Bioconjugated Hybrid Nanomaterials

Conjugated denatured BSA not only controls the crystal growth but also stabilizes the colloidal crystals by forming an electric double layer at water-solid interface. In this chapter, we have explored the possibility of further using this conjugated BSA as hot spots on Se surface to attract and direct the nucleation of Au/Ag atoms in order to produce bioconjugated Se-Au/Ag hybrid materials. Though, some examples of such materials are already known in the literature\textsuperscript{1-6}, protein directed synthesis of hybrid materials is a novel aspect. The main importance of this study is related to its aqueous phase moderate temperature (at 80 °C, BSA is in unfolded state) control synthesis that allows not only to preserve the biofunctionalities of BSA but also helps to understand the fundamental basis of site specific nucleation. As both Au and Ag possess fcc geometry, therefore, we expect similar nucleation processes centred on BSA hot spots. Such hybrid materials are expected to have interesting optical properties\textsuperscript{5,6}.

Results and Discussion

5.1 UV-Visible Studies

The synthesis of hybrid nanomaterials is described in experimental section and the reactions were also simultaneously monitored with UV-visible measurements. Figures 5.1a and b, respectively, show the variation in the absorbance due to SPR of Au and Ag NPs around 600 nm and 440 nm. Both plots show prominent peaks within 10 minutes of the beginning of their respective reactions which subsequently diminish with the passage of time. It means that both Au(III) and Ag(I) ions are reduced by ascorbic acid (AA) into their respective atomic forms within 10 minutes of the reaction. Usually small sized NPs (> 20-30 nm) produce sharp absorbance in the visible region of the spectrum contrary to the broad absorbance seen in Figures 5.1a and b\textsuperscript{7}. Broad absorbance occurs due to two prominent factors i.e., either the large polyhedral geometries of NPs exceeding 100 nm or self-aggregation among small NPs\textsuperscript{7}. In view of the absence of any stabilizing agent to provide colloidal stability of freshly produced NPs, the self-aggregation among NPs is expected and that may produce broad absorbances. A regular fall in the absorbance of both Au (Figure 5.1a) and Ag NPs (Figure 5.1b) with the passage of time indicates either the self aggregated NPs settle down at the bottom or they prefer to deposit at some specific sites on the Se
**Figure 5.1.** a) UV-visible absorbance plots of Au NPs with respect to reaction time. Absorbance decreases with the passage of reaction time within the start of the reaction from 10 minutes to two hours. A broad absorbance close to 600 nm indicates the presence of large sized Au NPs of aggregates. (b) Similar reaction in the presence of Ag NPs under the same experimental conditions. A broad absorbance close to 420 nm is due to the presence of Ag NPs. Here too, the absorbance of Au NPs decreases with reaction time. (c) Plots showing the variation in the absorbance at 590 nm and 420 nm due to Au and Ag NPs with respect to reaction time. (see details in text)
rods. In both cases, a decrease in the number density of colloidal Au/Ag NPs in their respective solution will reduce their absorbance. A plot of intensity versus time is shown in Figure 5.1c. It is interesting to note that both Au/Ag NPs follow a similar trend of a decrease in their SPR with time and it takes about 50 minutes to reach a minimum value which then tends to level off. It means that the self aggregation of Au NPs or their adsorption on the Se NRs is really a time dependent process. DLVO theory explains the stability of colloidal particles in terms of flocculation and coagulation. Charge and steric stabilizations provide potential energy of electrostatic repulsions ($V_R$) necessary to maintain the colloidal stability. The colloidal stability is only achieved when $V_R$ predominates the potential energy of electrostatic attractions ($V_A = -Aa/12H$, where $A$ is Hamaker constant, $a$ is the particle radius, and $H$ is the inter-particle distance). $A = \pi^2 C \rho_1 \rho_2$, is further related to the number of atoms per unit volume of two interacting particles and particle-particle pair interactions parameter, $C$. Thus, lower the value of $A$ or greater the value of $H$ will keep the electrostatic interactions at less magnitude in comparison to that of $V_R$ and hence maintain the colloidal stability. But on the contrary, as in the present case, coagulation sets in with greater value of $A$ and lower value of $H$. $A$ is expected to increase because even the tiny nucleating centres undergo autocatalytic process which will increase $\rho_1$, $\rho_2$, and $C$. Electrical double layer around each growing NP provides necessary $V_R$ but cannot maintain its predominance over $V_A$ for a growing NP and consequently NPs lose stability. Hence, a coagulation process also leads to a decrease in the viscosity ($\eta$) of the dispersion medium which is inversely related to the rate of second order process i.e., $k_2^o = 4kT/3\eta$. A decrease in the absorbance of colloidal Au/Ag NPs with respect to time in Figure 5.1c can considered to be analogous process with second order rate constant values of $8.78 \times 10^{-4}$ s$^{-1}$ and $8.72 \times 10^{-4}$ s$^{-1}$ for Au and Ag NPs, respectively. The presence of self aggregates of NPs or their selective adsorption on the Se NRs can be best evaluated with the help of microscopic studies.

### 5.2 SEM and TEM Studies

Figure 5.2a shows a dark field SEM image of Se-Au hybrid NCs synthesized with a mole ratio of [Se]/[Au] = 6. Most of the Au NPs exist in the self assembled ball shaped aggregates but many NPs have grown on the Se NRs. Self-aggregated balls do not exist independently rather they are further arranged in a typical pearl-necklace
Figure 5.2. (a) Dark field SEM image of hybrid Se-Au NCs prepared with 0.5 mM of HauCl₄. White arrows indicate the presence of Se NRs laden with small Au NPs. Self aggregated Au NPs are also visible. (b), (c), and (d) indicate the close up TEM images of Au NPs (dark shaded) embedded in the surface of Se NRs. (e), (f), and (g) are the corresponding dark field TEM images of hybrid Se NRs-Au NCs. White arrow in frame (g) indicates the alignment of small Au NPs on the twin boundary of hexagonal Se NR. (see details in text)
type arrangement. Figures 5.2(b-d) show bright field TEM images of dark Au NPs grown over Se NRs at different places whereas complementary dark field images in Figures 5.2(e-g) further authenticate this association. Se is a non-metal and therefore less susceptible in comparison to contrast in comparison to Se. Thus, the bright coloured particles grown on Se NRs shown in the dark field TEM images are Au NPs. It is to be noted that Au NPs are grown at some specific places where BSA capping is available on the Se NR surface, because Se NRs in fact are synthesized in the presence of BSA. BSA is also known for its reducing ability\(^\text{11}\) and in the aqueous phase it is expected to attract some of the gold ions due to electrostatic interactions with protonated amino acid residues. Adsorbed BSA predominantly exists in the unfolded state with broken disulfide bridges and exposed reducing amino acids like cysteine which will instantaneously reduce the gold ions into atoms and hence will trigger the initiation of nucleating centres only on those places at the Se NRs where BSA is present. Most of the Au NPs can be seen either grown on the ends of Se NRs (Figure 5.2d) or along the twin crystal planes of hexagonal geometry of Se NRs (Figure 5.2g). Both places are expected to have crystal defects or grain boundaries\(^\text{12}\) which act as active sites for BSA adsorption.

This sample has been further analysed by detailed EDS analysis. Figure 5.3a shows bundles of Se NRs with attached balls of aggregated Au NPs. A careful selection of one of the Se NR for EDS analysis shows the presence of 78.6 atomic % Se apart from Si and C due to carbon coated silicon surface which means this NR is entirely made up of Se and no adsorbed Au NPs are present. Likewise, similar analysis was done by choosing a single ball of self aggregated NPs which shows the presence of only Au in 70.3 atomic % with no Se. On the other hand, if we select Se NR bearing Au NPs as shown in Figure 5.3b, it produces emissions due to both Se and Au, though the amount of Se emission is much higher than Au due to its larger surface area and high mole ratio of [Se]/[Au] = 6. Note both images (Figures 5.3a and b) do not show any independent Au NPs or their self assembled balls which means that all Au NPs are either grown on the Se NRs or self assembled in large balls. Figure 5.4 shows similar images of Se-Au hybrid NCs with increased amount of Au and a lower ratio of [Se]/[Au] = 3. A dramatic increase in the number density of adsorbed Au NPs on Se NRs is observed. Figure 5.4a shows a typical example of TEM image of Se NR bearing several Au NPs of different sizes. A dark field image (Figure 5.4b) further
Figure 5.3. (a) SEM image of hybrid Se-Au NCs prepared with 0.5 mM of HAuCl₄. A careful EDS analysis of a NR and self-aggregated NPs indicate that the emissions are only due to Se and Au, respectively. (b) shows long Se NRs bearing Au NPs. By selecting an area of Se NR bearing Au NPs for EDS analysis shows the emissions due to both Se and Au. Dotted circles enclose non-aggregated as well as aggregated Au NPs on Se NRs. (see details in text)

confirms the presence of several NPs as bright spots on a single Se NR. Even the SAED image (inset) gives two different distinct patterns due to the presence of crystalline Se and Au. They are simultaneously analysed by EDS analysis in Figure 5.4c which authenticate the presence of pure Se NR bearing balls of Au NPs.

Similar experiments were performed with AgNO₃ to generate Ag NPs in the presence of Se NRs with mole ratio of [Se]/[Ag] = 6. Due to several similarities in the crystal structure of Au and Ag, similar behaviour of Ag NPs is expected in the formation of Se-Ag hybrid NCs to that of Au. Figure 5.5a demonstrates a low resolution image of different Se-Ag hybrid NCs where all Se NRs are covered with Ag NPs as small dots (Figure 5.5a). No self-aggregated Ag NPs in the form of balls are present. Figure 5.5b represents a close up image and Ag NPs as small dots are
Figure 5.4. (a) and (b) show the bright and dark field TEM images of hybrid Se-Au NCs prepared with 1 mM of HAuCl₄. SAED images indicate the crystalline nature of both Se NRs as well as Au NPs. (c) A careful selection of only Se NR and self aggregated ball of Au NPs for EDS analysis reveals respective emissions due to Se and Au. (see details in text)

visible on almost all NRs. Dark field image (Figure 5.5c) differentiates between the contrast of Se NR surface and Ag NPs on the basis of electron emission where one can see Ag NPs as bright dots embedded in the surface of Se NR. EDS analysis further
Figure 5.5. (a) A low resolution image of various Se NRs loaded with small Ag NPs (as small dots) prepared with 0.5 mM of AgNO$_3$. (b) Similar image at higher magnification. (c) A high resolution image of small Ag NPs as bright spots embedded in a single Se rod. A simultaneous EDS analysis confirms the emission due to both Se and Ag. (see details in text)

confirms the presence of both Se and Ag in their 28.2 and 8.6 atomic %, respectively. An increase in the amount of AgNO$_3$ with mole ratio [Se]/[Ag] = 3 (Figure 5.6), significantly increases the number density of Ag NPs. Now the Se NRs are almost entirely covered by the Ag NPs, and mostly Ag NPs are predominantly in monodispersed and singly distributed state (Figure 5.6a). A low magnification dark field image (Figure 5.6b) further confirms a thorough distribution of Ag NPs on all Se NRs. By carefully choosing one Se NR, EDS analysis was performed with emissions due to Se and Ag in their 33.9 and 24.2 atomic %, were obtained. The emission due to Ag in this sample is much higher than the previous one because of a double amount of AgNO$_3$ used in this sample.
**Figure 5.6.** (a) and (b) show the bright and dark field close up images of hybrid Se-Ag NCs prepared with 0.5 mM of AgNO₃. Ag NPs are clearly visible as dark dots in (a). Inset in (a) further confirms the crystalline nature of hybrid NCs. (c) A single Se NR loaded with Ag NPs is chosen for the EDS analysis and emission due to Se and Ag is clearly visible.

### 5.3 Role of BSA

In order to understand the mechanism of Se-Au/Ag hybrid NC formation, it is important to authenticate the presence of capping BSA on Se NRs. As mentioned in the experimental section, Se NRs were synthesized by using BSA as capping and stabilizing agent. Thorough purification removes the excess of unreacted BSA since BSA is highly water soluble. 1g of carefully dried powder of Se NRs is taken for the reaction with Au/Ag salts. Following Bradford method, the amount of associated BSA with Se NRs is determined. An average value of 1.29 ± 0.8 µg for three
independent samples of 1 mg of Se NR powder is obtained which corresponds to a total concentration of BSA in 1 mg of Se NR powder is about 0.02 μM. It is an extremely low amount in comparison to the amount of Au/Ag salt (0.5/1 mM) used. That is why, greater amount of Au NPs exist in the self-assembled state rather than grown on the Se NRs. Adsorbed BSA as hot spots are expected to take part in the initial reduction of Au/Ag ions. Once the nucleation is initiated, it is followed by an autocatalytic process. BSA possesses several amino acids which can act as reducing agents but cysteine (Cys-34, HS-CH₂-) which resides between helical folds of h2 and h3 of sub-domain 1A is partially accessible to solvent and has high affinity for gold\(^{14,15}\). It exists as a reduced thiol or as a mixed disulfide with \(pK_{\text{SH}}\) is ~5.0\(^{16}\). A reaction temperature of 80 °C keeps BSA in its unfolded form and that further enhances the reducing ability of Cys-34 and consequently produces Au/Ag nucleating centres. It is proposed to work through a reaction mechanism illustrated in Figure 5.7\(^{17,18}\). In the first step, Au/Ag ions are attracted by protonated Cys residues (Figure 5.7b) of adsorbed BSA. There, they are reduced by Cys into atoms which then grow into nucleating centres while in contact with adsorbed BSA (Figure 5.7c). Nucleating centres find their way to the crystal defects on Se NR surface to further grow which are expected to be in good number at the ends or twin boundaries of Se NRs where they further grown in size due to autocatalytic process. On the other hand, ascorbic acid produces independent nucleating centres in aqueous phase which upon self-aggregation produce large balls of aggregated Au NPs due to a lack of an appropriate stabilizing agent. Such large balls with relatively little amount of VR and greater probability of VA are highly prone to coagulation and hence exist in the form of a typical pearl-necklace arrangement (Figure 5.2a). A relatively little amount of adsorbed BSA will not have the ability to bind them through thiol linkages of cystein residues.

On the contrary, this is not so in the case of Ag NPs, where no balls of aggregated NPs are observed. It means that the total amount of reduced Ag is accommodated on the Se NRs and that is quite evident from Figures 5.5 and 5.6. Here, apart from the reduction of Ag\(^+\) ions into Ag\(^0\) by ascorbic acid, another parallel liquid-solid interfacial reduction related to the underpotential deposition of Ag on Se NR surface cannot be ruled out. In fact, the latter reaction is facilitated especially on
{110} crystal planes rather than {100} or {111} just like that on gold NRs\textsuperscript{19,20} in comparison to the former reduction, which is entirely taking place in the aqueous phase. The present Se NRs\textsuperscript{13} are grown along {100} crystal planes and are bound with [110] facets (Figures 5.8a and b) with sufficiently large surface area as far as their size in the micrometer length scale is concerned. This large surface provides preferential catalytic properties especially at elevated temperature of 80 °C to facilitate the underpotential deposition (Figure 5.8c) which is considered to be promoted due to relatively low reduction potential of Ag in comparison to that in the presence of Au. In addition, single crystal nature of a crystalline surface is always preferred over the polycrystalline surface for underpotential deposition and it is especially true in the present study where all Se NRs are single crystals (Figure 5.8b). Such a deposition

\textbf{Figure 5.7.} Proposed mechanism of the formation of Au/Ag nucleating centres specifically on the hot spots created by the adsorption of the capping BSA. (a) Cysteine residues interact electrostatically with Au/Ag ions (b) and thereby reduce them to respective centres (c) initiating the formation of hybrid Se-Au/Ag NCs.
usually carried out in the form of a monolayer formation in the first place, but once the Ag nucleating centres are created, they continue the subsequent reduction through autocatalytic process in order to produce well-defined NPs. A close up

Figure 5.8. (a) and (b) TEM images of a single Se NR and its HRTEM image are taken from reference 13. (c) illustrates a schematic representation of simple reduction of Ag\(^+\) ions on Se NR surface to generate Ag NPs. See details in the text.
image of Figure 5.5a approves this mechanism as NPs are just embedded up to the skin (few monolayers) of Se NR surface.

**Conclusions**

The results present a simple synthesis of semiconductor-noble metal hybrid nanomaterials by using a low molecular weight protein, BSA, as hot spots. BSA capped Se NRs were synthesized first and then used further to grow Au/Ag nucleating centres on BSA hot spots present on Se NRs due to BSA capping. It was carried out by a simple reduction of Au/Ag salt by ascorbic acid in the presence of fixed amount of BSA capped Se NRs. This allowed time behaviour of Au/Ag nuclei to attach preferentially on the hot spots for further growth. In addition, cystein residues of unfolded BSA were also expected to take part in the reduction process which further facilitated the synthesis of hybrid nanocrystals.
References