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Green Synthesis of Protein-Protected Fluorescent Gold Nanoclusters (AuNCs): Reducing the Size of AuNCs by Partially Occupying the Ca^{2+} Site by La^{3+} in Apo-α-Lactalbumin

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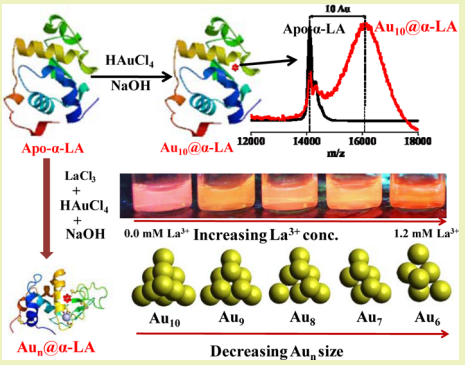
Supporting Information

ABSTRACT: In this paper, we report the studies relevant to controlling the size of protein-protected gold nanoclusters (AuNCs). In order to demonstrate this, we have chosen bovine apo α-Lactalbumin (apo-α-LA), which has a specific binding site for Ca^{2+}, and La^{3+} occupies this position when apo-α-LA is treated with lanthanum trichloride. When the Apo-α-LA is treated with Au^{3+}, it results in the formation of a protein-coated Au_{10} nanocluster where the protein reduces Au^{3+} to Au^0 and protects the nanoclusters (apo-α-LA-AuNCs) which are luminescent. In these protein-protected luminescent gold nanoclusters, the protein is involved both in reduction as well as protection, thereby supporting green synthesis. As La^{3+} occupies the Ca^{2+} site in apo-α-LA, the size of AuNCs formed is reduced to smaller than the Au_{10}, where the size is dependent on the extent to which La^{3+} is bound to the protein with a concomitant increase in luminescence. The apo-α-LA-AuNCs and the same formed in the presence of different concentrations of La^{3+}-bound protein were all characterized by analytical, spectral, and microscopy techniques. Control of the size of AuNCs formed was also studied by using Gd^{3+} instead of La^{3+} and found similar results. In particular, the size variation of AuNCs was clearly demonstrated by MALDI-TOF-MS and HRTEM. Thus, the apo-α-LA protein-coated gold nanocluster is a useful green material for suitable applications.



KEYWORDS: *Apo-α-lactalbumin, Protein-protected gold nanoclusters, Cluster size control, Isothermal titration calorimetry, MALDI-TOF-MS, HRTEM*

INTRODUCTION

The nanoscopic noble metal clusters can be grossly categorized into three different types based on their size, such as large nanoparticles, small nanoparticles, and nanoclusters.[1] Gold nanoclusters (AuNCs), by definition, are made up of a few to a hundred atoms, being 2−3 nm size, known as quantum clusters or nanoclusters.[2−4] For such clusters, their small core size can be compared to the Fermi energy (0.8 nm) of the free electrons.[5,6] As a result, the clusters show a significant difference in their optical and electronic properties such as quantum-confined, size-tunable electronic transitions from the other two categories.[7−9] Recently, protein-protected nanoclusters emerged as useful and functional materials possessing interesting properties.[10,11] The literature started witnessing reports on the development of green synthesis of AuNCs, where no external reducing and stabilizing agents were used,[12−17] and instead some proteins and peptides are being used. This includes lysozyme, pepsin, trypsin, transferrin, human serum albumin, bovine serum albumin, insulin, and horse radish peroxidase.[18−24] An important outcome of all these studies is the near constancy of luminescence, emission wavelength irrespective of core size, and almost complete absence of any distinct features of nanocluster core absorption.[18,22−26] This prompted the nanocluster community to consider the emission as being dominated by an Au^+−ligand complex shell as an outer shell of an Au cluster.[27,28] While a large number of nuclei of nanoclusters have been analyzed by mass spectrometry, the difficulty associated with obtaining accurate mass analysis of such clusters has limited our understanding. Added to this is the poor electrospray ionization efficiency of protein-protected nanoclusters.[29,30]

Many proteins have a well-defined binding site for specific metal ions, and α-lactalbumin (α-LA) is one such globular protein from milk possessing a specific binding site for Ca^{2+}. It has 123 amino acids with a molecular weight of 14.2 kDa. α-LA is composed of eight cysteine residues which are present as four disulfide cross-links, in addition to the presence of four tryptophans and three tyrosines.[31−33] The protein α-LA has high affinity to bind to La^{3+} in the Ca^{2+} binding site. In this protein, the binding core is very attractive to synthesize gold

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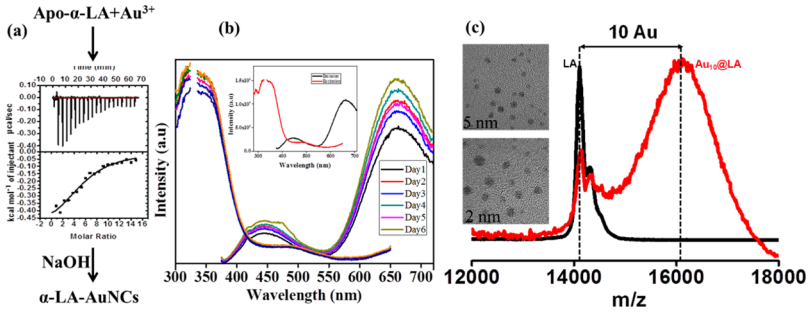

Figure 1. (a) ITC thermogram of apo-α-LA with Au^{3+}. (b) Time-dependent fluorescence spectra of α-LA-AuNCs at a molar ratio of 1:8; on excitation at 365 nm, inset emission and excitation spectra. (c) MALDI-TOF-MS spectra of α-LA:Au at 1:8 ratio, apo-α-LA (black) and α-LA-AuNCs (red) indicate that the presence of 10 gold atoms formed the core in the protein; inset HRTEM micrographs of α-LA-AuNCs.

nanoparticles since the site can be blocked with reasonable control by metalation using La^{3+}. This may potentially limit the gold cluster size formed by the protein.

In this paper, we report the first efficient synthesis of gold nanoclusters (AuNCs) using apo-α-LA, as well as the same upon metalation of the protein by La^{3+} to different extents since La^{3+} occupies the Ca^{2+} core in the protein. The effect of all these on controlling the growth of AuNCs cluster size (varying from 6 to 10 gold atoms) has been well studied by us in this work. Although two of the earlier papers report AuNCs coated with α-LA, the size control of the nanoclusters and their luminescent properties were not addressed.[13,34] Both the nanocluster size and their luminescence properties were well addressed and correlated in this study. α-LA-coated AuNCs showed red emission. Thus, the present study gives new insights into the protein-protected cluster system where the partial occupancy of the metal ion binding core leads to a newer dimension of AuNCs.

EXPERIMENTAL SECTION

Apo-α-lactalbumin (apo-α-LA), lanthanum chloride hexahydrate ($LaCl_3 \cdot 6H_2O$), tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$), and sinapic acid were procured from Sigma-Aldrich Chem. Co. and used without further purification. Milli-Q water was used for all the experiments.

Synthesis of α-LA-AuNCs. The protein-coated gold nanoclusters (α-LA-AuNCs) have been synthesized by adopting a procedure reported in the literature but with some modifications.[7] In order to make gold nanoclusters (α-LA-AuNCs), a mixture of 0.35 mM of apo-α-LA and 2.44 mM of $HAuCl_4 \cdot 3H_2O$ in equal volumes (1 mL of each) were taken to give a molar ratio of 1:8 (protein:Au^{3+}), and then, the mixture was treated with 1N NaOH (0.1 mL) that was added slowly dropwise, and the resultant solution was stirred for 12 h at room temperature.

Synthesis of α-LA-AuNCs in Presence of La^{3+}. Initially, a complex of α-LA with La^{3+} was prepared by adding different amounts La^{3+} ions ($LaCl_3$) to 0.35 mM of α-LA followed by stirring for 10 min. To this mixture, 2.43 mM $HAuCl_4$ (1 mL) and 1 N NaOH (0.1 mL) were added and continuously stirring for 12 h at room temperature. For the synthesis, four different concentrations, viz., 0.1, 0.4, 0.8, and 1.2 mM (40 μL), of La^{3+} were used, while the concentrations of apo-α-LA and Au^{3+} were kept constant. Synthesized protein-coated AuNCs showed red color luminescence under UV light and were used for further characterization.

Spectroscopy. UV−visible absorption studies were performed on a PerkinElmer Lambda 25 spectrometer. Two hundred microliters of the sample was taken and diluted to 2 mL using Milli-Q water in a cuvette, and the absorption spectra were measured at a scan rate of 200 nm/min in the region from 200 to 1100 nm. Fluorescence spectra were measured on a Jobin Yvon NanoLog fluorescence spectrometer with a slit width of 3 nm for both excitation and emission spectra. All the other details are same as those used for the absorption spectra. Far−UV CD spectra were recorded on a JASCO-810 using quartz cuvette with a 0.1 cm path length. CD spectra were accumulated at room temperature at a scan speed of 100 nm/min in the range of 190−270 nm. Each time, 0.5 mL of sample was taken for CD measurement. In order to confirm that the conformational changes were not affected by simple dilution, the CD spectrum of apo-α-LA was recorded in water at different concentrations and found no changes, indicating that the dilution has negligible effect.

MALDI-TOF-MS. For matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra, 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 positive mode, and an average of 250 shots was used for each spectrum. For the preparation of the matrix, 10 mg of sinapic acid is dissolved in a 1:2 ratio of acetonitrile and 0.1% trifluoroacetic acid. Here, 5 μL of the cluster sample was thoroughly mixed with 100 μL of sinapic acid matrix and drop-casted on the MALDI plate.

ICPAES. For inductively coupled plasma atomic emission spectroscopy (ICPAES), 1 mL of sample from the stock solution was taken and diluted to 5 mL with water. The sample was kept for dialysis for 12 h at RT by changing the water at least three times. The sample was analyzed with ICPAES.

Microscopy. High resolution transmission electron microscope (HRTEM) experiments have been carried out on a JEOL JEM 2100-F transmission electron microscope operating at 20−200 kV (resolution 2.4 Å). Carbon-coated copper grids with 200 mesh have been used as substrate. Here, 20 μL of sample was drop-casted on a TEM grid and allowed to air dry.

RESULTS AND DISCUSSIONS

Synthesis of Apo-α-LA-AuNCs. It is known from the literature that carboxylic- and amine-containing amino acids are responsible for Au^{3+} ion uptake to form protein−gold complexes in neutral to basic conditions. Further, by increasing the pH to 12, Au^{3+} ions are reduced to Au^0 by the concomitant oxidation of tyrosine and/or tryptophan residues, and the resultant gold cluster is stabilized by thio groups of the protein.[31−35]

α-LA-AuNCs were synthesized by the treatment of apo-α-LA with the Au^{3+} solution under alkaline conditions as given in the Experimental Section. In the present work, no $NaBH_4$ or any other chemical reducing agent is used for the preparation of any of AuNCs reported in this paper. Upon addition of Au^{3+} into the solution of apo-α-LA, Au^{3+} ions bind to the protein, and the binding was confirmed by an ITC study. The ITC data for apo-α-LA with Au^{3+} (Figure 1a) showed the number of binding sites (N) being 5.85 ± 0.46, with a binding constant of (K_a) (6.72 ± 1.7) × 10^3 M^{-1}. However, La^{3+} titration of this protein shows about a 20-fold stronger binding than that of Au^{3+} but

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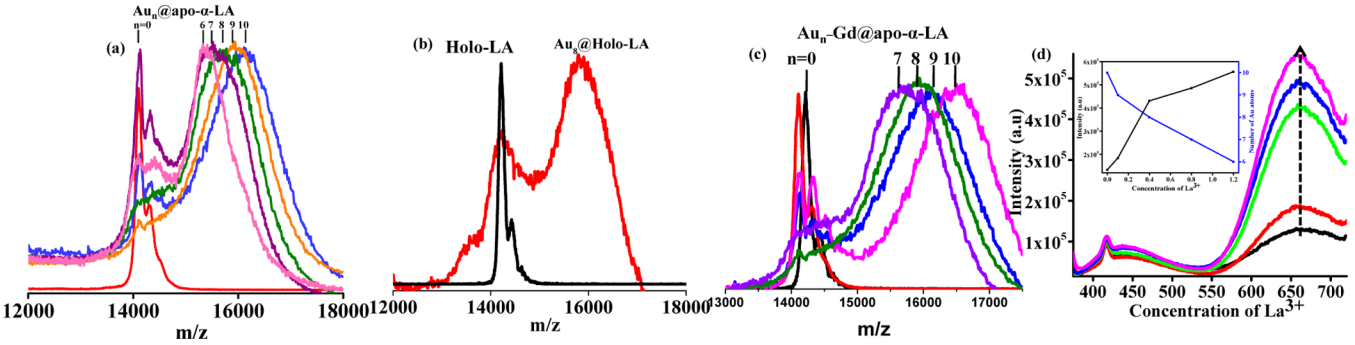

Figure 2. (a) MALDI-TOF-MS spectra of concentration-dependent La^{3+}-incorporated α-LA: apo-α-LA (black), apo-α-LA at pH 12 (red), α-LA-0.0 mM AuNCs (blue), α-LA-0.1 mM AuNCs (orange), α-LA-0.4 mM AuNCs (green), α-LA-0.8 mM AuNs (purple), and α-LA-1.2 mM AuNCs (magenta). (b) MALDI-TOF-MS spectra of holo-α-LA-bound AuNCs: holo-α-LA (black), Au_8-holo-α-LA (red). (c) MALDI-TOF-MS spectra of concentration-dependent Gd^{3+}-incorporated α-LA: apo-α-LA (black), apo-α-LA at pH 12 (red), α-LA-0.0 mM AuNCs (pink), α-LA-0.1 mM AuNCs (blue), α-LA-0.4 mM AuNCs (green), and α-LA-0.8 mM AuNs (purple). (d) PL spectra of concentration-dependent La^{3+}-incorporated α-LA-AuNCs: 0.0 mM La^{3+} (black), 0.1 mM La^{3+} (red), 0.4 mM La^{3+} (green), 0.8 mM La^{3+} (blue), and 1.2 mM La^{3+} (magenta). Inset: Plot of emission intensity at 660 nm and number of gold atoms vs concentration of La^{3+}.

with only one binding site. When the pH of the reaction mixture was adjusted to ∼12 by adding 1 M NaOH, the disulfide bonds break and the tyrosine residues are activated. This results in the reduction of Au^{3+} due to the involvement of these residues in the redox activity, and AuNCs thus formed are stabilized by the thio groups.[12]

Characterization of Apo-α-LA-AuNCs. Unlike metal nanoparticles, nanoclusters do not show any surface plasmon resonance (SPR) peak in their absorption spectrum due to their smaller core size resulting from the absence of any continuous density of states.[35] Although the absorption spectral results are not sufficient enough to understand the formation of nanoclusters, the absence of a clear SPR band supports that there are no larger-sized gold nanoparticles. The presence of the 280 nm band indicates that AuNCs are coated with the protein. As AuNCs are expected to emit light in the visible region upon excitation at 365 nm, fluorescence emission spectra were measured. This resulted in red luminescence by exhibiting two bands in the spectra, one at an emission maximum of 450 nm and the other at 660 nm (Figure 1b). While the former is attributed to the oxidized aromatic amino acid side chains,[36] the latter is attributed to the formation of subnanometer-sized AuNCs. The stability of α-LA-AuNCs was further demonstrated by measuring the fluorescence spectra over a period of 6 days, and the corresponding spectra are given in Figure 1b. As the concentration of La^{3+}-incorporated protein increases, the cluster core distribution narrows and thereby enhances the luminescence by a Foster resonance energy transfer (FRET) mechanism.[14] The fluorescence spectra showed no significant change over a period of time, either in its emission intensity or in its emission maximum supporting that AuNCs are stable and do not undergo any size variation or aggregation.

In the mass spectral analysis, apo-α-LA showed a well-defined peak for the molecular ion at m/z = 14,100 in the linear positive ion mode MALDI-TOF-MS spectrum. However, α-LA-AuNCs showed a broad band with the peak centered around m/z = 16,074 (Figure 1c) supporting that the core was formed with 10 gold atoms. The breadth of this peak is large ($\Delta\nu_{1/2}$ = ∼1300 Da) supporting the presence Au_n clusters, where n could range from 6 to 13 with the band position being centered at Au_{10}. Also, Figure 1c supports the presence of free or unused protein from the residual peak observed at m/z = 14,100. HRTEM studies showed the cluster size as small as ∼2 nm (Figure 1c, inset), and this is in agreement with the protein-protected clusters reported in the literature.[1] All the spectral, microscopy, and ITC data given in Figure 1 clearly support the formation of protein-protected AuNCs, viz., α-LA-AuNCs.

Synthesis and Characterization of α-LA-AuNCs in Presence of La^{3+}. Since La^{3+} was known to occupy the Ca^{2+} site in apo-α-LA, the formation of protein-coated AuNCs was studied in the presence of added La^{3+}. α-LA-La^{3+}−AuNCs were synthesized by treating apo-α-LA with different concentrations of La^{3+} followed by an Au^{3+} reaction under alkaline condition as given in the Experimental Section. All these solutions resulted in broad bands in mass spectra in support of the presence of Au_n clusters, where $\Delta\nu_{1/2}$ decreases with increasing concentration of La^{3+} ($\Delta\nu_{1/2}$ varies from 1250 to 925 Da) and the bands showed multiple maxima (Figure 2a). When only the presence of Au^0 is considered in these solutions, a systematic decrease in the AuNC core size was observed on increasing the added La^{3+} concentration from 0 to 1.2 mM as demonstrated by MALDI-TOF-MS, and the Au_n core size changes from Au_{10} to Au_6. However, when one considers the multiple maxima observed in these mass spectra, the presence of the La^{3+} ion can be invoked in these in addition to the fitting done using Au_n clusters. All this has been possible since La^{3+} occupies the site of Ca^{2+} in the protein which restricts the cluster growth region in addition to bringing some conformational changes. Even the Ca^{2+} occupancy does the same as explained later in this paper. To our knowledge, this is the first report demonstrating the size control of AuNCs protected by a protein that is partially occupied by a metal ion site using a green synthesis route since the protein acts as both reducing and protecting agents for AuNCs without the use of any external chemical reducing agent.

In order to see whether there is any La^{3+} ion present in the protein-protected NCs, ICP-AES studies were carried out for quantifying lanthanum as well as gold for the corresponding solutions after dialysis. The corresponding ICPAES data can be fitted to the Au^0 content of 8.3 ± 0.1, 7.9 ± 0.05, 6.5 ± 0.2, and 6.1 ± 0.03 ppm, which is in support of reduction in the gold cluster size upon incremental addition of La^{3+}. When no La^{3+} was added, ICPAES data fits to the Au^0 content of 10.2 ± 0.02 ppm. The ICPAES results for the presence of lanthanum are 0.6 ± 0.03, 1.1 ± 0.01, 1.3 ± 0.1, and 1.4 ± 0.05 ppm,

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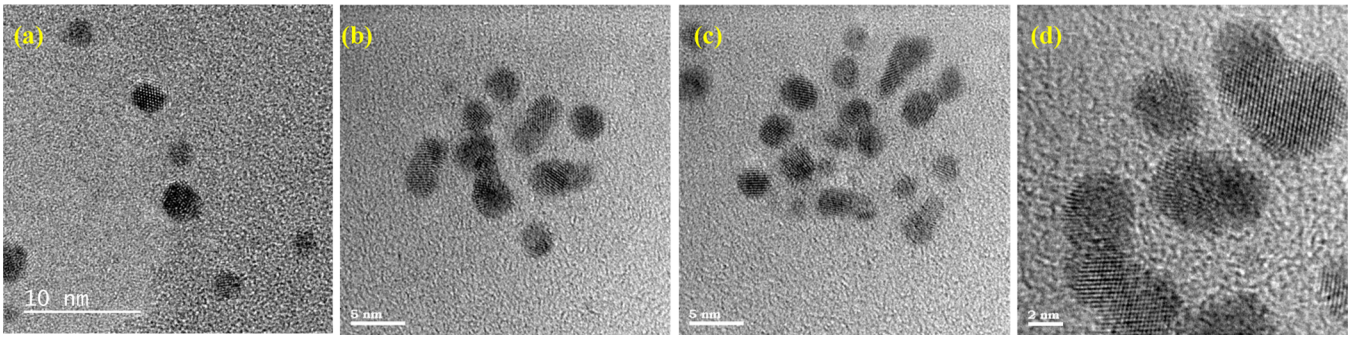

Figure 3. HRTEM micrographs of concentration-dependent La^{3+}-incorporated α-LA-AuNCs: (a) 0.1 mM La^{3+}, (b) 0.4 mM La^{3+}, (c) 0.8 mM La^{3+}, and (d) 1.2 mM La^{3+}.

supporting that in all the protein-coated AuNCs some amount of lanthanum ion is bound. All this can be explained; as La^{3+} occupies the Ca^{2+} binding core in apo-α-LA, the growth of AuNCs is restricted, thereby resulting in a smaller-sized cluster.

To further support this hypothesis, we have also performed AuNCs synthesis: (a) with the holo-α-lactalubmin (Ca^{2+} bound α-LA) and (b) in the presence of Gd^{3+} ions. In the case of (a), this resulted in AuNCs where the peak position in MALDI-TOF-MS fits well with Au_8 (Figure 2b), which is smaller than the Au_{10} cluster observed with simple apo-α-lactalbumin. In the case of (b), AuNCs are synthesized by using Gd^{3+} ions in place of La^{3+}, and this also showed clusters of Au_9 to Au_7 as noticed from the corresponding mass spectra given in Figure 2c. Corresponding HRTEM micrographs are given in the Supporting Information (Figure S02, a–d). Thus, when Ca^{2+} occupies the binding core of α-LA, the size of the Au_n formed is restricted. The fluorescence emission intensity of the band observed at 660 nm increases as the concentration of La^{3+} increases owing to the reduction in the size of AuNCs formed (Figure 2d). A decrease in the cluster size and increase in the fluorescence intensity are evident from the plots given in the inset of Figure 2d.

In all these cases, AuNCs were formed as supported by HRTEM. The corresponding micrographs are given in Figure 3a–d, and the line spacing of 0.242 ± 0.03 nm observed with these particles is consistent with that observed for AuNCs in the literature.[13] The TEM micrographs of apo-α-LA with La^{3+} forms vesicular structures ~60–70 nm in size as given in Figure S3. As La^{3+} is introduced into the protein prior to AuNCs synthesis, it tends to form vesicle-like structures. Further, the synthesis of AuNCs results in aggregation into the vesicular structures as can be noticed from the TEM micrographs given in Figure S4. The TEM micrographs of La^{3+}-incorporated α-LA-AuNCs are shown on a 50 nm scale in Figure S01, a–d. The XPS data clearly shows the presence of Au^0 at 84.0 eV, while the presence of Au^+ cannot be ruled out. All this can be clearly seen from Figure 4.

Secondary Structural Changes Observed in Protein When It Protects AuNCs. As the protein structure is sensitive to environmental effects, such as solvent, temperature, presence of metal ions, etc., one would expect changes in the secondary structure of the protein due to the development of AuNCs in the reaction. The protein oxidation was observed as a sign of increase in the absorbance at the 280 nm band and the bandwidth in the absorption spectra (Figure 5a), and further, the structural changes were monitored using CD and FTIR. The secondary structural changes observed based on CD spectra are significant. This data revealed a loss of 78% of α-

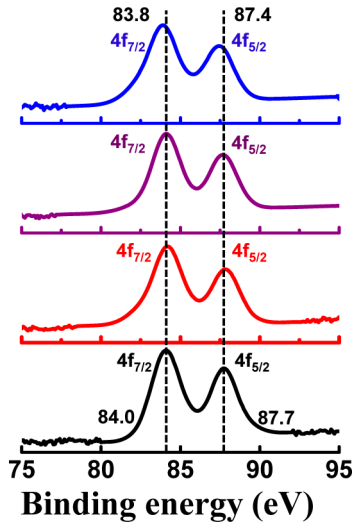

Figure 4. XPS data for AuNCs obtained from concentration-dependent La^{3+} (mM)-incorporated α-LA: 0.0 (black), 0.1 (red), 0.4 (purple), and 1.2 (blue).

helix (Figure 5b) during the synthesis of AuNCs. This is attributable to the breakage of the disulfide bonds in NaOH[37] and their concomitant involvement in stabilizing the α-LA-AuNCs. This was further supported by FTIR studies through changes occurring in the amide region. The shifts observed in the amide I, II, and III bands and the N–H and O–H stretching vibrations (Figure 5c and d) are suggestive of the secondary structural changes occurring in the protein upon capping AuNCs.

CONCLUSIONS AND CORRELATIONS

AuNCs were synthesized by adopting a green synthesis route using apo-α-LA as well as the same in the presence of La^{3+} ions. All AuNCs were well characterized by spectroscopy and microscopy. The fluorescence emission spectra showed two bands, one at 450 nm (due to the protein oxidation) and the other at 660 nm due to Au^{3+} reduction to Au^0 nanocluster, thus resulting in red luminescence. As the concentration of the added La^{3+} increases, the population of α-LA bound to La^{3+} increases in the solution. When the pH is raised to 12, the reaction mixtures undergo reduction of Au^{3+} to Au^0, and the clusters formed are Au_6 to Au_{10}. Although controlling the gold cluster size is reported in the case of small molecules,[38] ours is the first report with the use of proteins. Unlike the case of small molecules, in the present case, the size of AuNCs was

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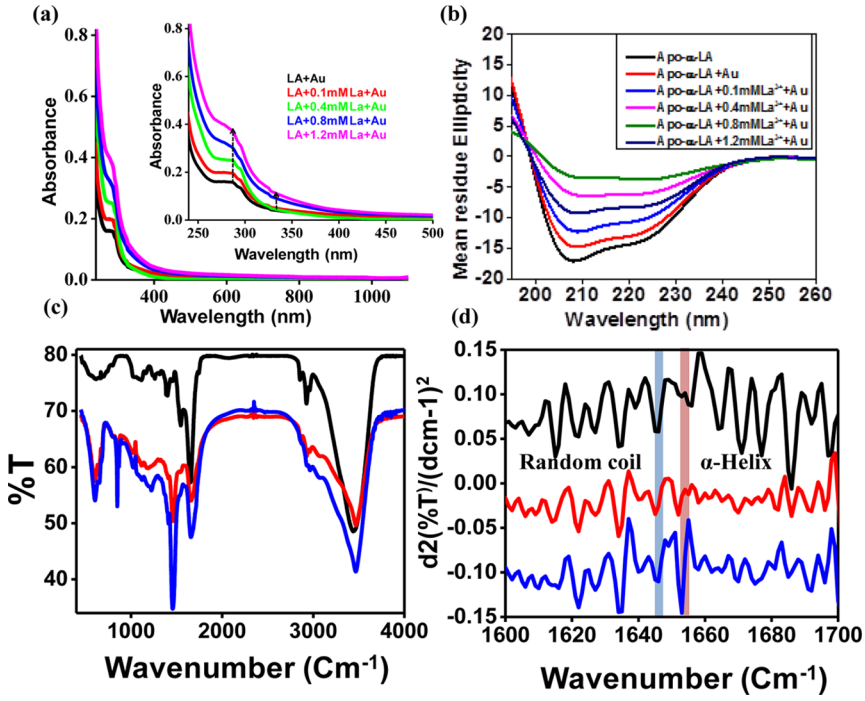

Figure 5. Spectra of concentration-dependent La^{3+}-incorporated α-LA: (a) absorption spectra, (b) CD, (c) FT-IR apo-α-LA (black), α-LA-AuNCs (red), α-LA-1.2 mM La^{3+}-AuNCs (blue), and (d) double derivative of FT-IR, highlighted portion with blue at 1654 cm^{-1} for random coil and pink at 1648 cm^{-1} for α-helix.

controlled by partially occupying the Ca^{2+} sites in apo-α-LA by La^{3+}, which in turn causes conformational changes in the protein structure.

As the concentration of the added La^{3+} increases, the fluorescence emission intensity of the 660 nm band increases and the size of AuNC cluster formed decreases. The MALDI-TOF-MS studies confirmed the formation of Au_n clusters with the core size ranging from n = 6 to n = 10 atoms of gold. A similar trend was observed in the Au_n cluster formed even when Gd^{3+} was used in place of La^{3+} and also when a holo-protein occupying Ca^{2+} was studied. In the absence of any La^{3+} ion in the solution, a core size of Au_{10} atoms was observed. The ICPAES results showed the presence of decreasing amounts of Au^0 going from the clusters prepared without La^{3+} to those prepared under different concentrations of La^{3+}. Although gold nanoclusters with different sizes are known in the literature, these are mostly formed from different proteins. The evolution of nanoclusters in protein templates depends on various features, such as size, structural complexity, and amino acid composition. Several amino acids present in the protein play important roles in the formation of AuNCs. Initially, the negatively charged amino acids such as Asp and Glu are responsible for the complexation with Au^{3+}, and the Tyr/Trp residues helps in reducing Au^{3+} to Au^+ and Au^0. Finally, the cysteine moieties would stabilize AuNCs through Au−SH interactions.[19]

In the literature, different sizes of AuNCs are produced mainly from different protein templates, for example, Au_{10} from the lysozyme,[18] Au_{25} from lactoferrin,[14] Au_{25} from BSA,[12] and Au_5, Au_8, Au_{13}, and Au_{25} from pepsin.[39] In case of pepsin, the reported Au_5 and Au_8 (blue emission), Au_{13} (green emission), and Au_{25} (red emission) exhibited different color emissions, and these clusters are formed due to the change in pH from 3 to 13. Similarly, clusters of type Au_4, Au_8, Au_{10}, Au_{13}, and Au_{25} were reported using BSA at different pH conditions.[40]

Therefore, AuNCs thus prepared by using apo-α-LA is a green material which can be used for different applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b00958.

Microscopy and spectral data. (PDF)

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Notes

The authors declare no competing financial interest.

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