Chapter 3

BIOCONJUGATION OF GOLD NANOPARTICLES WITH PROTEINS

3.1 Nanotechnology and biology

With the advent of nanotechnology in biology and medicine, the need for conjugation of nanoparticles (NPs) to biomolecules has grown recently. In particular, the attachment of NPs to proteins has found wide applications in imaging,\(^1\)\(^-\)\(^3\) in catalysis,\(^4\) in drug delivery,\(^5\) in the control of protein activity with an external field,\(^6,7\) and in understanding local structure in protein folding.\(^8\) NP-protein conjugates are not limited to biological applications, but have reached many other fields, such as material science, physics and energy, with the design of devices,\(^9,10\) such as nanosensors, biofuel cells\(^11\) and of new tools for assembly.\(^12,13\) Most research efforts in this area are focused on developing novel applications. However, efficient and useful NP labelling of proteins remains challenging, particularly due to the interface between the biomolecule and the nanostructure. Inorganic-biological interfaces have always been problematic on small length scales.\(^14\) Macroscopically, medical devices and implants must be compatible with living tissue. On the nanometer scale, surface effects become dramatically enhanced and could impair the integrity of biomolecules. A central issue is that both the structure and function of the protein must be maintained. Clearly, in order to create bioinorganic hybrid devices that properly function biologically, the interface must be optimized in such a manner so as to preserve the biological function of the biomolecule. Site-specific labelling of large and even very complex proteins with small dyes has been utilized for some time now, and has led to many advances in dynamics and structural studies as well as in imaging both \textit{in vitro} and \textit{in vivo}\(^15\) (e.g., fluorescence resonance energy transfer (FRET)). The labelling reaction can be done in living cells using enzymes that react specifically with a unique motif on the protein to label.\(^16,17\) It is possible to even crystallize the labelled complex and observe the effect on the structure.\(^18,19\) This has resulted in sophisticated understanding of cellular trafficking and protein behaviour and mechanism,\(^15,20,21\) along with new applications that exploit site-specific labelling. NPs have been of interest to the science community for many years because of their unique material and size dependent properties.\(^22\) It is therefore desirable to gain the same
Bioconjugation of gold nanoparticles with proteins

versatility with NPs as for small dyes. Although tremendous progress has been made, analogous capabilities for NP labelling are challenging. NPs are considerably larger than dyes and possess a lot of surface area which increase non-specific interactions of amino acid side chains of the protein with the NP ligand or surface. The details of these interactions are still unclear, because they are often numerous and difficult to physically and chemically characterize. As a result, the ability to control the site of NP attachment has been achieved primarily for GNPs, which have well-defined and controllable chemistry, and only on a limited set of proteins.23-25

A NP is more complex than a simple hard sphere, as it is often approximated. Instead, it has multiple surface coating ligands that have end groups and side chains that can interact with the protein. The surface of NPs surfaces represent a significant portion of the particle. Below ~2 nm the surface to volume atomic ratio can exceed 50%. NP surfaces are not uniform but have multiple crystal lattices, vertices and edges, each of which can have different chemical affinities for adsorption.26 This is further complicated by the fact that NP samples are typically polydisperse, with finite size distributions. Many spectroscopic techniques used to probe bulk surfaces are either not applicable or are difficult to implement on soluble NPs. Furthermore, surface-coating ligands on the NP are labile. They can adopt multiple conformations and can migrate to different sites on the NP surface. In fact, Rotello et al.27,28 have exploited this rearrangement of ligands to optimize the interaction with a protein. Consequently, bioconjugation strategies must consider the NP as a chemical system as complex as proteins.29

Furthermore, NP conjugation may result in unfolding of the protein. It is difficult to use most of the traditional techniques used for assaying the protein structure. It is technically more challenging to get a crystal structure of a conjugate, which is complicated by NP polydispersity. In addition, NMR studies require large amounts of samples. Therefore, the question arises as to how to characterize the structure of the protein when linked to a NP and identify the nature of its interactions with the NP surface atoms and ligands. These questions have begun to be probed for proteins that are non-covalently adsorbed on NPs and studies31-33 have revealed that the interface between the protein and NP can have significant biological ramifications.
Bioconjugation of gold nanoparticles with proteins

3.1.1. Linking chemistry

The strategies to link a protein to the NP have taken four main approaches (figure 1):

- electrostatic adsorption,
- conjugation to the ligand on the NP surface,
- direct conjugation to the NP surface

Issues involved in these labeling strategies include steric hindrance, or whether the protein can ‘get past’ the ligand to the NP surface or the relevant linking group. A choice of chemistries that result in a specific link (i.e., do not cause extensive cross-linking) and are stable for the desired purpose are also necessary considerations.

3.1.1.1 Electrostatic adsorption

The most widely used linkage approach consists of electrostatic adsorption (figure 3.1(a)). This is the simplest as it requires no chemical reaction, and is already used routinely as an electron dense marker in histology. Appropriate conditions for

Figure 3.1: NP–protein labeling strategies (a) electrostatic attachment of protein, (b) covalent attachment to the NP ligand and (c) direct linkage of amino acid on the NP core.
the NP ligands and protein side chains to become attracted to each other must be
determined. The interaction may be modulated by the pH or charge screening via
controlling the ionic strength of the medium. Since it is inherently a non-specific
interaction, the protein may interact with the NP at any of the labelling sites.
However, strategic modification of NP surface chemistry has enabled regio-specific
interactions with the protein.\textsuperscript{39}

3.1.1.2 Covalent Linkage

Another general method for NP–protein conjugation is covalently linking a
protein to the NP ligand (figure 3.1(b)). If the NP has multiple ligands that can react
with the protein, this can result in a distribution in the number of proteins on the NP.
The stoichiometry can be influenced by varying the ratio of the reaction. A population
of NP–protein conjugates with various protein:NP ratios is usually produced. This
approach has been greatly advanced by extreme control over the surface chemistry of
the NPs. For example, pioneering work by Hainfeld et al\textsuperscript{40} have been able to isolate
GNPs with exactly one reactive group by HPLC, which enables limiting the coverage
of proteins on the NP surface.

The synthesis of these gold clusters for labelling purposes has contributed to
the success of nanoprobe company. Although this chemistry was originally intended
for imaging by electron microscopy (EM), it has enabled capabilities well beyond EM
imaging such as electron transfer.\textsuperscript{10} Other strategies to restrict the possible number of
linking groups on a NP include control over the morphology of the surface ligands.
This has been demonstrated in strategic use of ligands that phase separate into
domains on the NP surface, creating only two sites on which a reactive ligand may be
placed.\textsuperscript{41} The synthesis of large ligands that coordinate in a particular way to the NP
surface has also permitted a precise number of linking groups.\textsuperscript{18,42-48} Another popular
labelling chemistry utilizes the covalent binding of primary amines with N-
Hydroxysuccinimide (NHS) esters or R-COOH groups via reaction with
ethyl(dimethylaminopropyl)carbodiimide (EDC) (figure 3.2).\textsuperscript{49,50} It involves an
aqueous two-step coupling process using EDC and NHS or sulfo-NHS to form an
amide bond with a protein or other molecules. It proceeds through an intermediate
sulfo-NHS ester, which has better reactivity for coupling amines than the initial EDC-
reactive ester. Organic solvent activation processes using NHS esters or acyl
Bioconjugation of gold nanoparticles with proteins

imidazole-reactive groups also can be used with solvent stable particles to result in the same product with an amine-containing molecule. NPs labelled with NHS esters can react to form covalent bonds with the primary amine of lysine on a protein. In addition, NPs coated with maleimide groups can react with the thiol of cysteine on a protein.

Figure 3.2: Aqueous two-step coupling process using ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) or sulfo-NHS to form an amide and organic solvent activation using dicyclohexylcarbodiimide (DCC) processes using NHS esters used with solvent stable particles to result in the same product with an amine-containing molecule.

3.1.1.3 Direct reaction with NP surface atoms

A direct reaction of a chemical group on the protein without the use of a linker\textsuperscript{23-25} is usually desired if the particle is used as a biosensor where Fluorescence Resonance Energy Transfer (FRET) or electron transfer is used. For gold nanoparticles, this can be achieved by the gold-thiol chemistry where a protein with a
Bioconjugation of gold nanoparticles with proteins

Cysteine (amino acid containing sulfur) covalently bonds to a GNP. The conjugation requires only incubation of the two species together as the Au–S bond is strongly favored. This results in a short, direct link from the protein side chain to the NP surface. Disulfides of self-assembled monolayers (SAMs) are known to break up in order to interact with gold because the Au–S bond strength approximates S–S strength (~40 kcal mol\(^{-1}\) compared to ~50 kcal mol\(^{-1}\)). This suggests that NP labelling can potentially break up dithiols of protein, which could potentially compromise the stability of the protein and lead to denaturation.

However, because disulfide bridges tend to be buried in the protein, this is unlikely if the protein remains folded upon NP attachment. In the case of proteins that have free cysteines close to the protein surface that are not tied up as dithiols, this is a highly convenient way to conjugate a protein to a GNP. Similarly, for sulphur containing NPs such as ZnS/CdSe, cysteine can directly form a disulfide bridge with the surface S atom. Direct linkages can also be achieved by Histidine tags, which can attach directly to Zn, Ni, Cu, Co, Fe, Mn atoms. Mattoussi et al. and other groups have exploited the fact that histidine (amino acid) groups can coordinate to Ni NPs and to the Zn on the surface of CdSe/ZnS or CdS/ZnS quantum dots, obviating the need for the addition of a metal ion.

An important concern in direct labelling of the NP core is steric crowding on the NP surface. It may be difficult for the targeted protein residue to reach the NP surface if the NP ligand is too densely packed on the surface or too long. For example, polyethylene glycol is a popular ligand for NPs as it prevents non-specific adsorption of protein side chains with the NP core. However, its long chain may hinder access to the conjugation site. Also, if the strength of interaction of the ligand with the NP surface is similar or higher than the bond-linking proteins to NPs, it may not be able to displace the ligand from the NP surface and attach the protein. In this case, a large excess of protein in the reaction is necessary.

3.1.2 Site-specific labeling

For numerous applications, it is important for the NP–protein conjugate function to label the protein at a particular site. For example, NPs are linked to proteins to sense when the protein binds to its substrate, typically by FRET. For this
purpose, it is crucial that the NP be placed in a particular site of the protein so that it is region-specific or even site specific. Also, for NPs used as EM imaging tags to localize a specific structure within a large protein, it is advantageous to attach the NP in close vicinity of the residues or motifs of interest. Even if one can control the stoichiometry of the resulting product (the NP:protein ratio), and assuming that the protein is fully folded upon labelling, most labelling chemistries do not have the ability to single out a single amino acid. Instead, there is a probability that the NP can be placed at any number of the available sites (figure 3.1), such as labelling the primary amines or carboxylic acids. This results in the protein assuming random orientations on the NP surface. Moreover, if one of these sites is in a non-ideal position, such as within the binding site, those proteins will not be able to bind to their substrate. If the experimental probe is an ensemble measurement, then one would not be able to distinguish a 20% decrease in activity due to all proteins having a lower activity due to the presence of the NP, or due to 20% of them being totally inactive. This distribution of labelling sites is also a problem if one wants to use the NP to obtain quantitative information about the protein function, such as via FRET pairs in which the distance of the NP to a chromophore matters.

This is further complicated by the fact that for larger proteins (more than approximately 100 residues) the possibility of having a unique amino acid that can link to a NP is difficult. For instance, if labelling is achieved via chemistry conjugating to the primary amine of lysine and a protein has multiple lysines, a distribution of labelling sites would result, some of which may be detrimental for the protein function. Nevertheless, labelling of NPs to a specific amino acid on a protein has been achieved by a variety of methods. Table 1 lists examples where proteins are linked directly to a NP core, and table 2 those which link to a ligand on the NP surface labelling.

### 3.1.3 Techniques for purification of conjugates

Once the NP–protein conjugation is achieved, it must be purified from both the free protein and free NP species. This has been achieved by a variety of approaches, such as spin columns, ion exchange, and gel filtration.
Bioconjugation of gold nanoparticles with proteins

In addition, multiple washes and centrifugation have been successful in isolating the NP–protein conjugate. If the NP is magnetic, magnetic separation can be used to purify the NP–protein conjugate away from free protein. This strategy has been developed for commercial use, and large beads containing multiple magnetic species can be used to purify proteins. However, from a labelling perspective, an introduction of a magnet precipitates all the magnetic NPs, including those which are unlabelled.

Table 1. Strategies for labeling a specific residue that involve direct linkage to the NP core.

<table>
<thead>
<tr>
<th>Labeled residue-NP material</th>
<th>Examples of labeled protein</th>
<th>Molecular weight</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-Au</td>
<td>Cytochrome c</td>
<td>12.6 kDa</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Ribonuclease S</td>
<td>13.7 kDa</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>70 s ribosome</td>
<td>2.5 MDa</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>scfv antibody</td>
<td>25 kDa</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Chaperonin</td>
<td>60 kDa</td>
<td>56</td>
</tr>
<tr>
<td>Cys-CdSe/ZnS</td>
<td>Chaperonin</td>
<td>60 kDa</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Human serum albumin</td>
<td>67.0 kDa</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>α-chymotrypsin</td>
<td>21.6 kDa</td>
<td>61</td>
</tr>
<tr>
<td>His Tag-CdSe/ZnS</td>
<td>Maltose binding protein</td>
<td>40.6 kDa</td>
<td>62</td>
</tr>
<tr>
<td>His Tag-Ni</td>
<td>Green fluorescent protein</td>
<td>27 kDa</td>
<td>57</td>
</tr>
</tbody>
</table>

While this is adequate for applications in which a particular protein needs to be pulled out of solution away from other proteins, it may not be feasible for synthesizing and purifying a NP–protein conjugate, because a mixture of both NPs linked to protein and free NPs will be obtained.
Table 2. Strategies for labeling a specific residue to the NP surface.

<table>
<thead>
<tr>
<th>Labeled residue-NP material</th>
<th>Examples of labeled protein</th>
<th>Molecular weight</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-dimercaptosuccinic acid</td>
<td>Cytochrome c</td>
<td>12.6 kDa</td>
<td>63</td>
</tr>
<tr>
<td>His Tag-Ni-NTA</td>
<td>Adenovirus serotype 12 knob</td>
<td>60.6 kDa</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>20S proteasome</td>
<td>750 kDa</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Streptopain</td>
<td>42 kDa</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Green fluorescent protein</td>
<td>27 kDa</td>
<td>65</td>
</tr>
<tr>
<td>His Tag-Co-NTA</td>
<td>Horseradish peroxidase</td>
<td>44 kDa</td>
<td>43</td>
</tr>
<tr>
<td>Lys-azide</td>
<td>Lipase</td>
<td>30 kDa</td>
<td>66</td>
</tr>
</tbody>
</table>

Nevertheless, adjustment of reaction stoichiometry may ameliorate this problem. HPLC has also been successful in isolating NP-protein conjugates.\textsuperscript{23,45} GNPs with aminoethanethiol ligands conjugated to yeast cytochrome c were separable from both free protein and free NP.\textsuperscript{23} Absorption spectra of the elution curves confirmed that the isolated species were indeed the conjugate. Gel electrophoresis has been used for many years. It was demonstrated in 2001 by Alivisatos et al\textsuperscript{69} that it can be used to purify NP-DNA conjugates of desired stoichiometry and soon after for NPs linked to proteins.\textsuperscript{70} Electrophoresis can be used both as a means to isolate the conjugate from other species, or as a means to assay the purity of a sample. It should be noted that the mobility of a sample is due to both its charge and size, and a particle size distribution and charge would lead to a smearing of bands, which can often obscure good separation. Electrophoresis utilizes the fact that the conjugate has a mobility that differs from both the free protein and free NP. This change in mobility is influenced by both a change in the size or charge.
3.2 Experimental details

3.2.1 Critical flocculation assay and conjugation of glutamic acid reduced GNPs with proteins

Bovine serum albumin (BSA) and immunoglobulin (IgG) were chosen as model proteins to study the GNP-biomolecular interactions. Glutamic acid reduced GNPs (40 nm) were used for the bioconjugation (figure 3.4). The minimum amount of protein required to stabilize the GNPs was optimized by employing a flocculation assay (figure 3.5).

The critical flocculation concentration (CFC) is indicated by a large decrease and or red shift of the maxima as a result of aggregation of GNPs. Critical flocculation concentration (CFC) was determined, as the threshold concentration of the electrolyte (NaCl) in the gold sol which caused rapid aggregation of the particles. In this assay, serial dilutions of protein solutions were prepared. 100 µL of each dilution was added to 1 mL of the GNPs solution. After 15 min, flocculation was induced by adding 100 µL of 10% NaCl and absorbance was measured at 580 nm.
The amount of protein necessary to prevent flocculation was deduced graphically from the concentration at which the absorbance becomes nearly constant.

For the determination of amount of protein bound to GNPs, BSA and IgG solutions were prepared at different concentrations (0.1–1 mg/mL) followed by spectrophotometric analysis. The initial concentration of the protein was checked using UV–vis spectrophotometer (Hitachi) by measuring the absorbance of protein solution at 280 nm.

**Figure 3.4:** Bioconjugation of Glutamic acid capped GNPs with protein molecules.

GNPs solution was mixed with protein solutions of different concentrations and the mixture was incubated under mild shaking for 2 hours at room temperature. After incubation, the mixture was centrifuged at 12,000 rpm for 30 minutes and washed several times with deionized water and the absorbance of the supernatant was measured at 280 nm (figure 3.6). The amount of protein bound to the GNPs was determined by subtracting the free protein in the supernatant from the total amount of the protein added initially. To determine the minimum amount of protein required for nanoparticle stabilization, flocculation assay was done for varying protein concentrations for the glutamic acid reduced GNPs (figures 3.6 (a) and (b)). The data
show that the adsorption of BSA and IgG to GNPs saturated at 20 and 40 µg/mL, respectively.

**Figure 3.5:** Synthesis of gold nanoparticle-protein conjugates.

**Figure 3.6:** Flocculation assay determining the amount of proteins (a) BSA and (b) IgG, required to stabilize the GNPs.
Bioconjugation of gold nanoparticles with proteins

Further, GNPs are highly polarizable metals with a large Hamaker constant. They are prone to aggregation in high ionic strength solutions in which the van der Waals attraction is stronger than the electrostatic or steric repulsion provided by surface-bound ligand. For confirmation of stability of GNP-protein complex, different concentrations of NaCl were used to induce aggregation in the GNP-protein complexes (figure 3.7). From these spectra, it was deduced that the glutamic acid reduced GNPs conjugated with proteins were stable to a higher extent (3 M) than the citrate stabilised particles (stable upto 2 M NaCl). This confirmed the high stability of GNPs towards electrolyte-induced aggregation.

**Figure 3.7:** UV-vis spectra of GNPs (~40 nm) incubated with varying concentrations of NaCl. (a) Citrate reduced GNPs (b) Glutamic acid reduced GNPs.

**Figure 3.8:** Appearance of solutions at each step in the conjugation process. From left to right: bare gold nanoparticles after synthesis, after addition of antibodies, pellet after centrifugation. Far right: insufficient amount of antibodies during conjugation can result in nanoparticles susceptible to aggregation.
The flocculation assays of gold nanoparticle suspensions followed the expected trends from DLVO theory; sol stability decreased with increasing salt concentration. According to DLVO theory, the stability of a gold colloid is determined by the balance of forces between electrostatic repulsion of like charged spheres and van der Waal’s attraction due to the large polarizability of gold. In general, the addition of electrolytes to gold nanoparticles causes flocculation as a result of the shielding of the repulsive double-layer charges. Proteins or polymers adsorbed to the particle surface or free in solution will also influence the stability of metal sols. Proteins adsorbed to the particle surface will stabilize the sol simply due to the large positive free energy input of protein desorption required prior to particle-particle fusion. Proteins (or other macromolecules) in solution can cause particle flocculation or stabilization. Flocculation occurs whenever colloidal particles approach so closely that the free protein is excluded from the interparticle region. This is an entropic effect; the protein leaves the interparticle region in response to the loss of configurational entropy upon compression by the particles. Loss of protein between the particles leaves behind pure solvent molecules.

A reduction in free energy is subsequently gained when the solvent also leaves the interparticle region to mix with the protein. This “depletion flocculation” will only occur by demixing protein chains and solvent in the interparticle region. In good solvents, demixing is thermodynamically unfavourable, and the free protein in solution will actually stabilize the sol by preventing close approach of two particles (depletion stabilization).

Figure 3.9 shows the absorption spectra for proteins (a: BSA and b: antibodies) containing solutions before and after reaction with the GNPs. At low protein concentration, the absorbance of the supernatant of the centrifuged protein solution after treating with colloidal GNPs essentially decreases to zero. Only after saturation of the nanoparticle surfaces with the protein molecules, the noticeable absorbance of supernatant is observed. For a fixed protein concentration (250 μg/mL), the amount of BSA adsorbed on GNPs was found to be 64 μg/mL. Similarly for IgG, the amount of protein adsorbed on GNPs was 149 μg/mL. The amount of protein molecules per gold particle (~40 nm) was calculated by measuring the difference of absorbance of the protein solution before and after incubation with GNPs.
Figure 3.9: Absorption spectra of supernatant of protein–GNPs solutions from 240-310 nm (a) GNP-IgG (b) GNP-BSA conjugates. The curve ‘a’ of each spectrum shows the absorbance of the protein only while curves ‘b–f’ indicate the spectra of the supernatant after conjugation of GNPs with different concentrations of protein solutions.

3.3 Characterization

3.3.1 Fluorimetric studies

Fluorescence spectroscopy was used to calculate the number of protein molecules bound to GNP.⁷⁶ For this, BSA was first labelled with a fluorescent dye, fluorescein isothiocyanate (FITC), and a standard calibration curve of fluorescence intensity versus FITC-BSA concentration was plotted. Stock solution of the dye, FITC (1 mg/mL) was prepared in DMSO. BSA stock solution of 1.5 mg/mL was prepared in phosphate buffer. 50 µL of FITC solution was added to it. The mixture was incubated for 1 hour at room temperature in dark and then dialyzed against phosphate buffer for 48 hours. The FITC:Protein ratio was calculated to be 5.65. So approximately 5 molecules of FITC are bound to BSA.

FITC labeled BSA was added to GNPs (250 µL) prepared in phosphate buffer (pH 7.0). To prevent aggregation, free BSA (20 µL) was added to each GNP solution, which acts as a stabilizer. The fluorescence of the supernatant after centrifugation at 12000 revolutions per minute (rpm) for 30 minutes was measured and compared with the fluorescence intensity of the standard FITC-BSA solution. Subsequently, the amount of FITC-BSA bound to the GNPs was correlated with the difference in the fluorescence intensities of free FITC-BSA in the supernatant and the total amount of the FITC-BSA added to the solution. Figure 3.10 (a) shows the standard calibration curve of fluorescence intensity of FITC-BSA versus concentration of protein ($R^2$...
Bioconjugation of gold nanoparticles with proteins

The number of BSA molecules bound to GNPs decreased gradually with decreasing concentration of the protein (figure 3.10 (b)). In a previous study, Xie et al.\textsuperscript{77} has reported that no more than 50 BSA molecules are adsorbed at monolayer coverage for 15 nm citrate stabilized GNPs.

![Figure 3.10](image)

**Figure 3.10:** (a) Standard curve of fluorescence intensity vs FITC-BSA concentration. The inset shows the number of BSA per GNPs with respect to the concentration of BSA. The number of particles of gold and protein were calculated from their respective molarities. (b) Plot of bound BSA vs. BSA added to the solution.

### 3.3.2 Electrophoretic studies

Gel electrophoresis (1% agarose) was used to qualitatively confirm binding of proteins (a: BSA and b: IgG) to carboxylated gold nanoparticles (figure 3.11). Each lane represents the increase in protein: GNP ratio. The gel shift decreased with increasing concentrations of protein in both the cases confirming binding of protein to the NPs.

![Figure 3.11](image)

**Figure 3.11:** Agarose gel electrophoresis of protein: GNPs conjugates. (a) BSA:GNP and (b) IgG:GNP. The lanes represent protein:GNPs conjugate: (1) control (GNPs), (2) 5:1, (3) 10:1, (4) 15:1 and (5) 20:1.
3.3.3 Fluorescence quenching studies

Specific noncovalent binding of nanoparticles to biological macromolecules, such as proteins, etc. due to quenching of fluorescence intensity of tryptophan residues of protein molecules enabled the determination of binding constant ($K_b$) of proteins (IgG and BSA) to GNPs. Figure 3.12 shows the relative fluorescence intensity of tryptophan residues of IgG and BSA with different concentrations of GNPs.

![Figure 3.12: (a) and (b) show the effect of conjugation of GNPs on proteins BSA and IgG, respectively. (A: GNP-BSA and B: GNP-IgG). Inset shows the double logarithmic plot of the quenching of the fluorescence by GNPs for the calculation of binding constant ($K_b$).](image)

The fluorimetric assay was chosen due to the high sensitivity of the technique in probing the intrinsic fluorescence of the tryptophan residue of IgG molecules, which is quenched by the binding of GNPs to the specific sites. This provides a strategy to investigate the interaction between GNPs and IgG through the evaluation of specific parameters that clearly describe the binding process as the binding constant ($K_b$). Both BSA and IgG caused a linear reduction in the fluorescence of tryptophan residues after the addition of increased concentrations of GNPs. The tryptophan residue fluorescence intensity ($F$) scales with the GNP concentration [GNP] through $(F_0 - F)/(F - F_{sat}) = ([GNP]/K_{dis})^n$. The binding constant $K_b$ was obtained by plotting $\log[(F_0 - F)/(F - F_{sat})]$ versus $\log$[GNP], where $F_0$ and $F_{sat}$ are the relative fluorescence intensities of the protein alone and the protein saturated with GNPs, respectively. The value of $\log$[GNP] at $\log[(F_0 - F)/(F - F_{sat})] = 0$ equals to the logarithm of the dissociation constant ($K_{dis}$). The reciprocal of ($K_{dis}$) is the binding constant $K_b$. Fluorescence intensity data corresponding to nanoparticles with a core...
Bioconjugation of gold nanoparticles with proteins

Size of roughly 40 nm is shown in figure 3.12. The inset represents the best fit in the data using \( \frac{(F_0 - F)}{(F - F_{sat})} = \frac{[\text{GNP}]}{K_d}\). The value of \( K_d \) obtained from the fitting for GNP-BSA and GNP-IgG was equal to \( 3.16 \times 10^{11} \) and \( 1.82 \times 10^{12} \) M\(^{-1} \), respectively.

3.3.4 Immunodot Assay Format

An immunodot based assay was developed using a nitrocellulose membrane (NC) (Amersham, India). 1 μL of each protein solution (BSA and IgG) prepared in phosphate buffer at different concentrations was spotted at the detection zone on NC membrane. After blocking with 10% skimmed milk at room temperature for 2 hours, the membrane was dipped in different dilutions of gold-protein conjugates (IgG-GNP and BSA-GNP) to react with the corresponding antigen/antibody coated on the membrane. The observed color due to binding of colloidal gold reagent at the site of test zone was found to be proportional to sample concentration (figure 3.13).

![Scheme 1](image1)

![Scheme 2](image2)

Figure 3.13: Immunodot assay format
3.3.5 Circular Dichroism (CD) studies

CD is one of the powerful analytical tools to study the interaction of protein to other molecules and to determine the protein conformation in solution or adsorbed onto other molecules. BSA has a high percentage of α-helical (67%) structure, which shows a characteristic CD signal in the far UV-region. Changes in the ellipticity at 208 nm and 222 nm are useful probes for visualizing varying α-helical content. Figures 3.14 shows the typical CD spectra of native BSA and BSA conjugated with GNP of different concentrations. The CD spectra were taken in a wavelength range between 197 and 250 nm, and the results were expressed as mean residue ellipticity (MRE) in deg cm² dmol⁻¹. The value of MRE was obtained using the equation MRE = [θ]208/10nIC where (θ208) is the CD in mdeg, n is the number of amino acid residues in protein (total 583 for BSA), l is the path length of cell (0.1 cm), and C is the concentration of the protein.79 The decrease in ellipticity values at 208 nm in the CD spectra of BSA indicates that there is loss of the α-helical content of protein after the conjugation with GNP with increasing concentration of GNP in the mixture (figure 3.14 (a)).

![Figure 3.14: CD spectra of (a): BSA (curve a) and BSA conjugated with GNP (curves b-f) of different concentrations. (b) IgG (curve a) and IgG conjugated with GNP (curves b-f) of different concentrations. The concentrations of GNP are (a) 0, (b) 2.5 X 10⁻¹¹M, (c) 5.0 X 10⁻¹¹M, (d) 10.5 X 10⁻¹¹M, (e) 15.0 X 10⁻¹¹M and (f) 20.5 X 10⁻¹¹M.](image)

It was attributed that the change in the helicity of BSA at different concentrations originated mainly from its different conformational states. The CD spectra of BSA-gold nanoparticle conjugates indicated a conformational transition from α-helix to β-sheet structure in the conjugated BSA. Similar conformational transition in the structure was observed when IgG molecules (Mw = 150,000) were
Bioconjugation of gold nanoparticles with proteins

conjugated with GNPs prepared at different concentrations (figure 3.14 (b)). The conjugation of whole IgG molecule with GNPs made significant changes in the conformation of IgG secondary structure, which affects the specificity of antigen binding of IgG molecules. Since IgG labelled GNPs are at the leading edge of the rapidly emerging field of immunodiagnostics and bioimaging, understanding of the conformational change in protein after conjugation with nanoparticles is important.

A comparative analysis of both the glutamic acid capped GNPs and citrate capped GNPs was done using CD and fluorimetric studies. Figures 3.15 (a and b) show the typical CD spectra of BSA and BSA conjugated with nanoparticles at pH 7.0 and 9.0 respectively, which correspond to different isomeric forms of BSA, i.e., N for the normal or native form and B for the basic form. To further explore the effect of GA capped GNPs on the conformation of BSA, conjugation studies were carried out both at pH 7 as well as pH 9. The conjugation of GNPs led to unfolding of proteins as evident from figure 3.16 (a). The effect was more pronounced in case of pH 9 as compared to pH 7 for the GA capped GNPs which showed significant decrease in the fluorescence intensity as depicted in figure 3.17 (a).

To study the effect of different concentrations of GNPs on the structure of BSA, both CD and fluorimetric techniques were used. Both at pH 7 as well as pH 9, there was a linear decrease in the CD value of the protein as evident from the CD curves (figures 3.16 (b) and 3.17 (b)). From the CD values, the % α helical content was calculated as described earlier. Also, a linear decrease in the fluorescence emission at 340 nm was obtained which was in perfect correlation with the CD data.
Bioconjugation of gold nanoparticles with proteins

(figures 3.16 (d) and 3.17 (d)). A decrease in the fluorescence emission as well as the CD values was also seen at pH 9. The change in α-helical content of protein was not significant at the optimum concentrations of GNPs, which implies that the protein could retain most of its helical structure after conjugation with GNPs.

![Graphs](figures)

**Figure 3.16:** Spectrometric analysis of GNP-BSA conjugates at pH 7.0

(a) Fluorescence spectra of native BSA, glutamic acid capped GNPs conjugated with BSA (GA-BSA) and citrate capped GNPs conjugated with BSA (cit-BSA).

(b) CD spectra of native BSA (curve a) and BSA conjugated with different concentrations of GA-GNPs (curves b–f).

(c) Plot of corresponding mean residual ellipticity (MRE) at 208 nm vs. the concentration of GNPs.

(d) Effect of conjugation of GNPs on fluorescence intensity of tryptophan residue in BSA. The concentrations of GNPs are (a) 0, (b) 2.5 X 10^{-11} M, (c) 5.0 X 10^{-11} M, (d) 10.5 X 10^{-11} M, (e) 15.0 X 10^{-11} M and (f) 20.5 X 10^{-11} M.
Bioconjugation of gold nanoparticles with proteins

Figure 3.17: Spectrometric analysis of GNP-BSA conjugates at pH 9.0
(a) Fluorescence spectra of native BSA, glutamic acid capped GNP s conjugated with BSA (GA-BSA) and citrate capped GNP s conjugated with BSA (cit-BSA).
(b) CD spectra of native BSA (curve a) and BSA conjugated with different concentrations of GA-GNPs (curves b–f).
(c) Plot of corresponding mean residual ellipticity (MRE) at 208 nm vs. the concentration of GNPs.
(d) Effect of conjugation of GNPs on fluorescence intensity of tryptophan residue in BSA. The concentrations of GNPs are (a) 0, (b) 2.5 X 10^-11M, (c) 5.0 X 10^-11M, (d) 10.5 X 10^-11M, (e) 15.0 X 10^-11M and (f) 20.5 X 10^-11M

BSA in the native form is known to possess the most compact form, while in basic form of BSA (B form), the molecules undergo expansion, and therefore, loss of secondary structure of the protein was expected for the basic form of BSA. This change in the original structure of the protein may result in loss of its biological activity or the activation of immune response.

The spectroscopic analysis of BSA-GNP conjugates done in the present study indicated that the protein did not undergo strong structural changes and could retain
Bioconjugation of gold nanoparticles with proteins

most of its helical structure after the conjugation. This is particularly important for the application of the protein-GNP conjugates in the biomedical applications where loss of the original structure of the protein in the bioconjugates may influence its use for such applications. The original structure of the protein is still retained owing to the higher curvature of the GNPs.

3.4 Bioconjugation of octadecylamine (ODA) functionalized GNPs with protein

3.4.1 Synthesis of conjugate

The amine capped GNPs (ODA capped GNPs) were successfully conjugated with Bovine Serum Albumin (BSA) in the following manner. BSA solution (100 μg/mL) was prepared in sodium acetate buffer (pH 3.36) followed by spectroscopic analysis. GNPs solutions of different concentrations were mixed with BSA solution and the mixture was incubated under mild shaking for 2 hours at room temperature. After incubation, the mixture was centrifuged at 12000 rpm for 30 min and washed several times with deionized water.

3.4.2 Determination of binding constant and the stoichiometry between GNP and protein

Binding constants of bound protein to functionalized GNPs were determined by a fluorescence based assay. In this assay, the protein solution (100 μg/mL) was mixed with different concentrations of GNPs. The fluorescence of protein-GNPs solutions was measured using a Perkin Elmer spectrofluorimeter (LS 50B) between 300 and 450 nm with an excitation at 290 nm. Analysis of fluorescence data was performed as mentioned earlier. The binding constant \( k_b \) was determined by plotting \( \log[(F_0 - F)/(F - F_{sat})] \) versus \( \log[GNP] \); where \( F \), \( F_0 \) and \( F_{sat} \) are the tryptophan residue fluorescence intensity of protein-GNP solution, the relative fluorescence intensity of the protein molecules alone, and the protein saturated with the GNPs, respectively. The slope of the double logarithm plot obtained from the experimental data is the number of equivalent binding sites \( n \), whereas the value of \( \log[GNP] \) at \( \log[(F_0 - F)/(F - F_{sat})] = 0 \) equals to the logarithm of the dissociation constant \( (k_{dis}) \). The reciprocal of \( k_{dis} \) is equal to the binding constant \( k_b \).

Figure 3.18 (a–c) shows the relative fluorescence intensity of tryptophan residues of BSA with different concentrations of GNPs of core size 28, 44 and 77 nm,
respectively. The fluorimetric assay has been chosen due to the high sensitivity of the technique in probing the intrinsic fluorescence of the tryptophan residue from BSA, which is quenched by the binding of GNPs to its specific sites. This provides a strategy to investigate the interaction between GNPs and BSA through the evaluation of specific parameters that clearly describe the binding process as the binding constant ($k_b$) and the binding stoichiometry of the complex ($n$). With increasing concentrations of GNPs in the bioconjugates, the fluorescence intensity decreased gradually.

Gold nanoparticles are known to exhibit efficient energy transfer behaviour as excited state quenchers. Thus, in the bioconjugates where protein is situated in the vicinity of the gold nanoparticles, efficient energy transfer occurs between the protein and GNPs. As a result, the emission of tryptophan residues in the protein is quenched. Fluorescence intensity data corresponding to nanoparticles with core sizes 28, 44 and 77 nm are shown in figure 3.18 (a–c).

Figure 3.18: Fluorescence spectra of BSA with (a) 28 nm GNPs, (b) 44 nm GNPs, and (c) 77 nm GNPs. Inset shows the corresponding straight-line curve for the calculation of the binding constant. The concentration of GNPs (in mol L$^{-1}$) from top to bottom:(a–f) correspond to 0, 2.62x10$^{-11}$, 7.89x10$^{-11}$, 15.88x10$^{-11}$, 22.85x10$^{-11}$, 27.98x10$^{-11}$ respectively, (g–l) correspond to 0, 1.35x10$^{-11}$, 6.22x10$^{-11}$, 12.45x10$^{-11}$, 19.63x10$^{-11}$, 25.29x10$^{-11}$ respectively and (m–r) correspond to 0, 1.92x10$^{-11}$, 5.83x10$^{-11}$, 13.59x10$^{-11}$, 18.44x10$^{-11}$, 21.85x10$^{-11}$ respectively.
Inset of figure 3.18 represent the best fit in the data using \((F_0-F)/(F-F_{sat}) = ([\text{GNP}]/k_{\text{diss}})_n\). Thus, the values of \(k_{\text{fn}}\) obtained from the fitting procedure are \(0.81 \times 10^{12} \text{M}^{-1} \text{(1.3165)}\), \(1.34 \times 10^{12} \text{M}^{-1} \text{(1.1059)}\) and \(1.818 \times 10^{12} \text{M}^{-1} \text{(1.0641)}\) for 28, 44 and 77 nm sized GNPs, respectively. These values show that the largest sized GNPs (77 nm) present the highest value of the binding constant but the least number of binding sites available for ligand binding. On the other hand, the smallest sized GNPs (28 nm) showed the lowest value of the binding constant but the largest value for the number of binding sites. The 44 nm sized particles thus proved to be the optimum ones for the efficient labelling with the protein molecules.

3.4.3 Circular dichroism measurements of the GNPs-protein conjugates

Circular dichroism spectroscopy is useful to obtain local information about the conformational and dynamic changes of protein. CD spectra of BSA and GNP-BSA samples prepared in PBS (10 mM, pH 7.4) showed a small disturbance on the BSA conformation on both the secondary and the tertiary levels with different core sizes of GNPs (figure 3.19). The data confirmed that the secondary as well as tertiary structure of protein remained almost intact after the conjugation with GNPs.

Keeping in mind, all these observations, it was concluded that approximately 40 nm GNPs were found to be the most optimum for efficient biolabelling.

Figure 3.19: CD spectra of BSA in the native state (curve d) and GNPs-BSA conjugates with GNP size of 28 nm (curve a), 44 nm (curve b), and 77 nm (curve c) respectively.
REFERENCES

Bioconjugation of gold nanoparticles with proteins

Bioconjugation of gold nanoparticles with proteins

Bioconjugation of gold nanoparticles with proteins


