This chapter covers the details about materials used, synthesis of bio-conjugated nanoparticles, film casting, methods used for the characterization of materials and monitoring the reactions.

2.1 Materials

Tetrachloroauric acid (HAuCl₄), zein protein, cadmium acetate (CH₃COO)₂Cd, (99.9 %), thioacetamide (TAA, 98%), acetic acid (99.5 %) were purchased from Aldrich. Glycerol and absolute ethanol were obtained from BDH chemicals. Double distilled water was used for all preparations. Proteins used for the direct synthesis of Au NPs as weak reducing as well as capping/stabilizing agents in the present study are listed in the following table.

<table>
<thead>
<tr>
<th>Material</th>
<th>Procured From (Cat. No.)</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (Lys) chicken egg white</td>
<td>Aldrich (L 7651)</td>
<td>14300 Da</td>
</tr>
<tr>
<td>Cytochrome c (Cyt,c) from bovine heart muscle</td>
<td>Aldrich (C 2037)</td>
<td>12400 Da</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Aldrich (A 7030)</td>
<td>66500 Da</td>
</tr>
</tbody>
</table>

2.1.1 Starch isolation

Kidney beans (100 g) were steeped overnight in aqueous toluene solution (2.2 ml/500 ml distilled water) at 40 °C. They were washed, peeled and ground with distilled water (1:10) to obtain slurry which was passed through a nylon cloth to remove fibers. The residue thus obtained was again ground with distilled water and the slurry was allowed to stand for 2-3 hours. Subsequent washings were given till the supernatant became clear and the starch was procured after drying at 40 °C. Amylose content of the isolated starch was determined by using the method of William et al.¹⁵⁰ and found to be 29.5 %.

2.2 Synthesis of Nanoparticles (NPs)

2.2.1 Lysozyme or Cytochrome c (Lys or Cyc,c) conjugated Au NPs

Aqueous mixtures (total 10 ml) of Lys/Cyc,c (7 - 80 μM) and HAuCl₄ (0.25 - 1.0 mM) were taken in screw-capped glass bottles. After mixing the components at room temperature, the reaction mixtures were kept in a water thermostat bath (Julabo F25) at precise 40 or 80 ± 0.1°C for six hours under static conditions. The color of the
solution changed from colorless to pink-purple and finally purple within half an hour and remained same thereafter in most of the cases.

After six hours, the samples were cooled to room temperature and kept for overnight. They were purified from pure water at least two times in order to remove unreacted protein. Purification was done by collecting the Au NPs at 10,000 rpm for 5 min after washing each time with distilled water.

2.2.2 Bovine Serum Albumin (BSA) conjugated Au NPs

Aqueous mixtures (total 10 ml) of BSA (0.0015 - 0.015 mM) and HAuCl₄ (0.25 - 1.0 mM) were taken in screw-capped glass bottles. After mixing the components at room temperature, the reaction mixtures were kept in a water thermostat bath (Julabo F25) at precise 40, 60 or 80 ± 0.1°C for six hours under static conditions. The color of the solution changed from colorless to pink-purple and finally purple within half an hour and remained same thereafter in most of the cases.

The following overall reaction (equation 1.1) was considered to take place where protein acts as a weak reducing agent due to the presence of several reducing amino acids like cysteine. The reduction progresses as more and more cysteine residues come in contact with aqueous phase during the unfolding of protein due to temperature or pH effects.

\[
\text{Au}^{3+} \text{(aq)} + \text{BSA} \text{(aq)} + 3e^- \rightarrow \text{Au}^0 \text{(s)} \tag{1.1}
\]

After six hours, the samples were cooled to room temperature and kept for overnight and purified as mentioned above.

2.2.3 Starch conjugated Au NPs

Different amounts of starch (0.5/1/2/5 %) were stirred in screw-capped glass bottles at 90 °C for 10 min with constant stirring. The suspension thus obtained in each case was cooled to room temperature and HAuCl₄ (0.25/0.5/1.0 mM) was added. The reaction mixture was kept in a water thermostat bath (Julabo F25) at precise 70 °C for six hours under static conditions. Color of the solution changed from colorless to pink-purple after 30 min and remained same thereafter in most of the cases indicating the formation of Au NPs. The following reaction is considered to take place.
After six hours, samples were cooled to room temperature and kept for overnight. The samples were washed with distilled water and then centrifuged at 10,000 rpm for 5 min at least two times in order to remove unreacted starch.

2.2.4 Starch conjugated CdS NPs

CdS NPs were synthesized by taking 10 mL of glycerol in a round bottom glass flask. Under constant stirring, 1 mL of 0.5 M aqueous acetic acid was added and followed by the addition of 0.5 mL of 0.1 M aqueous cadmium acetate solution. After mixing all the components at room temperature, reaction mixture was kept in an oil bath under precise temperature control of 150 °C at constant stirring. Then, 0.5 mL of aqueous 0.1 M thioacetamide was added and the reaction was carried out for 48 hours. Following reaction was considered to take place and the CdS NPs thus produced.

\[
\text{Cd(OOCCH}_3\text{)}_2 + \text{H}_2\text{O} \rightarrow \text{CdO} + 2\text{CH}_3\text{COOH}
\]

\[
\text{CH}_3\text{CSNH}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COONH}_4 + \text{H}_2\text{S}
\]

\[
\text{CdO} + \text{H}_2\text{S} \rightarrow \text{CdS} + \text{H}_2\text{O}
\]

The purified CdS NPs were collected by centrifugation at 10,000 rpm for 5 min after washing it 2-3 times with distilled water.

Different amounts of starch (1/2/5%) were stirred in screw-capped glass bottles at 90 °C for 10 min with constant stirring. The suspension thus obtained in each case was cooled to room temperature and as prepared purified CdS NPs (0.25/0.5/1.0 mM) added under constant stirring. The color of solution became yellow.
which indicated the formation of starch conjugated CdS NPs. Samples were kept for overnight. They were purified as mentioned above.

2.3 Film Casting

2.3.1 Zein protein film formation with Lys/Cyc,c/BSA conjugated Au NPs

Protein film casting was carried out by dissolving zein (10% w/v) in aqueous ethanol (90% v/v) along with glycerol (30% on zein weight basis) as plasticizer, and 10% of Lys/Cyc,c/BSA - Au NPs aqueous suspension. 5g of this filmogenic solution was placed in 9 cm diameter plastic Petri dish and gently swirled to coat the bottom of the dish. It was then placed without lid on a level surface (checked with a spirit level) in an oven at 40 °C for 24 hours. Dehydration of this filmogenic solution lead to the formation of a protein film with average thickness of 0.05 mm which was easily peeled off. Films made without glycerol were quite brittle, clear, and less elastic thus the addition of a small quantity of glycerol is required.

2.3.2 Starch films formation with Au or CdS NPs

Starch films embedded with Au or CdS NPs were made directly from the samples being prepared. 10 g of the respective colloidal NPs suspension was weighed in 9 cm diameter plastic petri dish along with 30% glycerol (starch weight basis) as a plasticizer. For CdS NPs sample, 30% glycerol was accounted for along with the as prepared colloidal NPs suspension. The suspension was then gently swirled to coat the bottom of the dish and was placed on a level surface in an oven at 40 °C for 24 hours. Three replicate films were cast for each sample. Dehydration of this filmogenic solution led to a film formation which was easily peeled off. All films were stored in a desiccator over P₂O₅ for at least two weeks to obtain constant weight.

2.4 Methods

2.4.1 UV-Visible Spectroscopy

UV-Visible spectra were recorded by UV spectrophotometer (Multiskan Spectrum, model no. 1500) in the wavelength range of 200-900 nm to determine the absorbance due to surface plasmon resonance (SPR). In addition, time dependent scans of some selective reactions at 40 and 80 ± 0.1°C at regular intervals were also carried out to understand the growth kinetics of Au NPs. Reactions were also
monitored over a wide temperature range from 20 to 70 °C in order to determine the influence of denaturation of protein on the synthesis of Au NPs.

2.4.2 Atomic Force Microscopy (AFM)

AFM measurements were carried out by Veeco diCaliber at room temperature. 25 μl of purified aqueous colloidal suspension was first spin coated (MTI corporation, PC100) at 500 rpm for 2 min on ultra cleaned glass cover slip and was left to dry in a dry-box. In case of starch film, 5 cm square piece of a starch film was placed on an ultra cleaned glass cover slip. It was then scanned with silicon nitride tips in contact mode to get amplitude and height images. Survey scanned images were processed and analysed by using SPM graphic software to obtain three-dimensional topography of protein β-sheet bearing Au NPs and of a starch film.

2.4.3 Transmission Electron Microscopy (TEM)

TEM measurements were carried out to characterize the colloidal NPs. Samples were prepared by mounting a drop of a sample on a carbon coated Cu grid and allowed to dry in air. They were observed with the help of a Philips CM10 Transmission Electron Microscope operating at 100kV. The diffraction as well as energy dispersive X-ray (EDX) analysis was also performed with the same instrument.

2.4.4 Scanning Electron Microscopy (SEM)

SEM analysis was carried out on a Zeiss NVision 40 Dual Beam FIB/SEM instrument. Photomicrographs were obtained in Bright Field Scanning/Imaging Mode, using a spot size of ~1 nm and 12 cm of a camera length.

2.4.5 X-ray Powder Diffraction (XRD)

XRD patterns were recorded by using Bruker-AXS D8-GADDS with T_{sec}=480. Samples were prepared on glass slides by putting a concentrated drop of aqueous sample and then dried in vacuum desiccator. Likewise, XRD patterns of starch films were determined by using separate attachment to hold the film.

2.4.6 pH Measurements

pH of some of the reactions was measured by using a pH-meter. Each reaction solution was taken in a glass flask with double walled jacket and temperature was
precisely controlled by externally circulating thermostated water (Julabo F25). pH measurements were carried out at regular intervals over a period of six hours.

2.4.7 Protein Assay

The total protein content in the BSA-NP conjugated suspension was determined by the Bradford method\textsuperscript{151}. First of all, standard BSA (reference) solutions of concentrations 0, 2, 4, 6, 8, and 10μg/μl were prepared in 100 μl pure water. 10 μl of each of these solutions was taken in triplicate in different wells of a UV-plate. The purified BSA-Au NPs conjugated suspensions were also taken in the same plate in another series of wells in doublet. Extra care was taken not to lose sample during the purification process. 20 μl of pure water and 170 μl of the Bradford reagent were mixed in all the wells so as to maintain a total volume of 200 μl. The absorbance of each solution was measured and from the absorbance values the amount of BSA conjugated to Au NPs in each sample was determined.

2.4.8 Fluorescence Spectroscopy (FL)

Steady state and time resolved FL of CdS NPs suspensions were carried out by using the PTI QuantaMaster and PicoMaster 2 TCSPC Lifetime Fluorometer, respectively. Both instruments are equipped with a thermoelectrically temperature controlled Cell Holder that allows to measure the spectrum at a constant temperature within ± 1 °C.

2.4.9 Differential Scanning Calorimetry (DSC)

TGA/DSC of the starch films was carried out on a TA instrument SDT Q600 using approximately 10 mg sample accurately weighed into an aluminum sample pan. An empty aluminum pan was used as reference. The sample was heated at 5 °C/min heating rate in the temperature range from 20 °C to 500 °C under air flow (100 mL/min).

2.4.10 Color coordinates

Color coordinate data of the films were determined using Hunter colorimeter Model D 25 optical sensor (Hunter Associates). The Hunter L, a, and b color space is a three-dimensional rectangular space based on the opponent color-theory. The \L* is a measure of “brightness” while a* and b* are the color coordinates that range from -90 to +90 in the color space ”circle”. For a*, -90 = green and +90 = red, and for b*, -90 = blue and +90 = yellow. To perform the color tolerance, the instrument (45°/0°
geometry, 10° observer) was calibrated against a standard red-colored reference tile
\((L_s = 25.54, a_s = 28.89, b_s = 12.03)\).

2.4.11 Mechanical Properties

The mechanical properties of the films were measured using a Texture
Analyzer (TAXT2, Stable Microsystems, Godalming, U.K.) with a 5 kg load cell. The
speed was kept constant at 1 mm/min. A well-defined geometry of each film (with
average thickness, width, and length of 0.05, 5, and 50 mm, respectively) was used to
measure the tensile properties of each film. The measurements were carried out by
fixing the ends of a film to two metal plates by using cyanoacrylate glue. It was then
single-edge-notched to a depth of 2.5 mm midway along the length. The mechanical
tests were performed to obtain force/displacement data, tensile strength (MPa), and
strain at failure. Tensile strength was measured using \( \sigma = \frac{F_{\text{max}}}{(w - a)t} \), where \( F_{\text{max}} \) is
the maximum force associated with failure, \( w \) is the width of the strip, \( a \) is the notch
length, and \( t \) is the thickness of the strip. Strain at failure was calculated as \( \varepsilon_{\text{fail}} = \frac{\Delta l_{\text{max}}}{l} \), where \( \Delta l_{\text{max}} \) is the change in length at \( F_{\text{max}} \) and \( l \) is the initial length.