Chapter 3

Green Synthesis of Gold Nanoparticles and its Interaction with Cancer Cells
3.1 INTRODUCTION

Nanoparticles such as nanospheres, nanorods, nanocubes, nanoplates, nanotetrapods, and nanoprisms can serve as building blocks for the synthesis of advanced materials, in a way analogous to that of atoms or molecules forming the basic constituents of matter. The size-dependent optical, magnetic, electronic, and catalytic properties of metal nanoparticles have attracted significant attention of researchers.\(^1\)\(^-\)\(^3\) Metal nanoparticles, particularly gold, are being considered in wide ranging applications, such as photonics, information storage, electronic and optical detection systems, therapeutics, diagnostics, photovoltaics, and catalysis.\(^4\)\(^-\)\(^7\)

Due to unique size, shape and special electrochemical behaviour gold nanoparticles (AuNP), show a variety of potential application in several fields such as catalysis,\(^8\) biotechnology,\(^9\) in making electronic devices\(^10\) and medicine.\(^11\) Several reports further indicated application of AuNP as antibacterial and antiviral reagent,\(^12\)\(^-\)\(^13\) in cancer cell imaging,\(^14\)\(^-\)\(^17\) photothermal therapy,\(^9\)\(^,\)\(^18\)\(^,\)\(^19\) drug delivery.\(^20\)\(^-\)\(^24\) In addition, the nanocomposites of organic dye molecules and nanoparticles showed high impact in biosensing and related fields.\(^25\)\(^-\)\(^28\) It is very attractive to use in bio-imaging, medicine and technology because of its chemical stability and unique propensity to bind thiol (-SH) group of proteins and similar other targets.\(^29\) To be used in different field, evolution in the synthesis and defining stability and optical sensitivity of gold nanoparticles continue with many reagents and proposed several solution condition.

Gold nanoparticles (GNPs) have recently emerged as an attractive candidate for delivery of various payloads into their targets.\(^30\)\(^,\)\(^31\) The payloads could be small drug molecules or large biomolecules, like proteins, DNA, or RNA. Efficient release of these therapeutic agents is a prerequisite for effective therapy. The release could be triggered by internal (e.g. glutathione (GSH)\(^32\), or pH\(^33\) or external (e.g. light\(^34\)) stimuli. Significantly the internal stimuli operate in a biologically control manner, whereas the external stimuli provide spatio-temporal control over the release. Drug delivery systems (DDSs) provide positive attributes to a ‘free’ drug by improving solubility, in vivo stability, and biodistribution. They can also alter the unfavorable pharmacokinetics of some ‘free’ drugs. Moreover, huge loading of pharmaceuticals on DDSs can render
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‘drug reservoirs’ for controlled and sustained release to maintain the drug level within the therapeutic window.\textsuperscript{35}

A common method for production of gold nanoparticles using typical chemical reduction methods including the classical Turkevich citrate reduction method\textsuperscript{36,37} and some modification are done using variety of organic solvents.\textsuperscript{38-40} In these procedures, typically, HAuCl\textsubscript{4} is reduced by citric acid which simultaneously functions as a stabilizing agent\textsuperscript{41} or is reduced by NaBH\textsubscript{4} in the presence of thiol or carboxylic acid stabilizers.\textsuperscript{42} Sometimes pernicious and highly volatile organic solvents are used as the reaction medium such as hydrazine hydrate reduction,\textsuperscript{43} polyl process and modified polyl process,\textsuperscript{44,45} photochemical reduction,\textsuperscript{46,47} electrochemical reduction,\textsuperscript{48} microwave induction.\textsuperscript{49} Most of these processes are employed through wet chemistry methods with the use of highly reactive inorganic reducing agents such as NaBH\textsubscript{4}, hydrazine hydrate (N\textsubscript{2}H\textsubscript{4}, H\textsubscript{2}O) etc. which are not eco-friendly. To avoid toxic reagents, eco-friendly solvent like water and biological materials as capping/stabilizing reagents have been used in these synthesis methods. Reports include use of amino acids,\textsuperscript{50} vitamins,\textsuperscript{51} use of plant extracts,\textsuperscript{52} and other environmentally benign biological compounds as reducing agents\textsuperscript{53} to exclude the use of toxic reagents.

In the present work we have presented a study to show synthesis of gold nanoparticle using bilirubin (BR) as both the reducing agent and stabilizing molecule. Further we have illustrated the binding interaction of adsorbed bilirubin on the gold nano surface and formation of BR stabilized gold nanoparticle (AuBR). Formation of BR assisted preparation of gold nanoparticles was reported,\textsuperscript{54} however, no other detail investigation regarding stability, reduction mechanism and binding interaction was investigated. The current manuscript has detailed the affinity of Au (III) ion towards BR, the mechanism of reduction of gold ion to produce nanoparticle, the role of BR in stabilizing the nanoparticles and its binding interaction and structure on the surface of gold nanoparticles.

The structure of BR on nano-surface may be a key factor in the understanding of nanoparticle formation and stabilization. FT-IR is an effective technique to investigate the interaction between the metal surface and the bound molecule.\textsuperscript{41,55-59} Changes in vibrational frequencies and the variation in intensities in the spectra of adsorbed molecules compared to that in free provide information on relative interaction and
binding stability of the ligand. FT-IR study confirmed that the intramolecular hydrogen bonds of BR broke out and carboxyl group ionized and remained bound to the metal surface. The bound BR facilitated the particle to gain extraordinary stability and monodispersity in aqueous solution.

3.2 EXPERIMENTAL SECTION

3.2.1 MATERIALS and METHODS

All chemicals used were of analytical grade or of the highest purity available. Hydrogen tetrachloroaurate (III) hydrate (HAuCl₄·3H₂O, 99.9%), bilirubin (C₃₃H₃₆N₄O₆, >98%) were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH) was purchased from Merck Millipore. All reagents were used as received. Glassware was carefully cleaned with aqua regia (3:1 HCl/HNO₃), and then rinsed several times with Mill-Q (18 MΩ.cm resistance at 25 °C) water prior to use under sonication. Mill-Q water was used to prepare all the solutions in this study.

3.2.2 CHARACTERIZATION

3.2.2.1 Absorption

Absorption spectra of prepared gold nanoparticles were measured with a JASCO V-630 Spectrophotometer (JASCO International Co. Ltd, Japan) in the range of 400 to 800 nm wavelength. High quality quartz cuvette was used to record the spectra.

3.2.2.2 Transmission Electron Microscopy

The size of the nanoparticles was studied using transmission electron microscope (TEM). For TEM imaging, a drop of aqueous suspension of the nanoparticle solution was placed on carbon coated 300-mesh copper grid (Allied Scientific Product, USA) and dried in a dust free atmosphere. The bright field electron micrographs of the samples were taken on a Tecnai G2 Spirit Biotwin (Type: FP5018/40) at an acceleration voltage of 80 kV.

3.2.2.3 FT-IR measurement

The FT-IR spectra of the samples were recorded on a Bruker TENSOR27 spectrometer using the KBr pellet technique. Solid samples (bilirubin/AuBR) were mixed with KBr in a clean glass pestle and mortar and compressed to obtain a pellet.
The spectra were recorded from 400–4000 cm\(^{-1}\). Background spectra were obtained with a KBr pellet for each sample. Bruker software was used for data processing.

### 3.2.2.4 Fluorescence

The Intrinsic fluorescence measurements of bilirubin were recorded on Agilent Technology, Cary Eclipse fluorescence spectrophotometer. The excitation wavelengths and emission measurement were selected according to the sample, with fixed excitation and emission slit widths of 5 nm each. The quartz cuvette (4 cm × 1 cm × 1 cm) with path length of 1 cm was used. All the experiments were carried out at room temperature (24°C).

For fluorescence quenching measurements, the intrinsic fluorescence of BR was measured in the presence and in the absence of Au (III). The fluorescence of BR was found to quench in the presence of Au (III). The quenching experiment was carried out simply by adding 2.5 μL aliquot (stock solution of gold salt 1.6 mM) solution to 2 mL BR. Small error due to dilution upon addition of the gold salt was neglected. Gold salt showed negligible absorbance at the excitation wavelength (470 nm) compared to BR absorption at this wavelength. Fluorescence intensities at 520 nm were recorded as a function of Au (III) concentration. To derive the binding parameters, obtained data were analyzed using modified Stern–Volmer equation.\(^{61}\)

### 3.3 RESULTS and DISCUSSION

#### 3.3.1 Synthesis of Gold Nanoparticles

Au (III) solution was prepared by dissolving required amount of hydrogen tetrachloro aurate (III) hydrate solution in the Mili-Q water in a 100 ml clean round bottle flask and adjusted the pH ~7 of the solution by the addition of aqueous 0.25M NaOH solution. Separately, bilirubin solution was prepared by dissolving required amount of bilirubin in Milli-Q water at pH ~8 in another clean round bottle flask covered with aluminium foil.\(^{60}\) Bilirubin solution was added slowly to the Au (III) solution with vigorous stirring at 1200 rpm under dim room light condition. Subsequently, to the reaction mixture 0.5M NaOH solution was added drop wise to raise the pH to 9.0. The final concentrations of both Au (III) and bilirubin in the reaction medium were 0.25mM and molar ratio of Au (III) / bilirubin was 1:1. The
stirring was continued for additional few hours. The reaction mixture subsequently was centrifuged at 18000 rpm for 15 min. The collected gold nanoparticle (AuBR) was pinkish red in color.

To examine the effect of pH on the production rate of AuBR experiment was also perform at higher pH. It was observed that with the increase of pH in the reaction mixture, rate of the particle formation increased and the completion time of the reaction was decreased. Keeping the concentration of starting materials same, approximate reaction time at pH 9, 10 and 12 were about 18 h, 15 h and 6 h, respectively. To wash the nanoparticles, the produced material was re-dispersed in deionized water and centrifuged. The process was repeated for three times to remove excess bilirubin and other by-products. This purified AuNPs was used for further investigation.

3.3.2 Absorption study

The UV-visible spectrum of gold nanoparticles prepared at pH 9.0 and dispersed in water is shown in Fig. 3.1. The band maxima appeared at ~525 nm which is due to the surface plasmon resonance (SPR) effect of gold nanoparticles. The peak position and shape of the band was related to the particle size. Relatively clean band at 525 nm indicated the average particle size ~ 20 nm in diameter. AuNP was also prepared at three other pH conditions and the suspended particles in all cases showed the similar plasmon resonance band at ~ 525 nm indicating that pH of the reaction mixture did not effect much on the shape and size of the produced nanoparticles.

![UV-vis spectra of bilirubin coated gold nanoparticles in milli-Q water at pH ~7.](image_url)

**Figure 3.1:** UV-vis spectra of bilirubin coated gold nanoparticles in milli-Q water at pH ~7.
3.3.3 X-ray diffraction study

Fig. 3.2 shows the XRD spectrum of the synthesized gold nanoparticles. The joint committee on powder diffraction standards Card no. 03-065-2870 of pure crystalline gold structures was used to define the crystalline nature of the gold particle. A comparison of the XRD spectrum confirmed that the synthesized gold nanoparticles were in the form of face-centered cubic nanocrystals with Fm3m symmetry, as evidenced by 20 values 38.27°, 44.33°, 64.92°, and 77.59° in the XRD pattern, corresponding to (111), (200), (220), and (311) planes, respectively.

![XRD Spectrum](image)

**Figure 3.2:** Powder XRD diffraction pattern of AuBR nanoparticles. The peaks are assigned based on JCPDS Card no. 03-065-2870.

3.3.4 Morphology by TEM analysis

A TEM image of AuBR prepared at pH 9 is showing in Fig. 3.3. The particles produced under basic conditions (pH 9.0, 10.0 and 12.0) were spheroidal and monodispersed. The average diameter of the nanoparticles was ~ 20 nm as confirmed by the TEM images. The plasmon resonance band that appeared at 525 nm also indicated that the average size of the particles was ~ 20 nm as reported by several research groups.⁶²,⁶³

![TEM Image](image)

**Figure 3.3:** Transmission electron microscopic (TEM) image of bilirubin coated gold nanoparticles. The scale bar is shown in the bottom and it’s 200 nm.
3.3.5 Bonding and interaction pattern of BR on gold nanosurface: molecular detail by FT-IR analyses

The FT-IR spectra of bilirubin and AuBR are shown in Fig. 3.4 and 3.5, respectively. In addition to the bands in the 600-1700-cm\(^{-1}\), the spectra show bands at high frequency region (2850-3450 cm\(^{-1}\)). The significant FT-IR bands of both BR powder and AuBR and their assignments were summarized in Table 1. Some of the characteristic FT-IR bands of strong and medium intensity appeared at 3406, 3267, 2912, 1695, 1645, 1611, 1250, 989 and 932 cm\(^{-1}\) and, other bands at 800-600 cm\(^{-1}\) were marked in the spectra. The bands were very consistent with the normal mode analysis of vibration frequency of BR\(^6\) and the assignments were made following the literature values.\(^6\)–\(^9\)

Based on the normal coordinate analysis data\(^6\)–\(^9\), the 3406 and 3267 cm\(^{-1}\) bands observed in BR powder were assigned to the stretching modes of the pyrrole N–H and the lactam N–H, respectively. Pyrrole N–H shifted to 3446 cm\(^{-1}\) and became broader on the nano surface. Asymmetric lactam N–H stretching frequency of BR on gold nano surface appeared as shoulder in a broad band of pyrrole N–H. Similarly, asymmetric CH\(_2\) stretching vibration at 2912 cm\(^{-1}\) of BR weakly appeared at 2915 cm\(^{-1}\) in AuBR. 1188 cm\(^{-1}\) band was assigned to pyrrole ring breathing mode and it was very weak in AuBR and might be shifted to 1165 cm\(^{-1}\) due to surface association of bilirubin with the gold nanoparticles.

BR contains two carboxyl side chains and produced intense carboxyl C=O stretching vibration at 1695 cm\(^{-1}\). However, this band was absent in BR bound to the nanosurface (AuBR). Two weak bands were observed at ~ 1563 and 1391 cm\(^{-1}\) and assigned to the asymmetric and symmetric stretching vibration of the carboxylate form (COO\(^{-}\)) of BR attached to gold nanoparticle. 1645 cm\(^{-1}\) band was assigned to lactam C=O vibration which was shifted to 1647 cm\(^{-1}\) on the nanosurface. The band at 1611 cm\(^{-1}\) was assigned to the C=C stretching of the conjugated system present in BR. On the nanosurface this band (1611 cm\(^{-1}\)) possibly blue shifted to 1628 cm\(^{-1}\). The bands at 1250, 989, and 932 cm\(^{-1}\) from the BR powder, were associated with the lactam systems (C–C, C–N vibrations) and they were broadened in AuBR. An interaction of carboxylate bound to the nano-surface could interact with water molecules adsorbed on the surface. The H\(_2\)O molecules complexed with COO\(^{-}\) often produce broad ν\(_{O-H}\) band.
at 3550–3414 cm$^{-1}$. The observed broad FT-IR in this region could be due to the presence of moisture in the AuBR powder.

Figure 3.4: FT-IR spectra of bilirubin powder in solid state using KBr pellet technique.

Figure 3.5: FT-IR spectra of lyophilized powder of AuBR in solid state using KBr pellet technique.
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Table 1. FT-IR peaks of BR and AuBR powder

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Bilirubin (3406)</th>
<th>Au-bilirubin (3446)</th>
<th>Vibration mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3406</td>
<td>3446</td>
<td>$\nu_{N\text{-}H}$</td>
</tr>
<tr>
<td>2</td>
<td>3267</td>
<td>3446</td>
<td>$\nu_{N\text{-}H}$ (lactam)</td>
</tr>
<tr>
<td>3</td>
<td>3008</td>
<td>2995</td>
<td>$\nu_{N\text{-}H}$</td>
</tr>
<tr>
<td>4</td>
<td>2912</td>
<td>2915</td>
<td>$\nu_{C\text{-}H}$</td>
</tr>
<tr>
<td>5</td>
<td>2856</td>
<td>2354</td>
<td>$\nu_{N\text{-}H}$</td>
</tr>
<tr>
<td>6</td>
<td>1695</td>
<td>1674</td>
<td>$\nu_{C\text{-}O}$ (COOH)</td>
</tr>
<tr>
<td>7</td>
<td>1645</td>
<td>1647</td>
<td>$\nu_{C\text{-}O}$ (lactam)</td>
</tr>
<tr>
<td>8</td>
<td>1611</td>
<td>1628</td>
<td>$\nu_{C\text{-}C}$</td>
</tr>
<tr>
<td>9</td>
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<td>1563, 1550</td>
<td>$\nu_{C\text{-}C}$</td>
</tr>
<tr>
<td>10</td>
<td>1499</td>
<td>1517</td>
<td>ring torsion</td>
</tr>
<tr>
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<td>1445</td>
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<td>bridge carbon def, ring torsion</td>
</tr>
<tr>
<td>12</td>
<td>1406</td>
<td>1391</td>
<td>$\nu_{C\text{-}N}, \text{CH}_3$ bending</td>
</tr>
<tr>
<td>13</td>
<td>1364, 1345</td>
<td>1340</td>
<td>C-H bending ($\text{CH}_3$)</td>
</tr>
<tr>
<td>14</td>
<td>1300</td>
<td>1311</td>
<td>bridge C-H bending, bridge $\nu_{C\text{-}C}$, $\nu_{C\text{-}N}$</td>
</tr>
<tr>
<td>15</td>
<td>1250</td>
<td>1250</td>
<td>ring</td>
</tr>
<tr>
<td>16</td>
<td>1219</td>
<td>1219</td>
<td>$\nu_{C\text{-}C}$</td>
</tr>
<tr>
<td>17</td>
<td>1188</td>
<td>1165</td>
<td>ring breathing</td>
</tr>
<tr>
<td>18</td>
<td>1108</td>
<td>1110</td>
<td>$\nu_{N\text{-}H}$</td>
</tr>
<tr>
<td>20</td>
<td>989</td>
<td>1020</td>
<td>ring (C-N)</td>
</tr>
</tbody>
</table>

3.3.6 Fluorescence study: Binding affinity of BR to Au (III)

The binding affinity of Au (III) for BR at pH 9.0 was measured by measuring the intrinsic fluorescence of BR in the presence of different concentration of the gold salt. Fig. 3.6A and Fig. 3.6B respectively illustrate the fluorescence quenching study of BR and Stern-volmer (S–V) plot determined in buffer solution at pH 9.0. BR shows a strong fluorescence with an emission peak at 530 nm. The fluorescence intensity gradually decreased with the increase of the Au (III) ion concentration.

Two mechanisms often used to explain the fluorescence quenching processes (i) dynamic fluorescence quenching and (ii) static quenching. Dynamic fluorescence quenching processes are primarily via a collision process, solvent viscosity and size of the particles play vital roles on the extent of quenching. In the static process, a close association of the fluorophore and the quencher lead to decrease of fluorescence. Substantial fluorescence quenching of BR by Au (III) indicated static fluorescence
quenching and this static quenching may be due to formation of a (dark) complex between BR and the ion.\textsuperscript{61,64,65} To determine the binding parameter, the fluorescence data obtained in the above experiments were analyzed using a modified S–V equation (Eq. 1) (Lakowicz 2006, 3rd edition). Fluorescence intensity at 520 nm of the BR solution at different concentration of Au (III) was fitted to a modified S–V equation as given below:

$$\frac{F_0}{\Delta F} = \frac{1}{fK[Q]} + \frac{1}{f}$$  \hspace{1cm} (1)

Where $F_0$ is the initial fluorescence intensity in the absence of Au (III), $\Delta F$ is the difference in fluorescence in the absence and presence of the Au (III) at concentration $[Q]$, $K$ is the S–V quenching constant, and $f$ is the fraction of the initial fluorescence which is accessible to the quencher (Lakowicz 2006). The plots of $F_0/\Delta F$ versus $1/[Q]$ yield $f^{-1}$ as the intercept, and $(fK)^{-1}$ as the slope. The intercept on y axis ($1/f$) indicated that ~100% of the total BR fluorescence was quenched. The S–V quenching constant as obtained from the modified S–V equation can be expressed as binding affinity constant, $K_a$.\textsuperscript{64, 65, 61, 66} The reciprocal of $K_a$ gives the dissociation constant, $K_d$, was found to be 2.3 $\mu$M. The value of $K_a$ was $4.3 \times 10^5$ M$^{-1}$.

3.3.7 Mechanism of of Au (III) Reduction by Bilirubin molecule

The binding of bilirubin (BR) to Au (III) and subsequent reduction of this ion is crucial for the formation of nano structure. So far no detail reduction mechanism of the metal ion by BR has been established. To start the reaction the aqueous solution of bilirubin was added drop wise to the Au (III) solution. At the beginning the solution transformed to blackish green from golden yellow color, indicating the reduction of Au
(III) to Au (0) and formation of very small particles. Afterwards, a significant color change was observed by vigorous stirring; then the solution turned deep red and indicated the formation of nanoparticles of bigger size (~20 nm). The reaction mixture was kept on stirring condition and drop wise NaOH solution (0.5 M) was added to raise the pH of the reaction mixture to 9.0. After 30 min, the color of the reaction mixture turned to reddish green and the appearance of this color indicated the formation of gold nanoparticles and the reaction was finished as per time mentioned below. As the pH of the solution and ionic environment of the reaction media play an important role in the nucleation process of gold atom from Au (III) state, the reaction was carried out at several pH conditions to establish the reaction mechanism. The formation rate of gold nanoparticle (AuNP) at pH 7.0, room temperature, was slow. Upon addition of NaOH solution, the reduction of HAuCl\textsubscript{4} by bilirubin was increased. At pH 9.0, the completion of the reaction took about 24 hours and however, it became quite fast at pH 12 and the reaction was completed in ~ 6 hours. However, at the lower pH (~ 6.1), the color of the solution did not change within 24h, indicating that the reduction was not completed, and no plasmon resonance band was observed.

![Figure 3.7: Hydrogen bond stabilized structure of 4Z,15Z-bilirubin IXα.](image)

We propose here a possible mechanism for the reduction of Au (III) by bilirubin in basic condition. The usual chemical structure of bilirubin (alpha form) is shown in Fig. 3.7 and the structure is not planar, rather involutes intramolecular hydrogen bond formation which provides stability to the molecule. It consists of four pyrrole rings, two
carbonyl groups and two carboxyl groups. The intramolecular hydrogen bond shields the hydrophilic sites of the bilirubin molecule and render it weakly soluble in water. However, in alkaline pH, solubility increased due to ionization of the COOH group. The reduction of Au (III) may be the direct hydrogen abstraction induced by the metal ion from bridge methylene in basic condition and this process may increase with the increase of \( \text{OH}^- \) ion concentration. The reduction mechanism is shown in the Fig. 3.8. Another possible way could be the reduction of metal precursor by the organic radicals formed in the reaction medium.

3.3.8 Stability Study of AuBR

Thermal stability of the nanoparticles in aqueous suspension was measured following the plasmon resonance band in different solution condition. BR coated gold nanoparticles (AuBR) were dried and stored under vacuum for weeks/months. Fig. 3.9A shows the plasmon peaks in water suspension of AuBR which was stored under vacuum for different interval of time. Close inspection of the plasmon resonance absorption band indicated a very small red shift (~3 nm) due to aging for a couple of months and also scattering level was not enhanced to a large extent. These indicated that AuBR was very stable under dry condition. In wet condition also the particles
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showed substantial stability and remained stable for days without much perturbation in the plasmon resonance band. The particles remained stable in aqueous solution (pH 7) for 120 days at 20°C and the UV-Vis optical band remained at 525 nm. The further stability test was performed in the presence of KCl and NaCl and it was observed that very high concentrations (~0.8 M) of these salts had no significant effect on the stability and dispersity of the nanoparticles. Fig. 3.9B and 3.9C showed that the plasmon resonance band was not broadened or increased in absorbance, suggesting their great stability in the salt solution.

Further experiment was performed to test the stability of the particles in different pH conditions. Fig. 3.10 presents the plasmon resonance band and color of the nanoparticles suspension in acidic and basic conditions. Freshly prepared gold nanoparticle was dispersed in 10 mM phosphate buffer and the pH of the solution was
varied from 3.0 to 12.0. The UV-Vis spectrum of the solution was taken after 30 minute incubation. The Plasmon resonance band appeared at 525 nm and no detectable shift in the position could be observed in the pH range of 5.0-12.0. The solution color in this pH range was remained pinkish red. These observations indicated uniformity in the solution state dispersity and stability of the particles in the above pH ranges.
The solution color of the dispersed nanoparticles, however, started to become wine (deep) red at pH below 5.0 as shown in Fig. 3.10. The plasmon resonance band was shifted to longer wavelength (red shift) and the band maxima appeared at ~555 nm at pH 3 after 15 min of incubation. A large red shift in plasmon absorption spectra is due to the formation of larger particle or the aggregation of the nanoparticles. The calculated particle sizes are mentioned in the figure. The largest size was ~80 nm at pH 3.0 as shown in Fig. 3.10. It was possible that bilirubin attached on gold nanosurface may promote coagulation of the particles. At low pH, acidic moiety of the BR might tend to be patented and could have a strong tendency to be released from the nano surface. Bare nanoparticles are more prone to form aggregates. Bigger particles showed red shift or low energy plasmon resonance band. Thus the synthesized particle was quite stable at room temperature and in a moderate pH range (pH 5.0-12.0). Also the added salt to the nanoparticles solution merely affect the monodispersity of the particles in this pH range and further indicated strong bonding between gold nano surface and the BR functional groups.

Current study illustrated mechanism and easy synthesis of gold nanoparticles by bilirubin. It also provided the binding details of the adsorbed bilirubin onto the metal surfaces. Without using any inorganic reducing and capping reagents and also avoiding use of microwave radiation, we demonstrated a single-step green synthesis of highly stable and well dispersed gold nanoparticle using bilirubin, a biological molecule, as the capping reagent. The XRD analysis confirmed that the synthesized gold nanoparticles were face centred cubic nanocrystals. In the present work we prepared gold nanoparticles from HAuCl₄ at different pH using bilirubin (BR) which acts as a mild reducing agent. The method is environment friendly and a kind of ‘green methods’ and avoided use of hazardous solvent, reagents and the elimination of toxic side products.

Our investigation suggested that two important parameters of the reaction were controlled by pH: (a) the reactivity of Au (III) complexes with OH⁻ group, which depends on the pH of the reaction mixture and (b) the reduction ability of bilirubin by introducing NaOH. Goia et al. reported that the reactivity of the Au (III) complexes with chloride and or hydroxide as the ligands reflected by their reduction potential. They found that the reactivity of Au (III) decreased upon increasing pH, owing to the
lower reduction potential of $\text{Au(OH)}_4^-$ (+0.56V) compare to $\text{AuCl}_4^-$ (+1.002V). It is obvious that the acceleration of reduction rate by NaOH in this system could not be attributed to the interaction between $\text{AuCl}_4^-$ and NaOH.

While rate of production of AuBR depend on the pH of the reaction solution, the size, shape and dispersity of the particle remained largely unchanged in neutral and alkaline pH. The absorption of BR rendered the nanoparticle to be mono dispersed and the size, shape and dispersity of the particle remained largely unchanged between pH 5 and 12. In strong acidic condition (pH below 4.0) the nanoparticles became unstable, visible color of the dispersed particle changed from pinkish red to blue violet. The plasmon resonance band shifted from 525 nm at pH 7.0 to 555 nm at pH 3.0 indicating its coagulation to larger size particles. At low pH due to possible protonation of several N and an acidic group of BR it may lose its strong affinity for the nanoparticles surface and got released into the solution and promotes coagulation to form a larger particle with a characteristic plasmon resonance band at 555 nm at pH 3.0.

The FT-IR absorption study confirmed the association of BR with the nanoparticle. Most of the BR bands in AuBR became broad, and shifted, and only could be identified in the expanded spectra. The carboxylic C=O stretching mode at 1695 cm$^{-1}$ in BR was largely affected and shifted to a much lower frequency. Asymmetric and a symmetric stretching vibrations for the carboxylate group often appear at 1500−1630 and 1305−1415 cm$^{-1}$, respectively. The FT-IR spectra of the washed AuBR showed possible asymmetric COO$^-$ stretching vibrations ($\nu$$_{asy}$ (COO$^-$)) at 1563 cm$^{-1}$, and another peak assigned to symmetric COO$^-$ stretching vibrations ($\nu$$_{sym}$ (COO$^-$)) at 1391 cm$^{-1}$ broad peak (Figure 3.5).

![Figure 3.11: Schematic representation of bilirubin attachment on the gold nanoparticle.](image-url)
For common acids the average carbonyl double bond (C=O), the stretching vibration modes appeared at ~1723 (± 20 cm\(^{-1}\)). It was observed that the acetic acid carbonyl adsorbed at 1706 cm\(^{-1}\). As carboxyl group of BR is hydrogen bonded with lactam rings the band was shifted to 1695 cm\(^{-1}\). However, the loss of this band on gold surface was attributed to the ionization of the carboxyl group and binding to the metal surface (Figure 4). The average position of the asymmetric and the symmetric vibration mode of carboxylate form (COO\(^-\)) appeared at 1580 (± 26 cm\(^{-1}\)) and 1406 (± 12 cm\(^{-1}\)). We observed the possible symmetric and asymmetric stretching frequencies at 1391 and 1563 cm\(^{-1}\) respectively. And the carbonyl C=O stretching in unionized COOH was absent. It indicated that BR interaction with gold nanoparticle surface involved COO\(^-\) group of BR.

Stretching vibration of the lactam system C=O appeared at 1645 cm\(^{-1}\) in BR, however in the AuBR the band was shifted to high frequency (1647 cm\(^{-1}\)). In addition, the stretching modes of the pyrrole N–H and the lactam N–H appeared at 3406 cm\(^{-1}\) and 3267 cm\(^{-1}\), respectively in free BR, however, the pyrrole N–H band shifted to 3431 cm\(^{-1}\). All the blue shifts mentioned above could be ascribed to the breaking of internal hydrogen bonds between two carboxyl groups and the four N–H bonds (two pyrrole and two lactam N–H bonds). The intramolecular H-bond involving various groups (pyrromethene rings and propionic acid) in BR were broken or became weaker as it binds to the gold nano surface. Two similar dipyrromethene groups separated by a methylene unit and about six intramolecular hydrogen bonds between the propionic acid groups and pyrrole N-H and between the propionic acid groups and lactam C=O/N–H bonds as shown in Fig. 3.7. These hydrogen bonds provide conformational stability. However, most of these hydrogen bonds were broken and the ionized COO\(^-\) strongly interacts with the gold nano surface, as shown in Fig. 3.11.

3.4 Cytotoxic Behavior

3.4.1 Cell culture preparation

Human breast cancer cell line MCF 7, kidney cancer cell line HEK 293, and neuroblastoma cell line Neuro 2a were procured from the National Centre for Cell Sciences (NCCS, Pune, India) and were grown in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin and gentamicin). Cells were
cultured at 37°C in 95% air and 5% CO₂ humidified incubators. MCF 7 cells were seeded at a density of 10⁵/well plated in 96 well plates. Cells were typically grown to 60–70% confluence and then rinsed in phosphate-buffered saline (PBS). After that they are placed into a serum-free medium for overnight prior to the treatments. After overnight incubation, the MCF 7 cells, HEK 293 cells, and Neuro 2a cells were treated with bilirubin, gold nanoparticles and bilirubin-coated gold nanoparticles separately at the concentration of 10 μM, 50 μM, 100 μM. After 48 hours the medium was discarded and 50 μl of fresh medium was added along with 10 μl of MTT (5mg/ml). MTT solution was slowly removed after 4 hours and the purple crystals were solubilised in 1.5 ml of DMSO. The absorbance was measured at test wavelength of 550 nm in Elisa Plate Reader made of Dynex Technologies, USA.

3.4.2 The MTT assay

The MTT assay determines the ability of metabolically active viable cells to reduce the yellow tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) to purple formazan crystals by mitochondrial dehydrogenase. The concentration of purple color formazan crystals were spectrophotometrically (in ELISA reader) determined when dissolved in an organic solvent, dimethyl sulfoxide (DMSO). Cultured primary MCF 7, HEK 293 and Hep G2 were treated with tryptophan, CuNPs, and tryptophan coated CuNPs separately (5 µM, 10 µM, 20 µM and 50 µM). The plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO₂. MTT was dissolved in PBS at a concentration of 5 mg/mL, filtered through a 0.22 µm filter to sterilize and remove insoluble residues, and then stored in the amber vials at 4 °C. After treatment the medium was removed and 50 µl of fresh medium was added along with 10 µl of MTT added in wells of the 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO₂. At the end of the incubation period, the media were discarded using a suction pump. In a 96-well plates 1.4 ml of DMSO were added to solubilize the purple crystals in different wells. The absorbance was measured at a test wavelength of 590 nm in an Elisa plate reader (Dynex MRX Microplate reader). The absorbance obtained from treated cells were expressed as percentages of absorbance obtained from untreated cells and are reported as mean ± Sd (n = 3).
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The in vitro cytotoxicity tests of the bilirubin, gold nanoparticles, and bilirubin-coated gold nanoparticles were performed with human breast cancer cell line MCF 7, kidney cancer cell line HEK 293, and neuroblastoma cell line Neuro 2a Fig. 3.12 A, B and C (lower panel) shows the cell viability of these cancer cell lines after 48 h incubation of the control cell lines and with different concentrations bilirubin, gold nanoparticles, and bilirubin-coated gold nanoparticles. It was observed that in all the cases (bilirubin, gold nanoparticles, and bilirubin-coated gold nanoparticles) does not show any significant change in the proliferation with a concentration up to 100 µM concentration with respect to the control, suggesting that all the three are nontoxic for these different cancer cell lines. Using different type of cultured cell lines, we showed the nontoxicity of the gold nanoparticles capped with bilirubin.

![Cell viability graphs](image)

**Figure 3.12:** Upper Panel showing the phase contrast images of MCF 7 cells viability with naked gold nanoparticles (A), bilirubin-coated gold nanoparticles (B) with respect to control cell lines (C) at 50.0 µM concentration of bilirubin . Lower Panel showing the cytotoxicity of AuNP on different cancer cell lines: (A) MCF7, (B) HEK 293 and (C) Neuro 2a cell lines respectively. Cells were incubated for 48 hours in presence of different samples.

Furthermore, cells were also examined under an inverted phase contrast microscope. For example, MCF 7 cells were treated with gold nanoparticles and bilirubin-coated gold nanoparticles for 48 h and phase contrast micrography was taken. As shown in Fig. 3.12A, 3.12B and 3.12C (upper panel), there was no cell death in response to this gold nanoparticles and bilirubin-coated gold nanoparticles as compared to the control cell line. It also confirms the nontoxicity of the gold nanoparticles and
bilarubin-coated gold nanoparticles and can be used as a carrier for delivering drugs to the target site.

**3.5 CONCLUSION**

Bilirubin is a biological breakdown byproduct and its use to prepare the nanoparticles was environmentally friendly and the produced particles showed a great uniformity and stability in a wide range of pH. While the rate of production of the AuBR depends on the pH of the reaction solution, the size, shape and dispersity of the particle remained largely unchanged in neutral and alkaline pH. The disparity of the particles was, however, affected at acidic pH (below pH 4.0) and the plasmon resonance band shifted to 555 nm with indications that some aggregation of the particles occurred. FT-IR study confirmed the strong binding of bilirubin to nanosurface with the ionized carboxylate (COO\(^{-}\)) side chain. BR being a biological molecule, the surface attachment of BR may provide biocompatibility of the nanoparticles in biological systems. Thus, the produced AuBR may be very useful for therapeutic purposes upon modification with appropriate ligands and can be used for anti-cancer drug delivery applications.

**REFERENCES**


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