therefore, for assigning cluster composition, mass spectrometry (MS) is often used, as it is possible to determine molecular weights of proteins and related systems with reasonable accuracy. For instance, Au/Ag/Au@Ag<sub>QC</sub>@BSA,  $^{28,32,34,46}$ Au/Ag<sub>QC</sub>@Lyz,  $^{24,47}$ Au<sub>QC</sub>@Lf,  $^{36,37}$  etc. (where the subscript QC refers to quantum clusters, another name for such clusters) have been studied using MS. However, it should be noted that matrix-assisted laser desorption/ionization (MALDI) MS involves studies in the gas phase, but the original synthesis of clusters occurs in the solution phase. Ions observed in MALDI MS need not necessarily correspond to the species in the condensed phase. So, it is essential to correlate the behavior of these clusters in both solution and gas phases. Thus comes the need for solution phase techniques like small-angle X-ray scattering (SAXS) and neutron scattering (SANS) to study the size and structural changes of protein protected clusters.

SAXS enables size analysis using small amounts of sample, even at very low concentrations. Unlike dynamic light scattering (DLS) studies, SAXS analysis does not require any special sample preparation steps (such as preconcentration or predilution) and can be performed at room temperature. Normally, transmission SAXS gives accurate information about species of size less than



Received: September 15, 2014 Revised: December 1, 2014 Published: December 29, 2014

50 nm. SAXS has been widely and successfully used to extract information about size, shape and also secondary structure of biomolecules, especially proteins, in solution. $^{48-51}$  Recently, the behavior of proteins like Lyz and BSA<sup>48,49</sup> in the presence of salts such as sodium dodecyl sufonate has been studied in detail using SAXS. Blanchet et al. and Lipfert et al. have described various aspects of SAXS investigations on biomolecules.<sup>52,53</sup> Besides macromolecules, many studies on the formation and growth of nanoparticles in various media exist. For example, Gang et al. have described DNA-mediated self-assembly of nanoparticles in heterogeneous systems where they programmed nanoparticlecoated DNA molecules to self-assemble into three-dimensional superlattices, and corresponding structural information was obtained from SAXS.<sup>54</sup> In another report from the same group, nanoparticle dimers linked via DNA were prepared, and the size and other information were collected via DLS and SAXS studies. One dimensional linear meso-structure of nanorod-DNA assembly has also been looked at by in situ SAXS.<sup>55</sup> The actual solution state mechanism for the formation of citrate-capped gold nanoparticles has also been demonstrated by Kraehnert et al.<sup>56</sup> using continuous flow SAXS measurement.<sup>57</sup> There are many other reports on the SAXS study of biological and material interfaces, 54,55,58-62 although until now very few reports existed on noble metal nanoclusters. In 1995, Murray and group studied alkanethiol protected gold clusters by SAXS and found that their core size was about 1.2 nm, consistent with the TEM data.<sup>63</sup> Recently SAXS study of Ag<sub>152</sub> cluster has been reported.<sup>56</sup> Most recently, the structure of Au<sub>68</sub> has been solved by electron microscopy and the size of the core has been validated by SAXS data.<sup>64</sup> However, in most cases, such studies have been limited to the basic characterization of the clusters.

Until now, none of the protein protected clusters could be crystallized, and they also have not been seen in electrospray ionization mass spectrometry (ESI MS). Although more than 300 papers have been published to date on protein-protected clusters, there is no direct way to observe these clusters in solution. Thus, it is necessary to acquire additional data on the cluster growth in solution using independent studies. As proteinprotected Au clusters are prepared in aqueous solutions, study of the dynamics and growth of proteins in the same phase is more useful. Since proteins are explored widely by SAXS, a study by this methodology has relevance. In this paper, we are reporting a combined MS-SAXS study of the evolution and growth of protein protected clusters for the first time. We have probed the growth of small gold clusters in a relatively small protein, Lyz and correlated the results with mass spectral data. The same procedure is followed for a larger protein, BSA, to validate our point. The sizes of the clusters derived from SAXS profiles are in good agreement with the previously reported cluster size as well as with the mass spectra. The results were further validated using dynamic light scattering.

#### 2. EXPERIMENTAL SECTION

**2.1. Reagents and Materials.** Lysozyme with >90% purity and sinapic acid with about 99% purity were purchased from Sigma-Aldrich. BSA at pH 6–7 of about 96% purity was purchased from SRL, India. Tetrachloroauric acid trihydrate (HAuCl<sub>4</sub>·3H<sub>2</sub>O) was purchased from CDH chemicals with 49% gold content. Sodium hydroxide (Rankem, India) was purchased locally. All the chemicals were used without further purification. Milli-Q water was used in all the experiments.

**2.2.** Synthesis. Au<sub>QC</sub>@Lyz was prepared by a method reported previously. Briefly, 1:2, 1:4, and 1:8 of Lyz:Au<sup>3+</sup> were

mixed and stirred for some time to get the Au<sup>+</sup>–Lyz adduct. After that, the pH of the solution was elevated to 12 by NaOH, and the solutions were further incubated to get red luminescent  $Au_{QC}$ @Lyz. This luminescence appeared over a period of 12 h. For adduct and nanoparticle formation, the same procedure was followed without the addition of NaOH to the system. For BSA protected clusters, 1:5, 1:10, and 1:20 BSA:Au<sup>3+</sup> were used, and the same procedure was followed, as described in the case of Lyz.

2.3. Instrumentation. For MALDI TOF MS analysis, an Applied Biosystems Voyager De Pro instrument was used with sinapic acid as the matrix. A pulsed nitrogen laser of 337 nm was used for ionizing the sample. Spectra were collected in the linear positive mode and an average of 250 shots was used for each spectrum. The matrix was prepared by dissolving 10 mg of sinapic acid in a 1:3 mixture of acetonitrile: 0.1% trifluoroacetic acid (overall volume of 1 mL). While preparing the samples for analysis, 5  $\mu$ L of the cluster solution (without dilution) was mixed thoroughly with 100  $\mu$ L of the matrix mixture. About 2.5  $\mu$ L of the resulting mixture was used for spotting. Luminescence measurement was carried out in a Jobin Yvon NanoLog fluorescence spectrometer with a band-pass of 3 nm for both emission and excitation spectra. UV-vis spectra were collected using a PerkinElmer Lambda 25 spectrometer in the range of 200-1100 nm. High-resolution transmission electron microscopic (HRTEM) images were taken using a JEOL 3010 instrument. DLS and zeta potential measurements were done using a Malvern Zetasizer ZSP instrument equipped with a 633 nm (He–Ne) laser.

2.4. Experimental Details of Transmission SAXS Measurements. Transmission SAXS experiments were performed to study the formation and growth of Au<sub>OC</sub> inside the protein, Lyz. Experiments were carried out as a function of incubation time. We have performed SAXS experiments at different pH and precursor concentrations to observe the change in the overall protein size in a wide time range. The SAXS profiles of the sample solutions were collected in transmission mode using a Rigaku Smart Lab X-ray diffractometer (9 kW; Cu $-K_{\alpha}$ radiation;  $\lambda = 1.54059$  Å). Transparent borosilicate capillary tubes (~1.5 mm internal diameter) containing the sample solutions were mounted at a fixed position on a sample holder. Sample alignment and premeasurement scan were carried out before each SAXS measurement. The maximum time taken for each SAXS data acquisition was ~20 min. The NANO-Solver software of Rigaku was used to solve the SAXS profiles. Prior to the data-fitting step, the SAXS profiles of the sample solutions were corrected for background contributions (originating from the solvent and the sample holder).

#### 3. RESULTS AND DISCUSSION

**3.1. Formation of Protein Protected Clusters.** As luminescent noble metal clusters protected with proteins have been investigated extensively, we introduce only their essential aspects in the following. They are formed upon incubation of the protein with the metal ions for 12 h at a basic pH, and the appearance of the cluster is manifested by the presence of an intense red luminescence upon UV excitation (Figure 1). These clusters in the case of Lyz show an emission at 690 nm when excited at 365 nm (see below, Figure 1).<sup>24</sup> As of now, the most widely applied property of such protein protected clusters is their strong luminescence, which has made them useful for sensing, biolabeling, and bioimaging. Cluster formation in such cases proceed through an Au(I)-Lyz complex that we call *adduct* in the subsequent discussion. Such adducts exist in the Au(I) form in

complexation with cysteine residues of the protein. A maximum of 10 Au attachments were seen in such cases with each Lyz molecule when the highest concentration of Au<sup>3+</sup> was used.<sup>65</sup> During cluster formation, these adducts get reduced to Au(0) involving interadduct metal transfer and consequent release of free protein (while some proteins contain clusters within them).<sup>24,36</sup> Depending on the protein involved in cluster formation, various gold cluster cores have been identified in different proteins; among which the most important are Au<sub>8,25</sub>@BSA,<sup>34,36</sup> Au<sub>13,25</sub>@Lf,<sup>36,37</sup> and Au<sub>10,11,12</sub>@Lyz<sup>24</sup> although many other proteins have been examined.

Proteins differ by their shape and size. Between Lyz and BSA, Lyz is more spherical,<sup>45</sup> therefore, we have used Lyz as a model protein for our study. Lyz is a small protein (molecular weight 14.3 kDa) with 129 amino acids among which 8 are cysteines forming 4 disulfide bonds. The overall surface structure of the protein shows nearly spherical geometry with mean diameter (considering three-dimensional orientation) of about 3.9 nm.<sup>45,47</sup> We have studied the formation and growth of gold clusters using SAXS, and the data were compared with mass spectrometric evolution of the clusters within the protein. It is known from a previous study that when protein interacts with Au<sup>3+</sup>, it unfolds to accommodate the Au ions inside. This can lead to the breakage of disulfide bonds and finally the protein's secondary structure is lost (partially).<sup>36,65</sup> When the protein interacts with Au ions, it may result in a free protein (without any Au attachment) or a protein with single or multiple Au attachments.<sup>36</sup> Considering the existence of all these species, we expect to get a broad size distribution due to the presence of various stages of unfolded proteins. This is reflected in the mass spectrum of Au<sup>+</sup>-Lyz, where we can see multiple peaks corresponding to Au attachments, including the parent protein peak.<sup>65</sup> When NaOH is added to the system, the Au ions start rearranging to form a cluster core, and we are supposed to get only a few possible species present in the system like, some free protein as well as Au attached proteins (not converted to cluster core) and mostly, cluster core containing proteins.<sup>24</sup> All these together should show reduced broadness in the size profile, largely because the cluster core distributions are narrower than the adduct distributions.

**3.2. Solution Phase SAXS Analysis of the Samples.** The as-prepared samples were used for SAXS analysis without further purification. Here we briefly describe the working principle of SAXS which may help understand of our results. The discussion is kept to the essential aspects. Particles in the range of 1-50 nm can efficiently scatter the incident X-ray. The larger the electron density difference between the solvent medium and solute particles ( $\Delta\rho$ ), the higher the scattering intensity [I(q)]. The scattering amplitude [A(q)] is calculated from the Born approximation as

$$A(q) = \sqrt{\int \Delta \rho(r) e^{iq \cdot r} dr}$$
(1)

$$q = (4\pi/\lambda) \cdot \sin(2\theta/2) \tag{2}$$

where, q is the scattering vector, V is the volume for the integral over the entire medium, and  $\lambda$  is the wavelength of the X-ray.

The scattering intensity is expressed as

$$I(q) = A(q)[A(q)]^*$$
(3)

If the particles possess definite shape, then I(q) is generalized in terms of the structure factor [S(q)] and the form factor [F(q)]as follows:

$$I(q) = |F(q)|^2 S(q) \tag{4}$$

The form factor, F(q), is related to the shape of the particle.

(i) For spherical particles with a radius of *R*, the form factor is expressed as

$$[F(q, R)]_{\text{sphere}} = \Delta \rho \cdot \frac{4\pi \{\sin(qR) - qR \cos(qR)\}}{q^3}$$
(5)

(i) In the cylinder model, form factor considers the contribution from aspect ratio (*a*) of the cylinder (or rod):

$$[F(q, \varphi, a, R)]_{\text{cylinder}} = \Delta \rho \cdot \frac{4\pi R^3 a \{\sin(q\alpha R \cos \varphi)(qR \sin \varphi\}}{(qR)^2 \sin \varphi \cos \varphi}$$
(6)

where,  $\varphi$  is the angle formed between the scattering vector and the normal to the surface of the rod.

For irregular particles or in case of particles of unknown shapes "Model Free Analysis" is often helpful, in which the B-spline function is first adopted to generate a "true" scattering profile with scattering intensity,  $I^{T}(q)$  from the raw SAXS profile as given in eq 7:

$$I^{T}(q) = \sum_{i=0}^{N} S_{i}(q - a_{i})^{p_{i}} \cdot (b_{i} - q)^{r_{i}}$$
(7)

Fourier transform of the true scattering profile generates the correlation function [C(R)], which relates to the interparticle interactions and the distance distribution function p(r) as follows:

$$C(R) = 1/R \cdot \{ \int q I^T(q) \sin(qR) \, \mathrm{d}q \div \int q^2 I^T(q) \, \mathrm{d}q \}$$
(8)

$$p(r) = 4\pi R^2 \cdot C(R) \tag{9}$$

The structure factor, introduced in eq 4, depends upon the interparticle distance and particle–particle arrangements, i.e., it indicates the correlation between particles in a system. Mathematically,

$$S(q) = 1 + \int \{n(r) - n_0\} \cdot e^{iq \cdot r} dr$$
(10)

where n(r) is the particle count density function. Usually, shortrange interparticle correlations are observed from SAXS analysis. In dilute solutions, the particles are considered to be randomly distributed such that the interparticle distances are very large.<sup>66</sup>

We chose the "sphere" and "cylinder" models of NANO-Solver software, and the simulated profiles are generated based upon eqs 5 and 6. We have considered S(q) = 1 while solving the SAXS profiles using these models.<sup>66</sup> The best profiles are those for which the residual between the raw and simulated profiles (residual factor) is the lowest. To minimize the error in evaluation of average sizes, we have considered only the best fit (simulated) profiles with residual factors less than or around 4%. Normalized dispersion value ( $\sigma$ ) is an important parameter that gives us an idea about the polydispersity of particles. For example, a solution containing monodisperse particles with narrow size distribution will have a low  $\sigma$  value. Also, correlation function [C(R)] and distance distribution functions [p(r)] have been evaluated through the "Model Free Analysis" program of Nanosolver to observe short-range interparticle interactions in the protein solutions under the reaction conditions. It is to be noted that "interparticle distance" shall be recognized as the

distance between centers of two neighboring particles, X and Y, arranged in any possible manner.

To select a suitable model for our interpretation of the solution phase growth of clusters in proteins, the SAXS profiles of pure Lyz and BSA solutions were fitted using both the models (See parts A and B of Supporting Information Figure S1). From the cylinder model, optimized simulated profiles could be generated only when the aspect ratio (a) was fixed in the range of 0.80-1.00for both Lyz and BSA. It can be clearly seen that by choosing either of these models similar fittings (with the same residual factor) could be obtained in each of the proteins as shown in Figure S1. However, the nature of the size distribution curves of Lyz (shown in Figure S1C) evaluated from the cylinder and the sphere models differs widely; the size of Lyz (and BSA; see Figure S1D) obtained from the sphere model is more in agreement with its reported value (3.9 nm). Moreover, a cylindrical model with aspect ratio in the range of 0.8-1.00 indicates that the particles are more or less spherical and so the simpler sphere model is a better choice. Thus, considering the above observations, we have used the sphere model in the subsequent analyses.45,47

**3.3. Evolution of Gold Cluster in Lyz.** Figure 1A shows that the simulated SAXS profile of Lyz changes significantly after the addition of Au<sup>3+</sup> in the system (Au<sup>+</sup>–Lyz and Au<sub>10</sub>@Lyz). Size profiles of Lyz, Au<sup>+</sup>–Lyz and Au<sub>10</sub>@Lyz have been presented in Figure 1B. Initially, pure Lyz solution shows a narrow size distribution with an average size of 3.9 nm along with the presence of large aggregates (~11.1%; not shown in the figure for simplicity). The average size of this adduct does not change significantly with time (see Figure S2) or at higher pH (Figure S3). However, upon addition of NaOH, the size profiles become relatively broader, possibly due to unfolding of the protein at basic pH (See Figure S4). In all the cases studied here, some amount of aggregates is always present (10–20%). Due to the broad distribution, it is not wise to conclude any size changes in the aggregate region (20 nm and above). The average size of

Lyz increases to 4.6 nm due to Au<sup>+</sup>-Lyz adduct formation. As multiple Au attachments (maximum 10 Au) are possible, we have observed a relatively broader size distribution in this case. Upon reduction of the adducts in basic medium, luminescent clusters (Figure 1C) form with an overall protein size of 4.7 nm (Au<sub>10</sub>@Lyz). Thus, the difference in size of Lyz protein before and after cluster formation is 0.8 nm (3.9 nm for Lyz). The cluster obtained in this process shows emission at 690 nm when excited at 365 nm as shown in Figure 1C. The as-formed cluster showed a mass difference of 10 atoms of Au from the parent protein peak in MALDI MS (Figure 1D). The core size remains the same without any further change with time. A core size of 0.8 nm from SAXS is consistent with the size obtained in TEM (1 nm as shown in Figure 1E). The C(R)profiles of Lyz shows two peaks at 6.31 and 10.82 nm corresponding to the nearest Lyz-Lyz interparticle distances (Figure S5 A). The C(R)-distance profile of Au<sub>OC</sub>@Lyz resembles that of the original Lyz solution and incubation of these Au<sub>OC</sub>@Lyz (up to 48 h) does not change the correlation pattern. This shows that the cluster formation in Lyz does not involve much change in its overall structure except for the expansion in its size observed from the size distribution profiles (see Figure 1B).

We have conducted a concentration-dependent SAXS study by varying the Au<sup>3+</sup> concentration, keeping Lyz concentration the same (see Figure 2 and Figure S6). During this study, the core size, analyzed by sphere model, does not change much.<sup>24</sup> This was explained in terms of the size of Lyz. Being a small protein, Lyz cannot accommodate larger cluster cores inside it. Less number of cysteines does not help in stabilizing a bigger core. The simulated SAXS profiles of different concentrations of Au<sup>3+</sup> reveal that the scattering pattern changes a bit and the corresponding size profile shows core sizes ranging from 0.7 to 0.9 nm (0.7, 0.8, and 0.9 nm for 1:2, 1:4, and 1:8 Lyz:Au<sup>3+</sup>, respectively). Mass spectral study shows that Au<sub>10-12</sub> cores are possible inside a single molecule of Lyz (see Table S1 for details)



**Figure 1.** (A) Difference in the SAXS profiles of Lyz, Au<sup>+</sup>-Lyz *adduct* and Au<sub>QC</sub>@Lyz. Size distribution obtained from the SAXS profile is shown in panel B. A 0.8 nm core is observed after cluster formation. The overall state of the protein is schematically represented in the background of each plot. (C) Fluorescence spectra of Au<sub>QC</sub>@Lyz. In the inset, photographs of Au<sub>QC</sub>@Lyz under UV and visible light are shown. (D) Comparative MALDI MS spectra of Lyz, Au<sup>+</sup>-Lyz and Au<sub>QC</sub>@Lyz. In the case of Au<sup>+</sup>-Lyz, distinct separation of peaks spaced by m/z 197 due to Au is observed. In Au<sub>QC</sub>@Lyz, however, a separation of 10 Au atoms from the parent protein peak due to an Au<sub>10</sub> core is seen. (E) A cluster core of 1 nm was observed in TEM. Some of them are circled.

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**Figure 2.** (A) Schematic representation of the concentration-dependent growth of  $Au^+$ –Lyz adduct resulting in  $Au_{QC}$ @Lyz. (B) SAXS patterns of  $Au_{QC}$ @Lyz samples after 24 h incubation, at various ratios of Lyz: $Au^{3+}$ . (C) Size distributions of different  $Au_{QC}$ @Lyz samples after 24 h of incubation.



**Figure 3.** (A) Time-dependent SAXS spectra of  $Au^+$ -Lyz up to 48 h of incubation, when nanoparticles are formed. (B) Corresponding size distribution showing a larger distribution in size, with reduced mean, indicating fragmentation of the protein. (C) UV-vis absorption spectra of  $Au^+$ -Lyz showing a plasmonic feature after 48 h due to nanoparticle formation. (D) TEM image of the Au nanoparticles formed after 48 h of incubation.

in this entire concentration range. These data clearly suggest that it is indeed possible to observe the solution phase growth of the cluster inside a protein, through SAXS.

The change in size was further supported by DLS, which gives the hydrodynamic diameter of the whole particle, including the protein. This study is very much sensitive to concentration (aggregation can happen at higher concentrations), pH and other factors. All the samples were analyzed at same experimental conditions to avoid errors. Lyz shows a size of 3.2 nm, which increases to 4.2 nm after  $Au^{3+}$  addition while  $Au_{QC}@Lyz$  has a size of 4.5 nm (see Table 1 and Figure S7). The data are in good agreement with the SAXS result. We have also probed the change in protein during cluster formation through zeta potential measurements. The same sample used for DLS analysis was used for



**Figure 4.** (A) SAXS profile of  $Au_{QCs}$ @BSA showing a completely different kind of scattering pattern from the parent BSA as well as Au<sup>+</sup>–BSA. (B) Corresponding size profile showing the presence of about 1.4 nm cluster core. (C) MALDI MS showing the presence of  $Au_{33}$  cluster core.

#### Table 1. Comparative Size (SAXS and DLS) and Zeta Potential for Lyz and Related Systems

sample	size from SAXS (nm)	size from DLS (nm)	zeta potential (mV)
Lyz	3.90	3.20	+12.2
Au <sup>+</sup> -Lyz	4.61	4.20	+39.3
Au <sub>10</sub> @Lyz	4.68	4.50	-29.9

zeta potential measurement. Lyz shows a positive zeta potential (+12.2 mV), which increases to +39.3 mV when excess positive charge is introduced in terms of  $Au^{3+}$  in the system. When clusters form at basic pH, zeta potential becomes negative (-29.9 mV) (see Table 1).

3.4. Growth of Au<sup>+</sup>-Lyz Adduct to Nanoparticles. When Au<sup>+</sup>-Lyz adducts are incubated for longer time (48 h) without changing the pH of the solution, they form Au nanoparticles, distinctly different from luminescent clusters. Formation of nanoparticles is indicated by the appearance of a purple colored solution. Corresponding changes were observed in the SAXS (as well as size) profiles. Size of protein decreases considerably when nanoparticles form (see Figure 3A,B). UV-vis absorption spectra show (Figure 3C) the presence of a plasmonic feature at 527 nm due to the presence of polydispersed Au nanoparticles (Au NPs). The core size of the particles was confirmed by transmission electron microscopic (TEM) analysis. Particles of different shape ranging from 5 to 20 nm in size were observed in TEM. When nanoparticles grow, due to the smaller cavity inside the protein, it cannot accommodate the growing particles within. As a result, nanoparticles come out from the protein cavity causing fragmentation of the protein molecules and

decrease in overall size of the protein. This was supported by increase in the size distribution with reduced mean size (see Table S1 for details).

**3.5. Cluster Growth in BSA.** To check the validity of SAXS in studying the growth of Au clusters inside proteins in general, we have performed similar experiments with another protein, BSA. It is a widely used serum protein (molecular weight 66.7 kDa) containing 583 amino acids. BSA contains 35 cysteine residues, which form 17 internal disulfide bonds, causing 9 internal loops in the tertiary structure resulting in three primary domains, each containing one small and two large loops. These disulfide bridges are the basis of its compact heart-shaped (equilateral triangle) structure. Two loops are of equal size of 9 nm while the other smaller one is 4 nm.<sup>44</sup> Considering the three-dimensional orientation of these different lobes, mean size comes around 7.3 nm.

The size distribution profiles of native BSA showed two peaks with the 7.4 nm as the major component (64% population) as shown in Figure 4A,B. Some fragments are observed with 2.7 nm in size in the parent solution itself. These fragments are also seen in MALDI MS, with about 13 kDa mass (see Figure 4C for details). Addition of  $Au^{3+}$  facilitates adduct formation; however, this did not show any considerable change in the overall SAXS profile other than an increase in overall size of the protein to 8.3 nm (Figure 5). Addition of NaOH to the as-formed adduct solution drastically changed the overall scattering pattern as well as the size distribution of BSA. After addition of NaOH to the adduct, size of BSA increased to 8.7 nm, which remained almost unchanged (8.8 nm) on further incubation for 24 h (after appearance of red luminescence). So the cluster core size is

about 1.4 nm (size of BSA is 7.4 nm); slightly more than the size observed in TEM reported for this cluster (1.1 nm). After 48 h, the size of BSA in was about 8.9 nm. From a previous study on the time-dependent evolution of BSA protected gold clusters, we know that these clusters grow to bigger cores (Au<sub>25</sub> to Au<sub>38</sub> and higher) with time and hence such minute increase in SAXS size is justified.<sup>36</sup> The C(R)-distance profiles generated from BSA, Au<sup>+</sup>-BSA adduct, and Au<sub>OC</sub>@BSA have been presented in Figure S5C. The C(R)-distance profile of BSA reveals several interparticle interactions at distances of 3.00, 5.30, 7.67, and 10.10 nm and the corresponding p(r)-distance shows numerous oscillations, characteristic to its multidomain nature. The C(R) pattern of Au<sup>+</sup>-BSA adduct (at pH = 12, at 0 h) is similar to the native BSA solution. Prominent change in the C(R)functions during the growth of Au<sub>OC</sub>@BSA (24-48 h) suggests conformational modifications in the structure of BSA under the reaction conditions. The isoelectric point of BSA is 4.7, and structural change is more likely to happen in this case during cluster formation. BSA also has higher amount of helical structures, which also get affected during cluster formation. This kind of conformational modification through loss in helicity has been reported already for such protein protected clusters.32

From the mass spectrum of  $Au_{QCs}@BSA$ , it is observed that at specific  $Au^{3+}$  concentration,  $Au_{33}$  core forms inside BSA, and the cluster can be assigned as  $Au_{33}@BSA$ . Being a bigger protein, BSA is capable of uptaking more Au inside it. Cluster core size varies linearly with concentration of  $Au^{3+}$  used. For higher Au concentration (1:20), 9.2 nm sized BSA was observed. SAXS data show that 0.6 to 1.7 nm Au cluster cores are possible when  $Au^{3+}$  concentration is varied from 1:5 to 1:20 (BSA: $Au^{3+}$ ; see Table 2).

After 48 h of incubation of the Au<sup>+</sup>–BSA adducts, nanoparticles form and the size of the protein decreases. The same kind of behavior was also observed for Lyz. Increase in Au concentration increases the amount of Au uptake, which is reflected from the increase in size of BSA. A clear increase in fragmentation can also be observed from MALDI MS data (see Figure S8). TEM analysis of these nanoparticles shows their polydispersed nature similar to the case of Lyz.

Formation of nanoparticles was further confirmed by UV-vis absorption spectrum showing the 540 nm plasmonic feature. DLS studies shows that the size of BSA (7.4 nm) increases to 8.4 nm when Au<sup>3+</sup> was introduced in the system (see Figure S9). Incubation of the Au<sup>+</sup>–BSA adduct resulted in nanoparticles after 48 h. Two different prominent peaks were observed when the nanoparticle sample was subjected to DLS. A 5.4 nm peak which corresponds to the fragmented protein and the other peak at 20 nm correspond to Au NPs. Change in protein was further monitored using zeta potential study and a comparison is shown in Table 3.

 Table 2. Comparative Size of BSA and Related Systems from

 SAXS Profile

sample	BSA:Au <sup>3+</sup>	time of incubation (h)	average particle size from SAXS (nm)
BSA		0	7.45 and 2.73
BSA+NaOH		0	7.08 and 3.06
Au <sup>+</sup> -BSA	1:10	0	8.29 and 3.63
Au <sup>+</sup> -BSA	1:10	24	8.61 and 3.52
Au <sup>+</sup> -BSA (nanoparticle)	1:10	48	6.81 and 2.50
Au <sub>QC</sub> @BSA	1:5	0	7.91 and 2.85
Au <sub>QC</sub> @BSA	1:5	24	8.04 and 2.69
Au <sub>QC</sub> @BSA	1:10	0	8.75 and 3.23
Au <sub>QC</sub> @BSA	1:10	24	8.84 and 3.28
Au <sub>QC</sub> @BSA	1:10	48	8.87 and 2.47
Au <sub>QC</sub> @BSA	1:20	0	9.16 and 3.32

# Table 3. Comparative Sizes (SAXS and DLS) and Zeta Potentials for BSA and Related Systems

sample	size from SAXS (nm)	size from DLS (nm)	zeta potential (mV)
BSA	7.30	7.40	-30.3
Au <sup>+</sup> -BSA	8.29	8.40	+18.6
After Au NP formation	6.81	5.40 and 20.30	+32.1
Au33@BSA	8.84	9.00	-39.3



**Figure 5.** Formation of Au NPs is schematically represented in A along with the corresponding size of BSA. B is showing time dependent SAXS profile of the formation of  $Au_{OC}$ @BSA. Change in SAXS profile due to  $Au^{3+}$  concentration is shown in C.

#### 4. SUMMARY AND CONCLUSIONS

In summary, we have presented a detailed SAXS and mass spectrometric study of the formation and growth of luminescent Au clusters inside a small protein, Lyz, for the first time. SAXS data shows the presence of a 0.8 nm core, which is in agreement with the Au<sub>10</sub> cluster seen in MALDI MS. Due to the smaller size and availability of less number of cysteine residues, the core size does not change significantly upon further increase in Au<sup>3+</sup> concentration, which was previously observed to be in the range of Au<sub>10-12</sub>. Nanoparticle growth can also be monitored using time dependent SAXS along with other microscopic (TEM) and spectroscopic (UV-vis) techniques. We have shown a similar kind of study of cluster growth with a larger protein, BSA. In this case, due to the unsymmetrical structure of the protein, we see an average three-dimensional distribution of the size. The data confirm the presence of Au clusters protected by proteins, in solution.

As of now, none of the protein-protected clusters could be crystallized. There is always a question of whether these clusters form inside a protein cavity or are they an artifact in the MALDI MS. This concern exists as ESI MS is not reported of the same clusters. The present work opens up new possibilities for studying solution phase structure of macromolecule-protected clusters. This study suggests that SAXS can be used to visualize cluster growth in solution regardless of the protein structure. Such studies may be extended to other proteins and several other macromolecules in which cluster growth has been observed.

#### ASSOCIATED CONTENT

#### **S** Supporting Information

SAXS profiles and corresponding size of BSA, Lyz and their Au adduct at different time and pH, correlation function and distance distribution function versus distance profiles in Lyz and BSA systems, DLS of BSA and Lyz systems, time-dependent MALDI MS of Au<sup>+</sup>-BSA, table of SAXS parameters of Lyz and Au<sup>+</sup>-Lyz at different conditions, and details of SAXS parameters of Au<sub>QC</sub>@Lyz at different conditions are shown in the Supporting Information. 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

A.B., J.S.M., and T.P. thank the Department of Science and Technology, Government of India for continuous support of our research program on nanomaterials. A.B. and A.M. thank the Council of Scientific and Industrial Research (CSIR) for fellowships. A.B. and J.S.M. thank Swathi R. for help in measuring DLS and zeta potential data. T.P. and H.L. thank an India–Korea research project (Department of Science and Technology, Government of India, in India and National Research Foundation, Korea) for funding.

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