3.1 Introduction

Inorganic nanoparticles have emerged as one of the best drug delivery system among the present engineered nanomaterials (Paleos et al., 2004; Kisak et al., 2004; Wu et al., 2005; Salem et al., 2003). They have low toxicity, rich functionality, wide bioavailability and good biocompatibility, potential capability of targeted and controlled delivery. However, it is noted that the cellular transfer efficiency with existing inorganic NPs is relatively low. Being chemically stable, inorganic NPs remain unaltered during the whole delivery process and they should not be biodegraded in plasma and cytoplasm of a human body (Xu et al., 2006).

Specifically, Gold nanoparticles (GNPs) have gained interest due to their special features such as optical and electronic properties, high stability, controllable morphology, size dispersion and easy surface functionalisation (Nie and Emory 1997; Rosi et al., 2006; Sperling et al., 2008; Grzelczak et al., 2008).

In addition, GNPs are considered as an ideal delivery system and their use especially for targeted delivery of drugs is dynamically increasing (Duncan et al., 2010; Pissuwan et al., 2011). GNPs themselves are nontoxic and compatible with biological materials (Connor et al., 2005; Yu et al., 2007). Though, the process of synthesis of GNPs poses potential environmental as well as biological risks. A simplest GNPs solution contains materials other than the core material (gold), such as, surface-bound stabilizing ligands and potential leftover chemicals after the synthesis (Alkilany and Murphy, 2010).

The observed toxicity of GNPs solution could be caused by any of these components and thus assessing the contribution of each component is crucial to understand the origin of toxicity (Alkilany et al., 2009). The typical known method
of GNPs synthesis usually involves the reduction of gold metal ions using harsh reducing agents such as hydrazine, sodium borohydrate, dimethyl formamide and CTAB (cetyl trimethyl ammonium bromide) which are highly reactive and toxic to both human cells and environment (Alkilany and Murphy, 2010; Alkilany et al., 2009; Nikoobakht and El-Sayed, 2003; Brust et al., 1994; Nikoobakht and El-Sayed, 2001; Sau and Murphy, 2004; Murphy et al., 2005). Consequently, reducing the use of these chemicals and effective replacement of these chemical ligands with biologically adaptable biomolecules will eventually enhance all biological applications of GNPs.

Other major issue which is found to be associated with GNPs is their stability as GNPs tend to quickly agglomerate and oxidize after synthesis (Raghuraman et al., 2007; Tiwari et al., 2011). Therefore, various stabilization methods were applied which includes the use of citrates and thiols for the storage of GNPs (Brust et al., 1994; Brewer et al., 2005; Sugunan and Dutt, 2005). However, citrates can be difficult to handle and use due to their strong acidic nature, while, thiols cannot be readily exchanged onto peptides or other biomolecules because of their strong interaction with gold metal (Raghuraman et al., 2012; Gittins and Caruso, 2002; Sperling and Parak, 2010)

To overcome issues related to synthesis and stabilization, the present study uses environmental friendly methods to allow the synthesis of monodispersed GNPs using nitrate reductase, bromelain (a naturally occurring cysteine protease) and trypsin as reducing agent and human serum albumin (HSA) as a capping agent in aqueous solution under controlled conditions.
Here, HSA has been used as a capping agent and linker between drug and NPs. HSA protein is acidic in nature, soluble in a wide range of pH, biodegradable and lacks toxicity and immunogenicity. There are many reports, where nanocarriers have been shown to be a very effective cargo of the drugs like gentamycin (Lecaroz et al., 2006), ampicillin (Fattal et al., 1989), amphotericin B (Uma maheshwari et al., 2004), tuberculosis drugs (Pandey et al., 2003) and anticancer drugs (Moghimi, 2006). There are many drugs which are required in very high concentration in human body and hence produce toxic effects (Schellie et al., 1999). Therefore, nano-conjugates will help in reducing the dose of certain drugs. The accurate effect of conjugated drug could be determined only if quantification of conjugated drug is exactly known. Hence, in this part of the study, we attempted to develop biosynthetic GNPs encapsulated with HSA as a delivery system for different drugs including secnidazole and compared the efficiency and stability of conjugated GNPs.

3.2 Materials and Methods

H[AuCl₄] (gold salt)  Sigma Aldrich
Na₂SO₃  Himedia
HSA  Sigma Aldrich
Bromelain  Merck
Trypsin  Himedia
Secnidazole  Sigma Aldrich
EDC  Himedia
HEPES buffer  Himedia
α-NADPH  Sigma Aldrich
3.2.1 *In Vitro* Synthesis of GNPs by Using Nitrate Reductase Enzyme With HSA as a Capping Agent

In this study *in Vitro* synthesis of GNPs was done by taking a total reaction mixture of 3 ml containing 1.0 mM each of freshly prepared H[AuCl₄] and Na₂SO₃, 350μg of HSA (Human Serum Albumin), 1.0 mM α-NADPH (α-Nicotinamide adenine dinucleotide phosphate, reduced disodium salt) and 1.66 U (100μg protein) of sulphite reductase (Kumar et al., 2007). The reaction mixture was incubated, under anaerobic conditions at 25°C. Reactions performed in the absence of α-NADPH enzyme, HSA as well as the inactivated enzyme was used as a control. Samples were removed at regular intervals and analyzed in UV-Vis spectroscopy to confirm the NPs formation. On completion of the reaction, GNPs were collected through centrifugation (30,000 g, 30 min), washed twice with Milli Q water and the unbound proteins were removed by treating with 50% v/v of ethanol and used for further characterization.

3.2.2 *In Vitro* Synthesis of GNPs by Using Bromelain at Varying Concentration and Temperature

*In vitro* synthesis of GNPs of different sizes at varying concentrations were done by taking total four reaction mixtures of 3 ml, each containing 1.0 mM H[AuCl₄] (prepared in 50mM Phosphate buffer) and different concentrations of freshly prepared Bromelain (i.e., 0.33mg/ml, 0.66mg/ml, 1.66mg/ml and 3.33mg/ml) (table 3.1). All the four reaction mixtures were incubated at 40°C for 48hrs. *In vitro*
synthesis of GNPs of different sizes at varying temperature were also done by taking four different reaction mixtures of 3 ml each containing 1.0 mM H[AuCl₄].

Table 3.1 Specific experimental conditions for the synthesis of GNPs of different sizes using 0.33mg/ml concentration of bromelain at different temperature.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Bromelain* (mg/ml)</th>
<th>PO₄²⁻-Buffer (50 mM)</th>
<th>H[AuCl₄] ** (1M)</th>
<th>Incubation</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 mg/ml</td>
<td>1.997 ml</td>
<td>3 μl</td>
<td>48 hrs</td>
<td>40°C</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1 mg/ml</td>
<td>1.997 ml</td>
<td>3 μl</td>
<td>48 hrs</td>
<td>50°C</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1 mg/ml</td>
<td>1.997 ml</td>
<td>3 μl</td>
<td>24 hrs</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1 mg/ml</td>
<td>1.997 ml</td>
<td>3 μl</td>
<td>12 hrs</td>
<td>70°C</td>
<td></td>
</tr>
</tbody>
</table>

*Final concentration of Bromelain in 3 ml reaction mixture was 0.33mg/ml.
**Final concentration of H[AuCl₄] is 1mM. *** MilliQ filtered distilled Water was used.

All four reaction mixtures were incubated at different temperature (i.e., 40°C, 50°C, 60°C and 70°C) separately (table 3.2). A reaction performed in the absence of Bromelain was used as control. Samples were removed at regular intervals and analyzed in UV-Vis spectroscopy to confirm for the NPs formation. On completion of the reaction, GNPs were collected by centrifugation (30,000 g, 30 min), washed twice with Milli Q water and unbound Bromelain was removed by treating with 50%
v/v of ethanol and used for further characterization (Khan et al., 2014). [Authors have filed a process patent (DEL/805/2012) for this methodology].

Table 3.2 Provides specific experimental conditions for the synthesis of GNPs of different sizes using different concentrations of bromelain.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Bromelain* (mg/ml)</th>
<th>PO₄²⁻ Buffer (50 mM)</th>
<th>H AuCl₄ ** (1M)</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1mg/ml</td>
<td>1.997ml</td>
<td>3μl</td>
<td>48hrs</td>
</tr>
<tr>
<td>II</td>
<td>2mg/ml</td>
<td>1.997ml</td>
<td>3μl</td>
<td>48hrs</td>
</tr>
<tr>
<td>III</td>
<td>5mg/ml</td>
<td>1.997ml</td>
<td>3μl</td>
<td>48hrs</td>
</tr>
<tr>
<td>IV</td>
<td>10mg/ml</td>
<td>1.997ml</td>
<td>3μl</td>
<td>48hrs</td>
</tr>
</tbody>
</table>

*Final concentration of Bromelain in 3ml reaction mixtures I, II, III and IV were 0.33mg/ml, 0.66mg/ml, 1.66 mg/ml and 3.33 mg/ml, respectively.**Final concentration of AuCl₄⁻ is 1mM.***MilliQ filtered distilled Water was used.

3.2.3 In Vitro Synthesis of NPs by Using Trypsin

In vitro synthesis of GNPs of different sizes were done by taking total three reaction mixtures of 3 ml, each containing 1.0 mM H[AuCl₄], 350μg of HSA and different concentrations of freshly prepared trypsin (i.e., 0.33mg/ml, 0.66mg/ml and 1.66mg/ml). All three reaction mixtures were incubated at 40°C. A reaction
performed in the absence of Trypsin was used as control. Samples were removed at regular intervals and analyzed in UV-Vis spectroscopy to confirm the formation of NPs. On completion of the reaction, GNPs were collected by centrifugation (30,000 g, 30 min), washed twice with Milli Q water and the unbound proteins were removed by treating with 50% v/v of ethanol and used for further characterization.

Table 3.3 Provides specific experimental conditions for the synthesis of GNPs of different sizes using different concentrations of Trypsin.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Trypsin* ((\text{mg/ml}))</th>
<th>PO\textsubscript{4}\textsuperscript{2-} Buffer ((50 \text{ mM}))</th>
<th>HSA ((10 \text{mg/ml}))</th>
<th>H\textsubscript{AuCl}_4 **(\text{1M}))</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1\text{mg/ml})</td>
<td>(1.962\text{ml})</td>
<td>(35\mu\text{l})</td>
<td>(3\mu\text{l})</td>
<td>(48\text{hrs}) (40^\circ\text{C})</td>
</tr>
<tr>
<td>I</td>
<td>(2\text{mg/ml})</td>
<td>(1.962\text{ml})</td>
<td>(35\mu\text{l})</td>
<td>(3\mu\text{l})</td>
<td>(48\text{hrs}) (40^\circ\text{C})</td>
</tr>
<tr>
<td>II</td>
<td>(5\text{mg/ml})</td>
<td>(1.962\text{ml})</td>
<td>(35\mu\text{l})</td>
<td>(3\mu\text{l})</td>
<td>(48\text{hrs}) (40^\circ\text{C})</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Final concentration of Trypsin in 3ml reaction mixtures I, II and III were 0.33mg/ml, 0.66mg/ml and 1.66 mg/ml, respectively. **Final concentration of \(\text{AuCl}_4^-\) is 1mM. ***MilliQ filtered distilled Water was used.

3.2.4 Bioconjugation of Biosynthetic NPs with Secnidazole

Eventually, \textit{in vitro} synthesized GNPs-HSA were bioconjugated to secnidazole with the free carboxylate group present on HSA by using the activator 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Timkovich, 1977; Hermanson, 1996), 5 ml reaction mixture containing 50 mM MES/HEPES buffer,
250 µg of Secnidazole and 250 µg of GNPs-HSA with 5 mM EDC in aliquots was used. Bioconjugation was performed at 30°C for a period of 3 hrs and the bioconjugates were parted from unconjugated GNPs-HSA by passing the reaction mixture through Biogel P-30 gel filtration column pre-equilibrated with 20 mM HEPES buffer (pH 6.0) containing 150 mM NaCl. The fractions were scanned between 200-900 nm and the pooled fractions which exhibit the absorbance at 320nm/525nm were dialyzed against distilled water and used for further characterization.

3.2.5 Determination of Loading Efficiency (LE) of Secnidazole Drug on Gnps-HSA by a High Performance Liquid Chromatography (HPLC)

The Loading efficiency (LE) of secnidazole drug on GNPs-HSA were determined by a high performance liquid chromatography (A Shimadzu-model HPLC equipped SPD-20A UV/VIS detector, LC-20AT pump, Rheodynamic injector fitted with a 20-µl loop was used and the data were documented and assessed using Spinchem software) at 25 °C with a reversed-phase C-18 column, Luna Column (5 µm, 250×4.6 mm inner diameter) using a mobile phase consisting of buffer: 0.01M KH₂PO₄: ACN (85:15) at a flow rate of 1 ml/min with UV detection at 228 nm. The mobile phase was filtered through 0.22-µm nylon filter prior to use. The experiments were performed in triplicate. Quantitative determination of secnidazole was performed according to the method described by Rivera et al. (2000). The pure drug calibration curve was plotted in the linear range of 2-25µg/ml. The percentage loading of secnidazole to GNPs was calculated by using RP-HPLC C-18 column. The standard curve of the pure secnidazole drug was established and unbound drug was calculated from the standard curve. Amount of bioconjugated drug was
calculated by subtracting unbound drug from the total amount of drug added. The exact amount of bioconjugated drug was calculated using the following equation:

\[
\% \text{ Bioconjugation} = \frac{\text{Amount of drug Bioconjugated}}{\text{Total drug added}} \times 100
\]

3.2.6 Characterization of In-vitro Synthesized GNPs by Various Techniques

3.2.6.1 UV/Vis spectroscopy

UV-Vis spectrophotometry measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

3.2.6.2 Circular Dichroism measurements (CD)

Far-UV circular dichroism (CD) measurements of native and GNPs encapsulated protein were recorded on J-815 Jasco spectropolarimeter (Japan) in the wavelength range of 200–250 nm. All scans were recorded at wavelength intervals of 1 nm.

3.2.6.3 Transmission Electron Microscopy (TEM)

Samples were prepared by drying a drop of GNPs solution on carbon coated TEM copper grids followed by measurements on (TEM) FEI Company, Tecnai™ G² Spirit BioTWIN operated at an accelerating voltage of 80kV.
3.2.6.4 Scanning Electron Microscopy (SEM)

GNPs-HSA were air dried on glass slides and then coated with gold. The morphology of NPs was examined under a scanning electron microscope (JEOL JSM 5200).

3.2.6.5 Dynamic Light Scattering (DLS)

The mean particle size (MPS) of HSA encapsulated biosynthesized GNPs (GNPs-HSA) was measured with a dynamic light scattering particle size analyzer (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instrument Ltd, Malvern, UK). The sample was taken in a DTS0112-low volume disposable sizing cuvette of 1.5 ml. The sample powder was diluted to a concentration of 0.5% (wt/v) in deionised water and sonicated for 1 min before measurement. Mean particle size was the average of triplicate measurements for a single sample.

3.2.6.6 High Performance Liquid Chromatography (HPLC)

The secnidazole drug on GNPs-HSA were determined by a high performance liquid chromatography (A Shimadzu-model HPLC equipped LC – 20 AT pump, SPD-20 A UV/ VIS detector, Rheodyne injector fitted with a 20-μl loop was used and the data were recorded and evaluated using Spinchrom software) at 25 °C with a reversed-phase C-18 column, Luna Column (5 μm, 250×4.6 mm inner diameter) using a mobile phase consisting of buffer: 0.01M KH2PO4: ACN (85:15) at a flow rate of 1 ml/min with UV detection at 228 nm. The mobile phase was filtered through 0.22-μm nylon filter prior to use.
3.3 Results and Discussion

A variety of reducing agents such as citrate and borohydrate have been reported to be used for the reduction of gold salt and these agents demonstrate a significant influence on the morphology of the GNPs synthesized (Young et al., 2011). Unfortunately, these reducing agents have some or the other disadvantages like toxicity, hindrance in surface modification and functionality of GNPs, etc. (Majzik et al., 2009; Shan et al., 2008). However, the method used in the present study involves a novel approach to synthesize monodispersed GNPs of controllable sizes using bromelain as a reducing agent as well as a capping agent in aqueous solution under controlled conditions. Bromelain was selected for the study because it was found to be highly interactive and a very good capping agent for GNPs. Bromelain is a cysteine protease (having cysteine residue in the active site) which belongs to papain super family and preferentially cleaves glycyl, alanyl and leucyl bonds where nucleophile of the catalytic site is part of histidine amino acid (covalent catalysis) which normally functions as a base (Bogre et al., 1996).
There were two different approaches adopted to synthesize GNPs of best qualities in terms of size, shape, monodispersity and stability by using bromelain. Figure 3.1 illustrates the schematic formation of different sizes of bromelain coated GNPs (GNPs-Bromelain) at varying concentration of bromelain as well as at different temperature of incubation. In the first approach variable concentrations of bromelain (0.33 mg/ml; 0.66 mg/ml; 1.66 mg/ml & 3.33 mg/ml) was incubated with constant concentration of gold salt (H[AuCl₄]) (1 mM) at constant temperature (40°C). This approach gave the optimum concentration of bromelain (0.33 mg/ml) at which best GNPs were produced among the others (Figure 3.2). These GNPs showed characteristic ruby red color Figure (3.2a inset) with fundamental surface plasma resonance at 522 nm Figure (3.2a) and hydrodynamic diameter 58.65 nm Figure (3.2b). These GNPs were found to be monodisperse and spherical with size 16.5 nm.
under SEM (Figure 3.2c). TEM micrograph (Figure 3.2d) Figure (3.3a, 3.3b and 3.3c) clearly revealed the trend and showed that the size of GNPs increases (from 8.59nm to 44.14nm) gradually with the increase in concentration of bromelain (from 0.33-3.33mg/ml) at 40°C. However, their stability got decreased with increase in size. It is a well known fact that bromelain rapidly deteriorates through self-digestion in aqueous solution (Maurer 2001). When concentration of bromelain is increased rate of deterioration increases and hence, effective concentration of bromelain decreases and at the same time the length of protein moieties present in solution doesn’t remain same. So, there will be several protein chains of different lengths available for capping. These different proteins, probably smaller in size, will not be proved as good capping agents for GNPs.

In the second approach optimum concentration (0.33 mg/ml) was kept constant and the temperature of incubation was varied from 40 °C; 50 °C; 60 °C to 70 °C. Here, the best incubation temperature for the production of monodisperse GNPs at 0.33 mg/ml concentration was found to be 40 °C. However, increase in temperature proportionally increases the rate of GNPs formation, but unfortunately their sizes were bigger with lower stability. Interestingly, it has been proved by researchers that heating facilitates the rapid synthesis of GNPs if gold solution is accomplished with reducing and capping agents (Merza et al., 2012; Li et al., 2011). At optimum concentration (0.33 mg/ml) of bromelain, the synthesis of GNPs at different temperatures 40°C, 50°C, 60°C and 70°C occurred after 48 hrs, 24 hrs, 12 hrs and 2 hrs of incubation, respectively (Figure 3.4 a, 3.4b and 3.4c). Though with increase in temperature the activity of bromelain decreases gradually due to degradation of protease (Rungtip et al., 2010). This degradation produces several non
active polypeptide chains of varied length and with an increase in temperature degradation also increases which consequently produces shorter polypeptide chains. Hence, proper capping agents were not present in the solution and good capping of GNPs could not be achieved. The sizes of GNPs at a given temperature are shown in Figure 3.4 as SEM images. SEM micrograph image of all the samples indicated spherical shape and monodispersity of GNPs.

Zeta potential of GNPs synthesized at different concentrations as well as temperatures was shown in inset of Figure (3.4a, 3.4b and 3.4c.) Evaluating Zeta Potential is considered as an effective, simplest and most straightforward way for predicting NPs stability and understanding the nanoparticle surface status. In fact, conclusions regarding concentration, distribution, exposure or shielding of charged moieties, ionization, adsorption (Rabinovich-Guilatt et al., 2004; Huynh et al., 2009) could be easily drawn from the analysis of zeta potential data. Fortunately, in the present study, Zeta potential of GNPs synthesized by using 0.33-3.33 mg/ml concentration of bromelain showed negative values (-16.6 to -6.47 mV) which eventually relates to its stability in aqueous solution (Figure 3.8d, 3.9a, 3.9b and 3.9c inset).

On the other hand, Zeta potential of GNPs synthesized at different temperature 50°C, 60°C and 70°C also showed negative values between -9.56 to -6.12 mV (Figure 3.10a, 3.10b and 3.10c inset). Table 3.4 and 3.5 shows comparative study of the two different approaches of the synthesis, thus, stability decreases (as zeta potential changes) with increase in the concentration of bromelain as well as with increase in the incubation temperature.
Figure 3.2. (a) UV-visible spectra (b) SEM micrograph (c) DLS results (Z-average mean diameter) (d) TEM micrographs (Zeta Potential in Inset) of GNPs synthesized by using 0.33 mg/ml concentration of Bromelain at 40°C.
Figure 3.3. TEM micrographs of GNPs synthesized using different concentration of Bromelain at 40°C temperature (Zeta Potential in Inset); 3.3a: GNPs synthesized using 0.66 mg/ml Bromelain; 3.3b: GNPs synthesized using 1.66 mg/ml Bromelain; 3.3c: GNPs synthesized using 3.33 mg/ml Bromelain.

Figure 3.4: SEM micrographs of GNPs synthesized using same concentration of Bromelain (0.33 mg/ml) at different temperature (Zeta Potential in Inset); 3.4a: GNPs synthesized at 50°C; 3.4b: GNPs synthesized at 60°C; 3.4c: GNPs synthesized at 70°C.
### Table 3.4. Comparative DLS, SEM and TEM analysis of GNPs synthesized by using 0.33mg/ml concentration of Bromelain incubated at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>DLS analysis Size Z-average diameter (nm)</th>
<th>SEM analysis Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40°C</td>
<td>58.65</td>
<td>16.5</td>
</tr>
<tr>
<td>50°C</td>
<td>155.9</td>
<td>22.6-31.1</td>
</tr>
<tr>
<td>60°C</td>
<td>176.9</td>
<td>39.5</td>
</tr>
<tr>
<td>70°C</td>
<td>200.7</td>
<td>50.8-62.1</td>
</tr>
</tbody>
</table>

### Table 3.5. Comparative DLS, SEM and TEM analysis of GNPs synthesized by using different concentration of bromelain incubated at 40°C.

<table>
<thead>
<tr>
<th>Bromelain Concentration (Mg/ml)</th>
<th>DLS analysis Size Z-average diameter (nm)</th>
<th>SEM analysis Size (nm)</th>
<th>TEM analysis Size (nm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.33 mg/ml</td>
<td>58.65</td>
<td>16.5</td>
<td>8.59-12.92</td>
</tr>
<tr>
<td>0.66 mg/ml</td>
<td>65.23</td>
<td>16.5-21</td>
<td>11.27-35.68</td>
</tr>
<tr>
<td>1.66 mg/ml</td>
<td>82.26</td>
<td>18.2-26.5</td>
<td>-</td>
</tr>
<tr>
<td>3.33 mg/ml</td>
<td>306.5</td>
<td>50.0-63.0</td>
<td>-</td>
</tr>
</tbody>
</table>

*TEM analysis of only best size NPs prepared using 0.33mg/ml and 0.66 mg/ml of Bromelain (Based on SEM and DLS analysis)

Moreover the present study provides a process of synthesizing GNPs using environmentally benign materials for stabilization in aqueous media towards use in pharmaceutical and biological applications. Further, in comparison to the methods used in the previous literature, the GNPs synthesis method of the present study is a facile single step method and includes significantly fewer chemicals, which are
biocompatible and ecofriendly too. Another ecofriendly method used in the present study involves a novel approach to allow the synthesis of monodispersed GNPs using trypsin as a reducing agent and HSA as a capping agent in aqueous solution under controlled conditions. In addition, GNPs having desired sizes were easily fabricated using varying concentrations of trypsin. Figure 3.5 illustrates the schematic formation of HSA coated GNPs (GNPs-HSA) by using trypsin as a reducing enzyme and HSA as a capping agent.

![Schematic Representation of Biosynthesis of HSA Capped GNPs-HSA](image)

**Figure 3.5.** Schematic representation of biosynthesis of HSA capped GNPs-HSA by using trypsin as reducing agent.

The plasmon-derived optical resonance of GNPs strongly relates to the dimensions and morphology of the NPs. Young et al., (2011). In the present study, the formation of the GNPs-HSA by different concentrations of trypsin has been confirmed by their representative surface plasmon resonance (SPR) band measured as measured by optical density in UV–Vis absorption spectroscopy. The SPR absorption spectra of GNPs-HSA were plotted in Figure 3.6
The UV-visible spectra of the GNPs solution prepared by different concentrations of trypsin, increasing from 0.33 to 1.66 mg/ml, shows an increase in absorbance which correlates to an increase in particle volume and concentration.

Figure 3.6 shows the pronounced red shifting of the plasmon, which was associated with increased nanoparticle size. This shifting effect was in corroboration with the prediction described by Mie theory (Link and El-Sayed, 1999; Mie, 1908; Hovel et al., 1993). For spherical NPs below 50nm diameter there will be a blue shift of absorption band caused by further splitting of energy levels and the further increase of energy gap with the decrease of the diameter because of the size effect of NPs (He et al., 2005). The linear regression equation ($\lambda_{\text{max}} = 515.04 + 0.3647d$) was
established by researchers He et al., (2005) to explain the relation between particle diameter and $\lambda_{\text{max}}$. The SPR absorption band for GNPs-HSA where trypsin concentration 0.33 mg/ml was used for the synthesis appeared at 522 nm, while, SPR absorption band for GNPs-HSA where trypsin concentration 0.66 mg/ml was appeared at 527 nm. However, decrease in intensity with a significant broadening was observed for GNPs-HSA which was synthesized by using 1.66 mg/ml concentration of trypsin where SPR absorption band appeared at 539 nm.

Figure 3.7. (a) TEM (b) SEM (c) DLS of GNPs-HSA synthesized by using 0.33 mg/ml concentration of trypsin

Figure (3.7a, 3.7b and 3.7c) shows TEM, SEM, DLS results of GNPs synthesized by using 0.33 mg/ml concentration of Trypsin. It reveals that the
particles are smaller in size and uniformly distributed. The particle size of the GNPs-HSA was calculated to be $8 \pm 2$ nm, although, the hydrodynamic diameter of NPs appears larger ($40 \pm 2$ nm) in DLS. It is well known that DLS obtain the hydrodynamic radius of the particle while TEM estimates the projected area diameter. (Berne and Pecora, 2000; Chu, 1991; Park et al., 2004) In fact, a thin electric dipole layer of the solvent adheres to the surface of a dispersed particle when it moves through a liquid medium. Consequently, the hydrodynamic diameter provides us information of the inorganic core along with coating material and the solvent layer attached to the particle. However, when size is estimated by TEM, this hydration layer is absent; so, we get information only about the inorganic core. Therefore, the hydrodynamic diameter observed by DLS always found to be greater than the size estimated by TEM. In agreement with these facts, our results also showed that the hydrodynamic diameter of GNPs-HSA appears larger in DLS as compared to size estimated by TEM.

TEM, SEM and DLS analysis of GNPs synthesized by using 0.66 mg/ml and 1.66 mg/ml concentrations of Trypsin are shown in Figure (3.8a, 3.8b and 3.c) and Figure (3.9a, 3.9b and 3.9c) respectively When 0.66 mg/ml concentration of trypsin was used for the synthesis, the particle size of Au-HSA was found to be $10 \pm 3$ nm, while, hydrodynamic diameter by DLS was estimated to be $75.65 \pm 4$ nm (Figure 3.8). In contrast, when 1.66 mg/ml concentration of trypsin was used, GNPs-HSA size was found to be in a range of 17-35 nm and hydrodynamic diameter by DLS was estimated as $110 \pm 5$ nm (Figure 3.9). With an increase in concentration of trypsin the proteolytic activity of trypsin against HSA increased, and hence, effective
concentration of HSA got decreased which in turn produce GNPs of larger size. Further, SEM micrograph image (Figure 3.7, 3.8 and 3.9) of all the samples indicated spherical shape and monodispersity of the GNPs. In principle, GNPs of different sizes may be produced in a quantitative way by controlling the experimental conditions like concentration or temperature. In the present study, GNPs of different sizes were fabricated by using increasing concentration of trypsin. Trypsin is a naturally occurring serine protease which contain a catalytic triad consisting of histidine-57, aspartate-102 and serine-195 at its active site (Polgár 2005). These three residues form a charge relay that serves to make the active site serine nucleophilic, which is further responsible for the proteolytic action of
trypsin. However, in the present study, trypsin was used to reduce gold salt to form GNPs. In fact, the energy generated by proteolytic activity of trypsin was used to reduce gold. Figure 3.6-3.9 validates two aspects in our study. Firstly, that trypsin

![Fig. 3.9a](image1.png) ![Fig. 3.9b](image2.png) ![Fig. 3.9c](image3.png)

**Figure 3.9.** (a) TEM (b) SEM (c) DLS of GNPs-HSA synthesized by using 1.66 mg/ml concentration of trypsin.

can synthesize GNPs and secondly, the size of GNPs increases with increase in concentration of trypsin. Before closing the discussion we find it appropriate to mention that the methodologies used in this study could estimate the efficiency of trypsin and bromelain in synthesizing the NPs of different sizes. However, further studies would be needed to observe the secondary and tertiary structure of protein encapsulated on GNPs to correlate well with the outcomes of this experimental study. Therefore, CD spectra studies were conducted on these GNPs.
The bromelain is predominantly an $\alpha$ helix and $\beta$ sheet protein with 23% $\alpha$ helix, 18% antiparallel $\beta$ sheet, 5% parallel $\beta$ sheet, 18% turns, and the remaining 35% as other structures (Reyna and Arana, 1995; Reyna et al., 1994). It has a molecular mass of 23,800 Da (212 amino acid residues), contains three disulfides and a single free cysteine residue. Figure (3.10) shows the far-UV-CD spectra of native (control) and bromelain GNPs. There was an almost 19.4% loss in the secondary structure after formation of GNPs of bromelain as compared to that of control bromelain as observed at 208 nm. This can be due to conformational change from the peptide native state in solution upon binding to the nanoparticle surface, and/or energy transfer from the peptide to the metal nanoparticle.

![Figure 3.10](image_url)

**Figure 3.10.** Far UV-CD spectra of Bromelain (—); Bromelain-gold nanoparticle (--) ; and gold particle (…..).
It is likely that the second possibility might be the dominant mechanism since the Bromelain peptide still retained its main helical features based on analysis of the CD spectra using CDPro software. Similar wavelength shifts has also been observed upon protein adsorption to metallic surfaces (Jiang et al., 2005). Furthermore, upon modification induced in the secondary structure of protein as a consequence of NPs formation, it leads to increase in mean residual ellipticity (MRE) value as compared to the control. It is interesting to note that although the secondary structure has lossed upon capping with GNPs; however the identity of secondary structure was retained as such as compared to the control. The structural retention is observed from the peaks at 208 and 222 nm of GNPs-bromelin. This result signifies that structure has opened/unfolded upon modification induced by GNPs.

Figure 3.10 shows near-UV CD spectra of stem bromelain in the presence and absence of GNPs. A positive peak around 280nm characterizes the native state of bromelin also reported elsewhere (Reyna et al., 1994; Kamphuis et al., 1984). As can be seen in the figure 3.10 there was insignificant loss in the tertiary structure of modified bromelain as compared to its control. It is interesting to note that trivial increase in ellipticity with the spectrum similar to that of native bromelain preparation, apparently due to the formation of a relatively compact structure.

All the above results taken together indicate that loss in secondary structure but substantial retention of tertiary structure of GNPs-bromelin. This implies that slight loss in secondary structure does not lead to tertiary structure loss. Hence, it could be implicated that upon the formation of the GNPs-bromelin, the functional properties as determined by the tertiary structure does not hampered, however its
secondary structure leads to the unfolding of the protein structure, which could cause its enhanced activity as supported by other experiments.

On the other hand, Figure 3.11 shows the far-UV CD spectra of native trypsin and complexed trypsin with GNPs. The native trypsin exhibited a major negative band at 207 nm and an ordinary negative band at 202 nm on its far-UV CD spectra curve, specifying that it is a double broad negative band character mainly attributable to the presence of mixtures of α-helix and β-structures (Wu et al., 1981).

![Figure 3.11](image)

**Figure 3.11.** Far UV-CD spectra of Trypsin pure (---); Trypsin-gold nanoparticle (.....)

The native trypsin was calculated and was composed of 16% α-helix, 35% β-sheet, 11% β-turn and 38% random coil respectively by the computer software K2D2. It was observed that the double-negative band of the native trypsin was trimmed as a single negative band at about 206 nm as complexed with GNPs. The percent changes induced by GNPs with the trypsin, was that; α-helix was found
decreased from 16% to zero, β-turn from 11% to 1%, β-sheet increased from 35 to 61 %, and the random kept unchanged basically.

Second part of this chapter was focused on development of novel delivery vehicle for secnidazole and estimation of the accurate amount of bioconjugated drugs on the GNPs. Figure 3.12 illustrates the schematic formation of GNPs-HSA by using nitrate reductase as a reducing enzyme, NADPH as a cofactor and HSA as a capping agent, and eventually their bioconjugation with secnidazole drug.

![Figure 3.12: Schematic representation of biosynthesis of HSA capped GNPs (GNPs-HSA) by using Nitrate Reductase as reducing agent and eventually their bioconjugation with secnidazole drug (GNPs-HSA-Snd).](image)

The formation of the GNPs-HSA has been established by their representative surface plasmon resonance (SPR) band measured by optical density in UV–Vis absorption spectroscopy. The SPR absorption spectra of GNPs-HSA and secnidazole–conjugated HSA GNPs (GNPs-HSA-Snd) are plotted in Figure 3.13.
Figure 3.13. UV-Vis spectra of GNPs-HSA, GNPs-HSA-Snd and pure secnidazole.

The SPR absorption band for GNPs-HSA-Snd appears at 525 nm, with a significant broadening and slight decrease in intensity compared to the plasmon band for GNPs-HSA at 527 nm. The plasmon resonance is a surface phenomenon which modifies after attachment of any ligand at the surface. The ligand binding significantly affects the absorption intensity and full width at half maximum (FWHM) of absorption band. The observed broadening showed in some blurring of the TEM micrograph. The hydrodynamic diameter of NPs appears large (18 ± 3nm) in DLS due to the interaction and binding of solvent to the surface of NPs. Eventually, GNPs–HSA-Snd appears bigger than non conjugated GNPs-HSA one due to subsequent enlargement of NPs.

The GNPs-HSA were found to be negatively charged with a zeta potential of -13.3 mV (Figure 3.14a, 3.14b, 3.14c and 3.14d), which can be one of the reasons for
their long term stability and the electrostatic repulsive forces between the NPs might protect them from getting closer and thereby preventing agglomeration or clumping in aqueous suspension. The zeta potential of GNPs-HSA-Snd was found to be -7.97 mV which is well in the range required for stable emulsion (Figure 3.15a, 3.15b, 3.15c and 3.15d).

Absorption band is attributed to the attachment of secnidazole drug at the surface of NPs. The absorption spectrum of GNPs-HSA-Snd exposes two peaks at 320 nm and 525 nm corresponding to secnidazole aromatic nitrite transitions and GNPs respectively, suggesting its binding to GNPs.

Figure 3.14a, 3.14b, 3.14c and 3.14d shows TEM, SEM, DLS, Size distribution and zeta potential of GNPs-HSA. It also reveals that the particles are smaller in size and uniformly distributed. The particle size of the GNPs-HSA was estimated to 7 ± 2 nm.

![Figure 3.14](image)

**Figure 3.14.** (A) TEM (B) SEM (C) Zeta Potential and (D) DLS of GNPs-HSA.
Figure 3.15. (A) TEM (B) DLS (C) Zeta Potential of Gnp-HSA-Snd.

Figure 3.15a, 3.15b and 3.15c shows TEM, DLS, Size distribution and zeta potential micrographs of GNPs-HSA-Snd. TEM micrographs of GNPs-HSA-Snd revealing the attachment of secnidazole (and consequent enlargement of the particles) as a biomolecular layer at the surface, resulting the decrease in zeta potential may be attributed to the decrease of the free amino groups on the GNPs-HSA-Snd after bioconjugation of the secnidazole drug to these groups. The quantitative estimation of conjugated secnidazole with GNPs-HSA was determined by RP-HPLC chromatography by using the C-18 column. The amount of bioconjugated secnidazole was found to be 70% indicating efficient binding of secnidazole with GNPs-HSA-Snd (figure 3.16 and 3.17).
Figure 3.16. Calibration curve of conjugated secnidazole drug

Figure 3.17. RP-HPLC chromatograms for (a) pure secnidazole drug and (B) GNPs-HSA-Snd.

The methodology of present study provides a simple, economic, ecofriendly and highly effective technique for synthesizing GNPs of different sizes which are
stable for long time at room temperature. In addition, effective drug delivery system through GNPs was formed successfully with increased efficacy.

3.4 Conclusion

This study provides a novel approach for the synthesis of GNPs using trypsin as reducing agent, nitrate reductase and human serum albumin (HSA) as a capping agent while bromelain as a reducing as well as capping agent. In this study, a comparative account of TEM, SEM and DLS analysis of GNPs synthesized by using different concentrations of bromelain at different temperature have been discussed. We found that when the concentration of bromelain as well as incubation temperature increased, the size of GNPs also got increased. 0.33 mg/ml concentration of bromelain at 40°C was found to be most effective in producing GNPs of smaller size, high monodispersity and higher zeta potential. Such information may aid in the design of versatile, potent and stable GNPs for future nano-medicinal studies. Further, protein structural studies of encapsulated proteins are required to explore the potential of GNPs synthesized in this study and to validate the findings presented herein. This study shows that bromelain, nitrate reductase and trypsin were successful in synthesizing stable GNPs of different sizes which can be kept at room temperature for months.

In addition, bioconjugation of secnidazole GNPs-HSA-Snd were developed as an ideal targeted drug delivery system to improve therapeutic effects significantly of secnidazole drug and diminish its side effects. This system not only makes the drug stable but also reduces the dose with enhanced potency.